Impacts of metallic pollutants on honey bees : from the colony to the brain

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Impacts of metallic pollutants on honey bees: from the colony to the brain

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Summary

Honey bees are crucial pollinators. A plethora of environmental stressors, such as agrochemicals, have been identified as contributors to their global decline. Especially, these stressors impair cognitive processes involved in fundamental behaviours. So far however, virtually nothing is known about the impact of metal pollutants, despite their known toxicity to many organisms. Their worldwide emissions resulting from human activities have elevated their concentrations far above natural baselines in the air, soil, water and flora, exposing bees at all life stages. The aim of my thesis was to examine the effects of metallic pollution on honey bees using a multiscale approach, from brain to colonies, in laboratory and field conditions. I first observed that bees exposed to a range of concentrations of three common metals (arsenic, lead and zinc) in the laboratory were unable to perceive and avoid, low, yet harmful, field-realistic concentrations of those metals in their food. I then chronically exposed colonies to field-realistic concentrations of lead in food and demonstrated that consumption of this metal impaired bee cognition and morphological development, leading to smaller adult bees. As metal pollutants are often found in complex mixtures in the environment, I explored the effect of cocktails of metals, showing that exposure to lead, arsenic or copper alone was sufficient to slow down learning and disrupt memory retrieval, and that combinations of these metals induced additive negative effects on both cognitive processes. I finally investigated the impact of natural exposure to metal pollutants in a contaminated environment, by collecting bees in the vicinity of a former gold mine, and showed that individuals from populations most exposed to metals exhibited lower learning and memory abilities, and development impairments conducing to reduced brain size. A more systematic analysis of unexposed bees revealed a relationship between head size, brain morphometrics and learning performances in different behavioural tasks, suggesting that exposure to metal pollutants magnifies these natural variations. Hence, altogether, my results suggest that honey bees are unable to avoid exposure to field-realistic concentrations of metals that are detrimental to development and cognitive functions; and call for a revision of the environmental levels considered as ‘safe’. My thesis is the first integrated analysis of the impact of several metal pollutants on bee cognition, morphology and brain structure, and should encourage further studies on the contribution of metal pollution in the reported decline of honey bees, and more generally, of insects.

Keywords: heavy metals, *Apis mellifera*, cognition, behaviour, morphometry

Résumé

Les abeilles sont des pollinisateurs essentiels. Une pléthore de facteurs de stress environnementaux, tels que les produits agrochimiques, a été identifiée comme contribuant à leur déclin mondial. En particulier, ces facteurs de stress altèrent les processus cognitifs impliqués dans les comportements fondamentaux. Jusqu'à présent, cependant, on ne sait pratiquement rien de l'impact de l'exposition à des métaux lourds, dont la toxicité est avérée chez de nombreux organismes. Pourtant, leurs émissions mondiales résultant des activités humaines ont élevé leurs concentrations bien au-dessus des niveaux naturels dans l'air, le sol, l'eau et la flore, exposant ainsi les abeilles à tous les stades de leur vie. Le but de ma thèse était d'examiner les effets de la pollution métallique sur l'abeille domestique en utilisant une approche multi-échelle, du cerveau à la colonie, en laboratoire et sur le terrain. J'ai d'abord observé que les abeilles exposées à une gamme de concentrations de trois métaux communs (arsenic, plomb et zinc) en laboratoire étaient incapables de percevoir et éviter des concentrations usuelles, néanmoins nocives, de ces métaux dans leur nourriture. J'ai ensuite exposé de façon chronique des colonies à des concentrations réalistes de plomb dans la nourriture et démontré que la consommation de ce métal altérait la cognition et le développement morphologique des abeilles. Comme les polluants métalliques se trouvent souvent dans des mélanges complexes dans l'environnement, j'ai exploré l'effet des cocktails de métaux, montrant que l'exposition au plomb, à l'arsenic ou au cuivre seul était suffisante pour ralentir l'apprentissage et perturber le rappel de la mémoire, et que les combinaisons de ces métaux induisaient des effets négatifs additifs sur ces deux processus cognitifs. J'ai finalement étudié l'impact de l'exposition naturelle aux polluants métalliques dans un environnement contaminé, en collectant des abeilles à proximité d'une ancienne mine d'or, et montré que les individus des populations les plus exposées aux métaux présentaient des capacités d'apprentissage et de mémoire plus faibles, et des altérations de leur développement conduisant à une réduction de la taille de leur cerveau. Une analyse plus systématique des abeilles non exposées a révélé une relation entre la taille de la tête, la morphométrie du cerveau et les performances d'apprentissage dans différentes tâches comportementales, suggérant que l'exposition aux polluants métalliques amplifie ces variations naturelles. Ainsi, mes résultats suggèrent que les abeilles domestiques sont incapables d'éviter l'exposition à des concentrations réalistes de métaux qui sont préjudiciables au développement et aux fonctions cognitives, et appellent à une révision des niveaux environnementaux considérés comme «sûrs». Ma thèse est la première analyse intégrée de l'impact de plusieurs polluants métalliques sur la cognition, la morphologie et l'organisation cérébrale chez l'abeille, et vise à encourager de nouvelles études sur la contribution de la pollution métallique dans le déclin signalé des abeilles, et plus généralement, des insectes.

Mots-clés : métaux lourds, *Apis mellifera*, cognition, comportement, morphométrie

Declaration

I certify that this work is being submitted to Macquarie University and Université Toulouse 3 Paul Sabatier in accordance with the Cotutelle agreement dated 23/07/2019.

To the best of my knowledge and belief, the thesis contains no material previously published or written by another person except where due reference is made in the thesis itself.

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

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

Coline Monchanin (23/04/2021)

Preface

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

Publications included in this thesis:

Coline Monchanin, Jean-Marc Devaud, Andrew B. Barron, Mathieu Lihoreau (2021). **Current permissible levels of heavy metal pollutants harm terrestrial invertebrates.** *Science of the Total Environment,* 779, 146398.

- Included as **Chapter 1** (published version in **Appendix 1**).

CM and ML designed the study. CM collected the data, analysed the data, and wrote the first draft of the manuscript. All authors contributed substantially to revisions.

Coline Monchanin, Maria Gabriela de Brito Sanchez, Loreleï Lecouvreur, Océane Boidard, Grégoire Méry, David Baqué, Jérôme Silvestre, Arnaud Elger, Andrew B. Barron, Mathieu Lihoreau, Jean-Marc Devaud. **Honey bees cannot sense harmful concentrations of metal pollutants in food.** *Under review at Journal of Neurosciences.*

- Included as **Chapter 2**.

CM, AE, ML, JMD designed the study. CM, MGBS, LL, OB, GM, DB, JS collected the data. CM analysed the data and wrote the first draft of the manuscript. All authors contributed substantially to revisions.

Coline Monchanin, Amaury Blanc-Brude, Erwann Drujont, Mohammed Mustafa Negahi, Cristian Pasquaretta, Jérôme Silvestre, David Baqué, Arnaud Elger, Andrew B. Barron, Jean-Marc Devaud, Mathieu Lihoreau (2021). **Chronic exposure to trace lead impairs honey bee learning.** *Ecotoxicology and Environmental Safety*, 212, 112008.

- Included as **Chapter 3** (published version in **Appendix 2**).

CM, ABB, JMD and ML designed the study. CM, ABB, ED, MMN, JS, DB and AE collected the data. CM and CP analysed the data. CM wrote the first draft of the manuscript. All authors contributed substantially to revisions.

Coline Monchanin, Erwann Drujont, Jean-Marc Devaud, Mathieu Lihoreau, Andrew B. Barron (2021). **Metal pollutants have additive negative effects on honey bee cognition.** *Journal of Experimental Biology*, 224, jeb241859.

- Included as **Chapter 4** (published version in **Appendix 3**).

CM and ABB designed the study. CM and ED collected the data. CM analysed the data and wrote the first draft of the manuscript. All authors contributed substantially to revisions.

Coline Monchanin, Erwann Drujont, Jérôme Silvestre, Gaël Le Roux, Arnaud Elger, Philipp Lösel, Andrew B. Barron, Jean-Marc Devaud, Mathieu Lihoreau. **Environmental exposure to arsenic pollution impairs honey bee cognition and brain development.** *Paper in preparation for Ecology Letters.*

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CM and ML designed the study. CM, ED, JS, GLR, PL collected the data. CM analysed the data and wrote the first draft of the manuscript. All authors contributed substantially to revisions.

Coline Monchanin, Tamara Gomez-Moracho, Paul Marchal, Maria Eugenia Villar, Nicolas Doubovetzky, Patrick Schulteiss, Cristian Pasquaretta, Renaud Lebrun, Fabrice Lihoreau, Philipp Lösel, Andrew B. Barron, Jean-Marc Devaud, Mathieu Lihoreau. **Bees with larger heads have better olfactory learning and memory performance**. *Paper in preparation for Current Biology.*

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CM and ML designed the study. CM, TGM, PM, MEV, ND, PS, RL, PL collected the data. CM and CP analysed the data. CM wrote the first draft of the manuscript. All authors contributed substantially to revisions.

Stéphane Kraus, Coline Monchanin, Tamara Gomez-Moracho, Mathieu Lihoreau (2019). **Insect diet**, in: Jennifer Vonk, Shackelford Todd (Eds.), Encyclopedia of animal cognition and behaviour.

- Included as **Appendix 4**.

SK, CM, TGM and ML reviewed the literature. ML wrote the first draft of the manuscript. All authors contributed substantially to revisions.

Mathieu Lihoreau, Thibault Dubois, Tamara Gomez-Moracho, Stéphane Kraus, Coline Monchanin, Christian Pasquaretta (2019). **Putting the ecology back into insect cognition research**, in Russell Jurenka (Eds.), Advances in insect physiology.

- Included as **Appendix 5**.

ML, TD, TGM, SK, CM and CP reviewed the literature. ML wrote the first draft of the manuscript. All authors contributed substantially to revisions.

Théotime Colin, Coline Monchanin, Mathieu Lihoreau, Andrew B. Barron (2020). **Pesticide dosing must be guided by ecological principles**. *Nature Ecology & Evolution*, 4, 1575-1577.

- Included as **Appendix 6**.

TC and CM reviewed the literature. TC wrote the first draft of the manuscript. All authors contributed substantially to revisions.

Philipp D. Lösel*, Coline Monchanin*, Renaud Lebrun, Alejandra Rayme, Andrew B. Barron, Jean-Marc Devaud, Mathieu Lihoreau, Vincent Heuveline. **Large-scale quantitative comparative analysis of honey bees brain volume and organisation using micro-CT scanning techniques and the Biomedisa segmentation platform.** *Paper submitted to Nature Communications* [*Co-first authors]

- Included as **Appendix 7**.

PDL, CM and RL collected the data. PDL and CM wrote the first draft of the manuscript. All authors contributed substantially to revisions.

Amélie Cabirol*, Tamara Gomez-Moracho*, Coline Monchanin*, Cristian Pasquaretta, Mathieu Lihoreau. **Considering phenotypic variance in studies of environmental stressors can reveal potential for population resilience.** *Paper submitted to the Journal of Applied Ecology* [*Co-first authors]

- Included as **Appendix 8**.

AC, TGM and CM reviewed the literature. CM and CP analysed the data. AC, TGM, CM and ML wrote the first draft of the manuscript. All authors contributed substantially to revisions.

Mathieu Lihoreau, Coline Monchanin, Jean-Marc Devaud, Alexandre Dore, Thibault Dubois, Stéphane Kraus, Mathilde Lacombrade, Ana Moran, Tamara Gómez-Moracho. **How bumblebees became model species in apidology: a brief history and perspectives.** *Paper under review at Apidologie.*

- Included as **Appendix 9**.

ML, TD, SK, ML, CM, AM and TGM reviewed the literature and wrote the first draft of the manuscript. All authors contributed substantially to revisions.

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General introduction

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"They are the soul of the summer, […] they are the untiring wing on which delicate perfumes float, […] and their flight is the sure and melodious note, of all the myriad fragile joys that are born in the heat and dwell in the sunshine. […] To him who has known them and loved them, a summer where there are no bees becomes as sad and as empty as one without flowers or birds."

Maurice Maeterlinck, The Life of the Bee.

GENERAL INTRODUCTION

1. Metallic pollution

1.1. Environmental contamination

Metallic trace-elements (MTE)[1] are naturally occurring elements in the environment, characterized by their high atomic weight and a density above 5 $g.cm^{-3}$, their persistence and tendency to bioaccumulate. At low concentrations, some compounds, such as copper (Cu), iron (Fe), manganese (Mn), selenium (Se) and zinc (Zn) are essential micronutrients required for the proper function of biochemical processes in animals and plants (Fraga, 2005; Phipps, 1981). They function as cofactors of enzymes, components of antioxidants proteins, and as free ions in cellular signalling cascades (Hänsch and Mendel, 2009). Other MTE, like cadmium (Cd), lead (Pb), mercury (Hg) and arsenic (As), have no known physiological function and are toxic even in small concentrations (Tchounwou et al., 2012; Wright and Baccarelli, 2007).

MTE pollution has become an increasingly important ecological concern worldwide (Nriagu and Pacyna, 1988). Their widespread uses in domestic, industrial, agricultural, medical and technological applications (Bradl, 2005) have led to their wide distribution in the environment (Fig. 1). In addition to natural sources (volcanic activity, weathering of geological deposits, forest fires etc.), anthropogenic activities (mining and chemical industries, waste incineration, transport etc.) have considerably increased environmental concentrations of MTE far above natural baselines, contaminating air (Suvarapu and Baek, 2017), soils (Su et al., 2014; Wuana and Okieimen, 2011), water (Mance, 1987) and plants (Krämer, 2010), along with the nectar and pollen they produce (Eskov et al., 2015; Gutiérrez et al., 2015). The pattern of MTE contamination depends on the chemical element and is temporally and spatially highly variable. While lead

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[1] The term ‘heavy metals’ has been widely used as a group name for metals and metalloids associated with contamination and potential toxicity. However, this has no chemical or toxicological basis, and the use of this terminology does not seem sensible (Duffus, 2002). However, this term is so widely used that it is hardly possible to eliminate it (Appenroth, 2010). Therefore, as ‘heavy metals’, ‘metallic trace-elements’ and ‘metal pollutants’ terms are commonly accepted (Bánfalvi, 2011), I will use them in this thesis.

contamination has been recently declining in Europe and North America (Chadwick et al., 2011; Kierdorf and Kierdorf, 2004), notably due to the phase-out of leaded gasoline, its concentrations are increasing in Asia, Australia, South America and Antarctica (Li et al., 2012; Marx et al., 2016). Cadmium (Ruiz-Hernandez et al., 2017) and mercury (Pacyna et al., 2009) emissions in Europe and North America have recently decreased. But other studies report increasing emissions of mercury (Driscoll et al., 2013) and arsenic (Han et al., 2003). Nonetheless, even if metal pollution is decreasing in some part of the world, former high emissions of these metals lead to a legacy of pollution which remains a major public health concern (WHO, 2019). Since MTE are persistent for millennia and non-biodegradable in the environment (Demková et al., 2017; McConnell and Edwards, 2008), they accumulate and transfer from one environmental compartment to another (Järup, 2003) and through the food chains (Ali and Khan, 2019). In addition, because they share common emission sources (Vareda et al., 2019), they are often co-occurring in complex hazardous mixtures (Chen et al., 1999; Navas and Machín, 2002).

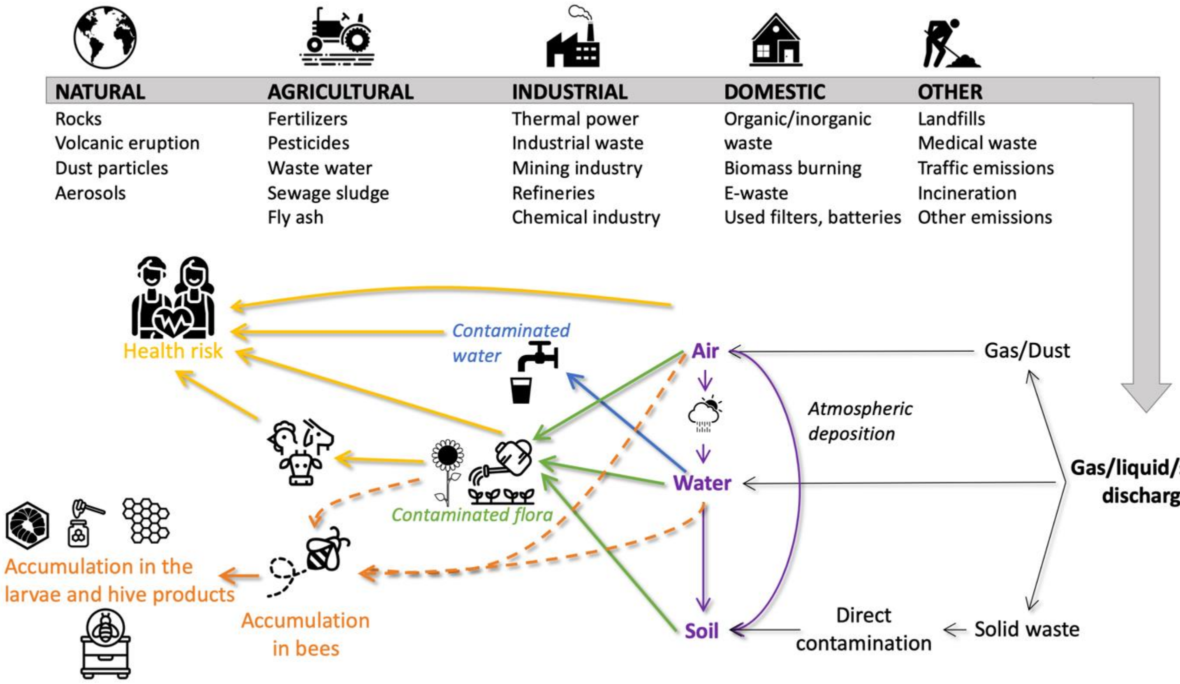

Figure 1: Environmental contamination by metal pollutants. Major anthropogenic sources can be classified into 5 categories: natural, agricultural, industrial, domestic and miscellaneous (Bradl, 2005). MTE pollute the air (as fine particulate matter), water and soil, cycling between environmental compartments (*in purple)*, eventually contaminating plants (*in green*). Humans are exposed through air, soil, dust and via the ingestion of contaminated water of food (*in yellow*). Metals bioaccumulate (*in orange*) in the bodies of bees that are exposed to metal pollution when foraging in the environment, as well as in the hive.

2.2. Toxicology of metal pollutants

Because of the hazards MTE pose to human health, they are ranked at the top of the priority list of substances with the most significant potential threat to human health (e.g. arsenic in 1st, lead in 2nd, mercury in 3rd, cadmium in 7th) (ATSDR, 2019). Their various toxic effects are well documented and are associated with dysfunction and deterioration in multiple organ systems (Järup, 2003; Tchounwou et al., 2012). Toxicity compromises the function and structure of organs directly exposed (e.g. skin, lungs, gastrointestinal tract) or that accumulate metals (e.g. bone, liver, kidney, brain). MTE are associated with cancers (Mishra et al., 2010; Yuan et al., 2016), neurodevelopmental and neurodegenerative diseases like autism spectrum disorder, Alzheimer's and Parkinson's diseases (Grandjean and Landrigan, 2006; Wright and Baccarelli, 2007), along with sublethal effects such as sensory impairments, neuromuscular dysfunction, learning and memory deficits, mood disorders (Neathery and Miller, 1975; Sankhla et al., 2016; Wright and Baccarelli, 2007).

Particularly worrisome is the fact that MTE transferred from abiotic environments to living organisms, are then accumulating in biota at different trophic levels eventually contaminating the whole food chain (Ali and Khan, 2019; Gall et al., 2015), of which human is the apex (Fig. 1). MTE are known to impact a variety of organisms and ecosystems, such as plants (Hagemeyer, 2004), microbes (Hiroki, 1992), invertebrates (Jensen and Trumble, 2003), fishes (Farombi et al., 2007), coral reefs (Al-Rousan et al., 2007), small (Drouhot et al., 2014; Shahsavari et al., 2019) and large (Alonso et al., 2002) terrestrial mammals, marine mammals (Kakuschke and Prange, 2007), etc.

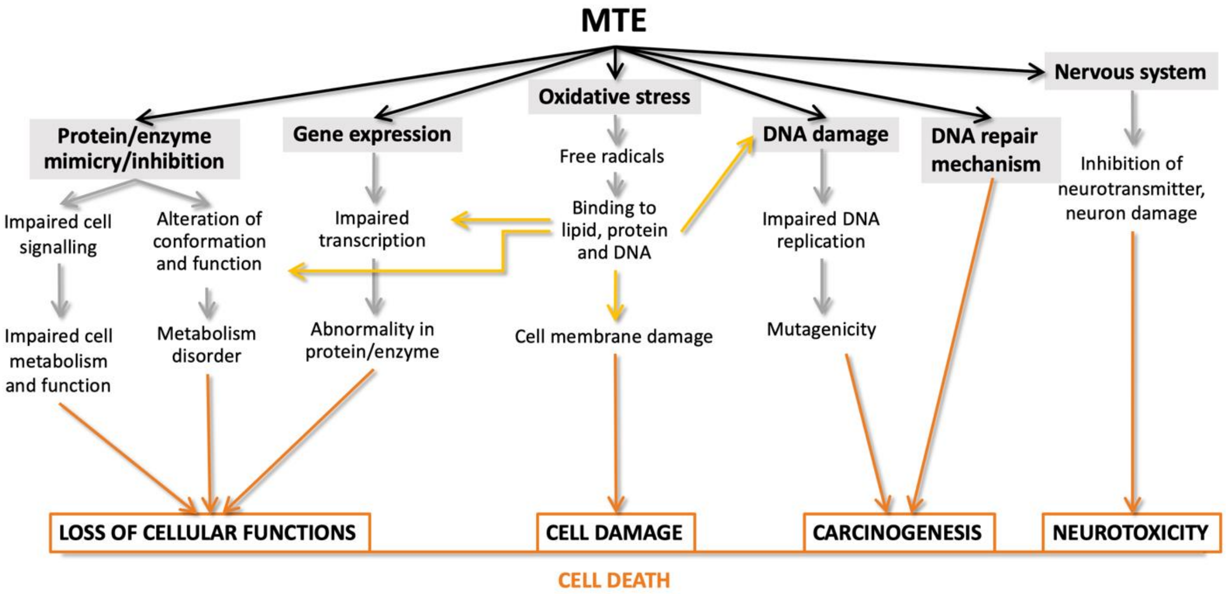

Figure 2: Mechanisms of action of MTE in humans. Some cellular pathways are common to different MTE and lead to DNA damage, oxidative stress, reactive oxygen species generation and apoptosis, resulting in cellular and tissue damages and leading to various adverse effects and diseases.

While many details of the mode of action of MTE toxicity have not yet been fully elucidated, some common physiological mechanisms are known to underlie their toxic effects (Azeh Engwa et al., 2019) (Fig. 2). Firstly, MTE can mimic the physiological role of another metal when there are similarities in the ions' size and charge (Bridges and Zalups, 2005; Clarkson, 1993), hence altering the metabolism. For instance, lead can play the role of calcium ions in calcium-dependent intracellular signalling cascades and cellular processes involved in neurotransmitter release (Gorkhali et al., 2017). Toxic MTE can also interact with or replace the native metal(loid)s in metalloenzymes or metalloproteins, thus inactivating or over-activating the protein (Dudev and Lim, 2014). Secondly, MTE cause oxidative stress, due to the generation of free radicals and peroxides in excess of the body's antioxidant capabilities (Valko et al., 2005). These free radicals damage DNA, proteins, lipids and other molecules, disrupting their structural integrity and impairing their function (Valko et al., 2006). For instance, copper at physiological concentrations is an important antioxidant by being a component of many antioxidant enzymes. But exposure to excessive copper causes oxidative stress, through free radical damage and lipid peroxidation (Gaetke, 2003). Thirdly, MTE may exert carcinogenic effects by causing epigenetic changes, due to their capacity to bind to the DNA and impair DNA repair and methylation (Brocato and Costa, 2013; Senut et al., 2014). For example, lead exposure has been reported to cause abnormal DNA methylation patterns in human embryonic cells (Senut et al., 2014). Eventually, MTE can interfere directly with the nervous system, by interacting with synaptic vesicles, ion channels and the metabolism of neurotransmitters (Marchetti, 2014; Sadiq et al., 2012) and by causing neuronal damages (Chen et al., 2016). Ultimately, all these mechanisms of action can lead to cell apoptosis (Wang and Shi, 2001).

Not only can MTE individually exert toxic effects, but as they are often present in complex mixture, they can also interact with each other (Lin et al., 2016), or with other chemicals present in the environment, such as pesticides (Singh et al., 2017). The risk assessment of combined exposure to multiple stressors has been identify as a current major challenge in the ecotoxicological field (Bopp et al., 2018).

2. The honey bee: an ecologically relevant study model

2.1. Bees provide a crucial ecosystem service but are declining

Pollination by wild animals is a key ecosystem service. By facilitating the sexual reproduction of many crops and wild plants (Klein et al., 2007), animal pollination plays a crucial role in food security and human welfare (van der Sluijs and Vaage, 2016), along with supporting ecosystem diversity (Biesmeijer et al., 2006). In Europe, 84% of crop production relies on pollinators (Williams, 1994), and the worldwide benefit of pollination is estimated to be 361 billion US$ (Lautenbach et al., 2012). Among insect pollinators, bees forage on more than 90% of the major global crops (Klein et al., 2007), rendering honey bees the leading managed pollinator worldwide (Rader et al., 2009).

Increasing evidence points towards a global decline in insect abundance and diversity (Goulson, 2019; Sánchez-Bayo and Wyckhuys, 2019), and bees are not spared (Potts et al., 2010). The health of managed and wild bees has been severely declining in Europe and the United States over the last decades (Ellis et al., 2010; Neumann and Carreck, 2010), and massive unexplained colony losses of domestic honey bees have been reported (vanEngelsdorp et al., 2009). In Europe, the overall number of managed honey bee colonies has increased since 1960, but high mortality rates have also been recorded (vanEngelsdorp and Meixner, 2010). More than half of the European wild bee species are classified as Data Deficient by the IUCN, which nonetheless reports that about 15% of the species are threatened (Nieto et al., 2014).

Multiple drivers of pollinators decline have been identified (Brown et al., 2016). Changes in land-use lead to habitat loss, fragmentation and homogenization (Williams and Osborne, 2009), increased urbanization (Grubisic et al., 2018) and reduced diversity of food resources (Burkle et al., 2013; Dietzsch et al., 2011). The intensive use of agrochemical products (Sánchez-Bayo et al., 2016) is considered a major threat. The diffusion of biological stressors, such as the ectoparasitic mite *Varroa* sp. (Le Conte et al., 2010), the microsporidian parasite *Nosema ceranae* (Fries, 2010), numerous viruses (Ellis and Munn, 2005), or invasive species, like the Asian hornet (Requier, 2019) impair bee health. Climate change impacts geographical ranges (Kerr et al., 2015; Williams et al., 2007), bee species richness (Dormann et al., 2008), potentially leading to the disruption of plant-pollinator interactions (Memmott et al., 2007). Electromagnetic (Shepherd et al., 2018), air (Lusebrink et al., 2015) and night-time light (Gaston et al., 2012) pollutions are also regarded as contributors to the decrease in pollinator biodiversity and biomass.

Not only are pollinators exposed to these manyfold pressures, but they are chronically exposed to many stressors simultaneously (Goulson et al., 2015). In general, the combined effects of multiple stressors are likely to be more harmful than one stressor alone, as each is likely to reduce the ability to cope with the others. Hence, the study of environmental stressors calls for a holistic approach (European Union, 2018), integrating individual and interacting effects, at different scales. For instance, co-exposures to various agrochemicals (Tosi and Nieh, 2019; Zhu et al., 2017), agrochemicals and pathogens (Alaux et al., 2010; Aufauvre et al., 2014), virus (Coulon et al., 2018), metal pollutants (Sgolastra et al., 2018), weather or landscape context

(Henry et al., 2014; Monchanin et al., 2019) or nutritional stress (Tong et al., 2019) constitute detrimental combinations for pollinators (Brown et al., 2016).

Because bees and other central-place foragers rely on precision in their navigational and cognitive abilities to forage, the deleterious effects at the individual level can have dramatic effects on the whole colony or population (Klein et al., 2017). Indeed, bees must gather pollen and nectar in a dispersed and changing environment, and return them to the nest to feed the brood. Accordingly, bees must learn to recognize flowers and orientate, navigate and learn foraging circuits (Lihoreau et al., 2012). Successful and efficient foraging relies on the integration and processing of sensory information across brain networks, and even subtle disturbances of neural function could have dramatic consequences on individual cognitive abilities. Consequently, disruption of key cognitive functions, and hence foraging performance, could ultimately threaten brood development and colony function survival (Klein et al., 2017).

2.2. Learning and memory abilities

The domestic honey bee *Apis mellifera* is an insect belonging to the Hymenopteran order, with a well-defined social organization within the nest. The labour division depends on the age and the reproductive status of the individual. The queen is usually the only reproductive member of the hive and lays thousands of eggs daily in the comb. The drones play no role in the hive activity, apart from reproduction during the mating flight of the newly born queen (Hartfelder and Engels, 1998). The workers, the most common caste within the hive, perform several different tasks, depending on their age (age polyethism). These include cleaning the nest, feeding and taking care of the brood, carrying food, and building combs, guarding the entrance and finally, foraging to bring back pollen, nectar and water to the nest (Calderone, 1998).

The domestic honey bee has been extensively used as an animal model. In addition to being a vital pollinator involved in the maintenance of ecosystem diversity and a good bioindicator species, the honey bee, equipped with a brain smaller than 1 mm^3, displays a rich behavioural repertoire and high-order cognitive capacities (Giurfa, 2007). Hence, it is well suited for behavioural studies allowing us to uncover the neural substrates of such complex behaviours and cognitive processes (Giurfa, 2013).

When foraging, honey bees are exposed to a stream of sensory information. They navigate over large distances to locate pollen and nectar sources and communicate those food locations to their nest mates (Farina et al., 2005; Grüter et al., 2006). This lifestyle makes honey bees suitable model organisms for studying the principles of learning, memory and navigation (Pahl et al., 2010).

Since the pioneering work of Karl von Frisch (von Frisch, 1967), a variety of conditioning protocols have been established, based on the acquisition of information regarding visual, olfactory or tactile stimuli (Scheiner et al., 2013). In the laboratory, the most widely used assay is appetitive olfactory conditioning of the proboscis extension response (PER) (Bitterman et al., 1983; Takeda, 1961), which is based on Pavlovian conditioning. In this task, harnessed bees are trained to associate an odorant (the conditioned stimulus) with a sucrose reward (the unconditioned stimulus). The behavioural response is the extension of the proboscis, with which the animal licks and draws nectar from flowers. Proboscis extension is triggered as a reflex by the stimulation of antennae with sugar. Honey bees quickly learn the association between the odorant and the paired sucrose presentation and end up responding to the odorant alone (Matsumoto et al., 2012).

Conditioning protocols in the lab enable the exploration of various levels of behavioural complexity (Giurfa, 2003). Simple learning protocols provide a non-ambiguous relationship between stimuli in training, such as absolute conditioning (where a single stimulus A is reinforced: A+) or differential learning (where one stimulus A is reinforced while another one, B, is not: A+ *vs.* B-). Reversal learning is considered an ambiguous task, in which the initial contingency learned through differential conditioning (A+ *vs.* B-) is reversed in a second learning phase (A- *vs.* B+). Achieving this task involves cognitive flexibility in order to override the response pattern established by the first trained contingency.

The neural pathways underlying Pavlovian learning in honey bees have been extensively studied (Fig. 3) (Giurfa and Sandoz, 2012). Olfactory information is detected by the olfactory sensory neurons located in the sensillae of the antennae, which project to the antennal lobes (AL, the primary olfactory centres (Hansson and Anton, 2000)). The information is then transmitted to the projection neurons which will send it to higher brain centres, such as the lateral horns (LH) and the mushroom bodies (MB). The latter are involved in the processing of multimodal (visual gustatory, mechanosensory) information (Fahrbach, 2006; Hammer and Menzel, 1995), and have been identified as being specifically required for the resolution of ambiguous learning tasks such as reversal learning (Boitard et al., 2015; Devaud et al., 2007). In addition, MB are also involved in memory formation (Lozano et al., 2001). The gustatory pathway relies on gustatory receptor neurons, contained within the gustatory sensillae, located on the antennae, mouthparts and tarsi (de Brito Sanchez, 2011). The antennal gustatory receptor neurons project to the subesophaegeal ganglion (SEG). Within the SEG, the ventral unpaired median maxillar 1 (VUM-mx1) neuron conveys the information through a wide arborization innerving the AL, MB and LH (Hammer, 1993).

Honey bees have evolved highly refined cognitive abilities and an optimized brain enabling them to efficiently forage and exploit complex and changing environments. Environmental stressors can, among other things, alter the proper function of various systems in the brain and disrupt the neural pathways supporting learning, memory and navigation. Amidst environmental stressors, the impact of metallic pollution, and its interaction with other stressors (e.g. agrochemicals (Singh et al., 2017)), on pollinators remains largely unexplored.

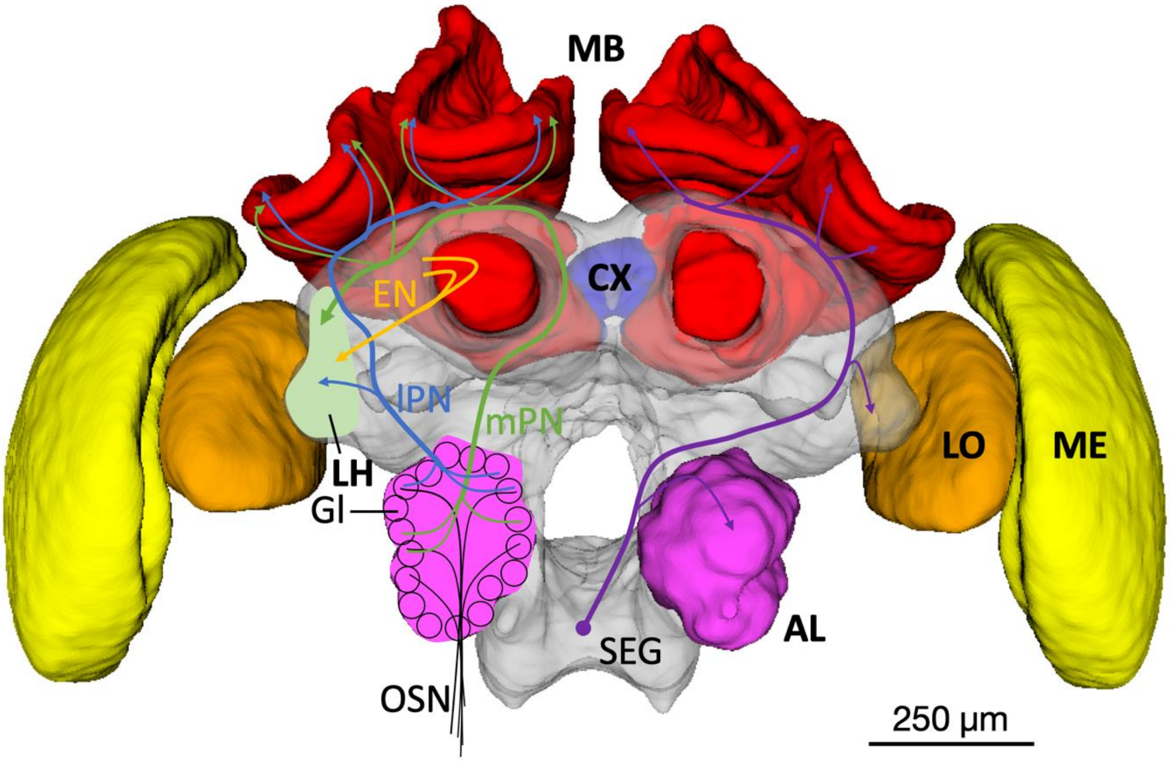

Figure 3: Neural pathways for olfactory and gustatory information in the honey bee brain (adapted from Giurfa and Sandoz (2012)). The honey bee brain comprises five main neuropilar regions: the antennal lobes (AL) (*in pink*), the mushroom bodies (MB) (*in red*), the medullas (ME) (*in yellow*) and lobulas (LO) (*in orange*), and the central complex (CX) (*in blue*). The AL receive input from olfactory sensory neurons (OSN) detecting odorants within sensillae from the antennae and convey processed olfactory information to higher brain through centres projection neurons (PN), initiating within the glomeruli (Gl). The medial tract (mPN) (*green arrow*) first projects to the MB and then to the lateral horn (LH). The lateral tract (lPN) (*blue arrow*) projects, in the reverse order, to the same structures. Extrinsic neurons (EN) (*orange arrow*) take information from the MB and project to the LH. Gustatory sensory neurons (located within the gustatory sensillae of the antennae, tarsi, and mouthparts) detect gustatory information. They projected to the subesophaegeal ganglion (SEG) and then to different regions of the brain (LH, AL, MB) (*purple arrow*). Note that not all neural pathways are shown.

2.3. Honey bee and metallic pollution

Honey bees are exposed to MTE pollutants when foraging (Fig. 1). They can collect contaminated pollen, nectar (Perugini et al., 2011; Xun et al., 2018) or water (Li et al., 2020), or gather particles while flying (Negri et al., 2015; Thimmegowda et al., 2020). These compounds accumulate in the bee’s bodies (Goretti et al., 2020) (Fig. 4A), are transferred to the larvae (Balestra et al., 1992; Exley et al., 2015), and eventually contaminate the hive products, such as honey (Satta et al.,

2012) (Fig. 4B), wax (Tlak Gajger et al., 2016) and propolis (Roman et al., 2011). Hence, honey bees and their products are considered one the most versatile and efficient bioindicators for many environmental pollutants, and metals are no exception (Cozmuta et al., 2012; Zhou et al., 2018).

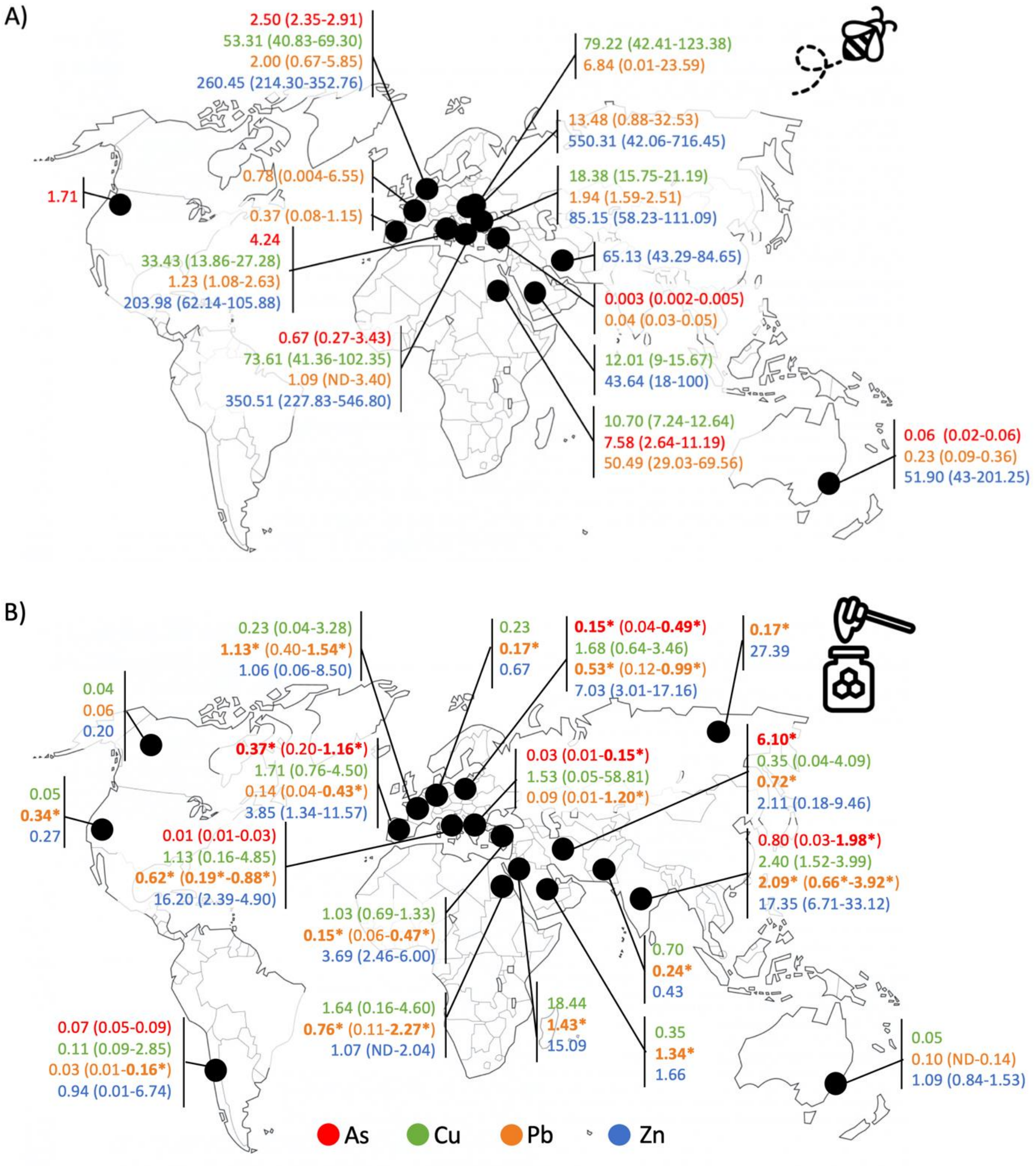

Figure 4: Concentrations of MTE in honey bees and honey worldwide. A) Mean (minimal-maximal, when available) concentrations of arsenic (*red*), copper (*green*), lead (*orange*), zinc (*blue*) in honeybee samples (mg.kg^{-1}). ND: not detected. Values retrieved from: Australia (Zhou et al., 2018); Bulgaria (Zhelyazkova, 2012); Czech republic (Veleminsky et al., 1990); Egypt: (Naggar et al., 2013); France (Lambert et al., 2012); Italy (Conti and Botrè, 2001; Giglio et al., 2017; Goretti et al., 2020; Leita et al., 1996; Perugini et al., 2011; Salvaggio et al., 2017; Satta et al., 2012); Moldova (Eremia et al., 2010); The Netherlands (van der Steen et al., 2012); Poland

(Roman, 2010); Romania (Cozmuta et al., 2012); Saudi Arabia (Taha et al., 2017); Serbia (Krunić et al., 1989; Zarić et al., 2016); Spain (Gutiérrez et al., 2015); Turkey (Matin et al., 2016); USA (Fisher, 1984). **B) Mean (minimal-maximal, when available) concentrations of arsenic (r*ed*), copper (*green*), lead (*orange*), zinc (*blue*) in honey samples (mg.L^{-1}). Asterisks (*) indicate concentrations of arsenic or lead above the maximal level authorized in food (0.14 mg.L^{-1}) (Codex Alimentarius, 2015)**. ND: not detected. Values retrieved from: Australia (Bibi et al., 2008; Zhou et al., 2018); Canada (Bibi et al., 2008); Chile (Bastías et al., 2013; Fredes and Montenegro, 2006); Croatia (Bilandžić et al., 2011); Egypt (Naggar et al., 2013); France (Devillers et al., 2002); Germany (Bibi et al., 2008); India (Aggarwal, 2017; Buldini et al., 2001; Chandrama et al., 2014); Iran (Aghamirlou et al., 2015; Samimi et al., 2001); Italy (Buldini et al., 2001; Frazzoli et al., 2007; Leita et al., 1996; Pisani et al., 2008; Satta et al., 2012); Jordan (Atrouse et al., 2004); Pakistan (Bibi et al., 2008); Poland (Formicki et al., 2013; Przybylowski and Wilczynska, 2001; Roman et al., 2011); Russia (Eskov et al., 2015); Saudi Arabia (Al-Khalifa and Al-Arify, 1999; Bibi et al., 2008); Spain (Bratu and Georgescu, 2005; Frías et al., 2008; González-Miret et al., 2005; Herrero-Latorre et al., 2017; Terrab et al., 2005, 2004); Turkey (Leblebici and Aksoy, 2008; Silici et al., 2016; Tuzen et al., 2007; Uren et al., 1998; Yarsan et al., 2007); USA (Bibi et al., 2008).

While agrochemicals have been identified as a major contributor to the widespread pollinator decline and have received a lot of attention over the last decades, MTE have been largely overlooked, and there is currently a paucity of information concerning their effects on bees (Fig. 5). Copper, cadmium, lead (Di et al., 2016), selenium (Hladun et al., 2013) and arsenic (Fujii, 1980) induce larval and adult mortality. Exposure to cadmium, copper or lead led to significant changes of gene expression, enzyme activity, and redox status, and those effects are metal and dose dependent (Nikolić et al., 2019, 2016). Cadmium was found to reduce the immunocompetence of bees (Polykretis et al., 2016), and selenium to affect the bee microbiome (Rothman et al., 2019) and to induce oxidative stress (Alburaki et al., 2019). Honey bees also seem to possess a system of detoxification of metals, involving metallothioneins (Salvaggio et al., 2017), which are induced following metal exposure in controlled conditions (Gauthier et al., 2016) or with the degree of anthropogenic pollution of the environment (Badiou-Bénéteau et al., 2013).

In addition to physiological effects, behavioural disruptions have also been reported. Copper, lead and cadmium can modify the bee’s feeding behaviour (Burden et al., 2019). Copper is rejected by the bees’ antennae but is readily consumed, while cadmium is rejected by both bees’ antennae and proboscis. Lead on the other hand appears to be detected at some concentrations only. Honey bees seem able to regulate their intake of certain MTE, be it a key nutrient (e.g. potassium, calcium etc.) or a nutrient at low concentration only (e.g. copper, zinc etc.) (Teixeira De Sousa, 2019). Chronic exposure to aluminium disrupts bees' motility and circadian rhythmicity (Chicas-Mosier et al., 2019). Foraging behaviour alterations have been reported following exposure to MTE. Manganese ingestion induces a precocious foraging activity and alters brain biogenic amine levels (Søvik et al., 2015). An acute exposure to aluminium is sufficient to affect the floral choices of honey bees, potentially by altering sucrose perception, increasing activity level or reducing the likelihood of foraging on safer resources (Chicas-Mosier et al., 2017). The presence of nickel in plant nectar discourages bumblebees from visiting flower, while aluminium in nectar does not influence foraging patterns (Meindl and Ashman, 2013). Bees' flower visitation rate is not affected by soil lead contamination, and bees seem unable to distinguish between flowers grown in lead-contaminated soil, or not (Sivakoff and Gardiner, 2017). By altering the foraging behaviour of pollinators such as bees, MTE in nectar can eventually impact the plant fitness (Xun et al., 2018).

While the neurotoxicity of MTE is well established in mammals, only one study investigated the impact on bee cognition (Fig. 5). Acute exposure to selenium disrupts learning and long-term memory performance of honey bees (Burden et al., 2016). These impacts at the individual level are reflected at the colony scale, with decreased brood production and honey yield following controlled exposure to cadmium, copper, lead or selenium (Hladun et al., 2016), or natural exposure to arsenic and cadmium in a polluted area (Bromenshenk et al., 1991).

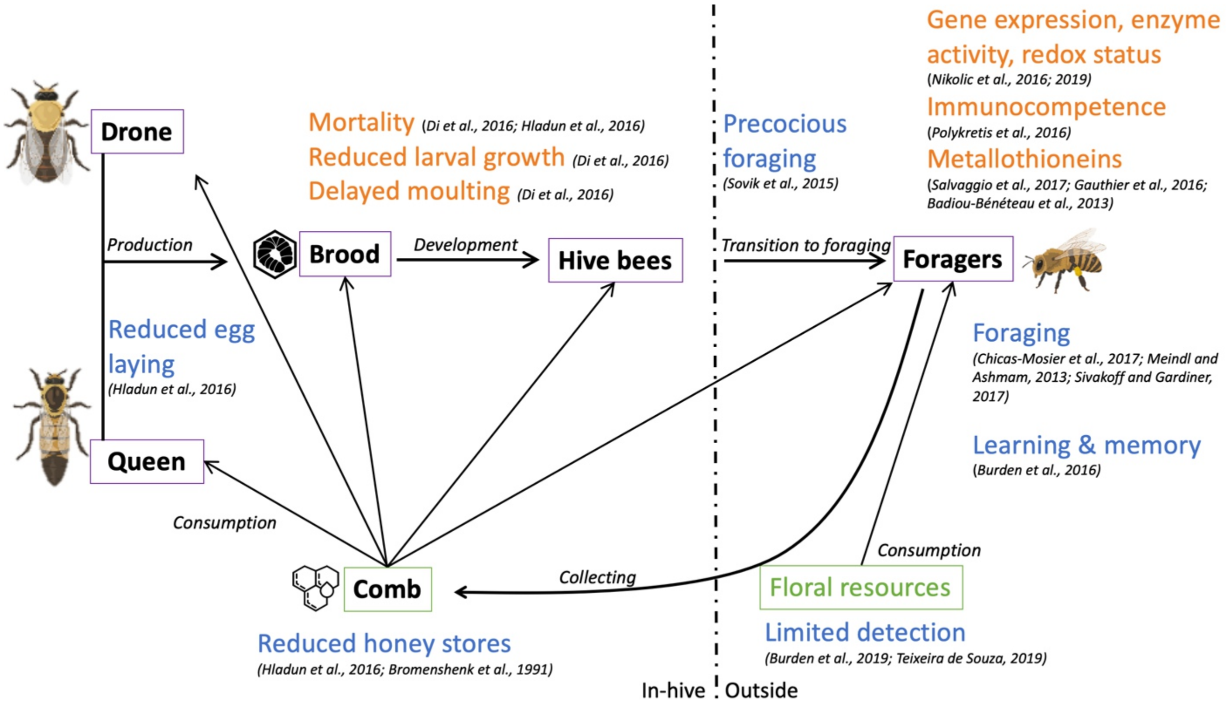

Figure 5: Effects of MTE on honey bee physiology, behaviour and colony dynamics. The brood (eggs, larvae and pupae) develops into in-hive bees that later start foraging. Foragers gather nectar and pollen from floral resources for storage in the hive comb. The food stores are then consumed by the queen, the drones, the larvae and the adult workers. Bees can be exposed to MTE at different life stages (*in purple*), by consuming contaminated resources (*in green*), potentially disrupting the whole colony dynamics. MTE accumulate in all castes (*in purple*) and hive products, both can be used as biomonitors of the environmental quality. MTE are known to induce mortality and impact the bee physiology and development (*in orange*). Behavioural alterations (*in blue*) are also reported: MTE reduce brood production, induce precocious foraging, affect the cognitive functions and reduce the food gathering. Adapted from (Klein et al. 2017).

Several gaps in knowledge can be identified from the current available literature. Firstly, MTE represent a wide range of chemical elements that are not equally studied. Secondly, there is a need for more studies on the sublethal effects leading to long-term impacts on the population. The paucity of information regarding behavioural and cognitive effects on bees is striking when considering the well-known neurotoxic effects of MTE on many organisms. Finally, the study of the combined effects of MTE has been largely overlooked. To our knowledge, only two studies addressed this question (Di et al., 2020; Nisbet et al., 2018), and the ecologically-relevant issue of cocktails of MTE needs to be tackled.

Thesis prospectus

This thesis aimed to study the sublethal effects of metallic pollution on honey bees. I examined the individual cognitive abilities and colonial behaviour of bees contaminated with various MTE using a combination of laboratory experiments and field observations (Fig. 6). I developed an integrative approach relying on multiscale studies using the domestic honey bee as a model organism. I also studied different MTE, that are considered required trace elements for the metabolism when at low concentrations (e.g. zinc, copper), or are toxic even at low concentrations (e.g. arsenic, lead). The thesis is organised in six chapters.

In **Chapter 1** (**Appendix 1**), I argued, on the basis of a review of the scientific literature, that metal pollutants, related to industrial activities, are currently overlooked but widespread invertebrate stressors. I provided evidence of their harmful effects on a diversity of terrestrial invertebrates, and in particular on species with key ecological functions such as pollinators. Most worryingly, I showed that many such species are negatively impacted by metallic pollutants at levels below those considered safe for humans, and thus challenge our current understanding of ‘safe’ levels of metal contamination.

In **Chapter 2**, I explored whether bees were able to perceive MTE (i.e. arsenic, lead and zinc) in food and if they can use such information to avoid exposure. I present behavioural observations coupled with electrophysiological recordings in an attempt to evaluate the hazard MTE poses to foraging honey bees in contaminated environments, which seem unable to detect low, yet harmful concentrations.

In **Chapter 3** (**Appendix 2**), I exposed hives to two field-realistic concentrations of lead in food for ten weeks. The consumption of this single metal impaired bee cognition and morphological development. In particular, foragers developed smaller heads, which have may constrained their cognitive functions as I showed a general relationship between head size and learning performance.

In **Chapter 4** (**Appendix 3**), I tackled the ecologically relevant issue of MTE cocktails. MTE often co-occur in complex mixtures, but how they might act in combination has received very little attention. Here, I showed that field-realistic concentrations of lead, copper and arsenic slowed down appetitive learning and disrupted memory retrieval. Combinations of metals induced additive negative effects on these cognitive processes. These results highlight the need to further characterize the toxicity of metallic mixtures.

In **Chapter 5**, I conducted a field study in the vicinity of a former gold mine highly polluted with MTE, especially arsenic. I combined behavioural data, morphological measurements and quantitative analysis of the volume of different brain compartments to assess the impact of chronic MTE exposure in natural conditions. I showed that environmental exposure to MTE disrupted learning and memory retrieval and that bees closer to the pollution source developed smaller heads and smaller brains.

In **Chapter 6**, I investigated the relationship between head/brain morphometrics and learning/memory performances in tasks involving different contexts (appetitive, aversive) and modalities (olfactory, visual).

By studying how MTE impact on individual behaviour and morphological development, and by unravelling a relationship between morphology and cognitive performances, this work helps us to better understand the consequences of metallic pollution for pollinator insects.

In **Appendices 4 to 9**, I present six papers, for which I was not the instigator or intellectual leader: in **Appendix 4**, a book chapter reviewing the basis of insect nutrition; in **Appendix 5**, a book chapter highlighting the need to reconsider insect cognition into an ecological context; in **Appendix 6**, a review paper on the rethinking of insecticides doses guided by ecological principles; in **Appendix 7**, a technical paper on the large-scale quantitative analysis of bee brain data; in **Appendix 8**, a review paper on the effects of environmental stressors on bee behavioural variance; in **Appendix 9**, a review paper on bumblebees as a model species in apidology.

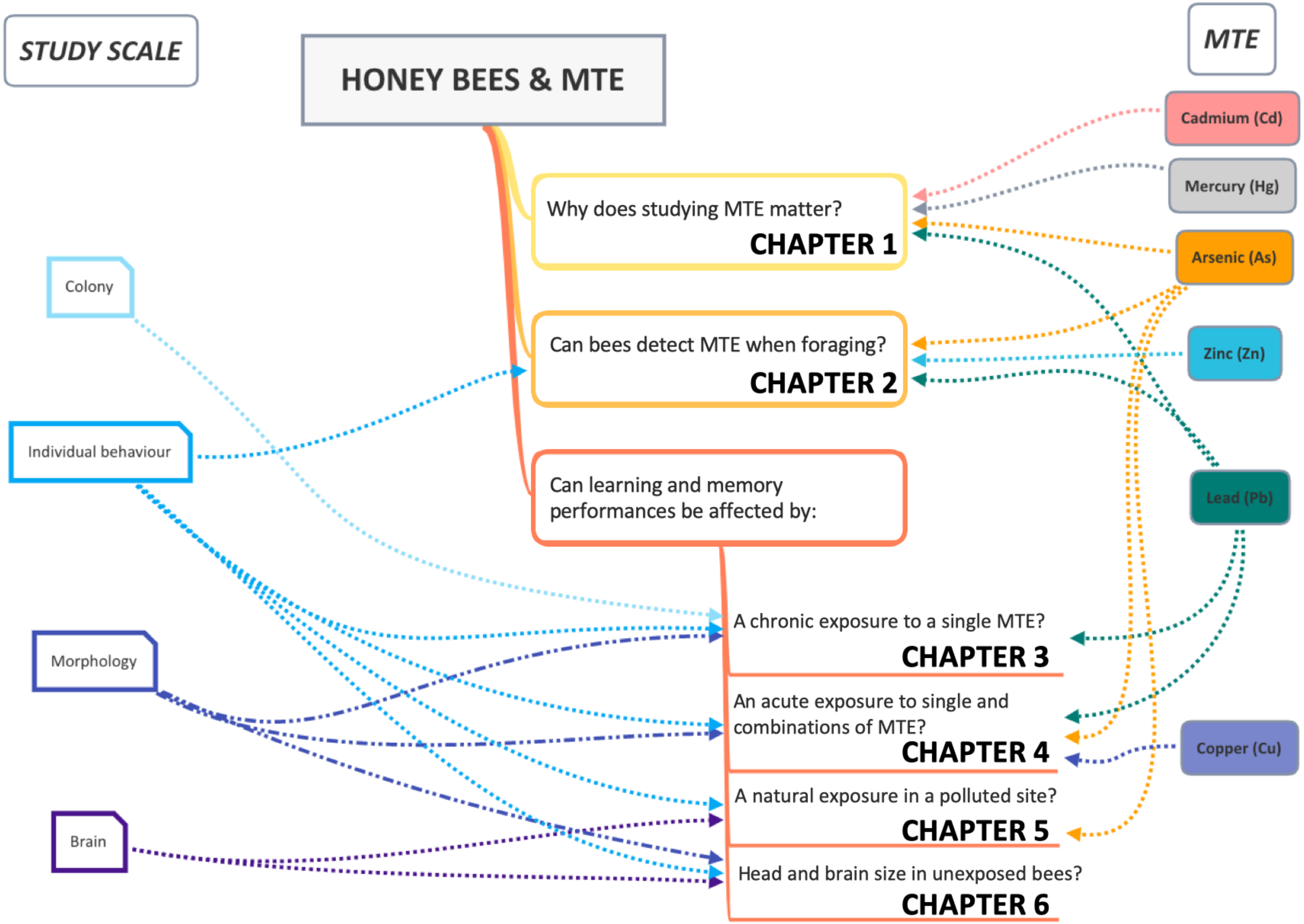

Figure 6: Synthesis of the thesis prospectus. I use the domestic honey bee as a model organism to study the impacts of several MTE at different scales.

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Chapter 1

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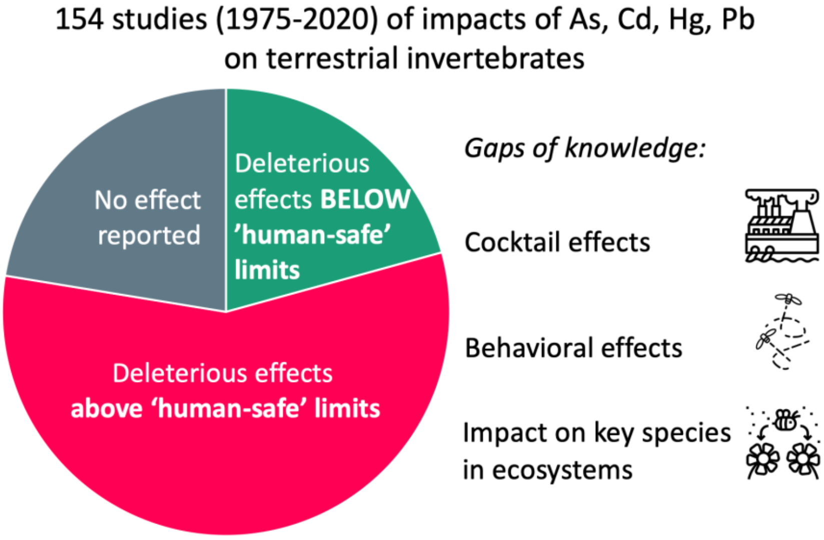
Current permissible levels of metal pollutants harm terrestrial invertebrates

Highlights:

- The current decline of invertebrates worldwide is alarming.
- Major pollutants, like metallic trace-elements in the air, soils and water, are a potential cause, so far overlooked.
- We reviewed the scientific literature on the effects of As, Cd, Pb and Hg on terrestrial invertebrates.
- These well-studied pollutants impact invertebrates even at levels below those recommended as ‘safe’ for humans.
- Our results call for a revision of the regulatory thresholds to protect terrestrial biodiversity.



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CHAPTER 1: Current permissible levels of metal pollutants harm terrestrial invertebrates

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Abstract

The current decline of invertebrates worldwide is alarming. Several potential causes have been proposed but metal pollutants, while being widespread in the air, soils and water, have so far been largely overlooked. Here, we reviewed the results of 527 observations of the effects of arsenic, cadmium, lead and mercury on terrestrial invertebrates. These four well-studied metals are considered as priorities for public health and for which international regulatory guidelines exist. We found that they all significantly impact the physiology and behavior of invertebrates, even at levels below those recommended as ‘safe’ for humans. Our results call for a revision of the regulatory thresholds to better protect terrestrial invertebrates, which appear to be more sensitive to metal pollution than vertebrates. More fundamental research on a broader range of both compounds and species is needed to improve international guidelines for metal pollutants, and to develop conservation plans to protect invertebrates and ecosystem services.

Keywords: heavy metals, metalloids, invertebrate decline, international guidelines, environmental pollution

1. Introduction

Terrestrial invertebrate bioabundance and biodiversity are declining (Wagner, 2020). Since invertebrates are basal to terrestrial food webs and provide key ecosystem services, the short-term ecological consequences of invertebrate decline could be very severe (Goulson, 2019; Sánchez-Bayo and Wyckhuys, 2019). The rate of decline is especially alarming as it has been estimated that land-dwelling insects abundance has been declining at a rate of ca. 1% every year for a century (van Klink et al., 2020). Many factors have been proposed to explain this loss. These include climate change (Wilson et al., 2007), habitat reduction due to intensive agriculture and urbanization (Dudley and Alexander, 2019; Fattorini, 2011), introduced pathogens, predators and competitors (Goulson et al., 2015), as well as chronic exposure to agrochemicals (van Lexmond et al., 2015).

Here we argue that metallic pollution is a major, yet currently overlooked, stressor of insects and other terrestrial invertebrates that needs urgent attention from scientists and stakeholders. At trace levels, metals such as cobalt, copper, iron, manganese, selenium and zinc are essential micronutrients for animals and plants (Phipps, 1981; WHO/FAO/IAEA, 1996). Others, such as cadmium, chromium, mercury, lead and nickel, have no useful biological function and exert toxic effects even at low concentrations (He et al., 2005; Tchounwou et al., 2012). This is also the case for the metalloid arsenic, which we here also refer to as a metal pollutant for the sake of simplicity. While all of them are naturally present in the Earth’s crust, their environmental concentrations have considerably increased above natural baselines (Zhou et al., 2018), due to mining and smelting operations, combustion of fossil fuels, industrial production, domestic and agricultural use of metals and metal-containing compounds (Bradl, 2005). This elevated and widespread contamination of air (Suvarapu and Baek, 2017), soils (Wuana and Okieimen, 2011), water (Mance, 1987) and plants (Krämer, 2010) has generated major public health concerns.

There are many detrimental impacts of metal pollutants on vertebrates, which include cellular damage, carcinogenesis and neurotoxicity (Chen et al., 2016; Tchounwou et al., 2012). Many local initiatives exist to reduce their emissions (e.g. lead: (Chadwick et al., 2011), cadmium:

(Hayat et al., 2019), mercury: (Pacyna et al., 2009)). Even so, environmental metallic pollution is still high (Järup, 2003), calling for a more systematic assessment on the impact on biodiversity. For example, in 2019 the World Health Organization (WHO) stated that there was no safe level of lead for vertebrates (WHO, 2019), yet the majority of industrial activities are increasing the level of lead in the environment (Järup, 2003; Li et al., 2014). The recent report that bees and flies in densely urbanized areas suffer from exposure to metallic air particles (Thimmegowda et al., 2020) suggests that the consequences of metallic pollution on terrestrial invertebrates could be extremely important and widespread (for a review on aquatic invertebrates see (Rainbow, 2002)).

Here, we assessed the impact of metal pollutants on terrestrial invertebrates through a review of the scientific literature on four well-studied metals over the past 45 years. We found that these metals have detrimental effects on a wide diversity of species at levels below those considered safe for humans. We discuss the need for more fundamental research into the impacts of metal pollutants on insects to improve international guidelines for the regulation of metal pollutants, and better inform conservation plans.

2. Results

2.1. Few studies focus on species delivering important ecological function

The 527 observations extracted from the literature covered 100 species (83% Arthropoda, 15% Annelida, 1.2% Rotifera, 0.4% Tardigrada, 0.2% Mollusca; Fig. 1B). Studies were biased toward pest species with an economic impact (34% of observations; e.g. the gypsy moth *Limantria dispar,* the grasshopper *Aiolopus thalassinus,* the beet armyworm *Spodoptera exigua*) and model species in biology (10%; e.g. fruit fly *Drosophila melanogaster,* large milkweed bug *Oncopeltus fasciatus*). Other groups were comparatively under-represented, including important bioindicator species, such as decomposers (15%; e.g. *Lumbricus terrestris, Eisenia fetida* and *E. andre),* predators (10%; e.g. ants *Formica* spp., spiders *Araneus* spp. and *Pardosa* spp.) and pollinators (13%; e.g. the honey bee *Apis mellifera*). Some taxonomic orders that include large numbers of species involved in nutrient cycling (e.g. proturans, diplurans, earwigs), soil aeration (e.g.

centipedes), or pollination (e.g. thrips) were not represented at all. Research is thus needed on these important invertebrate orders with key ecological functions to get a more accurate picture of how metallic pollution disturbs ecosystems (Skaldina and Sorvari, 2019).

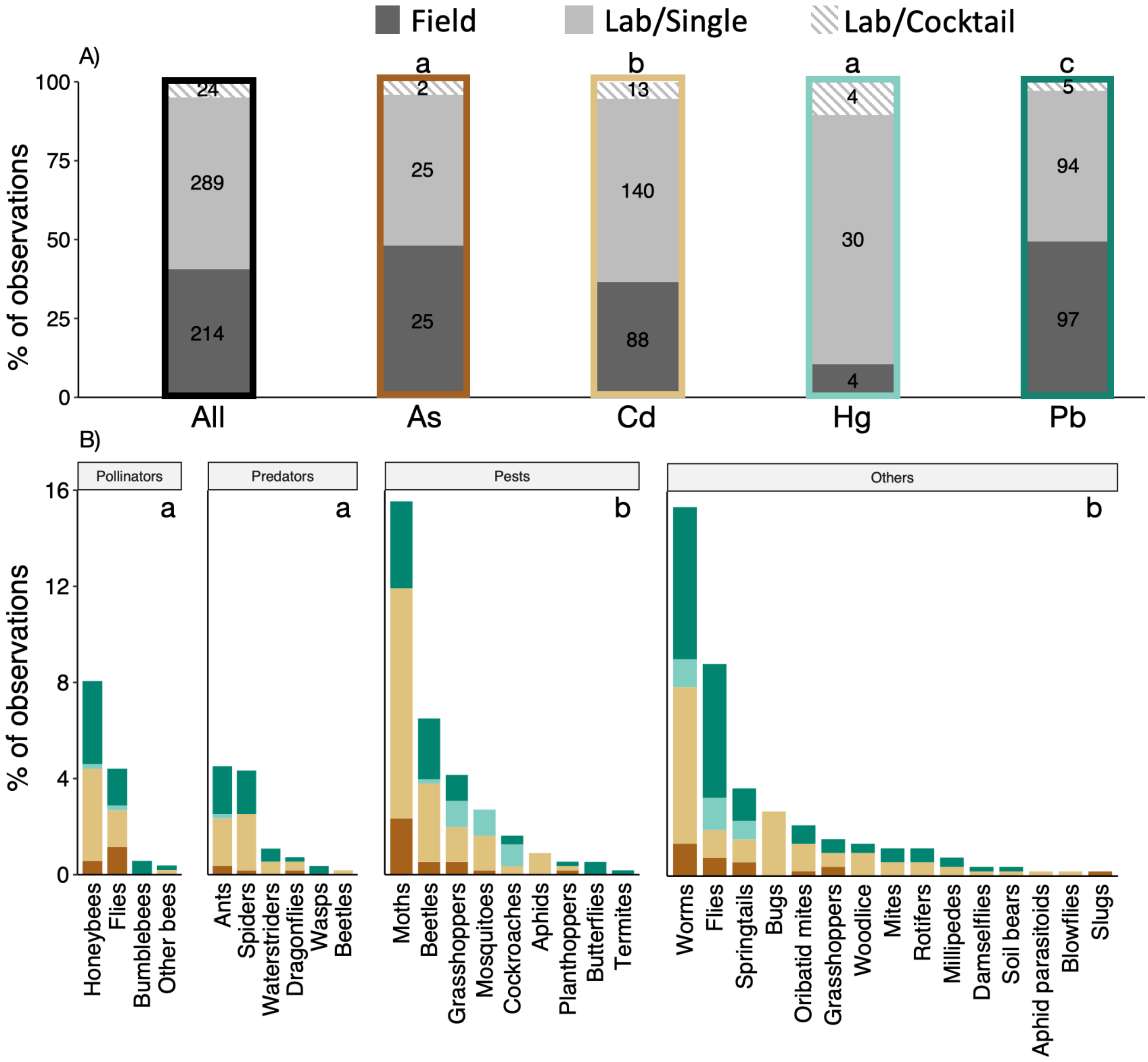

Figure 1: Summary of invertebrate and experimental diversity in the surveyed literature. A) Percentage of observations conducted in the field (dark grey) or in the lab (light grey) per metal pollutant. Observations with mixtures of pollutants in the lab are displayed in textured light grey. Numbers of observations are shown in bars. Letters show statistical significance from chi-square test of homogeneity of proportions of observations per metal pollutant (Chi^2=315.88, df=3, $p<0.001$). **B)** Diversity of invertebrate groups classified by broad categories according to their ecological function and economic importance (based on (Skaldina and Sorvari, 2019)).

Observations with different metal pollutants are marked using the same color code as Table 1 (As: brown, Cd: beige, Hg: light green, Pb: dark green). Letters show statistical significance from chi-square test of homogeneity of proportions of observations per functional group (Chi^2=180.83, df=3, p<0.001).

2.2. Metal pollutants have detrimental effects below permissible limits

Deleterious effects were reported in 84% of the laboratory observations (N=263 out of 313) and 49% of the field observations (N=104 out of 214), thus representing an average of 70% (N=367 out of a total of 527; Fig. 2A). These negative effects were observed following chronic (69%) or acute (79%) exposure (resp. N=348 out of 503 and 19 out of 24).

We then compared the doses at which these effects were observed to international permissible limits (i.e. recommended maximum concentrations) based on human toxicity data and determined by the World Health Organization (WHO) and the Food and Agriculture Organization (FAO) of the United Nations (see Methods). These toxic levels were determined for food, but also water and soils to which arthropods are in direct contact.

When considering only the observations reporting deleterious effects (N=367), 73% of these effects (N=269) were measured at concentrations above the maximal estimated permissible limit (see Table 1). Yet, 12% (N=45) were measured in between the regulatory thresholds and 15% (N=53) below the minimal estimated limit (Fig. 2A). In addition, a majority (57%, N=53 observations out of 93) of the observations using at least one concentration below the minimal estimated permissible limit found a negative effect at that low level, irrespective of the metal.

When considering only the laboratory studies, in which exposure concentrations were controlled (Fig. 2B-C), only 32% of the studies (N=98 out of 313) used at least one concentration below or in between permissible limits. 57% of the studies that examined levels below the maximal permissible limits (N=56 observations out of 98) reported deleterious effects on invertebrates below the permissible limits. Of the laboratory studies investigating acute exposure below the maximal permissible limits (N=16), ten found deleterious effects (Fig. 2B). Hence, acute exposure, while presumably rare in nature, can have deleterious effects on invertebrates below current permissible exposure levels. This suggests that the permissible limits designed for humans are not appropriate for terrestrial invertebrates, who seem to be more sensitive to metal pollutants.

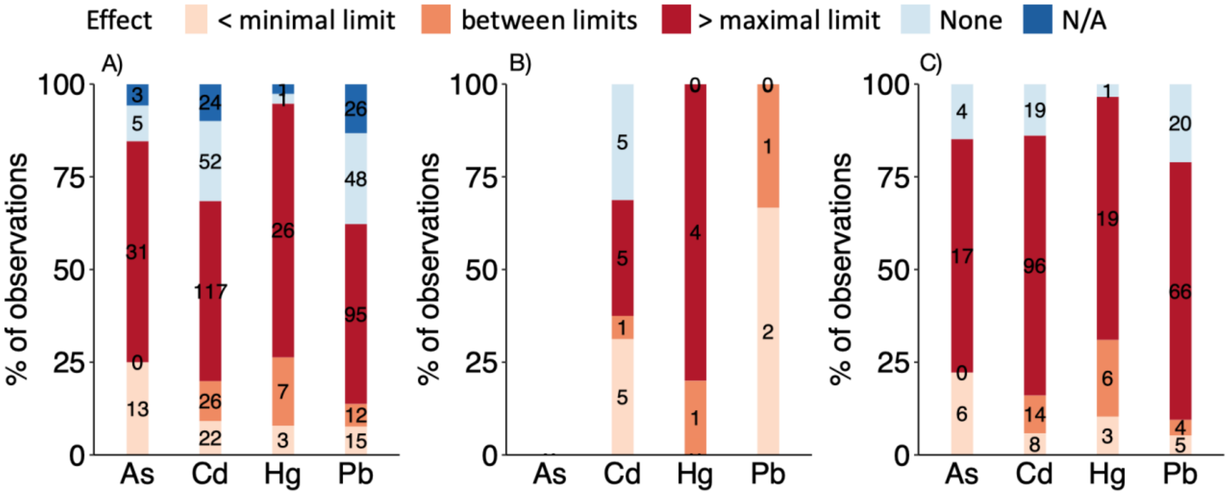

Figure 2: **Effects observed according to permissible limits**. We defined the following ranges below the minimal estimated limit, between the minimal and the maximal estimated limits, or above the maximal estimated limit. **A)** All studies (N=527). **B)** Laboratory studies with acute exposure (N=24) and **C)** chronic exposure (N=288). None: no observable effect, N/A: no conclusion available. Sample sizes are in black. Concentration ranges were marked using the same color code as Table 1.

Table 1: Permissible limits (ppm) for metal pollutants in food, water and soil. For each metal, we defined three concentration ranges: below the minimal estimated permissible limit (beige), between the minimal and maximal estimated permissible limits (orange), and above the maximal estimated permissible limit (red).

Matrices	**Arsenic (As)**			**Cadmium (Cd)**			**Mercury (Hg)**			**Lead (Pb)**		
Food	<0.1	0.1-0.2	>0.2	>0.05	0.05-2	>2	<0.5	0.5-1	>1	<0.01	0.01-3	>3
Water	<0.01	0.01-0.1	>0.1	<0.003	0.003-0.01	>0.01	<0.001	NA	>0.001	<0.01	0.01-5	>5
Soil	<20	NA	>20	<0.9	0.9-3	>3	<0.03	0.03-2	>2	<30	30-50	>50

2.3. Few studies address the behavioral effects of metal pollutants

79% of the 154 studies we found were published after 2007 (Fig. 3A). About half of the observations focused on physiology (52%), followed by studies on development (17%), survival (13%), population dynamics (6%), reproduction (6%) and behavior (6%) (Fig. 3B). It has become increasingly clear that understanding the sublethal behavioral effects of a stressor (e.g. mobility, navigation, feeding behavior, learning, memory) is crucial to assess the long-term impact of that stressor on invertebrate populations (Mogren and Trumble, 2010). This has become evident for bees, for instance, for which any impairment of the cognitive functions involved in foraging can result in a disruption in food supply to the colony compromising larval growth (Klein et al., 2017). In our review, 33 experiments reported behavioral effects (Fig. 3B), but only two explored cognitive effects (Philips et al., 2017; Piccoli et al., 2020). This is a very low number considering the well-known neurotoxic effects of the four metals on humans (Chen et al., 2016; Wright and Baccarelli, 2007) and other animals, including aquatic invertebrates (Salanki, 2000).

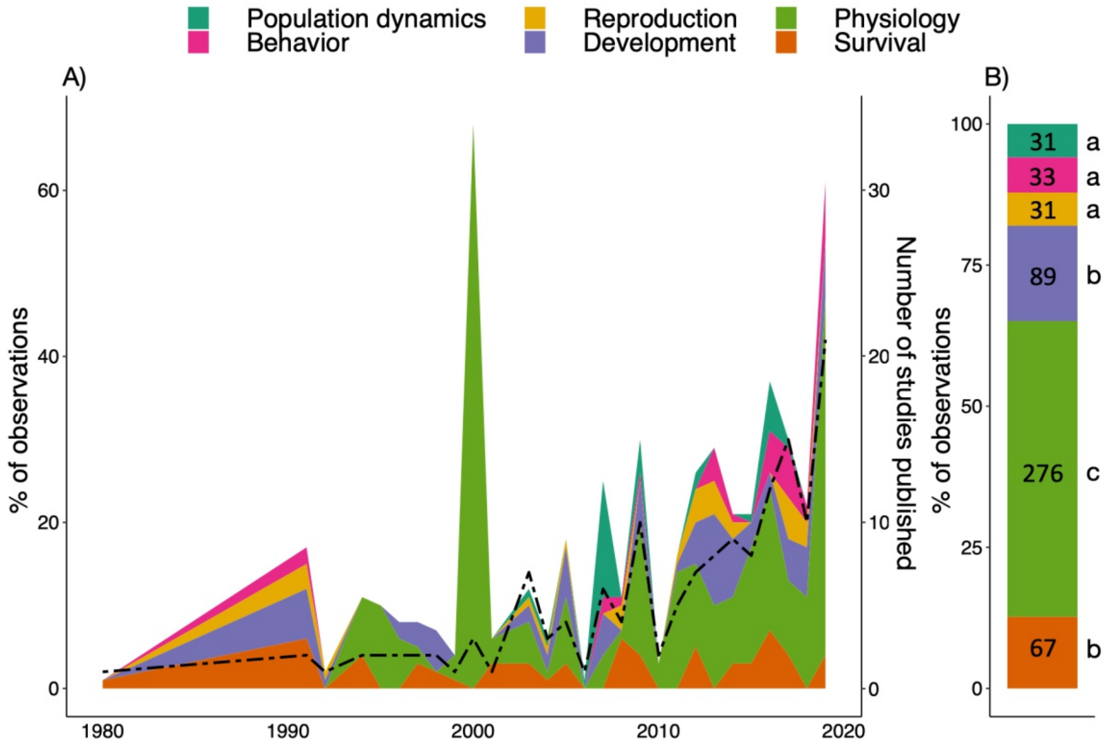

Figure 3: Biological variables measured. A) Area chart of the number of observations per biological variable (year 2020 was omitted). The peak in 2000 is due to three large studies of physiological effects in the field (38 observations). The black dashed line represents the number

of studies published yearly. **B)** Overall proportions of observations per biological variable (numbers of observations in black). Letters show statistical significance from chi-square test of homogeneity of proportions (Chi^2=619.02, df=5, p<0.001).

2.4. Few studies investigated co-occurrences despite clear synergistic effects

Only 7 out of the 154 studies addressed the question of combined effects of metal pollutants in laboratory conditions (Fig. 1A). Nonetheless the effects are clear: 55% of the observations (N=10) reported synergistic detrimental consequences. For instance, ants (*Formica aquilonia*) chronically exposed to both cadmium and mercury failed to develop compensatory mechanisms to maintain energetic balance, causing colony collapse, while being able to cope when exposed to each metal alone (Migula et al., 1997). Similarly, the lethal effects of cadmium and zinc on aphids (*Myzus persicae*) were potentiated when the two metals were combined, which led to accelerated extinction of the treated population (Stolpe and Müller, 2016). These two metals were reported to be either synergistic or antagonist on earthworms (*E. fetida*) depending on their concentrations (Wu et al., 2012). Finally, the joint exposure of honey bees (*A. mellifera*) to cadmium and copper caused an increased development duration, elevated mortality, and decreased food intake and sucrose response (Di et al., 2020). Thus, the effects of metal co-exposure are complex and variable. The paucity of studies may be because they require more sophisticated experimental designs, larger sample sizes (factorial designs) and may yield results that are more difficult to interpret. Yet, these studies are crucial if we are to revise the current regulations which presently only consider permissible limits for metals in isolation (Tables 1 and S2).

3. Discussion

Our review of the literature on lead, arsenic, cadmium and mercury shows many negative effects of these metal pollutants on terrestrial invertebrates. Excessive exposure to these compounds lead to a plethora of consequences, such as cytotoxicity (Braeckman, 1997), carcinogenic and/or mutagenic effects (Kheirallah et al., 2019), and disruption of metabolic processes (Ortel, 1995). Particularly worrisome are the reports of negative effects observed at doses below permissible limits in most of the studied taxa. There are reported lethal effects on grasshoppers (Schmidt et al., 1991), moths (Andrahennadi and Pickering, 2008), flies (Massadeh et al., 2008) and other groups (Osman et al., 2015; Polykretis et al., 2016; Stolpe et al., 2017). Metal exposure causes a number of sublethal effects, sometimes difficult to assess, such as impaired fertility (grasshoppers: (Schmidt et al., 1991); springtail: (Crouau and Pinelli, 2008); earthworm: (Konečný et al., 2014)), developmental defects (blowfly: (Nascarella et al., 2003); moth: (van Ooik et al., 2007); ant: (Skaldina et al., 2018)), resistance to pathogens (ant: (Sorvari et al., 2007); honey bee: (Polykretis et al., 2016)) and also altered feeding behavior (aphid: (Stolpe et al., 2017); honey bee: (Burden et al., 2019)).

3.1. The impact of metal pollutants is poorly understood

At present, it is likely that the severity of these effects is underestimated. Many laboratory experiments gave animals rather limited exposure times, rarely reaching the duration of a complete life cycle. Besides, most studies overlooked any consequences of exposure to multiple metal contaminants, which would be a common occurrence in nature. There is now growing interest in assessing the sublethal impacts of metals. This trend echoes the recent shift seen in pesticide research on beneficial insects, especially pollinators, which has moved from decades of standard survival assays to experimental designs aiming at characterizing the effects on behavior and cognition (Desneux et al., 2007; Klein et al., 2017). Just like pesticides, metal pollutants have subtle, but potentially serious, effects on pollinators' behavior by disturbing foraging activity (Sivakoff and Gardiner, 2017; Xun et al., 2018), food perception (Burden et al., 2019) and the learning and memory abilities required for efficient foraging (Burden et al., 2016; Monchanin et al., 2021). Through all of these mechanisms, exposure to metal pollutants can compromise food supply to the offspring, and hence the viability of a colony or population.

There are potentially complex interactions between behavior and pollutant exposure. Since an animal’s behavior can influence how much metal pollution it is exposed to (Gall et al., 2015; Mogren and Trumble, 2010), behavioral disturbances may affect exposure and sensitivity to metals. For example, impaired locomotion may reduce the capacity of individuals to avoid contaminated sites (Hirsch et al., 2003) and indiscriminate oviposition may jeopardize the survival of offspring if they are deposited on an unfavorable food plant (Cervera et al., 2004; Tollett et al., 2009). It is thus likely that we are currently underestimating the impact of metal pollution on invertebrates, due to a lack of understanding of their sublethal effects on most species.

In nature, pollutants rarely occur alone. Metals are no exception since they share common emission sources (Vareda et al., 2019). For instance, cadmium, copper, zinc and lead frequently co-occur due to the output from smelters, or the application of sewage sludge as fertilizer (Bradl, 2005). High positive correlations between chromium, cadmium and arsenic amounts have been found in soil samples (Chen et al., 1999; Navas and Machín, 2002), and many studies have shown the co-accumulation of several trace metals in insects (Goretti et al., 2020; Nummelin et al., 2007; Wilczek and Babczy, 2000). As such, co-occurring metals could have additive, antagonistic or synergistic effects (Jensen and Trumble, 2003). These interactive effects may also be influenced by the presence of other environmental stressors, such as pesticides or parasites (Alaux et al., 2010).

3.2. Multiple possible causes of invertebrates’ high sensitivity to metal pollution

Our survey of the literature suggests that invertebrates may be more sensitive to the damaging effects of metal pollutants than the mammals (e.g. humans, rodents) typically used to determine “safe” environmental levels. This may be explained by differences in sensitivity to pollutants that can vary between species and with different metals (Malaj et al., 2016). Some species can discriminate metal contaminated food from uncontaminated food (Mogren and Trumble, 2010), but other species seem unable to (Burden et al., 2019; Stolpe et al., 2017). This is particularly critical for animals feeding on resources that can accumulate metals, such as leaves (Krämer,

2010) or nectar (Gutiérrez et al., 2015). Perhaps more importantly, there is emerging evidence that invertebrates may have higher levels of exposure to metal pollutants in the field than large mammals. Surveys of terrestrial biotopes show that non-essential metals tend to be accumulate at higher levels in invertebrates than in vertebrates (Hsu et al., 2006). This seems to also be the case for aquatic taxa (Xin et al., 2015). Due to their small size, their relatively high surface area/volume ratio and the niches they occupy, invertebrates are frequently in intimate contact with soils and vegetation, or could get contaminated by specific feeding modes such as filter-feeding or deposit-feeding (De Lange et al., 2009). Their limited dispersal capacities may reduce their ability to move away from polluted areas, even if they can detect harmful levels of trace elements. As a result, metals accumulate in the bodies of individuals (Goretti et al., 2020; Mukhtorova et al., 2019; Nannoni et al., 2011; Schrögel and Wätjen, 2019) and in the nests of social species (Skaldina et al., 2018; Veleminsky et al., 1990). Some terrestrial invertebrates (e.g. ants, earthworms, bees, Isopoda) could therefore be relevant and sensitive bioindicators of metal pollution due to their particular vulnerability to metal contamination.

Invertebrates do have mechanisms to process metal pollutants. Excessive metals can be eliminated through feces (Przybyłowicz et al., 2003), accumulated in insect exoskeleton before molting (Borowska et al., 2004), or stored in specific organs (Nica et al., 2012) like the Malpighian tubules (the excretory system of invertebrates) (Rabitsch, 1997). They can also induce expression of proteins involved in metal excretion and/or detoxification, like metallothioneins (for reviews, see (Janssens et al., 2009; Merritt and Bewick, 2017)). Yet, while these detoxification mechanisms may protect species to a point, they are unlikely to spare them from the sublethal effects of metal pollutants. This can impair brain or organ function, especially since invertebrates nervous systems are size constrained with brains containing relatively few neurons (Niven and Farris, 2012). Cellular damage or death in the insect brain can result in severe consequences for the individual (Klein et al., 2017). We clearly need a better characterization of the physiological and molecular mechanisms underlying metal transfer, toxicity and tolerance in invertebrates in order to better understand their sensitivity to metal pollutants.

3.3. A need to revise guidelines of safe environmental levels of metal pollutants

Since metals are such widespread and persistent pollutants in the environment, it is a priority to develop a better assessment of their impacts on invertebrates. Our most concerning finding is the evidence that terrestrial invertebrates are highly sensitive to metal pollutants. In particular, a high percentage of studies of arsenic reported toxic effects below international permissible limits, thus pointing toward the need for more research on this specific metal (Ng et al., 2003). Our review of the literature also highlights important gaps in our knowledge. We need to study a larger diversity of species, and have more systematic investigation of doses below permissible limits. We should consider potential cocktail effects, and extend studies beyond the four metals addressed here. Although our study focuses on four metal pollutants that are well studied and considered as priority for public health concerns, other metallic compounds have been reported to negatively impact terrestrial invertebrate populations at low doses, such as selenium (deBruyn and Chapman, 2007), zinc (Cheruiyot et al., 2013), copper (Di et al., 2016), cobalt (Cheruiyot et al., 2013), nickel (Cheruiyot et al., 2013), manganese (Ben-Shahar, 2018) and chromium (Sgolastra et al., 2018). Characterizing the impacts of metal pollutants on insect fitness is going to demand an integrative and interdisciplinary research agenda, just like what has been established to assess pesticide impacts on beneficial insects. For example, focusing awareness on the sublethal effects of neonicotinoids on pollinators (Crall et al., 2018; Henry et al., 2012), triggered a revision of the risk assessments scheme and their ban in the European Union in 2018.

3.4. Concluding remarks

This survey of the existing literature clearly indicates that terrestrial invertebrates appear particularly vulnerable to arsenic, cadmium, lead and mercury, and that most existing standards are not suited to protect them. We now need more integrative toxicological studies, on a broader range of metal pollutants and invertebrate species to better assess their impact on fitness, and to update the current environmental regulation. Only by addressing these important challenges will we be able to mitigate consequences on ecosystems and food safety, in a context of rapid and widespread decline of invertebrate biodiversity.

4. Methods

4.1. Literature review and data extraction

We focused on the four most hazardous metals documented for humans (ATSDR, 2019), for which international regulatory implementations exist (Table 1): arsenic (As), cadmium (Cd), mercury (Hg) and lead (Pb). We searched articles in the ISI Web of Knowledge database (search performed on 25/03/2020) using keywords combined with Boolean operators: *Topic=(heavy metal* OR metalloid* AND (insect* OR invertebrate* AND (cadmium OR lead OR arsenic OR mercury)*. The search was restricted to articles published between 1975 and 2020 (maximum available year range on ISI Web of Knowledge). Among the 460 hits, we selected those studies focusing on terrestrial invertebrates (i.e. protostomes) from the abstracts, and excluded review articles. This filtering yielded a subset of 154 articles from which we extracted 527 observations investigating effects of metal pollutants on terrestrial invertebrates.

From each observation, we extracted: (1) the name of targeted invertebrate species, (2) the metal(s) used, (3) the experimental conditions (field, laboratory), (4) the mode of exposure to the metal (food, water, soil), (5) the type of exposure (acute: < 24 h, chronic: > 24 h), (6) the range of metal concentrations tested (min- max in ppm), (7) the biological responses measured (e.g. survival, reproduction, behavior), and (8) the lowest metal concentration for which an effect was observed. Heterogeneity of proportions was assessed using chi-square test.

Briefly, the vast majority of the observations focused on cadmium (46%) and lead (37%), while less information was available on arsenic (10%) and mercury (7%) (Fig. 1A). 59% of the observations were obtained in field surveys and 41% in laboratory experiments with controlled exposure. Since the effects can greatly vary depending on the duration of exposure and time of assessment, here we considered as acute exposure any case where individuals were exposed to a single dose and assessed within 24 h. Despite the diversity of protocols, most studies used chronic exposure (95%), through the diet (49%) or the soil (43%).

4.2. Concentration ranges

All permissible limits are based on human toxicity data. Levels were determined from the international standards set by the World Health Organization (WHO) and the Food and Agriculture Organization (FAO) of the United Nations. The permissible limits are recommended values for: 'food and drinking water', as defined in the Codex Alimentarius (Codex Alimentarius, 2015, p. 2), to deal with 'contaminants and toxins in food and feed' and to be 'applied to commodities moving in international trades' (Codex Alimentarius, 2015); guidelines for water quality in irrigation (Ayers and Westcot, 1994); critical values in soil based on the Organization for Economic Co-operation and Development (OECD) risk assessment studies (de Vries et al., 2003) and FAO standards (WHO/FAO, 2001). These limits vary across types of food, water (i.e. drinking, irrigation) and soils (i.e. allotment, commercial, residential, agricultural). Local guidelines (see S1 Table), when they exist, can vary across countries and are less conservative (higher thresholds) than the international standards, especially for soils and water. For each of these matrices, we thus considered the minimal and the maximal estimates of permissible limits. We defined three concentration ranges: below the minimal estimated limit, between the minimal and maximal estimated limits, and above the maximal estimated limit (Table 1). Whenever only one threshold value was defined, no intermediate range could be defined (NA: not applicable). Note that for water, whenever possible, we considered the minimal value for drinking water and the maximal value for irrigation water.

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Supporting materials

S1 Table. Permissible limits (ppm) for metal pollutants in food, water and soil according to international and local standards. International standards used are displayed in bold.

Matrices	Area of application	Arsenic (As)	Cadmium (Cd)	Mercury (Hg)	Lead (Pb)	Source
Food	**International**	**0.1-0.2**	**0.05-2**	**0.5-1**	**0.01-3**	**[1]**
	European Union	0.1-0.2	0.05-3	0.1-1	0.02-3	[2,3]
	USA	NA	NA	NA	NA	The U.S. Food and Drug Administration has not established regulatory limits for trace metals in finished food products other than bottled water.
	China	0.5	0.05	0.01	0.2	Retrieved from [4]
Drinking water	**International**	**0.01**	**0.003**	**0.001**	**0.01**	**[1]**
	European Union	0.01	0.005	0.01	0.001	[5]
	USA	0.01	0.005	0.002	0.015	[6]
	China	0.01	0.005	0.001	0.01	[7]
Irrigation water	**International**	**0.1**	**0.01**	**NA**	**5**	**[8]**
	USA	NA	0.005-0.01	NA	5	[9]
	China	0.05	NA	0.01	NA	Retrieved from [10]
Soil	**International**	**20**	**0.9-3**	**0.03-2**	**30-50**	[11,12]
	European Union	NA	NA	NA	NA	The European Union has not established limits for heavy metals in soils.

						There is however on-going policy to manage contamination, see [13] which states limit values in sludge for use in agriculture (Cd: 1-3 ppm; Hg:1-1.5 ppm; Pb:50-300 ppm)
	Finland	5-100	1-20	0.5-5	60-750	[14]. The Finnish standard values represent a good approximation of different national systems in Europe have been applied in an international context for agricultural soils as well.
	UK	32-640	10-230	1-3600	450-750	[15]
	USA	0.11	0.48	1	200	[16] [17] stated limit values in sludge for use in agriculture (As: 75 ppm; Cd: 85 ppm; Hg: 420 ppm; Pb: 840 ppm)
	China	20-40	0.3-0.6	0.3-1	80	[18]

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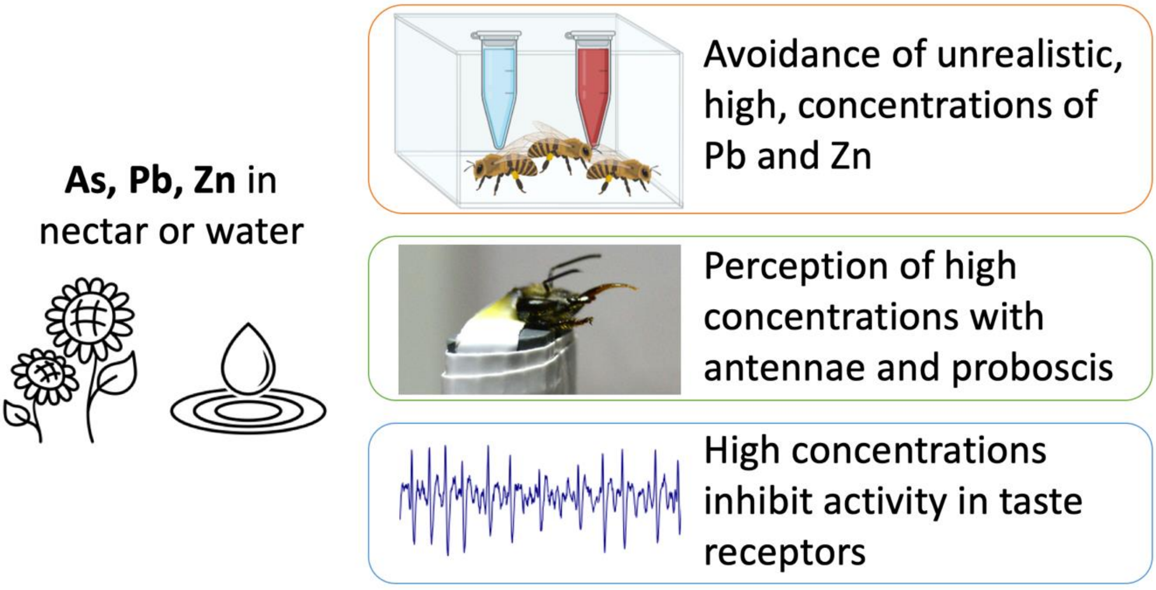
Chapter 2

Honey bees cannot sense harmful concentrations of metal pollutants in food

Highlights:

- Metal pollution represents a global ecological and public health concern worldwide.
- Whether bees can actively perceive and avoid metal-contaminated food is a fundamental question.
- Bees are only repelled by high unnatural concentrations of lead and zinc.
- Based on electrophysiological recordings, bees only perceive a range of concentrations.
- Undetected low, yet harmful, concentrations of metals may be a threat to foraging bees.



Chapter under review at Journal of Neurosciences

CHAPTER 2: Honey bees cannot sense harmful concentrations of metal pollutants in food

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Abstract

Whether animals can actively avoid food contaminated with harmful compounds through taste is key to understanding their ecotoxicological risks. Here, we investigated the ability of honey bees to perceive and avoid food resources contaminated with common metal pollutants known to impair their cognition at low concentrations (lead, zinc and arsenic). In behavioural assays, bees showed no aversive response to food contaminated with field-realistic concentrations of these metals. Bees only reduced their food consumption and displayed aversive behaviours at very high, unrealistic concentrations of lead and zinc that they perceived through their antennae and proboscis. Electrophysiological analyses confirmed that sucrose solution containing the three metals at high concentrations induced a reduced response to sucrose in their antennae. Our results thus show that honey bees can avoid metal pollutants in their food only at very high concentrations, above regulatory levels. Their inability to detect lower, yet harmful, concentrations in a field-realistic range suggests that the presence of metal pollutants in contaminated environments is a major threat for bee populations.

Keywords: *Apis mellifera*, metal pollution, feeding behaviour, PER conditioning, electrophysiology, taste

1. Introduction

Pollinators play major economic and ecological roles by facilitating the reproduction of many plants. However, pollinating insects are declining due to many stressors derived from human activities, among which are pesticides, reduced floral diversity, pests and viruses (Potts et al., 2010). Exposure to metal pollutants may have additional impact, though largely overlooked despite raising ecological and public health concern worldwide (Monchanin et al., 2021b). The release of metal pollutants into the environment, as a result of industrial manufacturing and mineral extraction, has resulted in their accumulation in ecosystems at levels far beyond concentrations that would be considered natural (Bradl, 2005; Nriagu and Pacyna, 1988). Because metal pollutants cannot be degraded and can be poisonous at low levels, they represent a potential threat to animals exploiting contaminated resources (Monchanin et al., 2021b).

In the case of pollinators, such as bees, the effects of metal pollutants could have ecosystemic consequences (Monchanin et al., 2021b). Bees are exposed to metal pollutants while flying (Thimmegowda et al., 2020) and collecting food resources (water, pollen and nectar) (Gutiérrez et al., 2020; Roman, 2007).The metals then bio-accumulate in the bodies of the bees (Balestra et al., 1992; Goretti et al., 2020), as well as in hive products (Satta et al., 2012; Zhou et al., 2018). The deleterious effects of metal pollutants on mammals (Domingo, 1994), and specifically on human health (Tchounwou et al., 2012), are well known, and there is clear evidence that exposure to metals have deleterious effects on the survival (Di et al., 2016), physiology (Nikolić et al., 2019, 2016) and behaviour (Burden et al., 2019; Chicas-Mosier et al., 2017) of bees. However, whether bees can detect metal pollutants in food is not known.

Bees can detect natural deterrent substances produced by plants and recognize them as harmful, at least in specific experimental conditions (Ayestaran et al., 2010; de Brito Sanchez et al., 2005; Guiraud et al., 2018; Wright et al., 2010). Even when ingested, such substances trigger subsequent aversive responses due to a delayed malaise-like state (Ayestaran et al., 2010; Guiraud et al., 2018; Lai et al., 2020; Wright et al., 2010). If endowed with sensitivity to harmful metal concentrations, bees could actively avoid contaminated food. By contrast, they could still consume food containing low doses of metals resulting harmless, or even profitable as some are micronutrients needed for physiological functions (Herbert and Shimanuki, 1978).

Metal ions can distort the function of peripheral chemoreceptors involved in taste-mediated feeding behaviour (Koul, 2008), particularly by reducing the sensitivity of gustatory neurons to sugars in some insect species (Hodgson, 1957; Schoonhoven and Jermy, 1977). Honey bees can recognize a variety of potentially noxious substances through gustatory receptor cells located on their antennae, mouthparts and forelegs (de Brito Sanchez, 2011), but their capacity to detect and/or avoid metals in their food seems limited. In a study of the proboscis extension reflex, restrained bees willingly consumed solutions containing field-realistic levels of selenium (Hladun et al., 2012) or cadmium (Burden et al., 2019) with no behavioural indications of avoidance. Copper and lead solutions appeared to be palatable at certain concentrations, and only lead solutions induced any aversive responses (Burden et al., 2019). Field studies have reported either no discrimination between flowers grown in lead-contaminated or uncontaminated soils (Sivakoff and Gardiner, 2017), or increased visitation of zinc- and lead-treated flowers (Xun et al., 2018). Thus, it appears that the ability to detect and reject potentially toxic substances varies greatly with their chemical identities and concentrations, the body parts in contact with them (mouthparts, antennae or tarsi), and the experimental or ecological context (e.g. harnessed vs. free-flying individuals). Whether bee taste receptors actually respond to metals has never been tested to our knowledge, so that the mechanisms of metal perception remain unknown (Burden et al., 2019).

Here, we tested whether bees could detect common metals in food. We focused on salts of zinc (an essential nutrient at low concentrations (Brodschneider and Crailsheim, 2010)), as well as of lead and arsenic (two major environmental pollutants (ATSDR, 2019)). We first assessed whether bees modified their consumption of sucrose solutions containing metal pollutants in choice and no-choice conditions. We then investigated whether bees could detect metal pollutant salts through their antennae and proboscis. Finally, we tested the capacity of gustatory antennal neurons to respond to metal pollutant salts delivered alone or in combination with sucrose.

2. Materials and methods

2.1. Bees and metals

We collected honey bees (*Apis mellifera*) from fourteen hives at our experimental apiary (University Paul Sabatier – Toulouse III, France) between January 2019 and August 2020. For the experiments, we used lead ($PbCl_2$; CAS #7758-95-4 and $PbC_4H_6O_4$ 3H2O; CAS #6080-56-4), zinc ($ZnCl_2$; CAS # 7646-85-7 and $ZnC_4H_6O_4$; CAS #557-34-6) and arsenic ($NaAsO_2$; CAS #7784-46-5) (all from Sigma-Aldrich, St Louis, MO) that were either dissolved in 30% (w/v) sucrose solution (for feeding, proboscis responses and electrophysiological assays) or in mineral water (for antennal responses and electrophysiological recordings). We tested both chloride or acetate salts of lead (Pb) and zinc (Zn). For arsenic (As) (for the sake of simplicity, we will refer to it as a metal pollutant), we chose arsenite as it is the chemical form derived from smelting, and that occurs in insecticides (Bissen and Frimmel, 2003). We used nominal concentrations of 0.001, 0.013, 0.129 and 12.83 µM of As; 0.36, 3.60, 35.96 µM and 3.6 mM of Pb; and 0.012, 0.12, 1.22 and 122.3 mM of Zn (see Table S1 for correspondences in ppm and $mg.L^{-1}$). These concentrations were chosen so that the three lower concentrations of Zn and Pb and all concentrations of As have been reported in field studies of metal pollution (Table S1). The highest concentrations were above the regulatory levels in food as defined by the WHO (Codex Alimentarius, 2015, 1984), and were assessed through chemical analysis, which gave a good recovery rate (Table S1).

2.2. Feeding assays

We tested the ability of bees to discriminate metal salts in food in assays in which groups of bees could self-select foods over several hours (Kessler et al., 2015). We collected workers of unknown age at the colony entrance of five different hives, as they returned from foraging. The bees were cold-anaesthetized and placed in groups of 20 in plastic cages (80 x 50 x 40 mm), for 3 days in an incubator (dark, 28 °C ± 1°C, 60% relative humidity). Each cage contained two 2 mL feeding vials (Eppendorf) pierced with two 2 mm holes at the bottom to allow drinking of the sucrose solutions they contained. In the no-choice condition, bees were offered only one type of food:

either 30% sucrose solution or 30% sucrose solution containing either As, Pb or Zn salts at one of the concentrations in Table S1. In the choice condition, bees were offered one feeder containing pure sucrose and one feeder containing a sucrose and metal salt solution. Feeding vials were weighed prior to be placed in the experimental cages, then removed, weighed and replaced by fresh ones every 24 h during 3 days. Cages without bees were used to measure the evaporation rate from the feeding vials. The amount of solution consumed daily was estimated by measuring weight loss in each vial every 24 h. The average value for evaporation of each treatment was subtracted from this final value for each vial. The number of dead bees in each cage was counted every hour (from 9 am to 5 pm), thus allowing the calculation of the mean daily consumption per bee (daily consumption divided by the mean number of bees alive in the cage).

2.3. Devaluation assays following antennal stimulation

We tested the ability of bees to perceive metal salts diluted in water using a devaluation assay that assesses whether repeatedly pairing a previously rewarding odour to contaminated water delivered to the antennae could lead to the devaluation of this odour, thus meaning that it would be perceived as aversive (Ayestaran et al., 2010). Workers of unknown age were collected from the top of the frames of eight different hives, cooled on ice, and harnessed in individual plastic holders allowing free movements of their antennae and mouthparts. We fixed their head to the holder using a droplet of melted bee wax, fed them 5 μL of sucrose solution (50% w/v) and let them rest for 3 h in an incubator (dark, 28 ± 1 °C, 60% relative humidity). Before starting the experiment, we checked for intact proboscis extension reflex (PER) by gently touching the antennae with a toothpick soaked in 50% (w/v) sucrose solution without subsequent feeding. Bees that did not exhibit the reflex were discarded.

The first phase of the assay started with three trials pairing an odour (pure 1-nonanol) with a 50% (w/v) sucrose solution reward. The second phase consisted of 10 trials where a presentation of the trained odorant was followed by the stimulation of the antennae with a metal solution, or just water for the control group. For the second phase of the assay, we only kept bees that performed a PER response to 1-nonanol (92% of the bees). The odour was presented via an automated odour delivery system with a continuous air-stream as described in (Aguiar et al., 2018). For each trial, the harnessed bee was placed in the conditioning set-up for 15 s to allow familiarization, then 1-nonanol was released for 6 s. Four seconds after odour onset, the antennae were stimulated with 50% (w/v) sucrose solution (phase 1) or metal solutions or water (phase 2) for 2 s followed by 1s of feeding with sucrose. The bee was left in the conditioning setup for 20 s before being removed. Inter-trial interval was 15 min for both phases. We recorded the proboscis extension response at each trial (extension=1, no extension=0).

2.4. Devaluation assay following proboscis stimulations

We assessed whether bees were able to perceive metal salts through their proboscis (Wright et al., 2010), by testing their potential devaluating effect when applied to the proboscis. We collected workers of unknown age from the top of the frames of four different hives, harnessed and fed them with 5 µL of sucrose solution (50% w/v) and left them to rest for 3 h (dark, 28 ± 1°C, 60% relative humidity). For 12 trials, bees were conditioned to associate 1-nonanol with ingestion of the sucrose-contaminated stimulus: after application of a droplet of 30% (w/v) sucrose onto the antennae to trigger PER, a 0.4 µL droplet of a metal-spiked sucrose solution was delivered to the proboscis. We recorded the proboscis extension response upon odour delivery for each of the 12 trials (extension=1, no extension=0). Here again, we expected that any decrease of response frequency would reveal innate aversion to some stimuli, triggered by their detection at the proboscis level and/or post-ingestive consequences (i.e. malaise-like state). In addition, we collected bees in the same conditions, harnessed and fed them with 4.8 µL (12 times 0.4 µL) of each solution and monitored their survival for 150 min (i.e. the duration of the proboscis response assay).

2.5. Electrophysiological recordings

We performed electrophysiological recordings on chaetic sensilla (Esslen and Kaissling, 1976; Hodgson et al., 1955), which can be easily identified by their external morphology (Whitehead and Larsen, 1976) (Fig. 3A). We focused on the antennae, the organs concentrating the highest number of taste sensilla (de Brito Sanchez, 2011), and specifically on the tip ventral zones (Haupt, 2004), which are devoid of olfactory sensilla (Esslen and Kaissling, 1976). Irrespective of responses to metal pollutant salts, we identified two main response profiles that lead us to distinguish two functional categories of sensilla: those responding to both sucrose and KCl (Type I, 722 recordings) and those responding to sucrose only (Type II, 953 recordings).

We immobilized the antennal flagellum with a metal thread stuck with wax and a glass electrode (ext. diameter 10–20 μm) was placed over a single taste sensillum (de Brito Sanchez, 2011). We used a silver wire inserted into the contralateral eye as grounded reference electrode. Electrodes were pulled from borosilicate glass capillaries, filled with different solutions and stored in a humid chamber before use. We prepared 30 mM sucrose solutions, contaminated or not with metal pollutant salts (Table S1), in 1 mM KCl, which ensures the necessary conductivity for recording, and kept at 4 °C (1mM KCl was used as the reference). We stimulated taste sensilla in the following order: 1mM KCl, 30 mM sucrose, then 30 mM sucrose containing increasing concentrations of metal pollutant salts. In a separate experiment, increasing concentrations of KCl (1 mM, 10 mM, 50 mM and 500 mM), diluted in 30 mM sucrose, were also tested. All stimuli were applied for 2s, with an interstimulus interval of 1min. The recording and reference electrodes were connected to a preamplifier (TasteProbe—SYNTECH, Kirchzarten, Germany). The electric signals were amplified (×10) using a signal connection interface box (Syntech, Kirchzarten, Germany) in conjunction with a 100-3000Hz band passfilter. Experiments started when the recording electrode contacted the sensillum under study, which triggered data acquisition and storage on a hard disk (sampling rate: 10kHz). We then analysed these data using Autospike (Syntech) and quantified the number of spikes after stimulus onset.

2.6. Statistical analysis

We performed all statistical analyses in R (RStudio Team, 2015). For the choice assay, we analysed the consumption preference (difference between mean daily consumptions of each food: g/bee) with linear mixed effect models (LMMs; lme4 package (Bates et al., 2015)), against zero (no preference). For the no-choice assay, we analysed the daily consumption of solution (g/bee) with LMMs. Models were fitted with treatment as a fixed effect and cages nested in hive as a random effect. Models were followed by pairwise comparisons (multcomp package (Hothorn et al., 2008)). We analysed the survival probability over three days using a Cox regression model (Therneau, 2020).

For antennal and proboscis response assays, we scored the PER of each bee as a binary variable (response=1, no response=0), and analysed the mean score (averaged over the trials: 10 for antennal responses, 12 for proboscis responses) using a binomial generalised linear mixed model (GLMM, lme4 package (Bates et al., 2015)), with treatment as fixed effect, trial number as a covariate, individual identity nested in the colony, and trial as random grouping variable. For proboscis responses we also applied GLMMs separately for each trial, with treatment as fixed effect and individual identity nested in the colony, to better capture the temporal dynamics of responses. We analysed the survival probability over 150 min using a Cox regression model.

Electrophysiological data were analysed by comparing frequencies of recorded spikes using a negative binomial GLMM using Template Model Builder (Brooks et al., 2017), with treatment as a fixed effect and bee identity as random variable to take into account the repeated measurements per individual. Models were followed by pairwise comparisons (Hothorn et al., 2008).

3. Results

3.1. *Bees only avoided high concentrations of Pb and Zn in food*

The highest concentrations of Zn salts (both chloride and acetate) in food were toxic, inducing high mortality after 24 h (Cox model: $p<0.001$ and $p=0.010$ respectively, Fig. S1). Therefore, we compared food consumptions across all treatments and for choice and no-choice feeding assays over the first 24 h only.

We first tested whether bees discriminated metals in food when given a choice between two accessible sucrose solutions, one of which contained one out of four concentrations of either As, Pb or Zn (Fig. 1A). None of the As solutions were avoided or preferred when compared to pure sucrose solution. Similarly, there was no difference in consumption of pure sucrose and sucrose solutions containing low concentrations of Pb and Zn (Table S2A). However, the highest concentrations of Pb (3.6 mM, LMM: $p<0.001$ for both chloride and acetate) and Zn (122.3 mM, LMM: $p<0.001$ for both salts) were consumed significantly less by bees.

We then tested whether bees would still avoid their consumption of metals in food when they had no alternative choice (Fig. 1B). Bees showed similar consumption of food containing either As (all concentrations), low concentrations of Pb and Zn, or no metal pollutant salts (control) (LMM: $p>0.05$). However, they reduced their total food consumption by 40% when it contained the highest concentration of Pb (3.6 mM, LMM: $p<0.001$ for both salts), and by 87% when it contained the highest concentration of Zn (122.3 mM, LMM: $p<0.001$ for both salts) (Table S2B). These effects were independent of the chemical forms (acetate vs. chloride) of Pb and Zn (LMM: $p>0.05$ for pairwise comparison for each concentration).

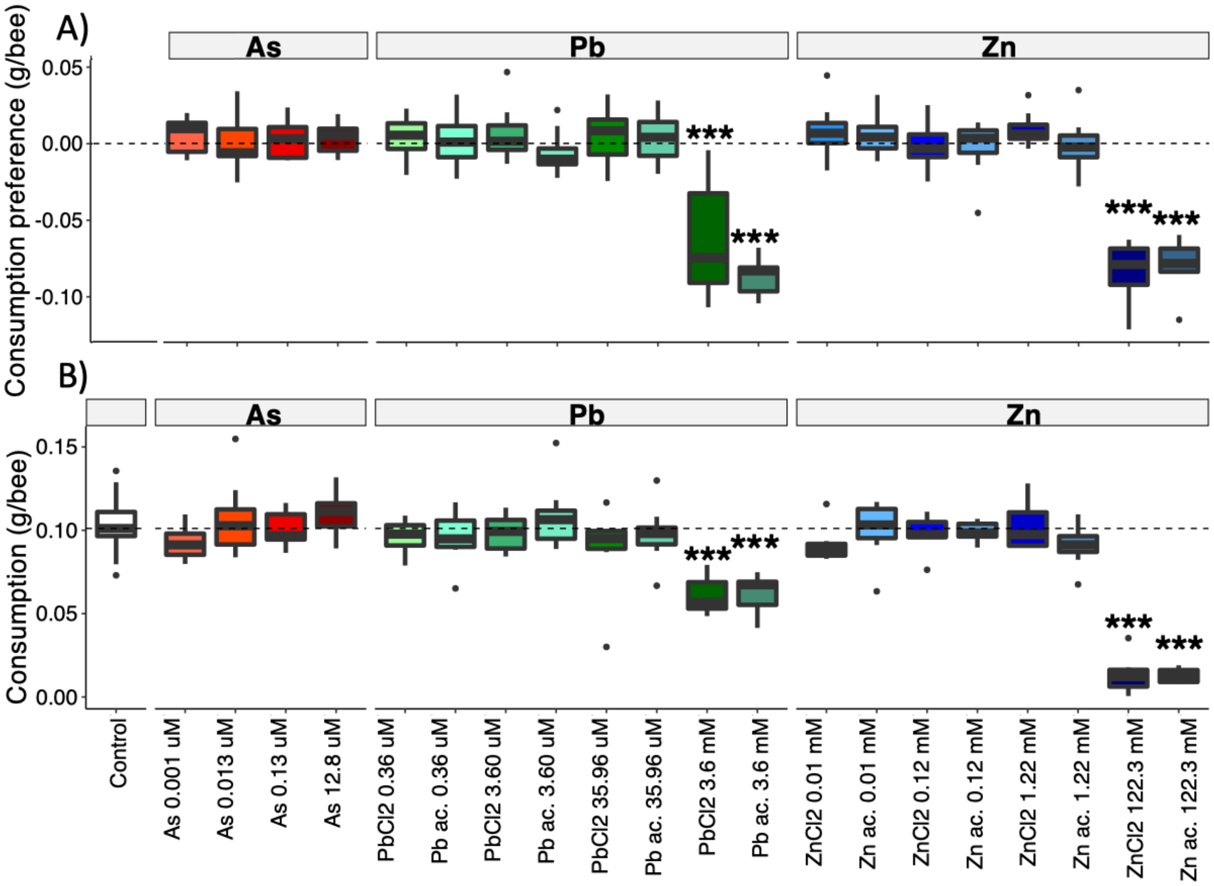

Figure 1: Feeding assays. A) Choice experiment. Consumption preference (difference in daily consumption between the two solutions) are plotted. Positive values: preference for the metal pollutant solution; zero (dotted line): no preference; negative values: preference for the pure sucrose solution. N=8 cages of 20 bees per treatment. **B) No-choice experiment.** Daily food consumption of each solution; the dotted line indicates the median value for control bees (plain sucrose solution, white). N=8 cages per treatment and N=27 cages for control bees. In both experiments we used three metals (arsenic - red, lead - green, zinc - blue) at four concentrations each. Box plots show median (horizontal line), 25th to 75th percentiles (box), smallest and highest values within 1.5*inter-quartile range of the hinge (error bars), and outliers (dots). *p<0.05, **p<0.01, ***p<0.001: differences with zero (A) or control bees (B), LMMs (Table S2)

3.2. Bees perceived only high concentrations of the three metals, with their antennae and proboscis

We tested whether bees were able to perceive metal salts through their antennae in a devaluation experiment. Since the conditioning odour had been associated with sucrose in a first phase, we expected a progressive decrease of the rate of conditioned PER over subsequent unrewarded (water) presentations (phase 2) in all groups. If bees perceive metal salts in water, the decrease of response to the metal solution should be stronger than with water. Overall, antennal stimulation with solutions containing metal pollutant salts affected PER responses (Fig. 2A, Table S3). The mean PER rate was significantly reduced for the two highest concentrations of As (12.8 µM, 0.13 µM), Zn chloride and acetate (122.3 mM, 1.22 mM), Pb acetate (3.6 mM, 35.96 µM) and only for the second highest concentration of Pb chloride (3.6 mM). We found no overall effect of the chemical form (acetate vs. chloride) of Pb or Zn (Binomial GLMM: $p>0.05$ for pairwise comparison for each concentration). Therefore, bees perceived the highest concentrations of each metal salt through their antennae and reduced their appetitive response.

A devaluation paradigm was also used, in which bees were trained, over 12 trials, to associate an odour with a sucrose presentation on the antennae (to induce PER) followed by delivery of a lower concentration of sucrose to their proboscis, thus leading to a progressive reduction of PER rate. Here, metals were diluted in sucrose instead of water to ensure their ingestion. Bees that received sucrose containing metals to the proboscis reduced their PER response more than controls (Fig. 2B). Zn-treated bees showed significantly lower levels of PER as early as the 3rd trial (Binomial GLMM: $p=0.001$ for Zn chloride, $p=0.036$ for Zn acetate). By contrast, the response levels of Pb and As groups initially reached a maximum PER response that was similar to the controls, then decreased responding from the 6th trial onwards with Pb (Binomial GLMM: $p=0.009$ for chloride, $p=0.044$ Pb acetate), and from the 8th trial onwards for As (Binomial GLMM: $p<0.001$). These effects were independent of the chemical forms of Zn and Pb (Tukey HSD: $p>0.05$). Thus, bees seemed to evaluate negatively all three metals, through their proboscis and/or post-ingestive effects, as they eventually responded to all contaminated solutions by markedly decreased PER rates (GLMM: mean PER response: $p<0.001$ for all treatments). The ingested volumes of metal pollutant solutions were not sufficient to impact survival over the duration of the experiment (Fig. S3).

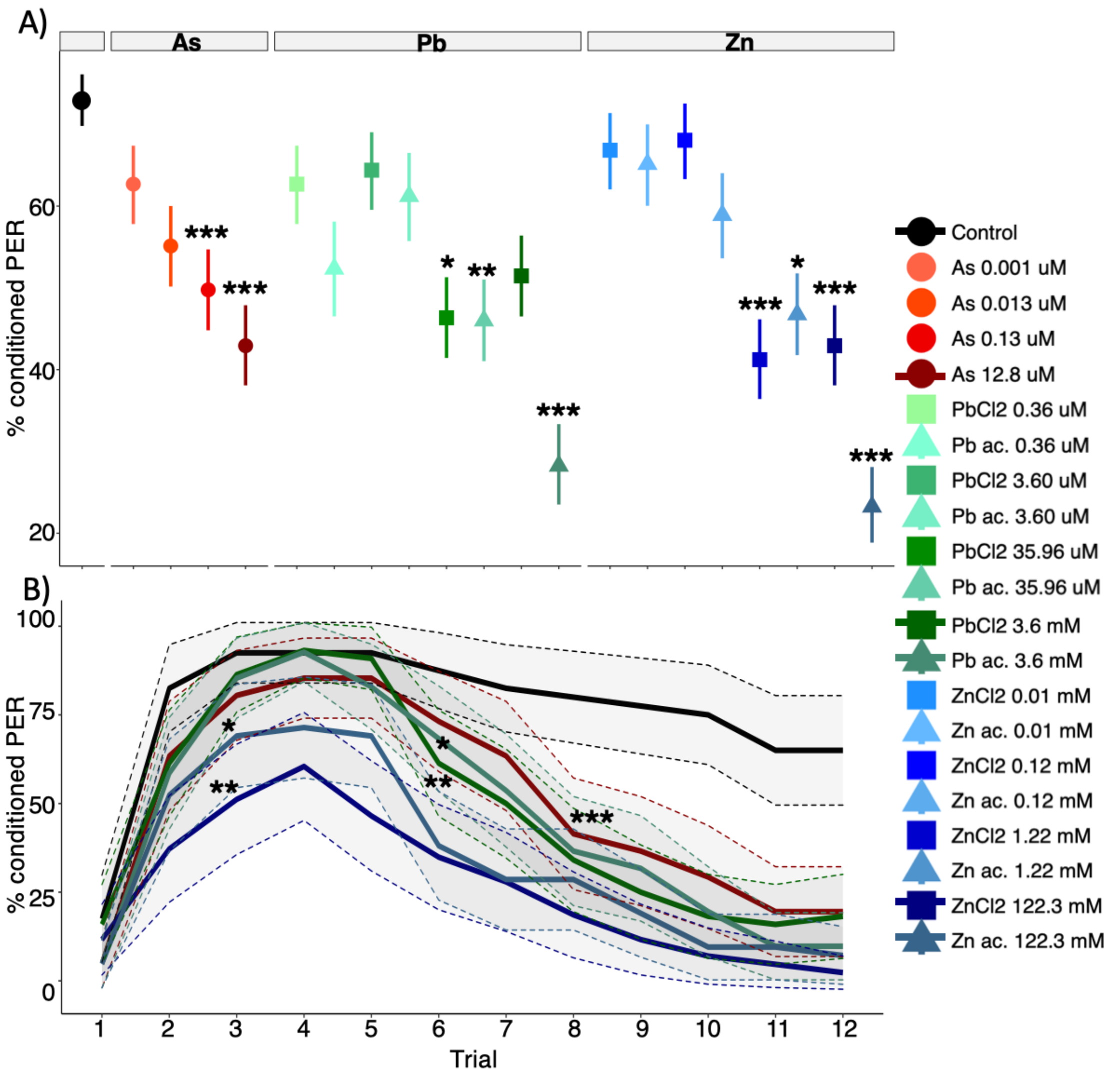

Figure 2: Devaluating effects of metal salts. Mean conditioned proboscis extension response (PER) and 95% confidence intervals (bars in A, shaded in B) across devaluation trials, for each treatment. **A) Application on the antennae.** For lead and zinc, chemical forms are shown by the mean point shape, square for chloride (Cl_2) and triangle for acetate ($C_4H_6O_4$). N=35-41 bees/treatment. $*p<0.05$, $**p<0.01$, $***p<0.001$: binomial GLMM, compared to controls (N=79)). **B) Application on the proboscis.** N=40-42 bees/treatment. $*p<0.05$, $**p<0.01$, $***p<0.001$: differences with controls (N=40), displayed only for the first trial showing significant differences (binomial GLMM).

3.3. Highly concentrated metals inhibit sucrose-evoked activity in taste receptors

We finally performed electrophysiological recordings to investigate the mechanisms by which bees detect metal salts, focusing on neurons in antennal gustatory sensilla (Fig. 3A-B), which are mostly tuned to detect sugars and salts (de Brito Sanchez, 2011).

We recorded electrophysiological responses to ascending concentrations of each metal pollutant salt diluted in 30% mM sucrose (Fig. 3C). Some sensilla responded equally to both sucrose and KCl (type I sensilla), but showed a drop in spike frequency in response to high metal concentrations. This is a specific response to metal salts since adding a nutrient salt such as KCl to sucrose had the opposite effect (Fig. S4). Other sensilla responded much more to sucrose than to KCl (Type II sensilla) and showed a similar reduction in their activity in response to all metal pollutant salts, when compared to pure sucrose. Overall, the chemical form of Pb or Zn had no effect (GLMM: $p>0.05$; except for 3.60 μM Pb on type I sensilla: $p<0.001$; and 0.12m Zn on type II sensilla: $p<0.001$). Thus, the presence of metal pollutant salts at high levels in sucrose solution could be detected by antennal gustatory neurons, which reduced their activity.

We then asked whether metal salts could be detected independently of the presence of sucrose, and thus used water solutions as stimuli (Fig. 3D). Type I sensilla responded to low concentrations of all metal salts similarly to KCl or sucrose. By contrast, they reduced their spike frequency when stimulated with high metal concentrations, as compared to both KCl and sucrose. Type II sensilla failed to show marked activity in response to most metal solutions, as they did for KCl. Thus, metal salts did not trigger a specific response pattern by themselves, but rather reduced sucrose-triggered responses when added at high concentration to the sucrose solution.

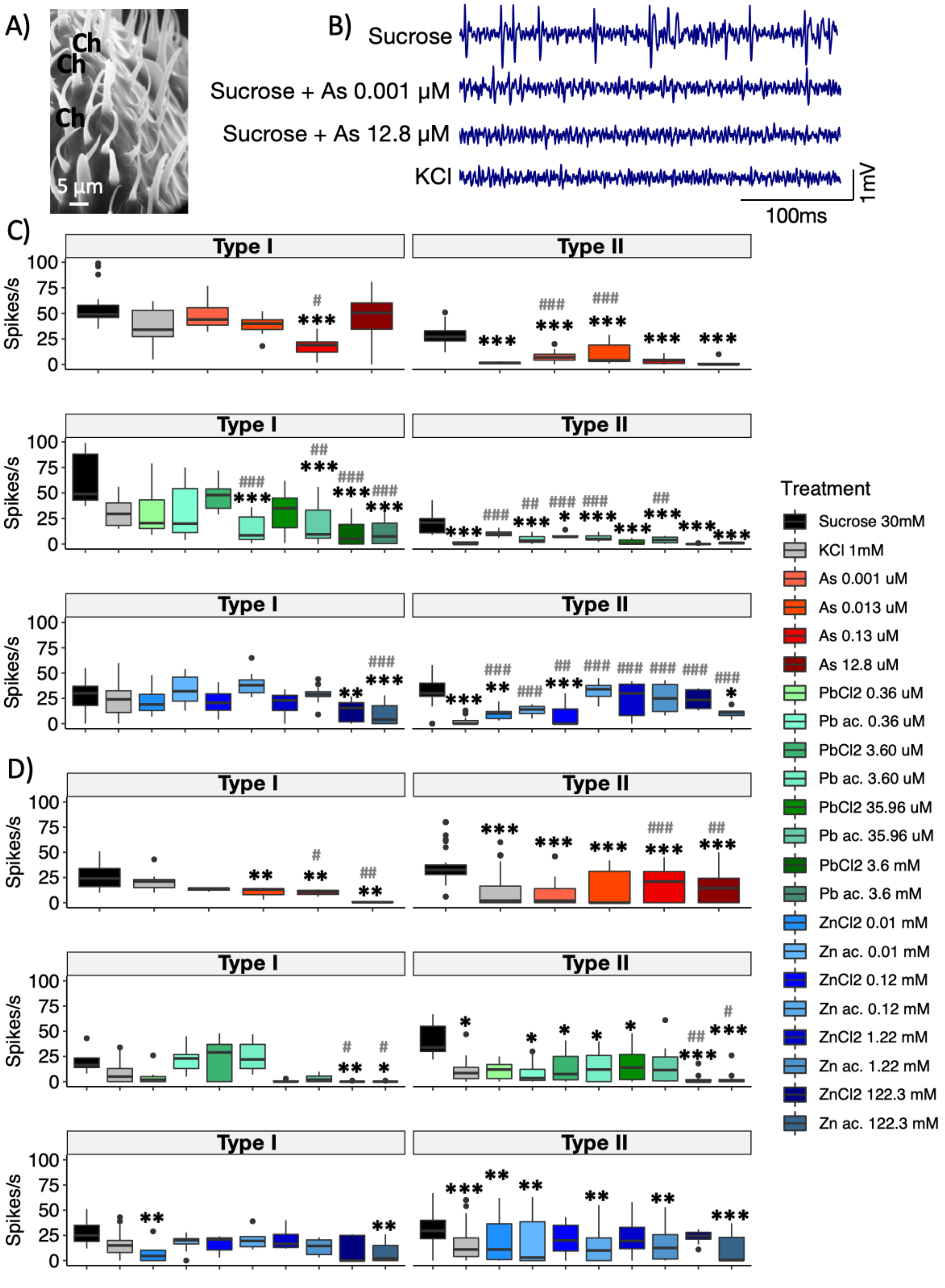

Figure 3: Electrophysiological recordings of the gustatory neurons from the antennae. A) Scanning electron microscope picture of the surface of the antenna showing chaetic sensilla (Ch) chosen for recording. **B)** Examples of spike trains recorded from a type II sensilla in response to various stimuli. Note the decreased spike frequency induced by the presence of As in the sucrose solution. **C, D)** Boxplots of the spiking responses to sucrose (black), KCl (grey), and increasing

concentrations of arsenic (red), lead (green) and zinc (blue), for a type I sensilla (responding to both KCL and sucrose, left) or a type II (responding to sucrose only, right). **C)** Stimulation with metal salts diluted in sucrose solution (As: N=4; Pb: N=5; Zn: N=6). **D)** Stimulation with metal salts diluted in water (As: N=4; Pb: N=5; Zn: N=6). (#/*p<0.05, **p<0.01, ***p<0.001: differences with sucrose (star) or KCl (hash) (pairwise comparisons following GLMM).

4. Discussion

Pollinators are impacted by metal pollutants that significantly impair physiology, behaviour and cognition (Burden et al., 2019; Monchanin et al., 2021a, 2021c; Thimmegowda et al., 2020). Here we showed that bees have only a limited capacity to detect and avoid these poisons in food. Honey bees perceived very high, unrealistic, concentrations, of Pb and Zn through their proboscis and antennae, and avoided ingesting them. Sucrose containing concentrated As was detected, but still consumed. By contrast, lower, yet harmful, field-realistic concentrations of the metal pollutants were neither avoided nor detected in our conditions. Electrophysiological recordings from gustatory neurons confirmed that bees can only taste a limited concentration range of metal pollutants.

Bees avoided Zn and Pb (but not As) at very high concentrations, above most environmental levels, even in the absence of alternative food sources. This observation is consistent with previous reports of decreased food consumption following exposure to high Zn or Pb levels (Burden et al., 2019; Di et al., 2016; Teixeira De Sousa, 2019). However, honey bees ingested sucrose solutions containing all three metal salts at concentrations similar to those found in nectar (Hajar et al., 2014; Maiyo et al., 2014). While Pb and As are toxic, Zn at low concentrations is an essential micronutrient. The absence of any behavioural responses to these solutions at field relevant concentrations suggests that honey bees are incapable of discriminating between toxic and essential metals. This result is consistent with studies reporting indiscriminate visits on metal-contaminated flowers (Chicas-Mosier et al., 2017; Hladun, 2013; Sivakoff and

Gardiner, 2017). At these realistic concentrations, metal pollutants alter development (Di et al., 2016), impair learning and memory functions (Monchanin et al., 2021a, 2021c), and disrupt metabolism (Nikolić et al., 2019, 2016) and antioxidative responses (Gauthier et al., 2016).

Stimulations of gustatory organs with metal solutions demonstrated that high concentrations were perceived through the antennae and the proboscis. This devaluating effect, occurred with antennal stimulation only, thus independently of potential post-ingestive effects such as those observed with other toxic substances (Ayestaran et al., 2010; Lai et al., 2020; Wright et al., 2010). While methodological differences make difficult a direct comparison of devaluation responses with and without ingestion, they did not seem to be much stronger when metals were delivered to the proboscis and ingested, thus indicating that any post-ingestion effect would have been minimal.

The detection of metals by taste receptors was sufficient to reduce appetitive behaviour. The decreased responsiveness to repeated stimulations with contaminated sucrose on taste receptors likely results from a mismatch between expected and obtained rewards, possibly because peripheral detection of metals actively inhibited appetitive behaviour and/or because sucrose-sensitive taste receptors were inhibited. Both mechanisms have been involved in the feeding suppression triggered by plant-derived deterrents (Koul, 2008), but electrophysiological data was lacking to confirm the implication of either process in these and previous behavioural effects of metal pollutants (Burden et al., 2019; Teixeira De Sousa, 2019). Here, we show that concentrated Pb, As and Zn decrease sucrose-evoked spike frequencies in bee taste receptors' response to sucrose, irrespective. This effect was specific as it was observed irrespective of the metal salts used (acetate vs. chloride), and in a different concentration range as for common salts (e.g. KCl). By contrast, we found no clear evidence of specific detection systems, consistently with the limited molecular repertoire of gustatory receptors in this species (Robertson and Wanner, 2006). Thus, such effect might result from non-specific effects detrimental to neural activity such as toxic effects, e.g. oxidative stress and ion channel dysfunction (Garza-Lombó et al., 2019; Marger et al., 2014). While the exact mechanism remains to be determined, very high metal concentrations (rarely encountered even in contaminated environments) can trigger rejection of food sources that would be toxic at short term, as already observed for naturally deterrent compounds (e.g. bitter substances) (de Brito Sanchez et al., 2014, 2005). However, such anti-feeding action of many phytochemicals may have been selected as a plant defence mechanism against phytophagous insects (Koul, 2008), it appears rather inefficient for bees to avoid field–relevant doses.

5. Conclusion

Our study echoes to the recent findings that bees cannot detect harmful insecticides through taste (Arce et al., 2018; Kessler et al., 2015) and calls for further research to better characterize the response of bees to heavy metal pollutants. Since metal pollutants are undetected and consumed by bees, low amounts can bioaccumulate, which may lead to long-term detrimental effects on individuals and colony health (Klein et al., 2017). Evidence of hazards of heavy metals on terrestrial wildlife worryingly accumulate (Monchanin et al., 2021b). It has become an urgent issue to account for such effects in order to adjust permissible levels of environmental metal pollution accordingly (Monchanin et al., 2021b).

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Supporting materials

Table S1: Comparison of the concentrations tested and field measures. Theoretical concentrations are given in molarity, ppm and mg.L^{-1}. The highest concentrations of metals in sucrose solutions, used for subsequent dilutions, were analysed [1]. For this, solutions were acidified at 3% HNO_3 with ultra-pure 69% HNO_3 to avoid precipitation or adsorption in containers and then diluted with a HNO_3 3% solution to reduce the spectral interference and viscosity effects. Solutions were then analysed using Inductively Coupled Plasma Emission Spectroscopy (ICP-OES, quantification limit: 5-20 µg.kg^{-1}, precision measure: 1-5%; AMETEK Spectro ARCOS FHX22, Kleve, Germany). Mean (minimal-maximal) concentrations of arsenic, lead and zinc recorded in honey and flower samples worldwide. ND: not detected. Values in bold show concentrations above the international permissible values in food as per WHO and FAO (As: 0.2 ppm; Pb: 3 ppm; Zn: 60 ppm [2,3])

Metal	Nominal concentration (molarity)	Actual concentration (molarity) (recovery percentage given)	Nominal concentration (ppm)	Nominal concentration (mg.L^{-1})	Concentration recorded in honey samples (ppm)	Concentration recorded in flower samples (ppm)
As	0.001 µM		0.0001	0.000096		
	0.013 µM		0.001	0.00096	0.007 (0.003-0.02) [4]	
	0.129 µM		0.010	0.0096	0.015 (0.002-0.03) [5]	0.098 (0.075-0.12) [6]
	12.83 µM	8.72 µM (68%)	**0.853**	**0.96**	0.56 (0.019-1.39) [7] 0.52 (ND-1.93) [8]	0.31 [9]
Pb	0.36 µM		0.07	0.075	0.07 (0.01-0.84) [10] 0.08 (0.03-0.24) [11]	
	3.60 µM		**0.66**	**0.75**	0.62 (0.61-0.63) [12]	0.61 [13]
	35.96 µM		**6.61**	**7.45**	0.720 (ND-4.78) [14] 14.59 (10-18) [15]	8.05 [16] 1.53 (0.13-7.68) [15]

	3.6 mM	$PbCl_2$ 3.83 mM (94%) $PbC_4H_6O_4$ 3.06 mM (85%)	**661**	**745**		
Zn	0.012 mM		0.71	0.80	0.75 (0.04-5.96) [17] 0.75 (ND-1.43) [18]	0.42 (0.05-0.63) [13]
	0.12 mM		7.09	8.00	6.39 (1.37-22.15) [14] 7.76 (4.17-22.30) [19]	17.8 (1.15-49.12) [20]
	1.22 mM		**70.94**	**79.95**	9.33 (0.23-73.60) [21] 43.88 (4.7-174) [22]	79.0 [23]
	122.3 mM	$ZnCl_2$ 114.4 mM (94%) $ZnC_4H_6O_4$ 386.6 mM (71%)	**7094**	**7995**		

Table S2: Parameter estimates from the LMMs for the feeding assay after 24 h. A) For the consumption preference (g/bee) of the choice experiment, compared to 0 (i.e. no preference). **B)** For the food consumption (g/bee) of the no-choice experiment compared to control bees. Significant p-values are shown in bold. SE: standard errors.

	A) Choice experiment		**B) No-choice experiment**	
	Estimate ± SE	p-value	Estimate ± SE	p-value
As 0.001 µM	0.0055 ± 0.0064	0.393	-0.0106 ± 0.0053	0.918
As 0.013 µM	0.0006 ± 0.0065	0.987	0.0038 ± 0.0053	1
As 0.13 µM	0.0026 ± 0.0064	0.691	-0.0034 ± 0.0054	1
As 1.8 µM	0.0040 ± 0.0064	0.537	0.0061 ± 0.0053	0.999
$PbCl_2$ 0.36 µM	0.0034 ± 0.0064	0.598	-0.0068 ± 0.0053	0.999
$PbC_4H_6O_4$ 0.36 µM	0.0024 ± 0.0064	0.707	-0.0093 ± 0.0054	0.981
$PbCl_2$ 3.60 µM	0.0062 ± 0.0065	0.344	-0.0034 ± 0.0054	1
$PbC_4H_6O_4$ 3.60 µM	-0.0057 ± 0.0064	0.380	0.0006 ± 0.0055	1
$PbCl_2$ 35.96 µM	0.0038 ± 0.0065	0.552	-0.0171 ± 0.0053	0.151
$PbC_4H_6O_4$ 35.96 µM	0.0030 ± 0.0064	0.647	-0.0079 ± 0.0053	0.997
$PbCl_2$ 3.6 mM	-0.0629 ± 0.0065	**<0.001**	-0.0417 ± 0.0053	**<0.01**
$PbC_4H_6O_4$ 3.6 mM	-0.0866 ± 0.0065	**<0.001**	-0.0428 ± 0.0054	**<0.01**
$ZnCl_2$ 0.01mM	0.0079 ± 0.0064	0.220	-0.0106 ± 0.0053	0.914
$ZnC_4H_6O_4$ 0.01 mM	0.0051 ± 0.0065	0.435	-0.0057 ± 0.0055	1
$ZnCl_2$ 0.12mM	-0.0018 ± 0.0065	0.787	-0.0049 ± 0.0053	1
$ZnC_4H_6O_4$ 0.12 mM	-0.0029 ± 0.0064	0.655	-0.0052 ± 0.0053	1
$ZnCl_2$ 1.22mM	0.0093 ± 0.0064	0.153	-0.0041 ± 0.0053	1
$ZnC_4H_6O_4$ 1.22 mM	-0.0005 ± 0.0064	0.939	-0.0138 ± 0.0054	0.548
$ZnCl_2$ 122.3mM	-0.0839 ± 0.0065	**<0.001**	-0.0878 ± 0.0053	**<0.01**
$ZnC_4H_6O_4$ 122.3 mM	-0.0791 ± 0.0065	**<0.001**	-0.0920 ± 0.005	**<0.01**

Table S3: Parameter estimates from the GLMM for the mean proboscis extension response, compared to control bees, of the antennal response assay. Significant p-values are shown in bold. SE: standard errors.

	Estimate ± SE	p-value
As 0.001 µM	-1.6609 ± 0.6752	0.631
As 0.013 µM	-2.1685 ± 0.6541	0.106
As 0.13 µM	-2.5849 ± 0.6306	**<0.001**
As 1.8 µM	-3.1880 ± 0.6266	**<0.001**
$PbCl_2$ 0.36 µM	-1.5803 ± 0.6734	0.717
$PbC_4H_6O_4$ 0.36 µM	-1.7026 ± 0.7482	0.766
$PbCl_2$ 3.60 µM	-1.2555 ± 0.6897	0.964
$PbC_4H_6O_4$ 3.60 µM	-1.1799 ± 0.7439	0.992
$PbCl_2$ 35.96 µM	-2.6603 ± 0.6261	**<0.001**
$PbC_4H_6O_4$ 35.96 µM	-2.4830 ± 0.6751	**0.034**
$PbCl_2$ 3.6 mM	-1.9016 ± 0.6507	0.287
$PbC_4H_6O_4$ 3.6 mM	-3.9365 ± 0.6832	**<0.001**
$ZnCl_2$ 0.01mM	-1.3833 ± 0.6765	0.893
$ZnC_4H_6O_4$ 0.01 mM	-0.7728 ± 0.7460	1
$ZnCl_2$ 0.12mM	-0.9943 ± 0.6923	0.998
$ZnC_4H_6O_4$ 0.12 mM	-0.9144 ± 0.7362	0.999
$ZnCl_2$ 1.22mM	-3.1825 ± 0.6204	**<0.001**
$ZnC_4H_6O_4$ 1.22 mM	-2.5721 ± 0.6806	**0.023**
$ZnCl_2$ 122.3mM	-3.1551 ± 0.6315	**<0.001**
$ZnC_4H_6O_4$ 122.3 mM	-4.5625 ± 0.6839	**<0.001**

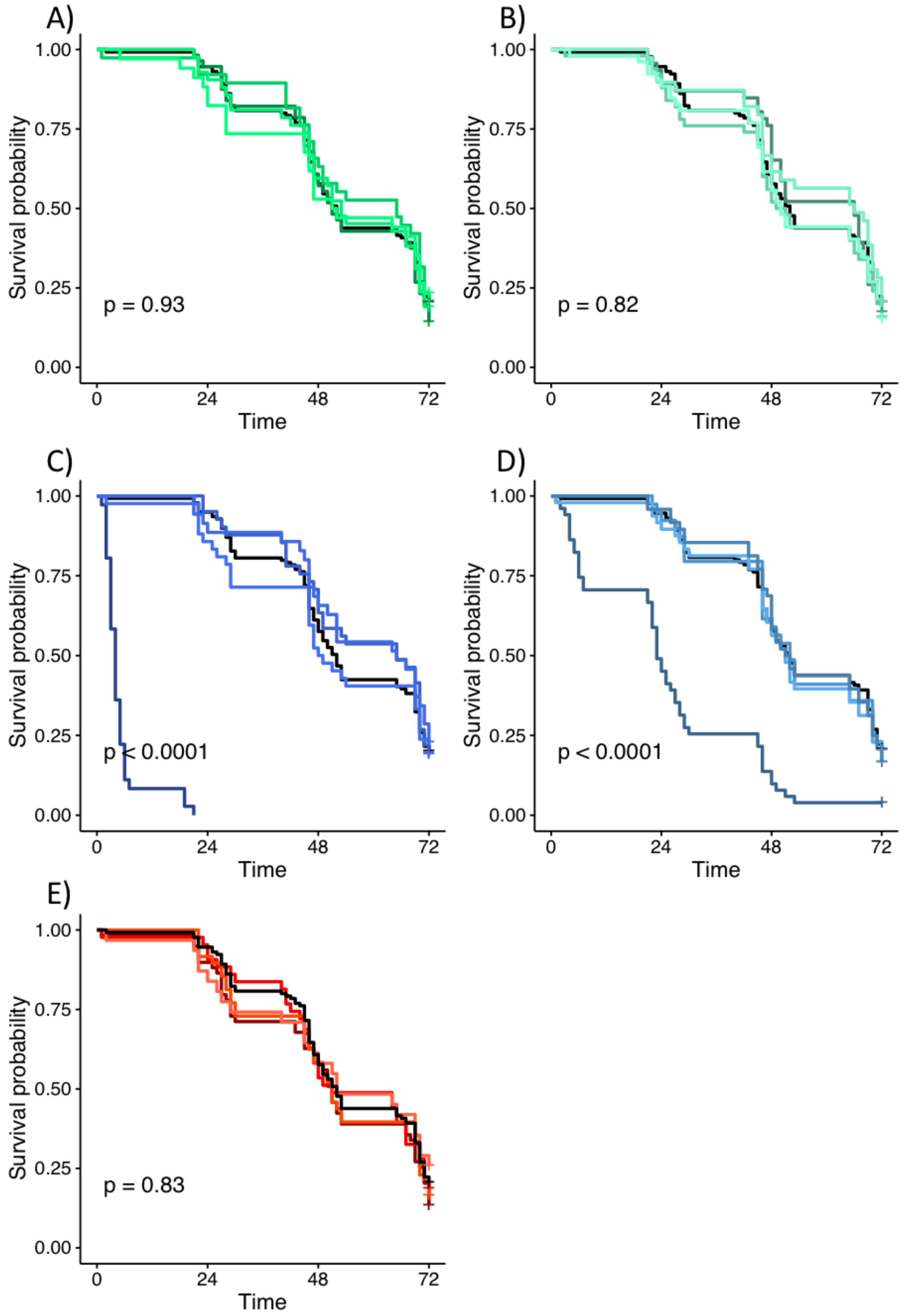

Figure S1: Survival probability over the 3 days of the no-choice experiment. A) Lead chloride (0.36 µM-3.6 mM of Pb). **B)** Lead acetate (0.36 µM-3.6 mM of Pb). **C)** Zinc chloride (0.012-122.3 mM of Zn). **D)** Zinc acetate (0.012-122.3 mM of Zn). **E)** Arsenic (0.001-12.83 µM of As). Controls are displayed in black. P-values were obtained from Cox regression models compared to control.

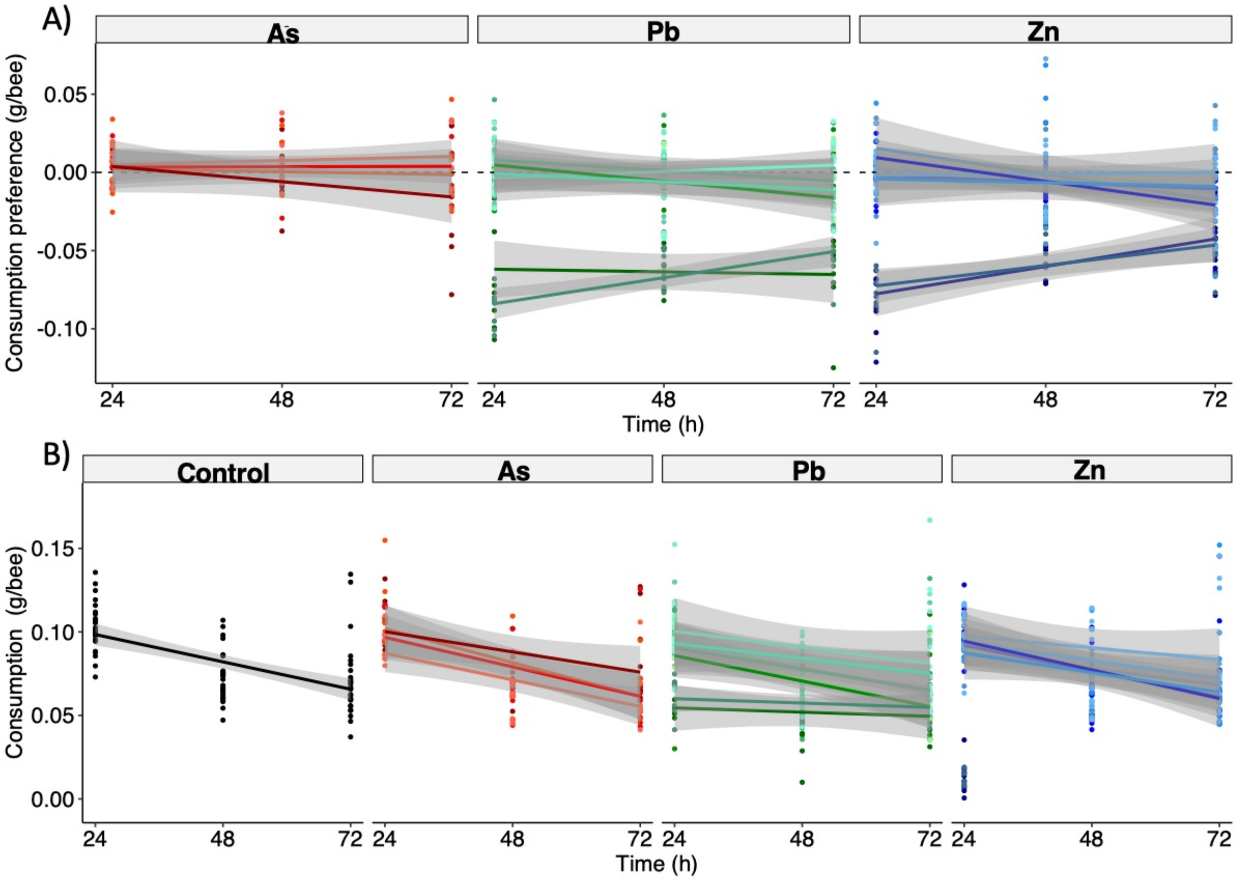

Figure S2: Feeding assay. A) Choice experiment. Food consumption preference (g/bee) over the 3 days of experiment. Values over 0 show preference for sucrose-metal diets; values below zero indicate preference for uncontaminated sucrose solution. Dotted line represents no preference. N=8 cages of 20 bees per treatment **B) No-choice experiment.** Food consumption (g/bee) over the 3 days of experiment. N=8 cages per treatment and N=27 cages for control bees. We used three metals (arsenic - *red*, lead - *green*, zinc - *blue*) at four concentrations each.

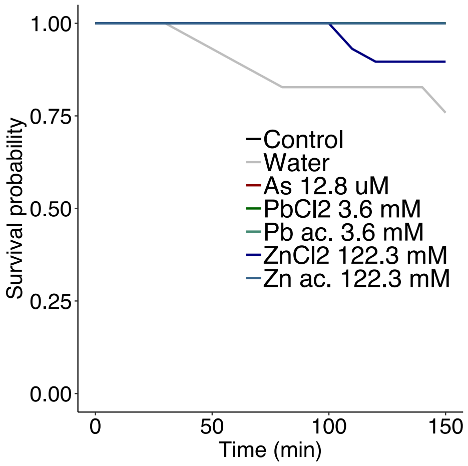

Figure S3: Survival probability over the duration of the proboscis response assay. Bees were fed 4.8 µL (equivalent of 0.4 µL ingested during each of the 12 trials) of solutions. As, Pb and Zn acetate treatments had no effect on survival. Bees exposed to Zn chloride exhibited mortality, but not different from the control bees. Bees fed with water only exhibited the highest mortality rate (Cox regression models: p<0.05).

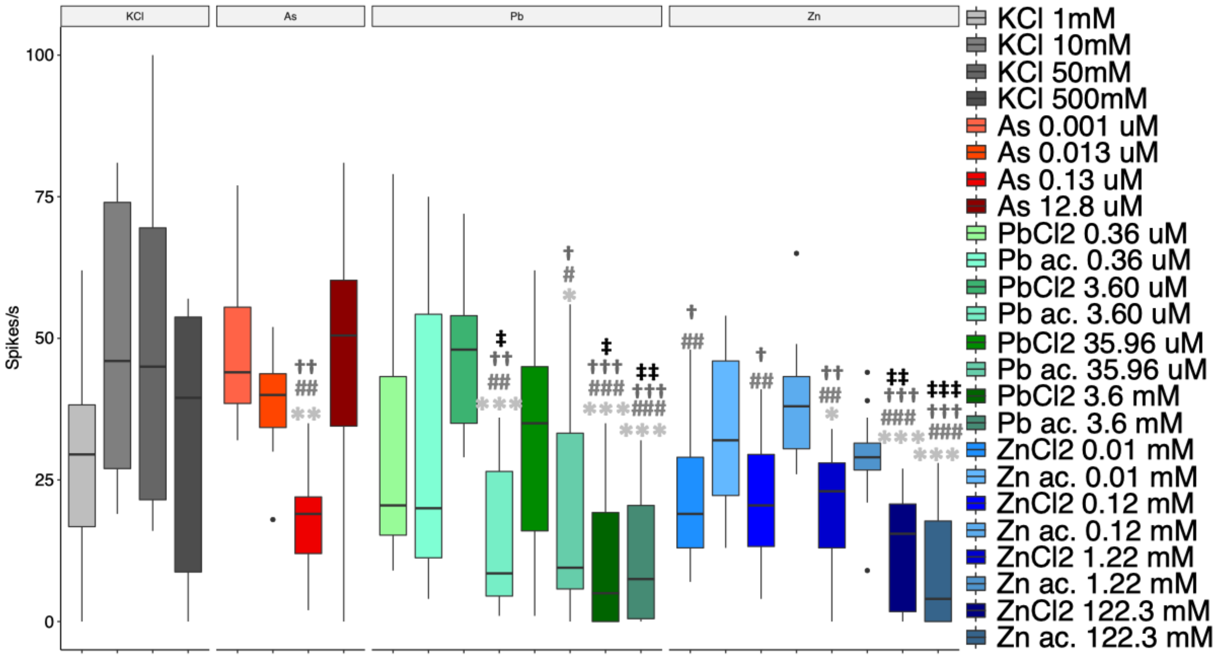

Figure S4: Electrophysiological recordings of gustatory neurons from antennal type I sensilla. Comparison of spike frequencies following stimulation with 30 mM sucrose containing either a common salt (KCl, *grey*) or metal salts (arsenic, *red*; lead, *green*; zinc, *blue*). P-values were obtained from GLMM, and comparisons to KCl 1mM (*), 10mM (#), 50 mM (†) and 500 mM (‡) are displayed (*p<0.05, **p<0.01, ***p<0.001).

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Chapter 3

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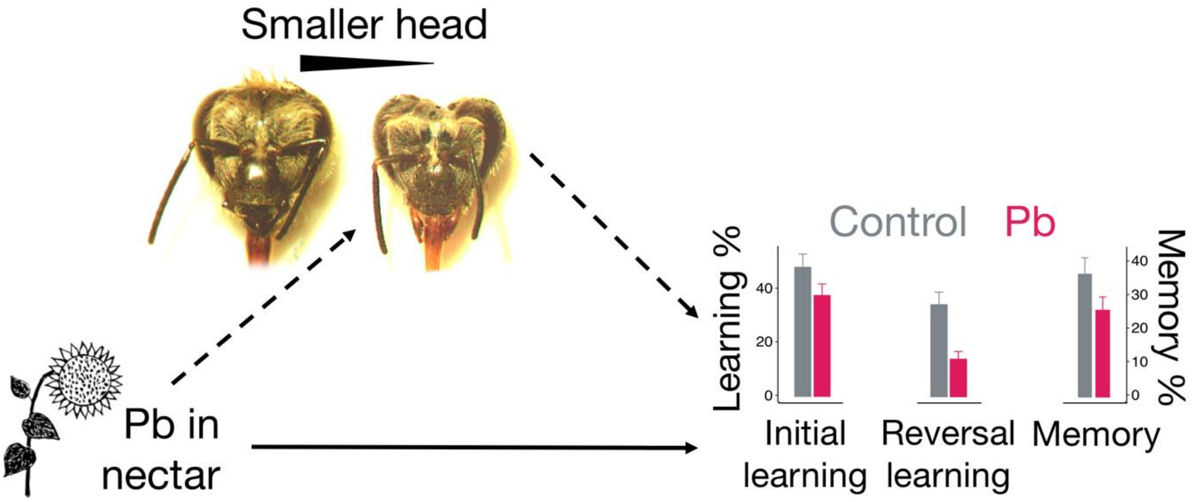
Chronic exposure to trace lead impairs honey bee learning

Highlights:

- Agrochemicals have been identified as important causes of pollinator declines.
- But major pollutants, like metallic trace elements, have received less attention.
- We exposed honey bee colonies to field-realistic concentrations of lead in food.
- Treated bees had reduced head size and cognitive performances.
- These sublethal effects, at trace levels, can impact populations and pollination.



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CHAPTER 3: Chronic exposure to trace lead impairs honey bee learning

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Abstract

Pollutants can have severe detrimental effects on insects, even at sublethal doses, damaging developmental and cognitive processes involved in crucial behaviours. Agrochemicals have been identified as important causes of pollinator declines, but the impacts of other anthropogenic compounds, such as metallic trace elements in soils and waters, have received considerably less attention. Here, we exposed colonies of the European honey bee *Apis mellifera* to chronic field-realistic concentrations of lead in food and demonstrated that consumption of this trace element impaired bee cognition and morphological development. Honey bees exposed to the highest of these low concentrations had reduced olfactory learning performances. These honey bees also developed smaller heads, which may have constrained their cognitive functions as we show a general relationship between head size and learning performance. Our results demonstrate that lead pollutants, even at trace levels, can have dramatic effects on honey bee cognitive abilities, potentially altering key colony functions and the pollination service.

Keywords: *Apis mellifera*, heavy metal pollution, PER conditioning, reversal learning, morphometry

1. Introduction

Honey bees and other central-place foraging pollinators rely on their cognitive abilities (learning and memory) to efficiently forage on flowers (Klein et al., 2017; Lihoreau et al., 2011). Yet, these abilities can be easily disrupted by some environmental stressors, even at low exposure levels (e.g. neonicotinoid insecticides: Colin et al., 2019b; Desneux et al., 2007; Henry et al., 2012). In theory, any stressor impairing brain development and/or learning processes may have subtle effects on individual's foraging capacity, with dramatic consequences on colony function, if food supply is compromised (Perry et al., 2015). Here, we focused on the possible sublethal effects of lead (Pb), a metallic trace element (MTE) with well-established neurotoxic properties in vertebrates (Chen et al., 2016; Mason et al., 2014), but whose effects on invertebrates are still poorly documented.

MTE are naturally present in the environment (Bradl, 2005). However, their widespread use in industrial and domestic applications has elevated their levels far above natural baselines in and around urbanised or industrial areas (Hladun et al., 2015; Wuana and Okieimen, 2011). Lead, in particular, is a worldwide pollutant (Cameron, 1992), which can occur at high and persistent concentrations in soils (Han et al., 2002) and in plant nectar between 0.001 and 0.075 $mg.kg^{-1}$ (Gutiérrez et al., 2020). Lead is one among the few MTE for which international permissible limit values exist (Codex Alimentarius, 2015). However, soil contamination levels are unlikely to decrease in a near future (Marx et al., 2016) and these limits defining acceptable levels of lead pollution for humans may not apply for other animals (Codex Alimentarius, 2015). Insect pollinators may be particularly exposed to airborne particles while flying (Thimmegowda et al., 2020) and to contaminated water, nectar and pollen when foraging (Formicki et al., 2013). Lead bio-accumulates in the insect body (Mertz, 1981) and it can contaminate pollen, honey and wax in the bee hive (Zhou et al., 2018) and be transferred with food to the larvae (Balestra et al., 1992). Thus, it is likely that pollinators foraging in many urbanised environments are exposed to lead at different life stages.

Lead is known to impact the survival (Hladun et al., 2016), physiology (Gauthier et al., 2016; Nikolić et al., 2019), and development of bees (Di et al., 2016), leading to adults with smaller body sizes. While exposure to lead has also been reported to impair some foraging capacities (Sivakoff and Gardiner, 2017; Xun et al., 2018), the impact on cognition has not been assessed. For bees, efficient foraging requires the capacity to associate floral cues (e.g. odorant) with the presence of food (e.g. nectar) in order to develop preferences for profitable resources (Giurfa, 2007). Since the nectar status of flowers changes with time, any such associations must be continually updated with new experience. This demands cognitive flexibility, i.e. the capacity to modify behaviour in response to environmental changes (Scott, 1962). Such flexibility, often assessed with reversal learning paradigms (Izquierdo et al., 2017), is sensitive to many sources of stress and can be impaired in humans exposed to sublethal MTE levels (Mergler et al., 1994; Rafiee et al., 2020). In honey bee foragers, reversal learning performance develops during adulthood and significantly improves at foraging onset, as does the maturation of the underlying brain circuits (Cabirol et al., 2018, 2017). We therefore hypothesised that a chronic exposure to lead could yield alterations in development and learning performances in foraging bees, as it does in mammals (Giordano and Costa, 2012; Grandjean and Landrigan, 2006; Mason et al., 2014).

Here, we tested this hypothesis by exposing caged honey bee colonies to field-realistic (low) concentrations of lead for 10 weeks and monitored impacts on the morphology and reversal learning abilities of foraging bees. Given the known impact of lead on morphological development (Di et al., 2016), we also evaluated a potential basal relationship between body size and cognitive performances in non-contaminated and uncaged bees foraging on natural plant resources.

2. Materials and methods

2.1. Bee colonies

Experiments on the effects of lead on morphology and cognition were conducted from 14/06/2019 (day 1) to 23/08/2019 (day 70), using caged bees from nine colonies of *Apis mellifera* (Buckfast) maintained in 5 frame hives (Dadant). Each colony was placed in an outside tent (3 m x 3 m) at our experimental apiary (University Paul Sabatier, France) to control the food intake and the foraging experience of bees. Each tent contained two 500 mL feeders. One feeder was filled with sucrose solution (with or without lead, see below) and the other with water. The two feeders were located 1 m apart, 2 m in front of the hive entrance. Caged colonies were given pollen patties (Icko, Bollène, France) once a week directly into the hives.

The experiments on the basal relationship between morphology and cognition were conducted from 02/2018 to 04/2018, by randomly collecting uncaged bees from a pool of 15 colonies (*A. mellifera*, Buckfast) as they foraged on an outside feeder in the same apiary. These non-contaminated bees had free access to natural plant resources.

2.2. Lead exposure

Caged colonies were assigned to one of three lead treatments (three colonies per treatment): 1. unexposed (hereafter ‘control bees’), 2. exposed to a low (0.075 $mg.L^{-1}$) concentration of lead (‘L bees’), 3. exposed to a high (0.75 $mg.L^{-1}$) concentration of lead (‘H bees’). Bees were exposed to lead by them ingesting 50% (w/v) sucrose solution from the feeder, to which lead (II) chloride ($PbCl_2$) (Sigma-Aldrich, Lyon, France) was added. The low and high lead concentrations fell within the range of concentrations measured in natural flowers (Eskov et al., 2015; Gutiérrez et al., 2020; Maiyo et al., 2014; Uren et al., 1998) and honey (Ajtony et al., 2007; Naggar et al., 2013; Satta et al., 2012). Both concentrations are sublethal to adult honey bees (LC_{50}: 345 $mg.L^{-1}$) (Di et al., 2016). Control hives were fed 50% (w/v) sucrose solution. Feeders were refilled daily so that bees had an *ad libitum* access to food.

Caged hives were maintained in these conditions for 70 days. This duration was long enough for colonies to store contaminated food, so that nectar foraging bees sampled for the cognitive assays were likely to have ingested lead during their development. On average, colonies consumed 8.5±0.6 (SE) kg of sucrose solution and 616±25 (SE) g of pollen during the experiment (N=9). During this period, we kept track of the foraging experience of all the nectar foragers (number of days since the onset of foraging) by paint-marking bees with a colour code while feeding on the sucrose solution feeder (Posca pen, Tokyo, Japan). Each day was encoded with a new combination of colours. This operation was repeated twice everyday (1 h in the morning, 1 h in the afternoon).

2.3. Lead quantification

Lead levels were analysed in samples of the sucrose solution and bees from caged hives using Inductively Coupled Plasma Emission Spectroscopy (ICP-OES, quantification limit: 5 – 20 μg.kg^{-1}, precision measure: 1–5%; AMETEK Spectro ARCOS FHX22, Kleve, Germany).

Our ability to detect lead was first verified by assaying the lead level in our high lead concentration sucrose solution (0.75 mg.L^{-1}). The solution was acidified at 3% of HNO_3 with ultra-pure 69% HNO_3 to avoid precipitation or adsorption in containers. The solution was then diluted with a HNO_3 3% solution to reduce the spectral interference and viscosity effects. With this method, the amount of lead was recovered at 96% (nominal concentration: 0.75 mg.L^{-1}, actual concentration: 0.71 mg.L^{-1}).

The fact that bees exposed to different concentrations of bio-accumulated lead in a dose-dependent manner was then verified. Lead content was assessed in bees collected 30 days after the start of the exposure (i.e. midway through the experiment). For each sample, bees were pooled in batches of five. Each batch was rinsed with 5 mL HNO_3 at 3% for 30 s. Bees were wet mineralised in 50 mL polypropylene tubes using a Digiprep system (SCP Science, Quebec, Canada) with 5 mL of 69% nitric acid, following a protocol for athropods (Bur et al., 2012; Astolfi et al. 2020). This consisted of a digestion phase carried out at room temperature overnight, followed by a second phase of heating at 80 °C for 60 min. The nitric acid was evaporated, and the samples were diluted with 9 mL of 3% HNO_3. Final solutions were at 3% HNO_3 and total dissolved solids below 5%.

Certified reference materials (CRMs) were used as quality controls to validate the protocol of mineralization and multi-elementary ICP analysis: waters (SLRS-6, SUPER-05, ION-96.4) and a solid arthropod CRM (PRON-1 river prawn reference material). Recovery coefficients (ratios measured *vs.* certified values) for major and trace elements ranged between 85% and 115%.

2.4. Colony dynamics

The effect of lead exposure on colony dynamics was assessed in the caged colonies through continuous measurement of hive parameters in the caged colonies. Hive weight (± 0.01 kg) was recorded every hour with an electronic scale (BeeGuard, Labège, France) below each hive. Every two weeks hives were opened and pictures of both sides of each frame were taken with a Panasonic Lumix DMC-FZ200 equipped with a F2.8 25–600 mm camera lens. From the pictures, areas of capped brood and food stores were estimated using CombCount (Colin et al., 2018). Each frame was weighted, after gently removing the adult bees, and the total weight of adult bees (total adult bee mass) was determined by subtracting the tare of the hive and the weight of the frames from the weight of the hive.

2.5. Learning assays

The cognitive performances of bees from caged and uncaged colonies were assessed using olfactory conditioning of the proboscis extension reflex (PER; Giurfa & Sandoz, 2012). Overall, 268 bees from caged colonies were tested (84 control bees, 84 L bees, 100 H bees). These bees were exposed to lead for their whole life (foragers exposed from larvae to foraging age, collected between days 46 and 70 from the start of lead treatment) and originated from 8 of the 9 colonies (one control hive showed very low foraging activity). We focused on new foragers (between 24 and 48 h after the onset of foraging) to avoid inter-individual cognitive variation caused by differences in foraging experiences (Cabirol et al., 2018). Another 149 bees from uncaged colonies were tested. Neither the age nor the foraging experience of these bees were controlled.

All bees were submitted to a reversal learning task, i.e. a two-stage task assessing the cognitive flexibly of bees in response to changes in flower rewards (Raine and Chittka, 2007). This test mimics the natural situation where one floral species ceases producing nectar before another species starts doing so. Phase 1 is a differential learning phase, in which the bees must learn to differentiate an odour A reinforced with sucrose (50% w/v in water) and an odour B not reinforced (A+ *vs*. B-). Phase 2 is a non-elemental learning phase, in which the bees must learn the opposite contingency (A- *vs*. B+). We used pure limonene and eugenol (Sigma-Aldrich, Lyon, France) as odours A or B alternately on successive days, so that each contingency was used for about half of the bees for each treatment.

On the morning of each test, foragers (24–48 h after onset of foraging) were collected on the feeders, cooled on ice and harnessed in restraining holders that allowed free movements of their antennae and mouthparts (Matsumoto et al., 2012; Fig. 1A). Turning of the head was prevented by fixing the back of the head with melted bee wax. All bees were then tested for PER by stimulating their antennae with 50% sucrose solution. Only those that responded for the conditioning phases (77% of all bees tested) were kept for the experiments. These bees were fed 5 µL of sucrose solution and left to rest in a dark incubator for 3 h (temperature: 25±2°C, humidity: 60%).

Bees were then trained using an automatic stimulus delivery system (Fig. 1A; Aguiar et al., 2018). Each training phase included five trials with the reinforced odorant and five trials with the non-reinforced odorant in a pseudo-random order with an eight-minute inter-trial interval. Each conditioning trial (37 s in total) started when a bee was placed in front of the stimulus delivery system, which released a continuous flow of clean air (3,300 mL.min^{-1}) to the antennae. After 15 s, the odour was introduced to the airflow for 4 s. For rewarded odours, the last second of odour presentation overlapped with sucrose presentation to the antennae using a toothpick soaked in sucrose solution (Fig. 1A) and sucrose feeding by presenting the toothpick to the mouthparts for 4 s. For the unrewarded trials, no sucrose stimulation was applied. The bee remained another 15 s under the clean airflow. Bees were kept in the incubator for 1 h between the two learning phases (A+ *vs*. B- and A- *vs*. B+).

During conditioning, we recorded the presence or absence of a conditioned PER to each odorant at each trial (1 or 0). Each bee was given a learning score for phase 1 (1 if the bee responded to A+ and not to B- in the last trial of phase 1, 0 otherwise) and for phase 2 (1 if the bee responded to B+ and not to A- on the last trial, 0 otherwise) (Cabirol et al., 2018). Short-term memory (1 h) was assessed by comparing the responses at the last trial of phase 1 and the first trial of phase 2. Each bee was given a memory score for the two odorants (1 if the bee still responded appropriately to the A+ and B- on the first trial of the phase 2, 0 otherwise).

2.6. Morphometry

Developmental differences among bees was evaluated by conducting morphometric measures on frozen individuals (-18 °C) from caged and uncaged hives.

To test the effect of lead exposure on morphology in caged bees, foragers of unknown age were collected on the day before lead exposure (day 0 of the experiment), during lead exposure (day 53 of the experiment) and at the end of the experiment (day 70 of the experiment), and their head length and head width were measured (Fig. 2A). Emerging adult bees were also sampled every week from each hive (before exposure, during exposure, and at the end of the exposure period). For each bee, the fresh body weight (± 0.001 g) (precision balance ME103T, Mettler-Toledo Gmbh, Greifensee, Switzerland) and eight morphometric parameters were recorded: head length, head width, forewing length, forewing width, femur length, tibia length, basitarsus length, basitarsus width (Fig. 2A; De Souza et al., 2015; Mazeed, 2011).

To test for a relationship between morphology and cognitive performances in the uncaged bees, the head length and head width of the conditioned bees hives were measured after the conditioning experiments. All measurements (± 0.01 mm) were taken using a Nikon SMZ 745T dissecting scope (objective x0.67) with a Toupcam camera model U3CMOS coupled to the ToupView software.

2.7. Statistics

All analyses were performed with R Studio v.1.2.5033 (RStudio Team, 2015). Raw data are available in Dataset S1. Lead content of bees was compared using a Kruskal-Wallis test (package

FSA; Ogle et al., 2019). The effects of lead exposure on colony parameters were evaluated with a multi-model approach (MMI), with treatment, time since the beginning of the exposure (standardised using rescale function, package arm; Gelman & Su, 2013) and their interaction as fixed effects, and hive identity as random factor. A model selection (package MuMIn; Barton, 2020) was run and conditional model average was applied to evaluate the effects of the different factors on the response variables. A MMI was run followed by a conditional model average to assess the effects of treatment, time of exposure and their interactions on brood area (square-root transformed), food stores area and total adult bee mass.

For learning assays, proportion tests were used, followed by pairwise comparisons with a Bonferroni correction (package RVAideMemoire; Hervé, 2020), to evaluate whether lead exposure changed sucrose responsiveness (i.e. proportions of unresponsive bees across treatments). Generalised linear mixed-effects models (GLMM) (package lme4; Bates et al., 2015) were performed to evaluate the effect of treatment on the behavioural variables (PER responses, learning, reversal and memory scores). Proportions of successful responses during the fifth trial of each learning phase were compared using a binomial GLMM, with odorants, treatments and their interactions as fixed effects, and bee identity nested in the hive identity as random factors. A similar GLMM was run to compare the learning, reversal and memory scores, with hive identity as random factor.

For the morphometric analyses on caged bees, LMMs were used for each parameter, considering treatment as a fixed effect, and hive identity as a random factor. To assess the global effect of lead, the nine parameters were collapsed into a principal component analysis (PCA) (package FactoMineR, Lê et al., 2008). Bees were clustered into subgroups based on PCA scores, and clusters were compared with a permutational multivariate analysis of variance (PERMANOVA; package vegan; Oksanen et al., 2019). A LMM was run on individual coordinates from the PCA, with treatment as a fixed effect, and hive identity as a random factor. To assess the effect of head size on the cognitive performances of uncaged bees, head width and head length measures were collapsed into the first component of a PCA and a binomial GLMM was run on learning, memory and reversal scores, with individual coordinates from the PCA as fixed effect, and test day as random factor.

3. Results

3.1. Exposure to high lead concentration reduced learning performance

We assessed the effect of lead exposure on cognitive flexibility by conducting reversal learning assays in caged bees. The proportion of bees that responded to the antennal stimulation of sucrose was similar across treatments (control bees: 74% N=113; L bees: 69% N=122; H bees: 76% N=132; Chisq=1.423, df=2, p=0.491), indicating that lead exposure did not affect appetitive motivation or sucrose perception.

Treatment had no significant effect on learning phase 1, although H bees tended to perform less well (Fig. 1B-C). Upon the last trial of phase 1, bees from all treatments discriminated the two odorants (Binomial GLMM: $p < 0.001$ for all treatments), and exhibited similar response levels to odour A (Binomial GLMM: L bees p=0.877; H bees p=0.206) and B (Binomial GLMM: L bees p=0.331; H bees p=0.459). The proportions of bees that learned to discriminate the two stimuli (learning score equals to 1) were similar across treatments (Control: 48%; L bees: 43%; H bees: 37%) (Fig. 1C; Table S1). These results were independent of the odours used as stimuli A+ and B- (Binomial GLMM: $F_{1,266=}0.905$, p=0.526). The proportion of learners at the end of the first phase was similar across hives, within each treatment group. Therefore, exposure to lead, had no significant effect on performance in the differential conditioning task.

Treatment did not significantly affect short-term memory between the two phases neither (Fig. 1D). Bees from all treatments had similar memory scores (Binomial GLMM: L bees p=0.873; H bees p=0.115). However, H bees had a reduced percentage of correct responses between the two phases (25% compared to 36% for control bees).

By contrast, treatment had a clear effect on learning in phase 2 (Fig. 1E-F). Upon the last trial, control and L bees were able to discriminate the two odorants (Binomial GLMM: Control p<0.001; L bees p=0.007), but not H bees (Binomial GLMM: p=0.075). The response level to

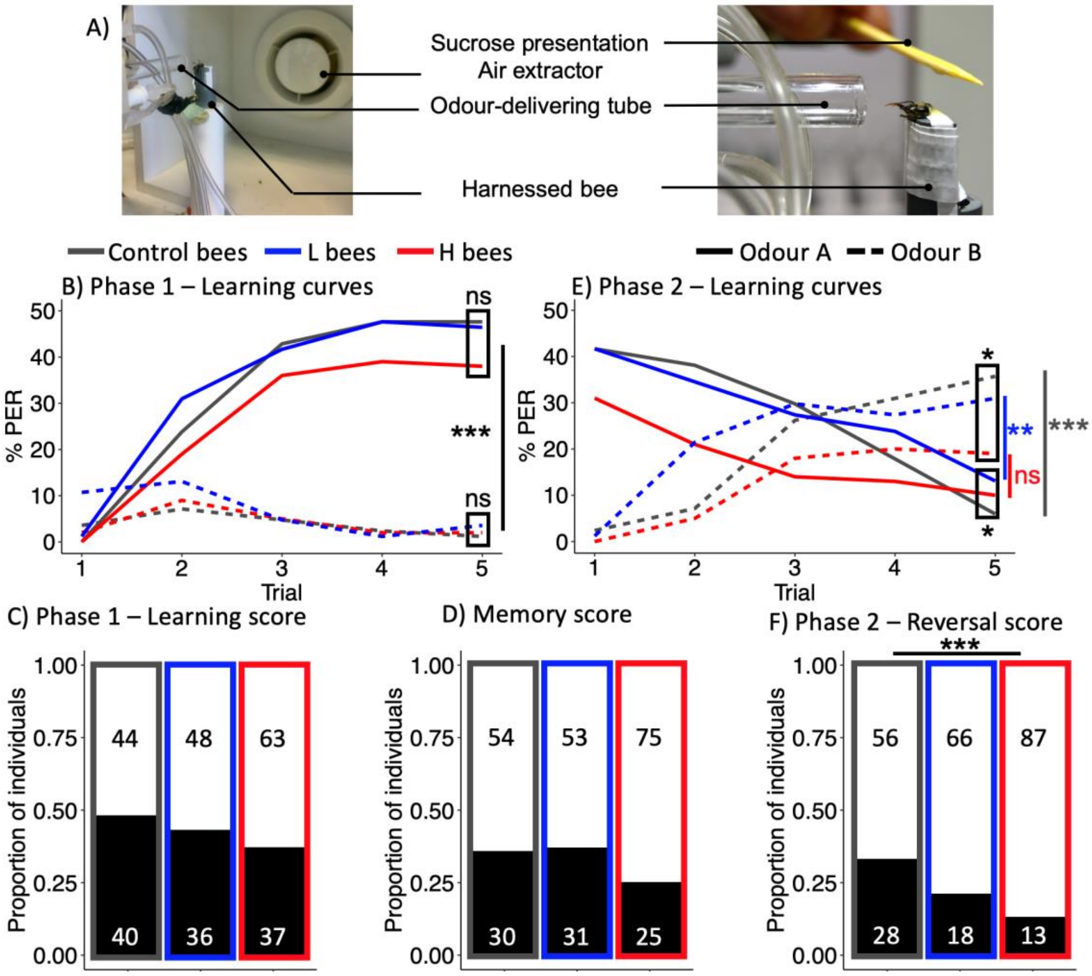

Figure 1: Learning and memory performances of bees from caged hives exposed to lead treatments. A) Picture of a harnessed bee in the conditioning set-up. **B), E)** Line plots show the percentage of proboscis extension responses (PER) elicited by odour A (solid line) and odour B (dashed line) during phase 1 **(B)** and phase 2 **(E)** of reversal learning. Control bees (N=84, dark grey), bees exposed to a low concentration of lead (L bees: 0.075 mg.L^{-1}; N=84, blue) or a high concentration of lead (H bees: 0.75 mg.L^{-1}; N=100, red). Statistical comparisons of the response level at the last trial were obtained with p-values from the binomial GLMM (see details in Table S1). **C), D), F)** Bar plots show the proportions of learners (black) and non-learners (white) in the last trial of phase 1 **(C)** and phase 2 **(F)**, with sample size displayed. **D)** Bar plots show the proportions of bees remembering (black) or not (white) during the 1 h memory recall, with sample size displayed. Statistical comparisons were obtained with p-values from the binomial GLMM (Table S1) (ns: non-significant, p>0.05; *p<0.05; **p<0.01; ***p<0.001).

odours A and B was similar between control and L bees (Binomial GLMM: odour A p=0.097; odour B p=0.513), but H bees responded less to odour B (Binomial GLMM: p=0.012) and more to odour A (Binomial GLMM: p=0.032) compared to control. Consequently, H bees exhibited lower reversal scores (13% of learners) than L bees (21%) and controls (33%) (Binomial GLMM: L bees, p=0.086; H bees, p=0.001) (Table S1, Fig. 1F). There was no effect of the odours used as stimuli A- and B+ (Binomial GLMM: $F_{1,266=}1.300$, p=0.636), nor of the hive, on the proportion of learners within treatment groups. Therefore, exposure to a high concentration of lead reduced the performance of bees in the reversal learning task.

The dose-dependent effect of lead exposure on bee cognition was correlated with dose-dependent bio-accumulation of lead in bees. Control bees and L bees showed no difference in lead content (controls: 0.126±0.031 mg.kg^{-1} d.m., N=3; L bees: 0.130±0.002 mg.kg^{-1} d.m., N=3; Kruskal-Wallis: H=7.636, df=1, p=0.712), whereas H bees accumulated significantly more lead (H bees: 0.809±0.044 mg.kg^{-1} d.m., N=5; Kruskal-Wallis: H=7.636, df=1, p=0.039). This result was also independent from any influence of the state of the colony, since lead treatment had no effect on colony measures (syrup and pollen consumption, dynamics of brood production, size of food stores, total adult bee mass, colony weight; LMM: Treatment effect: p > 0.05 for all parameters; for further details see Fig. S1).

3.2. Bees exposed to the high lead concentration were shorter with smaller heads

Given the observed effects of chronic exposure on the cognitive flexibility of foragers, we asked whether this might result from compromised development. We measured head size in individuals from the different caged hives. Foragers of unknown age collected on the day before the beginning of treatment (day 0) had similar head measurements irrespective of treatment (LMM: L bees: head length p=0.296, head width p=0.287; H bees: head length p=0.333, head width p=0.394). Foragers collected in the middle (day 53) and at the end (day 70) of the experiment had significantly smaller heads than controls (LMM: L bees: head length p=0.017, head width p=0.456; H bees: head length p<0.001, head width p=0.040; Table S2).

To better assess this developmental impact of lead exposure, we also collected bees at adult emergence, thereby considering only the preimaginal period. For this analysis, we included different body measures in addition to head length and width (Fig. 2A), and used them to perform a PCA (Fig. 2B, Table S3). Two PCs explaining 58% of the variance were sufficient to separate control bees and H bees into two distinct clusters, while L bees were intermediate (PERMANOVA: Pseudo-F=5.575, p=0.002; control bees *vs.* L bees: p=0.975; C bees *vs.* H bees: p=0.003; L bees *vs.* H bees, p=0.189). We focused on PC1 which explained 45.8% of the total variance and was associated with general body size. PC1 was negatively correlated with lead concentration (LMM: p=0.042), so that the H bees tended to be smaller than L bees and control bees (Table S4). H bees displayed a rather homogeneous decrease in most parameter values, resulting in a notable weight loss of ca. 8.33% (Table S4).

The fact that emerging and foraging bees exhibited a similar decrease in head size (LMM: age effect p>0.05; Tables S2, S4) suggests that most of the impact of lead exposure on morphology occurred before the adult stage.

3.3. Unexposed bees with larger heads showed better learning performance

Because the above data suggests a link between lead-induced learning impairment and alterations of head development in our caged bees, we tested the possibility of a general correlation between performance at adulthood and head size, irrespective of lead treatment. For this, we submitted unexposed adult bees from uncaged hives to a reversal learning task (N=149). We separated bees according to their learning, memory and reversal scores (see Methods), in order to compare the morphometric characteristics of bees with different levels of performance. We ran a PCA on this subset of bees, and used the first component (PC1, 73% of the morphological variance), which collapsed head width and length, as a proxy of overall head size (Fig. 3). In phase 1 of reversal learning, the proportion of learners (79% N=118) increased with head size (Fig. 3A), as did the short-term memory recall (46% N=68) (Fig. 3B). In phase 2, the proportion of learners (18% N=27) also increased with head size (Fig. 3C). Therefore, bees with larger heads showed better learning and memory performances in absence of any cage confinement or lead treatment.

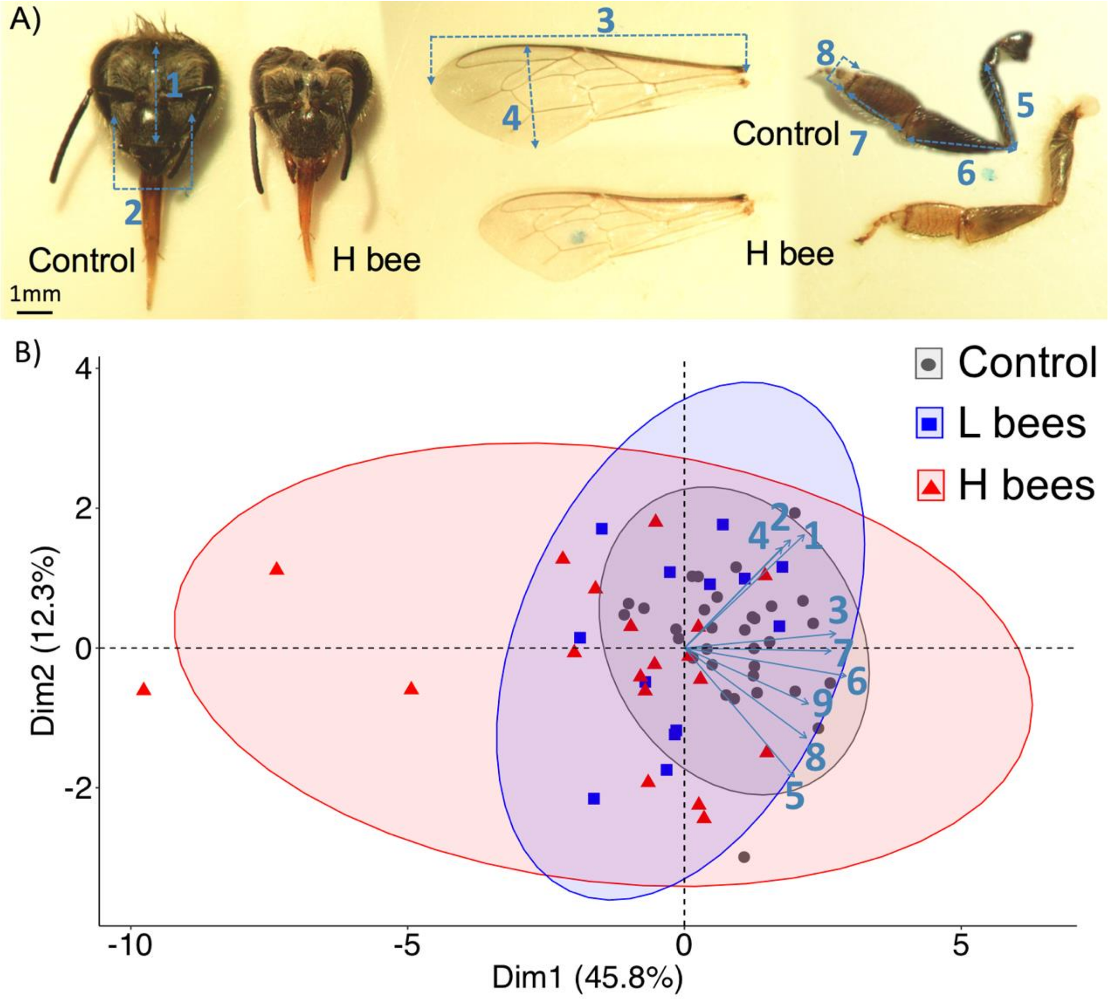

Figure 2: Morphometric analysis of bees from caged hives exposed to lead treatments. A) Details of the parameters measured. This example shows morphological differences in emerging bees. (1) Head length, (2) Head width, (3) Wing length, (4) Wing width, (5) Femur length, (6) Tibia length, (7) Basitarsus length, (8) Basitarsus width, (9) Bee weight (not shown). **B)** Principal component analysis (PCA) map shows the relationship among the morphometric measures (same number code as in A). 95% confidence ellipses of the mean are displayed for each treatment. Controls: bees unexposed to lead (N=32); L bees: bees exposed to the low concentration of lead (0.075 mg.L^{-1}) (N=13); H bees: bees exposed to the high concentration of lead (0.75 mg.L^{-1}) (N=19).

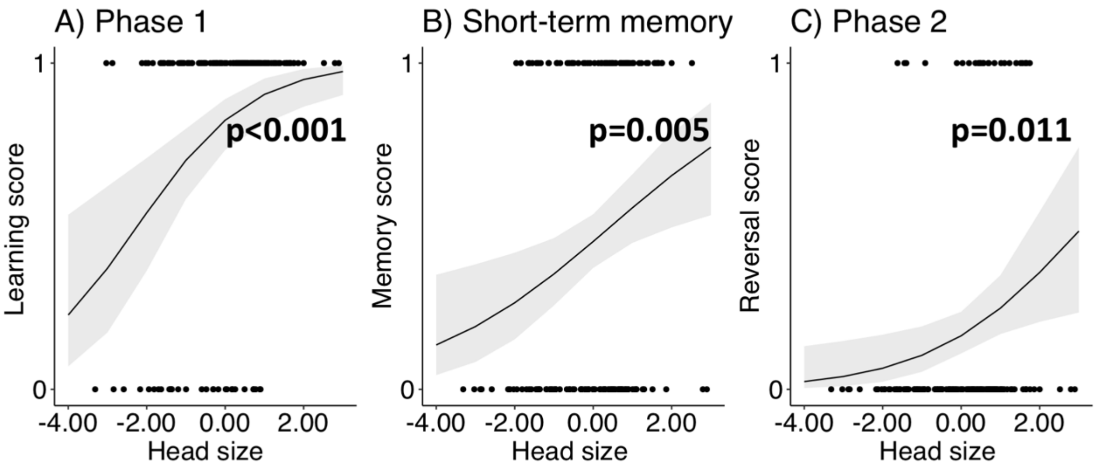

Figure 3: Relationship between head size and cognitive performance in bees from uncaged hives unexposed to lead treatments. Data points represent the individual data for learners (learning score=1) and non-learners (learning score=0). Fitted lines of head size effect are displayed in black with 95% confidence intervals in grey. N=149 bees. **A)** Learning score at the end of phase 1. **B)** Short-term memory score. **C)** Reversal score at the end of phase 2. Statistical comparisons were obtained with p-values from the binomial GLMM testing bees coordinates in PC1 on cognitive scores, significant values (<0.05) are shown in bold. Increasing head size significantly enhanced the learning performances in phase 1 (Binomial GLMM: estimate±SE, 0.693±0.188, p<0.001) and phase 2 (0.523±0.205, p=0.011), as well as short-term memory recall (0.415±0.149, p=0.005).

4. Discussion

Recent studies suggest that MTE can have sublethal effects on individual bees, with potential detrimental consequences for colonies and the pollination service through altered foraging behaviour (Burden et al., 2019, 2016; Skaldina and Sorvari, 2019; Søvik et al., 2015). Here, we found that honey bees chronically exposed to trace concentrations of lead in food have reduced body sizes and learning abilities. The positive correlation between head size and learning performances in unexposed bees suggests that consumption of lead affects bee development, by reducing head size and cognitive function, and thus constitutes a significant neurocognitive stressor for bees at field realistic levels.

Chronic exposure to trace lead led to reduced cognitive performance in an olfactory appetitive condition task. This assay reproduces a foraging context in which bees need to learn olfactory cues signalling the presence or absence of nectar. Neither differential learning (first learning phase) nor short-term memory were affected. However, we found a decreased performance in reversal learning (second learning phase). Thus, the treatment we used did not induce a general impairment of olfactory discrimination nor a decreased motivation for sucrose. This contrasts with the decreased responsiveness to sucrose exhibited in bees acutely treated with lead at similar concentrations (Burden et al., 2019), suggesting a different impact of chronic lead exposure on bees. The specific impairment of reversal learning indicates a loss of cognitive flexibility, which is crucial for bee foragers to switch preferences for flowers whose value changes over time (Ferguson et al., 2001). Over the long-term, this sublethal impact on individual cognition may compromise the overall foraging efficiency of a colony exploiting changing resources, and thus its survival.

Reversal learning has been shown to be more strongly affected by lead exposure than seemingly simpler differential learning in rats (Hilson and Strupp, 1997), monkeys (Bushnell and Bowman, 1979) and humans (Evans et al., 1994). These tasks measuring cognitive flexibility are particularly sensitive to the adverse effects of stressful stimuli, or of neurodevelopmental disorders (Dajani and Uddin, 2015). Just like mammals (Schoenbaum et al., 2000), honey bees rely on specific brain regions to perform reversal learning, which are not essential for simple differential conditioning (i.e. phase 1 of the conditioning task in our protocol). These are the mushroom bodies (MBs) (Boitard et al., 2015; Devaud et al., 2007), whose maturation over adulthood relates to the acquisition of the capacity for reversal learning (Cabirol et al., 2017, 2018). Interestingly, adult MB organisation is altered following exposure to several forms of stress in bees (Cabirol et al., 2017; Peng and Yang, 2016) and other insects (Jacob et al., 2015; Wang et al., 2007). Thus, the specific reversal impairment of lead-exposed bees might be due to neural circuits being more sensitive to the impact of lead in the MBs than in other brain regions.

Lead exposure is known to impair brain excitation/inhibition balance during development, through multiple effects such as loss of GABAergic interneurons (Stansfield et al., 2015), altered maturation of GABAergic neurons (Wirbisky et al., 2014), decrease in GABA and glutamate release (Xiao et al., 2006) or transport (Strużyńska and Sulkowski, 2004), or inhibition of post-synaptic glutamatergic action (Neal and Guilarte, 2010). In insects, although no specific effect of lead on GABAergic signalling has been demonstrated yet, the effects of lead exposure on synaptic development (Morley et al., 2003), presynaptic calcium regulation (He et al., 2009) and acetylcholinesterase activity (Nikolic et al., 2019) are compatible with a disruption of the excitation/inhibition balance. It has been proposed that reaching an optimal value for such balance in MB circuits is what determines efficient reversal learning in mature adults (Cabirol et al., 2017, 2018). If this is somehow disrupted following lead exposure, that would explain the specific impairment observed only during the reversal phase of the task.

Importantly, all bees had undergone their larval and pupal stages during the exposure period, providing ample opportunity for the detrimental effects of lead to be caused by larval ingestion of contaminated food brought by foragers. Lead alters larval development in flies and bees (Cohn et al., 1992; Di et al., 2016; Safaee et al., 2014). Further evidence supports the hypothesis of a developmental effect of lead, since bees exposed to the highest concentrations developed lighter bodies, with shorter wings, and smaller heads. In bees, head width is correlated with the volume of the brain (honey bee foragers: Gronenberg & Couvillon, 2010; bumblebees: Riveros & Gronenberg, 2010) and the MBs (honey bee foragers: Mares et al., 2005; bumblebees: Smith et al., 2020). Here, we also found that for bees that had not been exposed to lead, those with smaller and shorter heads had a lower learning performance. This suggests there is a general relationship between head size and cognitive performance in a reversal learning task. We did not control for the age of the measured individuals in this part of the study. However, possible age variations among foragers are unlikely to cause any significant head size changes, since this would be expected to stabilise once the adult cuticle is hardened. In addition, reversal learning performance tend to decrease with foraging experience (Cabirol et al, 2018). It is thus unlikely that bees with larger heads in our sample were those that foraged for shorter times. Our results do not necessarily suggest that such a relationship should be expected for all cognitive tasks. Because control bees with larger heads performed better in both phases of the task, and exposed bees with larger heads only performed better in the reversal task, we assume that lead altered brain development in a specific way resulting in a stronger impact on development or performance of MB neural networks.

Continuous exposure to environmentally realistic amounts of lead resulted in bioaccumulation of the metal in the bees' bodies. This is likely to have impaired aspects of head and brain development during larval and pupal stages, resulting in adults with deficits in cognitive flexibility in an ecologically relevant cognitive task. Although this mechanistic hypothesis remains to be confirmed, our results clearly indicate a sublethal impact of lead exposure with potential consequences on foraging efficiency. Importantly, the lead contents measured in the bodies of exposed bees in our experiments ranged within the measurements from bees in field conditions (Goretti et al., 2020). The two concentrations of lead in the sucrose solutions used for chronic exposure (0.075 and 0.75 mg.L^{-1}) fell below the maximum level authorised in food (3 mg.kg^{-1}; Codex Alimentarius, 2015) and irrigation water (5 mg.L^{-1}; Ayers & Westcot, 1994), and the lowest concentration was under the threshold set for honey by the European Union (0.10 mg.kg^{-1}; Commission Regulation (EU) 2015/1005, 2015). This indicates that the cognitive and developmental impairments identified in our experimental conditions may be affecting bees foraging on flowers in many contaminated environments.

Although our experiment and recent similar approaches (Hladun et al., 2016) did not capture any consequences on colony dynamics, these individual effects observed over several weeks might ultimately alter colony function, in particular if lead exposure impairs a broader range of behaviours (e.g. communication, feeding, defence). Thus, differences in colony performances could be predicted over longer term (Klein et al., 2017), which might contribute to collapse, as observed for pesticide exposure at sublethal concentrations (Colin et al., 2019a; Meikle et al., 2016). Our results thus call for future studies to better characterise the impact of lead exposure in bee populations, including in combination with other MTE as such cocktails are often found in contaminated areas (Badiou-Bénéteau et al., 2013; Goretti et al., 2020). More generally, a better assessment of the contribution of heavy metal pollutants to the widespread decline of insects has become an urgent necessity for preserving ecosystem services.

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Supporting materials

Table S1: Parameter estimates from the binomial GLMM for response levels at the end of both learning phases, and for learning, reversal and memory score models in bees from caged hives exposed to lead treatments. Significant p-values (<0.05) are shown in bold. SE=conditional standard errors.

Conditional average	Estimate	SE	p-value
PER response at the end of Phase 1			
Intercept	-0.1002	0.2303	0.664
Low concentration	-0.0505	0.3266	0.877
High concentration	-0.4146	0.3277	0.206
Odour B-	-4.4110	1.0472	**<0.001**
Low concentration:Odour B-	1.1762	1.2091	0.331
High concentration:Odour B-	0.9418	1.2733	0.459
PER response at the end of Phase 2			
Intercept	-2.7600	0.4611	**<0.001**
Low concentration	-0.2146	0.3280	0.513
High concentration	-0.8622	0.3418	**0.012**
Odour A-	-2.1722	0.5143	**<0.001**
Low concentration:Odour A-	1.0820	0.6518	0.097
High concentration:Odour A-	1.4250	0.6638	**0.032**
Learning score			
Intercept	-0.0953	0.2185	0.663
Low concentration	-0.1924	0.3104	0.535
High concentration	-0.4369	0.3010	0.147
Memory score			
Intercept	-0.5878	0.2277	**0.010**
Low concentration	0.0515	0.3209	0.873
High concentration	-0.5108	0.3243	0.115
Reversal score			
Intercept	-0.6931	0.2315	**0.003**
Low concentration	-0.6061	0.3525	0.085
High concentration	-1.2078	0.3768	**0.001**

Table S2: Analysis of the morphological parameters of forager bees from caged hives exposed to lead treatments. Median, minimum and maximal values of each morphological parameter of forager bees from caged hives, per treatment and percentage of variation between medians compared to control bees. Estimated regression parameters, standard errors (SE) and p-values of the linear mixed effects models. Significant differences with control group ($p<0.05$) are shown in bold.

Morphological parameters	**Treatment**	**Median (min-max)**	**Variation compared to control**	**Estimate±SE**	**p-value**
Head length (mm)	Control	2.88 (2.55-3.07)			
	Low concentration	2.78 (2.33-2.99)	-3.60%	-0.1054±0.0432	**0.017**
	High concentration	2.69 (2.42-2.87)	-7.06%	-0.1877±0.0395	**<0.001**
Head width (mm)	Control	2.42 (2.27-2.62)			
	Low concentration	2.41 (2.16-2.52)	-0.41%	-0.0294±0.0354	0.456
	High concentration	2.30 (2.18-2.48)	-4.99%	-0.0990±0.0324	**0.040**

Table S3: Principal component analysis (PCA) on the morphometry of emerging bees from caged hives exposed to lead treatments. Correlation coefficients >0.4 in absolute value are shown in bold.

Variable	**PC1**	**PC2**
Bee weight	**0.654**	-0.233
Head length	**0.633**	**0.474**
Head width	**0.560**	**0.452**
Wing length	**0.799**	0.060
Wing width	**0.516**	**0.421**
Femur length	**0.580**	**-0.539**
Tibia length	**0.854**	0.117
Basitarsus length	**0.773**	-0.012
Basitarsus width	**0.644**	-0.376
% Total variance	45.84	12.32
Cumulative proportion of total variance	45.84	58.17

Table S4: Analysis of the morphological parameters of emerging bees from caged hives exposed to lead treatments. Median, minimum and maximal values of each morphological parameter of emerging bees from caged hives, per treatment and percentage of variation between medians compared to control bees. Estimated regression parameters, standard errors (SE) and p-values of the linear mixed effects models. Significant differences with control group (p<0.05) are shown in bold.

Morphological parameters	Treatment	Median (min-max)	Variation compared to control	Estimate±SE	p-value
Bee weight (g)	Control	0.12 (0.10-0.14)			
	Low concentration	0.11 (0.06-0.12)	-9.35%	-0.0108±0.0064	0.142
	High concentration	0.11 (0.06-0.013)	-8.33%	-0.0173±0.0058	**0.029**
Head length (mm)	Control	2.89 (2.67-3.03)			
	Low concentration	2.90 (2.47-2.97)	0.34%	-0.0712±0.0679	0.365
	High concentration	2.65 (2.15-3.01)	-9.06%	-0.2021±0.0615	**0.050**
Head width (mm)	Control	2.42 (2.24-2.71)			
	Low concentration	2.41 (2.23-2.58)	-0.21%	-0.0339±0.0501	0.530
	High concentration	2.32 (2.02-2.47)	-4.09%	-0.1624±0.0452	**0.022**
Wing length (mm)	Control	8.79 (8.42-9.08)			
	Low concentration	8.84 (8.39-9.03)	0.62%	-0.0030±0.1199	0.981
	High concentration	8.75 (7.57-8.96)	-0.40%	-0.2846±0.1086	**0.048**
Wing width (mm)	Control	3.12 (2.71-3.35)			
	Low concentration	3.10 (2.72-3.34)	-0.81%	-0.0547±0.0506	0.285
	High concentration	3.13 (2.64-3.38)	0.16%	-0.0331±0.0447	0.462
Femur length (mm)	Control	2.30 (2.15-2.53)			
	Low concentration	2.26 (2.08-2.40)	-1.77%	-0.0680±0.0491	0.227
	High concentration	2.27 (1.90-2.46)	-1.32%	-0.0718±0.0442	0.178
Tibia length (mm)	Control	3.04 (2.90-3.18)			
	Low concentration	3.04 (2.81-3.15)	0%	-0.0532±0.0608	0.430
	High concentration	3.04 (2.60-3.14)	0%	-0.0916±0.0560	0.189

Basitarsus length (mm)	Control	2.06 (1.94-2.24)			
	Low concentration	2.05 (1.95-2.15)	-0.73%	-0.0247±0.0537	0.665
	High concentration	2.04 (1.63-2.21)	-1.23%	-0.0634±0.0492	0.264
Basitarsus width (mm)	Control	1.16 (1.05-1.40)			
	Low concentration	1.16 (1.04-1.25)	0%	-0.0066±0.0371	0.868
	High concentration	1.10 (0.95-1.27)	-5.45%	-0.0690±0.0337	0.118
Bees coordinates in PC1	Control	1.01 (-1.09-2.63)			
	Low concentration	-0.17 (-1.89-1.77)		-0.9940±0.9397	0.346
	High concentration	-0.65 (-9.77-1.49)		-2.6526±0.8607	**0.042**

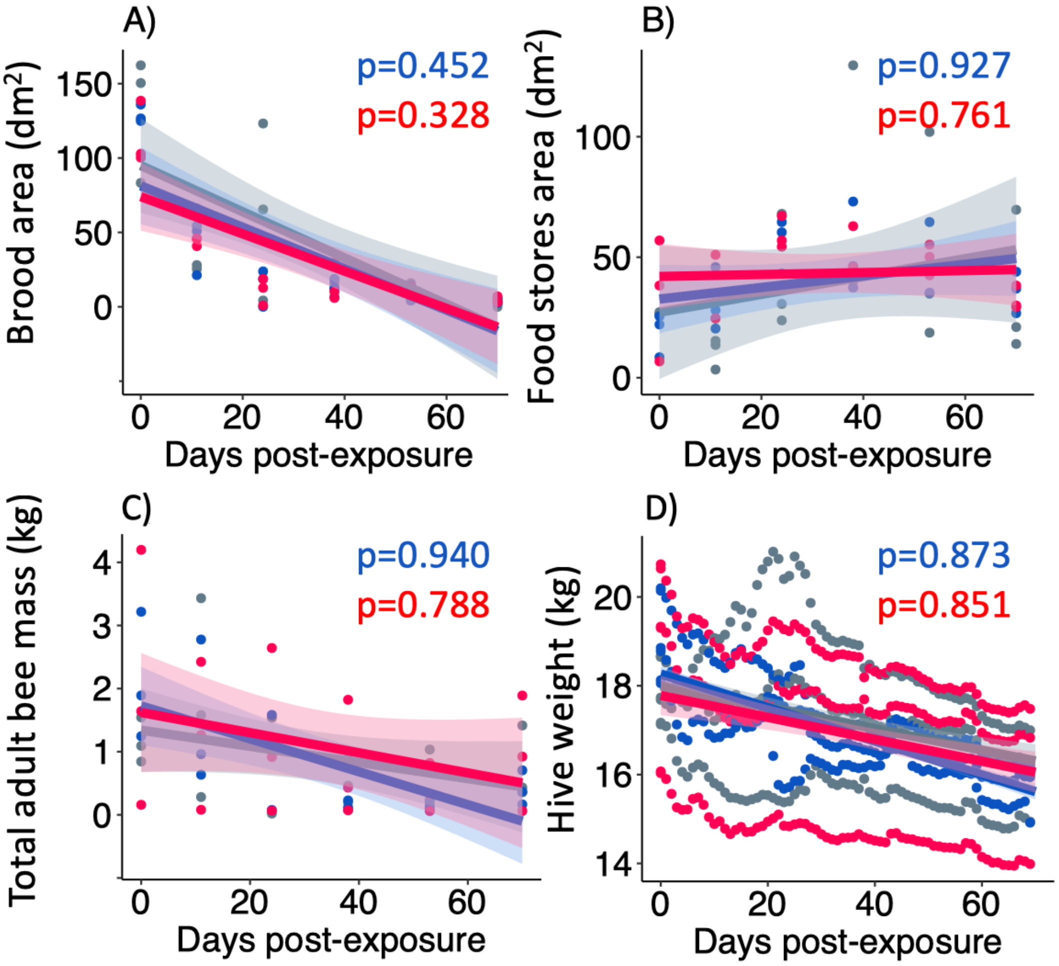


Figure S1: Amount of brood, food stores, total bees mass, hive weight for caged hives exposed to lead treatments throughout the experiment. Control colonies (N=3, grey), colonies exposed to a low concentration (0.075 mg.L^{-1}; N=3, blue) or a high concentration (0.75 mg.L^{-1}; N=3, red) of lead. Evaluations for brood, food stores and bees were conducted every 15 days for all hives. Total adult bee mass was recorded every hour and averaged on a daily basis. **A)** Area of capped brood cells. **B)** Area of food (honey and pollen) stores. **C)** Total adult bee mass. **D)** Hive weight. Estimate trends are displayed in solid lines. 95% confidence level interval are displayed in the same colour code as treatment. P-values were obtained from LMMs and are displayed for the treatment effect.

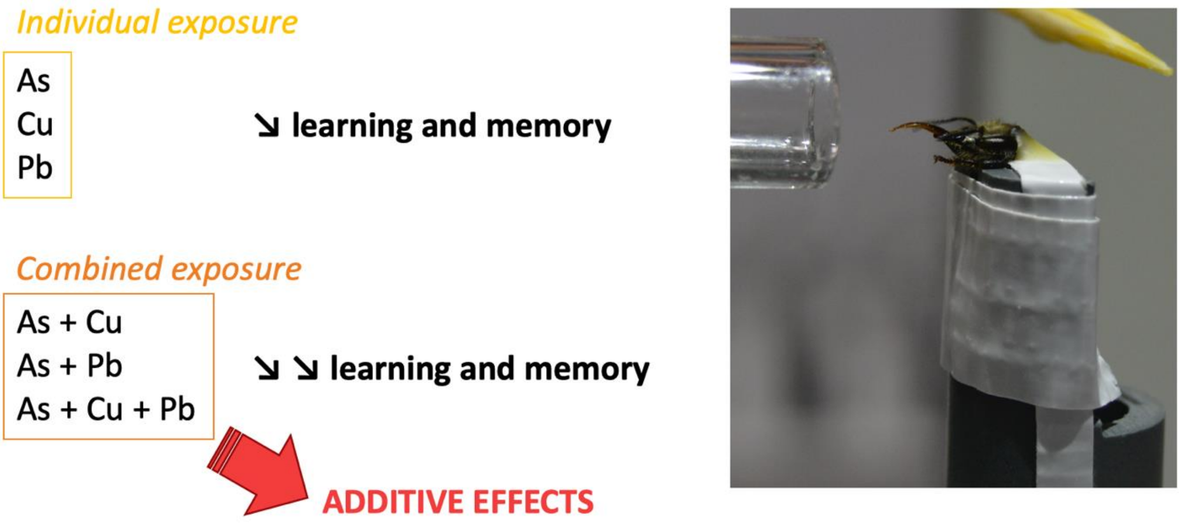
Chapter 4

○○○○●○○○

Metal pollutants have additive negative effects on honey bee cognition

Highlights:

- Environmental stressors can alter cognitive functions, underlying crucial behaviours.
- Metal pollutants are naturally co-occurring in the environment.
- We assessed the effects of combinations of arsenic, copper and/or lead on bee learning and memory.
- While acute exposure to one of these metals reduced learning and memory, exposure to combinations of these metals exerted additive effects.



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CHAPTER 4: Metal pollutants have additive negative effects on honey bee cognition

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Abstract

Environmental pollutants can exert sublethal deleterious effects on animals. These include disruption of cognitive functions underlying crucial behaviours. While agrochemicals have been identified as a major threat to pollinators, metal pollutants, which are often found in complex mixtures, have so far been overlooked. Here we assessed the impact of acute exposure to field-realistic concentrations of three common metal pollutants, lead, copper and arsenic, and their combinations, on honey bee appetitive learning and memory. All treatments involving single metals slowed down learning and disrupted memory retrieval at 24 h. Combinations of these metals had additive negative effects on both processes, suggesting common pathways of toxicity. Our results highlight the need to further assess the risks of metal pollution on invertebrates

Keywords: *Apis mellifera*, PER conditioning, pollutant interaction, arsenic, lead, copper

1. Introduction

Metal pollution is of increasing concern for both ecosystem and public health (Nriagu and Pacyna, 1988). Over the last century, the widespread use of metals in domestic, industrial and agricultural applications (Bradl, 2005) has considerably elevated their concentrations in water (Mance, 1987) and terrestrial habitats (Krämer, 2010; Su et al., 2014) up to potentially toxic levels.

Pollinators, such as honey bees, are directly exposed to metal pollutants when foraging on contaminated nectar and pollen (Perugini et al., 2011; Xun et al., 2018), and while flying through air containing suspended particles (Thimmegowda et al., 2020). Metals accumulate in the bodies of adults (Giglio et al., 2017) and larvae (Balestra et al., 1992), as well as in hive products (Satta et al., 2012). For instance, bioaccumulation of arsenic (As), copper (Cu) and lead (Pb), resulting from metal production industries (Kabir et al., 2012) and mining (Khaska et al., 2018; Lee et al., 2005), is common in both honey bees (Badiou-Bénéteau et al., 2013; Giglio et al., 2017; Goretti et al., 2020) and their honey (Pisani et al., 2008; Terrab et al., 2005).

The deleterious effects of metals on humans (Tchounwou et al., 2012) and some model animals (mice: Cobbina et al., 2015; flies: Doğanlar et al., 2014) are well-known. As, Cu, Pb and other metals have neurotoxic effects that induce neural and neuromuscular alterations, sensory impairments and many other behavioural dysfunctions (Chen et al., 2016). Deficits in cognition and memory have been reported for As (e.g. humans: Tolins et al., 2014; mice: Tyler et al., 2018; Wu et al., 2006), Pb (e.g. mice: Anderson et al., 2016; humans: Mason et al., 2014) and Cu (e.g. mice: Lamtai et al., 2020; Pal et al., 2013; flies: Zamberlan, 2020). Recent studies showed that low doses of Pb (Monchanin et al., 2021a) and selenium (Se) (Burden et al., 2016) also impair behaviour and cognition in honey bees, suggesting a widespread impact on pollinators. So far, however, very little attention has been given to the potential combined effects of co-exposure to different metals (Monchanin et al., 2021b).

Interactions among stressors are commonly classified as antagonistic (when the effect of one stressor reduces the effect of the other one), additive (when stressors have simple cumulative effects) or synergistic (when stressors together have a greater effect than the sum of their individual effects) (Folt et al., 1999). Additive effects of As, Cu and Pb have been described for humans (Lin et al., 2016), rats (Aktar et al., 2017; Mahaffey et al., 1981; Schmolke et al., 1992) and fishes (Verriopoulos and Dimas, 1988). In rats, for example, co-exposure to Pb and As disrupted brain biogenic amine levels (Agrawal et al., 2015). In humans, it has been hypothesized that combined exposure to Pb and As, or other metal pollutants, have additive or synergistic toxic responses leading to cognitive dysfunction (Karri et al., 2016). To our knowledge, two studies have addressed the impact of metallic cocktails on bee physiology. Honey bees simultaneously exposed to Pb, cadmium (Cd) and Cu accumulated significant levels of these metals in their bodies and had lower brain concentrations of dopamine compared to control honey bees (Nisbet et al., 2018). Cd and Cu exerted a weak synergistic effect on honey bee survival (Di et al., 2020). However, none of these studies investigated potential effects of combined exposure on cognition.

Here we compared the effects of exposure to single metals or ecologically relevant combinations of these metals on honey bee learning and memory. We hypothesised that combinations of metals may have synergistic negative effects, as has been found with pesticides (Yao et al., 2018; Zhu et al., 2017). We tested individual honey bees in a standard protocol of proboscis extension reflex (PER) conditioning following acute exposure to As, Pb and Cu alone or in combination. We tested three concentrations of As, considered the most toxic substance (ATSDR, 2019), and added one concentration of Cu or Pb (binary mixtures), or both (tertiary mixture), to reach the molarity of the As solutions.

2. Materials and methods

2.1. Metal solution

Arsenic ($NaAsO_2$), lead ($PbCl_2$) and copper ($CuCl_2 2H_2O$) were purchased from Sigma-Aldrich Ltd (Lyon, France) and diluted in 50% (w/v) sucrose solution. Control honey bees were fed 50% sucrose solution. Three concentrations of As were used (Table 1): a low concentration (0.13 µM) corresponding to the maximal permissible value in drinking water (0.01 $mg.L^{-1}$) (Codex

Alimentarius, 2015), a high concentration (0.67 μM) corresponding to half the maximal permissible value in irrigation water (0.1 mg.L^{-1}) (Ayers and Westcot, 1994), and an intermediate concentration (0.40 μM). This range of concentrations was reported in water sampled from polluted areas (e.g. mining sites) and in honey (Table S1). For Pb and Cu, we chose 0.27 μM (0.055 mg.L^{-1}of Pb and 0.017 mg.L^{-1} of Cu) so that the binary combinations (As 0.13 μM + Cu 0.27 μM or As 0.13 μM + Pb 0.27 μM) could be compared to the As intermediate concentration (0.40 μM), and the tertiary combination (As 0.13 μM + Pb 0.27 μM + Cu 0.27 μM) to the As high concentration (0.67 μM) (Table 1). These concentrations of Pb and Cu have also been reported in honey samples (Table S1). The mass consumed for As and the concentrations for Cu and Pb fell within sublethal ranges for the honey bee: the LD50 of elemental As for $NaAsO_2$ ranged from 0.330 to 0.540 μg per bee (Fujii, 1980), the LC50 of Cu is 72 mg.L^{-1} (Di et al., 2016) and that of Pb is 345 mg.L^{-1} (Di et al., 2016).

Table 1: Concentrations used. Combined treatments are shown in grey.

Treatment	Molarity (μM)	Concentration (mg.L^{-1})			Ingestion of 5μL (ng/bee)		
		As	Cu	Pb	As	Cu	Pb
Control	0	0	0	0	0	0	0
Low [As]	0.13	0.01	0	0	0.05	0	0
[Cu]	0.27	0	0.02	0	0	0.09	0
[Pb]	0.27	0	0	0.06	0	0	0.28
Med [As]	0.40	0.03	0	0	0.15	0	0
[As+Cu]	0.40	0.01	0.02	0	0.05	0.09	0
[As+Pb]	0.40	0.01	0	0.06	0.05	0	0.28
High [As]	0.67	0.05	0	0	0.25	0	0
[As+Cu+Pb]	0.67	0.01	0.02	0.06	0.05	0.09	0.28

2.2. Bee exposure to metals

We collected honey bees (*Apis mellifera* Linnaeus 1758) returning from foraging trips at the entrance of five different hives in mornings during August 2020. We then anesthetised the bees on ice and harnessed them in plastic tubes, secured with tape and a droplet of wax at the back of the head (Matsumoto et al., 2012). We tested all bees for an intact proboscis extension (PER) by stimulating their antennae with 50% sucrose. We then fed the responding honey bees 5 μL of 50% sucrose solution (see Table 1), making sure they consumed the whole droplet, and left them to rest for 3 h in the incubator (temperature: 25±2°C, humidity: 60%). Honey bees that did not respond to the sucrose solution were discarded.

2.3. Absolute learning

Prior to conditioning, we tested all honey bees for the PER by stimulating their antennae with 50% sucrose solution, and kept only those that displayed the reflex. We then performed olfactory absolute conditioning according to a standard protocol using an automatic stimulus delivery system (Aguiar et al., 2018). Honey bees had to learn to respond to an olfactory conditioned stimulus (CS, 1-nonanol, Sigma-Aldrich Ltd, Lyon, France) reinforced with the unconditioned stimulus (US, 50% sucrose solution), over five conditioning trials with a ten-minute inter-trial interval. Each trial (37 s in total) began when a bee was placed in front of the stimulus delivery system, which released a continuous flow of clean air (3,300 $mL.min^{-1}$) to the antennae. After 15 s, the odour was introduced into the airflow for 4 s, the last second of which overlapped with sucrose presentation to the antennae using a toothpick. This was immediately followed by feeding for 4 s by presenting the toothpick to the proboscis. The bee remained for another 15 s under the clean airflow. We recorded the presence or absence (1/0) of a conditioned PER in response to the odorant presentation during each conditioning trial. Honey bees spontaneously responding in the first conditioning trial were discarded from the analysis. The sum of conditioned responses over all trials provided an individual acquisition score (between 0 and 4), and honey bees responding at the last trial were categorized as learners.

2.4. Long-term memory

Only honey bees that had learnt the task were kept for the analysis of memory performance. After conditioning, these honey bees were fed 15 µL of 50% sucrose solution, left overnight in the incubator, and fed another 5 µL of sucrose solution the following morning. Three hours later (24 h post-conditioning), we performed the retention test, consisting of three trials similar to conditioning except that no sucrose reward was presented. In addition to the odour used during the conditioning (CS), we presented two novel odours, in randomized order, to assess the specificity of the memory: nonanal was expected to be perceived by honey bees as similar to 1-nonanol, while 1-hexanol was expected to be perceived differently (Guerrieri et al., 2005). We recorded the presence or absence (1/0) of a conditioned PER to each odorant at each memory retention trial. We classified honey bees according to their response patterns: response to the CS only, response to the CS and the similar odour (low generalization level), response to all odours (high generalization level), no or inconsistent response.

2.5. Statistics

We analysed the data using R Studio v.1.2.5033 (RStudio Team, 2015). Raw data are available in Dataset S1. We performed binomial generalised linear mixed-effects models (GLMM) (package lme4; Bates et al., 2015), with hive and conditioning date as random factors and treatment as a fixed effect. Using the GLMMs, we evaluated whether molarity or treatment impacted the initial response to antennal stimulation, the spontaneous response in the first conditioning trial, the response in the last trial, the response to each odorant during the memory test, the proportion of honey bees per response pattern in the retention test, and the survival at 24 h. Acquisition scores were standardised and compared with GLMMs using Template Model Builder (Brooks et al., 2017). For all response variables, we compared (1) the treated groups to the control, (2) groups exposed to concentrations of the same molarity (e.g. Med [As], [As+Cu] and [As+Pb]), (3) the separate and joint effects of the treatments (e.g. Low [As], [Cu] and [As+Cu]) in order to identify interactive effects (antagonistic, additive, synergistic).

3. Results and discussion

3.1. *Exposure to metals did not impact appetitive motivation*

The proportion of honey bees that responded to the initial antennal stimulation with sucrose was similar among treatments (GLMM: $p > 0.05$). Therefore, treatment did not affect appetitive motivation or sucrose perception. Consistent with our observations, the ingestion of similar concentrations of Pb and Cu had no effect on responsiveness to increasing concentrations of sucrose (Burden et al., 2019). By contrast, Di et al. (2020) found that honey bees exposed to increasing concentrations of a mixture of Cu and Cd exhibited a decreased ability to distinguish sucrose concentrations, but this may be explained by the much higher (at least 600 times) concentrations used in that study. Thus, in our conditions any impact on appetitive learning is unlikely due to a decreased motivation for sucrose or sucrose perception.

3.2. *Individual and joint exposures to metals reduced learning performance*

Two out of the 381 honey bees subjected to the absolute learning task spontaneously responded to the first odour presentation and were therefore discarded. In all groups, the number of honey bees showing the conditioned response increased over trials, thus showing learning (Fig. 1A). However, fewer honey bees exposed to metals learned the task when compared to controls (GLMM: $p < 0.05$, except for Low [As], $p = 0.082$). Accordingly, the acquisition scores of honey bees from all treatments were lower than those of controls (Fig. 1B). Honey bees exposed to Med [As] (GLMM: -0.610 ± 0.246, $p = 0.013$), High [As] (GLMM: -0.639 ± 0.241, $p = 0.008$) and [As+Cu+Pb] (GLMM: -0.592 ± 0.244, $p = 0.015$) had acquisition scores significantly lower than those of controls. Honey bees exposed to [As+Pb] had similar acquisition scores to bees exposed to Med [As] (GLMM: 0.299 ± 0.234, $p = 0.201$), but honey bees exposed to [As+Cu] performed better (GLMM: 0.596 ± 0.241, $p = 0.013$). Honey bees exposed to High [As] and [As+Cu+Pb] exhibited similar acquisition scores (GLMM: $p = 0.810$). We found no difference in the acquisition scores and the proportions of learners between honey bees treated with a single metal and mixed treatments (GLMM: $p > 0.05$), that would have indicated non-additive effects (i.e. antagonistic or synergistic). Thus, exposure to metals significantly reduced learning performance, and combined exposure appeared to exert simple additive deleterious effects.

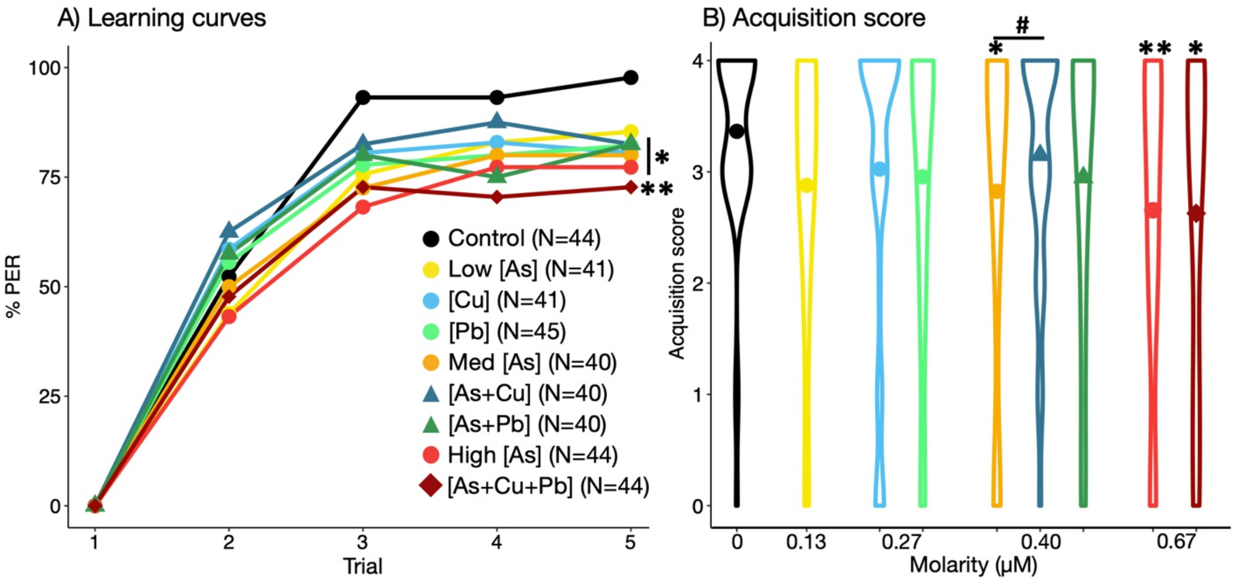

Figure 1: Learning. A) Learning curves show changes in the percentages of honey bees displaying the conditioned proboscis extension response (PER) over five training trials. Asterisks indicate significant differences in response rates at the last trial compared to those for control honey bees. **B)** Violin plots of acquisition score values (sum of conditioned responses for each honey bee). Symbols (*circle*: single exposure; *triangle*: binary mixture; *diamond*: tertiary mixture) indicate the mean score for each treatment. Significant differences between groups exposed to solutions of the same molarity (#) or with respect to control honey bees (*) are indicated (#/*$p<0.05$, **$p<0.01$; GLMM).

3.3. Individual and joint exposures to metals reduced long-term memory specificity

To examine possible effects of metal exposure on memory retention, we tested memory 24 h past training. Only honey bees that had learned the CS-US association at the end of conditioning were tested. 167 out of the 379 honey bees subjected to the absolute learning task did not learn and were therefore not included in the memory test.

We found no effect of treatment on survival at 24 h (GLMM: $p>0.05$). However, long-term memory was significantly affected (Fig. 2). Overall, treated honey bees responded less to the learned odorant (CS) than did controls, as indicated by a significant effect of exposure to metals on retention levels (GLMM: $p<0.05$) (Fig. 2A). Yet, this decrease was not significant for honey bees exposed to Med [As] (GLMM: -0.260±0.628, p=0.679) and High [As] (GLMM: -1.023±0.570, p=0.073). Finally, there was no clear dose effect on responses to the CS among treated groups (GLMM: -0.576±0.579, p=0.320).

Individual response patterns (Fig. 2B) revealed a loss of memory specificity. While honey bees from all treatments responded similarly to the similar odour (GLMM: $p>0.05$), those exposed to higher doses responded more frequently to all odorants, indicating a higher degree of response generalization (GLMM: 1.954±0.775, p=0.012). This was accompanied by a significantly lower proportion of specific (CS-only) responses for honey bees exposed to [Pb] (GLMM: -1.795±0.690, p=0.009), low [As] (GLMM: -1.313±0.589, p=0.026) and [As+Cu+Pb] (GLMM: -1.200±0.588, p=0.041). Exposure also significantly increased the frequency of inconsistent responses as compared with that in controls (GLMM: $p<0.05$). This was the case for each individual treatment except for Med [As] (p=0.293). Thus, exposure to metals had a negative impact on memory performance at 24 h. The analysis of individual response patterns also revealed additive effects as they did not differ among groups exposed to solutions with the same molarity, nor between single and mixed metal treatments (GLMM: $p>0.05$). Thus, most treatments reduced memory performance at 24 h.

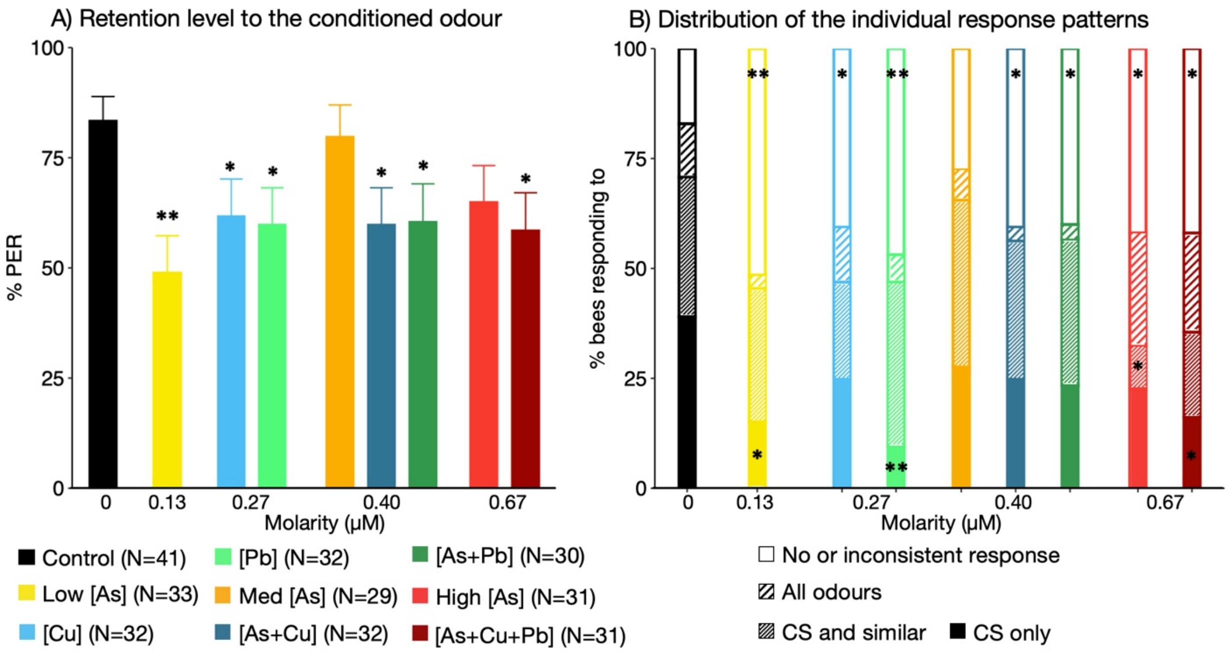

Figure 2: Long-term memory. A) Percentages of responses to the conditioned stimulus (CS) odour in the 24 h-memory retention test (means ± s.e.m). **B)** Distribution of honey bees according to their individual response pattern during the long-term memory test: response to CS only; response to CS and similar odour; response to all odours; no or inconsistent response. Significant differences from controls are indicated (*p<0.05, **p<0.01; GLMM).

3.4. The additive effects of metal mixtures may be explained by common pathways of toxicity

Although many mechanisms of metal toxicity have not yet been elucidated, some points of consensus are emerging from the literature. Firstly, interactions between metals can occur in the environment of the organism (Grobelak and Kowalska, 2020; Noyes and Lema, 2015), and during uptake into the organism, leading to potentially toxic processes of speciation, absorption, binding, transport and distribution (Wu et al., 2016). Once metals enter an organism, they can induce, alter or inhibit a range of biological responses and metabolic pathways. For example, by mimicking other essential metals (Bridges and Zalups, 2005) or damaging the permeability of biological membranes (Rothshein, 1959), metals enable the uptake or loss of other compounds from intracellular compartments (Viarengo, 1994). Metals are also known to disrupt signalling and calcium homeostasis (particularly important in neurons) by interfering with calcium channels (Bridges and Zalups, 2005; Chavez-Crooker et al., 2001; Tamano and Takeda, 2011). This might lead to dysfunction and cytotoxicity as a result of the disruption of cell signalling and calcium homeostasis. Genotoxicity (Doğanlar et al., 2014) may be achieved through covalent binding to DNA (Brocato and Costa, 2013; Senut et al., 2014). Eventually, oxidative stress and lipid peroxidation of the cell membrane may lead to neuronal death. Additionally, metals in mixtures could interact at target sites, but the effect on toxicity of that interaction is largely unknown (Svendsen et al., 2011). Metal mixtures could change the bioavailability (Gong et al., 2020), toxicokinetics and toxicodynamics (Gao et al., 2016) of each metal, which could impact the toxicity for the organism (Løkke et al., 2013). Based on these shared mechanisms of toxicity that include oxidative stress (Nikolić et al., 2016; Zaman et al., 1995), apoptosis (Raes et al., 2000) and interference with neurotransmitters (Nisbet et al., 2018), the toxic effects of metal pollutants in mixtures is expected to be additive (von Stackelberg et al., 2013). Of note, these conclusions emerge from studies mostly conducted on vertebrates, thus possibly leaving aside specificities of meta actions in invertebrate organisms.

Mixtures of metals may affect many aspects of neural activity and brain function in honey bees, as in other species (Karri et al., 2016). Here, we focused on learning and memory of olfactory cues because they play crucial roles in the behavioural ecology of honey bees and other pollinators, for the identification of food resources. Our results in controlled laboratory conditions suggest that exposure to sublethal combinations of toxic elements in the field might alter individual foraging efficiency, and in turn jeopardize survival of pollinator populations. While we could not identify interactive effects in such conditions, this will need to be confirmed in field experiments where exposure conditions will differ and affect a broader range of behavioural responses (flight activity, navigation). Our approach aims to fill a gap in the evaluation of combined actions of metals (Meyer et al., 2015), which appears necessary to better assess the risks they represent (Nys et al., 2018; Otitoloju, 2003) and better inform regulatory frameworks (European Commission, 2012). Current risk assessment guidance mainly assesses the effect of exposure to individual metals, which fails to capture potential interactive effects. This is of particular importance for honey bees and many other species, where contaminated food is transferred and shared among individuals. Hence, evaluation of the impact of metal mixtures and their modes of action needs to be developed (Sasso et al., 2010). Additionnally, interactions between toxic metals and environmental factors (Naqash et al., 2020) as well as with other chemicals (EFSA Scientific Committee et al., 2019) (e.g. pesticides (Sgolastra et al., 2018; Singh et al., 2017), volatile organic compounds (Sasso et al., 2010) etc.) should be implemented in an integrated research framework.

4. Conclusion

In summary, we demonstrated that As, Pb, Cu or combinations of these metals, at levels found in the environment, slow down appetitive learning and reduce long-term memory specificity in honey bees. These metals show simple additive effects as we found no differences in effects between different solutions of the same molarity, suggestive of possible non-linear effects (synergism or antagonism). Thus, regarding effects on learning and memory, concentration seems to be more important than the identity of any specific metal. Given that learning and memory of olfactory cues play crucial roles in the behavioural ecology of honey bees, acute exposure to mixtures of metal pollutants could impair fundamental hive function and population growth.

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Supporting materials

Table S1: Concentrations of As, Cu and Pb reported in water, honey and honey bee worldwide. Mean (minimal-maximal) values are reported. NA: not available.

Matrix	Location	As	Cu	Pb	Concentration
Water ($mg.L^{-1}$)	Former mining area, France	0.02 (0.002-0.11) 2.56 (0.03-7.6)	0.003 (0.0005-0.006) NA	0.28 (0.0003-0.0009) NA	(Khaska et al., 2018) (Guerin et al., 2000)
	Mining area, Turkey	0.10	0.07	0.01	(Sasmaz et al., 2015)
Honey ($mg.L^{-1}$)	Croatia	0.03 (0.01-0.15)	1.53 (0.05-58.81)	0.09 (0.01-1.20)	(Bilandžić et al., 2011)
	Hungary	0.03	0.29	0.07	(Ajtony et al., 2007)
	Italy	0.01 (0.004-0.029)	1.29 (0.25-8.41)	0.11 (0.04-0.43)	(Pisani et al., 2008)
	Post-mining area, Italy	NA	NA	0.06 (0.01-0.21)	(Satta et al., 2012)
	Industrial area, Serbia	0.05 (0.03-0.08)	NA	NA	(Krunić et al., 1989)
	Turkey	NA	0.02 (0.01-0.05)	0.02 (0.01-0.09)	(Silici et al., 2008)
	Worldwide	0.07 (0-0.14)	2.84 (0.07-24.65)	0.61 (0.001-4.61)	(Solayman et al., 2016)
Honey bee ($mg.kg^{-1}$ d.w)	Urban area, Italy	0.027 (0.07-0.34)	12.82 (7.44-27.09)	0.113 (0.02-0.39)	(Giglio et al., 2017)
	Industrial site, Italy	1.95 (1.51-2.68)	5.42 (3.22-7.77)	0.16 (0.06-0.28)	(Salvaggio et al., 2017)
	The Netherlands	0.714 (0.67-0.83)	15.21 (11.65-19.77)	0.571 (0.19-1.67)	(van der Steen et al., 2012)

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Chapter 5

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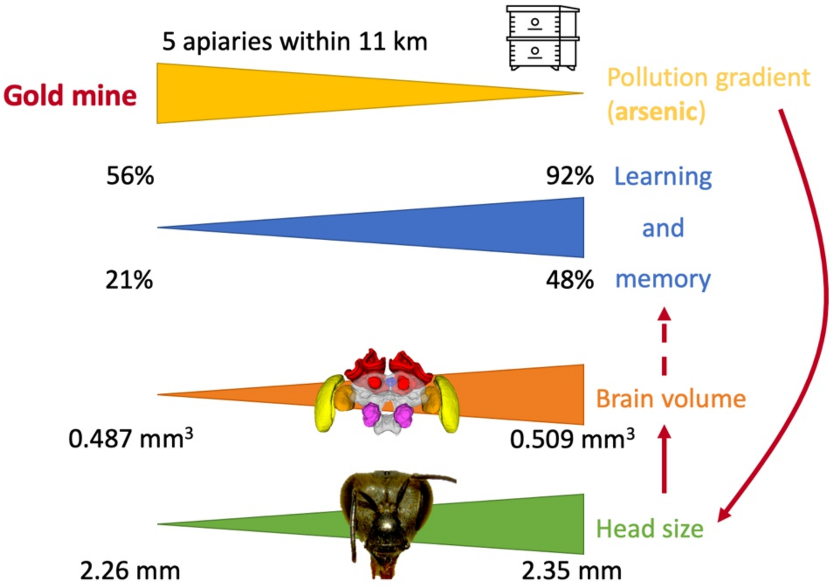
Environmental exposure to arsenic pollution impairs honey bee cognition and brain development

Highlights:

- We collected honey bees in the vicinity of a former gold mine, an area highly polluted with arsenic.
- Bees closer to the mine had reduced cognitive performances and developed both smaller heads and smaller brain components.
- The positive contribution of antennal lobes' volume to learning was weakened for the bees close to the mine.
- These sublethal effects of metal pollution on bee cognition is a major threat for bee populations and pollination.



In preparation for Ecology Letters

CHAPTER 5: Environmental exposure to arsenic pollution impairs honey bee cognition and brain development

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Abstract

Recent laboratory studies report that metal pollutants have detrimental effects on invertebrate behaviour and cognition, even at low levels. Here, we report the impacts of environmental exposure to the metalloid arsenic on a key sentinel species, the honey bee. More than 1,000 bee foragers were sampled in five apiaries within 11 km of the world's largest gold mine in Southern France, an area highly polluted with arsenic, amidst other metals. Bees collected close to the mine exhibited decreased olfactory learning and memory performances and developed smaller heads, with smaller brains. 3D scans of bee brains revealed that the size of the olfactory brain areas of bees sampled close to the mine was negatively correlated with cognitive performances, indicating functional impairment of the brain. Our findings unravel serious concerns about the cognitive health of honey bees in metal-polluted areas, which could ultimately jeopardize colony function and the pollination service.

Keywords: *Apis mellifera*, heavy metals, PER conditioning, morphometry, micro-computed tomography scanning

1. Introduction

Metals and other metalloids are naturally found in the earth crust and water and occur as a result of natural phenomena, like volcanic eruptions and soil erosion. Pollution from industrial activity and mining has raised environmental levels of metal and metalloid pollutants far above baseline levels (Han et al., 2003). In particular, historical and modern mining operations (Demková et al., 2017) and metal smelters have led to elevated concentrations of highly toxic pollutants such as arsenic, lead and mercury in superficial soils (Su et al., 2014) and water (Nordstrom, 2002) across large areas worldwide. Consequently, humans and wildlife can be exposed to toxic concentrations of these metals through inhalation of air and dust, and contaminated food, water, and soil (ATSDR, 2019).

These effects could be assessed by scrutinising physiological and behavioural traits in sentinel species, such as honey bees (Herrero-Latorre et al., 2017). Indeed, as pollinators they are exposed to heavy metals in plant nectar and pollen (Krunić et al., 1989), and these compounds are then transferred to honey (Bastías et al., 2013), wax (Tlak Gajger et al., 2016), propolis (Maragou et al., 2017) and ultimately ingested by larvae.

Recent studies showed that that controlled exposure of honey bees to a single metal or a cocktail of heavy metals in the lab led to developmental and cognitive deficits (Burden et al., 2016; Di et al., 2016; Monchanin et al., 2021a, 2021d). Chronic exposure to realistic concentrations of lead resulted in bees with smaller heads and reduced olfactory learning abilities, suggesting a developmental effect of heavy metal pollution on the bee brain (Monchanin et al., 2021a). Bees seem unable to detect field-realistic concentrations of heavy metals (Monchanin et al., 2021b, Chapter 2). In polluted areas, bees may therefore collect contaminated food, potentially impairing key cognitive abilities and whole colony dynamics (Klein et al., 2017).

Here, we explored the effects of environmental exposure of honey bee colonies to heavy metal pollutants on morphological development, brain growth and cognition, by collecting foragers from five apiaries along a gradient of metal pollution within 11 km of a former gold mine in the south of France, in Salsigne. Following the discovery of gold in 1892, the mine became an important precious metal producer. It became the largest arsenic mine worldwide, supplying up to 25% of the world's arsenic need, until its closure in 2004 (Trueb, 1996). This field site was particularly contaminated with arsenic, but also lead, cadmium, copper, nickel, zinc, at exceptionally high levels (Pérez and Valiente, 2005), above the international permissible limits (Ayers and Westcot, 1994; de Vries et al., 2003; WHO/FAO, 2001), and the contamination has persisted even after closure and partial remediation of the site (Khaska et al., 2018, 2019). More worryingly, recent floods in 1999 (Gaume et al., 2004) and 2018 (JO Sénat, 2019) contributed to spread metal pollutants in the whole valley, raising concerns about human health which were supported by alarming arsenic levels measured from children in the area (ARS, 2019). Monitoring environmental pollution and health issues in the region is of national, if not international, importance (Elbaz-Poulichet et al., 2017), and the remediation strategy (combining chemical and phytostabilisation technology) developed at Salsigne is being adopted in other polluted sites (Gruiz et al., 2005). Hence, the Salsigne area is considered as "critically polluted". Arsenic leads to neurodevelopmental and cognitive disorders, including learning and memory impairments (reviewed in Tolins et al., 2014). Because the latter are critical processes used by honey bees to find, identify and exploit environmental resources, we tested the hypothesis that bees foraging in the area would be exposed to high levels of arsenic that might prove detrimental to their learning and/or memory abilities.

In experiment A, we investigated the learning and short-term (1 h) memory abilities of foragers collected from different apiaries, and in experiment B, we further assessed the memory specificity for both short and long-term (24 h) memories. In addition, we measured body parameters and quantified brain size.

2. Results

2.1. *Bees closer to the mine showed lower learning performances*

Forager bees, collected from five apiaries within 11 km of the former gold mine, at different distances expected to correspond to a gradient of environmental arsenic levels (Fig. 1), were brought to the laboratory and trained to associate an odorant to a sucrose reward, using the paradigm of proboscis extension response (PER) conditioning. Prior to conditioning, we tested all bees for an intact proboscis extension reflex upon antennal stimulation with 50% (w/v) sucrose solution. The proportions of responding bees were similar among sites (ANOVA: Chi^2=0.255, df=4, p=0.993) (Site A: 97.8%, N=136; Site B: 98.5%, N=135; Site C: 97.9%, N=140; Site D: 98.3%, N=121; Site E: 100%, N=144). Therefore, site location did not affect appetitive motivation nor sucrose perception by bees.

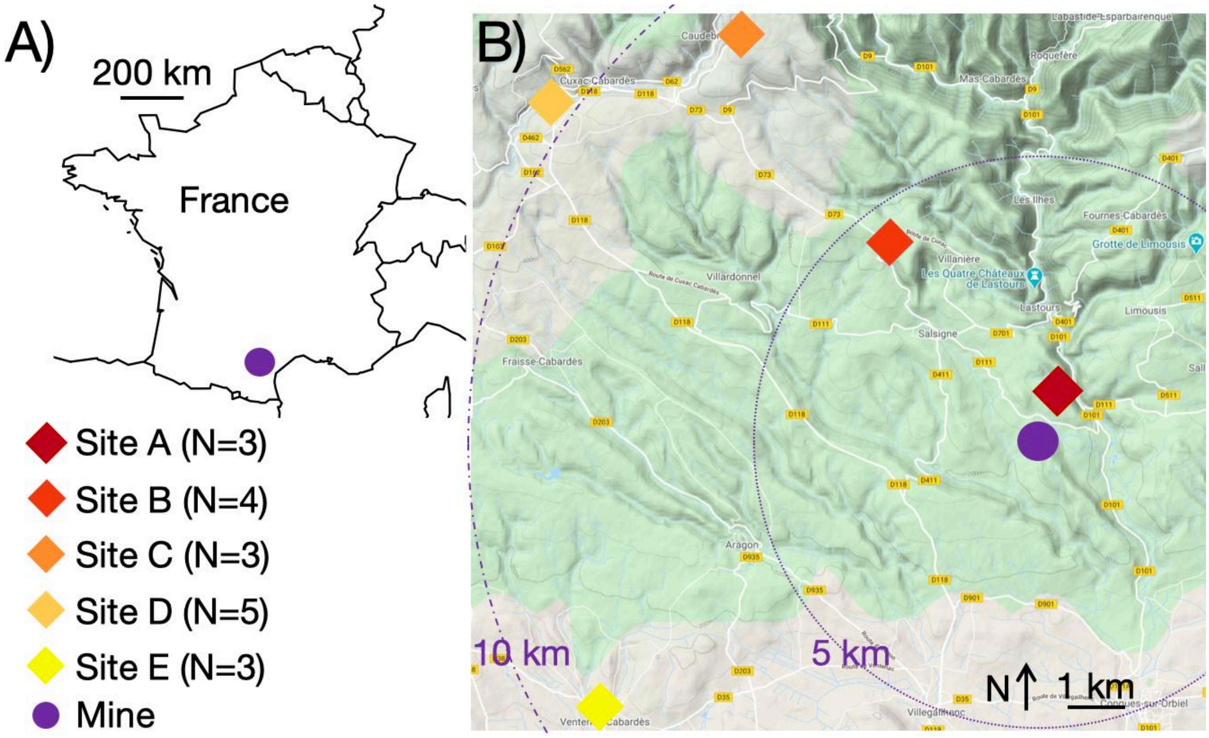

Figure 1: Location of the study sites. A) Former gold mine located in the south of France. **B)** Location of the five apiaries (number of hives displayed) where foragers were collected. Distance from the mine is displayed by the dotted (5 km) and dashed (10 km) lines.

We trained 673 bees in a five-trial absolute learning task, during which we recorded conditioned responses. Bees spontaneously responding at the first odour presentation were discarded (N=42), without effect of location on the proportion of such spontaneous responses (ANOVA: Chi^2=4.560, df=4, p=0.336). Among the resulting 631 bees kept for analysis, in all sites the proportions of individuals showing conditioned responses to the odour increased with learning trials, thus showing learning (Fig. 2A). However, by the last conditioning trial, a lower proportion of bees from sites A (GLMM: -2.130±0.777, p=0.006) and C (-1.572±0.780, p=0.044) had finally learned the task, as compared to those from the farthest site E (A: 56.45%, B: 73.39%, C: 68.94%, D: 78.90%, E: 91.55%). When measured individually, learning performances were affected accordingly since bees from site A had lower acquisition scores (sum of the conditioned responses during the conditioning) (Fig. 2B) than bees from site B (GLMM: -1.018±0.477, p=0.033), site D (-1.055±0.489, p=0.031) and site E (-1.287±0.581, p=0.027). We found no effect of the odorant used (either limonene or eugenol) on bees' responses (GLMM: p=0.141). Thus, site proximity to the former mine significantly reduced the learning performances of bees.

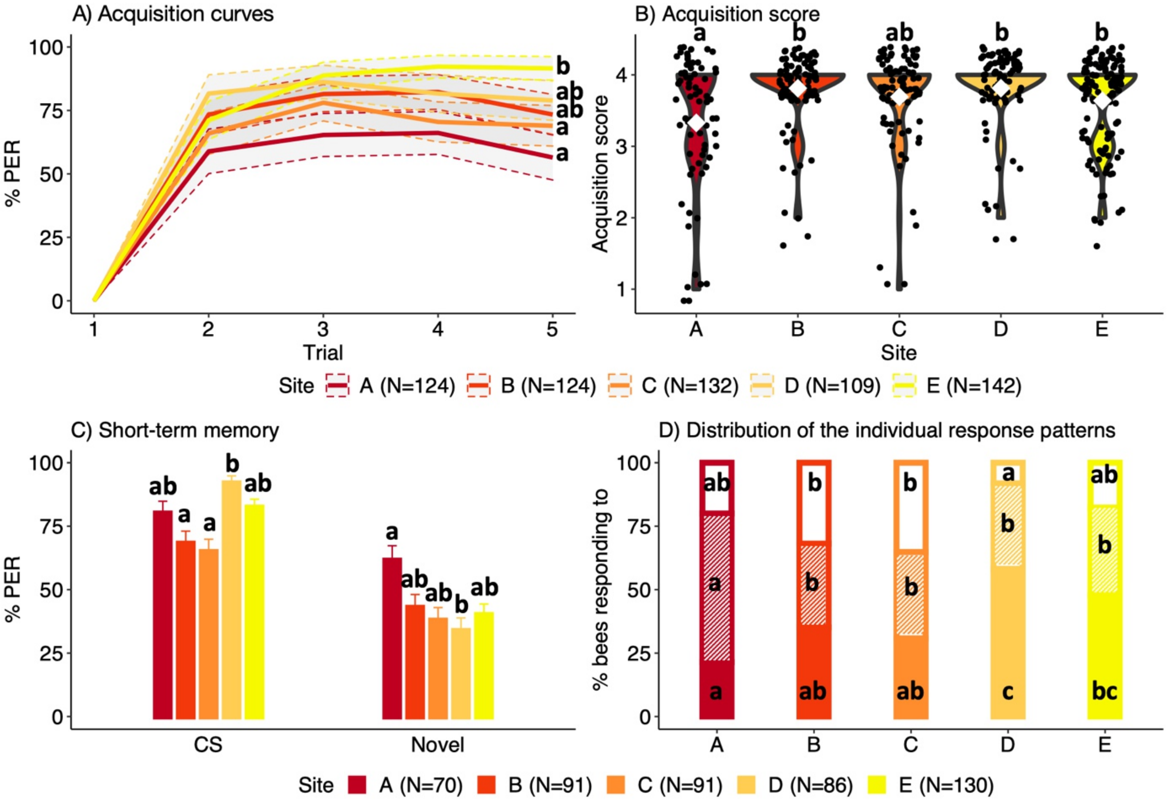

Figure 2: Learning and short-term memory (experiment A). A) Acquisition curves show changes in the percentages of bees displaying conditioned proboscis extension responses (PER)

over the five trials. Shaded areas show 95% confidence intervals. Letters indicate significant differences in response proportions at the last trial between sites (binomial GLMM). **B)** Violin plots of individual acquisition scores (sum of conditioned responses for each bee, white diamonds display mean average values). Letters indicate significant differences between sites (GLMM). **C)** Percentages of responses to the two odours during the one-hour memory retention test (mean ± s.e.m). Significant differences in response levels for each odour, obtained from GLMM, are displayed. **D)** Distribution of bees according to their individual response patterns during the memory test: CS-specific responses (coloured), generalized responses to both odours (hatched) and inconsistent or absent responses (white). Letters indicate significant differences between sites for each response pattern.

2.2. Bees closer to the mine showed reduced short-term memory specificity

As short-term memory processes are involved in decision-making during foraging trips, we next assessed one-hour memory recall by recording conditioned PER response to odorants, without sucrose reward. In addition to the conditioning stimuli (CS) used during conditioning, we presented a novel odorant, to evaluate the specificity of the memory (response to CS only).

Among bees that had effectively learnt the task, one-hour memory recall of the odour-reward association was also dependent on site location. Bees from site D responded more to the CS (91.86%) than bees from closer sites B (68.13%) and C (64.85%) (GLMM: 1.591±0.504, p=0.016 and 1.795±0.500, p=0.003 respectively). Additionally, bees from the closest site (A) responded more to the novel odour than bees from sites D (GLMM: 1.196±0.411, p=0.036) (Fig. 2C). The individual response patterns of individuals (Fig. 2D) also showed a clear effect of site location on the proportion of bees displaying CS-specific memory (ANOVA: Chi^2=15.054, df=4, p=0.005) (A: 21.43%, B: 35.16%, C: 30.77%, D: 58.14%, Site E: 47.69%), with significantly less specific responses recorded in bees from site A than D and E (GLMM: resp. -1.625±0.477, p<0.001; -1.210±0.477, p=0.011); and in bees from site B than C and D (resp. 1.162±0.441, p=0.008 and 0.898±0.422, p=0.033). Moreover, bees from site A showed higher generalization of their response to the novel odour as compared to those from all other sites (ANOVA: Chi^2=11.757, df=4, p=0.019; A: 58.57%, B: 32.97%, C: 34.07%, D: 33.72%, E: 34.62%). Site proximity to the mine thus had a pronounced negative effect on memory specificity.

2.3. Bees closer to the mine showed reduced short-term memory specificity at 1 h and a loss of memory at 24 h

In order to investigate the memory impairments more specifically, we conducted additional behavioural assays on bees from sites A and E, the two extremes of the metallic pollution gradient. We compared their performances in both short-term (1 h) and long-term (24 h) memories, the latter guiding bee foraging and participating in communication between nestmates within the hive. Here, to better capture the effect on olfactory generalization, we used two novel odours, in addition to the CS, one perceptually similar (low generalization level) and one dissimilar (high generalization level) (experiment B).

As previously, the proportions of bees initially exhibiting intact PER was similar between both sites (ANOVA: Chi^2=, df=1, p=1; A: 96.17%, E: 100%). These represented 261 bees that were submitted to the absolute learning task, of which only 4 spontaneously responded to the first odour presentation, thus resulting in a total of 257 analysed bees. By the end of the five conditioning trials, a lower proportion of bees from site A had learned the task compared to site E (GLMM: -2.197±0.866, p=0.011) (A: 62.00%, E: 92.52%) (Fig. 3A). While they also exhibited a lower mean acquisition score (mean ± s.e.m: A: 2.27 ± 0.14, E: 3.22 ± 0.11), the difference was not significant (GLMM: -0.848±0.526, p=0.107) (Fig. 3B). Thus, like in the first experiment, proximity to the former mine significantly reduced learning performances.

In the short-term memory retrieval test (1 h), bees from both sites responded similarly to the CS (A: 67.94%, E: 68.25%) (GLMM: 0.123±0.579, p=0.846). However, bees from site A had more generalization responds to novel odours (Fig. 3C), although only significantly to the similar one (GLMM: 1.3231±0.305, p<0.001) (similar: 69.89% *vs.* 40.40%; dissimilar: 16.13% *vs.* 3.03%). Patterns of individual responses also showed a clear effect of the proximity to the mine on the selectivity of responses (Fig. 3D). Bees from site A displayed less CS-specific responses (12.90% *vs.* 37.37%; GLMM: -1.400±0.387, p<0.001), generalised more to other odours, both similar (51.61% *vs.* 35.35%; GLMM: 0.668±0.295, p=0.024) and dissimilar (16.13% *vs.* 3.03%; GLMM: 1.848±0.795, p=0.020). Hence, bees closer to the mine exhibited higher levels of generalization and less specific memory.

When tested for long-term memory (24 h), bees from both sites responded in similar proportions to all three odours (Fig. 3E): the CS (A: 51.72%, E: 66.67%; GLMM: -0.630±0.482, p=0.191), the similar odour (A: 48.28%, E: 39.39%; GLMM: 0.352±0.411, p=0.392) and the dissimilar one (A: 20.69%, E: 11.11%; GLMM: 0.803±0.625, p=0.199). Regarding their individual response patterns (Fig. 3F), bees from site A displayed less CS-specific responses (A: 10.34%, E: 29.29%; GLMM: -0.865±0.414, p=0.037) and more inconsistent or absent responses (A: 49.43%, E: 35.35%; GLMM: 0.850±0.412, p=0.039). Generalization responses were equally frequent in bees from both sites, to the similar odour (Site A: 25.29%, E: 26.26%; GLMM: -0.224±0.339, p=0.508) as well as to the dissimilar one (A: 14.94%, E: 9.09%; GLMM: 0.469±0.696, p=0.501). Therefore, while at 24 h the hive location did not impact on generalization levels, a shorter distance to the mine was associated with lower levels of memory specificity, as for short-term memory.

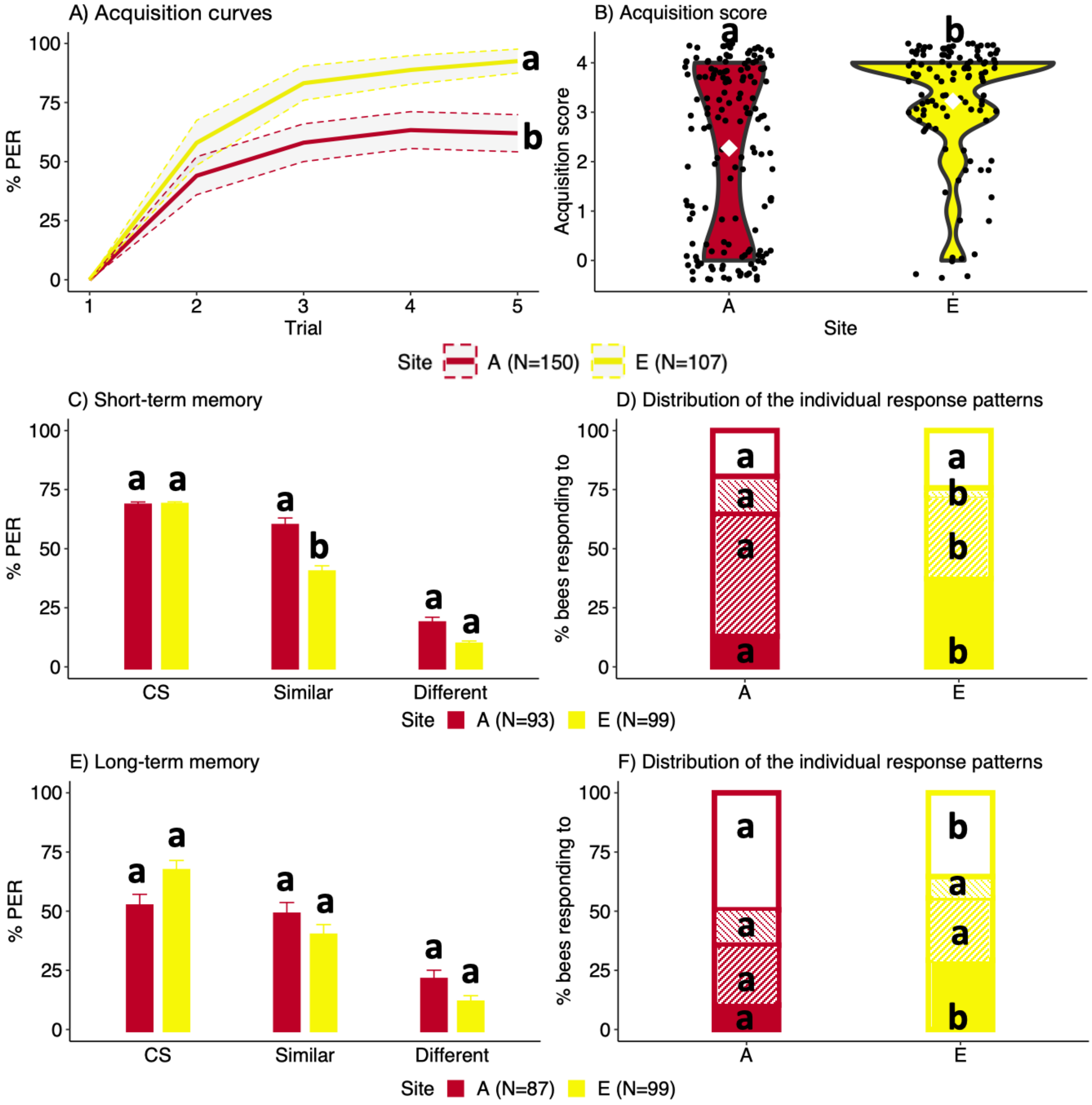

Figure 3: Learning, short-term and long-term memory (experiment B). A) Acquisition curves show changes in the percentages of bees displaying conditioned proboscis extension responses (PER) over the five trials. Shaded areas show 95% confidence interval. Significant differences in responses at the last trial (from GLMM) between sites is displayed. **B)** Violin plots of individual acquisition scores (sum of conditioned responses for each bee, white diamonds display average values). Letters indicate significant differences between sites (GLMM). **C)** Percentages of responses to the three odours during the one-hour memory retention test (mean ± s.e.m). Significant differences in response levels for each odour, obtained from GLMM, are displayed. **D)** Distribution of bees according to their individual response patterns during the short-term

memory test: CS-specific responses (coloured), generalized responses to the similar odour (dense hatches), generalized responses to both similar and dissimilar odours (hatched), and inconsistent or no responses (white). Letters indicate significant differences between sites for each response pattern. **E)** Percentages of responses to the three odours during the 24 h-memory retention test (mean ± s.e.m). Significant differences in response levels for each odour, obtained from GLMM, are displayed. **F)** Distribution of bees according to their individual responses during the long-term memory test, as in D. Letters indicate significant differences between sites for each response pattern.

2.4. Bees closer to the mine had smaller heads

To assess the potential developmental impact of mine proximity on bee development, we measured five morphological parameters (Fig. 4A) on the bees that underwent the behavioural assays (see Figs. 2-3). We assessed the effect of site location on overall morphology with a PCA including all parameters (Fig. 4B). Two PCs explaining 59% of the variance discriminated overall larger bees *vs.* smaller bees (1st PC) and bees with larger *vs.* smaller heads (2nd PC) (PERMANOVA: Pseudo-F=10.923, p=0.001). Bees from site A were morphologically different from those from all other sites (pairwise PERMANOVA: p=0.01 for all comparisons). Their heads were not longer than those of other bees (ANOVA: Chi^2=1.224, df=4, p=0.354; Fig. 4C), but significantly narrower that in sites D and E (resp. LMM: -0.080±0.034, p=0.037 and -0.084±0.037, p=0.045; Fig. 4D). This resulted in bees from the closest site (A) having overall smaller heads than bees from the farthest sites (D and E) (resp. LMM: -0.940±0.414, p=0.044 and -1.011±0.455, p=0.049; Fig. 4E). By contrast, (Fig. 4F-G), bees from all sites exhibited similar femur and wing length (ANOVA: resp. Chi^2=0.311, df=4, p=0.866; Chi^2=0.336, df=4, p=0.849). Finally, there was no clear relationship between weight and hive location (ANOVA: Chi^2=3.13, df=4, p=0.054; Fig. 4H). Therefore, proximity to the mine influenced the bee development and resulted in significant morphological differences, especially leading to a reduced head size.

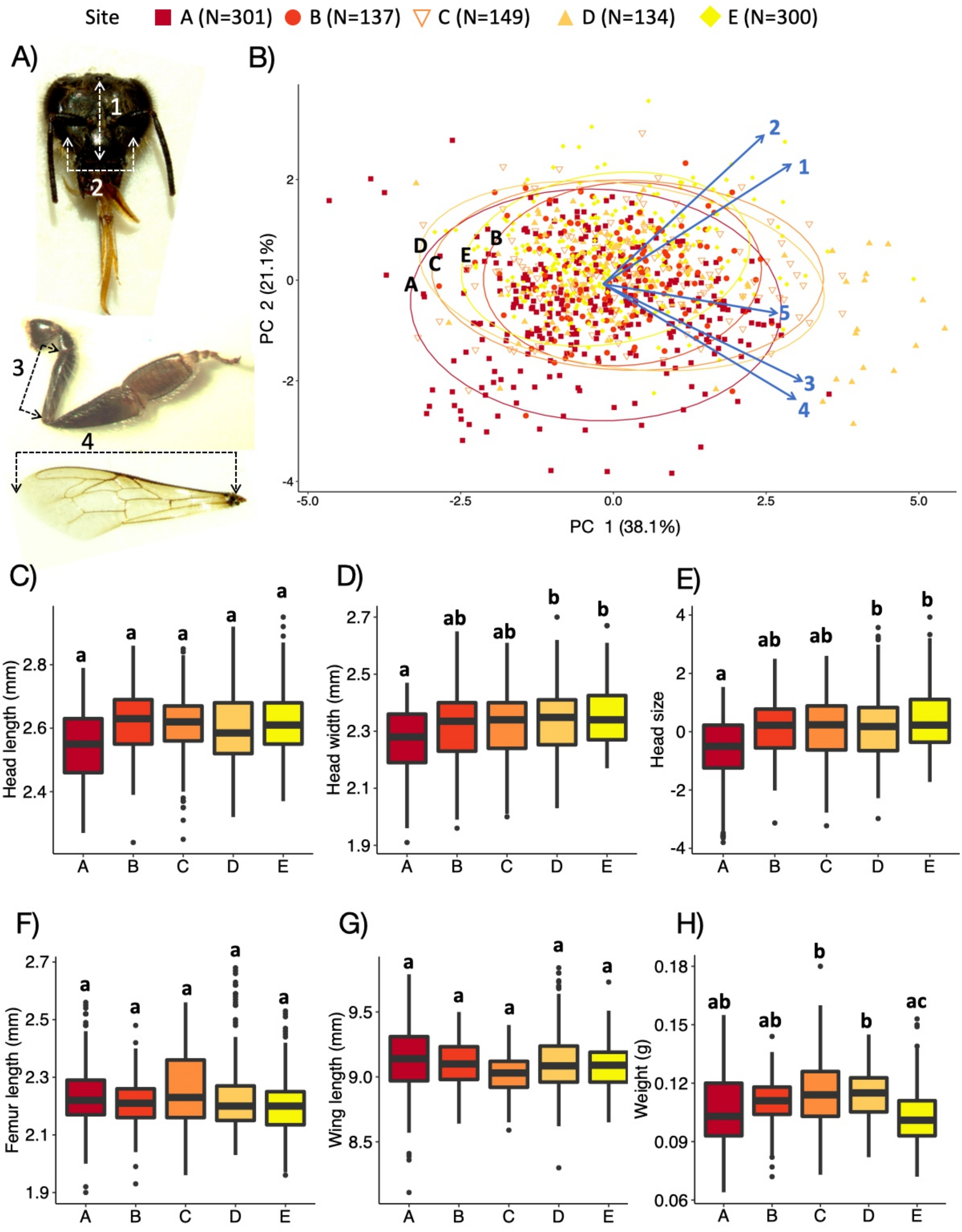

Figure 4: Morphometric measurements of forager bees. A) Details of the parameters measured. (1) head length, (2) head width, (3) femur length, (4) wing length, (5) bee weight (not shown). **B)** Principal component analysis (PCA) map shows the distribution of individuals along the two principal components (PC1, PC2) and the relationship among the morphometric measures

(same number code as in A). 95% confidence ellipses of the mean are displayed for each site. **C)** Head length. **D)** Head width. **E)** Head size. **F)** Femur length. **G)** Wing length. **H)** Body weight. Letters indicate significant differences between sites (LMM).

2.5. Bees closer to the mine tended to have a reduced brain volume

Given the observed variations in head size, we next assessed whether proximity to the mine could alter the size of the brain size, as well as whether a reduced size of specific brain centers might be related to learning and memory deficits (Fig. 5). For this, we quantified volumes from 3D reconstructions (Fig. 5A) obtained from bees from sites A and E used in the behavioural experiment B (see Fig. 3). As expected, brain size was smaller in bees from site A (albeit not significantly) (Fig. 5B) and was positively correlated with head size (Fig. 5C). All neuropil volumes increased with total brain volume (AL: $R^2=0.32$, $p=0.052$, MB: $R^2=0.56$, $p=0.006$; OL: $R^2=0.78$, $p<0.001$; CX: $R^2=0.30$, $p=0.075$). Interestingly as they play a major role in olfactory learning, the antennal lobes (ALs) were also significantly impacted: bees from site A had smaller ALs (Fig. 5D), whose volume correlated with head size (Fig. 5E). By contrast, the mushroom bodies (MBs) were not (Fig. 5F-G), although they support olfactory memory retrieval. In addition, the optic lobes (OLs) varied like total brain size (Fig. 5H-I), but no visual task was performed here to identify possible behavioural correlates. Finally, the central complex (CX), involved in navigation, did not vary (Fig. 5J-K)

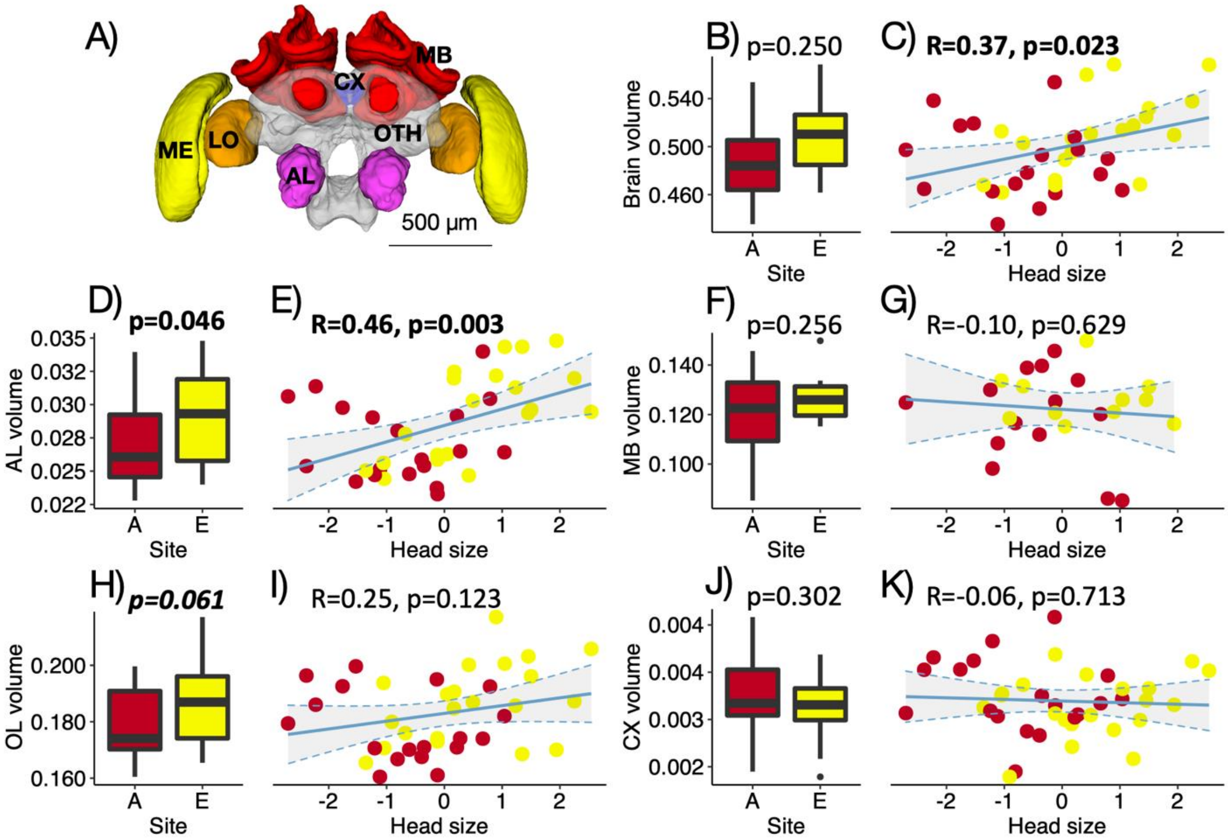

Figure 5: Brain area volumes (mm^3) of forager bees from site A and E. A) Example of a reconstructed brain (frontal view) showing ALs, MBs, OLs (combining ME and LO), CX. **B)-C)** Total brain in function of the site **(B)** and head size **(C)**. **D)-E)** Antennal lobes (AL) in function of the site **(D)** and head size **(E)**. **F)-G)** Mushroom bodies (MB) in function of the site **(F)** and head size **(G)**. **H)-I)** Optic lobes (OL) in function of the site **(H)** and head size **(I)**. **J)-K)** Central complex (CX) in function of the site **(J)** and head size **(K)**. Statistical comparisons for the neuropil volume between sites (Site A: N=18; Site E: N=20) were obtained with p-values from LMM. Shaded areas show 95% confidence interval of the regression line (in blue). Pearson correlation coefficient (R) and p-value are given. Significant p-values are displayed in bold.

2.6. Proximity to the mine reversed the relationship between learning performances and antennal lobe volumes

We finally explored whether the observed differences in brain size and neuropil volumes were associated with differences in learning scores in the bees sampled at different locations. Like previously observed on the larger dataset (Fig. 2), within the subset of CT-scanned bees acquisition scores were lower in bees from site A than from site E (GLMM: -1.166±0.370, p=0.002; Fig. 6A), as were the proportions of learners (i.e. bees responding correctly at the fifth conditioning trial; GLMM: -1.791±0.707, p=0.011). In addition, head size showed a similar trend (LMM: -1.256±0.444, p=0.099). We found no effect of brain volume on acquisition score (Fig. 6B). We found a significant positive association between acquisition score and the volume of ALs, but not of other neuropils (LMM: 1.203±0.470, p=0.011; Fig. 6C), indicating that bees with larger AL showed better learning performances. However, we found a significant negative interaction between antennal lobes volume and proximity to the mine on the acquisition score (LMM: -2.284±0.729, p=0.002; Fig. 6C). We were unable to further assess the relationship between brain volumes and memory performances, as only 6 bees from site A managed to learn the task.

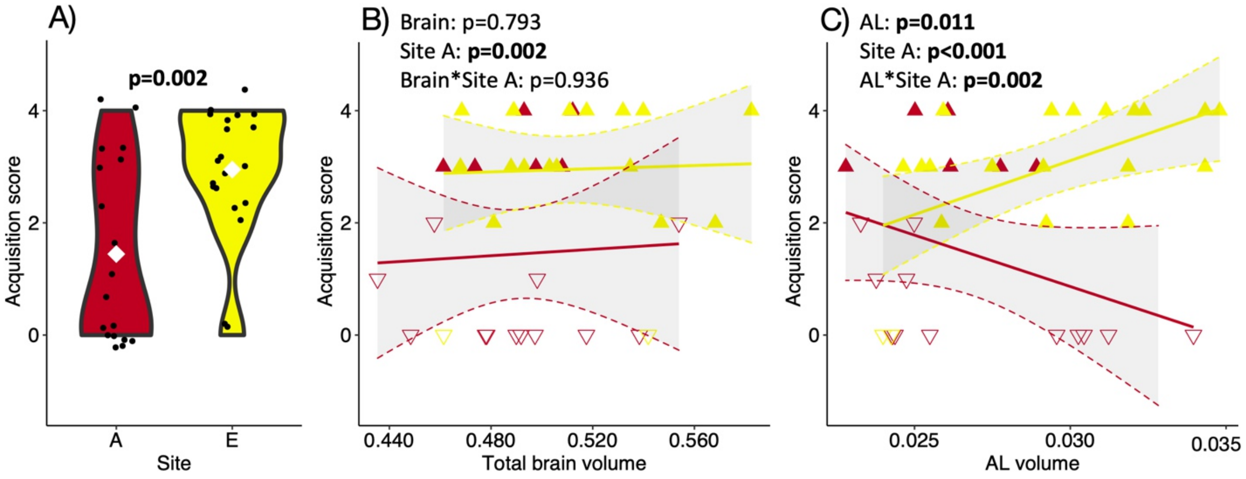

Figure 6: Acquisition score according to site location and to antennal lobes and total brain volumes (mm^3). A) Violin plots of acquisition score values (sum of conditioned responses for each bee) per site. White diamonds display mean acquisition score. **B-C)** Acquisition score in function of the total brain (**B**) and antennal lobes (AL) (**C**) volumes per site. Non-learner bees are shown by empty triangles, learners by coloured triangles. Shaded areas show 95% confidence interval from LMM.

3. Discussion

Here, we assessed these effects in honey bees exposed to gradient of environmental arsenic contamination around a unique polluted site. We showed that bees closer to the mine, exposed to higher doses, exhibited poorer olfactory learning abilities and memory specificity, and that this was associated with decreased head and brain size, as well as reduced volume of brain regions supporting learning.

Since proper learning and memory functions are crucial to the behavioural and chemical ecology of honey bees, our data suggest that exposed bees may be less efficient at foraging, since this behaviour relies on learning olfactory cues associated with flowers providing nectar or pollen (Reinhard and Srinivasan, 2009) and remembering such cues to revisit these profitable food sources (Chittka and Raine, 2006). Finally, bees need to remember, transfer and retrieve these information when communicating with nestmates (Farina et al., 2005; Grüter et al., 2006), insuring an efficient recruitment. Altogether, this suggests that the collective capacity of the colony to feed, grow and/or survive may be jeopardized by environmental exposure to arsenic (Klein et al., 2017).

Our study provides the first results regarding the fate of honey bees in a metal-polluted area. A few studies demonstrated impacts on physiological (Murray et al., 2000) or developmental (Leveque, 2013) traits in other insect species, but behavioural data were still lacking. Our results could be used as baseline data for future studies on bees, and more generally other insects, foraging in metal polluted environments, such as anthropized areas (Badiou-Bénéteau et al., 2013), industrial areas (e.g. copper processing plant (Krunić et al., 1989), mining area (Zhou et al., 2018), industrial districts (Matin et al., 2016)).

Although the impacts of contamination around the former gold mine of Salsigne have been little studied, our conclusions are consistent with epidemiological data obtained in the area, showing an excessive mortality rate among mine workers (Simonato et al., 1994) and arsenic-specific cancer attributed to environmental contamination (Dondon et al., 2005). Impacts on biota were also reported, with small mammals collected in this area having bioaccumulated significant amount of arsenic (Drouhot et al., 2014).

Here, forager bees closer to the mine developed smaller heads, with smaller brains, and smaller antennal lobes. Such neurodevelopmental impairments are reminiscent of those observed in mammals following exposure to arsenic (Tolins et al., 2014; Tyler et al., 2018; Wu et al., 2006), as well as on other insects. When feeding on arsenic-contaminated food, grasshoppers exhibited a decreased body weight (Rathinasabapathi et al., 2007), while moths had reduced larval survival and increased pupal stage duration (Andrahennadi and Pickering, 2008). Oribatid mites collected on a pollution gradient, where arsenic was recorded, showed leg deformities (Eeva and Penttinen, 2009). Ants sampled along a metal pollution gradient developed smaller heads (Grześ et al., 2015), and so did midges fed with cadmium or copper (Martinez et al., 2003). Exposure to stressors during development can reduce bee head growth, e.g. infestation by *Varroa* ectoparasites (Belaïd et al., 2017) or chronic exposure to lead (Monchanin et al., 2021a); and brain growth, e.g. pesticides (Smith et al., 2020).

The effects of arsenic exposure on brain development are likely to contribute to the reduced cognitive abilities observed in our results, since increasing environmental doses correlate with more severe growth defects as well as learning and memory deficits. Importantly, previous work already pointed out that bees perform better in olfactory tasks when they have bigger heads, even in the absence of contamination (Gronenberg and Couvillon, 2010; Monchanin et al., 2021a). Of particular interest is the association between reduced AL size and impaired olfactory learning, as these primary olfactory centers support olfactory (Menzel et al., 1996). Interestingly, we found that OLs were affected in a very similar way, thus suggesting that at least visual perception and/or learning might be impaired as well. Further experimental studies assessing the capacity for bees to learn about visual cues (Scheiner et al., 2013) are needed to verify this hypothesis. Surprisingly, we found no effect of MBs size on learning performances. However, behavioural experiments showed memory defects, and MBs are known support olfactory memory retrieval (Menzel, 2001).

Hence, subtle changes in synaptic connectivity within MBs (Cabirol et al., 2018) could affect memory specificity (Groh and Rössler, 2020), even without changes in overall volume.

These behavioural and developmental impairments might explain previous observations on the dynamics of colonies along a gradient of arsenic and cadmium pollution. (Bromenshenk et al., 1991) reported a significant decrease of the number of bees and honey yield, with bees accumulating high levels of metal(loid)s, when getting closer to the industrial sources. Especially, as metal pollutants occur in complex mixtures in the environment, they can interact, potentially leading to additive or synergistic pernicious effects (von Stackelberg et al., 2013), as reported for honey bees, but in controlled conditions only (Di et al., 2020; Monchanin et al., 2021d; Nisbet et al., 2018).

Our results call for more studies to characterize the impact of industrial pollution on both managed and wild bee populations. More generally, there is an urgent need to further assess the contribution of metal pollutants to the widespread decline of insects (Monchanin et al., 2021c).

4. Materials and methods

4.1. Field sites

This study was carried out in the vicinity of a former gold mine located in Salsigne, France (43°18’41’’N, 2°22’44’’E). The ores procession, to extract mainly gold and silver, led to major contamination by arsenic in the surroundings of the industrial plant, in soils (Drouhot et al., 2014; Pérez and Valiente, 2005) and water (Guerin et al., 2000), far beyond maximal permissible limits (Ayers and Westcot, 1994; Codex Alimentarius, 2015; WHO/FAO, 2001). Five apiaries, installed for at least two months before the beginning of the experiment (Table 1), were selected within 11 km of the former gold mine (sites A-E in Fig. 1, Table 1).

Table 1: Location of the study sites and details on the hives.

Site	Distance to mine	Coordinates	Number of hives	Hive history
Site A (Lastours)	1 km	43°19'12''N, 2°22'57''E	3	Queens from 2019. Installed in May 2020
Site B (Villanière)	4.5 km	43°20'40''N, 2°20'49'' E	4	Queens from 2020. Installed in April 2020
Site C (Caudebronde)	9.2 km	43°22'46'' N, 2°18'50''E	3	Queens from 2019. Installed in May 2020
Site D (Cuxac)	10.4 km	43°21'57'' N, 2°16'26''E	5	Queens from 2020. Installed in May 2020
Site E (Ventenac)	9.10 km	43°16'3'' N, 2°17'2''E	3	Queens from 2019. Installed in 2019

4.2. Bees

Between July and August 2020, we collected returning forager honey bees (*Apis mellifera*, Buckfast strain) at the hive entrance on the day before the behavioural experiments. We housed bees in plastic boxes containing groups of 20 individuals with access to 400 µL of 50% (w/v) sucrose solution (thus ca. 20 µL per bee following trophallaxis). We kept the plastic boxes overnight in an incubator (28 ± 1 °C, 70% humidity) (Villar et al., 2020). In the morning of the training day, we cooled bees on ice and harnessed them in plastic tubes, secured with tape and a droplet of wax at the back of the head (Matsumoto et al., 2012). We then fed them 5 µL of 50% sucrose solution and left them to rest for 3 h in the incubator.

4.3. Conditioning

We first tested the proboscis extension reflex (PER) of all bees by stimulating their antennae with 50% sucrose solution, and kept only those that responded for the conditioning. We performed olfactory absolute conditioning, in which bees must learn to associate an odour (conditioned stimulus, CS) delivered by an automatic stimulus delivery system (Aguiar et al., 2018) with a 50% sucrose reinforcement, according to a standard protocol (Matsumoto et al., 2012). We ran two different sets of experiments. In experiment A, we used pure limonene and eugenol (Sigma-Aldrich Ltd, Lyon, France) alternately on successive days, so that each combination was used for about half of the bees. In experiment B, we used 1-nonanol (Sigma-Aldrich Ltd, Lyon, France).

The conditioning included five trials with a ten-minute inter-trial interval. Each conditioning trial (37 s in total) started when a bee was placed in front of the stimulus delivery system, which released a continuous flow of clean air (3,300 $mL.min^{-1}$) to the antennae. After 15 s, the odour was introduced to the airflow for 4 s, the last second of which overlapped with sucrose presentation to the antennae using a toothpick and subsequent feeding for 4 seconds. The bee remained another 15 s under the clean airflow. We recorded the presence or absence of a conditioned PER to each odour at each conditioning trial (1 or 0), and the sum of conditioned responses was used to calculate an individual acquisition score ranging between 0 and 4.

4.4. Memory tests

After the last conditioning trial, we put the bees back into the incubator for 1 h, before submitting them to a short-term memory retrieval test.. In addition to the odour used during the conditioning (CS), a novel odour was presented following the same dynamics of the conditioning trial but with no sucrose reward. We recorded the presence or absence of a conditioned PER to each odour at each trial (1 or 0).

We also tested some groups of conditioned bees for long-term retrieval, dependant of protein synthesis in the bee brain (Lefer et al., 2012). For this, bees were fed 15 µL of 50% sucrose solution after the short-term memory test, left overnight in the incubator, and fed the following morning with 5 µL of sucrose to ensure their survival. This second test was performed using the same procedure as for short-term retrieval, 24 h after the end of conditioning.

On the morning of the test, bees were collected, harnessed and fed 5 µL of 50% sucrose solution. Three hours after, we submitted them to an absolute conditioning using 1-nonanol as CS. Retention tests were performed at 1 h and 24 h post-conditioning, using 1-nonanol, nonanal (similar odour) and 1-hexanol (different odour).

In experiment A, only short-term memory was assessed, and bees were presented limonene and eugenol (each being the CS or the novel odorant, depending of the individual). We classified bees according to their response during the memory test: response to CS only, response to both odours, no or inconsistent (response to novel odour only) response. In experiment B, we presented nonanal and 1-hexanol in addition to the CS, 1-nonanol. Nonanal is perceived as similar to 1-nonanol and induces a high level of generalization, while 1-hexanol is perceived as dissimilar and yields little generalization (Guerrieri et al., 2005). Here, we classified bees as: responding to the CS only, showing generalization toward the similar odorant (i.e. responding to the CS and the similar odour, low generalization level), showing generalization to both the similar and dissimilar odorants (i.e. responding to all odours, high generalization level), no or inconsistent response. Note that only bees that learnt the task were kept for the analysis of the memory performances.

4.5. Morphometry

All bees were frozen after the behavioural assays and stored -18°C. An experimenter blind to bee identity measured their fresh body weight (±0.001g) (precision balance ME103T, Mettler-Toledo Gmbh, Greifensee, Switzerland) and took measures (±0.01 mm) under a Nikon SMZ 745T dissecting microscope (objective x0.67) with a Toupcam camera model U3CMOS coupled to the ToupView software. We measured the head length (distance between the upper edge of the labrum and the lower part of the median ocellus), head width (distance between the two lower corners of the composed eyes), wing length and femur length (Fig. 4A) (Monchanin et al., 2021a).

4.6. Brain scanning and volume measurements

We performed micro-computed tomography (micro CT) scanning of 47 foragers from experiment B. We removed the front part of the head (just above the labrum) (Smith et al., 2016) and fully submerged the heads in 5% phosphtungstic acid solution (5 mg.L^{-1} in a 70/30% ethanol/water solution) for 15 days. Each head was scanned with a resolution of 5 µm using a micro CT station EasyTom 150/RX Solutions (Montpellier Ressources Imagerie, Montpellier, France). Raw data for each brain scan was reconstructed using X-Act software (RX Solutions, Chavanod, France). We then re-oriented to the same plane-of-view the reconstructed scan, and each brain was re-sliced into a new series of two-dimensional images. Based on the staining and segmentation quality, we kept 38 brains (Site A: N=18; Site E: N=20) from the 47 scanned. We then measured the volume of the whole brain and of several of its main neuropils (Brandt et al., 2005). The antennal lobes (AL), the mushroom bodies (MB) (comprising medial and lateral calyx, peduncle and lobe), the central complex (CX) (comprising the central body, the paired noduli and the protocerebral bridge), the medulla and lobula (altogether referred to as 'optic lobe' (OL)). We performed the segmentation and volume analysis using AVIZO 2019.1 (Thermo Fisher Scientific, Waltham, USA). We first manually performed the segmentation of the structures on every 5 slices of 26 brains. These data were then used to train a neural network implemented in the Biomedical Image Segmentation App (Lösel et al., 2020). We next used the trained neural network to predict the segmentation of every brain, which output was manually checked by an experimenter (Lösel et al., 2021, Appendix 7). Neuropil absolute volume was calculated using the voxel count function of AVIZO, with relative volume calculated by dividing absolute volumes by the total brain volume.

4.7. Statistics

We analysed the data using R Studio v.1.2.5033 (RStudio Team, 2015). All data are available in Dataset S1.

For the conditioning trials, we performed generalized linear mixed-effects models (GLMM) (package lme4; Bates et al., 2015), fitted with binomial family, with hive and conditioning date as random factors and site as fixed effect. Using GLMM, we evaluated whether site location would impact the percentage of initial responses to antennal stimulation, spontaneous responses at the first conditioning trial, conditioned responses at the last conditioning trial and responses to each odour during memory test, as well as the proportion of individual response patters during retrieval. GLMMs were followed by F-tests to test the significance of fixed categorical variables using anova function (car package; (Wox and Weisberg, 2019)). Acquisition scores were standardized and compared with GLMM using Template Model Builder (Brooks et al., 2017), and fixed categorical variables significance was tested using Anova.glmmTMB function of that package.

For the morphometric analyses, we conducted a PCA (package FactoMineR, (Lê et al., 2008)) on the five parameters measured, and clusters were compared with permutational multivariate analysis of variance (PERMANOVA; package Vegan; (Oksanen et al., 2019). In addition, head width and length measures were collapsed into the first component of a PCA, which was used as a proxy of the head size. Linear mixed-effects models (LMM) were run for each morphological parameter, considering site as fixed effect, and hive and date as random factors.

For the brain analyses, we conducted, for each neuropil, LMM with hive as random factor and site as fixed effect. To analyse how brain and brain component volumes influenced acquisition score, we used GLMM (Brooks et al., 2017), with an interaction term between site and volume, for each component. Similar GLMM were conducted to assess the interaction between site and lateral volume for paired neuropils.

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Chapter 6

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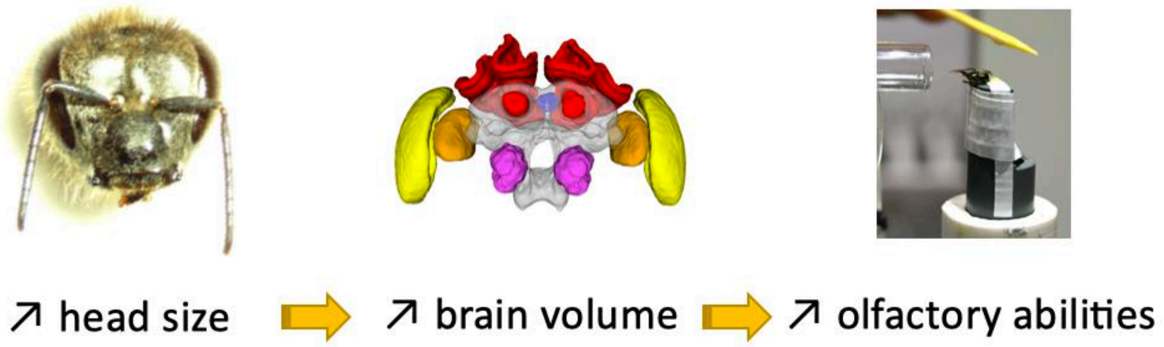
Bees with larger heads have better olfactory learning and memory performance

Highlights:

- Whether head and brain sizes are correlated to better cognition is a long debated question.
- Social insects, such as bees, display high levels of inter-individual morphological, behavioural and cognitive variability which may be adaptive for division of labour.
- We explored the relationships between head size, brain composition and cognitive performances in bees.
- Bees with larger heads showed higher olfactory and learning abilities, and developed larger brains, with larger olfactory brain area.



In preparation for Current Biology

CHAPTER 6: Bees with larger heads have better olfactory learning and memory performance

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Abstract

Whether and how brain size is correlated with cognitive ability is unclear. Here we explored the relationships between variations in head and/or brain size and inter-individual variability in learning performance in bees. We compared head size and cognitive performances of 1,600 honey bees, using original and published datasets. Head size varied by about 30% among honey bees and individuals with a larger head showed higher olfactory learning and memory performance. Analyses of 3D reconstructed brains showed that better learners had the largest antennal lobes. This effect was independent of the size of sensory organs (eyes, antennas), did not affect sensitivity to conditioning stimuli (sucrose reward, odour, light, electric shock), and was not observed in visual learning tasks. Similar results were obtained in bumblebees, suggesting that cognitive variability associated to brain size variation may be a widespread phenomenon in bees.

Keywords: *Apis mellifera*, *Bombus terrestris*, cognition, variability, morphometry, micro-computed tomography scanning

1. Introduction

A positive association between brain volume and intelligence in humans has been suspected since the 19th century (Galton, 1889), and brain size is still used as a predictor of cleverness by some authors (Gibson, 2002). Correlations between brain size and ‘intelligence’ have been also reported for other animals, such as mammalian carnivores (Benson-Amram et al., 2016), birds (Møller, 2010) or fishes (Edmunds et al., 2016; Kotrschal et al., 2013). However many studies have shown that brain size was not such a good proxy of cognitive abilities (Schoenemann et al., 2000), and this remains controversial (Chittka and Niven, 2009). In fact, the determinism of cognitive performances is multifactorial in many species, and inter-individual differences may be due to many environmental, developmental (Tautz et al., 2003) and genetic (Liang et al., 2012) factors, and can vary according to specific life history traits like e.g. social complexity (Dunbar, 1998; Dunbar and Shultz, 2007) or foraging strategy (Harvey et al., 1980; Sayol et al., 2020).

While insects have miniature brains, they are able to operate with this size constraint (Muscedere et al., 2014), and display various forms of sophisticated cognition (Perry et al., 2017) while exhibiting diverse levels of sociality (O’Donnell et al., 2019). Social insects exhibit division of labour relying on inter-individual behavioural variability, which is adaptive to allow for fast collective responses and resilience of colonies to environmental changes (Jandt et al., 2013; Jeanson and Weidenmüller, 2014). Differences in behavioural repertoire related to task allocation can be associated with differences in morphology (e.g. size polymorphism in bumblebees, Jandt and Dornhaus (2009)); age (e.g. age polyethism in honey bees, Robinson (1987)); physiology (e.g. maturation polyethism in ant, Fénéron et al. (1996)) and genetic background (Chole et al., 2019). Behavioural variability also arises on a finer scale, within a single caste (Chittka et al., 2003; Klein et al., 2017; Perry et al., 2016), whose underlying mechanisms are still poorly understood. Yet, correlations between increasing head size and enhanced cognitive performances are reported for wasps (van der Woude et al., 2018) and ants (Gronenberg, 2008), but are highly debated (Chittka and Niven, 2009; Lihoreau et al., 2012). Studies on bumblebees suggest that morphometric variations may be associated with different cognitive performances among workers. Body size appear to correlate with task allocation within workers (Garófalo, 1978), foraging efficiency (Klein et al., 2017) and visual learning speed (Frasnelli, 2020; Riveros and Gronenberg, 2012; Worden, 2005) in bumblebees. Larger workers tend to have larger eyes with more ommatidia (Kapustjanskij et al., 2007) resulting in enhanced visual sensitivity and resolution (Spaethe and Chittka, 2003), longer antennae with more sensillae enabling increased antennal sensitivity to odours and olfactory accuracy (Spaethe et al., 2007), and larger brains (Riveros and Gronenberg, 2010), with most brain components showing a similar size increase as the overall brain (Mares et al., 2005). Unlike bumblebees, honey bees show an age-based division of labour correlated with brain maturation, in the absence of obvious morphological changes. Within the brain, antennal lobes (ALs) (Winnington et al., 1996) and mushroom bodies (MBs) (Withers et al., 1993) undergo volume changes with age, and exhibit an experience-dependant plasticity (Durst et al., 1994; Farris, 2016). The ALs are the primary olfactory center, and the MBs a multimodal integration center accounting for learning and memory (Giurfa and Sandoz, 2012).

Honey bees show little variability in body size (Waddington, 1989). However recent studies suggest that learning performance in different conditioning assays (Gronenberg and Couvillon, 2010; Monchanin et al., 2021a) is correlated with head or brain size. However, these conclusions are limited to a small range of olfactory appetitive tasks, thus raising the question of whether this is a general relationship across tasks of variable difficulty and sensory modalities, and whether it affects proper learning processes or its modulation by sensitivity to the stimuli (conditioned and unconditioned) used to assess learning.

Here we addressed the question in an unprecedently large dataset with various cognitive tests and high resolution brain data. We used original and published datasets in honey bees and bumblebees (Table S1) to explore whether head size variation could explain general learning and memory variability in bees for various tasks involving different contexts (appetitive, aversive) and modalities (visual, olfactory). In order to better understand the functional meaning of the identified relationships, we next focused on a specific learning task to investigate the potential contributions of volume variations in the whole brain as well as in identified neuropils.

2. Results and discussion

2.1. Head size varied significantly between honey bees

We found significant head size variation among of the 1,593 measured honey bees (Fig. 1A). Antenna length varied by 14% (mean±s.e.m: 2.73±0.001 mm; min-max: 2.51-2.92 mm), head width by 28% (2.35±0.002 mm; 1.99-2.78 mm), eye length by 34% (2.07±0.003 mm; 1.67-2.54 mm) and head length by about 38% (2.71±0.004 mm; 2.03-3.25 mm). These size ranges are consistent with previous observations, despite occasional methodological differences (Belaïd et al., 2017; Gowda and Gronenberg, 2019; Monchanin et al., 2021a; Steijven et al., 2017; Streinzer et al., 2013). Head length and head width were positively correlated (Fig. 1B). Because we were interested in the global effect of the head size, we collapsed the head width and length of each bee into a component of a principal component analysis (PCA; Fig. S1). PC1 explained 71% of the variance and discriminated bees with wide and large heads. Thereafter, we used the individual coordinates from PC1 as a proxy of head size. Head size was positively correlated with the length of eyes (Fig. 1C) and antennae (albeit weakly for the latter, Fig. 1D), which were also weakly correlated together ($R=0.17$, $p<0.001$).

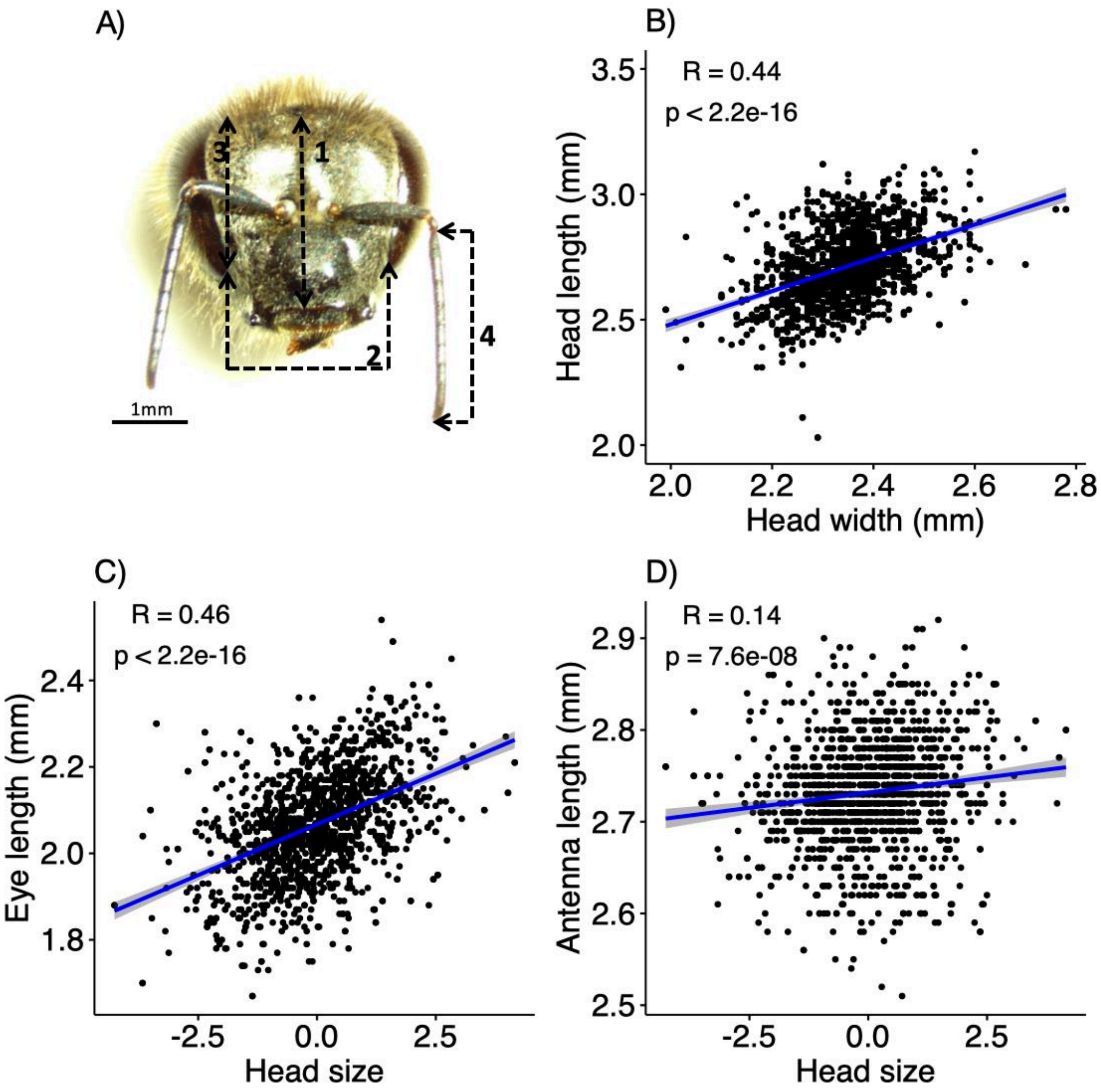

Figure 1: Morphometric measurements of honey bee heads (N=1593). A) Details of the parameters measured: (1) head length, (2) head width, (3) eye length, (4) antenna length. **B)** Correlations between head length and width, **C)** Head size (PC1) and eye length, **D)** Head size (PC1) and antenna length. Regression lines are displayed in blue with confidence intervals in grey. Pearson correlation coefficient (R) and p-value (p) are given.

2.2. Honey bees with larger heads had better olfactory learning performances

A previous study reported relationship between head size, brain size and learning performance as assessed in an olfactory absolute conditioning assay where one odorant was rewarded (Gronenberg and Couvillon, 2010). We tested whether such a relationship was general, rather than specific to a given task, using data obtained from various olfactory learning tasks, all based on the standard conditioning of the proboscis extension response (PER), where bees must learn to associate odours to a sucrose reward (Giurfa and Sandoz, 2012). We first compared results from absolute conditioning (association odour A/reward: A+, as in (Gronenberg and Couvillon, 2010)) with differential conditioning, involving two odours (one rewarded and one non-rewarded: A+ *vs.* B-). In the former task, head size was positively correlated to acquisition (p-values for the acquisition scores analysis are given in the figures, here Fig. 2A) and increased proportion of learners (GLMM: $p<0.001$), consistently with previous results (Gronenberg and Couvillon, 2010). So did antenna length on acquisition score (Fig. 2B) and proportion of learners (GLMM: $p=0.050$), but not eye length (acquisition score: Fig. 2C; proportion of learners: GLMM $p=0.316$). Similarly, bees with larger heads performed better at differential conditioning (Fig. 2D), with higher proportion of learners (GLMM: $p<0.001$), although in this case neither antenna nor eye length had a significant effect (Fig. 2E-F). We then asked whether this head size effect could extend to other olfactory learning tasks which rely on different brain circuits. While absolute and differential conditioning are MB dependent tasks, other tasks such as reversal learning and negative patterning require functional MBs to be achieved (Boitard et al., 2015; Devaud et al., 2007). Reversal learning consists of two successive differential tasks involving opposite associations (first A+ *vs.* B-, then A- *vs.* B+). The capacity to achieve the second, reversal phase, also correlated to head size, be it the acquisition score (Fig. 2G) or the proportion of learners (GLMM: $p=0.010$). Yet, here we found a negative correlation between antenna length and the proportion of learners (GLMM: $p=0.031$), but not with the acquisition score (Fig. 2H), nor for eye length (Fig. 2I). Contrary to the three previous tasks, negative patterning is a non-elementary task, in which bees need to respond to single rewarded odorants but not to their unrewarded mixture (A+, B+ *vs.* AB-); for this they need to treat the mixture as a stimulus different from the mere sum of its elements (configural strategy) (Boitard et al., 2015; Devaud et al., 2015; Giurfa, 2013). Despite this distinction, this task again revealed the correlation between head size and acquisition score (Fig. 2J), but not with the proportion of learners (GLMM: $p=0.106$), while antenna and eye length remained insignificant (Fig. 2K-L).

Importantly, this effect of head size in these four olfactory tasks was unlikely driven by differences in odour perception since neither head size (GLMM: p=0.562), antenna length (GLMM: p=0.536) nor eye length (GLMM: p=0.264) influenced odour sensitivity (Fig. S2A-C). Thus, overall, honey bees with larger heads learned better in olfactory tasks. This shows that this relationship, previously reported only for absolute conditioning (Gronenberg and Couvillon, 2010), appear consistent across tasks, irrespective of the number of odours, the underlying brain circuits or the cognitive strategies (elemental vs. configural) they involve.

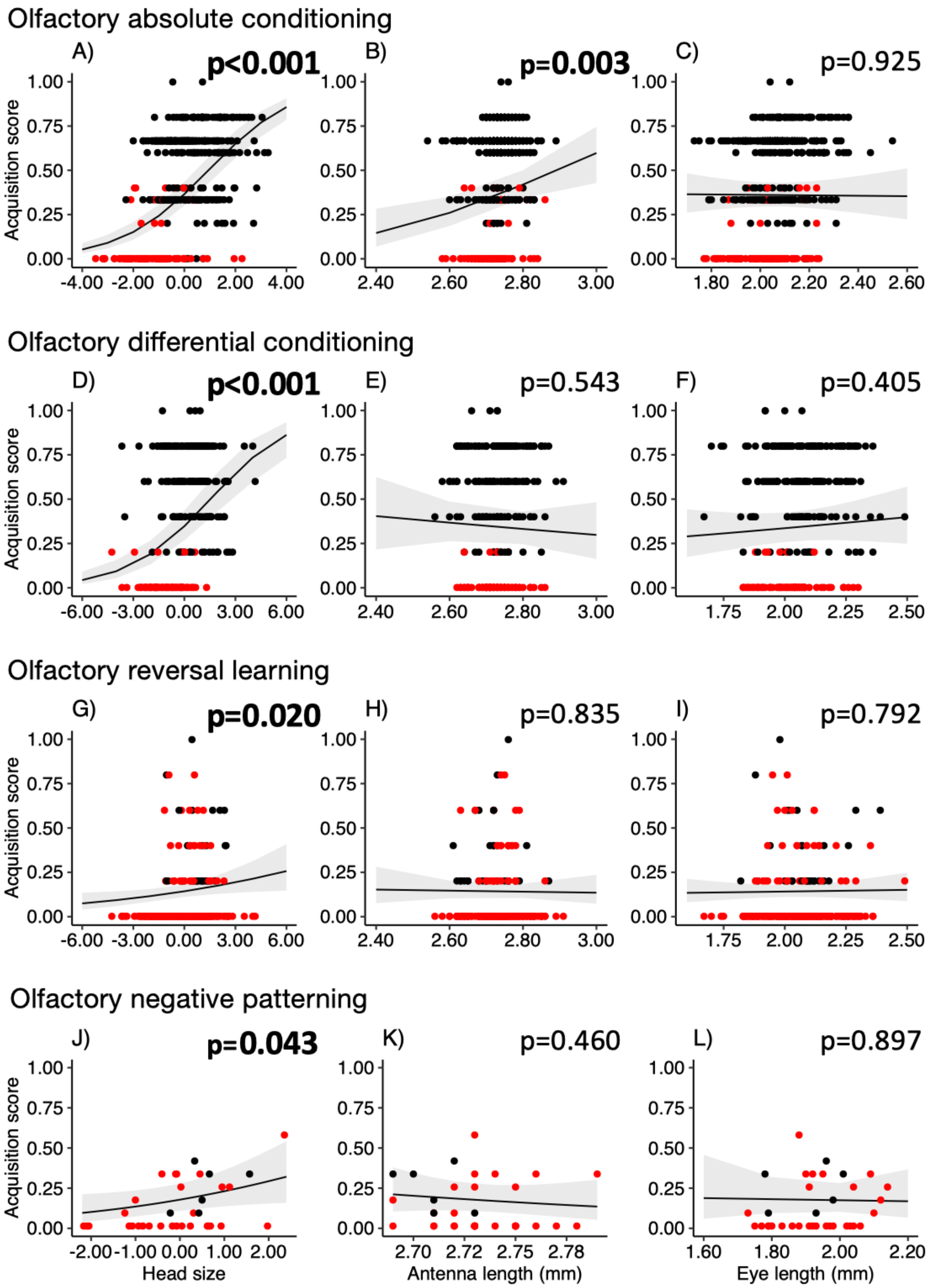

Figure 2: Acquisition scores per olfactory conditioning task relative to head size (PC1), antenna length and eye length in honey bees. Points represent the individual data for learners (learning=1; black) and non-learners (learning=0; red). Fitted lines of each morphological variable effect are displayed in black with 95% confidence interval in grey. **A-C)** Olfactory absolute conditioning (N=496). **D-F)** Olfactory differential conditioning (N=270). **G-I)** Olfactory reversal learning (N=234). **J-L)** Olfactory negative patterning (N=35). P-values were obtained from GLMM on acquisition scores and are displayed in bold when significant (Table 1). See details of data sources in Table S1.

Table 1: Parameter estimates (estimate±standard error) and p-values from mixed-effect models for acquisition, learning and memory specificity scores of honeybees and bumblebees. Significant p-values (<0.05) are shown in bold.

Species	**Modality**	**Task**	**N**	**Parameter**	**Head size**	**Flagellum length**	**Eye length**	**Social role** (in-hive *vs.* forager)
Honey bees	Olfactory	Absolute	N=496 (N=210 foragers + N=286 in-hive)	Acquisition	0.586±0.056 **p<0.001**	3.603±1.196 **p=0.003**	-0.052±0.546 p=0.925	0.285±0.192 p=0.138
				Learning	2.360±0.284 **p<0.001**	7.673±3.907 p=0.050	-1.887±1.883 p=0.316	1.771±0.603 **p=0.003**
		STM	N=174 (foragers only)	Specificity	0.740±0.194 **p<0.001**	0.684±6.290 p=0.913	0.882±1.821 p=0.628	NA
		LTM	N=201 (N=155 foragers + N=63 in-hive)	Specificity	0.202±0.142 p=0.155	-4.900±3.374 p=0.146	-0.430±1.415 p=0.761	0.445±0.497 p=0.370
		Differential	N=270 (N=121 foragers + 149 in-hive)	Acquisition	0.410±0.061 **p<0.001**	-0.778±1.281 p=0.543	0.538±0.646 p=0.405	0.209±0.240 p=0.384
				Learning	1.395±0.236 **p<0.001**	-1.129±1.830 p=0.769	1.045±1.829 p=0.568	1.299±0.626 **p=0.038**
		STM		Specificity	0.549±0.144 **p<0.001**	6.056±2.859 p=0.034	1.009±1.389 p=0.468	-0.506±0.429 p=0.339
		Reversal	N=234 (N=85 foragers + 149 in-hive)	Acquisition	0.121±0.052 **p=0.020**	-0.238±1.142 p=0.835	0.154±0.586 p=0.793	0.438±0.160 **p=0.006**
				Learning	0.440±0.171 **p=0.010**	-1.263±1.855 p=0.496	1.045±1.829 **p=0.031**	1.734±0.717 **p=0.016**

		Negative patterning	N=35 (N=10 foragers + N=25 in-hive)	Acquisition	0.325±0.161 **p=0.043**	-5.372±7.275 p=0.460	-0.223±1.726 p=0.897	-0.517±0.343 p=0.132
				Learning	1.175±0.727 p=0.106	-79.299±41.171 p=0.054	-5.448±5.657 p=0.336	1.338±1.636 p=0.414
		LTM		Specificity	1.619±1.224 p=0.186	-1.892±33.129 p=0.954	-5.999±8.389 p=0.475	21.119±19.068 p=0.999
	Visual	Appetitive differentiall	N=30 (foragers only)	Acquisition	-0.164±0.219 p=0.455	115.742±6.850 **p=0.022**	1.198±1.320 p=0.364	NA
				Learning	-0.440±0.623 p=0.480	34.325±22.306 p=0.124	3.177±3.799 p=0.403	NA
		Aversive differential	N=65 (N=33 foragers + N=32 in-hive)	Acquisition	-0.170±0.100 p=0.123	-0.474±1.152 p=0.681	3.552±1.119 **p=0.002**	-0.065±0.269 p=0.809
				Learning	0.414±0.487 p=0.396	-2.504±4.897 p=0.609	2.150±4.393 p=0.625	-0.712±0.845 p=0.400
		LTM		Specificity	0.099±0.180 p=0.581	0.605±1.976 p=0.760	-0.816±1.763 p=0.644	0.596±0.326 p=0.067
Bumblebees	Olfactory	Absolute	N=173	Acquisition	0.792±0.193 **p<0.001**	-0.147±0.301 p=0.626	-1.015±0.435 **p=0.020**	NA
				Learning	2.071±0.504 **p<0.001**	-0.236±0.716 p=0.742	-2.463±0.995 **p=0.013**	NA
		STM	N=54	Specificity	1.137±0.814 p=0.163	-0.039±1.314 p=0.976	-2.750±2.224 p=0.216	NA
		LTM	N=74	Specificity	-0.188±0.622 p=0.763	0.638±0.994 p=0.521	-0.764±1.325 p=0.564	NA
		Differential	N=124	Acquisition	0.365±2.237 p=0.251	-0.094±0.414 p=0.820	-0.473±1.010 p=0.0640	NA
				Learning	0.976±0.879 p=0.267	-0.240±1.019 p=0.814	-1.649±2.620 p=0.529	NA
		STM		Specificity	-0.531±0.831 p=0.523	0.734±1.160 p=0.527	0.754±2.683 p=0.779	NA
		Reversal	N=124	Acquisition	-0.217±0.282 p=0.441	-0.413±0.361 p=0.253	0.875±0.954 p=0.359	NA
				Learning	0.015±0.943 p=0.988	-1.805±1.313 p=0.169	0.782±2.981 p=0.793	NA
	Visual	Appetitive differential	N=157	Acquisition	0.382±0.158 **p=0.016**	-0.262±0.273 p=0.338	1.436±0.507 **p=0.005**	NA
				Learning	0.277±0.332 p=0.404	0.083±0.565 p=0.883	1.203±1.083 p=0.267	NA
		STM		Specificity	-0.144±0.333 p=0.666	0.120±0.555 p=0.828	1.644±1.088 p=0.131	NA

2.3. Honey bees with larger heads displayed better short-term olfactory memory

We next sought a potentially new relationship, between head size and memory performance when bees where tested for their capacity to recall the odour-reward associations they had learnt in either of the four tasks. In this case, trained bees were presented odours in absence of any reward, either 1 h and 24 h after conditioning, depending on the experiment. These delays correspond to different memories: the former is typically used to assess short-term memory (which is independent of protein synthesis), while the latter enables evaluating long-term memory, which requires protein synthesis (Menzel, 2001). In all cases, comparing the levels of responses to the previously rewarded stimulus and to novel (absolute learning) or previously unrewarded odours (differential conditioning, negative patterning) allows assessing the level of generalization to other odours, and thus the odour specificity of the expressed memory. When tested at short term, recall performance increased with head size following both absolute (GLMM: $p<0.001$) and differential conditioning (GLMM: $p=0.012$), as did memory specificity (Fig. 3A-F). While eye length had no influence, increasing antenna length was positively correlated with better recall performance and memory specificity (Fig. 3E), but only after differential conditioning. By contrast, long-term memory recall did not correlate with any of the morphological parameters, following either absolute learning (GLMM: $p=0.066$) (Fig. 3G-I) or negative patterning (GLMM: $p=0.262$) (Fig. 3J-L).

Because bees that actually learned the task are more likely to recall the association when tested for their memory, we asked whether the relationship between head size and memory could be observed independently of variations in learning scores. When considering only the 490 bees that successfully learned (learning score=1), head size had no effect on memory performance in either of the 4 recall conditions (Fig. S3). Thus, the relationship between head size and memory performance is probably the mere consequence of that observed during acquisition. Indeed, considering only learners selects for subsets of bees with much larger heads, by 42% in absolute learning (0.920 vs. 0.535 for all bees), and by 108% in differential learning (0.440 vs. -0.024 for all bees).

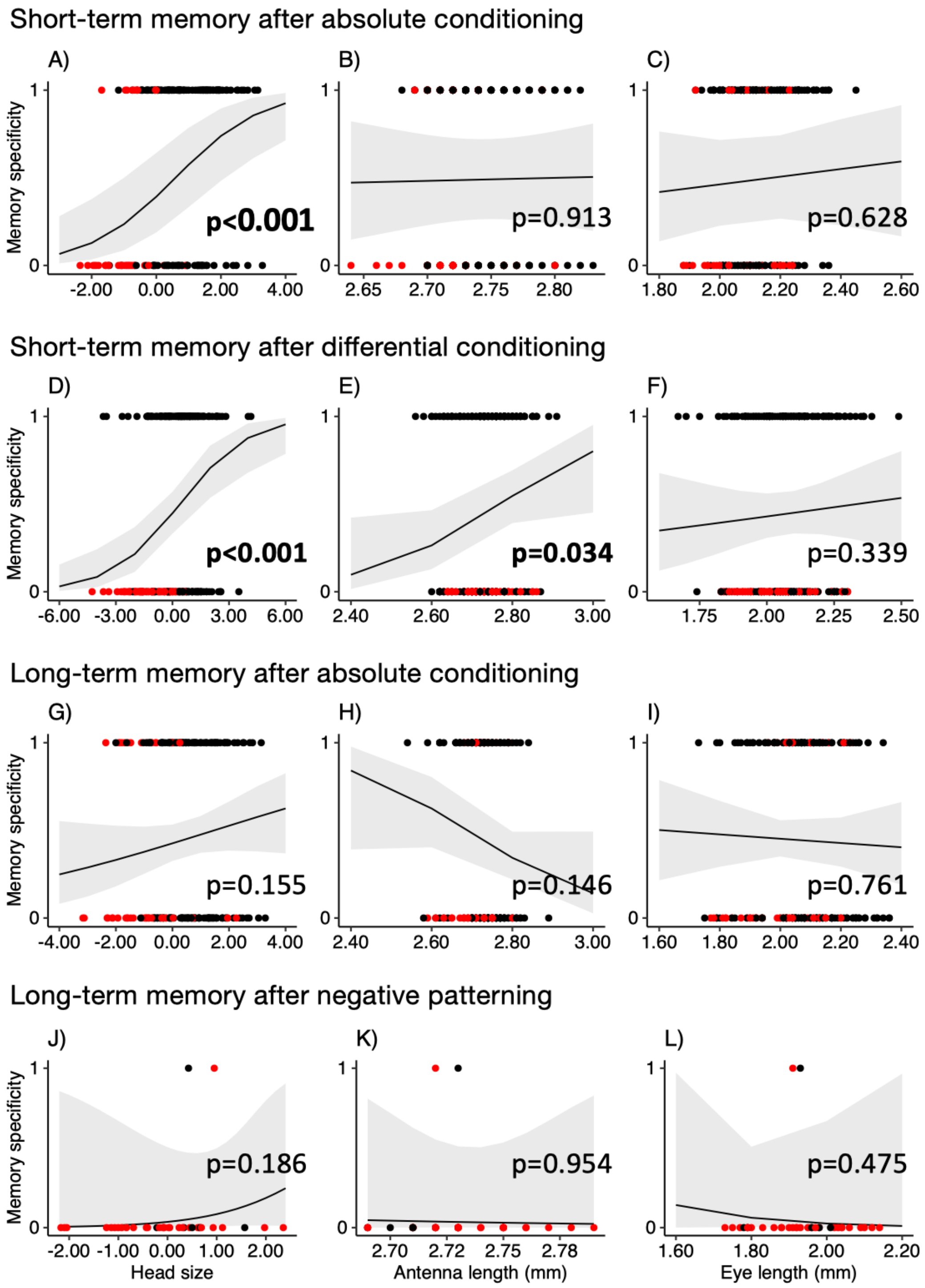

Figure 3: Olfactory memory specificity scores relative to head size, eye and antenna length in all conditioned honey bees. Points represent the individual data for learners (learning=1; black) and non-learners (learning=0; red) upon the last trial of learning. Fitted lines of each morphological variable effect are displayed in black with 95% confidence interval in grey. **A-C)** Short-term memory after absolute conditioning (N=174). **D-F)** Short-term memory after differential conditioning (N=270). **G-I)** Long-term memory after absolute conditioning (N=201). **J-L)** Long-term memory after negative patterning (N=35). P-values were obtained from GLMM

on memory specificity scores and are displayed in bold when significant (Table 1). See details of data sources in Table S1.

2.4. Honey bees with larger heads did not perform better in visual cognitive tasks

We then asked whether the observed effect of head size on learning and memory could be independent of the sensory modality used for learning. Hence we explored this relationship for the first time in visual modality tasks. For this, bees were trained in two differential conditioning assays with colours as visual stimuli: one where they were restrained as in previous olfactory assays, but with an electric shock as the unconditioned stimulus (aversive task), and the other where they could walk in a virtual reality setup, with sucrose (appetitive) or quinine (aversive) associated with each conditioned stimulus. Contrary to our observations in olfactory differential conditioning, for neither of these two tasks did head size correlate with acquisition scores (Fig. 4A, D) or proportion of learners (GLMM: p=0.480 and p=0.396 respectively). By contrast, longer antennae (Fig. 4B, E) and eyes (Fig. 4C, F) were correlated with higher acquisition scores, respectively in the task using both positive and negative reinforcement and in the aversive one. As for olfactory assays, we checked that sensitivity to light was independent of all three morphological parameters in a phototaxis assay (GLMM: head size p=0.488, antenna length p=0.806, eye length p=0.608; Fig. S2D-F). Hence, although one could argue that the lack of correlation in our dataset on visual learning might be due to much smaller sample sizes, our data suggest that the relationship between head size and learning performance may not be a general trend across all learning tasks. Importantly, we checked that sensitivity to neither sucrose (Fig. S2G-I) nor electric shocks (Fig. S2J-L) were related to neither of the 3 parameters (GLMM: resp. p=0.700 and p=0.759 for head size; p=0.620 and p=0.057 for antenna length; p=0.702 and p=0.142 for eye length). Thus, the correlations involving learning performance are likely to affect learning processes *per se* rather than changes in the sensitivity to the conditioned stimuli (odours, light) or in the motivation for the unconditioned stimuli (sucrose, shocks).

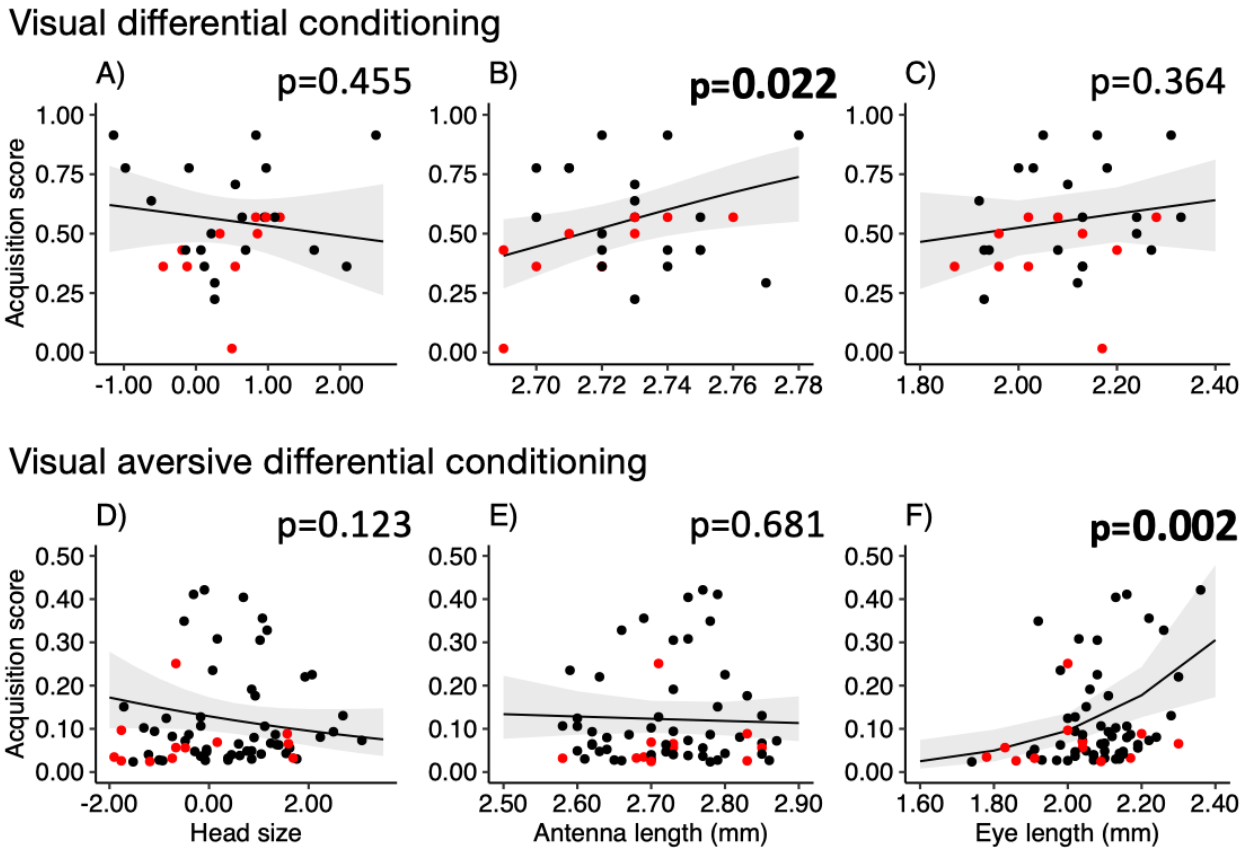

Figure 4: Acquisition scores per visual conditioning task relative to head size (PC1), antenna length and eye length in honey bees. Points represent the individual data for learners (learning=1; black) and non-learners (learning=0; red). Fitted lines of each morphological variable effect are displayed in black with 95% confidence interval in grey. **A-C)** Visual differential conditioning (N=30). **D-F)** Visual aversive differential conditioning (N=65). P-values were obtained from beta regression models on acquisition scores and are displayed in bold when significant (Table 1). See details of data sources in Table S1.

2.5. Head size effect on olfactory learning was independent of the social role or season

To test whether the head size effect on olfactory learning was linked to division of labour, we compared the head size and cognitive scores of two groups of honey bees in our datasets (Datasets 4, 8, 10, 12 in Table S1): those collected in colonies during autumn or winter (in-hive bees, N=609) and those collected at the hive entrance or at feeders during spring or summer (foragers, N=426). These two groups did not differ for any of the three morphometric parameters (head size:

GLMM: p=0.065; eye length: p=0.078; antenna length: p=0.568). Hence, social role had no influence on bees morphometric measurements, as previously reported (Monchanin et al., 2021a). In-hive bees had higher learning performances in absolute (GLMM: p=0.003), differential (p=0.038) and reversal learning (p=0.016) compared to foragers, but we found no effect on the acquisition scores, nor on negative patterning performances. In an olfactory absolute learning task, foragers had higher acquisition scores and learning by the end of the task than nurses (in-hive bees) (Scheiner et al., 2017), while nurses performed better than foragers in an olfactory reversal learning task, but had similar performances in differential learning (Ben-Shahar et al., 2000).

2.6. Higher absolute olfactory learning performances are linked to differences in antennal lobe volumes

To further explore the relationship between head size and olfactory learning performances, we micro-CT scanned the brains of 90 honey bees tested in absolute olfactory learning (Fig. 5A; Dataset 6 in Table S1). As previously described by Gronenberg and Couvillon (2010), we found that bees with larger heads developed larger brains (LMM: p=0.002). With increasing head size, they had larger antennal lobes (ALs) (p=0.016) and mushroom bodies (MBs) (p=0.015). However, head size did not relate to the volume of the central complex (CX) (LMM: p=0.875) or optic lobes (OLs) (LMM: p=0.325; p=0.453 for ME; p=0.151 for LO) (Fig. 5B, S4).

We next asked whether the learning performance related to the size of the brain or some brain regions. Interestingly, both acquisition and learning scores were higher in bees with larger brains (GLMM: resp. p=0.004 and p=0.006) (Fig. 5C), as well as larger ALs (resp. p=0.038 and p=0.041). By contrast, learning performance was independent of the volume of the other components (MB, OL, CX) had no influence on the learning performances. Gronenberg and Couvillon (2010) found that total brain volume was correlated with acquisition score in an absolute olfactory task. In particular, they highlighted the contribution of the mushroom body calyces, but for Africanised honey bees only, as it has been reported for bumblebees (Smith et al., 2020) or butterflies (Sivinski, 1989).

For short-term memory (Fig. 5D), when considering all bees, we also found a positive effect of brain volume (GLMM: p=0.046), but not of AL volume (GLMM: p=0.356). Short-term memory specificity was enhanced with decreasing relative volume of MBs (GLMM: p=0.019). The volume of the other component had no effect on short-term response to CS nor memory specificity.

On the contrary, response to CS during long-term memory test was not affected by brain (GLMM: p=0.402) or any neuropils volumes (GLMM: p>0.05), and nor was memory specificity (GLMM: p>0.05).

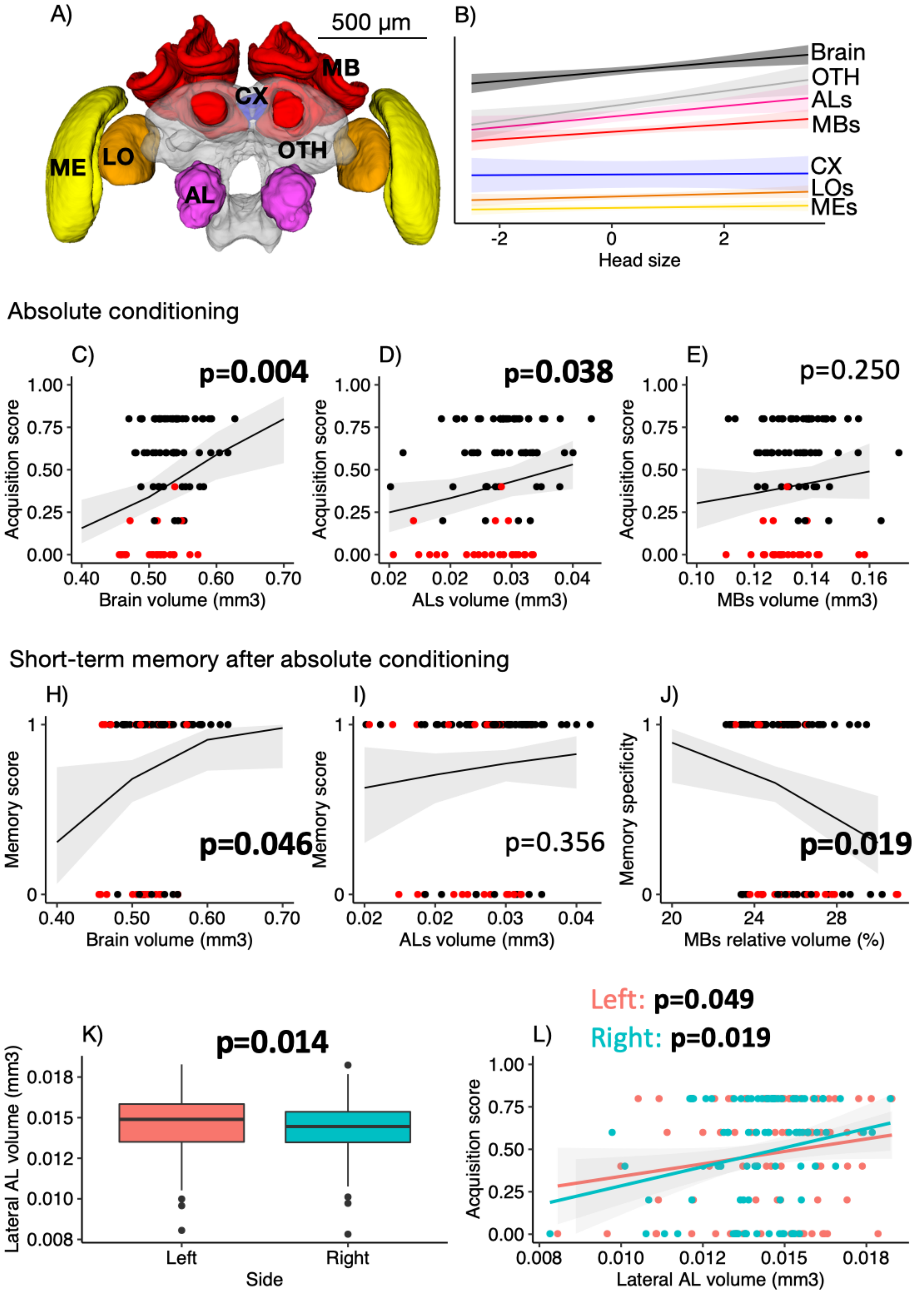

Figure 5: Relationship between brain composition and cognitive performance (N=90 bees). **A)** Example of a reconstructed brain (frontal view). AL: antennal lobes, MB: mushroom bodies, CX: central complex, ME: medullae, LO: lobulae, OTH: other neuropils. **B)** Neuropils volumes

(mm^3) in function of the head size (y-axis not given: differ for each neuropils). Fitted lines of each neuropils volume are displayed (using the same colour code as in A, total brain is shown in black) with 95% confidence interval. **C-E)** Olfactory absolute learning acquisition score, in function of the brain **(C)**, ALs **(D)** and MBs **(E)** volumes. **H-J)** Short-term memory score, in function of the brain **(H)**, ALs **(I)** volumes and MBs **(J)** relative volume. Points represent the individual data for learners (learning=1; black) and non-learners (learning=0; red) upon the last trial of conditioning. Fitted lines of each neuropil volume effect are displayed in black with 95% confidence interval in grey. P-values were obtained from beta regression models (for acquisition scores) or GLMM (for memory scores) and are displayed in bold when significant. **K)** Boxplot show median volumes (intermediate line) and quartiles (upper and lower lines) for left and right antennal lobe volume (mm^3). **L)** Olfactory absolute learning acquisition score. Fitted lines of each lateral AL (left – pink; right – blue) effect are displayed in colour with 95% confidence interval in grey. P-values were obtained from LMM (for volume comparison) or beta regression models (for acquisition scores) and are displayed in bold when significant. See details of data sources in Table S1.

2.7. Bumblebees with larger heads also showed better learning and memory performances

We next asked whether the association between head size and learning performance could be observed in other species than honey bees. For this, we addressed this question for the first time in bumblebees, and analysed data from 706 individuals trained in different olfactory and visual assays, similar to some used for honey bees. Overall, head measurements showed variation of a higher amplitude than for honey bees, as previously demonstrated and consistently with the known size polymorphism in this species (Mares et al., 2005). Head width varied by 50% (mean±s.e.m: 2.61±0.016 mm; min-max:1.89-3.75 mm), head length by 55% (2.76±0.016 mm; 1.68-3.72 mm), antenna length by 57% (3.23±0.017 mm; 1.89-4.26 mm) and eye length by 60% (2.26±0.013 mm; 1.45-3.64 mm). These levels and ranges of morphological variability are coherent with previous studies (Klein, 2018; Riveros and Gronenberg, 2010; Spaethe et al., 2007).

Again, we collapsed head width and length in a PCA. Here, PC1 explained 79% of the variance, and described bees with elongated heads *vs.* bees with large but short heads. PC2 discriminated bees with overall large head, and was used as a proxy of head size. Overall head size (PC2) was correlated with eye length (R=0.49, p<0.001), but not with antenna length (R=0.18, p<0.001), contrary to Spaethe et al. (2007) who reported a positive correlation between head width and antenna length. However, antenna length was negatively correlated to eye length (R=-0.50, p<0.001).

As in honey bees, proportion of learners in an absolute olfactory conditioning increased with head size (GLMM: p<0.001; Fig. 6A), independently of any correlation with sucrose sensitivity (GLMM: p=0.536). However, there were discrepancies between both species. First, antenna length (Fig. 6B) had no influence, while we found that eye length negatively impacted both acquisition score (Fig. 6C) and proportion of learners (GLMM: p=0.013), irrespective of any effect on sucrose sensitivity (p=0.627). However, none of the head measurements related to performance in differential conditioning (Fig. 6D-F) or reversal learning (Fig. 6G-I). We also found no influence on the short-term memory following absolute learning (Fig. 6J-L) or differential learning (Fig. 6M-O), nor on the long-term memory following absolute learning (Fig. 6P-R).

Importantly, unlike for honey bees, head size correlated with performance in a visual learning task (appetitive differential conditioning), where bumblebees with larger heads showed higher acquisition scores (Fig. 6S), but had no effect on the proportion of learners (GLMM: p=0.404). This confirms previous results established in a colour discrimination task (Worden, 2005). Antenna length remained without effect on both acquisition score and proportion of learners (Fig. 6T), while increasing eye length only enhanced acquisition score (Fig. 6U; proportion of learners GLMM: p=0.267). Short-term memory was also unaffected by the head measures (Fig. 6V-X). This effect is unlikely due to a bias of attraction to visual stimulus as suggested by previous studies in which there was no correlation between body size and phototaxis (Merling et al., 2020).

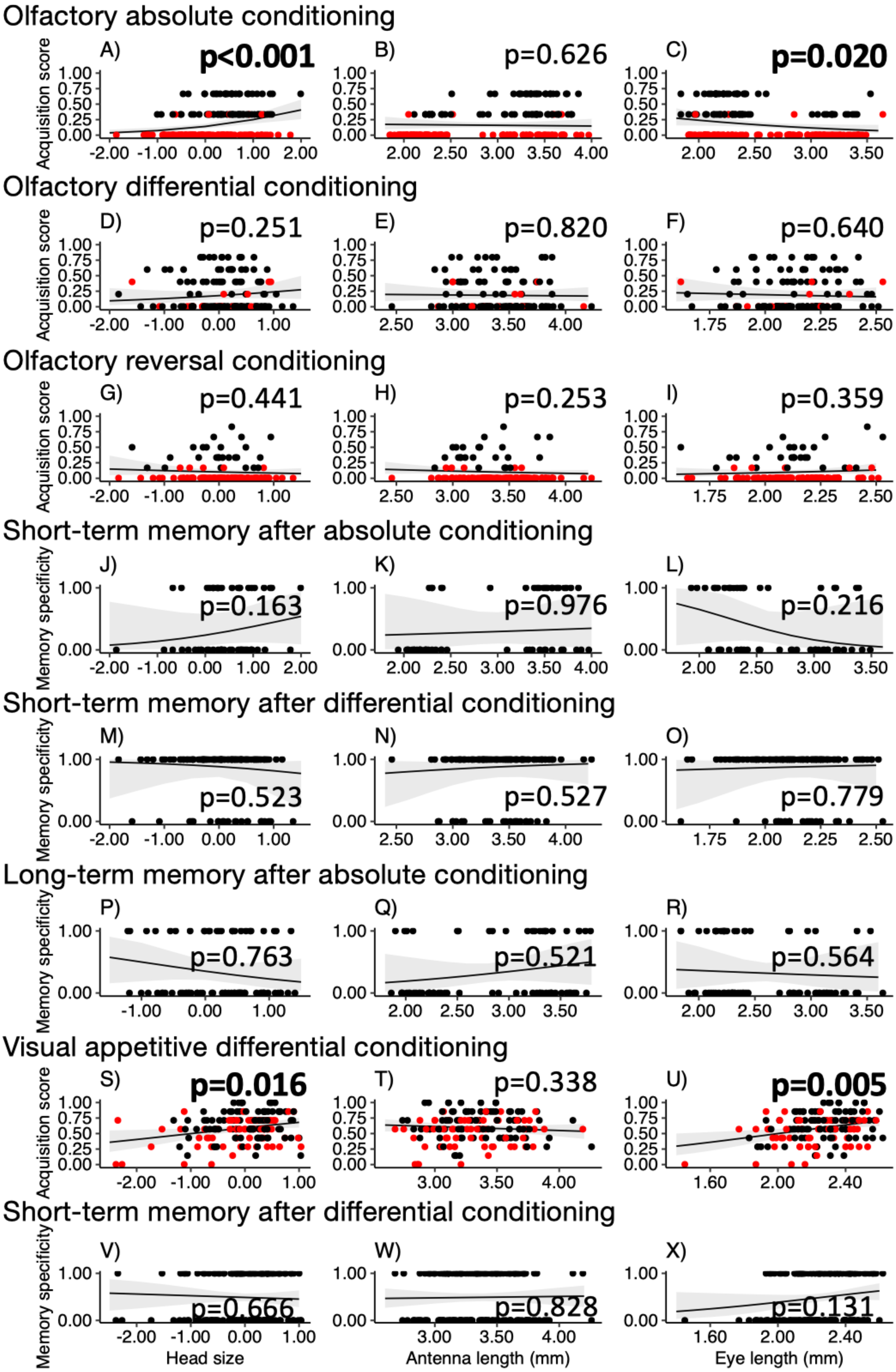

Figure 6: Acquisition scores and memory performances per conditioning task relative to head size (PC2), antenna and eye length in bumblebees. Points represent the individual data

for learners (learning=1; black) and non-learners (learning=0; red) upon the last trial of conditioning. Fitted lines of each morphological variable effect are displayed in black with 95% confidence interval in grey. **A-C)** Olfactory absolute conditioning (N=173). **D-F)** Olfactory differential conditioning (N=124). **G-I)** Olfactory reversal learning (N=124). **J-L)** Olfactory short-term memory specificity after absolute conditioning (N=54). **M-O)** Olfactory short-term memory specificity after differential conditioning (N=124). **P-R)** Olfactory long-term memory after absolute learning (N=74). **S-U)** Visual appetitive differential conditioning (N=157). Estimate trends are displayed in solid lines. 95% confidence level interval are displayed in grey. **V-X)** Visual short-term memory after differential conditioning (N=157). P-values were obtained from beta regression models (for acquisition scores) or GLMM (for memory scores) and are displayed in bold when significant (Table 1). See details of data sources in Table S1.

3. Conclusions

It has been argued that insect brains and cognitive capacities were shaped by their level of sociality (Gronenberg and Riveros, 2009) and by the role of individuals within societies (Chittka et al., 2012; Feinerman and Korman, 2017). In principle, division of labour should allow a reduction in inter-individual brain variability within a given caste, since all caste members perform a narrower range of behaviours (Gronenberg and Riveros, 2009). Here, we used a unprecedently large morphological and behavioural dataset on bees showing that variations in honey bee head size explain some inter-individual variability in olfactory learning and memory performances among workers. Bees with larger heads and brains perform better in olfactory, but not visual conditioning, irrespective of the task. By contrast, the size of sensory organs shows no marked correlation, nor do visual or olfactory sensitivities (or responsiveness to unconditioned stimuli).

Adult bees are known to show structural changes in brain area volumes and organisation with age (Ismail et al., 2006; Withers et al., 1993) and foraging experience (Withers et al., 2008). However, this might reflect dendritic branching and do not provide a precise measure of the synaptic connectivity therein: new synapses can be formed on pre-existing boutons or on new boutons (Cabirol et al., 2017). Similar observations were made on butterflies, with mushroom-bodies showing age-dependant increase in volume, while changes in antennal lobes volume were experience-related (Eriksson et al., 2019). Our results suggest that body size variation support cognitive variation at the basis of personalities, such as their inclination for social interaction contributing to subcaste division of labour (Walton and Toth, 2016). Behavioural diversity between individuals of the same castes also arise. Between- and within-individual speed-accuracy trade-offs lead to bees making consistently rapid choices but with low precision *vs.* other bees that are slower but more accurate (Chittka et al., 2003). Inter-individual variability within the forager caste also contributes to the optimization of colony-level foraging effort, with some individuals being elite foragers in response to unexpected colony needs (Tenczar et al., 2014).

Interestingly, similar results were observed in honey bees and bumblebees, two central-place foraging species whose social organization differ greatly (Bourke, 1988). Honey bees long-lived colonies rely on age-based division of labour, whereas bumblebees live in smaller colonies characterized by a body size-based division of labour. However, we observed for both species that increasing head size positively correlated with better learning performances, in some specific tasks at least. In both species, head size enhanced absolute olfactory learning and eye length visual differential learning. However, head size had no influnce on learning performance in olfactory tasks of higher complexity for bumblebees, while it positively correlated with learning in differential, reversal and negative patterning tasks for honey bees. Yet, some correlations may have been hidden due to the smaller sample sizes we assessed in some tasks (e.g. visual learning included 95 honey bees). The fact that we observe the same in honey bees and bumblebees suggests that this may be a process common to bees and probably other insects.

4. Materials and methods

4.1. Bees

We conducted all the experiments with honey bees (*Apis mellifera*, Buckfast) from our experimental apiary (University Paul Sabatier – Toulouse III, France) and bumblebees (*Bombus terrestris*) from a commercial supplier (Koppert, France) between 2018 and 2021.

4.2. Olfactory learning and memory

We collected individuals on the morning of each test, cooled them on ice and harnessed them. We harnessed honey bees in cylinders allowing free movements of their antennas and mouthparts. Head movements were prevented by fixing the back of the head using a droplet of melted wax (Matsumoto et al., 2012). We harnessed bumblebees in capsules crafted from 2mL Eppendorf tubes, with the head passing through a hole at the extremity (Toda et al., 2009). Bees were fed 5 µL of 50% sucrose solution (here and all subsequent concentrations of sucrose solutions are given in weight/volume) and left to rest 3 h in an incubator (dark; temperature: 25±2°C, humidity: 60%).

We assessed olfactory learning performance in several assays based on PER conditioning. For all experiments, we used a conditioning setup (Aguiar et al., 2018) where the trained individual was placed during each trial, facing a continuous flow of clean air (3,300 mL.min^{-1}). After 15 s of familiarization, the odour was presented during 4s, and the antennae were stimulated with a 50% (w/v) sucrose solution 3 s after the onset of the odour. Bees were rewarded by allowing them to feed on the sucrose for 2 s. Finally, bees remained another 15 s under the clean airflow. We recorded the presence or absence of a conditioned PER response at each trial (scored 1 or 0).

Sucrose sensitivity. We analysed data from 127 honey bees and 128 bumblebees (Datasets 1-2 in Table S1). During the assay, the bee's antennae were briefly stimulated with increasing concentrations of sucrose (0.1%, 0.3%, 1%, 3%, 10%, 30% w/v in water) (Scheiner et al., 2013). Prior to each sucrose trial, the antennae were stimulated with water, to prevent sensitization. The presence or absence of PER was recorded for each water and sucrose trial. If individuals did not answer to a subsequent 50 % sucrose stimulation, they were discarded, along with bees responding to water stimulation. Individual gustatory score (Scheiner et al., 2013) was calculated as the sum of PER throughout the assay (score between 0 and 6).

Odour sensitivity. We analysed data from 109 honey bees (Dataset 3 in Table S1). Olfactory stimuli were prepared by placing 1, 2.5, 5, 7.5 and 10 μL of pure geraniol (Sigma-Aldrich, Lyon, France), an attractive odour to honey bees (Free, 1962; Waller, 1970), on a 1 cm^2 piece of filter paper inserted in a 10 mL plastic syringe that was used to deliver the odour-filled air to the antennae. During the assay, odours in increasing concentrations (1, 2.5, 5, 7.5, 10 $\mu L.cm^{-2}$) were presented to harnessed bees during 4 s, interspersed with trials with clean air (syringe containing a piece of filter paper with no odorant) to prevent sensitization. The presence or absence of PER was recorded for each trial, and then summed throughout the assay (score between 0 and 5). Bees that did not respond at any trial were discarded.

Absolute conditioning. We analysed data from 496 honey bees and 172 bumblebees (Datasets 4-7 in Table S1). In absolute learning, bees must associate an odour to a reward (Matsumoto et al., 2012). One odour (nonanal or 1-nonanol, Sigma-Aldrich, Lyon, France) was reinforced with a sucrose reward (A+) across three (Datasets 4, 7 in Table S1) or five trials (Datasets 5, 6 in Table S1) (inter-trial interval of 10 min) (Matsumoto et al., 2012). We tested memory recall at 1 h (short-term memory) and at 24 h (long-term memory), by recording the response to the conditioned stimulus and to a novel odour to test for generalisation and thus assess memory specificity, both stimuli presented without reward.

Differential conditioning. We analysed data from 270 honey bees and 124 bumblebees (Datasets 8-9 in Table S1). In differential learning, bees must learn to respond differently to two odorants (A+ *vs.* B-) (eugenol and limonene, Sigma-Aldrich, Lyon, France) (Cabirol et al., 2018). One odour is reinforced with sucrose while the other odour is not, and each odour is presented 5 times in a pseudo-random order. We assessed memory recall at 1 h (short-term memory) by recording the response to both odorants, without reward.

Reversal learning. We analysed data from 234 honey bees and 124 bumblebees (Datasets 10-11 in Table S1). Following a first differential conditioning assay, bees must learn the opposite association. The two phases, separated by a 1 h rest, included 5 trials with the reinforced odour and five trials with the non-reinforced odour in a pseudo-random order (same odours as for differential conditioning) (Devaud et al., 2007).

Negative patterning. We analysed data from 35 honey bees (Dataset 12 in Table S1). In negative patterning, bees must learn to respond to the presentation of two distinct odours (limonene and 2-octanol, Sigma-Aldrich, Lyon, France), but not to the simultaneous presentation of both odorants (A+, B+ *vs.* AB-). The assay included x presentations of each rewarded odour and y of the unrewarded mixture, in a pseudo-random order (Deisig et al., 2001). We assessed long-term memory 24 h after conditioning, by presenting the three olfactory stimuli, without reward.

Each bee was given: (1) an acquisition score, which is the sum of correct responses divided by the number of trials. It represents how frequently bees showed that they had learned the association; (2) a learning score, 1 if they responded correctly upon the last trial of the cognitive task, elsewise 0; (3) a memory score, 1 if they responded to the conditioned stimulus during the memory test, elsewise 0, (4) a memory specificity score, 1 if they responded only to the conditioned stimulus during the memory test, elsewise 0.

4.3. Visual learning and memory

Phototaxis. We analysed data submitted 32 honey bees (Dataset 13 in Table S1). Bees were collected in the morning, fed ad libitum with 50% sucrose and left to rest for 3 h. They were then transferred into a 30 x 30 x 4.5 cm arena to measure their phototactic response. Each bee entered the arena individually through an opening at one corner, with a white cold-light source (200 lux) visible in the opposite corner. The rest of the arena was illuminated with red light, invisible to bees. The roof of the arena consisted of transparent plastic, and the bee's behaviour in the arena was filmed from above for 5 min (25 f.s-^{1}). The floor of the arena was wiped down with 70% ethanol between bees. Positions of the bees were averaged over 25 video frames, resulting in a sampling rate of 1/s for further analysis. For each bee, we calculated the latency until reaching within 10 cm of the light source (if a bee did not approach the light source this close, we recorded the maximum possible duration), expressed as a proportion of the total trial duration (5 min).

Shock sensitivity. We analysed data from 54 honey bees (Dataset 14 in Table S1). Bees, covered with conductance gel (Spectra 360 Electrode Gel, Parker Laboratories, Fairfield, USA), were harnessed on individual holders designed for aversive stimulation via delivery of an electric shock (Roussel et al., 2009). The bees were then fed 5 µL of 50% sucrose and left to rest for 1 h. They were then transferred to the electric shock delivery setup, consisting of a Plexiglas box where the brass plates of the holder could be connected to an electric stimulator (50 Hz-AC current), with an air extractor behind the holder. Each trial lasted 30 s, with 20 s of familiarization in the setup, 2 s of electric shock and remained for 8 s. Bees were stimulated with a sequence of increasing voltages (0.25, 0.5, 1, 2, 4 and 7 V). Placement trials, in which bees were placed in the setup without receiving any shock, were interspersed between each trial to avoid sensitization. The presence or absence of sting extension response (SER) was recorded for each trial. We then summed the number of SER throughout the assay (score between 0 and 5).

Aversive differential conditioning. We analysed data from 65 honey bees tested in a differential aversive visual learning task (Dataset 15 in Table S1) in which the bee must learn the association between a visual stimulus (blue or green light) and an electric shock (Marchal et al., 2019). Bees were collected at feeders each day and housed in small plastic boxes with 50% (w/w) sucrose solution available for feeding and placed in an incubator (28°C and 70% humidity) for at least 30 min before the beginning of the experiment. Before the 8 trials of the training sessions, bees first underwent 5min of familiarization in the set up. A conditioning trial began when the compartment not occupied by the bee was illuminated with blue light. When the bee entered into it, it received an electric shock during 200msec and then the light was switched to red for 1 min (intertrial interval). If the bee had not entered the blue-illuminated chamber within 5 min, the light was turned off and the trial finished without electric shock. At the end of training, each bee was placed in a pierced syringe with sucrose solution and put in the incubator overnight. A long-term memory test was performed at 24 h, during which the latency of bees to enter the blue-lit compartment (without electric shock) was recorded. Latencies were expressed as proportions compared to the maximal duration of each trial (5 min). Acquisition score was determined by summing the latencies to enter into the blue-compartment over the last 7 training trials. Bees were given a learning score of 1 if the latency at the last trial was greater than the latency at the first one, elsewise 0.

Differential conditioning. We analysed data from 30 honey bees tested in a visual differential learning task in a virtual reality set-up (Dataset 16 in Table S1). Foragers were collected the day before the experiment, housed in small plastic boxes with 50% (w/w) sucrose solution available for feeding and placed in an incubator (28°C and 70% humidity) overnight. On the morning of the experiment, bees were cooled on ice for 5 minutes and attached to the tethers for the virtual reality set-up (Schultheiss et al., 2017) for 3 h of familiarization. The visual stimuli used were two cubes, one blue and one green, which became larger as the bee moved closer to them. The bee needed to come within 3 cm of touching the cube and be positioned so it was facing the direct center of it for a choice to be recorded by the software. The bee was then provided a reward of 50 % sucrose solution for a correct choice or a punishment of 60 mM quinine solution for an incorrect choice. Bees underwent 10 trials, separated by 1 min of blank black screen. Acquisition score was determined as the sum of correct answers divided by the number of trials. Learning score was set at 1 if the bee learnt the task upon the last trial, 0 elsewise.

Appetitive differential conditioning. We analysed data from 157 bumblebees tested in a visual task (Dataset 17 in Table S1) using the free-moving proboscis extension response protocol (Muth et al., 2018). We isolated bumblebees from their colony and let them to rest 3 h in conditioning tubes (25 x 95 mm) where they could walk. The assay started with a preference trial, in which the bumblebee was presented two unrewarded visual stimuli (painted toothpick – blue or yellow) simultaneously. The bee was then tested in 7 training trials, in which one stimulus (unpreferred colour in trial 1) was rewarded (soaked in 50% sucrose solution) and the other stimuli (preferred colour in trial 1) was not (soaked in water). The short-term memory was assessed by presenting both stimuli an hour after the last training trial, without reward. We calculated an acquisition score as the sum of correct responses divided by the number of trials, a learning score of 1 if the bees successfully managed the task upon the last trial, and a memory score (1 if the bee still responded appropriately to the previously rewarded odour and not to the unrewarded).

4.4. Head measurements

All bees were frozen and kept at -18°C. We took measures (±0.01 mm) under a Nikon SMZ 745T dissecting microscope (objective x0.67) with a Toupcam camera model U3CMOS coupled to the ToupView software. We measured the head length (distance between the upper edge of the labrum and the lower part of the median ocellus) (De Souza et al., 2015; Monchanin et al., 2021a), head width (distance between the two lower corners of the composed eyes) (De Souza et al., 2015; Monchanin et al., 2021a; Spaethe et al., 2007), right eye length (distance between the lower corner and the upper corners of the compound eye) (Kapustjanskij et al., 2007) and left antennal length (flagellum length, (Spaethe et al., 2007)) (Fig. 1A).

4.5. Brain microtomography

We performed micro-computed tomography (micro CT) scanning of 100 honey bee foragers that underwent olfactory absolute learning and memory tests. We removed the front part of the head (just above the labrum) (Smith et al., 2016) and fully submerged the heads in 5% phosphtungstic acid solution (5 mg.L^{-1} in a 70/30% ethanol/water solution) for 15 days. Each head was scanned with a resolution of 5 µm using a micro CT station EasyTom 150/RX Solutions (Montpellier Ressources Imagerie, Montpellier, France). Raw data for each brain scan was reconstructed using X-Act software (RX Solutions, Chavanod, France). We then re-oriented to the same plane-of-view the reconstructed scan, and each brain was re-sliced into a new series of two-dimensional images. In total, 100 honey bee brains were scanned and 90 scans were kept based on staining and segmentation quality. We considered six neuropils: the antennal lobes (AL), mushroom bodies (MB) (comprising medial and lateral calyx, peduncle and lobe), central complex (CX) (comprising the central body, the paired noduli and the protocerebral bridge), medullae (ME) and lobulae (LO) (combined as 'optic lobe') and other neuropils (OTH) (protocerebral lobes and subesophageal ganglion) (Fig. 5A).

We analysed the volumetric growth of those six neuropils. We performed the segmentation and volume analysis using AVIZO 2019.1 (Thermo Fisher Scientific, Waltham, USA). We first performed the segmentation of the 6 neuropils on every 5 slices of 26 brains, followed by smart interpolation between the pre-segmented slices using Biomedical Image Segmentation App (Lösel et al., 2020). Those data were then used to train a neural network implemented in the Biomedical App (Lösel et al., in preparation - Appendix 7). We next used the trained neural network to predict the segmentation of every brain, which output was manually checked by an experimenter. Neuropil absolute volume was calculated using the voxel count function, with relative volume calculated by dividing absolute volumes by the total brain volume. For paired neuropils (e.g. AL, MB, ME, LO), we calculated the volume of the right and left structures.

4.6. Statistical analysis

All analyses were done with R Studio v.1.1.463 (RStudio Team, 2015).

Morphometric data. Because we were interested in the global effect of the head size, we collapsed the head width and length of each bee into a component of a principal component analysis (PCA; Fig. S1) using FactoMineR package (Lê et al., 2008). The individual coordinates from the PCA were then used as fixed effects in the following models.

Cognitive data. We evaluated the effects of the morphometric measurements on two types of cognitive parameters. Acquisition scores, and scores for sucrose, odour, phototaxis and shock sensitivity tests were analysed using beta regression mixed models (package glmmTMB; (Brooks et al., 2017)) with head size (from PCA), antenna and eye length as fixed effect, and date as random factor. Learning, memory and memory specificity scores were analysed with generalized linear mixed-effects models (GLMM) (package lme4; (Bates et al., 2015)) with a binomial distribution error, and the same fixed and random effects. For models regarding honey bees, we first tested the bee's social role (in-hive or outdoor) in interaction with all the head measures, but this interaction remained insignificant in all models, and did not improve them. Therefore, we kept the models with the social role as an additive fixed effect.

Scan data. We first assessed the relationship between head size and brain and neuropils volumes using linear mixed-effects models, with date as random factor, and head size as fixed effect. To assess the influence of brain and brain neuropils volumes on absolute olfactory learning and memory performances, we ran glmmTMB on acquisition scores and binomial GLMM on learning and memory scores, with date as random factor, and brain or neuropil volumes as fixed effect. To compare the lateral contribution for paired neuropils, we ran glmmTMB and GLMM for each lateral volume, and compared the models using Akaike's information criteria (AIC).

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Supporting materials

Table S1: Summary of the datasets analysed with type of cognitive test, species, sample size (N) and dataset statue (original/published).

Dataset	Type of test	Species	Social role	N	Dataset status
Olfactory cognition					
1	Sucrose sensitivity	Honey bees	In-hive	127	Original
2	Sucrose sensitivity	Bumblebees	NA	128	Original
3	Odour sensitivity	Honey bees	Foragers	109	Original
4	Absolute conditioning (3 trials)	Honey bees	Foragers In-hive	36 286	Original
5	Absolute conditioning (5 trials)	Honey bees	Foragers Foragers	33 41	Original Published (Monchanin et al., 2021b)
6	Absolute conditioning (5 trials)	Honey bees	Foragers	100	Original
7	Absolute conditioning (3 trials)	Bumblebees	NA	172	Original
8	Differential conditioning	Honey bees	In-hive Foragers	149 85	Original Published (Monchanin et al., 2021a)
9	Differential conditioning	Bumblebees	NA	124	Original
10	Reversal learning	Honey bees	In-hive Foragers	149 85	Original Published (Monchanin et al., 2021a)
11	Reversal learning	Bumblebees	NA	124	Original
12	Negative patterning	Honey bees	Foragers In-hive	10 25	Original
Visual cognition					
13	Phototaxis	Honey bees	In-hive	32	Original
14	Shock sensitivity	Honey bees	Foragers	54	Original
15	Aversive differential conditioning	Honey bees	Foragers In-hive	33 32	Original
16	Differential conditioning	Honey bees	Foragers	30	Original
17	Appetitive differential conditioning	Bumblebees	NA	157	Original

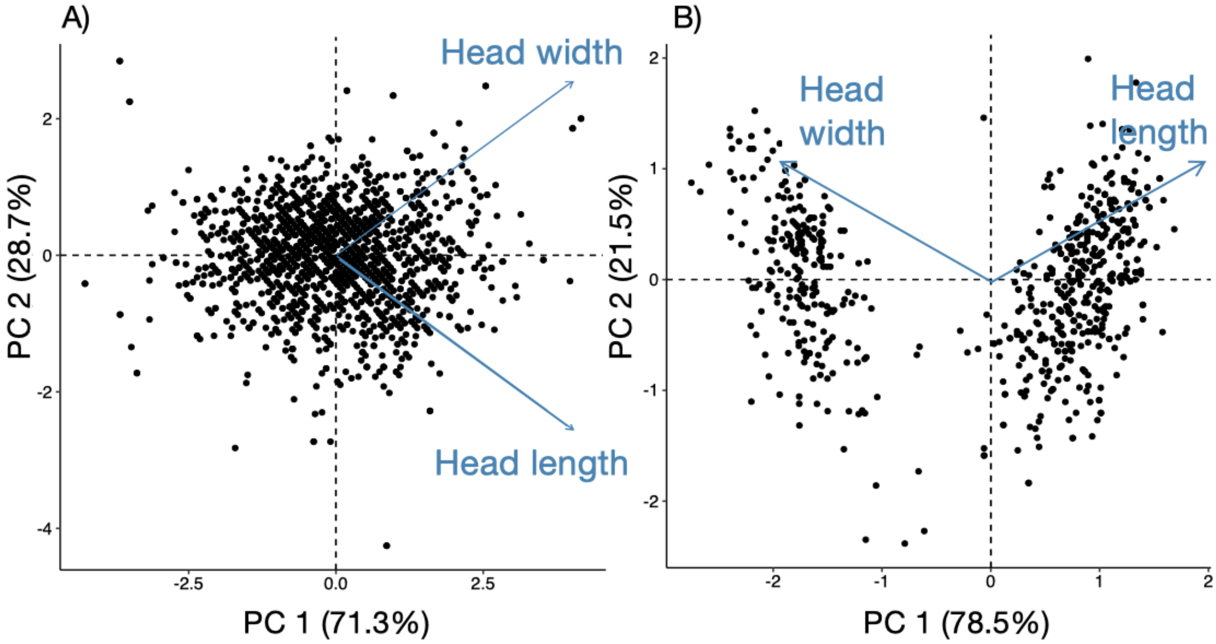

Figure S1: Principal component analysis map showing the relationship between head length and width. A) Honeybees (N=1593). **B)** Bumblebees (N=706).

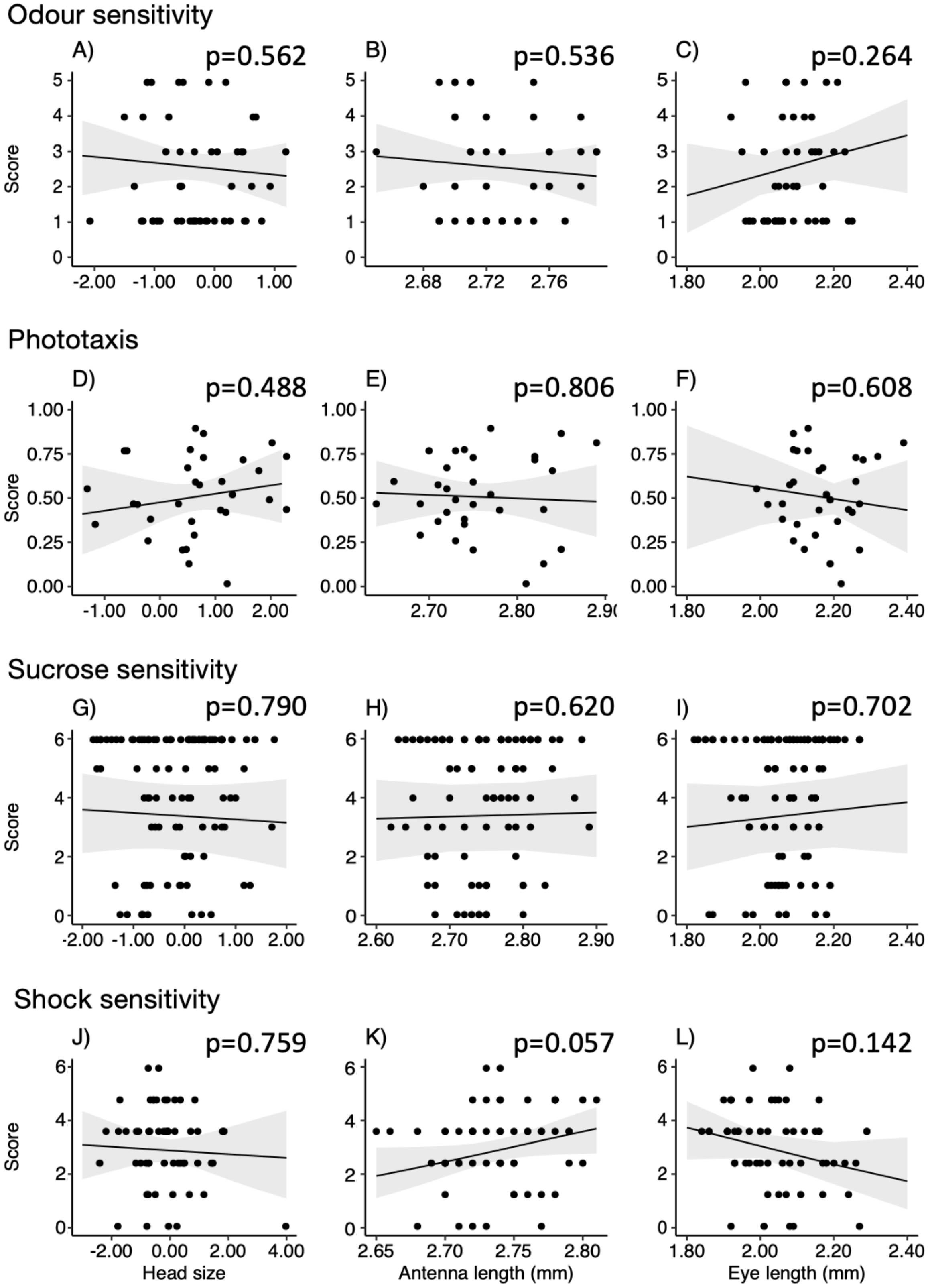

Figure S2: Sensitivity scores relative to head size, antenna and eye length in honey bees. Points represent the individual data. Fitted lines of each morphological variable effect are displayed in black with 95% confidence interval in grey **A-C)** Odour sensitivity test (N=50). **D-F)** Phototaxis assay (N=32). **G-I)** Sucrose sensitivity test (N=95). **J-L)** Shock sensitivity test (N=58). P-values were obtained from beta regression models and are displayed in bold when significant.

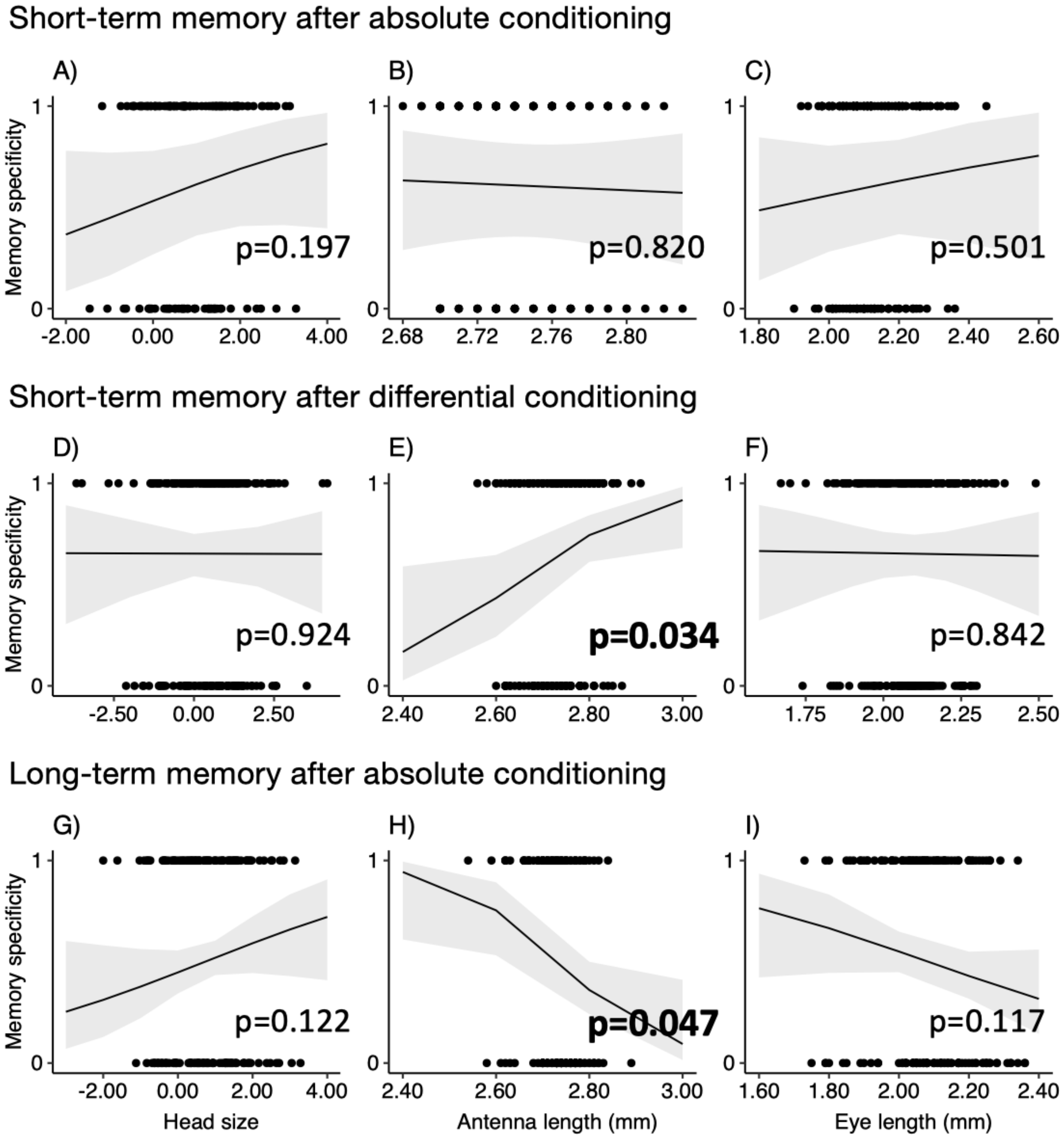

Figure S3: Olfactory memory specificity scores relative to head size, eye and antenna length in learner honey bees. Points represent the individual data for learners (learning=1; black). Fitted lines of each morphological variable effect are displayed in black with 95% confidence interval in grey. **A-C)** Short-term memory after absolute conditioning (N=139). **D-F)** Short-term memory after differential conditioning (N=193). **G-I)** Long-term memory after absolute conditioning (N=158). P-values were obtained from GLMM on memory specificity scores and are displayed in bold when significant (Table 1). See details of data sources in Table S1.

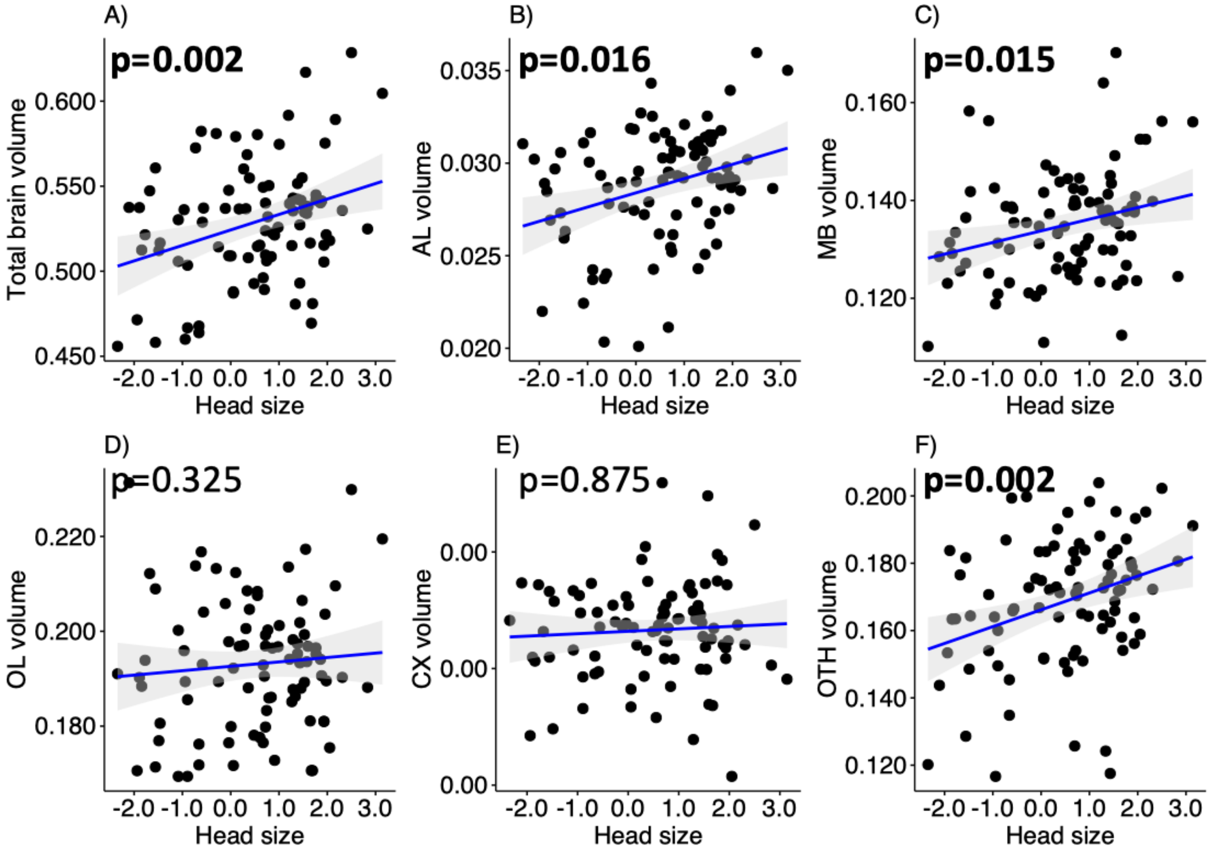

Figure S4: Relationship between brain composition (volume in mm^3) and head size. A) Total brain volume. **B)** Antennal lobes volume. **C)** Mushroom bodies volume. **D)** Optic lobes volume. **E)** Central complex volumes. **F)** Other neuropils volume. Fitted lines are displayed in blue with 95% confidence interval in grey. P-values were obtained from and are displayed in bold when significant.

General discussion

○○○○○○○●

“Poursuivant mon œuvre, je vais chanter le miel aérien, présent céleste. Je t’offrirai, à partir de tous petits êtres, un spectacle admirable. Quand le soleil d’or a mis l’hiver en fuite, et l’a relégué sous la terre, quand le ciel s’est rouvert à l’été lumineux, aussitôt les abeilles parcourent les fourrés et les bois, butinent les fleurs vermeilles [...]. Transportées alors par je ne sais quelle douceur de vivre, elles choient leurs couvées [...] et composent le miel.”

Virgile, Les Géorgiques.

GENERAL DISCUSSION

In light of my results (summarized in Fig. 7), here I discuss the impacts of metallic pollution on the behaviour of the honey bee, and more generally its potential contribution to the global biodiversity collapse.

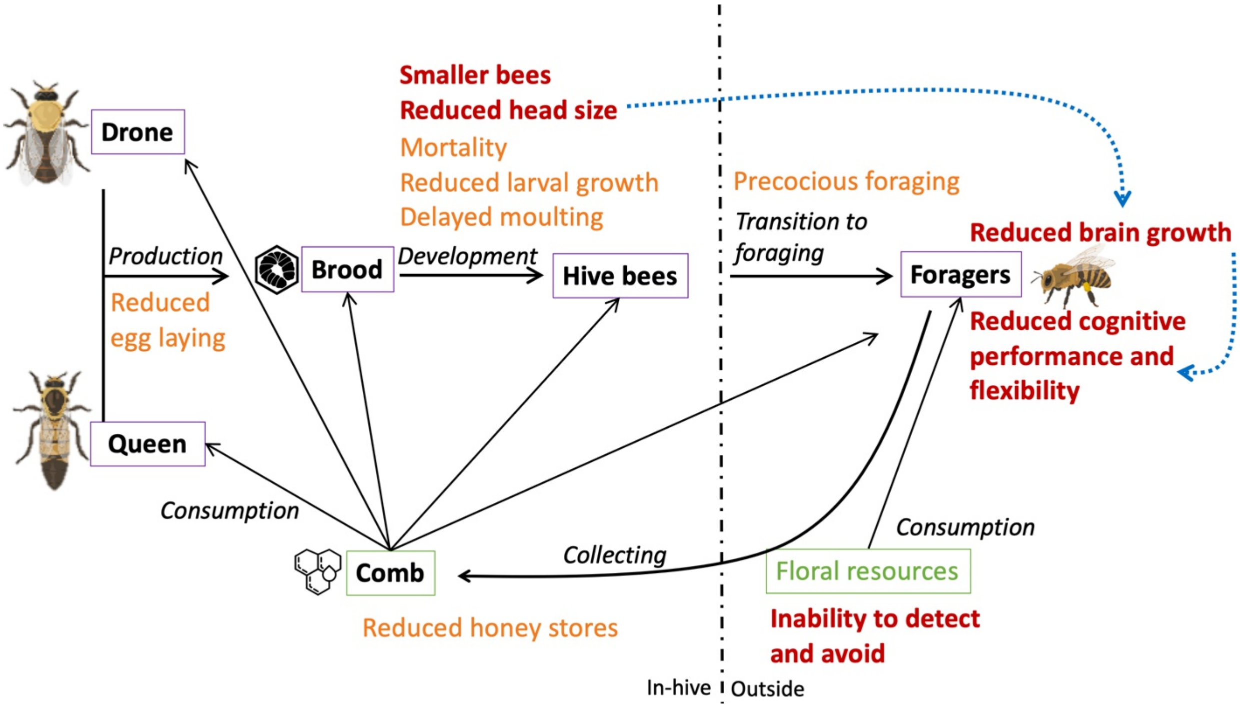

Figure 7: Effects of MTE on honey bee behaviour and colony dynamics. The brood (eggs, larvae and pupae) develops into in-hive bees that later start foraging. Foragers gather nectar and pollen from floral resources for storage in the hive comb. The food stores are then consumed by the queen, the drones, the larvae and the adult bees. Bees can be exposed to MTE at different life stages (*in purple*), by consuming contaminated resources (*in green*), potentially disrupting the whole colony dynamics. MTE accumulate in all castes (*in purple*) and hive products and are known to reduce brood production, alter development, induce precocious foraging and reduce the food gathering (*in orange*). The results produced during my thesis (*in red*) show that MTE can alter the proper head and brain development, affect the cognitive performance of foragers that are unable to control their exposure to metal-contaminated food resources. In unexposed bees, I unravelled a relationship between head size, brain composition and learning performance, which may be negatively affected by MTE (*in blue*). Adapted from (Klein et al., 2017a).

MTE have multiple origins, both natural (e.g. volcanic area (Bastías et al., 2013), rock erosion (Facchinelli et al., 2001)) and anthropogenic (e.g. urban growth (Rodríguez Martín et al., 2015), industrial (Shen et al., 2019; Zhou et al., 2018b) and agricultural activities (Huang et al., 2007)). MTE contaminate all environmental compartments, such as soil (Yang et al., 2018), water (Mance, 1987), air (Suvarapu and Baek, 2017), eventually accumulating in plants (Clemens and Ma, 2016). They are transferred through the food chains (Gall et al., 2015), with a potential for biomagnification (i.e. increasing concentration in tolerant organisms as they travel up the food chain) (Ali and Khan, 2019), ultimately jeopardizing human health (Hapke, 1996). MTE are persistent in the environment for millennia (McConnell and Edwards, 2008) in the form of complex mixtures (Anyanwu et al., 2018; Otitoloju, 2003) involving dozens of elements with several chemical forms (Hughes, 2002) and different levels of toxicity (Hladun et al., 2012). While some MTE are essential for the organisms at low doses (e.g. humans (WHO/FAO/IAEA, 1996); insects (Dow, 2017)), other are toxic even at trace levels (e.g. humans (Tchounwou et al., 2012); insects (**Chapter 1**)). Hence, they can exert toxic effects alone (**Chapters 2, 3**), but also when co-occurring (**Chapters 4, 5**) or combined with other environmental stressors such as pesticides (Singh et al., 2017) or microplastic pollution (Naqash et al., 2020). Notably, climate change alters many environmental conditions (pH, temperature etc.) that could impact metal speciation (i.e. the chemical form of the metal) and biotransformation, and consequently bioavailability, accumulation (Grobelak and Kowalska, 2020) and toxicity towards wildlife (Noyes and Lema, 2015) or humans (Blankholm et al., 2020). Hence, MTE represent a ubiquitous and complex source of pollution, and below I discuss their pernicious effects on honey bees and ecosystems in general.

1. How do metallic trace-elements impair crucial developmental and cognitive processes in honey bees?

1.1. Bees forage in contaminated environments

Metallic pollution is a widespread issue (Agarwal, 2009), and honey bees, among others insects, are on the front line when foraging in contaminated environments (**Chapter 1**). Understanding how insect feeding behaviour is modified by MTE exposure is necessary to assess the full scope and importance of MTE contamination (Mogren and Trumble, 2010). High metal concentrations seem to be repellent for various species (e.g. flies, grasshoppers) that will prefer food less contaminated, or not contaminated at all (Bahadorani and Hilliker, 2009; Migula and Binkowska, 1993; Rathinasabapathi et al., 2007), but whether this is a general trend is not known. This fundamental question of perception, detection and avoidance of metal-contaminated food resources by honey bees was previously little explored. Honey bees seem to perceive and readily ingest several essential minerals (e.g. sodium, potassium) but are deterred by higher, potentially toxic, concentrations of the same minerals (Teixeira De Sousa, 2019). While field-realistic concentrations of selenium (Hladun et al., 2012) or cadmium (Burden et al., 2019) were willingly consumed, copper and lead were only palatable at certain concentrations (Burden et al., 2019).

During my thesis, I demonstrated the inability of bees to perceive low, yet harmful, concentrations of arsenic, lead and zinc in food, which suggests that the presence of these metals in the environment represent a sizeable hazard to foraging bees (**Chapter 2**) (Fig. 7). This could explain why honey bees were unable to distinguish between sunflowers grown in lead-contaminated or uncontaminated soil (Sivakoff and Gardiner, 2017). Previous studies showed that honey bees spend less time foraging on metal-treated flowers, but visit them more (Xun et al., 2018). Honey bee floral decision are affected by the presence of aluminium in nectar (Chicas-Mosier et al., 2017). While the presence of aluminium in nectar has no influence on the foraging patterns of bumblebees, they visit for shorter time flowers containing nickel (Meindl and Ashman, 2013), which was also observed for other bees and flies (Meindl and Ashman, 2014). My observation that honey bees are unable to avoid metal-contaminated food resources, and therefore that metallic pollution could pose a serious threat to foraging bees, echoes the recent findings about the neonicotinoids' palatability (Kessler et al., 2015; Muth et al., 2020) and neonicotinoid-driven foraging preferences (Arce et al., 2018). Hence, my thesis emphasizes the need for further research into the mechanisms underlying bees' responses to metals, to better determine how metallic pollution might operate on foraging behaviours in the real world for different species of bees.

My work also questions the fate of honey bees inhabiting polluted environment such as urban areas with intense traffic (Papa et al., 2021; Zugravu et al., 2009), industrial and mining zones (Bastías et al., 2013; Matin et al., 2016; Satta et al., 2012; Taha et al., 2017), in which they bioaccumulate significant amount of MTE. Notably, urban beekeeping is presently booming in several cities. Not only such increase in urban apiary numbers leads to competition for floral resources that negatively affect wild pollinators (Ropars et al., 2019), but urban bees accumulate higher levels of insecticides and MTE compared to rural bees (Mahé et al., 2021). This raises issues concerning the health of honey bees in urban areas, but also the quality and food safety of urban honey that may exhibit a potential hazard to human health (Bosancic et al., 2020; Jovetić et al., 2018). Especially, the European Union very recently established regulations on the honey content in lead only (maximum level of 0.10 $mg.kg^{-1}$) (Codex Alimentarius, 2015), but other toxic metals were not considered. More generally, beekeepers should select more carefully apiary locations (Hladun et al., 2016), and avoid proximity to anthropized areas such as industrial facilities (Matin et al., 2016), busy roads (Gutiérrez et al., 2015), airports (Perugini et al., 2011) etc.

1.2. MTE disturb bee development

If honey bees are unable to control their exposure to metallic pollutants, they can collect contaminated nectar, pollen or water, bring them back to the hive, where they accumulate in bee bodies (**Chapter 3**), larvae (Balestra et al., 1992; Exley et al., 2015; Hladun et al., 2016) and hive products (Formicki et al., 2013; Roman et al., 2011). During my thesis, I found that chronic exposure to field-realistic levels of lead (**Chapter 3**) or complex mixtures found in a polluted area (**Chapter 4**) lead to a reduced honey bee larval growth, with smaller emerging honey bees, notably developing smaller heads (Fig. 7). This is consistent with reported effects of other stressors like pesticides (Gajger et al., 2017) or *Varroa destructor* mites (Belaïd et al., 2017). MTE have been reported to reduce the head size of other insects, such as ants (Grześ et al., 2015) or midges (Martinez et al., 2003). The developmental toxicity of MTE towards insects is well documented (**Chapter 1**). MTE delay development (Safaee et al., 2014) by altering carbohydrate and lipid metabolisms (Bischof, 1995), with a subsequent reduced growth rate (Ali et al., 2019) leading to smaller individuals (Cervera et al., 2004), as well as potential malformations (Eeva and Penttinen, 2009; Osman and Shonouda, 2017). In addition to mortality at adult age (**Chapter 2**) (Di et al., 2016), exposure to MTE during honey bee larval and pupal stages can induce mortality (Bromenshenk et al., 1991; Di et al., 2016; Hladun et al., 2016) or negatively affect bee development, by reducing larval growth rate (Di et al., 2020, 2016). Natural MTE exposure has also been reported to cause developmental asymmetries in wild bees (Szentgyörgyi et al., 2017). The susceptibility to MTE during development could be directly due to metal toxicity, through the alteration of nutrients metabolism (**Appendix 4**) (Ortel, 1995), the inhibition of enzyme activity (van Ooik et al., 2007), or due to detoxification costs counterbalancing the energy allocation to body growth (Mireji et al., 2010; Shephard et al., 2020).

1.3. MTE alter cognition and memory

In addition to developmental and physiological disruption (**Chapter 1**), MTE can impair insect behaviour (Mogren and Trumble, 2010). In controlled experiments, I used mostly concentrations falling below the permissible values in food and water (Ayers and Westcot, 1994; Codex Alimentarius, 2015), thus mimicking a realistic foraging scenario. For honey bees, acute exposure to field-realistic concentrations of arsenic, copper or lead slowed learning and reduced long-term memory recall in an olfactory absolute task (**Chapter 4**). Chronic exposure to realistic concentrations of lead under semi-field conditions (**Chapter 3**) also disrupted performance in a reversal learning, a task of ecological relevance (Izquierdo et al., 2017) that reveals cognitive flexibility (Scott, 1962). Finally, I also demonstrated that combinations of MTE in natural conditions (**Chapter 5**) or in the lab (**Chapter 4**) lead to similar dysfunctions of olfactory absolute learning and short- and long-term memory processes (Fig. 7). While the neurotoxicity of MTE towards humans is well established (Chen et al., 2016), the behavioural (e.g. mobility, navigation, feeding etc) and cognitive effects of MTE on insects is virtually unexplored. Exposure to lead altered butterflies cognitive processes (Philips et al., 2017) and mercury induced dysfunction in behavioural tasks for cockroaches (Piccoli et al., 2020). To our knowledge, only one study investigated the impact of MTE on honey bee cognitive functions: acute exposure to selenium was reported to disrupt olfactory differential learning and memory recalls, both short- and long-term (Burden et al., 2016). My results thus suggests that many MTE may have similarly negative impacts on bee behaviour, and pave the way for further analyses of the effects of MTE on insect cognition.

1.4. Cognitive performance is linked to head size and brain organization

I showed that cognitive performances of unexposed honey bees, in several behavioural tasks, were correlated to head size (**Chapters 3, 6**) and brain volume (**Chapters 6**). While this relationship was previously described in the case of absolute olfactory learning (Gronenberg and Couvillon, 2010), I extended these findings to learning tasks of increasing complexity (reversal learning, negative patterning) or involving other modalities (visual). I also unravelled a similar relationship between head size and cognitive abilities for bumblebees, for which previous studies reported correlations between body size and division of labour (Garófalo, 1978), foraging efficiency (Klein et al., 2017b) and visual learning speed (Frasnelli, 2020; Riveros and Gronenberg, 2012; Worden, 2005). Such correlations between brain size and cognitive abilities, while being vividly discussed (Chittka and Niven, 2009; Healy and Rowe, 2013; Lihoreau et al., 2012), have been reported for mammals (Benson-Amram et al., 2016; Dunbar, 1998), birds (Møller, 2010) or fishes (Edmunds et al., 2016), but also in insect species, such as ants (Gronenberg, 2008), butterflies (Snell-Rood et al., 2009) or wasps (van der Woude et al., 2018). Our converging findings between honey bees and bumblebees (**Chapter 6**) suggests that this may be common to several bee species.

A major innovation of my work was to study the brains of honey bees of different sizes. In particular, I showed, beyond the general trend of learning performance increasing with brain size, the significant contribution of antennal lobes (ALs) size to absolute olfactory learning performances (**Chapters 4, 6**). While the contribution of the mushroom bodies (MBs) was highlighted for honey bees (Gronenberg and Couvillon, 2010) and particularly of their calyces for bumblebees (D. B. Smith et al., 2020), we found that increasing ALs volume was a contributor to higher olfactory absolute learning success. ALs are the first olfactory centers in the brain, and participate in odour coding and olfactory learning (Marachlian et al., 2021; Menzel et al., 1996). Neural activity and synaptic plasticity within the ALs have been largely studied across insect species, e.g. moths (Hansson et al., 2003), flies (Silbering et al., 2008), wasps (Smid et al., 2003), bumblebees (Mertes et al., 2021) or honey bees (Peele et al., 2006). In honey bees, not only ALs are involved in sensory odour processing (Deisig et al., 2010), but also in associative odour-reward learning (Hammer and Menzel, 1998), optimizing odour representation (Denker et al., 2010) and facilitating odours discrimination (Rath et al., 2011). Besides MBs (Ismail et al., 2006; Withers et al., 2008, 1993), the ALs are the only brain structures showing changes associated with aging and behavioural maturation, as reported for honey bees (Brown et al., 2004) and butterflies (Eriksson et al., 2019). In addition, I showed a structural lateralization of the bee brain (**Appendix 7**), which might explain previous behavioural reports (Letzkus et al., 2008, 2006). Hence, my findings demonstrate the importance of brain and ALs volumes for olfactory learning in honey bees, and call for more research to better understand how brain organization in different bee species can influence learning performances in various paradigms and contexts.

Additionally, I demonstrated that CT-scan (Smith et al., 2016), coupled with automated prediction, appears to be a cost-effective and time-efficient technology to describe, with high-resolution, key composite structures of soft tissues, such as the insect brain (**Chapters 5, 6; Appendix 7**). Such techniques could be easily used to address evolutionary (Trautwein et al., 2012) and ecological questions related to cognition (e.g. effects of pesticides on brain development (D. B. Smith et al., 2020)), and could be more broadly used to investigate the impacts of MTE pollution on morphological impairments or metal accumulation (Courtois et al., 2021).

1.5. MTE may have multilevel sublethal pathways of action

Exposure to MTE, amidst other stressors (**Appendix 8**), reduced the cognitive abilities measured in adult honey bees (**Chapters 3, 4, 5**). I therefore wondered if these cognitive variations were due to a direct impact of MTE on adult brain function and/or an indirect impact through overall growth alterations (Fig. 7).

MTE can affect both neuronal structure and cellular physiology (neuronal excitability, synaptic transmission), but also alter subcellular mechanisms such as the biological actions of calcium (e.g. interference with calcium channels, disruption of calcium homeostasis, direct effect on calcium-mediated processes) or the function of proteins (e.g. neurotransmitter receptors or enzyme activity) (Audesirk, 1985). In insects, MTE are known to accumulate in brain tissues (e.g. grasshoppers (Augustyniak et al., 2006; Schmidt and Ibrahim, 1994); flies (Tylko et al., 2005)), where they can be traced (Yang et al., 2020), and to affect the nervous system, for instance by disrupting synaptic development (Morley et al., 2003), presynaptic calcium regulation (He et al., 2009), acetylcholinesterase activity (Schmidt and Ibrahim, 1994) or biogenic amines pathways (Søvik et al., 2017). In honey bees, perturbation of the acetylcholinesterase activity (Badiou-Bénéteau et al., 2013; Khalifa et al., 2020; Nikolić et al., 2019) or of biogenic amine levels (Nisbet et al., 2018; Søvik et al., 2015) have been reported.

The impact of MTE on adult cognition could also be indirect, via developmental alterations resulting in smaller heads (**Chapters 3, 5**) and brains (**Chapter 5**) in exposed insects. Indeed, unexposed bees performed better in olfactory tasks when they have bigger heads (**Chapters 3, 6**). Especially, in an absolute olfactory conditioning, honey bees with larger heads, thus larger brains, learned better (**Chapter 6**), as previously reported (Gronenberg and Couvillon, 2010). There is evidence that MTE reduce head growth in insects such as ants (Grześ et al., 2015), midges (Anderson et al., 1980; Martinez et al., 2003), wasps (Skaldina et al., 2020), moths (Jiang and Yan, 2017; Xiaowen et al., 2019) or honey bees (**Chapters 3, 5**), but contradicting results have been reported for ants (Eeva et al., 2004; Grześ et al., 2019) or isopods (Wadhwa et al., 2017). Decreased brain volumes were reported following exposure to manganese in humans (Chang et al., 2013), and/or lead in rats (Chandra et al., 1983), or zinc in honey bees (Milivojević et al., 2015).

My thesis thus calls for more work in order to clarify our hypotheses about the potential link between MTE, brain development, and cognitive dysfunctions. For instance, one could feed honey bee colonies with MTE-spiked food, explore the learning behaviour of adults that had experienced in-hive exposure during brood and/or early-stage adult development (D. B. Smith et al., 2020) while controlling their previous foraging experience using Radio Frequency Identification devices (Cabirol et al., 2018), and ultimately assess their morphology and measure their brain volume using micro-CT scanning techniques (**Appendix 7**).

1.6. MTE are overlooked contributors to bee decline

Overall, I showed that, at field-realistic concentrations, honey bees cannot avoid metal-contaminated food (**Chapter 2**), and that their exposure to different MTE, either chronically (**Chapters 3, 5**) or acutely (**Chapter 4**), whether in laboratory (**Chapter 4**), semi-field (**Chapter 3**) or natural conditions (**Chapter 5**), can alter learning and memory of olfactory cues (Fig. 7). Both processes play crucial roles in the behavioural ecology of honey bees for the identification of profitable food resources, social interactions and the recruitment of nestmates (Farina et al., 2005; Grüter et al., 2006). In addition, MTE, such as manganese, can lead to precocious and less efficient foraging activity (Søvik et al., 2015). Hence, MTE could impact colony function by destabilising the normal age-based division of labour, and by reducing foraging efficiency, as it has been reported following exposure to pesticide (Colin et al., 2019). Ultimately, MTE pollution could impair hive function and population growth via a snowball effect (Klein et al., 2017a). There is currently a paucity of information regarding the impact at the colony level of MTE. Under semi-field conditions, realistic levels of lead had not impact on colony function (**Chapter 3**), while hives fed with syrup, pollen and water containing selenium, cadmium, copper or lead displayed changes in colony dynamics and survival (Hladun et al., 2016). (Bromenshenk et al., 1991, 1985) reported reduced hive productivity for colonies in the vicinity of industrialized regions, contaminated with arsenic and cadmium. Urban lead contamination appeared to limit bumblebee colony growth (Sivakoff et al., 2020). Increasing concentrations of MTE negatively impacted the growth of wild bee populations (Moroń et al., 2014, 2012), which may be even more sensitive to

MTE pollution because they cannot rely on new cohorts of workers to replace contaminated individuals (Klein et al., 2017a).

My thesis highlights the need to better characterize sublethal effects (e.g. locomotion, reproduction) using field-realistic concentrations of MTE and to study MTE joint exposure (Otitoloju, 2003) to mimic realistic foraging exposure (**Chapter 1**), echoing the same recent trend as pesticide research (Benuszak et al., 2017). Especially, MTE are known to impact insect reproduction (**Chapter 1**) (e.g. reduced mating (Ved Chauhan et al., 2017), fecundity (Kenig et al., 2013), hatching rate (Luo et al., 2019)), hence slowing down the intrinsic rate of population increase (Skubała and Zaleski, 2012; Spurgeon et al., 2003); but no study has yet investigated this aspect on honey bees so far. Given that the threat of metal contamination may be very harmful to pollinators (**Chapter 1**), it is also necessary to further explore the effects on population dynamics. For instance, modern sensors enable the continuous monitoring of colony parameters (e.g. weight, temperature, foraging activity) and environmental conditions (temperature, humidity, pollutants) using connected hives (Marchal et al., 2019). Monitoring connected hives located in differentiated environments (e.g. natural, urban, industrial) characterized by increasing MTE pressure, among other pollutants, would be really informative.

In addition, MTE pollution should be taken into account when considering combined effects of multiple stressors, as they are known to interact with pesticides (Dondero et al., 2011; Sgolastra et al., 2018) or pathogens (Jiang et al., 2021) for instance, and should be implanted in mechanistic models used (Becher et al., 2018) to explore the multilevel impacts of populations under stress.

Finally, MTE contribution, while currently overlooked, should be better considered in the framework of bee decline. Findings from honey bee studies could be, to some extent (Wood et al., 2020), used as a starting point for extending them to other pollinator species (Thompson and Hunt, 1999). However, this would not bypass the urgent need to study other pollinator taxa (bumblebees (**Appendix 9**), wild bees, solitary bees etc.), to better understand what risk MTE may pose, and to get a more accurate picture of how they may disturb pollination service. Efficient pollination service relies on functional complementarity between wild bees and honey bees (Isaacs and Kirk, 2010), yet in some cases wild bee pollination appears more effective (Blitzer et al., 2016; MacInnis and Forrest, 2019). Beyond the crucial role of bees in crop pollination service (Kleijn et al., 2015) which underlies food safety (Hristov et al., 2020), conserving the biological diversity of bees is crucial in providing ecosystem resilience in the face of future environmental changes, by contributing to biodiversity maintenance and ecosystem structure, in supporting cultural and moral values (Senapathi et al., 2015), and might be an hidden weapon in the fight against climate change (Ollerton, 2021).

2. Metallic-trace elements, an underestimated threat in the global picture of biodiversity collapse?

2.1. Biomonitoring MTE pollution helps to establish geographical and/or temporal variations

Improved and timely implemented ecotoxicological risk assessment programmes may prevent further ecosystem poisoning, and the potential of many animal species to be used as reliable bioindicators and biomonitors of environmental metal contamination has been assessed (Stankovic et al., 2014). A metal biomonitor characterizes a species which accumulates MTE in its tissues, which may be used as a measure of MTE bioavailability in ambient habitats. For terrestrial ecosystems, this is the case for mammals (Tataruch and Kierdorf, 2003; Wren, 1986), birds (Berglund, 2018), bats (Zukal et al., 2015), ants (Skaldina et al., 2018), wasps (Urbini et al., 2006), but also plants (Markert et al., 1999; Sawidis et al., 2011). In aquatic ecosystems, corals (Hanna and Muir, 1990), sea turtles (Sakai et al., 1995), fishes (Authman, 2015) and their parasites (Sures et al., 1999), marine invertebrates (Chiarelli and Roccheri, 2014), for instance, are regarded as good biomonitors of the presence and relative magnitude of different metal sources (Rainbow, 1995).

Honey bees forage within up to 10 km from the hive and visit each an average of 1,000 flowers a day (Leita et al., 1996). Hence, they come into contact with a large number of pollutants, in all the environmental compartments of an area of about 7 km^2 surrounding the apiary. They can sample MTE, among other contaminants (Celli and Maccagnani, 2003), that will be stored in the nest, thereby exposing all colony members (Goretti et al., 2020; van der Steen et al., 2012) and accumulating in the hive products (Conti and Botrè, 2001). Especially, isotopic characterization in honey (Smith et al., 2021) and honey bees (Smith and Weis, 2020) can be used to discern MTE environmental and anthropogenic origins (Zhou et al., 2018b, 2018a), making them useful geochemical bioindicators (Smith et al., 2019). Honey can also be used as a biomonitor after an acute pollution event (e.g. the fire at Notre-Dame cathedral in Paris (K. E. Smith et al., 2020)). Not only honey bees and their products can be used to detect and measure environmental pollutants, but they can also be used as ecological bioindicators, i.e. indicative of the environmental quality, by assessing biological parameters in natural conditions at different scales: colony performance (colony growth, temperature regulation), and physiology of individuals (immune function, cognitive function) (**Chapter 5**; **Appendix 5**) (Quigley, 2019). Biomonitoring variations of the MTE bioavailability in the environment offers time-integrated measurements, that are of direct ecotoxicological relevance (**Chapter 1**).

2.2. MTE represent a serious threat for many ecosystems and organisms

While some MTE (e.g. iron, copper, zinc) have a suite of biological functions, high concentrations can lead to direct or indirect deleterious effects. Others metals exert toxicity, even at low levels. Some organisms are less susceptible to MTE stress and can develop tolerance towards MTE. Firstly, they can develop behavioural adaptation to avoid contamination (**Chapter 2**) (Wentsel et al., 1977). Organisms have also evolved mechanisms to deal with excessive metal pollutants

(Merritt and Bewick, 2017). In insects, metals can be eliminated through faeces (Przybyłowicz et al., 2003), accumulated in the exoskeleton before moulting (Borowska et al., 2004), or stored in specific organs such as the Malpighian tubules, their excretory system (Rabitsch, 1997). In addition, they can possess metallothioneins, which are proteins involved in the detoxification of MTE through binding and sequestering of metal ions, hence keeping low the bioavailability of those metals (Janssens et al., 2009). Metallothioneins have been identified in mammals (Sakulsak, 2012), marine mammals (Das et al., 2000), flies (Egli et al., 2006), grasshoppers (Liu et al., 2014) or honey bees (Purać et al., 2019).

Nonetheless, while those evolved behaviours or detoxification mechanisms may protect a species to a point, they are unlikely to spare them from the sublethal effects of these pollutants. Negative effects are reported for humans (Azeh Engwa et al., 2019; Briffa et al., 2020), marine mammals (López-Berenguer et al., 2020), corals (Howards and Brown, 1984), fishes (Govind and Madhuri, 2014), flies (Bahadorani and Hilliker, 2009) among other species. Especially, insects may be particularly sensitive to MTE pollution (**Chapter 1**). Their low dispersal capacity exposed them to continuous selection pressure (Migula et al., 2004). Sequestering or expelling excess metals involves metabolic costs (Morgan et al., 2007), which can limit the organisms' immune functions (Feldhaar and Otti, 2020; Sorvari et al., 2007) or ability to cope with other stressors, e.g. pathogens (Feldhaar and Otti, 2020; Jiang et al., 2021) or pesticides (Stone et al., 2001).

Many insects and invertebrates occupy a key place in the food chain, and have been found to provide an important link in transferring MTE from plants to carnivores (Zhuang et al., 2009). Ultimately, humans are being exposed (Briffa et al., 2020) through air, soil dust, but also via the ingestion of contaminated water (Fernández-Luqueño et al., 2013) or food, being meat (Wang et al., 2019), fish (Has-Schön et al., 2006), seafood (Golestani et al., 2019), plants (Hu et al., 2017) or insects (van der Fels-Klerx et al., 2018).

2.3. Perspectives

Firstly, my results call for an improvement of the international guidelines for the regulation of metal pollutants (**Chapter 1**). International guidelines are established for a couple of metals (i.e. As, Cd, Hg, Pb) only (Codex Alimentarius, 2015; WHO/FAO, 2001), and those thresholds, considered as ‘safe’ for humans are not restrictive enough to protect insects (**Chapters 1, 3, 5**). Over the last decade, an integrative and interdisciplinary research agenda has characterized the sublethal impacts of pesticides on beneficial insects, triggering a revision of the risk assessment scheme and their ban in the European Union in 2018 (Henry et al., 2012), even if there is still place for improvement (**Appendix 6**). There is an urgent need for policy-makers to address the issue of metallic pollution, as a major public health concern, update the current environmental regulation and develop conservation plans to mitigate global invertebrate and biodiversity declines and preserve associated ecosystem services. In addition to the necessity for more strict international thresholds (Hosono et al., 2011), there is a potential for further reduction of the MTE emissions (Pacyna et al., 2007; UNO, 2013) involving, for example, changes in industrial (Zheng et al., 2020) and agricultural practices (Puschenreiter et al., 2011), reduced traffic densities (Duong and Lee, 2011) and bioremediation plans to remove excessive metals (Rascio and Navari-Izzo, 2011; Wu et al., 2010) (e.g. the remediation strategy implemented at Salsigne (**Chapter 5**) (Gruiz et al., 2005)).

Secondly, my work highlights the need for more research on MTE (**Chapter 1**). For instance, the potential contribution of MTE to biodiversity changes across marine and terrestrial ecosystems (Bowler et al., 2020; Knapp et al., 2017) and to the reported decline in abundance and diversity of mammals (Yackulic et al., 2011), birds (Loss et al., 2015) or insects (Raven and Wagner, 2021; Sánchez-Bayo and Wyckhuys, 2019; Sánchez-Bayo and Wyckhuys, 2021; Wagner, 2020), such as butterflies (Warren et al., 2021) and pollinators (Brown et al., 2016) is barely considered. MTE concerns were also absent from recent roadmaps to develop insect conservation plans (Harvey et al., 2020) or from a proposal for the sustainability of European Common Agricultural Policy (Pe’er et al., 2020). Integrative ecotoxicological studies on a broader range of species and metal pollutants, also considering cocktail effects, between metals but also with other stressors (Otitoloju, 2003), are urgently needed (**Chapter 1**). The acknowledgement of the occurrence, importance and effects of contaminants on food chains and whole ecosystems (Boyd, 2010) will lead to the development of monitoring research and public awareness (Hsu et al., 2006) to protect not only humans, but also ecosystems in general.

Conclusion

This work constitutes the first integrated analysis of the impact of several MTE on cognition, morphology and brain organization of the domestic honey bee. I demonstrated that MTE, while being largely overlooked environmental stressors, pose a sizeable hazard to foraging bees and can impair crucial cognitive functions and disrupt honey bee development and brain growth. I hope this thesis will encourage further studies on the contribution of metal pollution to the jeopardized bees' fitness and health, and more generally, to the widespread insect decline and biodiversity collapse, and associated disruption of ecosystem services. A proper research agenda will help address these important challenges, while informing conservation plans to better protect ecosystems, underlying food security and public health. I believe my work can trigger awareness of the perniciousness of MTE pollution towards ecosystems and pave the way for further improvement of the environmental levels considered as 'safe'.

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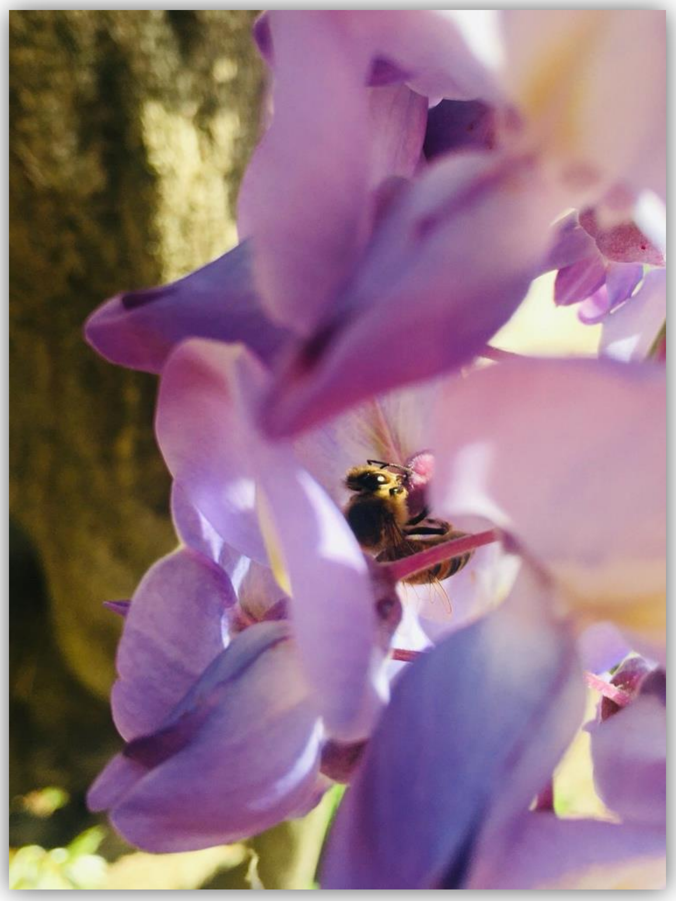
Appendices



“Anoche cuando dormía
soñé ¡bendita ilusión!
que una colmena tenía
dentro de mi corazón;
y las doradas abejas
iban fabricando en él,
con las amarguras viejas,
blanca cera y dulce miel.”

Antonio Machado.

Appendix 1A: Current permissible levels of metal pollutants harm terrestrial invertebrates

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Current permissible levels of metal pollutants harm terrestrial invertebrates

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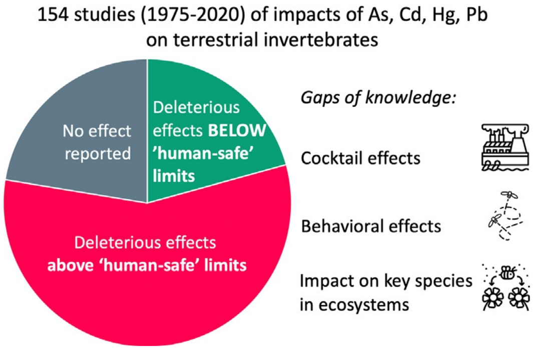
HIGHLIGHTS

- The current decline of invertebrates worldwide is alarming.
- Major pollutants, like metallic trace elements in the air, soils and water, are a potential cause, so far overlooked.
- We reviewed the sceintific litterature on the effects of As, Cd, Pb and Hg on terrestrial invertebrates.
- These well-studied pollutants impact invertebrates even at levels below those recommended as ‘safe’ for humans.
- Our results call for a revision of the regulatory thresholds to protect terrestrial biodiversity.

GRAPHICAL ABSTRACT



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ABSTRACT

The current decline of invertebrates worldwide is alarming. Several potential causes have been proposed but metal pollutants, while being widespread in the air, soils and water, have so far been largely overlooked. Here, we reviewed the results of 527 observations of the effects of arsenic, cadmium, lead and mercury on terrestrial invertebrates. These four well-studied metals are considered as priorities for public health and for which international regulatory guidelines exist. We found that they all significantly impact the physiology and behavior of invertebrates, even at levels below those recommended as ‘safe’ for humans. Our results call for a revision of the regulatory thresholds to better protect terrestrial invertebrates, which appear to be more sensitive to metal pollution than vertebrates. More fundamental research on a broader range of compounds and species is needed to improve international guidelines for metal pollutants, and to develop conservation plans to protect invertebrates and ecosystem services.

1. Introduction

Terrestrial invertebrate bioabundance and biodiversity are declining (Wagner, 2020). Since invertebrates are basal to terrestrial food webs and provide key ecosystem services, the short-term ecological consequences of invertebrate decline could be very severe (Goulson, 2019; Sánchez-Bayo and Wyckhuys, 2019). The rate of decline is especially

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alarming as it has been estimated that land-dwelling insects abundance has been declining at a rate of ca. 1% every year for a century (van Klink et al., 2020). Many factors have been proposed to explain this loss. These include climate change (Wilson et al., 2007), habitat reduction due to intensive agriculture and urbanization (Fattorini, 2011; Dudley and Alexander, 2019), introduced pathogens, predators and competitors (Goulson et al., 2015), as well as chronic exposure to agrochemicals (van Lexmond et al., 2015).

Here we argue that metallic pollution is a major, yet currently overlooked, stressor of insects and other terrestrial invertebrates that needs urgent attention from scientists and stakeholders. At trace levels, metals such as cobalt, copper, iron, manganese, selenium and zinc are essential micronutrients for animals and plants (Phipps, 1981; WHO/FAO/IAEA, 1996). Others, such as cadmium, chromium, mercury, lead and nickel, have no useful biological function and exert toxic effects even at low concentrations (He et al., 2005; Tchounwou et al., 2012). This is also the case for the metalloid arsenic, which we here also refer to as a metal pollutant for the sake of simplicity. While all of them are naturally present in the Earth's crust, their environmental concentrations have considerably increased above natural baselines (Zhou et al., 2018), due to mining and smelting operations, combustion of fossil fuels, industrial production, domestic and agricultural use of metals and metal-containing compounds (Bradl, 2005). This elevated and widespread contamination of air (Suvarapu and Baek, 2017), soils (Wuana and Okieimen, 2011), water (Mance, 1987) and plants (Krämer, 2010) has generated major public health concerns.

There are many detrimental impacts of metal pollutants on vertebrates, which include cellular damage, carcinogenesis and neurotoxicity (Tchounwou et al., 2012; Chen et al., 2016). Many local initiatives exist to reduce their emissions (e.g. lead: (Chadwick et al., 2011), cadmium: (Hayat et al., 2019), mercury: (Pacyna et al., 2009)). Even so, environmental metallic pollution is still high (Järup, 2003), calling for a more systematic assessment on the impact on biodiversity. For example, in 2019 the World Health Organization (WHO) stated that there was no safe level of lead for vertebrates (WHO, 2019), yet the majority of industrial activities are increasing the level of lead in the environment (Järup, 2003; Li et al., 2014). The recent report that bees and flies in densely urbanized areas suffer from exposure to metallic air particles (Thimmegowda et al., 2020) suggests that the consequences of metallic pollution on terrestrial invertebrates could be extremely important and widespread (for a review on aquatic invertebrates see (Rainbow, 2002)).

Here, we assessed the impact of metal pollutants on terrestrial invertebrates through a review of the scientific literature on four well-studied metals over the past 45 years. We found that these metals have detrimental effects on a wide diversity of species at levels below those considered safe for humans. We discuss the need for more fundamental research into the impacts of metal pollutants on insects to improve international guidelines for the regulation of metal pollutants, and better inform conservation plans.

2. Results

2.1. Few studies focus on species delivering important ecological function

The 527 observations extracted from the literature covered 100 species (83% Arthropoda, 15% Annelida, 1.2% Rotifera, 0.4% Tardigrada, 0.2% Mollusca; Fig. 1B). Studies were biased toward pest species with an economic impact (34% of observations; e.g. the gypsy moth *Limantria dispar*, the grasshopper *Aiolopus thalassinus*, the beet armyworm *Spodoptera exigua*) and model species in biology (10%; e.g. fruit fly *Drosophila melanogaster*, large milkweed bug *Oncopeltus fasciatus*). Other groups were comparatively under-represented, including important bioindicator species, such as decomposers (15%; e.g. *Lumbricus terrestris, Eisenia fetida* and *E. andre)*, predators (10%; e.g. ants *Formica* spp., spiders *Araneus* spp. and *Pardosa* spp.) and pollinators (13%; e.g. the honey bee *Apis mellifera*). Some taxonomic orders that include large numbers of species involved in nutrient cycling (e.g. proturans, diplurans, earwigs), soil aeration (e.g. centipedes), or pollination (e.g. thrips) were not represented at all. Research is thus needed on these important invertebrate orders with key ecological functions to get a more accurate picture of how metallic pollution disturbs ecosystems (Skaldina and Sorvari, 2019).

2.2. Metal pollutants have detrimental effects below permissible limits

Deleterious effects were reported in 84% of the laboratory observations (N = 263 out of 313) and 49% of the field observations (N = 104 out of 214), thus representing an average of 70% (N = 367 out of a total of 527; Fig. 2A). These negative effects were observed following chronic (69%) or acute (79%) exposure (resp. N = 348 out of 503 and 19 out of 24).

We then compared the doses at which these effects were observed to international permissible limits (i.e. recommended maximum concentrations) based on human toxicity data and determined by the World Health Organization (WHO) and the Food and Agriculture Organization (FAO) of the United Nations (see Methods and Table S2). These toxic levels were determined for food, but also water and soils to which arthropods are in direct contact.

When considering only the observations reporting deleterious effects (N = 367), 73% of these effects (N = 269) were measured at concentrations above the maximal estimated permissible limit (see Table 1). Yet, 12% (N = 45) were measured in between the regulatory thresholds and 15% (N = 53) below the minimal estimated limit (Fig. 2A). In addition, a majority (57%, N = 53 observations out of 93) of the observations using at least one concentration below the minimal estimated permissible limit found a negative effect at that low level, irrespective of the metal.

When considering only the laboratory studies, in which exposure concentrations were controlled (Fig. 2B–C), only 32% of the studies (N = 98 out of 313) used at least one concentration below or in between permissible limits. 57% of the studies that examined levels below the maximal permissible limits (N = 56 observations out of 98) reported deleterious effects on invertebrates below the permissible limits. Of the laboratory studies investigating acute exposure below the maximal permissible limits (N = 16), ten found deleterious effects (Fig. 2B). Hence, acute exposure, while presumably rare in nature, can have deleterious effects on invertebrates below current permissible exposure levels. This suggests that the permissible limits designed for humans are not appropriate for terrestrial invertebrates, who seem to be more sensitive to metal pollutants.

2.3. Few studies address the behavioral effects of metal pollutants

79% of the 154 studies we found were published after 2007 (Fig. 3A). About half of the observations focused on physiology (52%), followed by studies on development (17%), survival (13%), population dynamics (6%), reproduction (6%) and behavior (6%) (Fig. 3B). It has become increasingly clear that understanding the sublethal behavioral effects of a stressor (e.g. mobility, navigation, feeding behavior, learning, memory) is crucial to assess the long-term impact of that stressor on invertebrate populations (Mogren and Trumble, 2010). This has become evident for bees, for instance, for which any impairment of the cognitive functions involved in foraging can result in a disruption in food supply to the colony compromising larval growth (Klein et al., 2017). In our review, 33 experiments reported behavioral effects (Fig. 3B), but only two explored cognitive effects (Philips et al., 2017; Piccoli et al., 2020). This is a very low number considering the well-known neurotoxic effects of the four metals on humans (Chen et al., 2016; Wright and Baccarelli, 2007) and other animals, including aquatic invertebrates (Salanki, 2000).

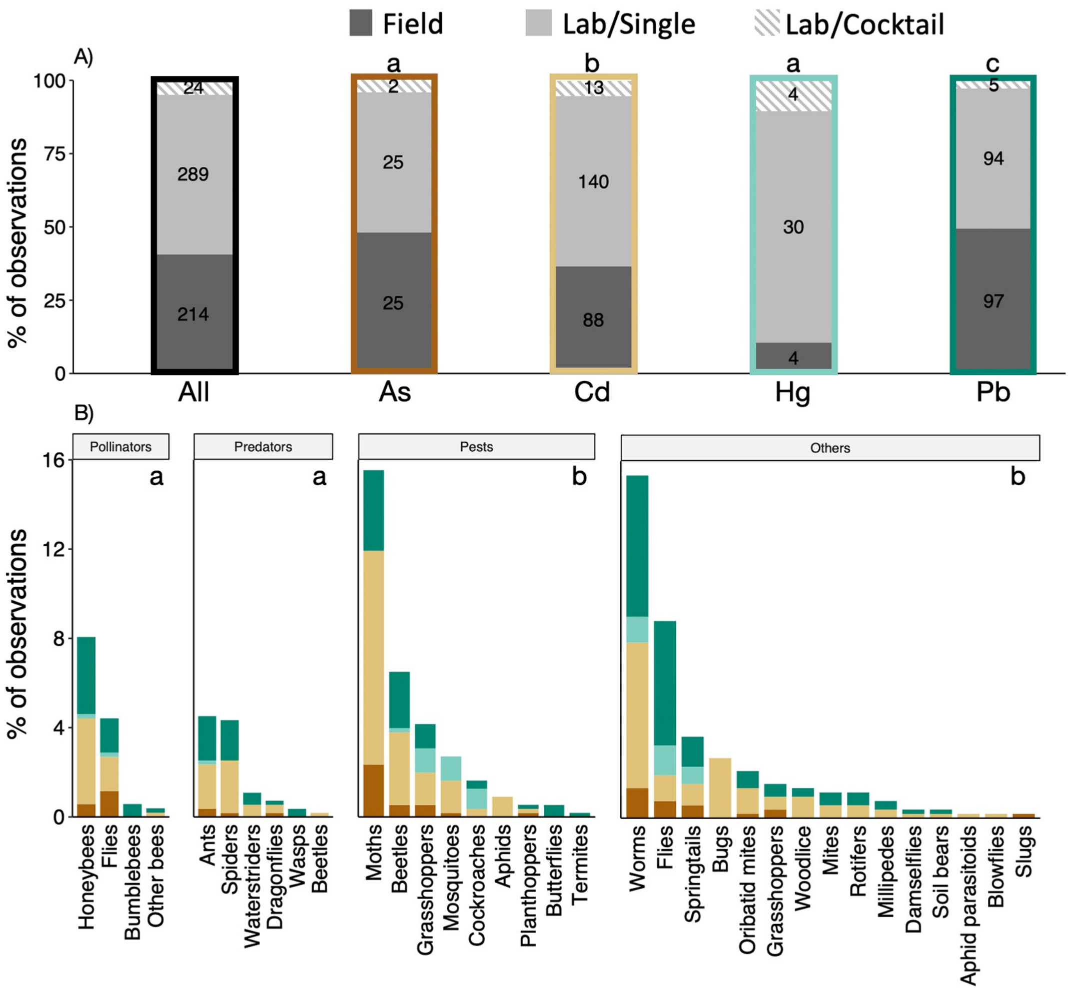

Fig. 1. Summary of invertebrate and experimental diversity in the surveyed literature. A) Percentage of observations conducted in the field (dark grey) or in the lab (light grey) per metal pollutant. Observations with mixtures of pollutants in the lab are displayed in textured light grey. Numbers of observations are shown in bars. Letters show statistical significance from chi-square test of homogeneity of proportions of observations per metal pollutant ($Chi^2 = 315.88$, $df = 3$, $p < 0.001$). B) Diversity of invertebrate groups classified by broad categories according to their ecological function and economic importance (based on (Skaldina and Sorvari, 2019)). Observations with different metal pollutants are marked using the same color code as Table 1 (As: brown, Cd: beige, Hg: light green, Pb: dark green). Letters show statistical significance from chi-square test of homogeneity of proportions of observations per functional group ($Chi^2 = 180.83$, $df = 3$, $p < 0.001$). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

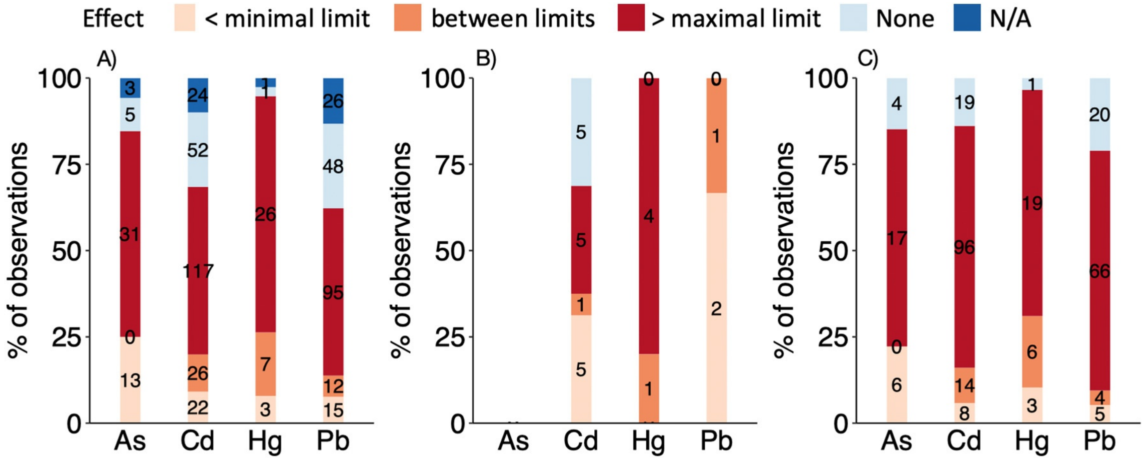

Fig. 2. Effects observed according to permissible limits. We defined the following ranges below the minimal estimated limit, between the minimal and the maximal estimated limits, or above the maximal estimated limit. A) All studies (N = 527). B) Laboratory studies with acute exposure (N = 24) and C) chronic exposure (N = 288). None: no observable effect, N/A: no conclusion available. Sample sizes are in black. Concentration ranges were marked using the same color code as Table 1.

Table 1
Permissible limits (ppm) for metal pollutants in food, water and soil. For each metal, we defined three concentration ranges: below the minimal estimated permissible limit (beige), between the minimal and maximal estimated permissible limits (orange), and above the maximal estimated permissible limit (red).

Matrices	Arsenic (As)			Cadmium (Cd)			Mercury (Hg)			Lead (Pb)		
Food	<0.1	0.1-0.2	>0.2	>0.05	0.05-2	>2	<0.5	0.5-1	>1	<0.01	0.01-3	>3
Water	<0.01	0.01-0.1	>0.1	<0.003	0.003-0.01	>0.01	<0.001	NA	>0.001	<0.01	0.01-5	>5
Soil	<20	NA	>20	<0.9	0.9-3	>3	<0.03	0.03-2	>2	<30	30-50	>50

2.4. Few studies investigated co-occurrences despite clear synergistic effects

Only 7 out of the 154 studies addressed the question of combined effects of metal pollutants in laboratory conditions (Fig. 1A). Nonetheless the effects are clear: 55% of the observations ($N = 10$) reported synergistic detrimental consequences. For instance, ants (*Formica aquilonia*) chronically exposed to both cadmium and mercury failed to develop compensatory mechanisms to maintain energetic balance, causing colony collapse, while being able to cope when exposed to each metal alone (Migula et al., 1997). Similarly, the lethal effects of cadmium and zinc on aphids (*Myzus persicae*) were potentiated when the two metals were combined, which led to accelerated extinction of the treated population (Stolpe and Müller, 2016). These two metals were reported to be either synergistic or antagonist on earthworms (*E. fetida*) depending on their concentrations (Wu et al., 2012). Finally, the joint exposure of honey bees (*A. mellifera*) to cadmium and copper caused an increased development duration, elevated mortality, and decreased food intake and sucrose response (Di et al., 2020). Thus, the effects of metal co-exposure are complex and variable. The paucity of studies may be because they require more sophisticated experimental designs, larger sample sizes (factorial designs) and may yield results that are more difficult to interpret. Yet, these studies are crucial if we are to revise the current regulations which presently only consider permissible limits for metals in isolation (Tables 1 and S2).

3. Discussion

Our review of the literature on lead, arsenic, cadmium and mercury shows many negative effects of these metal pollutants on terrestrial invertebrates. Excessive exposure to these compounds lead to a plethora of consequences, such as cytotoxicity (Braeckman, 1997), carcinogenic and/or mutagenic effects (Kheirallah et al., 2019), and disruption of metabolic processes (Ortel, 1995). Particularly worrisome are the reports of negative effects observed at doses below permissible limits in most of the studied taxa. There are reported lethal effects on grasshoppers (Schmidt et al., 1991), moths (Andrahennadi and Pickering, 2008), flies (Massadeh et al., 2008) and other groups (Osman et al., 2015; Polykretis et al., 2016; Stolpe et al., 2017). Metal exposure causes a number of sublethal effects, sometimes difficult to assess, such as impaired fertility (grasshoppers: (Schmidt et al., 1991); springtail: (Crouau and Pinelli, 2008); earthworm: (Konečný et al., 2014)), developmental defects (blowfly: (Nascarella et al., 2003); moth: (van Ooik et al., 2007); ant:

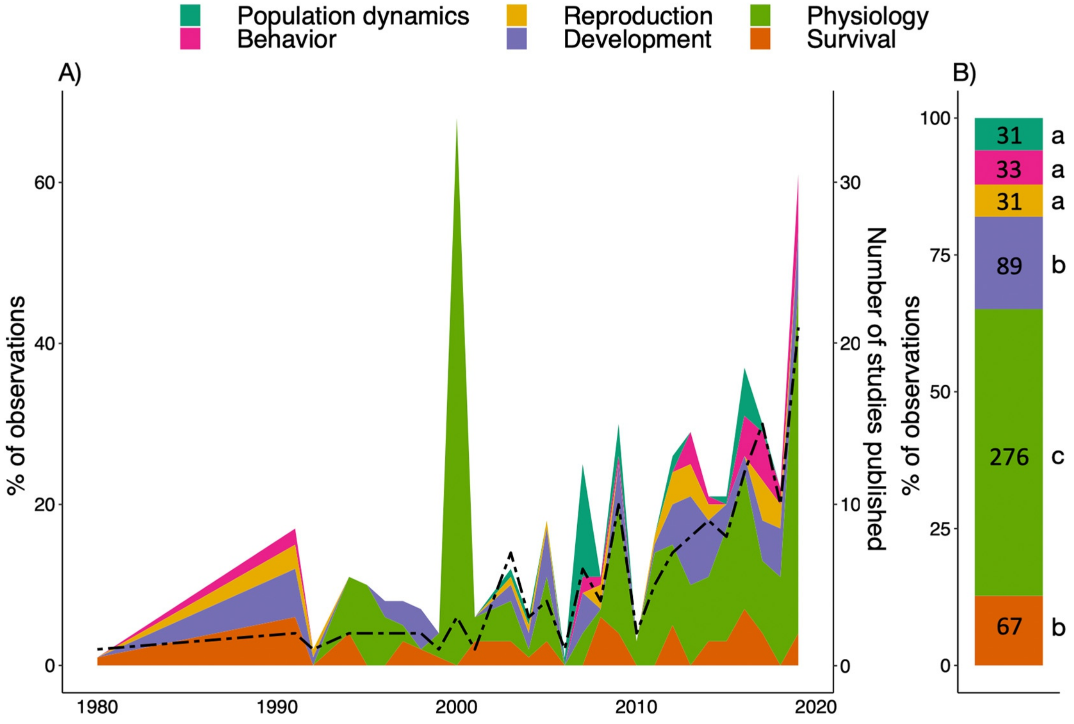

Fig. 3. Biological variables measured. A) Area chart of the number of observations per biological variable (year 2020 was omitted). The peak in 2000 is due to three large studies of physiological effects in the field (38 observations). The black dashed line represents the number of studies published yearly. B) Overall proportions of observations per biological variable (numbers of observations in black). Letters show statistical significance from chi-square test of homogeneity of proportions ($Chi^2 = 619.02$, $df = 5$, $p < 0.001$).

(Skaldina et al., 2018)), resistance to pathogens (ant: (Sorvari et al., 2007); honey bee: (Polykretis et al., 2016)) and also altered feeding behavior (aphid: (Stolpe et al., 2017); honey bee: (Burden et al., 2019)).

3.1. The impact of metal pollutants is poorly understood

At present, it is likely that the severity of these effects is underestimated. Many laboratory experiments gave animals rather limited exposure times, rarely reaching the duration of a complete life cycle. Besides, most studies overlooked any consequences of exposure to multiple metal contaminants, which would be a common occurrence in nature. There is now growing interest in assessing the sublethal impacts of metals. This trend echoes the recent shift seen in pesticide research on beneficial insects, especially pollinators, which has moved from decades of standard survival assays to experimental designs aiming at characterizing the effects on behavior and cognition (Klein et al., 2017; Desneux et al., 2007). Just like pesticides, metal pollutants have subtle, but potentially serious, effects on pollinators' behavior by disturbing foraging activity (Sivakoff and Gardiner, 2017; Xun et al., 2018), food perception (Burden et al., 2019) and the learning and memory abilities required for efficient foraging (Burden et al., 2016; Monchanin et al., 2021). Through all of these mechanisms, exposure to metal pollutants can compromise food supply to the offspring, and hence the viability of a colony or population.

There are potentially complex interactions between behavior and pollutant exposure. Since an animal's behavior can influence how much metal pollution it is exposed to (Mogren and Trumble, 2010; Gall et al., 2015), behavioral disturbances may affect exposure and sensitivity to metals. For example, impaired locomotion may reduce the capacity of individuals to avoid contaminated sites (Hirsch et al., 2003) and indiscriminate oviposition may jeopardize the survival of offspring if they are deposited on an unfavorable food plant (Cervera et al., 2004; Tollett et al., 2009). It is thus likely that we are currently underestimating the impact of metal pollution on invertebrates, due to a lack of understanding of their sublethal effects on most species.

In nature, pollutants rarely occur alone. Metals are no exception since they share common emission sources (Vareda et al., 2019). For instance, cadmium, copper, zinc and lead frequently co-occur due to the output from smelters, or the application of sewage sludge as fertilizer (Bradl, 2005). High positive correlations between chromium, cadmium and arsenic amounts have been found in soil samples (Chen et al., 1999; Navas and Machín, 2002), and many studies have shown the co-accumulation of several trace metals in insects (Wilczek and Babczy, 2000; Nummelin et al., 2007; Goretti et al., 2020). As such, co-occurring metals could have additive, antagonistic or synergistic effects (Jensen and Trumble, 2003). These interactive effects may also be influenced by the presence of other environmental stressors, such as pesticides or parasites (Alaux et al., 2010).

3.2. Multiple possible causes of invertebrates' high sensitivity to metal pollution

Our survey of the literature suggests that invertebrates may be more sensitive to the damaging effects of metal pollutants than the mammals (e.g. humans, rodents) typically used to determine “safe” environmental levels. This may be explained by differences in sensitivity to pollutants that can vary between species and with different metals (Malaj et al., 2016). Some species can discriminate metal contaminated food from uncontaminated food (Mogren and Trumble, 2010), but other species seem unable to (Stolpe et al., 2017; Burden et al., 2019). This is particularly critical for animals feeding on resources that can accumulate metals, such as leaves (Krämer, 2010) or nectar (Gutiérrez et al., 2015). Perhaps more importantly, there is emerging evidence that invertebrates may have higher levels of exposure to metal pollutants in the field than large mammals. Surveys of terrestrial biotopes show that non-essential metals tend to be accumulate at higher levels in invertebrates than in vertebrates (Hsu et al., 2006). This seems to also be the case for aquatic taxa (Xin et al., 2015). Due to their small size, their relatively high surface area/volume ratio and the niches they occupy, invertebrates are frequently in intimate contact with soils and vegetation, or could get contaminated by specific feeding modes such as filter-feeding or deposit-feeding (De Lange et al., 2009). Their limited dispersal capacities may reduce their ability to move away from polluted areas, even if they can detect harmful levels of trace elements. As a result, metals accumulate in the bodies of individuals (Goretti et al., 2020; Nannoni et al., 2011; Mukhtorova et al., 2019; Schrögel and Wätjen, 2019) and in the nests of social species (Skaldina et al., 2018; Veleminsky et al., 1990). Some terrestrial invertebrates (e.g. ants, earthworms, bees, Isopoda) could therefore be relevant and sensitive bioindicators of metal pollution due to their particular vulnerability to metal contamination.

Invertebrates do have mechanisms to process metal pollutants. Excessive metals can be eliminated through feces (Przybyłowicz et al., 2003), accumulated in insect exoskeleton before molting (Borowska et al., 2004), or stored in specific organs (Nica et al., 2012) like the Malpighian tubules (the excretory system of invertebrates) (Rabitsch, 1997). They can also induce expression of proteins involved in metal excretion and/or detoxification, like metallothioneins (for reviews, see (Janssens et al., 2009; Merritt and Bewick, 2017)). Yet, while these detoxification mechanisms may protect species to a point, they are unlikely to spare them from the sublethal effects of metal pollutants. This can impair brain or organ function, especially since invertebrates nervous systems are size constrained with brains containing relatively few neurons (Niven and Farris, 2012). Cellular damage or death in the insect brain can result in severe consequences for the individual (Klein et al., 2017). We clearly need a better characterization of the physiological and molecular mechanisms underlying metal transfer, toxicity and tolerance in invertebrates in order to better understand their sensitivity to metal pollutants.

3.3. A need to revise guidelines of safe environmental levels of metal pollutants

Since metals are such widespread and persistent pollutants in the environment, it is a priority to develop a better assessment of their impacts on invertebrates. Our most concerning finding is the evidence that terrestrial invertebrates are highly sensitive to metal pollutants. In particular, a high percentage of studies of arsenic reported toxic effects below international permissible limits, thus pointing toward the need for more research on this specific metal (Ng et al., 2003). Our review of the literature also highlights important gaps in our knowledge. We need to study a larger diversity of species, and have more systematic investigation of doses below permissible limits. We should consider potential cocktail effects, and extend studies beyond the four metals addressed here. Although our study focuses on four metal pollutants that are well studied and considered as priority for public health concerns, other metallic compounds have been reported to negatively impact terrestrial invertebrate populations at low doses, such as selenium (deBruyn and Chapman, 2007), zinc (Cheruiyot et al., 2013), copper (Di et al., 2016), cobalt (Cheruiyot et al., 2013), nickel (Cheruiyot et al., 2013), manganese (Ben-Shahar, 2018) and chromium (Sgolastra et al., 2018). Characterizing the impacts of metal pollutants on insect fitness is going to demand an integrative and interdisciplinary research agenda, just like what has been established to assess pesticide impacts on beneficial insects. For example, focusing awareness on the sublethal effects of neonicotinoids on pollinators (Henry et al., 2012; Crall et al., 2018), triggered a revision of the risk assessments scheme and their ban in the European Union in 2018.

3.4. Concluding remarks

This survey of the existing literature clearly indicates that terrestrial invertebrates appear particularly vulnerable to arsenic, cadmium, lead and mercury, and that most existing standards are not suited to protect them. We now need more integrative toxicological studies, on a broader range of metal pollutants and invertebrate species to better assess their impact on fitness, and to update the current environmental regulation. Only by addressing these important challenges will we be able to mitigate consequences on ecosystems and food safety, in a context of rapid and widespread decline of invertebrate biodiversity.

4. Methods

4.1. Literature review and data extraction

We focused on the four most hazardous metals documented for humans (ATSDR, 2019), for which international regulatory implementations exist (Table 1): arsenic (As), cadmium (Cd), mercury (Hg) and lead (Pb). We searched articles in the ISI Web of Knowledge database (search performed on 25/03/2020) using keywords combined with Boolean operators: *Topic = (heavy metal* OR metalloid* AND (insect* OR invertebrate* AND (cadmium OR lead OR arsenic OR mercury)*. The search was restricted to articles published between 1975 and 2020 (maximum available year range on ISI Web of Knowledge). Among the 460 hits, we selected those studies focusing on terrestrial invertebrates (i.e. protostomes) from the abstracts, and excluded review articles. This filtering yielded a subset of 154 articles from which we extracted 527 observations investigating effects of metal pollutants on terrestrial invertebrates (see raw data in S1 Table).

From each observation, we extracted: (1) the name of targeted invertebrate species, (2) the metal(s) used, (3) the experimental conditions (field, laboratory), (4) the mode of exposure to the metal (food, water, soil), (5) the type of exposure (acute: <24 h, chronic: >24 h), (6) the range of metal concentrations tested (min- max in ppm), (7) the biological responses measured (e.g. survival, reproduction, behavior), and (8) the lowest metal concentration for which an effect was observed. Heterogeneity of proportions was assessed using chi-square test.

Briefly, the vast majority of the observations focused on cadmium (46%) and lead (37%), while less information was available on arsenic (10%) and mercury (7%) (Fig. 1A). 59% of the observations were obtained in field surveys and 41% in laboratory experiments with controlled exposure. Since the effects can greatly vary depending on the duration of exposure and time of assessment, here we considered as acute exposure any case where individuals were exposed to a single dose and assessed within 24 h. Despite the diversity of protocols, most studies used chronic exposure (95%), through the diet (49%) or the soil (43%).

4.2. Concentration ranges

All permissible limits are based on human toxicity data. Levels were determined from the international standards set by the World Health Organization (WHO) and the Food and Agriculture Organization (FAO) of the United Nations. The permissible limits are recommended values for: 'food and drinking water', as defined in the Codex Alimentarius (Codex Alimentarius, 2015), to deal with 'contaminants and toxins in food and feed' and to be 'applied to commodities moving in international trades' (Codex Alimentarius, 2015); guidelines for water quality in irrigation (Ayers and Westcot, 1994); critical values in soil based on the Organization for Economic Co-operation and Development (OECD) risk assessment studies (de Vries et al., 2003) and FAO standards (WHO/FAO, 2001). These limits vary across types of food, water (i.e. drinking, irrigation) and soils (i.e. allotment, commercial, residential, agricultural). Local guidelines (see S2 Table), when they exist, can vary across countries and are less conservative (higher thresholds) than the international standards, especially for soils and water. For each of these matrices, we thus considered the minimal and the maximal estimates of permissible limits. We defined three concentration ranges: below the minimal estimated limit, between the minimal and maximal estimated limits, and above the maximal estimated limit (Table 1). Whenever only one threshold value was defined, no intermediate range could be defined (NA: not applicable). Note that for water, whenever possible, we considered the minimal value for drinking water and the maximal value for irrigation water.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2021.146398.

Data accessibility statement

Raw data used of the analyses are available in S1 Table.

CRediT authorship contribution statement

CM and ML designed the study. CM collected the data, analyzed the data, and wrote the first draft of the manuscript. All authors contributed substantially to revisions.

Declaration of competing interest

The authors declare no conflict of interest.

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Appendix 1B: The toxic error at the heart of efforts to curb heavy metal pollution

Popular science paper published in New Scientist

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Comment

A toxic error

Heavy metal pollution limits shouldn't just keep humans safe, but other animals too, say **Coline Monchanin** and **Mathieu Lihoreau**

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ACROSS the world, insects are in decline. Intensive pesticide use, new diseases, habitat destruction and climate change are all contributing. Sadly, we are increasingly discovering that there is another impact of our everyday activities that is just as important: heavy metal pollution.

These substances are all around us. They are naturally present in Earth's crust and are released at low levels through weathering of rock and volcanic activity. But this gets a significant boost from human activity. We release these metals in various ways, ranging from dust that comes from vehicle brakes to the burning of fossil fuels for power and transport. All of this raises concentrations above natural levels. And once metallic dusts are out there, they stay for millennia.

While some of the compounds of these metals are essential for living organisms, most of them are highly toxic even at low concentrations. There are international guidelines designed to protect us from such pollution, but it turns out they aren't strong enough to do the same for insects.

In our recent work, we surveyed the scientific literature from the past 45 years that looked at the most monitored metals: arsenic, cadmium, mercury and lead. From this, we were able to identify the concentrations of these that are harmful to terrestrial invertebrates, the majority of which are insects. We then compared them with thresholds for the metals recommended by international regulatory bodies, for food, water and soil.

Though amounts of these in the environment should be below "human-safe" limits, in almost half of the studies the levels in natural conditions exceeded these figures. Not so surprisingly, at these concentrations the metals almost always killed invertebrates. More alarmingly, 90 per cent of the studies investigating metal levels within "human-safe" limits reported harmful effects on insects.

What's more, while it is clear that contamination harms these animals, we only have a partial picture as studies focus on sentinel species such as moths and bees, so-called because we use them to give an idea of possible harm to people, and on organisms favoured in many lab studies such as *Drosophila*. While we expect these to reflect a broad pattern, they only represent a tiny fraction of insect biodiversity, which is by far the most abundant of the terrestrial animals on the planet. A deeper understanding is critical to find the best possible solutions to mitigate these effects.

We need to start now, so we are calling for urgent action. While many local initiatives have worked to reduce emissions of some specific metal pollutants and emission sources over the past 40 years, environmental metallic pollution globally is still high. Take lead, for example, which started to be banned from petrol in the 1980s, but remains high in the environment because of the processing of ore and metals, and use of leaded aviation gasoline.

We could effectively reduce metal emissions now by dramatically cutting the use of fossil fuels, gasoline vehicles and metal-based pesticides, which are all sensible ways to protect our environments.

Politicians and scientists also have to rethink guidelines about what a safe level of metal pollution is, to take account of non-human species. This kind of shift in thinking was achieved a few years ago for broad scale use of harmful pesticides in intensive agriculture in many parts of the world.

Metal pollution is still an underappreciated threat. Its potential contribution to the huge insect biodiversity decline, called "insectageddon" by some, hasn't been recognised in recent conservation plans, such as the proposal for a more sustainable European Union Common Agricultural Policy. It is time for that to change. ▮

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Appendix 2A: Chronic exposure to trace lead impairs honey bee learning

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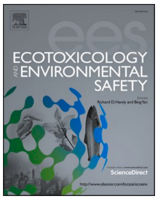

Chronic exposure to trace lead impairs honey bee learning

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ABSTRACT

Pollutants can have severe detrimental effects on insects, even at sublethal doses, damaging developmental and cognitive processes involved in crucial behaviours. Agrochemicals have been identified as important causes of pollinator declines, but the impacts of other anthropogenic compounds, such as metallic trace elements in soils and waters, have received considerably less attention. Here, we exposed colonies of the European honey bee *Apis mellifera* to chronic field-realistic concentrations of lead in food and demonstrated that consumption of this trace element impaired bee cognition and morphological development. Honey bees exposed to the highest of these low concentrations had reduced olfactory learning performances. These honey bees also developed smaller heads, which may have constrained their cognitive functions as we show a general relationship between head size and learning performance. Our results demonstrate that lead pollutants, even at trace levels, can have dramatic effects on honey bee cognitive abilities, potentially altering key colony functions and the pollination service.

1. Introduction

Honey bees and other central-place foraging pollinators rely on their cognitive abilities (learning and memory) to efficiently forage on flowers (Klein et al., 2017). Yet, these abilities can be easily disrupted by some environmental stressors, even at low exposure levels (e.g. neonicotinoid insecticides: Colin et al., 2019b; Desneux et al., 2007; Henry et al., 2012). In theory, any stressor impairing brain development and/or learning processes may have subtle effects on individual's foraging capacity, with dramatic consequences on colony function, if food supply is compromised (Perry et al., 2015). Here, we focused on the possible sublethal effects of lead (Pb), a metallic trace element (MTE) with well-established neurotoxic properties in vertebrates (Chen et al., 2016; Mason et al., 2014), but whose effects on invertebrates are still poorly documented.

MTEs are naturally present in the environment (Bradl, 2005). However, their widespread use in industrial and domestic applications has elevated their levels far above natural baselines in and around urbanised or industrial areas (Hladun et al., 2015; Wuana and Okieimen, 2011). Lead, in particular, is a worldwide pollutant (Cameron, 1992), which can occur at high and persistent concentrations in soils (Han et al., 2002) and in plant nectar between 0.001 and 0.075 mg kg^{-1} (Gutiérrez et al., 2020). Lead is one among the few MTEs for which international permissible limit values exist (Codex Alimentarius, 2015). However, soil contamination levels are unlikely to decrease in a near future (Marx et al., 2016) and these limits defining acceptable levels of lead pollution for humans may not apply for other animals (Codex Alimentarius, 2015). Insect pollinators may be particularly exposed to airborne particles while flying (Thimmegowda et al., 2020) and to contaminated water, nectar and pollen when foraging (Formicki et al., 2013). Lead bio-accumulates in the insect body (Mertz, 1981) and it can contaminate pollen, honey and wax in the bee hive (Zhou et al., 2018) and be transferred with food to the larvae (Balestra et al., 1992). Thus, it is likely that pollinators foraging in many urbanised environments are exposed to lead at different life stages.

Lead is known to impact the survival (Hladun et al., 2016), physiology (Gauthier et al., 2016; Nikolic et al., 2019), and development of bees (Di et al., 2016), leading to adults with smaller body sizes. While exposure to lead has also been reported to impair some foraging capacities (Sivakoff and Gardiner, 2017; Xun et al., 2018), the impact on

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cognition has not been assessed. For bees, efficient foraging requires the capacity to associate floral cues (e.g. odorant) with the presence of food (e.g. nectar) in order to develop preferences for profitable resources (Giurfa, 2007). Since the nectar status of flowers changes with time, any such associations must be continually updated with new experience. This demands cognitive flexibility, i.e. the capacity to modify behaviour in response to environmental changes (Scott, 1962). Such flexibility, often assessed with reversal learning paradigms (Izquierdo et al., 2017), is sensitive to many sources of stress and can be impaired in humans exposed to sublethal MTEs levels (Mergler et al., 1994; Rafiee et al., 2020). In honey bee foragers, reversal learning performance develops during adulthood and significantly improves at foraging onset, as does the maturation of the underlying brain circuits (Cabirol et al., 2017, 2018). We therefore hypothesised that a chronic exposure to lead could yield alterations in development and learning performances in foraging bees, as it does in mammals (Giordano and Costa, 2012; Grandjean and Landrigan, 2006; Mason et al., 2014).

Here, we tested this hypothesis by exposing caged honey bee colonies to field-realistic (low) concentrations of lead for 10 weeks and monitored impacts on the morphology and reversal learning abilities of foraging bees. Given the known impact of lead on morphological development (Di et al., 2016), we also evaluated a potential basal relationship between body size and cognitive performances in non-contaminated and uncaged bees foraging on natural plant resources.

2. Materials and methods

2.1. Bee colonies

Experiments on the effects of lead on morphology and cognition were conducted from 14/06/2019 (day 1) to 23/08/2019 (day 70), using caged bees from nine colonies of *Apis mellifera* (Buckfast) maintained in 5 frame hives (Dadant). Each colony was placed in an outside tent (3 m × 3 m) at our experimental apiary (University Paul Sabatier, France) to control the food intake and the foraging experience of bees. Each tent contained two 500 mL feeders. One feeder was filled with sucrose solution (with or without lead, see below) and the other with water. The two feeders were located 1 m apart, 2 m in front of the hive entrance. Caged colonies were given pollen patties (Icko, Bollène, France) once a week directly into the hives.

The experiments on the basal relationship between morphology and cognition were conducted from 02/2018 to 04/2018, by randomly collecting uncaged bees from a pool of 15 colonies (*A. mellifera*, Buckfast) as they foraged on an outside feeder in the same apiary. These non-contaminated bees had free access to natural plant resources.

2.2. Lead exposure

Caged colonies were assigned to one of three lead treatments (three colonies per treatment): 1. unexposed (hereafter 'control bees'), 2. exposed to a low (0.075 mg L^{-1}) concentration of lead ('L bees'), 3. exposed to a high (0.75 mg L^{-1}) concentration of lead ('H bees'). Bees were exposed to lead by them ingesting 50% (w/v) sucrose solution from the feeder, to which lead (II) chloride ($PbCl_2$) (Sigma-Aldrich, Lyon, France) was added. The low and high lead concentrations fell within the range of concentrations measured in natural flowers (Eskov et al., 2015; Gutiérrez et al., 2020; Maiyo et al., 2014; Uren et al., 1998) and honey (Ajtony et al., 2007; Naggar et al., 2013; Satta et al., 2012). Both concentrations are sublethal to adult honey bees (LC_{50}: 345 mg L^{-1}) (Di et al., 2016). Control hives were fed 50% (w/v) sucrose solution. Feeders were refilled daily so that bees had an ad libitum access to food.

Caged hives were maintained in these conditions for 70 days. This duration was long enough for colonies to store contaminated food, so that nectar foraging bees sampled for the cognitive assays were likely to have ingested lead during their development. On average, colonies consumed 8.5 ± 0.6 (SE) kg of sucrose solution and 616 ± 25 (SE) g of pollen during the experiment (N = 9). During this period, we kept track of the foraging experience of all the nectar foragers (number of days since the onset of foraging) by paint-marking bees with a colour code while feeding on the sucrose solution feeder (Posca pen, Tokyo, Japan). Each day was encoded with a new combination of colours. This operation was repeated twice everyday (1 h in the morning, 1 h in the afternoon).

2.3. Lead quantification

Lead levels were analysed in samples of the sucrose solution and bees from caged hives using Inductively Coupled Plasma Emission Spectroscopy (ICP-OES, quantification limit: 5–20 μg kg^{-1}, precision measure: 1–5%; AMETEK Spectro ARCOS FHX22, Kleve, Germany).

Our ability to detect lead was first verified by assaying the lead level in our high lead concentration sucrose solution (0.75 mg L^{-1}). The solution was acidified at 3% of HNO_3 with ultra-pure 69% HNO_3 to avoid precipitation or adsorption in containers. The solution was then diluted with a HNO_3 3% solution to reduce the spectral interference and viscosity effects. With this method, the amount of lead was recovered at 96% (nominal concentration: 0.75 mg L^{-1}, actual concentration: 0.71 mg L^{-1}).

The fact that bees exposed to different concentrations of bioaccumulated lead in a dose-dependent manner was then verified. Lead content was assessed in bees collected 30 days after the start of the exposure (i.e. midway through the experiment). For each sample, bees were pooled in batches of five. Each batch was rinsed with 5 mL HNO_3 at 3% for 30 s. Bees were wet mineralised in 50 mL polypropylene tubes using a Digiprep system (SCP Science, Quebec, Canada) with 5 mL of 69% nitric acid, following a protocol for athropods (Bur et al., 2012; Astolfi et al., 2020). This consisted of a digestion phase carried out at room temperature overnight, followed by a second phase of heating at 80 °C for 60 min. The nitric acid was evaporated, and the samples were diluted with 9 mL of 3% HNO_3. Final solutions were at 3% HNO_3 and total dissolved solids below 5%.

Certified reference materials (CRMs) were used as quality controls to validate the protocol of mineralisation and multi-elementary ICP analysis: waters (SLRS-6, SUPER-05, ION-96.4) and a solid arthropod CRM (PRON-1 river prawn reference material). Recovery coefficients (ratios measured vs. certified values) for major and trace elements ranged between 85% and 115%.

2.4. Colony dynamics

The effect of lead exposure on colony dynamics was assessed in the caged colonies through continuous measurement of hive parameters in the caged colonies. Hive weight (± 0.01 kg) was recorded every hour with an electronic scale (BeeGuard, Labège, France) below each hive. Every two weeks hives were opened and pictures of both sides of each frame were taken with a Panasonic Lumix DMC-FZ200 equipped with a F2.8 25–600 mm camera lens. From the pictures, areas of capped brood and food stores were estimated using CombCount (Colin et al., 2018). Each frame was weighted, after gently removing the adult bees, and the total weight of adult bees (total adult bee mass) was determined by subtracting the tare of the hive and the weight of the frames from the weight of the hive.

2.5. Learning assays

The cognitive performances of bees from caged and uncaged colonies were assessed using olfactory conditioning of the proboscis extension reflex (PER; Giurfa and Sandoz, 2012). Overall, 268 bees from caged colonies were tested (84 control bees, 84 L bees, 100 H bees). These bees were exposed to lead for their whole life (foragers exposed from larvae to foraging age, collected between days 46 and 70 from the start of lead treatment) and originated from 8 of the 9 colonies (one control hive showed very low foraging activity). We focused on new foragers (between 24 and 48 h after the onset of foraging) to avoid inter-individual cogntive variation caused by differences in foraging experiences (Cabirol et al., 2018). Another 149 bees from uncaged colonies were tested. Neither the age nor the foraging experience of these bees were controlled.

All bees were submitted to a reversal learning task, i.e. a two-stage task assessing the cognitive flexibly of bees in response to changes in flower rewards (Raine and Chittka, 2007). This test mimics the natural situation where one floral species ceases producing nectar before another species starts doing so. Phase 1 is a differential learning phase, in which the bees must learn to differentiate an odour A reinforced with sucrose (50% w/v in water) and an odour B not reinforced (A+ vs. B-). Phase 2 is a non-elemental learning phase, in which the bees must learn the opposite contingency (A- vs. B+). We used pure limonene and eugenol (Sigma-Aldrich, Lyon, France) as odours A or B alternately on successive days, so that each contingency was used for about half of the bees for each treatment.

On the morning of each test, foragers (24–48 h after onset of foraging) were collected on the feeders, cooled on ice and harnessed in restraining holders that allowed free movements of their antennae and mouthparts (Matsumoto et al., 2012; Fig. 1A). Turning of the head was prevented by fixing the back of the head with melted bee wax. All bees were then tested for PER by stimulating their antennae with 50% sucrose solution. Only those that responded for the conditioning phases (77% of all bees tested) were kept for the experiments. These bees were fed 5 μL of sucrose solution and left to rest in a dark incubator for 3 h (temperature: 25 ± 2 °C, humidity: 60%).

Bees were then trained using an automatic stimulus delivery system (Fig. 1A; Aguiar et al., 2018). Each training phase included five trials with the reinforced odorant and five trials with the non-reinforced odorant in a pseudo-random order with an eight-minute inter-trial interval. Each conditioning trial (37 s in total) started when a bee was placed in front of the stimulus delivery system, which released a continuous flow of clean air (3300 mL min^{-1}) to the antennae. After 15 s, the odour was introduced to the airflow for 4 s. For rewarded odours, the last second of odour presentation overlapped with sucrose presentation to the antennae using a toothpick soaked in sucrose solution (Fig. 1A) and sucrose feeding by presenting the toothpick to the mouthparts for

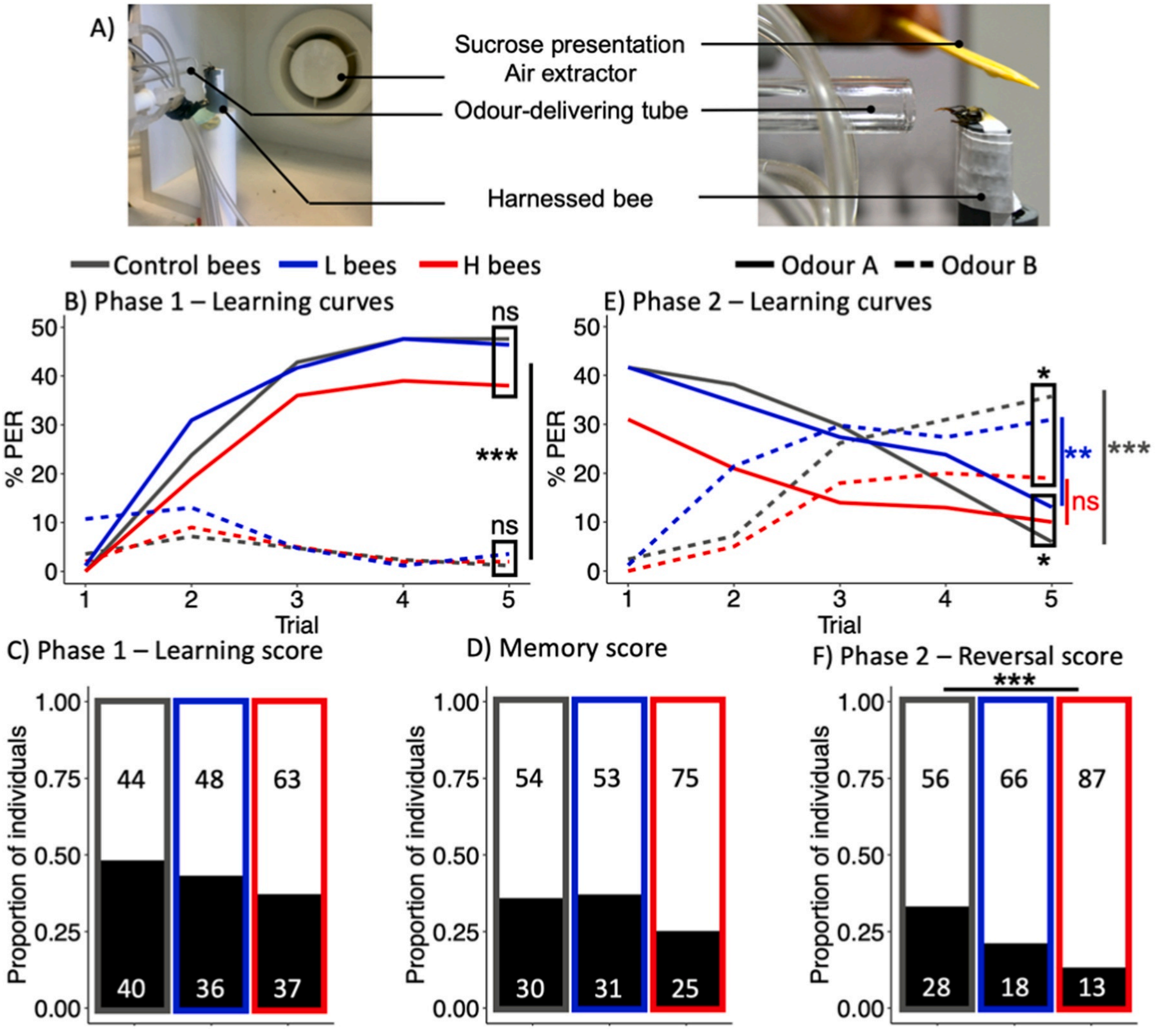

Fig. 1. Learning and memory performances of bees from caged hives exposed to lead treatments. A) Picture of a harnessed bee in the conditioning set-up. B), E) Line plots show the percentage of proboscis extension responses (PER) elicited by odour A (solid line) and odour B (dashed line) during phase 1 (B) and phase 2 (E) of reversal learning. Control bees (N = 84, dark grey), bees exposed to a low concentration of lead (L bees: 0.075 mg.L^{-1}; N = 84, blue) or a high concentration of lead (H bees: 0.75 mg.L^{-1}; N = 100, red). Statistical comparisons of the response level at the last trial were obtained with p-values from the binomial GLMM (see details in Table S1). C), D), F) Bar plots show the proportions of learners (black) and non-learners (white) in the last trial of phase 1 (C) and phase 2 (F), with sample size displayed. D) Bar plots show the proportions of bees remembering (black) or not (white) during the 1 h memory recall, with sample size displayed. Statistical comparisons were obtained with p-values from the binomial GLMM (Table S1) (ns: non-significant, $p > 0.05$; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$).

4 s. For the unrewarded trials, no sucrose stimulation was applied. The bee remained another 15 s under the clean airflow. Bees were kept in the incubator for 1 h between the two learning phases (A+ vs. B- and A- vs. B+).

During conditioning, we recorded the presence or absence of a conditioned PER to each odorant at each trial (1 or 0). Each bee was given a learning score for phase 1 (1 if the bee responded to A+ and not to B- in the last trial of phase 1, 0 otherwise) and for phase 2 (1 if the bee responded to B+ and not to A- on the last trial, 0 otherwise) (Cabirol et al., 2018). Short-term memory (1 h) was assessed by comparing the responses at the last trial of phase 1 and the first trial of phase 2. Each bee was given a memory score for the two odorants (1 if the bee still responded appropriately to the A+ and B- on the first trial of the phase 2, 0 otherwise).

2.6. *Morphometry*

Developmental differences among bees was evaluated by conducting morphometric measures on frozen individuals (−18 °C) from caged and uncaged hives.

To test the effect of lead exposure on morphology in caged bees, foragers of unknown age were collected on the day before lead exposure (day 0 of the experiment), during lead exposure (day 53 of the experiment) and at the end of the experiment (day 70 of the experiment), and their head length and head width were measured (Fig. 2A). Emerging adult bees were also sampled every week from each hive (before exposure, during exposure, and at the end of the exposure period). For each bee, the fresh body weight (± 0.001 g) (precision balance ME103T, Mettler-Toledo Gmbh, Greifensee, Switzerland) and eight morphometric parameters were recorded: head length, head width, forewing length, forewing width, femur length, tibia length, basitarsus length, basitarsus width (Fig. 2A; De Souza et al., 2015; Mazeed, 2011).

To test for a relationship between morphology and cognitive performances in the uncaged bees, the head length and head width of the conditioned bees hives were measured after the conditioning experiments. All measurements (± 0.01 mm) were taken using a Nikon SMZ 745T dissecting scope (objective x0.67) with a Toupcam camera model U3CMOS coupled to the ToupView software.

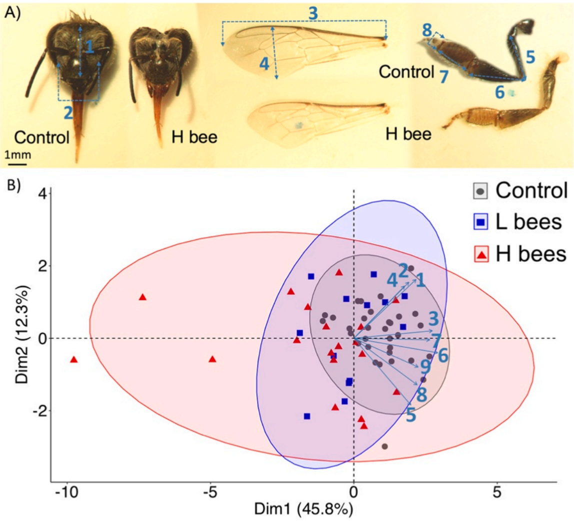

Fig. 2. Morphometric analysis of bees from caged hives exposed to lead treatments. A) Details of the parameters measured. This example shows morphological differences in emerging bees. (1) Head length, (2) head width, (3) wing length, (4) wing width, (5) femur length, (6) tibia length, (7) basitarsus length, (8) basitarsus width, (9) bee weight (not shown). B) Principal component analysis (PCA) map shows the relationship among the morphometric measures (same number code as in A). 95% confidence ellipses of the mean are displayed for each treatment. Controls: bees unexposed to lead (N = 32); L bees: bees exposed to the low concentration of lead (0.075 mg L^{-1}) (N = 13); H bees: bees exposed to the high concentration of lead (0.75 mg L^{-1}) (N = 19).

2.7. *Statistics*

All analyses were performed with R Studio v.1.2.5033 (RStudio Team, 2015). Raw data are available in Dataset S1. Lead content of bees was compared using a Kruskal-Wallis test (package FSA; Ogle et al., 2019). The effects of lead exposure on colony parameters were evaluated with a multi-model approach (MMI), with treatment, time since the beginning of the exposure (standardised using rescale function, package arm; Gelman and Su, 2013) and their interaction as fixed effects, and hive identity as random factor. A model selection (package MuMIn; Barton, 2020) was run and conditional model average was applied to evaluate the effects of the different factors on the response variables. A MMI was run followed by a conditional model average to assess the effects of treatment, time of exposure and their interactions on brood area (square-root transformed), food stores area and total adult bee mass.

For learning assays, proportion tests were used, followed by pairwise comparisons with a Bonferroni correction (package RVAideMemoire; Hervé, 2020), to evaluate whether lead exposure changed sucrose responsiveness (i.e. proportions of unresponsive bees across treatments). Generalised linear mixed-effects models (GLMM) (package lme4; Bates et al., 2015) were performed to evaluate the effect of treatment on the behavioural variables (PER responses, learning, reversal and memory scores). Proportions of successful responses during the fifth trial of each learning phase were compared using a binomial GLMM, with odorants, treatments and their interactions as fixed effects, and bee identity nested in the hive identity as random factors. A similar GLMM was run to compare the learning, reversal and memory scores, with hive identity as random factor.

For the morphometric analyses on caged bees, LMMs were used for each parameter, considering treatment as a fixed effect, and hive identity as a random factor. To assess the global effect of lead, the nine parameters were collapsed into a principal component analysis (PCA) (package FactoMineR, Lê et al., 2008). Bees were clustered into subgroups based on PCA scores, and clusters were compared with a permutational multivariate analysis of variance (PERMANOVA; package vegan; Oksanen et al., 2019). A LMM was run on individual coordinates from the PCA, with treatment as a fixed effect, and hive identity as a random factor. To assess the effect of head size on the cognitive performances of uncaged bees, head width and head length measures were collapsed into the first component of a PCA and a binomial GLMM was run on learning, memory and reversal scores, with individual coordinates from the PCA as fixed effect, and test day as random factor.

3. Results

3.1. *Exposure to high lead concentration reduced learning performance*

We assessed the effect of lead exposure on cognitive flexibility by conducting reversal learning assays in caged bees. The proportion of bees that responded to the antennal stimulation of sucrose was similar across treatments (control bees: 74% N = 113; L bees: 69% N = 122; H bees: 76% N = 132; Chisq = 1.423, df = 2, $p = 0.491$), indicating that lead exposure did not affect appetitive motivation or sucrose perception.

Treatment had no significant effect on learning phase 1, although H bees tended to perform less well (Fig. 1B-C). Upon the last trial of phase 1, bees from all treatments discriminated the two odorants (Binomial GLMM: $p < 0.001$ for all treatments), and exhibited similar response levels to odour A (Binomial GLMM: L bees $p = 0.877$; H bees $p = 0.206$) and B (Binomial GLMM: L bees $p = 0.331$; H bees $p = 0.459$). The proportions of bees that learned to discriminate the two stimuli (learning score equals to 1) were similar across treatments (Control:

48%; L bees: 43%; H bees: 37%) (Fig. 1C; Table S1). These results were independent of the odours used as stimuli A+ and B- (Binomial GLMM: $F_{1,266} = 0.905$, $p = 0.526$). The proportion of learners at the end of the first phase was similar across hives, within each treatment group. Therefore, exposure to lead, had no significant effect on performance in the differential conditioning task.

Treatment did not significantly affect short-term memory between the two phases neither (Fig. 1D). Bees from all treatments had similar memory scores (Binomial GLMM: L bees $p = 0.873$; H bees $p = 0.115$). However, H bees had a reduced percentage of correct responses between the two phases (25% compared to 36% for control bees).

By contrast, treatment had a clear effect on learning in phase 2 (Fig. 1E-F). Upon the last trial, control and L bees were able to discriminate the two odorants (Binomial GLMM: Control $p < 0.001$; L bees $p = 0.007$), but not H bees (Binomial GLMM: $p = 0.075$). The response level to odours A and B was similar between control and L bees (Binomial GLMM: odour A $p = 0.097$; odour B $p = 0.513$), but H bees responded less to odour B (Binomial GLMM: $p = 0.012$) and more to odour A (Binomial GLMM: $p = 0.032$) compared to control. Consequently, H bees exhibited lower reversal scores (13% of learners) than L bees (21%) and controls (33%) (Binomial GLMM: L bees, $p = 0.086$; H bees, $p = 0.001$) (Table S1, Fig. 1F). There was no effect of the odours used as stimuli A- and B+ (Binomial GLMM: $F_{1,266} = 1.300$, $p = 0.636$), nor of the hive, on the proportion of learners within treatment groups. Therefore, exposure to a high concentration of lead reduced the performance of bees in the reversal learning task.

The dose-dependent effect of lead exposure on bee cognition was correlated with dose-dependent bio-accumulation of lead in bees. Control bees and L bees showed no difference in lead content (controls: 0.126 ± 0.031 mg kg^{-1} d.m., $N = 3$; L bees: 0.130 ± 0.002 mg kg^{-1} d.m., $N = 3$; Kruskal-Wallis: $H = 7.636$, $df = 1$, $p = 0.712$), whereas H bees accumulated significantly more lead (H bees: 0.809 ± 0.044 mg kg^{-1} d. m., $N = 5$; Kruskal-Wallis: $H = 7.636$, $df = 1$, $p = 0.039$). This result was also independent from any influence of the state of the colony, since lead treatment had no effect on colony measures (syrup and pollen consumption, dynamics of brood production, size of food stores, total adult bee mass, colony weight; LMM: Treatment effect: $p > 0.05$ for all parameters; for further details see Fig. S1).

3.2. *Bees exposed to the high lead concentration were shorter with smaller heads*

Given the observed effects of chronic exposure on the cognitive flexibility of foragers, we asked whether this might result from compromised development. We measured head size in individuals from the different caged hives. Foragers of unknown age collected on the day before the beginning of treatment (day 0) had similar head measurements irrespective of treatment (LMM: L bees: head length $p = 0.296$, head width $p = 0.287$; H bees: head length $p = 0.333$, head width $p = 0.394$). Foragers collected in the middle (day 53) and at the end (day 70) of the experiment had significantly smaller heads than controls (LMM: L bees: head length $p = 0.017$, head width $=0.456$; H bees: head length $p < 0.001$, head width $p = 0.040$; Table S2).

To better assess this developmental impact of lead exposure, we also collected bees at adult emergence, thereby considering only the pre-imaginal period. For this analysis, we included different body measures in addition to head length and width (Fig. 2A), and used them to perform a PCA (Fig. 2B, Table S3). Two PCs explaining 58% of the variance were sufficient to separate control bees and H bees into two distinct clusters, while L bees were intermediate (PERMANOVA: Pseudo-F = 5.575, $p = 0.002$; control bees vs. L bees: $p = 0.975$; C bees vs. H bees: $p = 0.003$; L bees vs. H bees, $p = 0.189$). We focused on PC1 which explained 45.8% of the total variance and was associated with general body size. PC1 was negatively correlated with lead concentration (LMM: $p = 0.042$), so that the H bees tended to be smaller than L bees and control bees (Table S4). H bees displayed a rather homogeneous decrease in most parameter values, resulting in a notable weight loss of ca. 8.33% (Table S4).

The fact that emerging and foraging bees exhibited a similar decrease in head size (LMM: age effect $p > 0.05$; Tables S2, S4) suggests that most of the impact of lead exposure on morphology occurred before the adult stage.

3.3. *Unexposed bees with larger heads showed better learning performance*

Because the above data suggests a link between lead-induced learning impairment and alterations of head development in our caged bees, we tested the possibility of a general correlation between performance at adulthood and head size, irrespective of lead treatment. For this, we submitted unexposed adult bees from uncaged hives to a reversal learning task ($N = 149$). We separated bees according to their learning, memory and reversal scores (see Methods), in order to compare the morphometric characteristics of bees with different levels of performance. We ran a PCA on this subset of bees, and used the first component (PC1, 73% of the morphological variance), which collapsed head width and length, as a proxy of overall head size (Fig. 3). In phase 1 of reversal learning, the proportion of learners (79% $N = 118$) increased with head size (Fig. 3A), as did the short-term memory recall (46% $N = 68$) (Fig. 3B). In phase 2, the proportion of learners (18% $N = 27$) also increased with head size (Fig. 3C). Therefore, bees with larger heads showed better learning and memory performances in absence of any cage confinement or lead treatment.

4. Discussion

Recent studies suggest that MTEs can have sublethal effects on individual bees, with potential detrimental consequences for colonies and the pollination service through altered foraging behaviour (Burden et al., 2016, 2019; Skaldina and Sorvari, 2019; Søvik et al., 2015). Here, we found that honey bees chronically exposed to trace concentrations of lead in food have reduced body sizes and learning abilities. The positive correlation between head size and learning performances in unexposed bees suggests that consumption of lead affects bee development, by reducing head size and cognitive function, and thus constitutes a significant neurocognitive stressor for bees at field realistic levels.

Chronic exposure to trace lead led to reduced cognitive performance in an olfactory appetitive condition task. This assay reproduces a foraging context in which bees need to learn olfactory cues signalling the presence or absence of nectar. Neither differential learning (first learning phase) nor short-term memory were affected. However, we found a decreased performance in reversal learning (second learning phase). Thus, the treatment we used did not induce a general impairment of olfactory discrimination nor a decreased motivation for sucrose. This contrasts with the decreased responsiveness to sucrose exhibited in bees acutely treated with lead at similar concentrations (Burden et al., 2019), suggesting a different impact of chronic lead exposure on bees. The specific impairment of reversal learning indicates a loss of cognitive flexibility, which is crucial for bee foragers to switch preferences for flowers whose value changes over time (Ferguson et al., 2001). Over the long-term, this sublethal impact on individual cognition may compromise the overall foraging efficiency of a colony exploiting changing resources, and thus its survival.

Reversal learning has been shown to be more strongly affected by lead exposure than seemingly simpler differential learning in rats (Hilson and Strupp, 1997), monkeys (Bushnell and Bowman, 1979) and humans (Evans et al., 1994). These tasks measuring cognitive flexibility are particularly sensitive to the adverse effects of stressful stimuli, or of neurodevelopmental disorders (Dajani and Uddin, 2015). Just like mammals (Schoenbaum et al., 2000), honey bees rely on specific brain regions to perform reversal learning, which are not essential for simple differential conditioning (i.e. phase 1 of the conditioning task in our

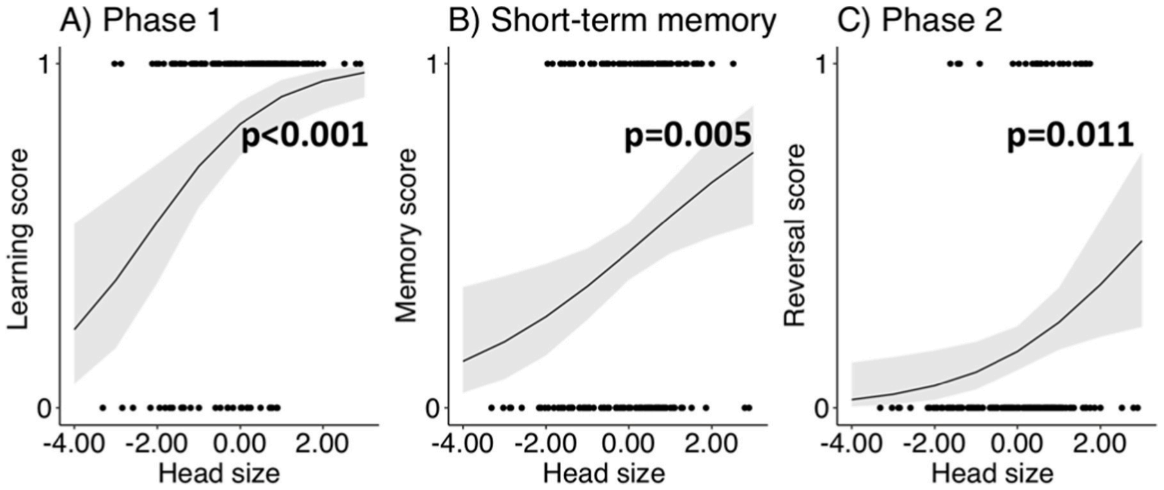

Fig. 3. Relationship between head size and cognitive performance in bees from uncaged hives unexposed to lead treatments. Data points represent the individual data for learners (learning score = 1) and non-learners (learning score = 0). Fitted lines of head size effect are displayed in black with 95% confidence intervals in grey. N = 149 bees. A) Learning score at the end of phase 1. B) Short-term memory score. C) Reversal score at the end of phase 2. Statistical comparisons were obtained with p-values from the binomial GLMM testing bees coordinates in PC1 on cognitive scores, significant values (< 0.05) are shown in bold. Increasing head size significantly enhanced the learning performances in phase 1 (Binomial GLMM: estimate ± SE, 0.693 ± 0.188, $p < 0.001$) and phase 2 (0.523 ± 0.205, $p = 0.011$), as well as short-term memory recall (0.415 ± 0.149, $p = 0.005$).

protocol). These are the mushroom bodies (MBs) (Boitard et al., 2015; Devaud et al., 2007), whose maturation over adulthood relates to the acquisition of the capacity for reversal learning (Cabirol et al., 2017, 2018). Interestingly, adult MB organisation is altered following exposure to several forms of stress in bees (Cabirol et al., 2017; Peng and Yang, 2016) and other insects (Jacob et al., 2015; Wang et al., 2007). Thus, the specific reversal impairment of lead-exposed bees might be due to neural circuits being more sensitive to the impact of lead in the MBs than in other brain regions.

Lead exposure is known to impair brain excitation/inhibition balance during development, through multiple effects such as loss of GABAergic interneurons (Stansfield et al., 2015), altered maturation of GABAergic neurons (Wirbisky et al., 2014), decrease in GABA and glutamate release (Xiao et al., 2006) or transport (Struzynska and Sulkowski, 2004), or inhibition of post-synaptic glutamatergic action (Neal and Guilarte, 2010). In insects, although no specific effect of lead on GABAergic signalling has been demonstrated yet, the effects of lead exposure on synaptic development (Morley et al., 2003), presynaptic calcium regulation (He et al., 2009) and acetylcholinesterase activity (Nikolic et al., 2019) are compatible with a disruption of the excitation/inhibition balance. It has been proposed that reaching an optimal value for such balance in MB circuits is what determines efficient reversal learning in mature adults (Cabirol et al., 2017, 2018). If this is somehow disrupted following lead exposure, that would explain the specific impairment observed only during the reversal phase of the task.

Importantly, all bees had undergone their larval and pupal stages during the exposure period, providing ample opportunity for the detrimental effects of lead to be caused by larval ingestion of contaminated food brought by foragers. Lead alters larval development in flies and bees (Cohn et al., 1992; Di et al., 2016; Safaee et al., 2014). Further evidence supports the hypothesis of a developmental effect of lead, since bees exposed to the highest concentrations developed lighter bodies, with shorter wings, and smaller heads. In bees, head width is correlated with the volume of the brain (honey bee foragers: Gronenberg and Couvillon, 2010; bumblebees: Riveros and Gronenberg, 2010) and the MBs (honey bee foragers: Mares et al., 2005; bumblebees: Smith et al., 2020). Here, we also found that for bees that had not been exposed to lead, those with smaller and shorter heads had a lower learning performance. This suggests there is a general relationship between head size and cognitive performance in a reversal learning task. We did not control for the age of the measured individuals in this part of the study. However, possible age variations among foragers are unlikely to cause any significant head size changes, since this would be expected to stabilise once the adult cuticle is hardened. In addition, reversal learning performance tend to decrease with foraging experience (Cabirol et al., 2018). It is thus unlikely that bees with larger heads in our sample were those that foraged for shorter times. Our results do not necessarily suggest that such a relationship should be expected for all cognitive tasks. Because control bees with larger heads performed better in both phases of the task, and exposed bees with larger heads only performed better in the reversal task, we assume that lead altered brain development in a specific way resulting in a stronger impact on development or performance of MB neural networks.

Continuous exposure to environmentally realistic amounts of lead resulted in bioaccumulation of the metal in the bees' bodies. This is likely to have impaired aspects of head and brain development during larval and pupal stages, resulting in adults with deficits in cognitive flexibility in an ecologically relevant cognitive task. Although this mechanistic hypothesis remains to be confirmed, our results clearly indicate a sublethal impact of lead exposure with potential consequences on foraging efficiency. Importantly, the lead contents measured in the bodies of exposed bees in our experiments ranged within the measurements from bees in field conditions (Goretti et al., 2020). The two concentrations of lead in the sucrose solutions used for chronic exposure (0.075 and 0.75 mg L^{-1}) fell below the maximum level authorised in food (3 mg kg^{-1}; Codex Alimentarius, 2015) and irrigation water (5 mg L^{-1}; Ayers and Westcot, 1994), and the lowest concentration was under the threshold set for honey by the European Union (0.10 mg kg^{-1}; Commission Regulation (EU) 2015/1005, 2015). This indicates that the cognitive and developmental impairments identified in our experimental conditions may be affecting bees foraging on flowers in many contaminated environments.

Although our experiment and recent similar approaches (Hladun et al., 2016) did not capture any consequences on colony dynamics, these individual effects observed over several weeks might ultimately alter colony function, in particular if lead exposure impairs a broader range of behaviours (e.g. communication, feeding, defence). Thus, differences in colony performances could be predicted over longer term (Klein et al., 2017), which might contribute to collapse, as observed for pesticide exposure at sublethal concentrations (Colin et al., 2019a; Meikle et al., 2016). Our results thus call for future studies to better characterise the impact of lead exposure in bee populations, including in combination with other MTEs as such cocktails are often found in contaminated areas (Badiou-Bénéteau et al., 2013; Goretti et al., 2020). More generally, a better assessment of the contribution of heavy metal pollutants to the widespread decline of insects has become an urgent necessity for preserving ecosystem services.

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CRediT authorship contribution statement

CM, ABB, JMD and ML designed the study. CM, AB-B, ED, MMN, JS, DB and AE collected the data. CM and CP analysed the data. CM wrote the first draft of the manuscript. CP, AE, ABB, JMD and ML contributed substantially to revisions.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data accessibility statement

Raw data are available in Dataset S1 (.xlsx file).

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ecoenv.2021.112008.

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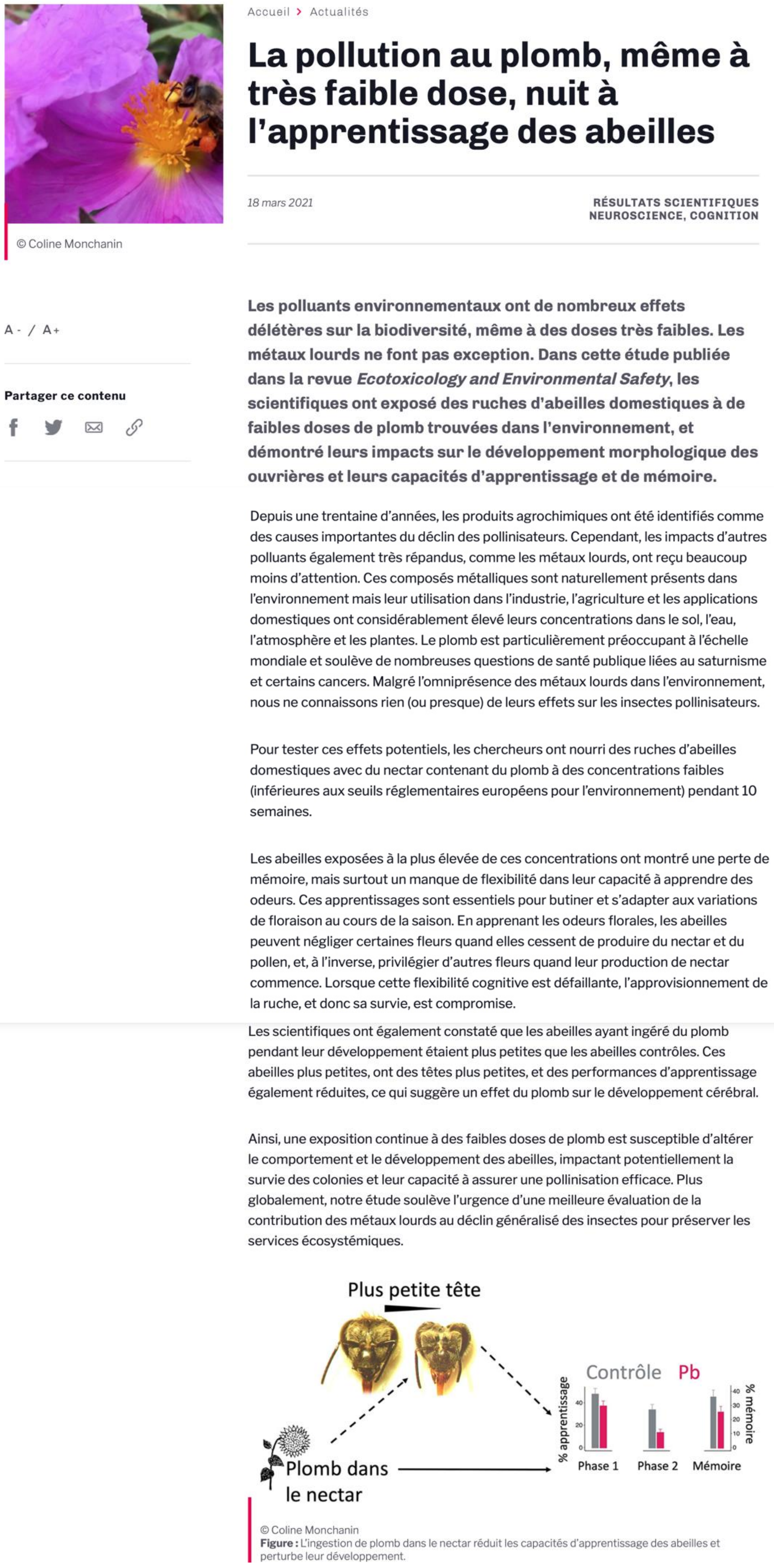
Appendix 2B: La pollution au plomb, même à très faible dose, nuit à l'apprentissage des abeilles

Press release from the CNRS
https://insb.cnrs.fr/fr/cnrsinfo/la-pollution-au-plomb-meme-tres-faible-dose-nuit-lapprentissage-des-abeilles



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Accueil > Actualités

La pollution au plomb, même à très faible dose, nuit à l'apprentissage des abeilles

18 mars 2021

RÉSULTATS SCIENTIFIQUES
NEUROSCIENCE, COGNITION

A - / A+

Partager ce contenu

Les polluants environnementaux ont de nombreux effets délétères sur la biodiversité, même à des doses très faibles. Les métaux lourds ne font pas exception. Dans cette étude publiée dans la revue *Ecotoxicology and Environmental Safety*, les scientifiques ont exposé des ruches d'abeilles domestiques à de faibles doses de plomb trouvées dans l'environnement, et démontré leurs impacts sur le développement morphologique des ouvrières et leurs capacités d'apprentissage et de mémoire.

Depuis une trentaine d'années, les produits agrochimiques ont été identifiés comme des causes importantes du déclin des pollinisateurs. Cependant, les impacts d'autres polluants également très répandus, comme les métaux lourds, ont reçu beaucoup moins d'attention. Ces composés métalliques sont naturellement présents dans l'environnement mais leur utilisation dans l'industrie, l'agriculture et les applications domestiques ont considérablement élevé leurs concentrations dans le sol, l'eau, l'atmosphère et les plantes. Le plomb est particulièrement préoccupant à l'échelle mondiale et soulève de nombreuses questions de santé publique liées au saturnisme et certains cancers. Malgré l'omniprésence des métaux lourds dans l'environnement, nous ne connaissons rien (ou presque) de leurs effets sur les insectes pollinisateurs.

Pour tester ces effets potentiels, les chercheurs ont nourri des ruches d'abeilles domestiques avec du nectar contenant du plomb à des concentrations faibles (inférieures aux seuils réglementaires européens pour l'environnement) pendant 10 semaines.

Les abeilles exposées à la plus élevée de ces concentrations ont montré une perte de mémoire, mais surtout un manque de flexibilité dans leur capacité à apprendre des odeurs. Ces apprentissages sont essentiels pour butiner et s'adapter aux variations de floraison au cours de la saison. En apprenant les odeurs florales, les abeilles peuvent négliger certaines fleurs quand elles cessent de produire du nectar et du pollen, et, à l'inverse, privilégier d'autres fleurs quand leur production de nectar commence. Lorsque cette flexibilité cognitive est défaillante, l'approvisionnement de la ruche, et donc sa survie, est compromise.

Les scientifiques ont également constaté que les abeilles ayant ingéré du plomb pendant leur développement étaient plus petites que les abeilles contrôles. Ces abeilles plus petites, ont des têtes plus petites, et des performances d'apprentissage également réduites, ce qui suggère un effet du plomb sur le développement cérébral.

Ainsi, une exposition continue à des faibles doses de plomb est susceptible d'altérer le comportement et le développement des abeilles, impactant potentiellement la survie des colonies et leur capacité à assurer une pollinisation efficace. Plus globalement, notre étude soulève l'urgence d'une meilleure évaluation de la contribution des métaux lourds au déclin généralisé des insectes pour préserver les services écosystémiques.

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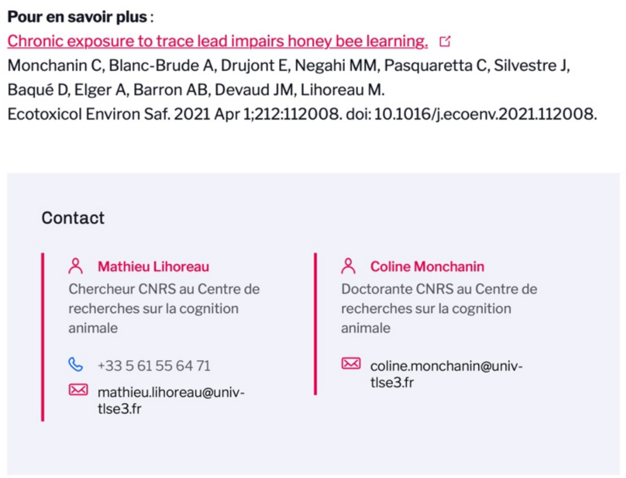
Figure : L'ingestion de plomb dans le nectar réduit les capacités d'apprentissage des abeilles et perturbe leur développement.



Pour en savoir plus :

Chronic exposure to trace lead impairs honey bee learning.

Monchanin C, Blanc-Brude A, Drujont E, Negahi MM, Pasquaretta C, Silvestre J, Baqué D, Elger A, Barron AB, Devaud JM, Lihoreau M.

Ecotoxicol Environ Saf. 2021 Apr 1;212:112008. doi: 10.1016/j.ecoenv.2021.112008.

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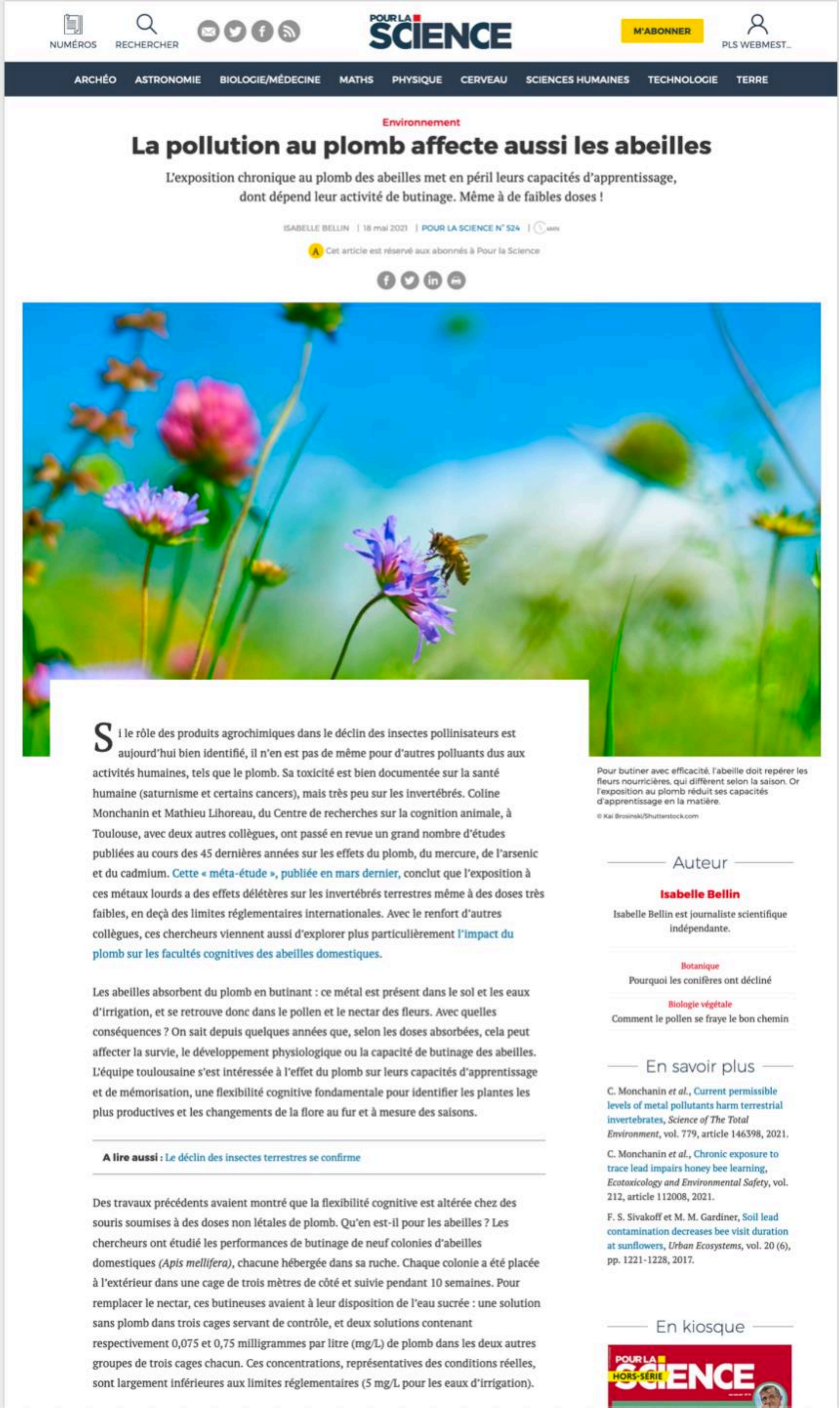
Appendix 2C: La pollution au plomb affecte aussi les abeilles

Popular science paper published in Pour La Science



Environnement

La pollution au plomb affecte aussi les abeilles

L'exposition chronique au plomb des abeilles met en péril leurs capacités d'apprentissage, dont dépend leur activité de butinage. Même à de faibles doses !

ISABELLE BELLIN | 18 mai 2021 | POUR LA SCIENCE N° 524 |

Cet article est réservé aux abonnés à Pour la Science

Si le rôle des produits agrochimiques dans le déclin des insectes pollinisateurs est aujourd'hui bien identifié, il n'en est pas de même pour d'autres polluants dus aux activités humaines, tels que le plomb. Sa toxicité est bien documentée sur la santé humaine (saturnisme et certains cancers), mais très peu sur les invertébrés. Coline Monchanin et Mathieu Lihoreau, du Centre de recherches sur la cognition animale, à Toulouse, avec deux autres collègues, ont passé en revue un grand nombre d'études publiées au cours des 45 dernières années sur les effets du plomb, du mercure, de l'arsenic et du cadmium. Cette « méta-étude », publiée en mars dernier, conclut que l'exposition à ces métaux lourds a des effets délétères sur les invertébrés terrestres même à des doses très faibles, en deçà des limites réglementaires internationales. Avec le renfort d'autres collègues, ces chercheurs viennent aussi d'explorer plus particulièrement l'impact du plomb sur les facultés cognitives des abeilles domestiques.

Les abeilles absorbent du plomb en butinant : ce métal est présent dans le sol et les eaux d'irrigation, et se retrouve donc dans le pollen et le nectar des fleurs. Avec quelles conséquences ? On sait depuis quelques années que, selon les doses absorbées, cela peut affecter la survie, le développement physiologique ou la capacité de butinage des abeilles. L'équipe toulousaine s'est intéressée à l'effet du plomb sur leurs capacités d'apprentissage et de mémorisation, une flexibilité cognitive fondamentale pour identifier les plantes les plus productives et les changements de la flore au fur et à mesure des saisons.

A lire aussi : Le déclin des insectes terrestres se confirme

Des travaux précédents avaient montré que la flexibilité cognitive est altérée chez des souris soumises à des doses non létales de plomb. Qu'en est-il pour les abeilles ? Les chercheurs ont étudié les performances de butinage de neuf colonies d'abeilles domestiques (*Apis mellifera*), chacune hébergée dans sa ruche. Chaque colonie a été placée à l'extérieur dans une cage de trois mètres de côté et suivie pendant 10 semaines. Pour remplacer le nectar, ces butineuses avaient à leur disposition de l'eau sucrée : une solution sans plomb dans trois cages servant de contrôle, et deux solutions contenant respectivement 0,075 et 0,75 milligrammes par litre (mg/L) de plomb dans les deux autres groupes de trois cages chacun. Ces concentrations, représentatives des conditions réelles, sont largement inférieures aux limites réglementaires (5 mg/L pour les eaux d'irrigation).

Pour butiner avec efficacité, l'abeille doit repérer les fleurs nourricières, qui diffèrent selon la saison. Or l'exposition au plomb réduit ses capacités d'apprentissage en la matière.

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Auteur

Isabelle Bellin

Isabelle Bellin est journaliste scientifique indépendante.

Botanique
Pourquoi les conifères ont décliné

Biologie végétale
Comment le pollen se fraye le bon chemin

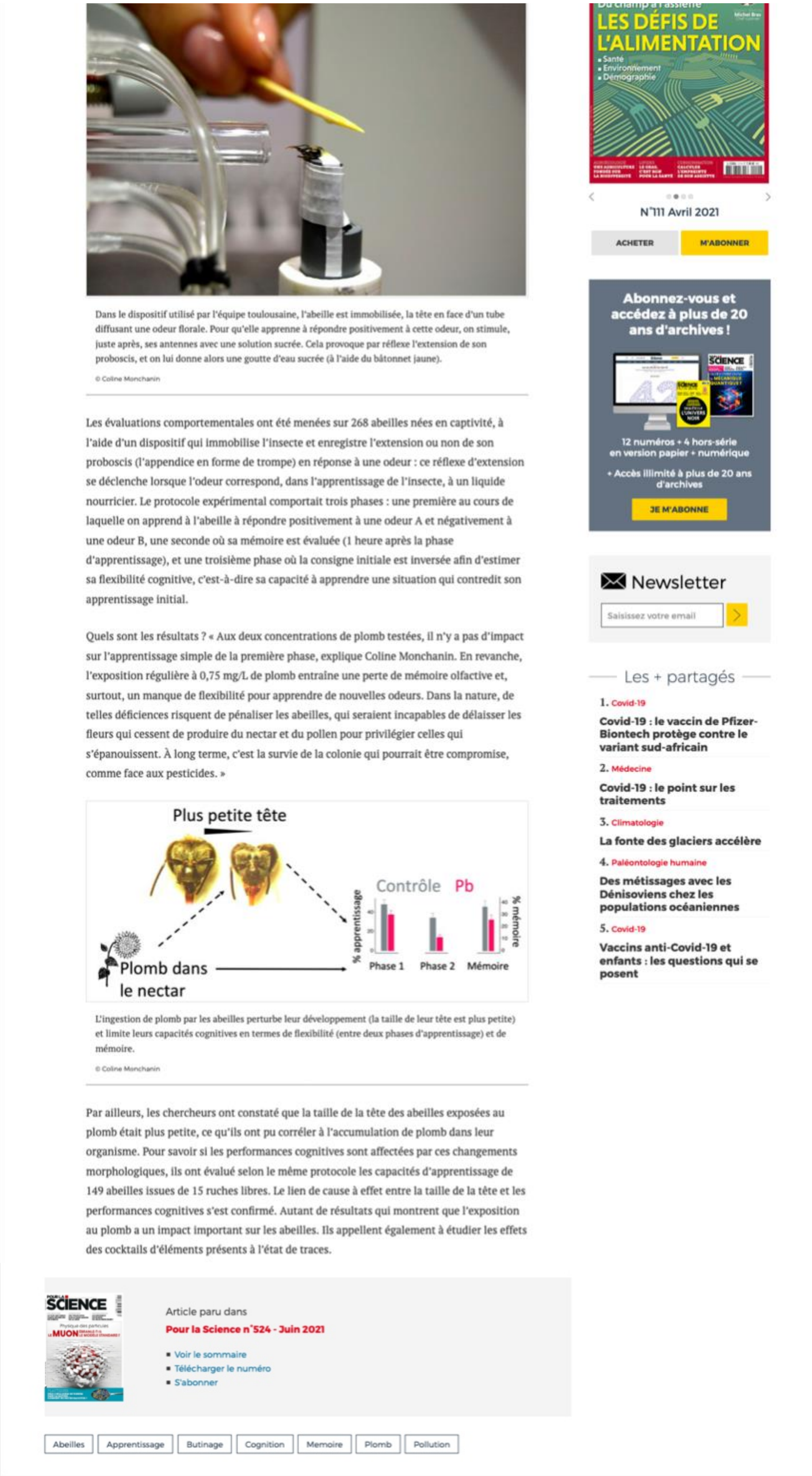
En savoir plus

C. Monchanin *et al.*, Current permissible levels of metal pollutants harm terrestrial invertebrates, *Science of The Total Environment*, vol. 779, article 146398, 2021.

C. Monchanin *et al.*, Chronic exposure to trace lead impairs honey bee learning, *Ecotoxicology and Environmental Safety*, vol. 212, article 112008, 2021.

F. S. Sivakoff et M. M. Gardiner, Soil lead contamination decreases bee visit duration at sunflowers, *Urban Ecosystems*, vol. 20 (6), pp. 1221-1228, 2017.

En kiosque



Dans le dispositif utilisé par l'équipe toulousaine, l'abeille est immobilisée, la tête en face d'un tube diffusant une odeur florale. Pour qu'elle apprenne à répondre positivement à cette odeur, on stimule, juste après, ses antennes avec une solution sucrée. Cela provoque par réflexe l'extension de son proboscis, et on lui donne alors une goutte d'eau sucrée (à l'aide du bâtonnet jaune).

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Les évaluations comportementales ont été menées sur 268 abeilles nées en captivité, à l'aide d'un dispositif qui immobilise l'insecte et enregistre l'extension ou non de son proboscis (l'appendice en forme de trompe) en réponse à une odeur : ce réflexe d'extension se déclenche lorsque l'odeur correspond, dans l'apprentissage de l'insecte, à un liquide nourricier. Le protocole expérimental comportait trois phases : une première au cours de laquelle on apprend à l'abeille à répondre positivement à une odeur A et négativement à une odeur B, une seconde où sa mémoire est évaluée (1 heure après la phase d'apprentissage), et une troisième phase où la consigne initiale est inversée afin d'estimer sa flexibilité cognitive, c'est-à-dire sa capacité à apprendre une situation qui contredit son apprentissage initial.

Quels sont les résultats ? « Aux deux concentrations de plomb testées, il n'y a pas d'impact sur l'apprentissage simple de la première phase, explique Coline Monchanin. En revanche, l'exposition régulière à 0,75 mg/L de plomb entraine une perte de mémoire olfactive et, surtout, un manque de flexibilité pour apprendre de nouvelles odeurs. Dans la nature, de telles déficiences risquent de pénaliser les abeilles, qui seraient incapables de délaisser les fleurs qui cessent de produire du nectar et du pollen pour privilégier celles qui s'épanouissent. À long terme, c'est la survie de la colonie qui pourrait être compromise, comme face aux pesticides. »

L'ingestion de plomb par les abeilles perturbe leur développement (la taille de leur tête est plus petite) et limite leurs capacités cognitives en termes de flexibilité (entre deux phases d'apprentissage) et de mémoire.

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Par ailleurs, les chercheurs ont constaté que la taille de la tête des abeilles exposées au plomb était plus petite, ce qu'ils ont pu corréler à l'accumulation de plomb dans leur organisme. Pour savoir si les performances cognitives sont affectées par ces changements morphologiques, ils ont évalué selon le même protocole les capacités d'apprentissage de 149 abeilles issues de 15 ruches libres. Le lien de cause à effet entre la taille de la tête et les performances cognitives s'est confirmé. Autant de résultats qui montrent que l'exposition au plomb a un impact important sur les abeilles. Ils appellent également à étudier les effets des cocktails d'éléments présents à l'état de traces.

Article paru dans
Pour la Science n°524 - Juin 2021

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Appendix 3: Metal pollutants have additive negative effects on honey bee cognition

Paper published in the Journal of Experimental Biology

© 2021. Published by The Company of Biologists Ltd | Journal of Experimental Biology (2021) 224, jeb241869. doi:10.1242/jeb.241869

SHORT COMMUNICATION

Metal pollutants have additive negative effects on honey bee cognition

Coline Monchanin[1,2,]*, Erwann Drujont[1], Jean-Marc Devaud[1], Mathieu Lihoreau[1] and Andrew B. Barron[2]

ABSTRACT

Environmental pollutants can exert sublethal deleterious effects on animals. These include disruption of cognitive functions underlying crucial behaviours. While agrochemicals have been identified as a major threat to pollinators, metal pollutants, which are often found in complex mixtures, have so far been overlooked. Here, we assessed the impact of acute exposure to field-realistic concentrations of three common metal pollutants, lead, copper and arsenic, and their combinations, on honey bee appetitive learning and memory. All treatments involving single metals slowed down learning and disrupted memory retrieval at 24 h. Combinations of these metals had additive negative effects on both processes, suggesting common pathways of toxicity. Our results highlight the need to further assess the risks of metal pollution on invertebrates.

KEY WORDS: *Apis mellifera*, **PER conditioning, Pollutant interaction, Arsenic, Lead, Copper**

INTRODUCTION

Metal pollution is of increasing concern for both ecosystem and public health (Nriagu and Pacyna, 1988). Over the last century, the widespread use of metals in domestic, industrial and agricultural applications (Bradl, 2005) has considerably elevated their concentrations in water (Mance, 1987) and terrestrial habitats (Krämer, 2010; Su et al., 2014) up to potentially toxic levels.

Pollinators, such as honey bees, are directly exposed to metal pollutants when foraging on contaminated nectar and pollen (Perugini et al., 2011; Xun et al., 2018), and while flying through air containing suspended particles (Thimmegowda et al., 2020). Metals accumulate in the bodies of adults (Giglio et al., 2017) and larvae (Balestra et al., 1992), as well as in hive products (Satta et al., 2012). For instance, bioaccumulation of arsenic (As), copper (Cu) and lead (Pb), resulting from metal production industries (Kabir et al., 2012) and mining (Khaska et al., 2018; Lee et al., 2005), is common in both honey bees (Badiou-Bénéteau et al., 2013; Giglio et al., 2017; Goretti et al., 2020) and their honey (Pisani et al., 2008; Terrab et al., 2005).

The deleterious effects of metals on humans (Tchounwou et al., 2012) and some model animals (mice: Cobbina et al., 2015; flies: Doğanlar et al., 2014) are well known. As, Cu, Pb and other metals have neurotoxic effects that induce neural and neuromuscular alterations, sensory impairment and many other behavioural dysfunctions (Chen et al., 2016). Deficits in cognition and memory have been reported for As (e.g. humans: Tolins et al., 2014; mice: Tyler et al., 2018; Wu et al., 2006), Pb (e.g. mice: Anderson et al., 2016; humans: Mason et al., 2014) and Cu (e.g. mice: Lamtai et al., 2020; Pal et al., 2013; flies: Zamberlan, 2020). Recent studies showed that low doses of Pb (Monchanin et al., 2021a) and selenium (Se) (Burden et al., 2016) also impair behaviour and cognition in honey bees, suggesting a widespread impact on pollinators. So far, however, very little attention has been given to the potential combined effects of co-exposure to different metals (Monchanin et al., 2021b).

Interactions among stressors are commonly classified as antagonistic (when the effect of one stressor reduces the effect of the other one), additive (when stressors have simple cumulative effects) or synergistic (when stressors together have a greater effect than the sum of their individual effects) (Folt et al., 1999). Additive effects of As, Cu and Pb have been described for humans (Lin et al., 2016), rats (Aktar et al., 2017; Mahaffey et al., 1981; Schmolke et al., 1992) and fishes (Verriopoulos and Dimas, 1988). In rats, for example, co-exposure to Pb and As disrupted brain biogenic amine levels (Agrawal et al., 2015). In humans, it has been hypothesized that combined exposure to Pb and As, or other metal pollutants, has additive or synergistic toxic responses leading to cognitive dysfunction (Karri et al., 2016). To our knowledge, two studies have addressed the impact of metallic cocktails on bee physiology. Honey bees simultaneously exposed to Pb, cadmium (Cd) and Cu accumulated significant levels of these metals in their bodies and had lower brain concentrations of dopamine compared with control honey bees (Nisbet et al., 2018). Cd and Cu exerted a weak synergistic effect on honey bee survival (Di et al., 2020). However, none of these studies investigated potential effects of combined exposure on cognition.

Here, we compared the effects of exposure to single metals or ecologically relevant combinations of these metals on honey bee learning and memory. We hypothesized that combinations of metals may have synergistic negative effects, as has been found with pesticides (Yao et al., 2018; Zhu et al., 2017). We tested individual honey bees in a standard protocol of proboscis extension response (PER) conditioning following acute exposure to As, Pb and Cu alone or in combination. We tested three concentrations of As, considered the most toxic substance (https://www.atsdr.cdc.gov/cep/index.html), and added one concentration of Cu or Pb (binary mixtures), or both (tertiary mixture), to reach the molarity of the As solutions.

MATERIALS AND METHODS

Metal solutions

Arsenic ($NaAsO_2$), lead ($PbCl_2$) and copper ($CuCl_22H_2O$) were purchased from Sigma-Aldrich Ltd (Lyon, France) and diluted in 50% (w/v) sucrose solution. Control honey bees were fed 50% sucrose solution. Three concentrations of As were used (Table 1): a low concentration (0.13 μmol l^{-1}) corresponding to the maximal

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permissible value in drinking water (0.01 mg l^{-1}) (Codex Alimentarius, 2015), a high concentration (0.67 μmol l^{-1}) corresponding to half the maximal permissible value in irrigation water (0.1 mg l^{-1}) (Ayers and Westcot, 1994), and an intermediate concentration (0.40 μmol l^{-1}). This range of concentrations was reported in water sampled from polluted areas (e.g. mining sites) and in honey (Table S1). For Pb and Cu, we chose 0.27 μmol l^{-1} (0.055 mg l^{-1} of Pb and 0.017 mg l^{-1} of Cu) so that the binary combinations (As 0.13 μmol l^{-1}+Cu 0.27 μmol l^{-1} or As 0.13 μmol l^{-1}+Pb 0.27 μmol l^{-1}) could be compared with the As intermediate concentration (0.40 μmol l^{-1}), and the tertiary combination (As 0.13 μmol l^{-1}+Pb 0.27 μmol l^{-1}+Cu 0.27 μmol l^{-1}) with the As high concentration (0.67 μmol l^{-1}) (Table 1). These concentrations of Pb and Cu have also been reported in honey samples (Table S1). The mass consumed for As and the concentrations for Cu and Pb fell within sublethal ranges for the honey bee: the LD$_{50}$ of elemental As for NaAsO$_2$ ranged from 0.330 to 0.540 μg per bee (Fujii, 1980); the LC$_{50}$ of Cu is 72 mg l^{-1} (Di et al., 2016) and that of Pb is 345 mg l^{-1} (Di et al., 2016).

Bee exposure to metals

We collected honey bees (*Apis mellifera* Linnaeus 1758) returning from foraging trips at the entrance of five different hives in mornings during August 2020. We anaesthetized the bees on ice and harnessed them in plastic tubes, secured with tape and a droplet of wax at the back of the head (Matsumoto et al., 2012). We tested all bees for an intact PER by stimulating their antennae with 50% sucrose. We then fed the responding honey bees 5 μl of 50% sucrose solution (see Table 1), making sure they consumed the whole droplet, and left them to rest for 3 h in the incubator (temperature: 25 ±2°C, humidity: 60%). Honey bees that did not respond to the sucrose solution were discarded.

Absolute learning

Prior to conditioning, we tested all honey bees for the PER by stimulating their antennae with 50% sucrose solution, and kept only those that displayed the reflex. We then performed olfactory absolute conditioning according to a standard protocol using an automatic stimulus delivery system (Aguiar et al., 2018). Honey bees had to learn to respond to an olfactory conditioned stimulus (CS, 1-nonanol; Sigma-Aldrich Ltd) reinforced with the unconditioned stimulus (US, 50% sucrose solution), over five conditioning trials with a ten-minute inter-trial interval. Each trial (37 s in total) began when a bee was placed in front of the stimulus delivery system, which released a continuous flow of clean air (3300 ml min^{-1}) to the antennae. After 15 s, the odour was introduced into the airflow for 4 s, the last second of which overlapped with sucrose presentation to the antennae using a toothpick. This was immediately followed by feeding for 4 s by presenting the toothpick to the proboscis. The bee remained for another 15 s under the clean airflow. We recorded the presence or absence (1/0) of a conditioned PER in response to the odorant presentation during each conditioning trial. Honey bees spontaneously responding in the first conditioning trial were discarded from the analysis. The sum of conditioned responses over all trials provided an individual acquisition score (between 0 and 4), and honey bees responding at the last trial were categorized as learners.

Table 1. Concentrations of heavy metals used

Treatment	Molarity (μmol l^{-1})	Concentration (mg l^{-1})			Ingestion of 5 μl (ng per bee)		
		As	Cu	Pb	As	Cu	Pb
Control	0	0	0	0	0	0	0
Low [As]	0.13	0.01	0	0	0.05	0	0
[Cu]	0.27	0	0.02	0	0	0.09	0
[Pb]	0.27	0	0	0.06	0	0	0.28
Int. [As]	0.40	0.03	0	0	0.15	0	0
[As+Cu]	0.40	0.01	0.02	0	0.05	0.09	0
[As+Pb]	0.40	0.01	0	0.06	0.05	0	0.28
High [As]	0.67	0.05	0	0	0.25	0	0
[As+Cu+Pb]	0.67	0.01	0.02	0.06	0.05	0.09	0.28

Int., intermediate. Combined treatments are shown in grey.

Long-term memory

Only honey bees that had learnt the task were kept for the analysis of memory performance. After conditioning, these honey bees were fed 15 μl of 50% sucrose solution, left overnight in the incubator, and fed another 5 μl of sucrose solution the following morning. Three hours later (24 h post-conditioning), we performed the retention test, consisting of three trials similar to conditioning except that no sucrose reward was presented. In addition to the odour used during the conditioning (CS), we presented two novel odours, in randomized order, to assess the specificity of the memory: nonanal was expected to be perceived by honey bees as similar to 1-nonanol, while 1-hexanol was expected to be perceived differently (Guerrieri et al., 2005). We recorded the presence or absence (1/0) of a conditioned PER to each odorant at each memory retention trial. We classified honey bees according to their response patterns: response to the CS only, response to the CS and the similar odour (low generalization level), response to all odours (high generalization level), no or inconsistent response.

Statistics

We analysed the data using R Studio v.1.2.5033 (http://www.rstudio.com/). Raw data are available from Dryad (Dataset S1, doi:10.5061/dryad.ghx3ffbms). We performed binomial generalized linear mixed-effects models (GLMM) (package lme4; Bates et al., 2015), with hive and conditioning date as random factors and treatment as a fixed effect. Using the GLMMs, we evaluated whether molarity or treatment impacted the initial response to antennal stimulation, the spontaneous response in the first conditioning trial, the response in the last trial, the response to each odorant during the memory test, the proportion of honey bees per response pattern in the retention test, and the survival at 24 h. Acquisition scores were standardized and compared with GLMMs using Template Model Builder (Brooks et al., 2017). For all response variables, we compared (1) the treated groups with the control, (2) groups exposed to concentrations of the same molarity (e.g. intermediate [As], [As+Cu] and [As+Pb]), (3) the separate and joint effects of the treatments (e.g. low [As], [Cu] and [As+Cu]) in order to identify interactive effects (antagonistic, additive, synergistic).

RESULTS AND DISCUSSION

Exposure to metals does not impact appetitive motivation

The proportion of honey bees that responded to the initial antennal stimulation with sucrose was similar among treatments (GLMM: $P>0.05$). Therefore, treatment did not affect appetitive motivation or sucrose perception. Consistent with our observations, the ingestion of similar concentrations of Pb and Cu had no effect on responsiveness to increasing concentrations of sucrose (Burden et al., 2019). By contrast, Di et al. (2020) found that honey bees exposed to increasing concentrations of a mixture of Cu and Cd exhibited a decreased ability to distinguish sucrose concentrations, but this may be explained by the much higher (at least 600 times) concentrations used in that study. Thus, in our conditions, any impact on appetitive learning is unlikely to be due to a decrease in motivation for sucrose or sucrose perception.

Individual and combined exposure to metals reduces learning performance

Two out of the 381 honey bees subjected to the absolute learning task spontaneously responded to the first odour presentation and were therefore discarded. In all groups, the number of honey bees showing the conditioned response increased over trials, thus showing learning (Fig. 1A). However, fewer honey bees exposed to metals learned the task when compared with controls (GLMM: *P*<0.05, except for low [As], *P*=0.082). Accordingly, the acquisition scores of honey bees from all treatments were lower than those of controls (Fig. 1B). Honey bees exposed to intermediate [As] (GLMM: −0.610±0.246, *P*=0.013), high [As] (GLMM: −0.639±0.241, *P*=0.008) and [As+Cu+Pb] (GLMM: −0.592±0.244, *P*=0.015) had acquisition scores significantly lower than those of controls. Honey bees exposed to [As+Pb] had similar acquisition scores to bees exposed to intermediate [As] (GLMM: 0.299±0.234, *P*=0.201), but honey bees exposed to [As+Cu] performed better (GLMM: 0.596±0.241, *P*=0.013). Honey bees exposed to high [As] and [As+Cu+Pb] exhibited similar acquisition scores (GLMM: *P*=0.810). We found no difference in the acquisition scores and the proportions of learners between honey bees treated with a single metal and mixed treatments (GLMM: *P*>0.05), that would have indicated non-additive effects (i.e. antagonistic or synergistic). Thus, exposure to metals significantly reduced learning performance, and combined exposure appeared to exert simple additive deleterious effects.

Individual and combined exposure to metals reduces long-term memory specificity

To examine possible effects of metal exposure on memory retention, we tested memory 24 h post-training. Only honey bees that had learned the CS–US association at the end of conditioning were tested; 167 out of the 379 honey bees subjected to the absolute learning task did not learn and were therefore not included in the memory test.

We found no effect of treatment on survival at 24 h (GLMM: *P*>0.05). However, long-term memory was significantly affected (Fig. 2). Overall, treated honey bees responded less to the learned odorant (CS) than did controls, as indicated by a significant effect of exposure to metals on retention levels (GLMM: *P*<0.05) (Fig. 2A). Yet, this decrease was not significant for honey bees exposed to intermediate [As] (GLMM: −0.260±0.628, *P*=0.679) and high [As] (GLMM: −1.023±0.570, *P*=0.073). Finally, there was no clear dose effect on responses to the CS among treated groups (GLMM: −0.576±0.579, *P*=0.320).

Individual response patterns (Fig. 2B) revealed a loss of memory specificity. While honey bees from all treatments responded similarly to the similar odour (GLMM: *P*>0.05), those exposed to higher doses responded more frequently to all odorants, indicating a higher degree of response generalization (GLMM: 1.954±0.775, *P*=0.012). This was accompanied by a significantly lower proportion of specific (CS-only) responses for honey bees exposed to [Pb] (GLMM: −1.795±0.690, *P*=0.009), low [As] (GLMM: −1.313±0.589, *P*=0.026) and [As+Cu+Pb] (GLMM: −1.200±0.588, *P*=0.041). Exposure also significantly increased the frequency of inconsistent responses as compared with that in controls (GLMM: *P*<0.05). This was the case for each individual treatment except for intermediate [As] (*P*=0.293). Thus, exposure to metals had a negative impact on memory performance at 24 h. The analysis of individual response patterns also revealed additive effects as they did not differ among groups exposed to solutions with the same molarity, nor between single and mixed metal treatments (GLMM: *P*>0.05). Thus, most treatments reduced memory performance at 24 h.

The additive effects of metal mixtures may be explained by common pathways of toxicity

Although many mechanisms of metal toxicity have not yet been elucidated, some points of consensus are emerging from the

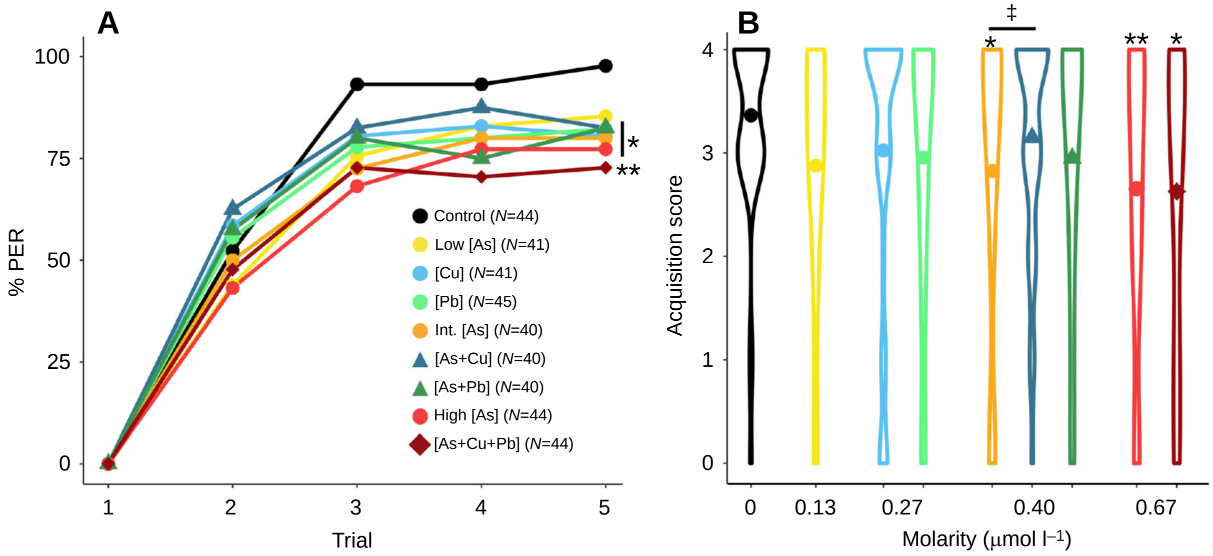

Fig. 1. Learning. (A) Learning curves show changes in the percentage of honey bees displaying the conditioned proboscis extension response (PER) over five training trials. Asterisks indicate significant differences in response rates at the last trial compared with those for control honey bees. (B) Violin plots of acquisition score values (sum of conditioned responses for each honey bee). Symbols (circle: single exposure; triangle: binary mixture; diamond: tertiary mixture) indicate the mean score for each treatment. Significant differences between groups exposed to solutions of the same molarity (‡) or with respect to control honey bees (*) are indicated (*,‡*P*<0.05, ***P*<0.01; GLMM).

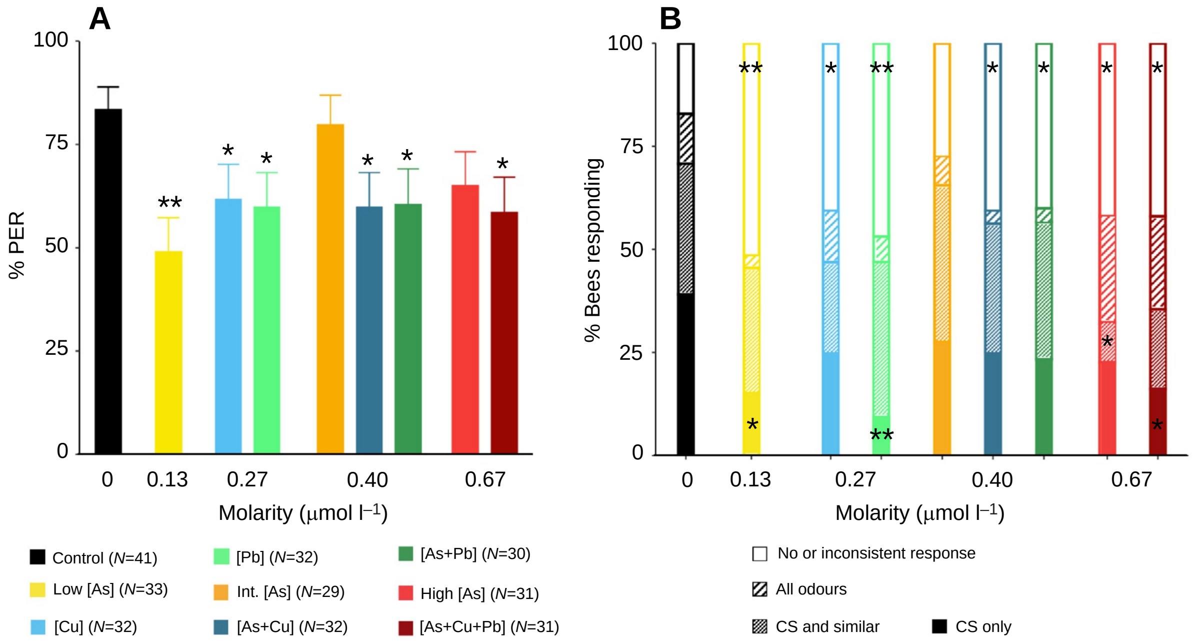

Fig. 2. Long-term memory. (A) Percentage of responses to the conditioned stimulus (CS) odour in the 24 h memory retention test (means±s.e.m.). (B) Distribution of honey bees according to their individual response pattern during the long-term memory test: response to CS only; response to CS and similar odour; response to all odours; no or inconsistent response. Significant differences from controls are indicated (*$P<0.05$, **$P<0.01$; GLMM).

literature. Firstly, interactions between metals can occur in the environment of the organism (Grobelak and Kowalska, 2020; Noyes and Lema, 2015), and during uptake into the organism, leading to potentially toxic processes of speciation, absorption, binding, transport and distribution (Wu et al., 2016). Once metals enter an organism, they can induce, alter or inhibit a range of biological responses and metabolic pathways. For example, by mimicking other essential metals (Bridges and Zalups, 2005) or damaging the permeability of biological membranes (Rothshein, 1959), metals enable the uptake or loss of other compounds from intracellular compartments (Viarengo, 1994). Metals are also known to disrupt signalling and calcium homeostasis (particularly important in neurons) by interfering with calcium channels (Bridges and Zalups, 2005; Chavez-Crooker et al., 2001; Tamano and Takeda, 2011). This might lead to dysfunction and cytotoxicity as a result of the disruption of cell signalling and calcium homeostasis. Genotoxicity (Doğanlar et al., 2014) may be achieved through covalent binding to DNA (Brocato and Costa, 2013; Senut et al., 2014). Eventually, oxidative stress and lipid peroxidation of the cell membrane may lead to neuronal death. Additionally, metals in mixtures could interact at target sites, but the effect on toxicity of that interaction is largely unknown (Svendsen et al., 2011). Metal mixtures could change the bioavailability (Gong et al., 2020), toxicokinetics and toxicodynamics (Gao et al., 2016) of each metal, which could impact the toxicity for the organism (Løkke et al., 2013). Based on these shared mechanisms of toxicity that include oxidative stress (Nikolić et al., 2016; Zaman et al., 1995), apoptosis (Raes et al., 2000) and interference with neurotransmitters (Nisbet et al., 2018), the toxic effects of metal pollutants in mixtures is expected to be additive (von Stackelberg et al., 2013). Of note, these conclusions emerge from studies mostly conducted on vertebrates, thus possibly leaving aside specificities of meta actions in invertebrate organisms.

Mixtures of metals may affect many aspects of neural activity and brain function in honey bees, as in other species (Karri et al., 2016). Here, we focused on learning and memory of olfactory cues because they play crucial roles in the behavioural ecology of honey bees and other pollinators, for the identification of food resources. Our results in controlled laboratory conditions suggest that exposure to sublethal combinations of toxic elements in the field might alter individual foraging efficiency, and in turn jeopardize survival of pollinator populations. While we could not identify interactive effects in such conditions, this will need to be confirmed in field experiments where exposure conditions will differ, and we should study a broader range of behavioural responses (flight, activity, navigation). Our approach aims to fill a gap in the evaluation of combined actions of metals (Meyer et al., 2015), which appears necessary to better assess the risks they represent (Nys et al., 2018; Otitoloju, 2003) and better inform regulatory frameworks (European Commission, 2012). Current risk assessment guidance mainly assesses the effect of exposure to individual metals, which fails to capture potential interactive effects. This is of particular importance for honey bees and many other species, where contaminated food is transferred and shared among individuals. Hence, evaluation of the impact of metal mixtures and their modes of action needs to be developed (Sasso et al., 2010). Additionally, interactions between toxic metals and environmental factors (Naqash et al., 2020) as well as with other chemicals (EFSA Scientific Committee et al., 2019) (e.g. pesticides: Sgolastra et al., 2018; Singh et al., 2017; volatile organic compounds: Sasso et al., 2010, etc.) should be implemented in an integrated research framework.

Conclusion

In summary, we have demonstrated that As, Pb, Cu or combinations of these metals, at levels found in the environment, slow down appetitive learning and reduce long-term memory specificity in honey bees. These metals show simple additive effects as we found no difference in effects between different solutions of the same molarity, suggestive of possible non-linear effects (synergism or antagonism). Thus, regarding effects on learning and memory, concentration seems to be more important than the identity of any specific metal. Given that learning and memory of olfactory cues play crucial roles in the behavioural ecology of honey bees, acute exposure to mixtures of metal pollutants could impair fundamental hive function and population growth.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: C.M., J.D., M.L., A.B.B.; Methodology: C.M., J.D., M.L., A.B.B.; Investigation: C.M., E.D.; Data curation: C.M.; Writing - original draft: C.M.; Writing - review & editing: C.M., J.D., M.L., A.B.B.; Visualization: C.M.; Supervision: J.D., M.L., A.B.B.; Funding acquisition: J.D., M.L., A.B.B.

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Data availability

Raw data are available from the Dryad digital repository (Monchanin, 2021): doi:10.5061/dryad.ghx3ffbms

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Appendix 4: Insect diet

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I

Insect Diet

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Definition

Insect diet refers to the food usually eaten by an insect for growth, tissue maintenance, and reproduction, as well as the energy necessary to maintain these functions.

Introduction

Most insects have qualitatively similar nutritional requirements in proteins, carbohydrates, lipids, vitamins, minerals, trace elements, and water (Table 1). These chemical compounds can either be synthetized by the insects themselves, provided by beneficial symbionts or acquired in food (Chapman 2012). Insect diets, and thus the fraction of nutrients acquired in food, can considerably vary among species and developmental stages of the same species, resulting from adaptations to particular environments in which access to nutrients is restricted by the types and diversity of foods available. Herbivores, which make up the majority of insects, eat plants and are typically trophic specialists, which means that they consume only one or a few plant species. By contrast, entomophagous, carnivorous, zoophagous, detritivorous, xylophagous, graminivorous, and omnivorous insects tend to be more generalists.

While the domestication of insects started somewhere around 5000 years ago, with the cultivation of silkworms and honey bees, research on insect nutrition only developed at the beginning of the twentieth century. Key discoveries were made possible through the multiplication of attempts for designing artificial diets, whose composition can be only partly (meridic diet) or fully (holidic diet) defined. In 1908, Bogdanov was the first to rear an insect (blowflies) entirely on an artificial diet made of peptone, meat extract, starch, and minerals. Since then, famous entomologists, such as Painter, Fraenkel, Dadd, Waldbauer, Dethier, Scriber, and Slansky and Bernays and Chapman, among others, have developed methods and concepts that set a revolution for research on animal nutrition (for a brief thematic history, see (Raubenheimer et al. 2009)). These experiments demonstrate that insects actively attempt to achieve of nutritional balance by carefully regulating their intake of several nutrients simultaneously either from artificial diets or natural foods. Beyond advancing fundamental knowledge on insect nutrition, this research also provided a framework for a series of pioneering nutritional ecology studies that helped establish major concepts of biology and ecology, such as, for instance, ecological niches.

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Insect Diet, Table 1 Minimal (irreducible) nutrients shown to be useful or essential to insects. Depending on species, some of these nutrients can be nonessential or even toxic (e.g., cellulose). (Modified from (Cohen 2015))

Proteins	Lipids	Carbohydrates	Vitamins	Minerals
Polypeptides	Sterols	Hexose	Water-soluble vitamins	Calcium
Glycoprotein	Cholesterols	Glucose	Ascorbic acid	Chlorine
Lipoprotein	β-Sitosterol	Fructose	Thiamine	Copper
Essential amino acids	Stigmasterol	Disaccharides	Riboflavin	Iron
Arginine	Campesterol	Sucrose	Pyridoxine	Magnesium
Histidine	24-methyl-cholesterol	Polysaccharides	Nicotinic acid	Manganese
Isoleucine	Phospholipids	Starch	Pantothenic acid	Phosphorus
Leucine	Fatty acids	Glycogen	Biotin	Potassium
Lysine	Linoleic acid	Cellulose	Folic acid	Sodium
Methionine	Linolenic acid		Choline	Sulfur
Phenylalanine			Cyanocobalamin	Zinc
Threonine			Inositol	
Tryptophan			Lipid-soluble vitamins	
Valine			Tocopherol	
			Vitamin A	

Nutrients

Foods are complex mixtures of nutritional and non-nutritional (sometimes toxic) compounds. For insects, these compounds typically involve macronutrients (proteins, carbohydrates, and lipids), micronutrients (vitamins and minerals), and water, which all directly participate to physiological functions (Cohen 2015). Some of these nutrients are essential, which means that insects lack the ability to synthetize them on their own and must acquire them in food of from beneficial symbionts (Table 1). Others, such as food additives (stabilizers, preservatives, bulking agents) and token stimuli (plant secondary compounds) have no direct nutritional function.

Proteins

Proteins are made of amino acids (organic compounds containing an amino (-NH2), carboxyl (-COOH) groups, and a specific chain) and are the principal source of nitrogen for insects. While free amino acids can be present in foods, most often they are linked together by peptide bounds to form proteins. Once assimilated, proteins are broken down into their amino acid components and turned into different proteins that can be used for a wide range of biological functions, such as cell structure, enzymes, transport and storage, or receptor molecules. Insects require nine to ten essential amino acids (Table 1). The others, non-essential, amino acids are generally synthetized in the fat body provided that precursors are available in the food, although other tissues can also be important (e.g., proline and glutamine are synthetized in the mosquito midgut). Most proteins contain approximately half essential and half non-essential amino acids.

Lipids

Lipids consist of fatty acids, phospholipids, and sterols. Lipids are an important source of energy, essential components of cell membranes, nutrient transporters, and defensive compounds, serve as pheromones, and are involved in hormone synthesis (e.g., sterols are involved in ecdysteroid or molting hormones and fatty acids in juvenile hormone). Insects can synthesize most fatty acids and phospholipids. However, to do so, polyunsaturated fatty acids are required in the diet. Sterols can serve for energy and the production of hormones and carbohydrates. The major essential sterol, the cholesterol, is abundant in animal tissues but only present in low quantities (if not present at all) in plants and fungal food. Therefore, most phytophagous insects must synthetize cholesterol via dealkylation of plant sterols (e.g., β-sitosterol and campesterol) (Behmer and Nes 2003).

Carbohydrates

Carbohydrates include simple sugars (e.g., the monosaccharides sucrose, fructose, glucose, maltose), starch, and other polysaccharides (e.g., cellulose). They serve as respiratory fuel, provide the carbon basis in molecular synthesis, and constitute building materials for the insect cuticle (e.g., polysaccharide chitin). Insects can synthesize glucose by gluconeogenesis from lipids or amino acids (Miyamoto and Amrein 2017) in such a way that some species can live without any sugar intake at all (e.g., wax moth and screwworm). By contrast, other insects require considerable amounts of carbohydrates in their diet (e.g., honey bee or locust). Not all sugars are usable by all insects (e.g., melibiose is digested by many flies but not by honey bees), and some monosaccharides can be toxic because they compete with other essential sugars (e.g., mannose blocks glucose pathway in bees). The digestive capability for carbohydrates also varies among insect species. For instance, flour beetles can hydrolyze a broad range of polysaccharides, whereas the grasshopper *Melanoplus* only accepts simple sugars. Digestibility of carbohydrates also varies between developmental stages of the same species (e.g., mosquito larvae use starch and glycogen while adults cannot). Cellulose cannot be digested by most insects and thus has no nutritional value.

Vitamins

Vitamins are organic compounds required in trace amounts for growth. Vitamins are classified in two groups depending on their solubility in water or lipid. Water-soluble vitamins have a relatively short half-life (excreted and lost from the insect's metabolic pool), while lipid-soluble vitamins remain compartmentalized in lipid stores. The main water-soluble vitamins include vitamin C (ascorbic acid) and the B vitamins. Vitamin C serves as phagostimulant and antioxidant and promotes the synthesis of collagen and the extracellular matrix in insects. The B vitamins are involved in many metabolic pathways, including ATP production (thiamine, riboflavin, niacin), acyl group transfer (pantothenate), and growth factor (biotin and folic acids). Some insects also require small quantities of other water-soluble vitamins such as choline (for the production of cell membrane), carnitine (for lipid metabolism), cyanocobalamin, and lipoic acid. Lipid-soluble vitamins essential to insects are the vitamin A complex (β-carotene and related carotenoids relatives) and vitamin E (tocopherols). Vitamin A is required for visual pigments function and formation. Vitamin E serves as fertility factor, including spermatogenesis and egg maturation.

Minerals

Calcium, chloride, copper, iron, magnesium, manganese, phosphorus, potassium, sodium, and zinc are vital in small quantities to many insects. These compounds are involved in the synthesis of coenzymes and metalloenzymes. Potassium, chloride, calcium, and sodium are essential for the excitability of tissues (e.g., muscle cell and neurons). Potassium and magnesium are major actors of the bioenergetics activity, respectively, via the ATP and glycolysis pathway. Pollutant minerals, present in the environment and passively ingested by insects, can replace dietary minerals and act as toxins.

Water

Water is essential to all insects. It provides the medium in which all metabolic processes proceed. As such it is necessary for the absorption of macronutrients. Water often contains naturally occurring micronutrients such as mineral salts. Insects actively ingest free water, have physiological mechanisms controlling thirst, and suffer fitness consequences if water is excessive or deficient in the diet. Meal size and inter-meal duration are both influenced by free water availability.

Consequences of Diet on Behavior and Physiology

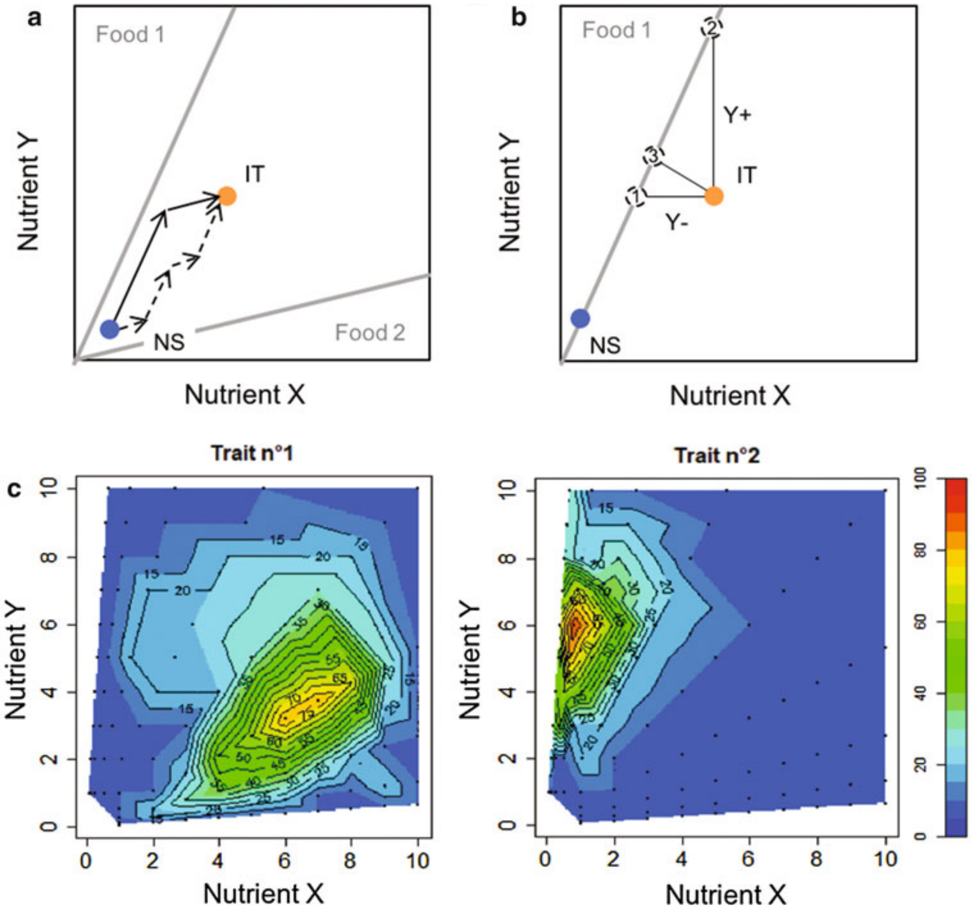
Insects have evolved behavioral and physiological strategies to acquire appropriate amounts and balances of the required nutrients from complex food mixtures. In most environments, no single food provides an optimally balanced diet for the insects. In such case, individuals must adjust their dietary choices and nutrient assimilation rates to reach and maintain a balanced diet (Simpson and Raubenheimer 2012).

Studying Nutrient Regulation: Nutritional Geometry

The study of nutrient regulation by insects took a significant step forward at the end of the twentieth century when Raubenheimer and Simpson (1993) introduced a unifying theoretical framework for nutrition studies known as “nutritional geometry.” This framework employs a state-space modeling approach taking into account the multiple interactions among mechanisms regulating the intake of different classes of nutrients. Individual insects, foods, and their interactions are represented graphically in a geometric space (a nutrient space) defined by two or more food components, typically carbohydrates and proteins (see examples Fig. 1). Foods are radials through the nutrient space at angles determined by the balance of nutrients they contain (nutritional rails). The insect’s state (nutritional state) is a point or region that changes over time. As the insect eats, its nutritional state changes along the rail for the chosen food. The functional aim for the insect is to eat foods in appropriate amounts and ratio to direct it to an optimal nutritional state (intake target). This intake target moves over time as the quantity and mix of nutrients change with activity, growth, development, reproduction, and senescence. For instance, in larval insects, early stages typically have diets richer in nitrogen than later stages. The intake target also shifts over evolutionary times as insects adapt to different diets. Insects that feed on other animals have high amino acid and fat requirements relative to carbohydrates, reflecting the relatively high protein and low carbohydrate content of animal tissues. Plant-feeding insects, however, generally require approximately equal amounts of amino acids and carbohydrates. Over the past decades, nutritional geometry has proved a powerful tool to understand how insects and other animals balance their diet across a wide diversity of taxa, feeding guilds and ecological of environments both behaviorally and physiologically (for a recent review, see (Raubenheimer and Simpson 2018)).

Behavioral Regulation

An insect can respond to a dietary imbalance in one of three ways. First, it can move from eating one food to another with a different nutrient balance. Hence, when given a choice between complementary foods (foods defining a nutrient space containing the intake target of the animal), most insects have been shown to self-compose a nutritionally balanced diet (Fig. 1a). This is possible because insects have separate appetites for different nutrients. Grasshoppers, caterpillars, aphids, flies, and cockroaches regulate their intake of protein and carbohydrates to a single intake target. Predatory ground beetles (Carabidae) have separate protein and lipid appetites. In locusts, levels of water are also regulated together with protein and carbohydrates (Clissold et al. 2014). This ability to adjust food intake to nutritional requirements implies some feedback of nutritional status on food selection and feeding behavior. In locusts, blood-borne nutrient feedback from eating a diet rich in amino acids depresses the sensitivity of peripheral contact chemoreceptors to amino acids in the diet but has no effect on the sensitivity of chemoreceptors to sucrose. Conversely, if the insect feeds on a diet with high levels of carbohydrates, the sensitivity of its receptors to sucrose is depressed. In species that heavily rely on learning and memory for foraging, such as bees, cognitive capacities are crucial for nutrient balancing by enabling insets to associate the quality of the food with feedback on their own nutrient status based on the visual, olfactory, and tactile characteristics of foods. In drosophila, protein appetite in mated females results of a sex peptide that is introduced with male’s seminal fluid during mating, which stimulates special sensory neurons in the female’s reproductive tract. An additional mechanism responding to the protein demands of egg development then controls how much yeast is eaten, involving TOR/S6 kinase and serotonin signaling pathways in the central nervous system (Vargas et al. 2010).



Insect Diet, Fig. 1 Examples of nutritional geometry models for hypothetical insects. (Modified from (Lihoreau et al. 2018)). (**a**) Nutritional rails (gray lines) represent the ratio of nutrients X and Y in foods. The blue dot is the nutritional state (NS) of the individual, and the red dot is its intake target (IT). Foods 1 and 2 are individually imbalanced but complementary (fall on opposite sides of the IT). The individual can reach its IT by combining its intake from the two foods (arrows). (**b**) The individual is restricted to a single imbalanced food and can (1) satisfy its needs for Y but suffer a shortfall of X; (2) satisfy its needs for X but over-ingest Y; and (3) suffer a moderate shortage of X and excess of Y. (**c**) Nutritional performance landscapes showing the effects of nutrients X and Y on fitness traits 1 and 2. In this example, trait 1 is maximized for a high X to Y ratio, whereas trait 2 is maximized for a low X to Y ratio

Second, insects feeding on imbalanced foods can also adjust the total amount ingested to acquire enough of the most limiting nutrients. Grasshoppers, caterpillars, cockroaches, aphids, and ants have been shown to increase the amount eaten if the entire nutrient composition of a diet is diluted with some inert non-nutritional substance. Insects can also selectively compensate for deficiencies in a class of nutrients by increasing the total amount eaten. When confined to a single, nutritionally imbalanced food (that does not allow the intake target to be reached), insects can compromise between overconsuming excess nutrients and eating too little of the nutrients in deficit. The form of this compromise in a nutrient space varies according to the nutrients involved and the ecology of species (Fig. 1b). For instance, when forced to ingest diets containing an unbalanced ratio of proteins and carbohydrates, specialist migratory locusts (*Locusta migratoria*) do not substantially overconsume the excess nutrient to decrease its deficit of the other nutrient.

In contrast, in the generalist desert locust (*Schistocerca gregaria*), individuals ingest a greater amount of either protein or carbohydrate to gain more of the more limiting nutrient. This pattern of host-plant generalists being more willing to tolerate nutrient excesses than host plant specialists is widespread across grasshoppers and caterpillars and is accompanied by a greater tendency by generalists to store excesses in body reserves (Behmer 2009).

Physiological Regulation

A third mechanism to face food nutritional imbalance is to adjust the efficiency with which the insect uses the ingested nutrients. The importance of such post-ingestive regulation has been best studied in locusts. These insects maintain a relatively constant increase in body nitrogen despite a threefold increase in the amount of nitrogen ingested. Most of the excess of protein is excreted as uric acid or some other unknown nitrogenous end product of catabolism. Other means of post-ingestive regulation include differential secretion of digestive enzymes to lower the efficiency of digestion excess carbohydrate or protein in the diet, the adjustment of the timing of gut emptying to alter the ratio of protein and carbohydrate absorbed from the gut, the increase of metabolic rate to burn off excess ingested carbohydrate, or the selection of environmental temperatures that favor the utilization of either proteins or carbohydrates.

Consequences of Diet on Fitness

Although some growth occurs on foods containing widely differing levels of nutrients, optimal performance requires the nutrient levels to be appropriately balanced. Ingesting and processing excessive quantities of food in order to obtain enough of a particular component that is present only in low concentration in the diet can prove costly in various ways. Firstly, some nutrient excess can be toxic or have deleterious effects (e.g., excess of carbohydrates can result in obesity-like syndromes in many insects). Secondly, interconversions from one compound to another can be metabolically costly and the rates at which they occur limited. These effects can affect a wide range of fitness traits such as growth, development, reproduction, immune responses, cognition, and life span. The interacting effects of nutrients on fitness traits are evident when mapping insect performances into nutrient spaces of nutritional geometry (Fig. 1c). Different traits often have different nutritional optima, which means that insects must make feeding decisions to trade-off between optimizing multiple traits simultaneously. The ability of insects to resolve these nutritional trade-offs has been first demonstrated in the fruit fly *Drosophila melanogaster* (Lee et al. 2008). When confided to 1 of 28 artificial diets varying in protein and carbohydrate content, female flies achieve a maximum life span on a diet containing a 1:16 ratio of protein to carbohydrate, while maximum egg laying rate is reached on a 1:2 protein to carbohydrate ratio. When allowed to self-select complementary foods, flies mix a diet comprising a 1:4 protein to carbohydrate ratio which maximizes lifetime egg production, a measure of global fitness. Similar trade-offs have been observed in many other insect species for life span and reproduction, immunity and reproduction, or even traits related to different stages of reproduction that cannot be attained simultaneously.

Consequences of Diet on Biotic Interactions

Upon its influence on the physiology, behavior, and fitness of individual insects, the diet can affect social behaviors and interspecific interactions (e.g., with symbionts, competitors, predators) and ultimately influence species assemblages and communities.

Social Behavior

In gregarious and social insects, diet influences collective behaviors and social structures (Lihoreau et al. 2018). At the most basic level, a deficit in key nutrients in the environment can generate mass movements. In the Mormon cricket, Anabrus the lack of proteins and mineral salts due to intense competition and food depletion during population outbreaks triggers intense cannibalistic interactions. By attempting to eat each other, crickets engage in mass migrations, whereby millions of individuals form marching bands extending over several kilometers (Simpson et al. 2006). In social caterpillars that forage in trails, differences in the nutritional states among trail members determine the identity of leaders (hungry) that guide the group and followers (well-fed) that follow behind. In more integrated insect societies, differences in early food experience can also mediate reproductive division of labor. This is the case in cooperative breeding burying beetles that feed on shared carcasses, where dominant females that access food in priority ingest more proteins and become breeders, whereas subordinate individuals that acquire relatively less proteins become sterile helpers. In eusocial insects, such as honey bees, differential nutrition of the larvae influences caste determination, so that female larvae fed a diet rich in royal jelly become reproductive queens, whereas female larvae fed lower levels of jelly become sterile workers.

Ecological Interactions

Symbionts

An estimated 10% of all insects utilize diets that are nutritionally so poor or unbalanced that they depend on beneficial symbiotic organisms for sustained growth and reproduction. These associations provide them with metabolic capabilities or additional nutrients (e.g., essential amino acids in insects feeding on plant sap, vitamins insects feeding on blood, and sterols in insects utilizing wood). In some insects, resident microorganisms degrade complex dietary components to a form that can be assimilated by the insect. This is the case of termites that rely on a rich gut microbiota community to degrade cellulose or soil matter (Bignell et al. 2010). In plant-sap feeding hemipterans that eat phloem and xylem low in essential amino acids, the microorganisms have a biosynthetic function. In *Drosophila*, where gut symbionts are acquired from the environment, variations in microbe communities can trigger different foraging strategies in the hosts that need to compensate for different nutrients (Wong et al. 2017). Other insect species have evolved ectosymbiotic associations. This is the case of fungus-farming termites and leaf-cutter ants that cultivate their own crop in well-protected gardens. In these social insects, foragers collect plant materials not digestible by the insects to feed a fungus that provides accessible key nutrients to the insects. In ants, workers regulate food intake to nourish the fungus with nutrient balances that maximize the production of edibles for the colony, at the expense of fungus reproduction (Shik et al. 2016).

Parasites and Pathogens

The diet can also affect the immunological responses of insects and their interactions with parasites and pathogens. For instance, caterpillars of the African cotton leafworm (Spodoptera littoralis) infected with either a bacterial or viral pathogen survive better as the ratio of protein to carbohydrate in the diet is increased despite the toxic effect of protein on life span. By contrast, uninfected larvae perform best on an intermediate nutrient ratio. When given a choice between multiple artificial diets, infected caterpillars tend to increase their consumption of protein, which has the consequence of enhancing the immune response, a nutritional behavior akin to self-medication (Lee et al. 2006).

Dietary Breath and Niche Partitioning

At a broader observation scale, variation in insect diet can determine the coexistence of species and shape local development of biodiversity. For instance, closely related species of generalist-feeding herbivores (grasshopper species in the genus *Melanoplus*) eat protein and carbohydrates in different absolute amounts and ratios even if they eat the same plant taxa (Behmer and Joern 2008). The existence of species-specific nutritional niches, such as this one, provides a cryptic mechanism that helps explain how generalist herbivores with broadly overlapping diets coexist.

Conclusions

There is a long history of developing artificial diets to culture insects for the food industry and academic research. This approach has showed that most insects have qualitatively similar requirements for macro- and micronutrients as other animals. Over the past decades, new concepts of nutritional ecology have moved the focus from the identification of essential and nonessential nutrients in insect diet to the study of interactions between food components and their consequences on fitness. Insects have evolved sophisticated behavioral and physiological strategies to reach and maintain nutrient balances and concentrations maximizing multiple fitness traits, and this is flexible throughout development. These consequences of diet can be observed at the individual level and beyond, across levels of biological organization. Integrative approaches of modern insect nutrition research offer a means for addressing more general problems in ecology, including the structuring of food webs, the regulation of food chain length, the flow of nutrients through ecosystems, and the dynamics of communities and ecosystems (Simpson et al. 2015).

Cross-References

- ▶ Amino Acid
- ▶ Arthropod Diet
- ▶ Digestive System
- ▶ Division of Labor
- ▶ Energy
- ▶ Feeding
- ▶ Fitness
- ▶ Food Industry
- ▶ Foraging
- ▶ Growth Diet
- ▶ Herbivore
- ▶ Hymenoptera Diet
- ▶ Lipid
- ▶ Local Resource Competition
- ▶ Nutrients
- ▶ Optimal Foraging Theory
- ▶ Protein
- ▶ Provisioning
- ▶ State Sensitive Model of Foraging

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Appendix 5: Putting the ecology back into insect cognition

Book chapter published in Advances in Insect Physiology

CHAPTER ONE

Putting the ecology back into insect cognition research

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Contents

Abstract

Over the past decades, research on insect cognition has made considerable advances in describing the ability of model species (in particular bees and fruit flies) to achieve cognitive tasks once thought to be unique to vertebrates, and investigating how these may be implemented in a miniature brain. While this lab-based research is critical to understand some fundamental mechanisms of insect brains and cognition, taking a more integrative and comparative view will help making sense of this rich behavioural repertoire and its evolution. Here we argue that there is a need to reconsider insect cognition into an ecological context in order to design experiments that address the cognitive challenges insects face in nature, identify competing hypotheses about the cognitive abilities driving the observed behavioural responses, and test them across different populations and species. Reconnecting with the tradition of naturalistic observations, by testing animals in the field or in ecologically-inspired setups and comparing

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their performances, is complementary to mechanistic research in the lab, and will greatly improve our understanding of the role of insect cognition, its diversity, and the influence of ecological factors in its evolution.

> *… que se passe-t-il dans ce petit cerveau d'hyménoptère? Y a-t-il là des facultés soeurs des nôtres, y a-t-il une pensée? Quel problème, si nous pouvions le résoudre; quel chapitre de psychologie, si nous pouvions l'écrire! […what happens in this little brain of Hymenoptera? Are there abilities similar to ours, is there a thought? What problem, if we could solve it; what chapter of psychology, if we could write it!]*
>
> ***Translated from Jean-Henri Fabre (Fabre, 1882, p. 405).***

1. Past and present of insect cognition research

Famous naturalists such as Réaumur, De Geer, Latreille, Fabre, Darwin, Lubbock, to name just a few, have played a considerable role in demonstrating that insects, just like large-brained animals, are capable of adapting to new situations through various forms of learning, memory and communication. In the 20th century, the first ethologists made invaluable contributions to our understanding of these processes, through experimental manipulations and quantifications of insect behaviour in the field. Von Frisch (1915), for instance, used artificial flowers to show colour discrimination by honey bees. This approach later facilitated the discovery of the symbolic communication by which foragers advertise the location of remote feeding sites to their nestmates by displaying dances on the vertical honey combs (von Frisch, 1967). Tinbergen manipulated the visual appearance of the nests of digger wasps with pine cones to demonstrate that wasps use visual memories to orient themselves and return home (Tinbergen, 1932).

Since then, generations of talented entomologists have described a rich diversity of cognitive abilities by which insects sample, process and use information from their environment to adapt their behaviour in different contexts (e.g., mate choice, foraging, egg laying, navigation) at different levels (e.g., as individuals and as groups) and in a variety of taxa (for recent reviews see: Collett et al., 2013; Feinerman and Korman, 2017; Giurfa, 2013, 2019; Papaj and Lewis, 2012; Perry et al., 2017). This research shows that model species (especially bees and fruit flies) achieve ever more impressive cognitive tasks despite their relatively simple neural system. At the individual level, bees are capable of learning concepts (Giurfa et al., 2001), counting (Howard et al., 2018), optimising paths (Lihoreau et al., 2012b), copying others (Alem et al., 2016), innovating (Loukola et al., 2017) and even self-assessing their chances to solve a task (Perry and Barron, 2013). Some wasps can recognize the faces of their nestmates (Sheehan and Tibbetts, 2011) and fruit flies can socially transmit mate choice preferences across several generations, possibly leading to local traditions (Danchin et al., 2018). At the collective level, ants and bees in colonies often make faster and more accurate decisions than alone when selecting food sources (Beckers et al., 1990) or a nesting site (Sasaki et al., 2013; Seeley, 2010), and can efficiently solve mazes (Goss et al., 1989) or transport large food items across complex environments (Gelbium et al., 2015).

Over the past decades, the development of new technologies and methods in neurosciences (Dubnau, 2014; Menzel, 2012) has progressively moved research in insect cognition from the description of sophisticated behaviour in the field to mechanistic investigations of cognitive processes and their neural correlates in the lab. Significant progresses in understanding insect brain organisation and function have been made using genetic mutants (e.g., GAL4/UAS, optogenetics), imaging techniques, drug injections or screening of gene expression in targeted neuropiles (Guo et al., 2019). We now have a fairly good idea of brain areas, neurons and molecular pathways involved in different forms of associative learning in model species such as fruit flies, honey bees, some ants, moths, cockroaches and crickets (Giurfa, 2013). In particular, the fruit fly *Drosophila melanogaster* has emerged as a key genetic model to address these questions both because of the relative simplicity of its nervous system (mapped at the level of synaptic connectivity, Zheng et al., 2018) and its rich behavioural repertoire both at the individual and collective levels (Sokolowski, 2010), allowing for the genetic dissection of sophisticated behaviours, such as place learning (Ofstad et al., 2011), flight control (Dickinson and Mujires, 2016), courtship (Pavlou and Goodwin, 2013), grooming (Hampel et al., 2015), memory-driven action selection (Owald and Waddell, 2015) and collective movements (Ramdya et al., 2014).

Although very insightful, the fast development of lab-based mechanistic studies has also reduced the scope of insect cognition research in several ways. First, the focus on the molecular and genetic bases of cognitive processes has limited investigations to few model species that may not express a cognitive repertoire representative of the estimated 5.5 million insect species (Stork, 2018). While it can be interesting to compare bees, ants and wasps when considering social evolution within the social Hymenoptera (e.g., Farris, 2016; Gronenberg and Riveros, 2009), the comparison with the more phylogenetically distant fruit flies may be less informative (Brenowitz and Zakon, 2015). Second, studies on the mechanisms of learning and memory often rely on hypotheses and paradigms inspired from human experimental psychology that may sometimes bias interpretations of the results, and limit the search for alternative (sometimes more parsimonious) explanations (e.g., Cheung, 2014; Guiraud et al., 2018). Third, research that is exclusively conducted in the lab presents the risk of disconnecting subjects, behaviours and cognitive traits of interest from their natural environment. Testing animals in very artificial setups in order to achieve a high level of control on information available to insects and their behavioural responses, does not always allow for the expression of the desired naturalistic behaviours (e.g., Niggebrügge et al., 2009). The questions or approaches used to study insect cognition are often very different from situations animals face in nature (e.g., study aversive learning using electric shocks, conditioning immobile harnessed insects, testing social insects in isolation). The animals themselves used for testing cognitive abilities often come from long-term laboratory or commercial cultures in which some traits may be inadvertently selected or counter selected (e.g., commercial bumblebees, *Drosophila* mutant strains). Fourth, the type and levels of stress animals are exposed to may be highly different in the lab and in the field. This can be problematic since several recent studies show that negative or positive experiences can induce emotion-like states in insects that have consequences on their behaviour and performances in cognitive tasks (e.g., *Drosophila*, Yang et al., 2013; honey bees, Bateson et al., 2011; bumblebees, Perry et al., 2016).

Here we argue that there is a need for complementing current lab-based insect cognition research with more ecologically inspired studies in order to fully understand the diversity and evolution of cognitive traits. In recent years, concepts of cognitive ecology have been embraced by behavioural ecologists and experimental psychologists working on vertebrates and proved successful to advance knowledge on the ecological role and evolution of bird and primate cognition (Dukas, 1998, 2008; Dukas and Ratcliffe, 2009; Morand-Ferron et al., 2015; Pritchard et al., 2016). In what follows, we advance that time is ripe for the development of a cognitive ecology of insects. First, we review conceptual frameworks that have been proposed for the evolution of the insect brain and cognition. We then explain how taking into account the ecological context in which cognitive traits are expressed in nature can help refine these frameworks by designing field-inspired experiments, testing wild animals, bringing lab-controlled protocols to the field, as well as comparing more species. Finally, we discuss how technological advances to study insect cognition in ecologically realistic conditions will help develop this comparative approach, by dramatically increasing the number of cognitive tasks and individuals that can be investigated.

2. The evolution of insect brains and cognition

While we are now getting a more accurate picture of what insects can and cannot do (Perry et al., 2017), and which are the brain areas and neural circuits involved in some of these cognitive operations (Giurfa, 2013), fundamental questions about why and how cognitive traits evolve in these animals remain poorly understood.

Both social and ecological factors are expected to fashion the evolution of brains and cognition (Shettleworth, 2009). Since early descriptions of the anatomy of the insect nervous system (Dujardin, 1850), many discussions about the evolution of insect brains and cognitive abilities have focused on the influence of social factors (Strausfeld, 2012). Following the "social brain hypothesis" developed to explain the evolution of large brains in social vertebrates, and in particular anthropoid primates (Byrne, 1996; Dunbar, 1998), two hypotheses were recently proposed for insects. Gronenberg and Riveros (2009) suggested that the transition from solitary to gregarious and colony-based social structures has required the expansion of brain regions related to communication, large behavioural repertoires and flexibility. By contrast, behavioural specialization in socially advanced species with division of labour may have led to reduced investment in brain regions underpinning a range of cognitive operations not required anymore, thereby predicting a quadratic relationship between increasing levels of social complexity and brain size (Gronenberg and Riveros, 2009). O'Donnell et al. (2015) proposed that group communication relaxes the need for individual information processing, resulting in a linear decrease of brain size (or brain size areas) with increasing levels of sociality.

Despite many attempts to correlate brain sizes with metrics of social complexity in different insect taxa, empirical supports for a social brain hypothesis are mixed (Farris, 2016; Gordon et al., 2019; Kamhi et al., 2016, 2019; O'Donnell et al., 2015; Riveros et al., 2012). Part of the problem may be methodological (e.g., coarse measures of social complexity and brain sizes, lack of phylogenetical approaches), thus calling for broader comparative analyses of neuro-anatomical and behavioural studies mapped on phylogenies (Godfrey and Gronenberg, 2019; Lihoreau et al., 2012a). Another difficulty in this hypothesis lies in the unverified assumption that larger behavioural repertoires require larger brains. In fact, many fundamental changes in the complexity of a nervous system may not result in measurable volumetric differences and novel behaviour can emerge from minimal rewiring of existing neurons (Chittka and Niven, 2009).

The strong focus on the importance of social factors for the evolution of brains and cognitive capacities (especially in Hymenoptera) has somehow neglected a number of alternative or complementary hypotheses that have been long developed by vertebrate biologists, such as the importance of diet (DeCasien et al., 2017), maternal care (Curley and Keverne, 2005) or spatial navigation (Jacobs et al., 1990). Ecological conditions are known to fashion the evolution of insect sensory systems and brain anatomy (e.g., vision (Briscoe and Chittka, 2001), and olfaction (Hansson and Stensmyr, 2011)). However, the links between ecological constraints and cognitive capacities have been little explored. In an attempt to test these alternative hypotheses in Hymenoptera, Farris and Schulmeister (2011) made a careful evaluation of the architecture of the mushroom bodies (central brain structures involved in various forms of visual, olfactory and bimodal memories (Strausfeld, 2012)) in a wide diversity of species and mapped their lifestyles and neural structure onto an established phylogeny. This analysis showed that relatively enlarged mushroom bodies, with elaborate structure and visual and olfactory inputs, evolved 90 million years prior to sociality, in solitary parasitoid wasps (Farris and Schulmeister, 2011). Presumably, the challenge of acquiring spatial memories for locating prey and provisioning larvae may have placed much higher cognitive demands in these first parasitoids than in their herbivorous ancestors. Only later, this cognitive adaptation to spatial orientation may have favoured the evolution of central place foraging and the development of large societies sustained by highly efficient visuo-spatial foragers (Farris, 2016).

3. Towards a cognitive ecology of insects

The emerging field of cognitive ecology provides a theoretical and methodological framework to study the ecology and evolution of animal cognition (for reviews see Dukas, 1998; Dukas and Ratcliffe, 2009). This involves designing new hypotheses and experiments based on field observations, testing wild animals, bringing lab-controlled experimental protocols into the field, taking into account the social context of the cognitive task, and comparing large numbers of species with known ecologies and phylogenetic relationships. While this approach has so far mainly been used for vertebrates, especially birds and mammals (Dukas, 2008; Morand-Ferron et al., 2015; Pritchard et al., 2016), below we highlight some key recent examples in insects.

3.1 Identifying new questions and hypotheses from field observations

Field observations are necessary to identify the types of problems animals must solve in their everyday life and how they might do so. The natural environment often contains much more relevant cues for the animals than typically assumed which structures the kind of information they can acquire. Observing freely moving insects in their natural environment is thus fundamental to design questions, identify competing hypotheses and develop experimental protocols, be the research later conducted in the lab.

Field observations are particularly important in insect navigation research since spatial orientation behaviours are not always easily expressed in lab setups with limited spatial scales and the incomplete set of environmental cues. In bees, field observations have recently moved the focus on simple nest-feeder navigation to more complex multi-destination route learning and optimisation (Lihoreau et al., 2013). In an attempt to study long-distance pollination by orchid bees in the Costa Rican rain forest, Janzen (1971) observed that some individuals often visited the same set of plants each day, probably in the same order. Given that bees are assumed to visit hundreds of flowers during a single foraging trip (von Frisch, 1967), this anecdotic observation has initiated several research programs investigating how bees develop routes between many familiar sites (Lihoreau et al., 2012b; Ohashi et al., 2007; Woodgate et al., 2017), for how long route memory is effective (Thomson, 1996), and how individuals achieve this behaviour while minimizing competition with other nectar foragers (Ohashi et al., 2008; Pasquaretta et al., 2019). In ants, field observations have raised new questions about how insects use environmental cues to solve orientation challenges. In the Australian desert, thermal turbulences due to solar heating of the ground create frequent wind gusts and it is not rare to see ants getting blown away from their familiar route. Even a small displacement of a few meters (i.e., several hundreds of body lengths for an ant) constitutes a big challenge for the ant to relocate its original position. Based on this observation, desert ants, *Melophorus bagoti*, were observed reorientating in the field after being experimentally displaced by wind gusts from a leaf blower into a dark pit (Wystrach and Schwartz, 2013). When released at windless unfamiliar locations, ants headed in a compass direction opposite to the one they had been blown away, thus functionally increasing their chance of returning to familiar areas. The encoding of wind direction relative to sun position occurs before being displaced, while clutching the ground to resist the wind (Wystrach and Schwartz, 2013). Field observations that ball-rolling dung beetles, *Scarabaeus lamarcki*, also appear to use wind in addition to the sun for spatial orientation have raised the question of how insects may use multimodal compass cues for navigation and inspired lab experiments in which sun and wind cues can be delivered in a tightly controlled manner (Dacke et al., 2019). In this setup, beetles were found to register information provided by the sun and the wind, and directional information can be transferred between these two sensory modalities, suggesting that beetles combine in the spatial memory network in their brain. This flexible use of compass cue preferences relative to the prevailing visual and mechanisms scenery provides a simple, yet effective, mechanism for enabling compass orientation at any time of the day when one type of cues may not be available (Dacke et al., 2019).

Field observations can also been pivotal to understand cognitive processes in populations of animals, such as the collective decisions underpinning the onset of insect swarms (Bazazi et al., 2008; Simpson et al., 2006). During population outbreaks Mormon crickets, *Anabrus simplex*, form marching bands of several kilometres long, comprising millions of individuals moving en masse (Sword et al., 2005). Observations of migratory bands indicated that many dead insects were left behind, as well as some carcasses of small vertebrates, suggesting that sedentary herbivorous crickets swarm in response to a local depletion of key nutrients (Simpson et al., 2006). Giving migrating crickets a choice between artificial diets varying in their nutritional composition in the field demonstrated that crickets in migratory bands are deprived of protein and mineral salts, which triggers their cannibalistic interactions. The crickets are in effect on a forced march, whereby individuals move ahead to try to eat conspecifics while escaping cannibalism by others behind them (Simpson et al., 2006).

3.2 Testing wild animals

Running experiments on wild animals offers the opportunity to assess important inter-individual variations in behaviour and cognition that are potentially shaped by environmental conditions, thereby providing a link between cognitive performances and the ecological context (Morand-Ferron et al., 2015).

In fruit flies, *D. melanogaster*, the utilization of wild-caught individuals for behavioural experiments has revealed the existence of natural allelic variations of the gene *foraging*, which encode a cGMP-dependant protein kinase (PKG) that affects the motor behaviour and social interactions of larvae and adults (Sokolowski, 1980). Sitter flies (for^S) are more sedentary and tend to aggregate within food patches, whereas rover flies (for^R) move more within and between food patches and are less gregarious (Sokolowski, 2010). These two natural behavioural variants are maintained at appreciable frequencies (ca. 70% rovers, 30% sitters) in nature (Sokolowski, 1980) and in the lab through negative frequency dependent selection (Fitzpatrick et al., 2007). Rovers and sitters also show important differences in their cognitive abilities. Rovers express stronger proboscis extension responses following a sucrose stimulation of their tarsi and show slower habituation of this response after multiple stimulations than sitters (Scheiner et al., 2004). Rovers develop better short-term aversive olfactory memory but poorer long-term memory than sitters (Mery et al., 2007). Interestingly, these two behavioural variants also differ in their ability to use social information. In a spatial task, where flies must learn to locate a safe zone in an aversively heated arena (i.e., invertebrate version of the Morris water maze), rovers rely more on personal information whereas sitters tend to primarily use social cues (Foucaud et al., 2013). These results suggest that both the utilization of information types and the cognitive performances of the two genotypes are co-adapted with their effects on foraging behaviour: the highly exploratory rovers could particularly benefit from fast learning based on individual information, whereas the more sedentary sitters should benefit more from social information and good long-term memory.

Wild populations are characterised by natural levels of genetic diversity that can greatly impact behavioural variability in cognitive tests. Experiments with German cockroaches, *Blattella germanica*, from different laboratory strains showed that individuals can discriminate between conspecifics with different genetic backgrounds, favouring aggregations with partners from the same strain (Rivault et al., 1998; Rivault and Cloarec, 1998) but mating with partners from different strains (Lihoreau et al., 2007). Intra-strain (kin) discrimination, however, could only be demonstrated in studies using wild-caught cockroaches sampled in separate geographic areas, showing that behavioural discrimination is based on quantitative differences in chemical signatures (i.e., cuticular hydrocarbon profiles) correlated with the genetic distance between individuals (Lihoreau et al., 2016a). The potential lack of genetic diversity in lab cultures maintained for long periods of time (highly inbred, no information about genetic background) may be a reason why kin recognition has been observed so rarely in insects (Fellowes, 1998; van Zweden and D'Ettorre, 2010).

3.3 Bringing experimental protocols into the field

Insect cognition research is largely based on well-defined paradigms designed to investigate specific cognitive traits (Giurfa, 2013). While this provides the advantage of allowing the identification of what animals can do, it may not, however, always reflect what animals actually do in the wild (Pritchard et al., 2016).

Firstly, important stimuli yielding information necessary for the expression of targeted behaviour may be absent in the lab. This is well illustrated by studies on visual cognition. Bees are capable of various forms of visual associative learning and memories used to locate and discriminate flowers, as well as developing routes between them (Avarguès-Weber et al., 2011). To control for the visual experience of bees, the spatial distribution of flowers and their rewarding value, bees spatial foraging strategies have been studied in the lab using artificial flowers in small flight arenas, flight rooms or greenhouses. In many bee species, foragers allowed to exploit an array of artificial flowers over several consecutive hours tend to develop repeatable flower visitation sequences (Lihoreau et al., 2010; Ohashi et al., 2007; Saleh and Chittka, 2007), a behaviour called "trapline foraging" (Thomson et al., 1997). Replicating these experiments in the field, using a harmonic radar to record the flight trajectories of individual bees at much larger ecologically relevant spatial scales, revealed that bees establish routes minimizing travel distances between all flowers and the nest based on long-term memories (Lihoreau et al., 2012b; Woodgate et al., 2017). In this case, both the increased spatial scales (e.g., longer travel distances associated to higher energetic costs) and the access to celestial cues (e.g., sun compass) have dramatically accelerated the dynamics of route formation and improved the optimization performance of bees in the field setup.

Another major advantage of adapting lab experiments to the field is to avoid potential sources of stress inherent to lab conditions. Even if insect species can be brought into the lab and the spatial scale and the information available to the insects were appropriate for understanding the behaviour of interest, the insect itself may still experience the lab task very differently than if it were presented with an analogous task in the wild. Again, research on bee visual cognition provides a good illustration of how lab-based protocols can be adapted to the field to tackle this problem. One of the most common paradigm for investigating learning and memory in honey bees is the conditioning of the proboscis extension reflex (PER), which tests for associations between an unconditional stimulus (sucrose reward) and a conditional stimulus (e.g., colour or scent) in harnessed bees (Takeda, 1961). This approach thus has the advantage of enabling the control for the timing of stimulus presentation (e.g., sequence of stimulus exposure, number of trials, inter-trial duration). However, while PER conditioning has been incredibly insightful to study olfactory cognition at the behavioural, neurobiological and molecular levels (Giurfa and Sandoz, 2012), it has always given poor or contrasted results with visual stimulations (e.g., some authors report the necessity to amputate antennae to obtain good learning, Kuwabara, 1957; Niggebrügge et al., 2009) and have never reached the usual levels observed in free-flying bees (Avarguès-Weber et al., 2011). Considering that bees predominantly use vision in flight, motion cues probably provide more natural visual context that participate to maintain a close dependence between visual and motor processing, and the immobilization of the bee in visual-PER studies undoubtedly disrupts this feedback loop (Avarguès-Weber and Mota, 2016). To address this issue, Muth et al. (2018) developed a field version of PER conditioning with freely moving insects in which animals reach high performance levels. This new protocol allows for testing visual associative learning and memory of different species of bees in a less stressful environment, while controlling for stimulus presentation as well as allowing tests in field conditions on wild populations (Muth et al., 2018).

3.4 Taking into account the social context

The difference between the lab and the natural conditions under which an animal usually learns is sometimes not just physical (Pritchard et al., 2016). While many standard cognitive tests are performed on isolated insects (Giurfa, 2013; Menzel, 2012), key model species such as *Drosophila*, honey bees, and ants live in groups (Sokolowski, 2010; Wilson, 1971). A number of social factors may thus influence what the insects can learn or how they express their behaviour.

Some behaviours are simply not expressed out of the social context. In an attempt to test the hypothesis that division of labour in social insects emerges from inherent inter-individual variation in response thresholds to environmental stimuli (i.e., the response threshold hypothesis, Beshers and Fewell, 2001), the behaviour of individual ants, *Temnothorax rugatulus*, was compared in different social contexts. When isolated, ants show highly variable responses to task-associated stimuli and these responses are not correlated to their behaviour in the colony, suggesting that testing ants outside of a social context alters the meaning or salience of the experimental stimuli and thus the observed behavioural response (Leitner et al., 2019). These social effects on insect cognition can also be developmental. In many gregarious insects, prolonged periods of social isolation can have dramatic developmental consequences and induce long-term behavioural disturbances known as "group effects" (Grassé, 1946). In the German cockroach, *B. germanica*, individuals experimentally reared in isolation during nymphal development show lower exploratory activities, foraging behaviour, and abilities to process social stimuli as adults (Lihoreau et al., 2009). This isolation syndrome can be partially rescued through social contacts artificially provided to cockroaches through mechanical stimulations (Lihoreau and Rivault, 2008; Uzsak and Schal, 2013).

Social interactions can also modulate learning and memory performances. In *D. melanogaster* social interactions facilitates the retrieval of olfactory memory (Chabaud et al., 2009). Flies trained to associate an electric shock to an odour in a T-maze develop two forms of long-lasting memories depending on inter trial intervals: long-term memory (LTM) is formed after spaced conditioning (short intervals), whereas anaesthesia-resistant memory (ARM) is formed after massed conditioning (long intervals) (Margulies et al., 2006). However, flies have higher ARM scores when tested in groups than in isolation (Chabaud et al., 2009). This social effect is independent of the social condition of training, of the experience of other flies in the group and is specific to ARM, indicating that it does not simply result from aggregation dynamics. Presumably, trained flies produce stress signals (e.g., CO_2, Yang et al., 2013) that alarms their conspecifics and enhances their attention or motivation to respond during memory retrieval. In honey bees, *A. mellifera*, social condition during breeding influences olfactory learning. Adults raised in large groups show better learning but no higher memory scores than conspecifics raised in small groups or in complete isolation (Tsvetkov et al., 2019). These differences are correlated with changes in dopamine levels in the brain suggesting that social interactions modulate learning through the biogenic amines.

Being in a group can also dramatically improve the speed and accuracy of decision-making through collective acquisition and processing of information, a phenomena known as "swarm intelligence" (Couzin, 2009; Feinerman and Korman, 2017; Seeley, 2010). In house hunting ants, *T. rugatulus*, collective decisions for the selection of a new nest site emerge from a competition between recruitment efforts by different individuals in the form of tandem running (i.e., an experienced ant drags a naïve ant towards a site) at different sites (Franks et al., 2002). When given a choice between potential nest sites varying in quality (e.g., light intensity), ant colonies can effectively compare a larger option set than individuals (Sasaki and Pratt, 2012) and are less vulnerable to irrational preference shifts induced by decoys (Sasaki and Pratt, 2011). However, this social advantage varies with the difficulty of the task (Sasaki et al., 2013). For a difficult choice (i.e., small differences of light intensity between nests), solitary ants have a relatively high probability of accepting the worst nest, because they rely on quality dependent acceptance probabilities that differ little for similar nests. Colonies do much better because the colony's choice emerges from a competition between recruitment efforts accentuated by a positive feedback loop and a quorum rule (Sasaki et al., 2013). For an easy choice (i.e., large differences in light intensity between nests), acceptance probabilities diverge rapidly with comparison, allowing solitary ants to make the right choice with high probability. Thus in this, case social information only adds little benefit to colonies.

3.5 Comparing species

Rigorous comparisons of the cognitive performances of individuals of the same species or different species that are either closely or distantly related can greatly enhance our understanding of how cognition is shaped by natural selection (Godfrey and Gronenberg, 2019).

Studies of closely related species with known ecologies is a powerful means to tease apart selective forces that drive the evolution of specific cognitive traits. In paper wasps such comparison demonstrates the importance of sociality in the evolution of visual cognition (Sheehan and Tibbetts, 2011). Queens of *Polistes fuscatus* cooperate to found, defend and provision their colony. These wasps live in strict hierarchical societies in which individuals recognise every other colony members based on long-term memories of facial masks (Sheehan and Tibbetts, 2008). By contrast, queens of *P. metricus* found colonies alone and do not require face recognition. When presented images of normal wasp faces, manipulated wasp faces, simple geometric patterns or caterpillars (i.e., the typical prey of these wasps) in an aversive conditioning paradigm in a Y-maze, *P. fuscatus* wasps learn to recognize correctly configured wasp faces more quickly and more accurately than they did with other images, indicating that learning is specific to faces in this species (Sheehan and Tibbetts, 2011). *P. metricus* wasps, however, perform better in pattern and caterpillar discrimination. In terms of gross neuroanatomy, there are no discernible differences between the visual system of *P. fuscatus* and closely related species that do not show face recognition (Gronenberg et al., 2008). It is therefore likely that the neural circuitry used by insects for prey recognition has been co-opted for face recognition, provided minor adjustments. In parasitoid wasps that lay eggs in animal hosts, differences in the spatial distribution of preys seems to determine major differences in olfactory memory dynamics (Smid et al., 2007). *Cotesia glomerata* and *C. rubecula* wasps coexist in the same environments and lay their eggs in caterpillars. These parasitoids are known to learn to associate plant odours with the presence of caterpillars during an oviposition experience on a plant (Lewis and Takasu, 1990). When wasps of both species are trained to oviposit on caterpillars on a neutral host plant and then given a choice between the neutral host plant and their preferred host plant (cabbage), *C. glomerata* show memory formation and consolidation after fewer trials than *C. rubecula* (Smid et al., 2007). This difference in memory dynamics reflects the difference in foraging ecology of the two species: *C. glomerata* exploits gregarious hosts and may benefit to learn from one massed experience on a single encounter with a plant, whereas *C. rubecula* exploit solitary hosts and may use more experiences and more time to evaluate information from many different plants before long-term memory is formed.

Comparing distantly related species can help identify cognitive traits that are conserved or are convergent across insect lineages. For example, in recent years, the finding that many insect taxa are capable of social learning, suggests that this cognitive ability once thought to be unique to vertebrates has evolved several times in insects. Forms of social learning have been demonstrated in insects exhibiting various levels of social organisation, including social bees that can learn new flower preferences (Worden and Papaj, 2005) or foraging techniques (Alem et al., 2016; Loukola et al., 2017), gregarious fruit flies that can learn preferences for oviposition sites (Battesti et al., 2015) or mating partners (Danchin et al., 2018), or even solitary field crickets that learn about the presence of danger (Coolen et al., 2005). This comparative research indicates that insect social learning is not a specific adaptation to social life but may rather involve fundamental associative learning processes used in an asocial context by many species (Leadbeater and Dawson, 2017).

4. Future directions

Perhaps with the exception of navigation research (Collett et al., 2013), ecologically-inspired studies of insect cognition are still relatively scarce, presumably because of the technical difficulties to run controlled experiments with many insect species in their natural environment (e.g., fast moving animals, large spatial scales, large numbers of individuals, etc.). However, several technological advances to quantify cognitive performances on freely moving insects in the field, or in field-realistic virtual environments in the lab, hold considerable promises for the development of an integrative insect cognitive research combining field and lab approaches.

4.1 Automated quantification of cognitive performances

A major limitation of current insect cognition research is that many experiments involve long learning protocols (e.g., training sessions over several days, Perry et al., 2016) with relatively low levels of success (e.g., low learning scores, Avarguès-Weber and Mota, 2016), often resulting in small sample sizes that do not enable for analyses of variability in cognitive traits. Developing a truly comparative analysis of cognitive performances within individuals through time, as well as between individuals, population and species, requires the development of non-invasive automated systems to record behavioural data on large numbers of insects over long periods of times.

This can be achieved by automatizing cognitive protocols. Although many standard protocols have been improved for automatically controlling the presentation of conditioned and unconditioned stimuli to animals (e.g., appetitive olfactory conditioning in bees Giurfa and Sandoz, 2012; aversive visual conditioning in bees, Kirkerud et al., 2013; and aversive olfactory conditioning in *Drosophila*, Jiang et al., 2016), the full automation of experimental setups for conducting cognitive tests is still rare. A recent successful example includes the development of arrays of automated feeders fitted with tracking systems to test flower choices, spatial learning and social interactions in freely flying bees in the lab (Ohashi et al., 2010) and in the field (Lihoreau et al., 2016b). In this approach, a large number of insects can self-train for several consecutive days without the intervention of an experimenter (Pasquaretta et al., 2019).

Advances in automated movement tracking systems now also enable to quantify the behaviour of individual insects, while walking or flying, at various spatial and temporal scales, in the lab and in the field. These include computer vision (e.g., Pérez-Escudero et al., 2014), radio frequency identification (e.g., Stroeymeyt et al., 2018), telemetry (e.g., Kissling et al., 2014), and radar tracking (e.g., Riley et al., 1996). Recent studies have begun to complement these behavioural measures with continuous recording of fitness data, population dynamics and environmental conditions (e.g., Crall et al., 2018). In bee research, for instance, connected hive systems (i.e., bee hives equipped with sets of sensors) can be used for the continuous monitoring of colony traits (e.g., temperature, humidity, weight, sound, traffic of foragers, social interactions, nectar and pollen collection) and environmental conditions (e.g., weather, air pollution) (Bromenshenk et al., 2015). This technological advance has opened the door for a real-time assessment of the link between insect cognitive performance, in-nest behaviour, colony health status, environmental quality and stress exposure (Meikle and Holst, 2015).

High-throughput monitoring of insect behaviour can only be insightful if combined with modern statistical methods to automatically analyse behavioural data. Machine learning and statistical physics are increasingly used to run unsupervised behavioural classification enabling to handle large behavioural datasets, discover features that humans cannot, and develop standard metrics for comparing data across species and labs with only few prior assumptions (Brown and de Bivort, 2018; Egnor and Branson, 2016).

4.2 Virtual reality on freely moving insects

The development of ecologically inspired lab-based experiments in which animals can express naturalistic behaviours under tightly controlled conditions is complementary to field research. While many classical protocols for testing learning and memory in the lab requires to immobilize insects (e.g., Giurfa and Sandoz, 2012), recent progresses in virtual reality techniques now provide unprecedented opportunities to test freely behaving animals in complex ecologically relevant virtual environments, in which cues can be manipulated independently, in ways that would be impossible to achieve in traditional experiments (Stowers et al., 2017). These new systems, in which the natural sensorimotor experience of animals is conserved, facilitate detailed investigations into neural function and behaviour. Virtual reality for freely moving animals has recently been used to elicit naturalistic object responses (e.g., make objects appear, disappear, or even be at apparent distances) in freely walking and flying insects. For instance, flying bumblebees, *Bombus terrestris*, can be trained to search for virtual feeding platform or avoid virtual obstacles displayed on a screen on the ground of a flight arena just as they would with real objects (Frasnelli et al., 2018).

Future developments of technologies to measure neural activities in freely moving insects will considerably advance investigations of brain function underpinning these naturalistic behaviours (Marescotti et al., 2018). Combining these technologies to virtual reality will allow researchers to study the mechanistic basis of behaviour under conditions in which the brain evolved to operate, thereby facilitating the dialogue between field and lab cognitive experiments in ecologically relevant conditions.

5. Concluding remarks

In the 1980s and the 1990s, the intersection of behavioural ecology and experimental psychology led to the new field of cognitive ecology (Dukas, 1998; Dukas and Ratcliffe, 2009) as researchers began to base their hypotheses on the natural history of different species to test predictions about the cognitive abilities of these animals. This approach has been taken with success by scientists working on large-brained animals (Morand-Ferron et al., 2015), but is still little embraced by entomologists. We argue that there is a need for developing an ecologically inspired research on insect cognition to develop a comprehensive understanding of both its mechanisms and evolution.

Beyond behavioural ecologists, such approach will benefit to the broad community of researchers interested in insect cognition. Considering the ecological context of cognition will likely help ethologists to make sense of the rich cognitive repertoire of insects observed in the lab (e.g., What does it mean for an insect colony to have optimistic and pessimistic foragers? Why should insects count?) and perhaps refine mechanistic explanations by asking alternative hypotheses inspired from field observations. Ecological considerations of cognition may also help neurobiologists and experimental psychologists interested in the evolution of cognition to understand the role of environmental factors in shaping animal behaviour and cognitive abilities. As the cognitive abilities of more species are studied in the environment in which such processes evolved, a truly comparative study of cognition becomes at reach. Importantly, the ecologically-inspired approach is complementary with land-based mechanistic explorations. Some of these explorations can also be performed in the field, for instance using selective drugs (Sovik et al., 2016) or inhibitor of gene expression (Cheng et al., 2015) to identify physiological pathways underpinning cognitive operations in conditions where animals may be in better position to fully express their cognitive repertoire.

Ultimately the dialogue between ecologically-based and lab-based approaches will help develop a more integrative understanding of insect cognition with the potential to illuminate broader scale ecological phenomena. For instance, detailed studies of the sublethal effects of pesticides on bee learning and memory (Stanley et al., 2015) combined with field monitoring of population dynamics (e.g., Henry et al., 2012) have provided a robust explanation for colony collapse and the broader declines of pollinator populations (Klein et al., 2017). Growing evidence show that cognitive processes observed in individual organisms result from complex interactions between components at different levels of organisation (gut microbiota, group, parasites and pathogens, environmental stressors) (Couzin, 2009; Cryan and Dinan, 2012). Considering these ecological interactions and their consequences throughout levels of organisations is a major challenge for insect cognition research in the decades to come.

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Appendix 6A: Pesticide dosing must be guided by ecological principles

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Pesticide dosing must be guided by ecological principles

Insecticide use could be reduced if dose recommendations move from a toxicological perspective (how much is needed to kill an insect pest) to an ecological perspective (how much is needed to protect a crop).

Théotime Colin, Coline Monchanin, Mathieu Lihoreau and Andrew B. Barron

Insect populations are in sharp decline, with potentially catastrophic consequences for ecosystem function[1]. This is a complex problem, but the widespread use of pesticides is certainly part of it[2,3]. Debates continue about whether some insecticides should be banned, but where bans have happened different insecticides have been substituted. These may be no less harmful to insects[4]. Agriculture needs to move away from such a heavy reliance on pesticides and adopt an integrated pest management (IPM) approach[5] and a better regulatory process[6], but this change will take time with the IPM approaches proposed to date. Here we argue that we could immediately reduce the amounts of insecticide applied to the environment without necessarily risking loss of crop yields if we rethink pesticide dosing recommendations based on ecological principles. This action alone will not solve the pesticide problem, but will reduce pesticide pollution to win time while we transition to a more sustainable agricultural model.

Since 1990 the amount (measured in weight) of insecticide applied to farmland in the United Kingdom has actually decreased, but this is because modern insecticides are far more toxic than older options[7]. For example, neonicotinoids are 10,000 times more toxic to insects than even dichlorodiphenyltrichloroethane (DDT)[8]! In the United Kingdom, the land area treated with insecticides has increased sharply since 2000, and the frequency and diversity of insecticide treatments has also increased[9]. Therefore, in recent decades the toxicity of the environment in the United Kingdom to insects has increased.

The justifications given by any pesticide supplier for their dose recommendations are seldom clear. The research performed to justify the dose is proprietorial and not in the public sphere[6], which is itself a problem. Usually dose guidelines, when given, refer to a measure of the LD_{50} (the dose lethal to 50%) of the active ingredient(s) of the pesticide against the target, and pesticides are recommended to be used at doses causing a fast death in the targeted pests (Table 1). We argue this kind of effect is not needed to control damage to crops from insect pests.

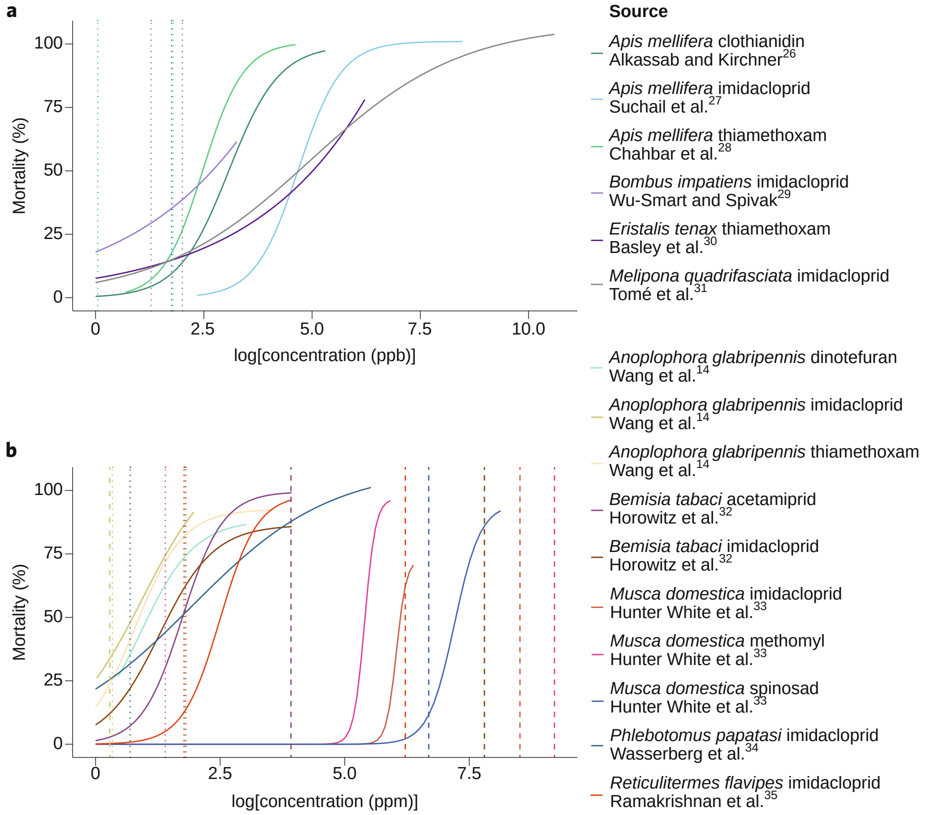

Fig. 1 | Examples of dose–response mortality curves for insects. a,b, Non-target (**a**) and target (**b**) insects[14,26–35]. Concentration is shown on a log scale. Colours represent different insect–insecticide combinations. Sublethal effects with impact on fitness (dotted lines) are often detected at doses well below the concentrations killing 50% or 100% of the population. Recommended doses for target insects (dashed lines, see Supplementary Methods) often exceed concentrations known to cause 100% mortality. Non-target insects are often more sensitive than targeted pests to insecticides[36], so concentrations are shown in parts per billion for non-target insects (**a**) and parts per million for target insects (**b**).

Currently, insecticides are applied at concentrations in the upper range of the mortality dose–response curve (Fig. 1, dashed lines) to deliver a rapid and total elimination of the pest. Mortality dose–response relationships are sigmoid[10]. As a consequence, a dose that yields even 90% mortality of the target organism can be far less than a dose yielding a promise of 100% mortality. For example, in the case of the cotton whitefly (*Bemisia tabaci*, Fig. 1b), a target of 90% mortality would reduce pesticide amount used by 75% from the current dose recommendation.

Table 1 | Research performed to justify the dose is mostly proprietorial, but usage guidelines for popular insecticides promise rapid eradication of pests

Manufacturer	Pesticide	Group	Marketed effect on pest
BASF	Dinotefuran	Neonicotinoid	"Control pyrethroid-resistant pests quickly"
Bayer	Spirotetramat	Keto-enol	"Suppression of woolly apple aphids", figure shows 80–100 "% control"
Bayer	Thiacloprid	Neonicotinoid	"Suppression of woolly apple aphids", figure shows 100% mortality
Corteva	Spinetoram and methoxyfenozide	Spinosyn, diacylhydrazine	"Faster knockdown and consistent control"
Corteva	Spinetoram and sulfoxaflor	Spinosyn, sulfoximine	"For control or suppression of listed pests"
Corteva	Chlorpyrifos and Lambda-cyhalothrin	Organophosphate, pyrethroid	"Fast knockdown and excellent residual control of a broad spectrum of insects"
Syngenta	Emamectin	Avermectin	Figures show 90 "% control" of a moth, and 100% bee mortality three hours after application
Syngenta	Pymetrozine	Pyridine	"Excellent control of aphid and suppression of whitefly populations"
Syngenta	Thiamethoxam	Neonicotinoid	"Death occurs by starvation within 24 hours"
Syngenta	Chlorantraniliprole and Abamectin	Ryanoid, avermectin	"Feeding stops within minutes, larvae start to wriggle then become paralysed, death follows after 48 hours"

See Supplementary Methods for references.

Sublethal effects of insecticides can be sufficient to eliminate economic damage to crops

Target insect pests may not need to be killed outright to prevent economic crop damage. Pesticides also have sublethal effects at low doses (Fig. 1), which can affect the feeding, vision, mobility, orientation, learning and fertility of insects[11]. These sublethal effects are known to severely reduce populations of beneficial insects[12,13], but they have been largely overlooked for the control of targeted pest insects.

There are examples of sublethal effects of commercial insecticides being sufficient to control target pest insects. Trees can be injected with highly concentrated doses of neonicotinoids to protect them against insect herbivores. Even when the insecticide doses injected into the trunks are highly concentrated, only sublethal concentrations end up in the leaves and twigs[14,15] (see purple dashed line in Fig. 1b for levels found in trees). These sublethal doses have nonetheless been shown to provide effective control against the Asian longhorn beetle[15] at doses 17 times less than the 100% lethal dose[14]. Two common insecticides, at sublethal doses, were found to cause the silverleaf whitefly to stop feeding and lay 75% fewer eggs[16], and tefluthrin inhibits feeding in the corn rootworm at a concentration causing only 20% direct mortality[17].

It is not necessary to kill all target insects to eliminate a pest population. The IPM paradigm has argued for decades that it is not necessary to treat a crop when the density of the pest is too low to cause any substantial economic damage[18]. Using additional principles from ecology[19], we further argue that for low density pest populations sublethal insecticide concentrations are probably sufficient to precipitate their extinction. Stochastic dynamics and Allee effects (the effect of population density on mean individual fitness) can be sufficient to drive small populations of pests to extinction[19]. The original description of the Allee effect came from a pest management study. Allee[19] reported that tsetse fly baits did not need to catch 100% of the flies to drive a local population to extinction. Allee effects in low density populations can be due to reduced foraging efficiency, mate finding, reduced predator dilution or from inbreeding[20]. Sublethal effects of pesticides can exacerbate these effects if they damage the capacity of insects to find food, mates or to avoid predators[20].

Insects are probably exposed to mixtures of pesticides[3]. Insecticide residues that accumulate in the environment, and other pesticides such as fungicides, can have additive or synergistic effects, including on beneficial insects[3]. Interactions between pesticides influence how much need be applied to control a pest. Fungicidal treatments are well known for their negative effects on invertebrates[3,21]. For example, two of the most common fungicides affect the development of Colorado potato beetle larvae, and can increase the susceptibility of the pest to imidacloprid[22]. If interactions between pesticides are better understood, it may be possible to reduce the amounts of pesticide used even further by applying principles from community ecology to pesticide application[23].

What pesticide dose is needed to prevent economic damage to a crop?

Few pesticides dosage guidelines are given in terms of economic outcomes for the crop, or assurance of yield. Most studies of insecticide dosing solely focus on lethality to the target insect rather than the economic benefits of the treatments (and lethality is widely used as a marketing argument, see Table 1). In fact, demonstrating economic benefits from insecticide treatments is not straightforward[2]. In the United Kingdom, no clear gains in crop yields have been seen linked to increased neonicotinoid use[2], perhaps because neonicotinoids are often applied prophylactically where no pests are present or because their effects on beneficial insects may negatively affect yields[2]. If we are not seeing benefits from high doses of insecticides, we have an even greater imperative to rethink insecticide doses.

Applying insecticides at the minimum dose needed to reduce the target pests' fitness to zero will also help manage insecticide resistance. This echoes lessons learned from managing antibiotic resistance: manage resistance by tightly controlling and minimizing antibiotic use[24]. By contrast, widespread prophylactic use of long-lived pesticides at high doses is alarmingly common[2], which may explain why insecticide resistance keeps increasing globally[2,25].

Controlling pest resistance on the long term will only be achieved by an IPM approach[25]. This should involve multiple IPM strategies, such as crop rotation, the use of short-lived pesticides and alternating pesticide treatments with different modes of actions[25]. Reducing the dose of pesticide will additionally slow the development of resistance in populations by reducing the extent and intensity of the selection pressure for insecticide resistance.

Rethinking necessary insecticide doses

Arguing to end-users that total eradication of pests is not needed to assure their economic returns will require changing expectations. It will take some serious re-education to reassure growers that a low pesticide dose that may leave some pests visible in a crop has worked to protect the crop. There is also work to be done to assure growers that a lower dose will be sufficient to protect their livelihoods. But a benefit to farmers will be that a lower insecticide dose will be cheaper to apply and cause less damage to beneficial insects such as pollinators.

We do not pretend that this will solve the problem of declining insect populations. Reducing insecticide dosing will not eliminate insecticide residues, but it will reduce the severity and the scale of the problem. This could be done now with no cost to crop yields. It may be a short-term, temporary and partial patch across a far larger and more complex problem, but perhaps this patch can help us win time to change pesticide regulatory processes, shift to an IPM culture and globally redesign the model of food production into a more sustainable form. ❐

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Competing interests

The authors declare no competing interests.

Additional information

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Appendix 6B: Insecticides, arrêtons de risquer l’overdose

Popular science paper published in the Exploreur
https://exploreur.univ-toulouse.fr/insecticides-arretons-de-risquer-loverdose

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Insecticides : arrêtons de risquer l'overdose

12 OCTOBRE 2020 | 4 MINUTES | PARTAGEZ

Pourquoi chercher à tuer systématiquement et instantanément 100% des insectes nuisibles dans toutes les cultures ? Pourquoi continuer à payer si chers des traitements qui impactent autant l'environnement ? De fortes concentrations ne sont pas nécessaires pour protéger les récoltes.

Par Mathieu Lihoreau, chercheur CNRS au Centre de Recherches sur la Cognition Animale (CRCA), **Théotime Colin**, de l'Institut agricole de Sydney, Université de Sydney et **Coline Monchanin**, doctorante de l'université Toulouse III – Paul Sabatier et de l'université Macquarie de Sydney.

La ré-autorisation récente de certains néo-nicotinoïdes pour la production de betteraves par le gouvernement français relance le débat sur l'utilisation des insecticides en général. On observe 25% d'insectes en moins qu'il y a 30 ans. Ce constat est accablant. De par leur action de pollinisateurs, d'ingénieurs des sols, de recycleurs de matières mortes et de prédateurs, les insectes sont essentiels au fonctionnement des écosystèmes et au maintien de la biodiversité.

Les insecticides sont dangereux pour l'environnement

L'utilisation massive d'insecticides dans l'agriculture intensive est souvent mise en cause dans le déclin massif des insectes. Les molécules les plus dangereuses pour l'environnement, aussitôt bannies, sont remplacées par d'autres qui ne s'avèrent souvent pas moins dangereuses. Bannir les insecticides n'est pas une solution en soi. Le monde agricole doit rapidement effectuer une transition vers des méthodes alternatives qui ne menacent plus l'environnement.

La plupart des insecticides sont développés dans les laboratoires et ciblent les insectes qui menacent les cultures. Leur marketing est parfois basé sur leur efficacité à tuer 100% des insectes nuisibles instantanément, et pour obtenir ce résultat, ils sont appliqués à de très fortes doses. Mais une fois passés des laboratoires aux champs, ils se retrouvent dans le sol, l'eau, le nectar et le pollen. Les abeilles, et autres insectes pollinisateurs, en sont les premières victimes collatérales, ce qui affecte forcément le rendement des champs. Ce risque est parfois injustifié, car les insecticides sont souvent appliqués de manière systématique et préventive, sans même vérifier que des insectes nuisibles sont présents dans ou autour des champs.

La transition vers de nouvelles pratiques agricoles est nécessairement lente

Pour stopper le déclin des pollinisateurs et des autres insectes bénéfiques, il est nécessaire de remplacer les insecticides chimiques par d'autres méthodes de contrôle des cultures. Des pièges à phéromones, un meilleur aménagement des zones agricoles, l'amélioration des variétés cultivées, et les auxiliaires des cultures (prédateurs) peuvent remplacer la plupart des insecticides.

Mais la transition vers ces méthodes est lente. Et l'ampleur du déclin des insectes est telle que chaque année les populations diminuent d'environ 10%. Il faut donc agir rapidement. Nous devons repenser l'usage des insecticides pour s'assurer de minimiser la contamination de l'environnement. Cela est déjà possible sans changer drastiquement les méthodes agricoles.

A court terme, il serait efficace de réduire drastiquement les doses

Il n'est en premier lieu pas nécessaire d'utiliser des doses létales si fortement concentrées. On sait, par exemple, que certains insecticides sont toxiques au point que des doses extrêmement faibles, difficiles à détecter, sont suffisantes pour paralyser des insectes ou les empêcher de manger, voir ou de s'orienter correctement. A ces faibles doses, le résultat contre les insectes nuisibles est le même : peu importe si un puceron est vivant, s'il est paralysé il n'affecte plus les tomates. Ceci a été très peu étudié jusqu'à présent, mais notre analyse de la littérature publiée dans *Nature Ecology & Evolution* indique que de nombreux insecticides sont probablement très efficaces à des doses bien plus faibles que celles employées aujourd'hui. A titre d'exemple, une dose 17 fois inférieure aux doses létales recommandées est suffisante pour contrôler efficacement des capricornes invasifs. De la même manière, les termites ingèrent 82 à 93 % moins de cellulose lorsqu'ils sont exposés à une dose 100 fois plus faible que certaines doses d'imidaclopride recommandées, ce qui pourrait suffire à réguler les colonies.

Un autre effet intéressant de l'utilisation de faibles doses d'insecticides est qu'elles affectent souvent la reproduction des insectes. Cela peut être suffisant pour protéger les cultures : quand il y a un très petit nombre de nuisibles dans un très grand champ, les dommages faits aux cultures sont négligeables. Empêcher ces insectes de se reproduire est donc suffisant pour s'assurer qu'il n'y a pas de pertes économiques pour le rendement agricole. Par exemple, la ponte des aleurodes du cotonnier est réduite de moitié lorsqu'ils sont traités avec une dose 490 fois plus faible que certaines doses d'imidaclopride recommandées en traitement par pulvérisateur. En quelques générations cela pourrait être suffisant pour que leurs dommages soient négligeables.

Les champs en agriculture intensive sont aussi souvent traités avec une série d'herbicides et d'antifongiques. Depuis peu, les chercheurs réalisent que ces pesticides affectent souvent aussi les insectes. Par exemple, certains antifongiques perturbent le très commun doryphore de la pomme de terre, et augmentent sa susceptibilité à l'insecticide imidaclopride. Si limiter la prolifération de mauvaises herbes et de champignons tuent les nuisibles, il ne devrait pas être nécessaire d'appliquer en même temps un insecticide.

Enfin, il faut contrôler l'utilisation des insecticides de manière plus stricte pour prévenir l'apparition d'insectes nuisibles résistants. À cause de l'utilisation systématique des insecticides, le nombre d'espèces résistantes pour lesquelles les molécules n'ont plus d'effets est en constante augmentation, ce qui force les chimistes à développer des insecticides de plus en plus toxiques. Cela peut être fait en évitant de sélectionner uniquement les individus résistants en utilisant des doses trop fortes, et en organisant une rotation des cultures et des insecticides.

Protéger les cultures, réduire l'impact sur l'environnement et faire des économies !

Utiliser moins de pesticides diminuera les coûts pour les agriculteurs, ce qui augmentera le rendement net des exploitations. Ces recommandations ne remplacent pas la nécessité de réformer les pratiques agricoles intensives vers des pratiques de luttes biologiques et mécaniques. Des doses même plus faibles risqueront toujours d'affecter la biodiversité. Mais elles peuvent être appliquées rapidement et avoir un effet positif immédiat pour le monde agricole et l'environnement, en attendant un monde meilleur …

CRCA : Centre de Recherches de la Cognition Animale - Centre de Biologie Intégrative (CNRS, Université Toulouse III - Paul Sabatier)

Référence bibliographique

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Appendix 7: Large-scale quantitative comparative analysis of honey bees brain volume and organisation using micro-CT scanning techniques and the Biomedisa segmentation platform

Paper submitted to Nature Communications

Large-scale quantitative comparative analysis of honey bees brain volume and organisation using micro-CT scanning techniques and the Biomedisa segmentation platform

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Abstract

Analysing large numbers of brain samples can reveal minor but statistically and biologically relevant variations addressing major issues related to behaviour, ecology and evolution. However, this approach is severely limited with non-model species due to the lack of easily manageable tools for high throughput imaging and analysis. Here we use non-invasive micro-CT scans of 110 honey bee brains in combination with the online segmentation platform Biomedisa to develop the largest comparative analysis of the inter-individual variability of brain morphologies and lateralization in an insect. We describe significant variations in overall bee brain size and report differences between hives and individuals. In addition, we describe architectural asymmetries of specific

neuropils (antennal lobes and lobulae), which may explain behavioral lateralizations previously reported for olfactory and visual learning. Our automated approach for insect brain image analyses based on deep learning holds considerable premises for the study of brain evolution across a wide range of taxa through large-scale quantitative neuroanatomical comparisons.

Introduction

Three-dimensional (3D) imaging is driving considerable progress in many scientific disciplines, such as medicine[1], fundamental biology[2,3], paleontology[4] and archaeology[5]. In particular, micro-computed tomography (micro-CT) enables non-destructive imaging of fine-scale internal structures of biological objects both in vivo and ex vivo. Ongoing improvements in micro-CT scanning technology are leading to higher resolutions down to micrometers and even sub-micron level, faster acquisition times[6,7], and a variety of user-friendly desktop and floor-standing devices, increasing the demand for accelerated image analysis. Image segmentation is often done manually and remains therefore the most labor-intensive and time-consuming task in 3D image analysis[8–11]. However, the conventional manual segmentation of slices followed by linear interpolation and manual correction often hinders the analysis of a large number of samples.

Comparative neurosciences is a striking example for which such an approach is lacking. Animals, from insects to humans, show a rich diversity of behavioural profiles that are underpinned by cognitive variability[12]. This variability is the basis for adapting to environmental changes. Understanding natural variations in brain sizes, architectures and cognitive performances is therefore a major challenge. However, due to the manual effort, studies of brain size and organisation are usually limited to a few samples and in model species for which brain atlases are available (e.g. rodents[13], primates[14], *Drosophila*[15]). However, low sample size makes it difficult to assess natural variability within populations, identify its biological drivers, and interpret its potential adaptive value which limits our understanding of the link between brain variation and cognitive variation. Only the analysis of a large number of brains could reveal minor, but statistically and biologically relevant, variations in brain sizes and architectures.

New brain imaging techniques, such as micro-CT scans can be easily applied to a wide range of species, including non-model species (e.g. ants[16], wasps[17], beetles[18], bees[19,20]). Automated segmentation approaches have also been developed. Biomedisa, for instance, is a cloud-based and user-friendly online platform that allows fast and accurate image segmentation, drastically reducing the manual effort typically required for segmentation of large volumetric image data[21–24]. It is accessible via a web browser and does not require complex and tedious software configuration or parameter optimization, making it accessible to scientists without the need for substantial computational expertise. Its semi-automatic segmentation method can be used to annotate training data, which can then be used to train the integrated deep neural network for subsequent automated segmentation of large numbers of samples.

These methodological advances for 3D image data acquisition and segmentation have opened the door to a more systematic comparative quantitative analysis that can address ecological and evolutionary questions related to neurosciences and cognition: Are bigger brains more performant[25,26]? What are the influences of social and ecological factors in brain evolution[12]? What's the effect of environmental stressors on brain development and cognition[19]?

Here, we present a large-scale comparative analysis of intraspecific variation in insect brain volumes and architecture, by combining micro-CT imaging with automated brain segmentation performed with Biomedisa[27]. We illustrate the workflow for applying Biomedisa to a large number of micro-CT scans using the honey bee brain as a model example (Fig. 1). Honey bees are equipped with brains smaller than 1 mm^3. Nevertheless, they show extensive inter-individual behavioural variability within and between colonies[28], making them ideal organisms for studying insect cognitive functions and underlying neural substrates[29,30]. This variability is central to division of labour and colony function. Some of this variability has been associated with maturation of specific brain areas due to development and foraging experience[31,32]. However, there is still no clear link between the neuro-architecture of bees and their behavioural variance, as data from comparative analyses are missing. Most studies so far have focused on a small sample size (10+ individual data) and studies at the population level are rare[33].

Since first reports on the bee brain architecture[34], modern imaging techniques have allowed accurate cartographies (e.g. the honey bee[35] and bumblebee[36] brain atlases). However, histological techniques still require invasive dissection, fixing, and staining, which sometimes lead to tissue distortion, desiccation, or damage that impede accurate quantification[37,38]. Microscopy eliminates the need for dissection and staining, but is limited in physical sample size[39] and provide data of lower resolution[40,41] with restricted discrimination of internal tissues[42]. In addition, 3D-reconstruction is often demanding and time-consuming (e.g. 250 hours for the reconstruction of the head of a beetle larva[43]).

We analysed the neuro-architecture of 110 bees from nine hives in two different apiaries using Biomedisa. Using this unprecedented large number of bee brains, we measured the variation in brain size, neuropil volumes, and architecture between bees, and described the lateralization of specific brain neuropils.

Results and discussion

Semi-automatic and automatic segmentation of honey bee brains

We performed micro-CT scanning of 120 forager honey bees (*Apis mellifera,* Buckfast) from two apiaries (Population A: 100 bees from 6 hives, population B: 20 bees from 3 hives) around Toulouse (France). Brain samples were prepared following[20] and CT-scanned at a resolution of 5.4 µm isotropic voxel size (see "Methods"). We analysed six major neuropils (without cell bodies) based on the 3D bee brain atlas[35]: the antennal lobes (AL), the mushroom bodies (MB) (comprising medial and lateral calyx, peduncle and lobe), the central complex (CX) (comprising the central body, the paired noduli and the protocerebral bridge), the medullae (ME) and lobulae (LO) (combined as 'optic lobe' (OL), retinae and laminae were not measured), and other neuropils (OTH) (protocerebral lobes and subesophageal ganglion) (Fig. 2). Among the 120 micro-CT scanned brains, 10 were damaged during manipulation and discarded, providing a total of 110 brains for our analysis. Image dimensions and image spacing varied across subjects and averaged 846 x 727 x 484 isotropic voxels and 0.0054 x 0.0054 x 0.0054 mm^3, respectively. Before processing, each dataset was manually cropped to the area of the neuropils (Fig. 2d) using AVIZO 2019.1 in order to reduce the size of the datasets, resulting in an average size of 451 x 273 x 166 voxels.

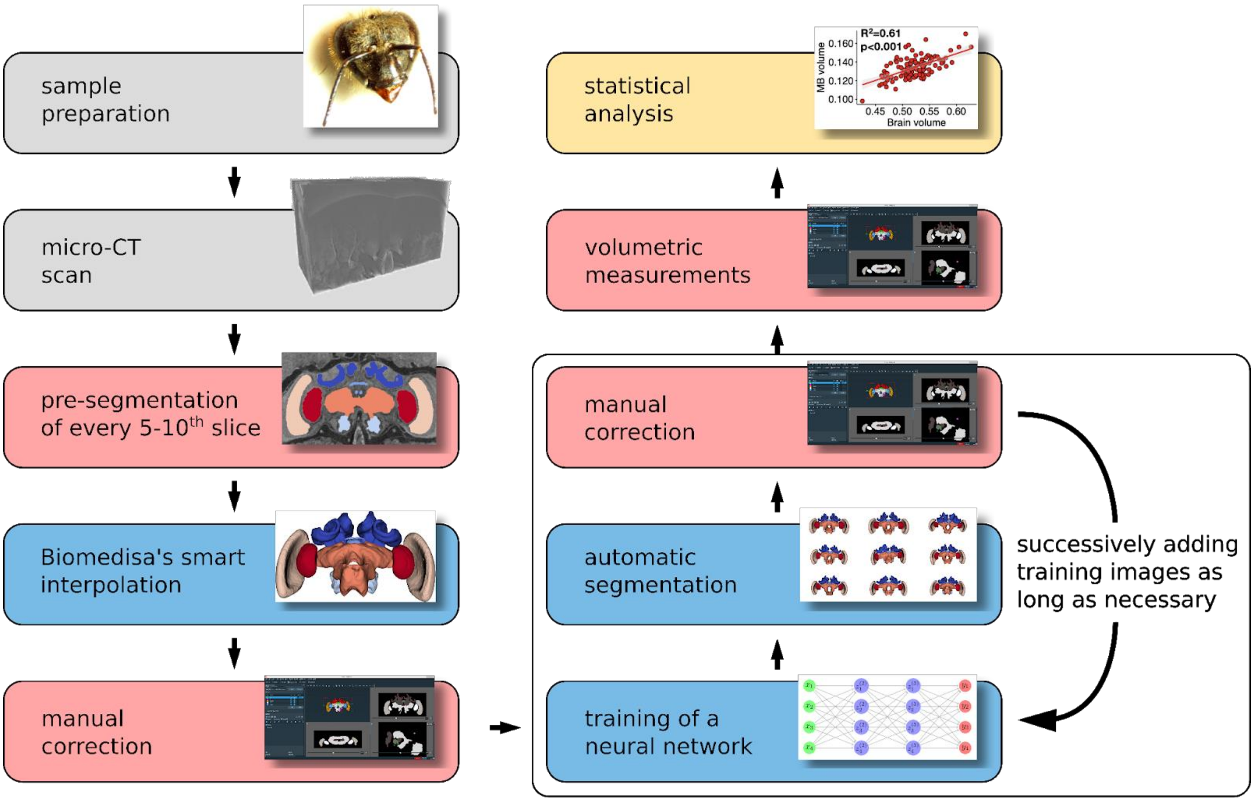

Fig. 1 Flowchart of the steps to perform large-scale quantitative comparative analyses of honey bee brain size and organisation using micro-CT imaging and the Biomedisa segmentation platform. After sample preparation and volume reconstruction (grey boxes), the micro-CT scans are segmented with AVIZO 2019.1 (red boxes) in combination with Biomedisa (blue boxes). Finally, the volumes are measured with AVIZO 2019.1 and statistically analysed with R Studio (yellow box).

The volumetric analysis required isolating the different neuropils from the CT scans by segmentation (Figs 1 & 2). In order to train a deep neural network for automatic segmentation, Biomedisa needs a set of fully segmented volumetric images. To create the initial training data, labels were assigned manually to the six neuropils in every 10th slice and every 5th slice in the interval containing CX (about 20 slices for each brain) within the 3D volume of three brains using AVIZO 2019.1 (Thermo Fisher Scientific, Waltham, USA). Biomedisa’s semi-automatic smart interpolation was then used to segment the remaining volume between the pre-segmented slices. Before interpolation, the image data was slightly smoothed using Biomedisa's denoise function (see “Methods”). Subsequently, outliers (i.e. unconnected voxels or islands) were removed and segmentation errors were corrected manually by an expert using AVIZO 2019.1.

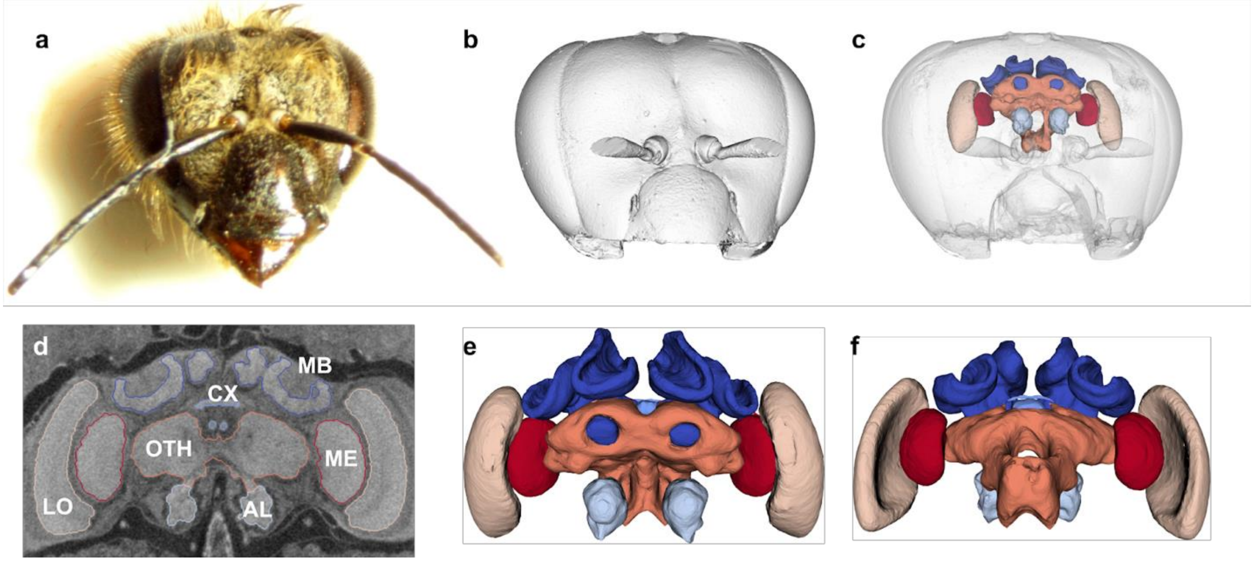

Fig. 2 Surface renderings of an example of CT-scanned honey bee’s head and reconstructed brain neuropils. a Frontal view of the head of a forager bee (hive H4). **b** Surface rendering of the head with the mandibles removed. **c** Overlay of the head and reconstructed neuropils. **d** Frontal cross-section of the tomogram with the segmentation boundaries of the mushroom bodies (MB), central complex (CX), antennal lobes (AL), medullae (ME), lobulae (LO) and other neuropils (OTH). **e** Frontal view of the reconstructed MB (*dark blue*), CX (*sky blue*), AL (*light sky blue*), ME (*red*), LO (*beige*) and OTH (*orange*). **f** Dorsal view of the reconstructed neuropils. B, C, E and F created with ParaView Glance integrated in Biomedisa’s online platform.

The segmentation results were then used to train a deep neural network (see “Methods”) in Biomedisa. Using the trained network, additional bee brains were segmented, corrected and added to the training data. By repeating this process, we successively trained deep neural networks using

3, 7, 12, 18 and 26 volumetric images, respectively, until the segmentation accuracy could no longer be significantly increased (Fig. 1 & Table 1). In each case, we used Biomedisa's default settings to train the network. The training times on 4 NVIDIA Tesla V100s were 1.5, 3.5, 6, 9, and 13 hours, respectively. Finally, we used the network trained on 26 images to automatically segment the remaining 84 micro-CT scans of honey bee brains. The automatic segmentation of the image stacks took an average of 21 seconds using 1 NVIDIA GeForce GTX 1080 Ti. Again, all results were checked and manually corrected by an expert.

Table 1: Average Dice scores of semi-automatic and automatic segmentation compared to manually corrected results. Outliers were automatically removed. Last column: amount of manual correction required.

Dataset	**AL**	**MB**	**ME**	**LO**	**CX**	**OTH**	**Total**	**Error**
AVIZO interpolation	0.926	0.926	0.924	0.921	0.847	0.946	**0.931**	**6.9%**
Semi-automatic-26	0.967	0.949	0.986	0.982	0.856	0.962	**0.967**	**3.3%**
Cross-validation-26	0.954	0.934	0.98	0.975	0.864	0.952	**0.957**	**4.3%**
Uncropped images	0.548	0.777	0.882	0.824	0.323	0.820	**0.819**	**18.1%**
3 training images	0.874	0.868	0.947	0.930	0.592	0.898	**0.907**	**9.3%**
7 training images	0.922	0.921	0.971	0.961	0.808	0.942	**0.946**	**5.4%**
12 training images	0.947	0.936	0.979	0.972	0.858	0.957	**0.959**	**4.1%**
18 training images	0.949	0.943	0.981	0.973	0.876	0.962	**0.963**	**3.7%**
26 training images	0.958	0.951	0.985	0.975	0.894	0.967	**0.969**	**3.1%**
26 training images (biased)	0.979	0.983	0.994	0.988	0.95	0.989	**0.989**	**1.1%**

AVIZO 2019.1 was used for pre-segmentation, correction of segmentation results and measuring the absolute neuropil volumes using the voxel count function. Biomedisa was used for smart interpolation to create the initial training data, training of the neural network and subsequent automatic segmentation. Since Biomedisa supports the AMIRA Mesh File format used by AVIZO and AMIRA, data can be easily transferred between the Biomedisa online platform and AVIZO 2019.1. For each image stack, it took about 5 to 7 minutes to import the data into AVIZO 2019.1, crop to the area of the neuropils (see “Methods”), export the data, upload it to Biomedisa, perform the automatic segmentation, download and import the segmentation result into AVIZO 2019.1, correct the segmentation result manually, and finally measure the volumes. Depending on the quality of the automated segmentation result, the manual correction of the results took 1 to 2 minutes, but much longer if the result was significantly flawed. Typical artifacts of the automatic segmentation are outliers (Supplementary Fig. 2b) which can be easily removed either with the Biomedisa cleaning function or with AVIZO 2019.1. In addition to AMIRA meshes (AM), Biomedisa supports many common data formats (such as DICOM, NifTI, TIFF, NRRD, MHA, MHD) and can therefore also be used in combination with many other segmentation tools.

Evaluation of automatic segmentation accuracy

To evaluate the accuracy of the segmentation results, we considered two metrics (see “Methods”): the Dice similarity coefficient (Dice) and the average symmetric surface distance (ASSD). Both are commonly applied metrics for evaluating performance in biomedical image segmentation challenges[44]. The Dice score quantifies the match of two segmentations and is between 0 and 1, where 0 means no overlap and 1 means a perfect match of the two segmentations. The ASSD is the average 3D Euclidean distance from a point on the surface of one segmentation to the closest point on the surface of the second segmentation, and vice versa. The smaller the ASSD, the closer the segmentations are.

Biomedisa is supposed to be a one-button solution. Therefore, the evaluation was based on the standard configuration. Only Biomedisa's cleanup function was used to automatically remove outliers or islands with a threshold of 0.1. The threshold value was chosen lower than the standard configuration (0.9) in order to avoid a partial deletion of paired neuropiles, since all objects of a label that are smaller than the default value (default is 90% of the size of the largest distinct object) are removed.

For the evaluation of the automatic segmentation, we considered two subsets of the image data, the 26 training images and the 84 remaining test images (see “Semi-automatic and automatic segmentation”). In order to evaluate the deep neural network trained on the 26 training images, we measured the accuracy of the segmentation results of the 84 test images without manual post-processing by comparing them with the ground truth data (i.e. segmentation results revised manually and corrected by an expert). In total a Dice score of 0.989 was achieved (Table 1). In 16% of the 84 test images, the automated segmentation result required little or no manual correction (error less than 0.01%, Supplementary Fig. 2a). In 68%, a slight manual correction was required lasting 1 to 2 minutes (error greater than 0.01% and less than 2%, Supplementary Fig. 2b). Only 7% of the segmentation results were significantly flawed (error greater than 4%, Supplementary Fig. 2c), which was usually caused by a significant deviation of the image data from the training data (e.g. degeneration of the brain) and required extensive manual correction or semi-automatic reconstruction using Biomedisa’s smart interpolation.

Overall, Biomedisa thus enabled high-precision and fast segmentation of the 84 test images. The segmentation results only required a manual correction of 1.1% (Table 1). While using an exclusively manual or semi-automatic segmentation approach, the segmentation would have taken several hours for each image stack. Major corrections were mostly only required for CX (5% total). Its fine structure in combination with low contrast often makes it difficult to detect CX in the CT scan.

Brain volumes varied significantly among bees

We generated and validated a unique dataset of 110 honey brain and neuropil volumes (Fig. 3). This is a much larger dataset than the 7 to 30 brains that few studies used to study honey bee brain size. Two of these studies used CT scan data[45,46]. The others used various microscopy techniques, such as stereo[32,47–49], confocal[35,40,50], or nuclear magnetic resonance[40] (Fig. 3). Our results overall conformed with data obtained with other techniques and are expected to be slightly more accurate since they were obtained from thinner slices (5.4 µm vs. 8[35]-60[51] µm) and by taking measurements on all successive slices, rather than leaving intermediate slices aside and interpolating (e.g.

Cavalieri principle[52]). Such technical discrepancies likely explain under- or over-estimated volumes in other studies compared to ours (Fig. 3).

Note that there are uncertainties in some measurements from other studies, e.g. on precisely which structures were included in the measurement of the CX, or inclusion or not of cell bodies in the total brain volume. Additionally, differences in the estimated structural volumes may arise from biological differences among samples taken from bees of different strains (e.g. European and Africanized honey bees[48]), ages and foraging experiences[32,53].

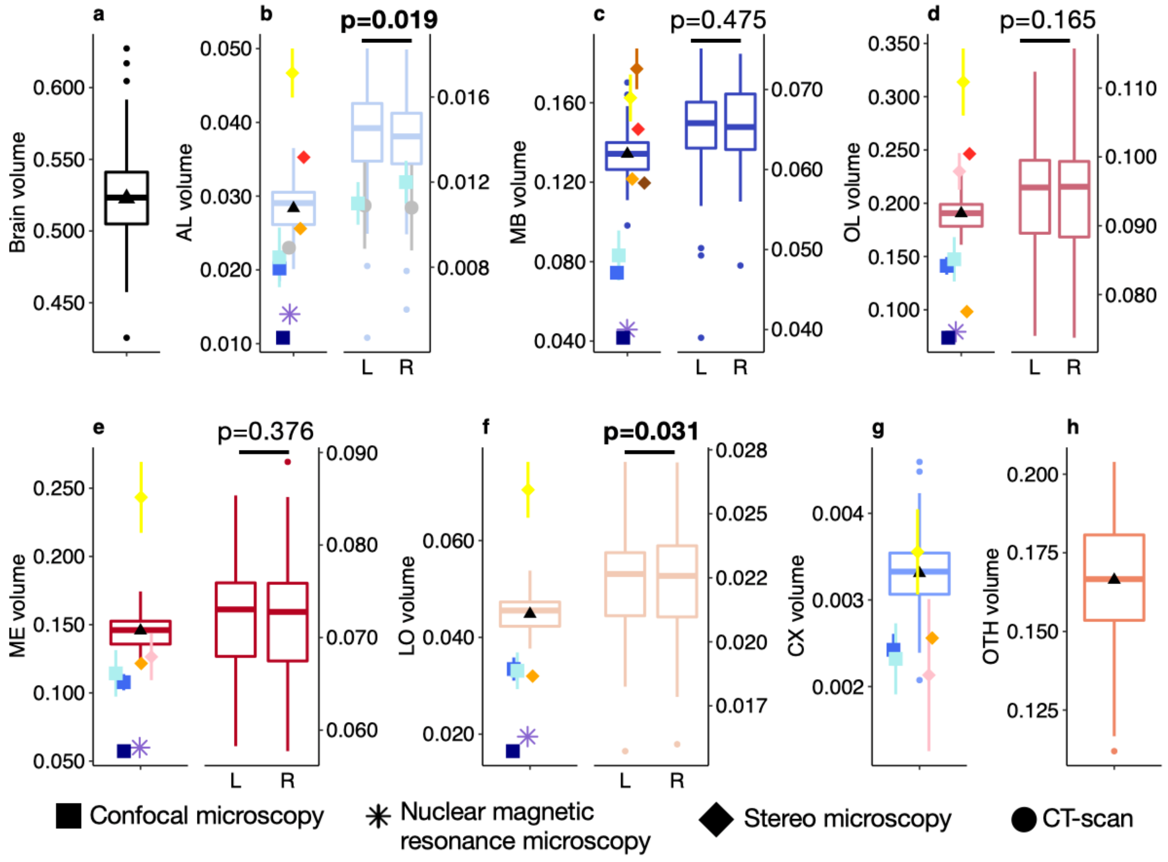

Fig. 3 Variation in brain and neuropils volumes (mm^3) and comparisons between left (L) and right (R) volumes (mm^3) for paired neuropils (N=110 honey bees). a Total brain. **b** Antennal lobes (AL). **c** Mushroom bodies (MB). Data for only 58 honey bees are shown in the left/right comparisons, as the two sides were segmented as a connected object and therefore could not be automatically differentiated from one another. **d** Optic lobes (OL). **e** Medullae (ME). **f** Lobulae (LO). **g** Central complex (CX). **h** Other neuropils (OTH). Boxplots show median volumes

(intermediate line) and quartiles (upper and lower lines). Black triangles display mean volumes. Statistical comparisons (p-values) for the neuropil volume between left and right side were obtained with LMMs, and are displayed in bold when significant. Colored symbols show mean (± s.d. when available) of neuropils volumes described for forager honey bees in other studies: using confocal microscopy (*square*): Brandt et al.[35] (N=20 bees - *turquoise*); Steijven et al.[50] (N=10 - *blue*); Haddad et al.[40] (*navy*); using nuclear magnetic resonance microscopy (*star*): Haddad et al.[40] (N=8 - *purple*); using stereo microscopy (*diamond*): Gowda & Gronenberg[47] (N=7 - *yellow)*; Gronenberg & Couvillon[48] (N=121 European and Africanized honey bees - *pink*); Mares et al.[49] (N=25 - *orange*); Maleszka et al.[51] (N=30 - *dark orange*); Withers et al.[32] (*red*); Durst et al.[53] (N=12 - *brown*); using CT scan (*point*): Greco & Stait-Gardner[45] (N=10 - *grey*). For the total brain volume, comparisons with other studies are not shown because of the cumulative uncertainties of the measurements.

Among our large dataset of 110 brains of forager bees from the same strain, total brain volume (i.e. sum of all measured neuropils) varied by 32% and neuropil volumes (Fig. 3a) by 30-45% (Table 2). We found a strong positive correlation between the absolute volumes of all neuropils and total brain volume (Fig. 4a-g). Most neuropils scaled isometrically with total brain volume (Fig. 4h), with only a lower correlation coefficient for the CX ($R^2=0.35$, $p<0.001$; Fig. 4f). Therefore honey bees with large brains also had larger neuropils. When considering relative volumes, we found a weak negative correlation with total brain volume for MB, OL, ME and CX (Supplementary Fig. 3).

Table 2: Mean (±standard deviation), minimal and maximal volume (mm³) and percentage of volume variation (N=110 bees). For paired neuropils, detailed data for both sides (left/right) are also given. Note that data for only 59 honey bees are shown in the left/right comparisons for MB due to difficulty to separate sides.

Volume (mm³)	Mean ± s.d.	Min	Max	% variation
Brain	0.523±0.036	0.426	0.627	32.16%
AL	0.0284±0.0033	0.0201	0.0365	44.94%
Left	*0.0143±0.0019*	*0.0096*	*0.0183*	*47.62%*
Right	*0.0140±0.0017*	*0.0095*	*0.0128*	*47.62%*
MB	0.134±0.011	0.098	0.170	42.30%
Left	*0.066±0.005*	*0.049*	*0.075*	*34.43%*
Right	*0.066±0.005*	*0.048*	*0.074*	*35.53%*
OL	0.191±0.014	0.161	0.228	29.41%
Left	*0.095±0.007*	*0.080*	*0.112*	*29.24%*
Right	*0.095±0.007*	*0.082*	*0.116*	*29.57%*
ME	0.146±0.011	0.122	0.174	29.95%
Left	*0.073±0.005*	*0.060*	*0.0853*	*29.41%*
Right	*0.073±0.006*	*0.062*	*0.0890*	*30.47%*
LO	0.045±0.004	0.038	0.054	29.94%
Left	*0.0223±0.0018*	*0.0183*	*0.0270*	*32.48%*
Right	*0.0225±0.0019*	*0.0179*	*0.0270*	*33.90%*
CX	0.0033±0.0004	0.0021	0.0046	54.83%
OTH	0.166±0.020	0.112	0.204	45.10%

When assessing the correlations between the absolute neuropil volumes, we found strong correlations between OL, LO and ME volumes (Table 3), as previously reported by Gronenberg & Couvillon[48]. However, in our dataset, AL was positively correlated with OL, ME and LO volumes, but not with MB, while Gronenberg & Couvillon found a negative correlation between AL and OL. We also found a weak correlation of CX and OTH with all the other neuropils. We next compared the relative neuropil volumes to search for relevant allometric relationships (Supplementary Table 1). We again found strong correlations between the relative volumes of OL, LO and ME, consistent with Gronenberg & Couvillon who reported a strong correlation between the relative volumes of ME and LO. We found that the relative volume of AL correlates positively with the relative volumes of OL, ME, and LO, while Gronenberg & Couvillon and studies on other bee species[54] suggest a negative correlation between AL and OL as a possible trade-off between visual and olfactory processing. We also found that the relative OTH volume was negatively correlated with all other neuropils, i.e. the larger the brain, the larger the OTH and the smaller the other neuropils (Supplementary Fig. 3h). Overall, the use of CT imaging in combination with automatic segmentation was found to be efficient for a quick measurement of brain volume and brain structure, and provided values similar to previous studies with other techniques, but based on a much larger sample size.

Table 3: Correlation between neuropils volumes. Pearson correlation coefficient and p-value are given. Strong correlations ($R^2>0.40$) and significant correlations ($p<0.05$) are displayed in bold.

	AL	**MB**	**OL**	**ME**	**LO**	**CX**
MB	0.18 (*p=0.064*)					
OL	**0.70** **(p<0.001)**	0.32 **(p<0.001)**				
ME	**0.68** **(p<0.001)**	0.30 **(p=0.002)**	**0.99** (**p<0.001)**			
LO	**0.70** **(p<0.001)**	0.35 **(p<0.001)**	**0.94** **(p<0.001)**	**0.89** **(p<0.001)**		
CX	0.27 **(p=0.005)**	0.10 (p=0.312)	0.30 **(p=0.001)**	0.28 **(p=0.003)**	0.35 **(p<0.001)**	
OTH	0.38 **(p<0.001)**	0.27 **(p=0.005)**	0.33 **(p<0.001)**	0.31 **(p=0.001)**	0.35 **(p<0.001)**	0.29 **(p=0.003)**

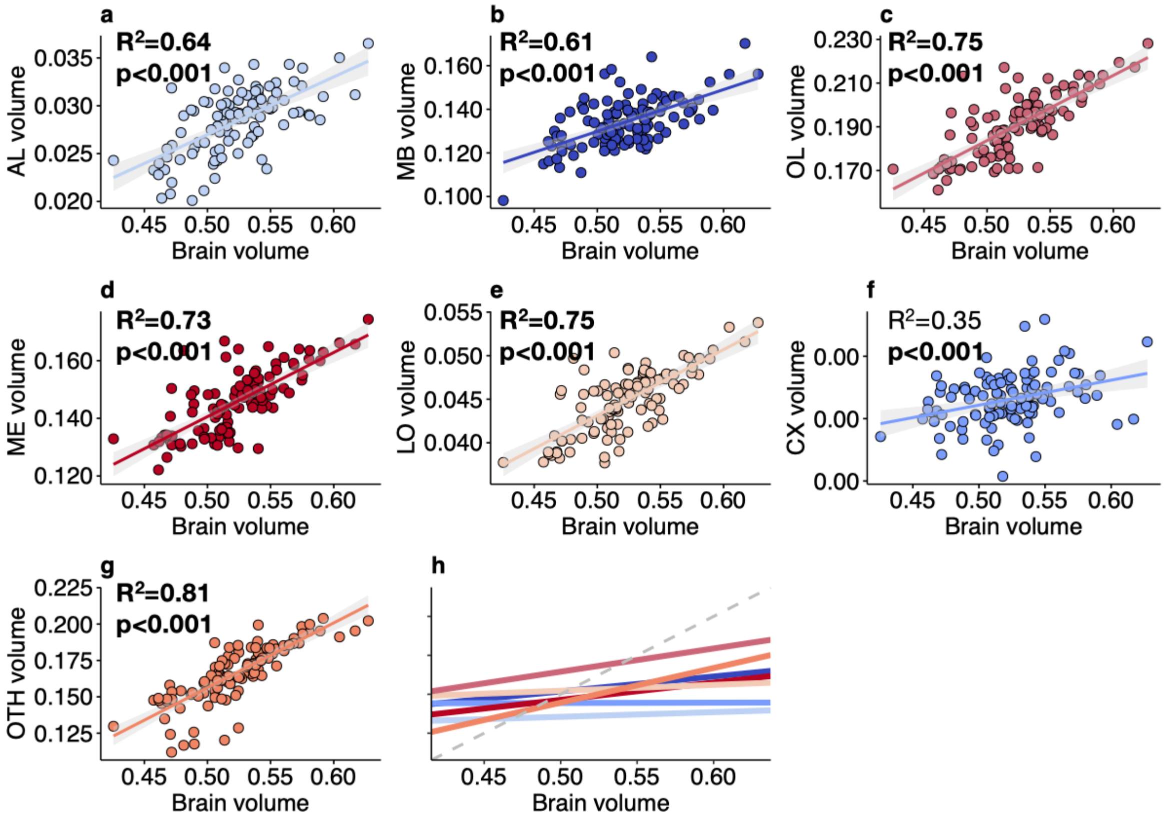

Fig. 4 Correlation between volumes of neuropils and whole brains (mm^3) (N=110 honey bees). a Antennal lobes (AL). **b** Mushroom bodies (MB). **c** Optic lobes (OL). **d** Medullae (ME). **e** Lobulae (LO). **f** Central complex (CX). **g** Other neuropils (OTH). Regression lines displayed with 95% confidence intervals. Pearson correlation coefficient (R^2) and p-value are given. Strong correlations (R^2>0.40) and significant correlations ($p<0.05$) are displayed in bold. **h** Linear correlations for the different neuropils (y-axis not given: differs for each neuropil). Grey broken line indicates true isometric correlation (slope=1).

Inter-individual variance was similar within and between colonies

We then explored inter-individual variability between and within colonies. Social bee colonies can be considered as superorganisms characterised by a division of labour among workers that is partly determined by the genetics, the age and the morphology of individuals[55]. In honey bees, MB volume increases with age[32] and foraging experience[53,56], and AL volume changes with behaviour and social role[57]. These changes in brain area volumes are believed to support division of labour, for instance by providing adult bees with higher abilities to learn spatial information and floral cues when foraging[53]. While some studies have investigated variations in brain composition between species[47] or variations due to treatments (e.g. pesticides[19], nutrition[50]), none have studied the magnitude of intra- and inter-colony variability in brain size variation.

Using our uniquely large dataset, we did not find significant inter-colony variations in brain volume (Fig. 5; Table 2), which varied by 32%. While ME (ANOVA: p=0.267; Fig. 5e) and LO (ANOVA: p=0.282; Fig. 5f) volumes were similar between the nine hives, the resulting OL volume (combination of ME and LO) differed (ANOVA: p=0.004; Fig. 5d). Bees from the nine colonies exhibited an overall similar brain volume (ANOVA: p=0.335; Fig. 5a), and neuropil volumes for AL (ANOVA: p=0.179; Fig. 5b), MB (ANOVA: p=0.282; Fig. 5c), CX (ANOVA: p=0.532; Fig. 5g) and OTH (ANOVA: p=0.635; Fig. 5h).

We also found relatively stable (18%) intra-colony variability in brain volumes (Table 4). CX and OTH volumes varied strongly between individuals of the same colony (resp. 11-51% and 11-43%, depending on the colony). Surprisingly, however, we have not observed any significant inter-individual variations in MB volumes or relative MB volumes that are known to increase by 15% in size with age and foraging experience[32].

Overall, we found only little variability in brain size and neuropil volumes within and between hives. This might be due to the fact that all of the bees used in this study belonged to the same strain (Buckfast) and behavioural caste (foragers).

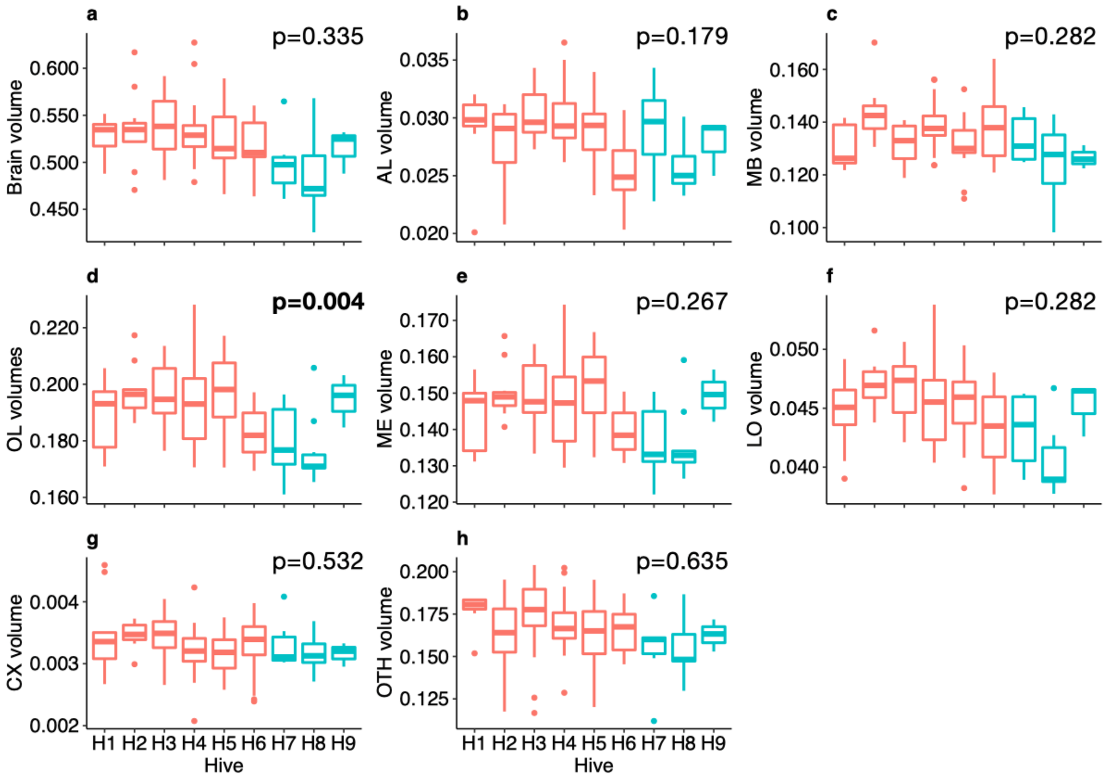

Figure 5: Variation in brain and neuropils volumes (mm^3) between hives (population A - *red*, N=6 hives; population B - *blue*, N=3 hives). a Total brain. **b** Antennal lobes (AL). **c** Mushroom bodies (MB). **d** Optic lobes (OL). **e** Medullae (ME). **f** Lobulae (LO). **g** Central complex (CX). **h** Other neuropils (OTH). Statistical comparisons (p-values) for the neuropil volume between hives were obtained from the F-test following LMMs and are displayed in bold when significant.

Table 4: Inter-individual variation in brain and neuropils volumes (%) within hives.

Population	**Hive**	**Number of bees**	**Brain volume**	**AL**	**MB**	**OL**	**CX**	**OTH**
A	H1	9	11.53%	37.23%	14.01%	16.9%	41.86%	17.49%
A	H2	11	23.72%	33.34%	23.26%	14.28%	19.75%	39.82%
A	H3	18	18.68%	20.51%	15.57%	17.36%	34.32%	42.79%
A	H4	19	23.64%	28.32%	20.86%	25.21%	50.98%	36.41%
A	H5	17	20.89%	31.38%	27.23%	21.44%	31.17%	38.44%
A	H6	16	17.23%	33.69%	26.29%	14.08%	39.91%	22.33%
B	H7	6	18.33%	33.6%	14.30%	17.98%	25.96%	39.70%
B	H8	11	25.09%	22.68%	31.30%	19.61%	26.45%	30.49%
B	H9	3	8.25%	15.00%	6.69%	9.09%	11.30%	11.02%

AL and LO show right/left asymmetries

Our whole brain dataset enabled the analysis of potential size asymmetries in paired neuropils on either side of the brain. Behavioural studies have shown honey bees to have lateralized learning abilities[58], but to date no study has reported potential asymmetries in brain size that could reveal neural adaptations that support the behavioural asymmetries observed in honey bees. The lack of data linking bee behavior and neural adaptations is likely due to the need to measure a variety of structures in order to identify possible significant and reproducible variations. However, the analysis of a large number of samples was prevented by the technical limits of the approaches used so far[57,59]. To answer this question, we overcome these limitations by analyzing a large and homogeneous biological data set using a high throughput approach.

We compared the volumes of right- and left-side paired neuropiles (i.e. AL, MB, ME, LO and OL) from our dataset (Table 2). The left AL (Fig. 3b) was significantly larger than the right AL (LMM: p=0.019), with 65% (71 of 108) of the bees having larger left AL. On the contrary, the right LO was consistently larger than the left (LMM: p=0.031; Fig. 3f), with 58% (63 of 108) of the bees having bigger right LO. These asymmetries contrast with the observation that left and right MB (Fig. 3c), LO (Fig. 3d), and ME (Fig. 3e) had similar volumes. This pattern was constant between bee colonies populations, since the interaction term between side and population remained non-significant for all paired neuropils (LMM: p>0.05)

Larger left ALs and larger right OLs in a majority of bees are consistent with previously reported behavioural lateralizations[60,61]. Bees can be trained to associate visual cues presented only to one eye with a food reward, and they perform better using their right eye[60]. Since the OL processes visual information[62], a larger OL might provide greater computational capacity on the right side and thus explain the behavioural data. Alternatively, if visual learning preferentially solicits the right visual pathway, a larger right OL might be a consequence of more pronounced activity-driven structural changes (e.g. synapse production). This latter hypothesis might also apply to the AL asymmetry as it would be consistent with lateralization in olfactory learning and memory[58]. Honey bees can be trained to differentially learn odour cues with the left and with the right antenna[63]. Bees perform better at learning rewarded odorants[61], and at remembering them at short delays[64] when using their right antenna. This is consistent with the lateralization of neural olfactory coding in the AL[65]: neural representations of odours are more separated in the right AL and bees are better in segregating an odour when using their right antenna. However, olfactory memories are better recalled on the long term (at least 24 h after training) when presenting the stimuli to the left antenna. Since antennal olfactory sensory neurons project unilaterally to the brain, this suggests that somehow the long-term memory trace is more accessible from the left hemisphere. Since such trace is associated with increased glomerular volume in the A[66], a larger left AL volume in many bees might reflect the storage of past olfactory experiences.

Conclusion

We used automated segmentation of micro-CT scans to carry out the largest quantitative comparative study to date of the variation in brain size in an insect. Our approach proves to be cost-effective and time-efficient for describing important composite structures of soft tissues such as the insect brain.The Biomedisa segmentation platform is extremely user-friendly and does not require computational expertise. There is no need to install a software or meet the hardware requirements when using Biomedisa online. Access to neural networks on Biomedisa's online segmentation platform makes it easier for researchers with no deep learning experience, while advanced users can modify the source code and optimize the models for their specific use cases. This approach will facilitate large-scale comparative analyses by biologists paving the way for high throughput comparative brain neuroanatomy research to address questions about the ecology and evolution of animal nervous systems. Beyond micro-CT scans of miniature brains, Biomedisa is suitable for many other types of volumetric image data, e.g. from confocal laser scanning microscopy (CLSM), focused ion beam scanning electron microscopy (FIB-SEM), histological imaging, magnetic resonance imaging (MRI), or ultrasound. We therefore anticipate our automatic segmentation approach can be used for a wide range of applications, from tumor and organ segmentation from medical images that enable patient-specific surgical planning and decision making, over segmentation of organoids and embryos from biological images that decode genotype-phenotype correlations or describe the morphological development, to other disciplines such as geology or materials science.

Methods

Sample preparation and CT scan. We performed micro-computed tomography scanning of 120 honey bees (*Apis mellifera*, Buckfast) collected from 9 hives located in two apiaries around Toulouse, France, in August 2020 (Population A: 100 bees from 6 hives, population B: 20 bees from 3 hives). Foragers returning to the colony were collected at the hive entrance, frozen and stored at -18°C. We stained the samples with phosphotungstic acid[20]. This staining agent is non-hazardous and does not lead to overstaining of the soft tissues, in contrast to other compounds previously used in CT scan studies of insect brains such as osmium-tetroxide[46] or iodine[67] For staining bee brains, we removed the front cuticle, just above the mandibles, and submerged the heads in phosphotungstic acid (5% in a 70/30% ethanol/water solution) for 15 days at ambient temperature[20]. Two heads were scanned at the same time (as both would fit in the field of view of the flat-panel imager) using a micro CT station EasyTom 150/RX Solutions (Montpellier Ressources Imagerie, France), with the following parameters: resolution of 5.4 µm isotropic voxel size, 15 minutes measurement times. Raw data for each brain scan was reconstructed using X-Act software (RX Solutions, Chavanod, France). The reconstructed volume was then re-oriented to the same (frontal) plane-of-view and each brain was re-sliced into a new series of two-dimensional images.

Statistical analysis

We analysed the parameters obtained from the reconstructed neuropils using R Studio v.1.2.5033[68]. We assessed correlations between brain neuropil volumes using the *rcorr* function from the *Hmisc* package[69]. To analyse the inter-colonial variations of brain volume, we conducted linear mixed models (LMMs) (*lme4* package[70]), with hive as fixed effect and population as random factor. LMMs were followed by F-tests to test the significance of fixed categorical variables using the *anova* function in the *car* package[71]. To assess the potential lateralization of paired neuropils, we conducted LMMs, with side as fixed effect, date as random factor and individual as repeated measure.

Artificial neural network architecture

Biomedisa uses Keras with TensorFlow backend. A patch-based approach is used in which 3D patches of the volumetric images are used instead of the entire CT-scan. The patches serve as input for a 3D U-Net and have a size of patches 646464 voxels. An overlapping of the patches is achieved by a stride size of e.g. 32 pixels that can be changed in Biomedisa. The network architecture of the deep neural network follows the typical architecture of a 3D U-Net[72]. It consists of a contracting and an expansive part with a repeated application of two 333 convolutions, each followed by batch normalization and a rectified linear unit (ReLU) activation layer. Each contracting block is followed by a 222 max pooling operation with stride 2 for downsampling. At each downsampling step, the number of feature channels is doubled, starting with 32 channels. Every step in the expansive part consists of an upsampling of the feature map and a concatenation with the corresponding cropped feature map from the contracting path, followed by two 333 convolutions, with each followed by batch normalization and a ReLU activation layer. At the final layer, a 111 convolution is used to map each feature vector to the desired number of classes. To train the network, stochastic gradient descent is used with a learning rate of 0.01, decay of 110^{-6}, momentum of 0.9, enabled Nesterov momentum, 200 training epochs, and a batch size of 24. All images are scaled to have the same mean and standard deviation.

Evaluation

To evaluate the initial creation of the first three training images, we compared the commonly used linear interpolation from AVIZO 2019.1 and the smart interpolation from Biomedisa (that was used to create the initial training data) for the 26 training images. For intervals including CX we used every 5th slice of the ground truth data, otherwise every 10th slice for each method. When using the same pre-segmented slices, Biomedisa's smart interpolation, which also takes the underlying image data into account, achieves a higher segmentation accuracy than the purely morphological interpolation from AVIZO 2019.1 (Table 1). This significantly reduces the manual work required to create the training data for a neural network.

To test the performance of the automatic segmentation, we evaluated the accuracy of the trained networks based on a successively increasing number of training images (3, 7, 12, 18 and 26). While increasing the number of training images increases the accuracy of the automatic segmentation and thus reduces the required manual post-processing, the gain in accuracy gradually slows down with an increasing number of training images (Table 1, Supplementary Fig. 1). Therefore, one has to weigh the gain in accuracy against the additional effort. Using our unique dataset, 12 to 20 training images were sufficient for adequate automatic segmentation.

For each number of training images, we used several subsets of the 26 training images in order to cover them all and calculated the mean accuracy (e.g. we used 9 subsets of 3 training images each, one image being used twice).

The last network of this sequence (26 training images) was trained and evaluated on the same images as the network with which the 84 test images were originally segmented. However, the accuracies of the two networks are significantly different (0.989 for “84 test images” and 0.969 for “26 training images”, Table 1). Since the training process is not a deterministic optimization, the results will always be slightly different (especially at the segmentation boundaries) and the small differences add up. As a result, the network used to generate the test data performs better because the results and ground truth data are closely related.

We also used a 5-fold cross-validation in which the 26 training images were split into 5 test groups. For each test group, a convolutional neural network was trained on all images excluding the images of the test group. The performance of each trained neural network was then evaluated by calculating the accuracy of the segmentation results of the images belonging to the test group. The accuracy was measured using the Dice score between the segmentation result and the expert-generated ground truth segmentation. On average, we achieved Dice scores of 0.954 for AL, 0.934 for MB, 0.980 for ME, 0.975 for LO, 0.864 for CX and 0.952 for OTH. Overall, the accuracy was 0.957, which equates to an error of 4.3% (Table 1).

The network was also trained and evaluated using the uncropped image data (i.e. full size images). Cropping the image data to the area of the neuropils significantly increased the segmentation accuracy of the neural network from a total Dice score of 0.915 for the uncropped image data to 0.969 for the cropped image data (Table 1). By default, Biomedisa scales each axis of the image data to a size of 256 pixels to facilitate processing. Thus, the cropped image data (average size of 451 x 273 x 166 voxels) were on average scaled by a factor of 0.57 along the x-axis, only marginal along the y-axis and by a factor of 1.54 along the z-axis. Without cropping the image data (average size of 846 x 727 x 484 voxels), a large amount of redundant information is added to the training data and the loss of resolution is greater compared to the cropped image data due to a greater reduction in size.

Evaluation metrics

For two segmentations X and X' consisting of n labels, the Dice similarity coefficient (Dice) is defined as

$$\mathrm{Dice} = \frac{2\sum_{i=1}^{n} |X_i \cap X_i'|}{|X| + |X'|},$$

where $|X|$ and $|X'|$ are the total number of voxels of each segmentation, respectively, and X_i is the subset of voxels of X with label i.

For the surfaces S_i and S_i' of the two segmentations, the average symmetric surface distance (ASSD) is defined as

$$\mathrm{ASSD} = \frac{1}{|S| + |S'|} \sum_{i=1}^{n} \left(\sum_{p \in S_i} d(p, S_i') + \sum_{p' \in S_i'} d(p', S_i) \right) ,$$

where

$$d(p, S_i') = \min_{p' \in S_i'} \|p - p'\|_2$$

is the Euclidean distance from a point p on the surface S_i of label i to the closest point p' on the corresponding surface S_i' of the second segmentation.

Denoising image data

Volumetric images are denoised using an arithmetic mean filter

$$A(x, y, z) = \frac{1}{|M|} \left(\sum_{i,j,k \in M} I(i, j, k) \right)$$

with a filter mask *M* of 333 voxels.

Data availability

Data will be made available from the corresponding authors upon reasonable request.

Code availability

The source code is freely available as part of the open-source software Biomedisa. It was developed and tested for Ubuntu 18.04 LTS and Windows 10. Any common browser can be used as an interface. Biomedisa can be downloaded at https://github.com/biomedisa/biomedisa and installed according to the installation instructions.

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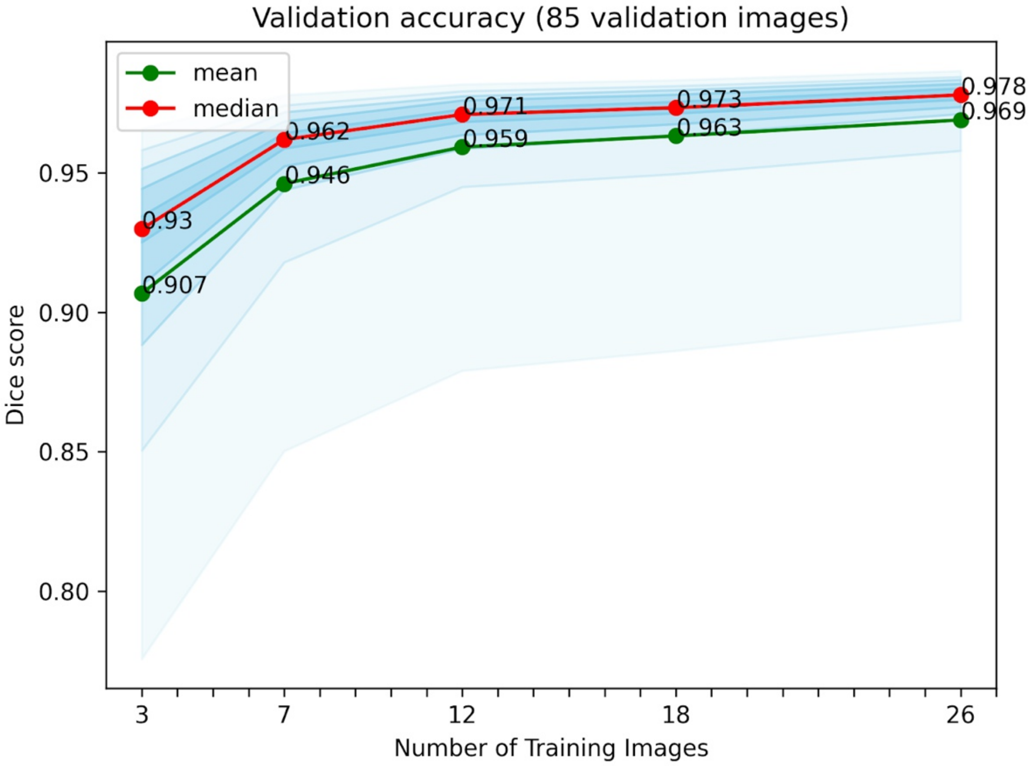
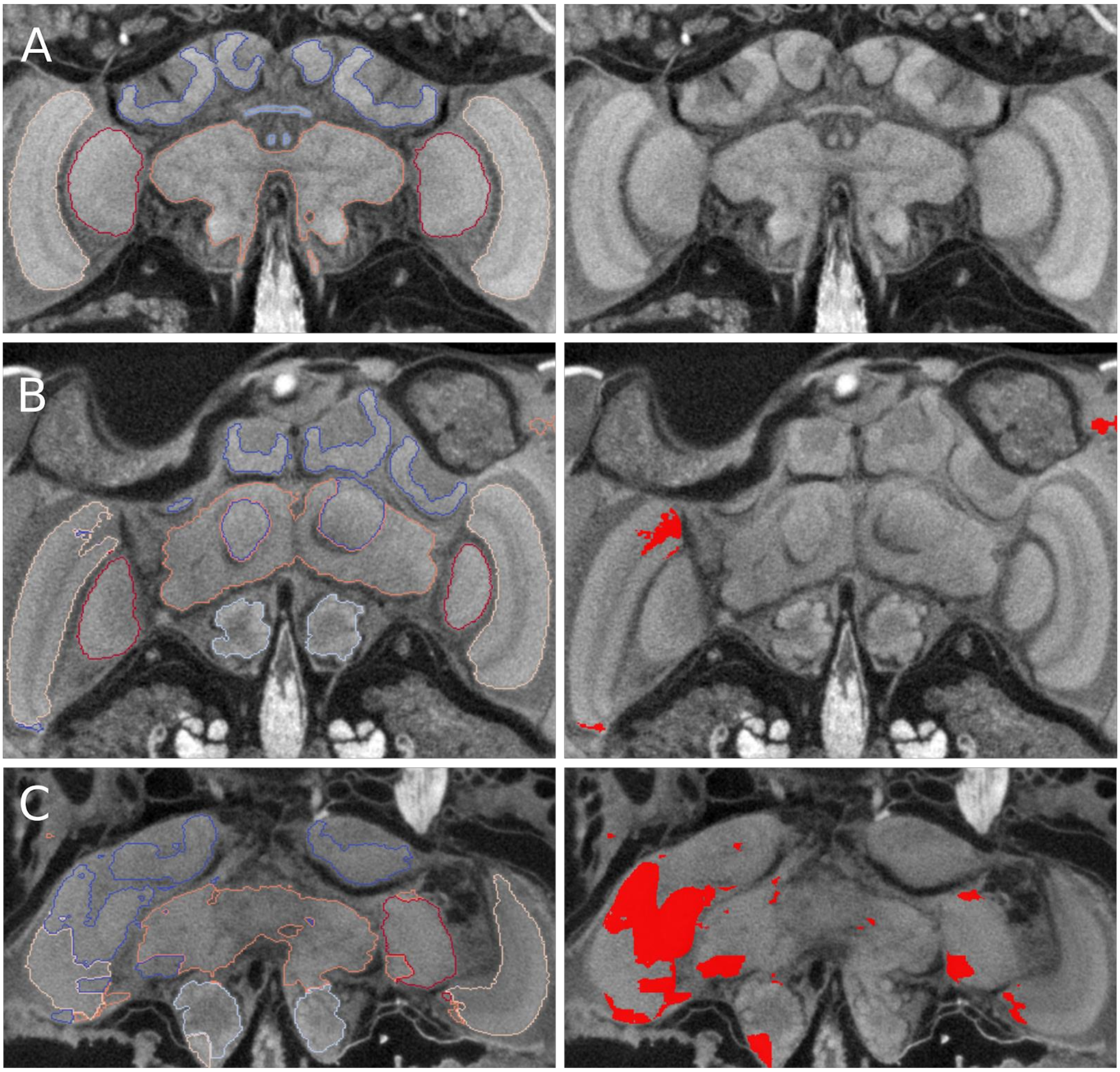
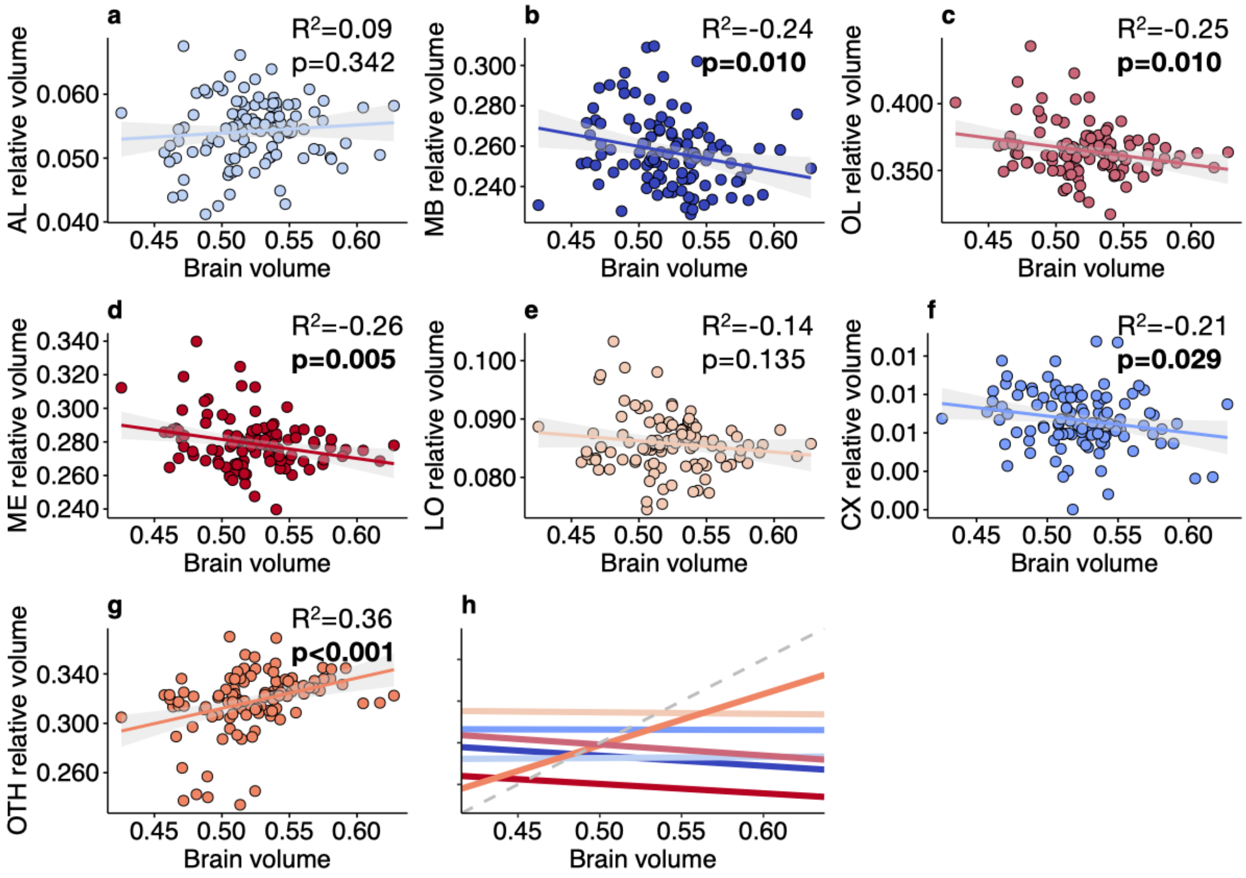
Supporting materials



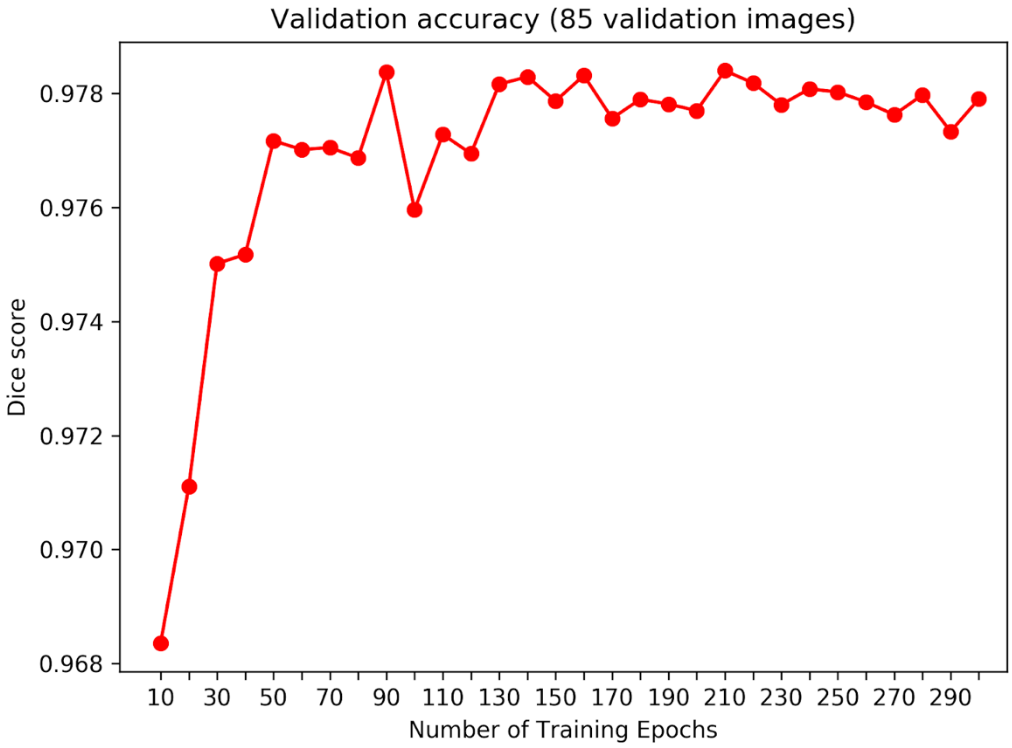
Supplementary Fig. 1: Average and median Dice scores of automatic segmentation results with respect to the number of training images. Reference data are 85 manually corrected results obtained from a deep neural network trained on the set of 26 training images. Outliers were automatically removed.



Supplementary Fig. 2: Segmentation results (left) and segmentation errors (right) of Biomedisa’s deep neural network trained on 26 images. A) Correct segmentation without errors. **B)** Partly flawed segmentation result with a typical outlier on the right edge of the image (segmentation accuracy: LO 97.6%, total 99.1%). **C)** Significantly flawed segmentation result (total segmentation accuracy 86.8%).



Supplementary Fig. 3: Correlation between relative neuropils volumes and brain volumes (mm^3) (N=120 honey bees). a Antennal lobes relative volume. **b** Mushroom bodies relative volume. **c** Optic lobes relative volume. **d** Medullae relative volume. **e** Lobulae relative volume. **f** Central complex relative volume. **g** Other neuropils relative volume. Regression lines displayed with 95% confidence intervals. Pearson correlation coefficient (R^2) and p-value are given. Strong correlations (R^2>0.40) and significant correlations ($p<0.05$) are displayed in bold. **h** Linear correlations for the different neuropils relative volume (y-axis not given: differs for each neuropil). Grey broken line indicates true isometric correlation (slope=1).



Supplementary Fig. 4: Validation accuracy depending on the number of training epochs.

Supplementary Table 1: Correlation between the relative volumes of neuropils. Pearson correlation coefficient and p-value are given. Strong correlations (R^2>0.40) and significant correlations (p<0.05) are displayed in bold.

	AL	**MB**	**OL**	**ME**	**LO**	**CX**
MB	-0.37 **(p<0.001)**					
OL	**0.40 (p<0.001)**	-0.18 *(p=0.067)*				
ME	**0.38 (p<0.001)**	-0.18 *(p=0.062)*	**0.99 (p<0.001)**			
LO	**0.41 (p<0.001)**	-0.13 (p=0.167)	**0.86 (p<0.001)**	**0.76 (p<0.001)**		
CX	0.03 (p=0.720)	-0.07 (p=0.462)	0.13 (p=0.173)	0.11 (p=0.260)	0.18 *(p=0.060)*	
OTH	-0.25 **(p<0.001)**	**-0.51 (p<0.001)**	**-0.74 (p<0.001)**	**-0.72 (p=0.001)**	**-0.66 (p<0.001)**	-0.09 (p=0.360)

Appendix 8: Considering phenotypic variance in studies of environmental stressors can reveal potential for population resilience

Paper submitted to the Journal of Applied Ecology

Considering phenotypic variance in studies of environmental stressors can reveal potential for population resilience

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Abstract

1. Environmental stressors have sublethal effects on animals, often affecting the mean of phenotypic traits within a population. However, the effects of stressors on phenotypic variance among individuals are much less understood. Since variance is the basis for adaptation, any effect of a stressor on phenotypic variance may have important implication for population resilience to stress.
2. We explored this possibility by analysing raw datasets from 24 studies on bees (6,913 bees) in which individuals were first exposed to stressors and then tested for various cognitive tasks.
3. While all stressor types decreased the mean cognitive performance of bees, their effect on the variance was more complex. Focusing on 15 pesticide studies, we showed that the level and the mode of exposure to stressors were critical to consider their effect on the variance. Variance decreased following a chronic exposure whereas it was not affected following an acute exposure. For both modes of exposure, the mean and the variance decreased with

increasing pesticide doses. Acute exposure to low doses therefore seems less damaging at the population level.

4. *Policy implications.* Current guidelines for the authorization of plant protection products (PPPs) on the European market prioritize acute toxicity assessments over chronic toxicity assessments on non-target organisms. By overlooking the consequences of a chronic exposure to PPPs, regulatory authorities may register new products that are harmful to bee populations. Our findings thus call for more research on stress-induced phenotypic variance and its incorporation to policy guidelines to help identifying the levels and mode of exposure which the population can cope with.

Keywords: *Apis cerana*, *Apis mellifera*, *Bombus impatiens*, *Bombus terrestris*, cognition, inter-individual variability, pesticides, pollinators

Introduction

Human activities have led to a dramatic increase in the extinction rates of animal species in the past 500 years (Barnosky et al. 2011; Dirzo et al. 2014; Wagner 2020). Human-induced stressors have partly been identified and act synergistically (Brook et al. 2008; Dirzo et al. 2014; Sánchez-Bayo and Wyckhuys 2019; Siviter et al. 2021). These include, but are not restricted to, pollution, habitat loss, and the introduction of invasive species. These stressors add to the ones naturally encountered by animals in their environment such as predation, pathogens, and parasites. Given the raising number of species threatened with extinction (Barnosky et al. 2011; Sánchez-Bayo and Wyckhuys 2019), it is urgent to understand how animal populations can cope with stressors to orient policies towards an efficient regulation of human activities affecting biodiversity.

Measuring the impact of stressors on animal populations is difficult to implement in the field because of the many confounding factors, and such experiments typically require important space and facilities. Instead, many studies focused on the effects of stressors on individual phenotypic traits (e.g. cognitive performance) (Badyaev 2005; Klein et al. 2017). Yet, the relevance of such risk assessment methods compared to field population-level studies has been questioned as mismatching conclusions often emerged from the two approaches (Thompson and Maus 2007; Henry et al. 2015). Even though stressors may affect individual phenotypic traits in the lab, life in a natural, sensory and socially enriched environment has been shown to buffer these effects (Cabirol et al. 2017; Lambert et al. 2016; Henry et al. 2015; Wright and Conrad 2008).

Here we argue that studying how stressors affect the variance of phenotypic traits, alongside their effect on the mean, will provide important information about the severity of stressors on animal populations and may reconcile results obtained in laboratory and field studies. It is well recognized that animals exhibit considerable variability in behavioural and physiological responses to stressors (Ebner and Singewald 2017; Mazza et al. 2019). Some individuals better cope with a stressor than others. Yet, an overlooked consequence of the stress-response is the variance of the phenotypic traits affected by the stressor (Nakagawa et al. 2015). If the variance is low in the population following stressor exposure, all individuals may suffer the consequences associated with the altered phenotype. On the contrary, if the variance remains high in the population, even though the mean is affected, some individuals may still exhibit an adaptive phenotype. In some cases, stressors may even increase phenotypic variance, a phenomenon suggested to promote the evolutionary diversification of species (Badyaev 2005). Stress-induced variation should therefore be considered when assessing the resilience of a population to a particular stressor.

To illustrate our point, we analysed the effect of human-induced stressors on the mean and variance of cognitive performances in bees. We focused on social bee species from the genera *Bombus* and *Apis*, as they are key pollinators worldwide known to be affected by multiple natural and human-induced stressors, and in particular pesticides (Potts et al. 2010; Goulson et al. 2015). These species live in colonies with a division of labour and are therefore characterized by a great inter-individual behavioural and cognitive variability (Jeanson and Weidenmüller 2014). Bee foragers, in particular, have evolved an impressively rich cognitive repertoire enabling them to locate and recognise plant resources, handle them, and navigate back to their hive to unload food for the colony (Giurfa and Menzel 2001). One of the most reported sublethal effect of stressors on bees is the decrease in their cognitive performance, which has been associated with a decreased foraging success and colony survival in the long-term (Klein et al. 2017). A recent meta-analysis confirmed that neonicotinoid pesticides, at field-realistic doses, consistently alter the mean olfactory learning and memory performance of bees (Siviter et al. 2018). Doses above field-realistic levels were even more detrimental. While acute and chronic exposures similarly decreased learning and memory scores, the effect on memory was stronger following a chronic exposure. The impact of stressor intensity (level and type of exposure) on the variance of the learning performance was not analysed.

We explored these effects by analysing the raw datasets from 24 studies (including two unpublished datasets). Our aim was to point out the importance of studying phenotypic variance in populations exposed to human-induced stressors. Although a decreased cognitive performance was expected in stressed bees, we predicted that the effect of stressors on the variance would depend on the stressor intensity (level and duration), which would help estimate the hazardous nature of a given stressor.

Material and methods

Search and selection of datasets

The search for scientific publications falling within the scope of our research question was performed in July 2020 using the PubMed database. The keywords used for the search were (“Stressor” OR “Pesticide” OR “Parasite”) AND (“Cognition” OR “Learning”) AND (“Pollinators” OR “Bees”). A total of 71 studies were found, of which 22 met our inclusion criteria. Two datasets belonging to the authors of this study were also included as they filled the inclusion criteria.

The studies included in our analyses measured the impact of stressors on the cognitive performance of adult bees. Cognitive performance was assessed using associative learning paradigms testing the ability of bees to associate an olfactory or/and visual stimulus with an appetitive or aversive reinforcement. The stressor type covered different pesticides, parasites, predator odours, alarm pheromones, and heavy metal pollutants. Studies performed with pesticides whose median lethal dose (LD50; i.e. dose that kills 50% of the population) could not be identified in the literature were excluded from our final selection. The bee species studied in the selected publications were the honey bees *Apis cerana* and *Apis mellifera*, and the bumblebees *Bombus impatiens* and *Bombus terrestris*. These species were not selected purposefully, but rather emerged from the refinement obtained with other inclusion criteria. All but three raw datasets were available online with the published material. Those three datasets were kindly provided by their authors. The list of the 24 selected studies is available in Table 1 and raw data are provided in Table S1.

Data organisation and normalisation of variables

The raw data from the selected studies were downloaded and saved as .csv files. A new dataset was created, which combined information on the species, the cognitive task studied, the type of stressor, the type of exposure (acute/chronic), and, in the case of pesticide studies, the dose (μg/bee) or concentration (ppb). To allow comparison across various cognitive tasks, a z-score was calculated for each individual on its cognitive performance by applying the function ‘scale’ in R (package {base}) which uses the mean and the standard deviation of the sample to scale each element. Within each study, the function ‘scale’ was applied on the cognitive performance of bees belonging to the same category of bee species, cognitive task, stressor type and exposure type. When learning performance was measured as a binary response (e.g. success vs. failure) across multiple trials, the raw data was first used to calculate a learning score for each individual corresponding to the number of successful trials. Such a calculation was required because the variance in binary variables can be mathematically predicted from the mean and sample size and does not reflect biological variance (Supplementary Fig. S1). For pesticide studies, the dose (acute exposure) and concentration (chronic exposure) were normalized using the LD50 of each drug.

The final dataset is available in Supplementary Table S1. Individual z-scores were used to calculate the mean and the variance of the z-scores for each control and stressed group. We thereafter refer to these variables as the “mean” and the “variance” of the cognitive performance. Each study may contain multiple control and stressed groups depending on the number of experiments performed and the number of stressors used. The final sample sizes are therefore larger than the number of studies and are displayed on the figures.

Table 1: Summary table of the studies used.

Stressor	**Genus**	**Exposure type**	**Reference**
Pesticide	*Apis*	Acute	(Ludicke and Nieh 2020)
Pesticide	*Apis*	Acute	(Hesselbach and Scheiner 2018)
Pesticide	*Apis*	Acute	(Urlacher et al. 2016)
Pesticide	*Apis*	Acute, chronic	(Tan et al. 2015)
Pesticide	*Apis*	Chronic	(Mustard et al. 2020)
Pesticide	*Apis*	Chronic	(Tan et al. 2017)
Pesticide	*Apis, Bombus*	Acute	(Siviter et al. 2019)
Pesticide	*Bombus*	Acute	(Muth et al. 2019)
Pesticide	*Bombus*	Acute, chronic	(Stanley et al. 2015)
Pesticide	*Bombus*	Chronic	(Smith et al. 2020)
Pesticide	*Bombus*	Chronic	(Lämsä et al. 2018)
Pesticide	*Bombus*	Chronic	(Phelps et al. 2018)
Pesticide, coexposure	*Apis*	Chronic	(Colin et al. 2020b)
Parasite	*Bombus*	Acute	Gomez-Moracho et al. (2021)
Parasite	*Bombus*	Acute	(Martin et al. 2018)
Pollution	*Apis*	Acute	Monchanin et al. (unpublished)

Pollution	*Apis*	Acute	(Monchanin et al. 2021b)
Pollution	*Apis*	Acute	(Leonard et al. 2019)
Pollution	*Apis*	Chronic	(Monchanin et al. 2021a)
Other	*Apis*	Acute	(Wang et al. 2016)
Other	*Apis*	Acute	(Shepherd et al. 2018)
Other	*Apis*	Chronic	(Shepherd et al. 2019)
Coexposure	*Apis, Bombus*	Chronic	(Piiroinen and Goulson 2016)
Coexposure	*Bombus*	Acute	(Piiroinen et al. 2016)

Data analyses

All analyses were conducted in R Studio v.1.2.5033 (RStudio Team 2015). Linear mixed-effects models (LMM; package {lme4}; Bates et al. 2015) were used to investigate the impact of stressors on the mean and the variance of the cognitive performance. The group (control vs. stressed), the type of stressor, the species or the type of tasks were defined as independent variables. The experiment's identifier was set as random factor.

Similar models were used to assess the impact of pesticides on the mean and variance of the cognitive performance. In the subset of pesticide studies (15 studies), Pearson correlation tests were also performed to assess the relationship between the mean and the variance of the cognitive performance within control and stressed groups. Finally, LMMs were conducted to study the influence of the pesticide dose (log-transformed) on individual z-scores, with the experiment's identifier set as random factor.

Results

Stressor types vary in their effect on the variance of the cognitive performance

We first explored the overall effects of stress on cognitive mean and variance in bee populations across the 24 studies. The mean cognitive performance of bees was severely impacted by exposure to environmental stressors, while the effects on the variance were less pronounced and overall, more heterogeneous (Fig. 1). Stressed bees exhibited a significantly lower mean cognitive performance than control bees (LMMs; *group effect: P* < 0.001) independently of the type of stressor (*group*stressor effect*: *P* = 0.453; Fig. 1A), the bee genus (*group*species effect*: *P* = 0.271; Fig. 1C) and the type of cognitive task (*group*task effect*: *P* = 0.511; Fig. 1E). By contrast the variance of the cognitive performance did not differ significantly between control and stressed bees (LMMs; *group effect*: *P* > 0.05 for all models). We found no effect of the bee species (*group*species effect*: *P* = 0.421; Fig. 1D) or the type of cognitive task (*group*task effect*: *P* = 0.533; Fig. 1F). There was a significant interaction between exposure to stressor and the type of stressor, thus showing a heterogeneous effect of stressors on the variance of the cognitive performance (*group*stressor effect: P* < 0.05; Fig. 1B). While variance decreased in stressed bees exposed to pesticides, it tended to increase in stressed bees exposed to other stressor types, compared to their respective control group.

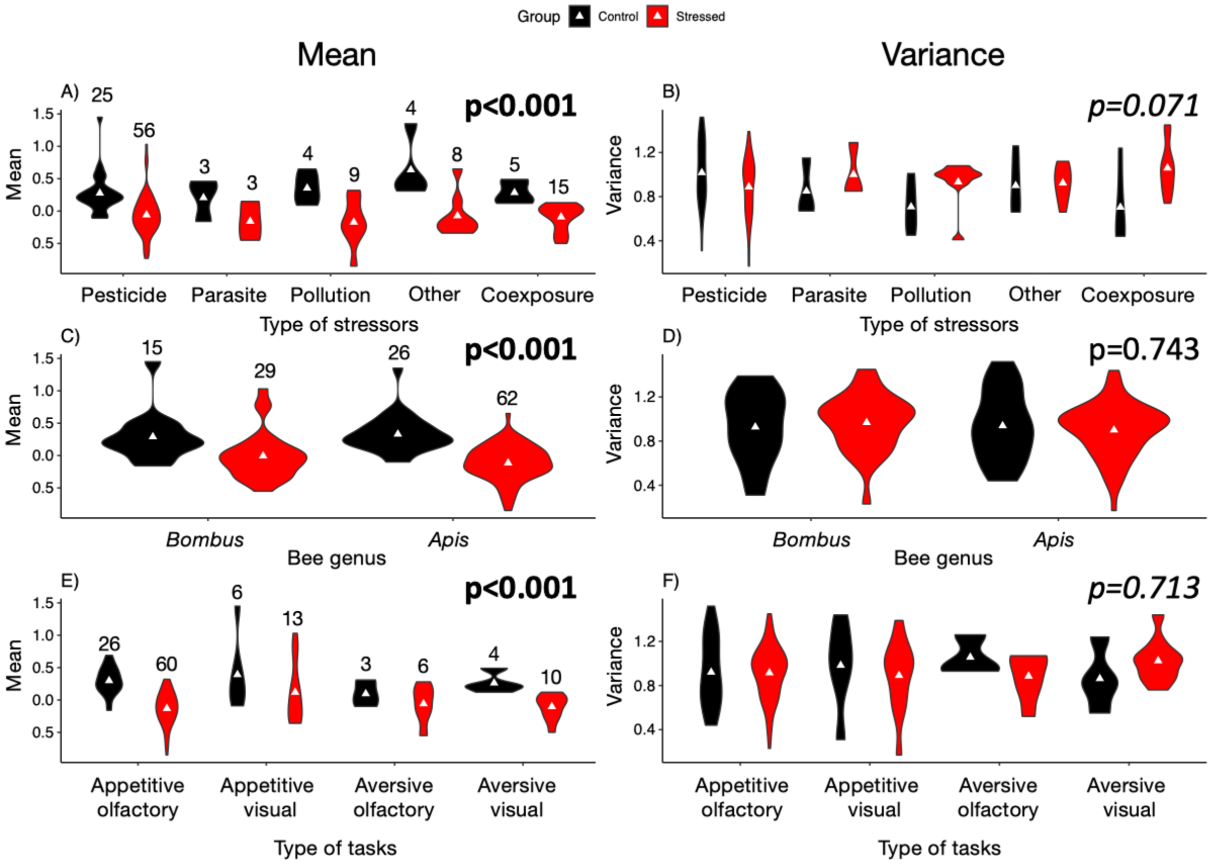

Figure 1. Stressors decrease the mean cognitive performance of bees, but not the variance. Violin plots showing the mean (left) and the variance (right) of the cognitive performance for control (black) and stressed (red) bees are displayed according to: **A-B)** the type of stressors; **C-D)** the bee genus; **E-F)** the type of cognitive tasks. White triangles represent the mean. Sample sizes are displayed above the violins. P-values from LMM are displayed for group effect only and are in bold when significant.

The mode of exposure to pesticides influences the variance of the cognitive performance

To investigate whether stressor intensity plays a role in the differential effects of stressors on the variance of the cognitive performance, we focused our analyses on the 15 pesticide studies of our dataset (Table 1). Pesticide studies were the most abundant in the literature and present the advantage that a normalization of stressor intensity across drugs was possible.

Both acute and chronic treatments reduced the mean cognitive performance (LMM; *acute:* mean±standard error: -0.232±0.095, $P < 0.05$; *chronic:* -0.465±0.083, $p < 0.001$). However, they affected the variance differently (Fig. 2). In the acute treatments, the decreased mean cognitive performance observed in stressed bees was not accompanied by a decrease in variance (-0.001±0.081, $P = 0.998$). In the chronic treatment, stressed bees exhibited a significantly reduced variance compared to the control bees (-0.317±0.107, $P < 0.01$).

A significant positive correlation between the mean cognitive performance and its variance was observed in both stressed groups (*acute:* $r = 0.437$, $P < 0.01$; *chronic:* $r = 0.657$, $P < 0.005$), but not in control groups (*acute:* $r = 0.057$, $P = 0.868$; *chronic:* $r = 0.072$, $P = 0.833$).

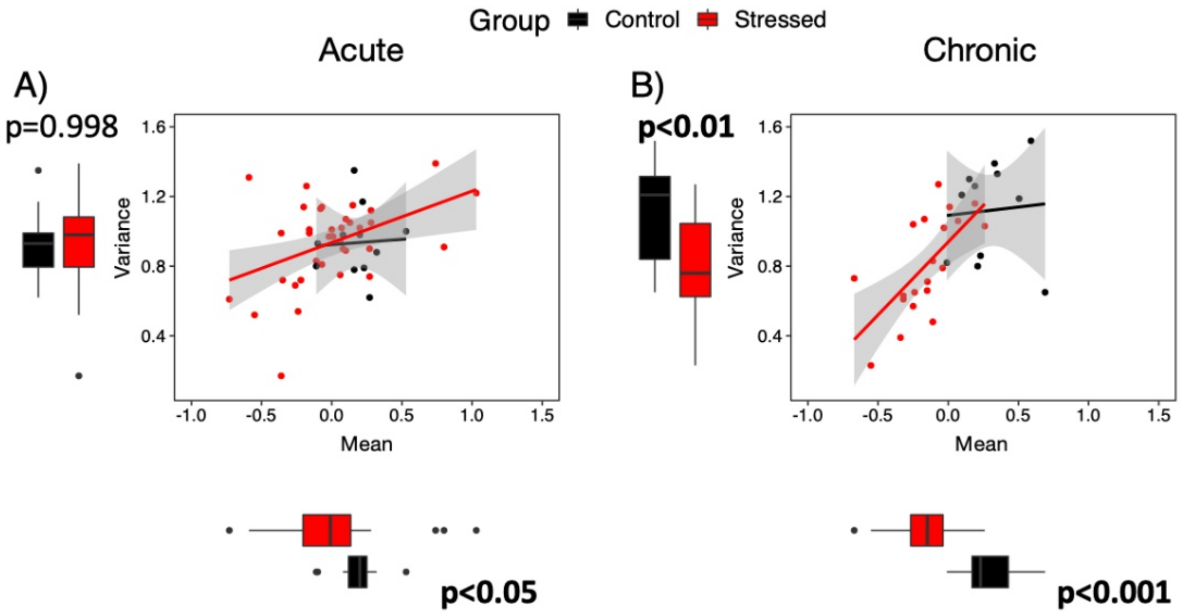

Figure 2. Pesticide exposure duration affects the variance of the cognitive performance. The mean and the variance of the cognitive performance are plotted for control (black) and stressed (red) bees following an **A)** acute (N = 13 controls, N = 36 stressed) or **B)** chronic (N = 11 controls, N = 20 stressed) exposure to pesticides. Horizontal and vertical boxplots represent the mean cognitive performance and its variance, respectively. P-values from LMM are displayed for group effect only and are in bold when significant.

Cognitive performance decreases as the pesticide level increases

To further explore whether the effect on mean and variance differed with stress magnitude, we analysed different levels of pesticides. A dose-dependent effect of pesticides exposure on cognitive performance was found for both acute and chronic exposure (Fig. 3). The individual z-scores significantly decreased with increasing levels of exposure (LMM; *acute:* -0.144±0.018, $P < 0.001$, Fig. 3A; *chronic:* -0.121±0.020, $p < 0.001$, Fig. 3B).With increasing pesticide levels, the reduced mean cognitive performance was accompanied by a decrease in the variance of cognitive performance for both acute and chronic exposure (Figs 3C-D). Most individuals in a population therefore seem to show a decreased cognitive performance following a treatment with high pesticide levels, whether they were exposed acutely or chronically.

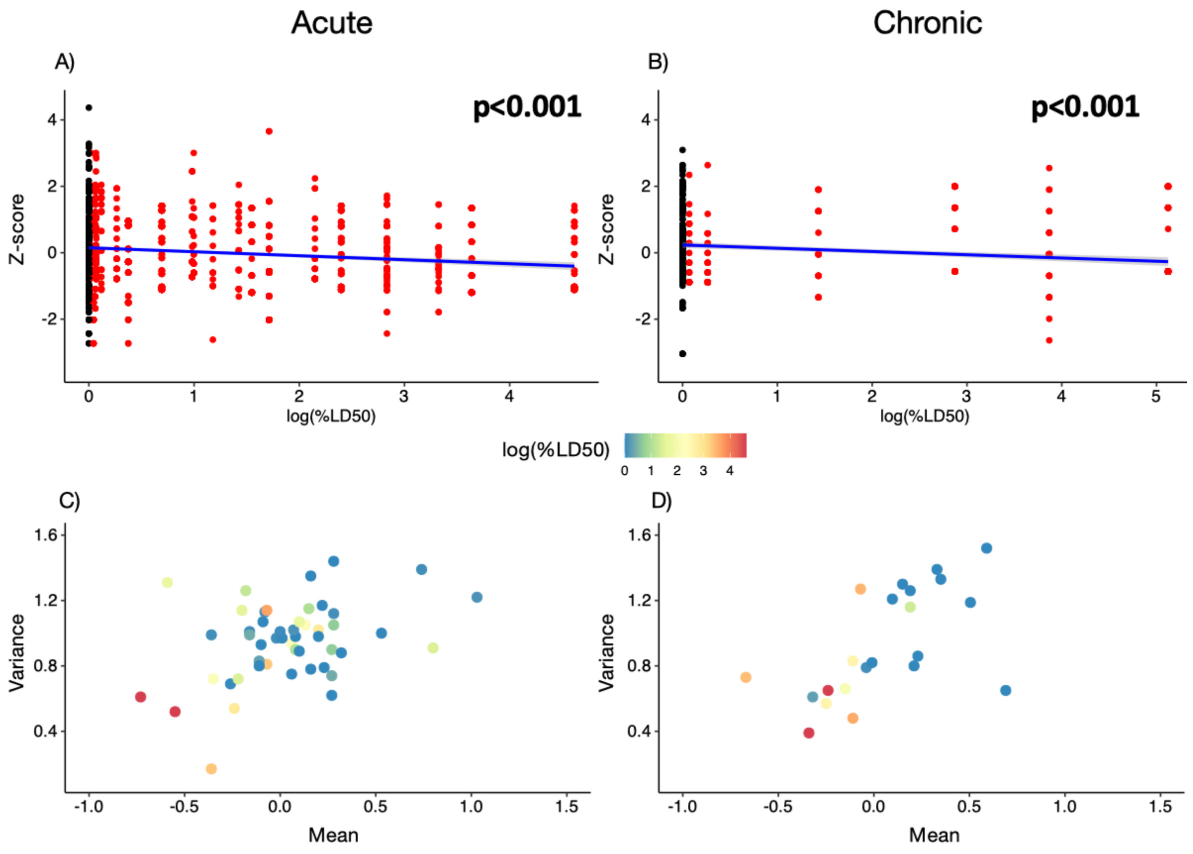

Figure 3. Effect of the pesticide level on cognitive performance. Individual z-scores are plotted relative to the normalized pesticide dose (logarithm of %LD50) for **A)** acute exposure (N = 2,141 bees) and **B)** chronic exposure (N = 1,026 bees). Estimate trends are displayed in solid blue lines. Plots showing the mean cognitive performance in function of the variance and coloured according to the normalized pesticide dose (logarithm of %LD50) are displayed for **C)** acute (N = 13 controls, N = 36 stressed) and **D)** chronic exposure (N = 11 controls, N = 11 stressed).

Discussion

Environmental stressors affect the behaviour and cognition of animals (Killen et al. 2013; Klein et al. 2017; Siviter et al. 2018). Most studies have typically focused on the effect of stress on mean cognitive performance. However, all individuals in a population are not similarly affected by the stressors they encounter, and the resulting variance in their cognitive performance might be key for stress resilience at the population level. We investigated the impact of environmental stressors on the cognitive performance of bees using raw datasets of 24 studies and found different effects on mean and variance depending on the stress level and exposure mode.

Focusing on pesticide studies revealed that the mean cognitive performance was altered for both chronic and acute exposures, which is consistent with previous findings (Siviter et al. 2018). However, the variance was only decreased after a chronic exposure. This means some bees were able to cope with an acute pesticide exposure, but not after a chronic exposure. In some individuals, homeostatic physiological processes might counteract the effect of an acute exposure to the drug, which is only present in the body for a short duration (Cohen 2006). Most pesticides indeed act on the nervous system of bees whose plasticity to maintain homeostasis is well-known (Turrigiano and Nelson 2000; Cabirol and Haase 2019). The lasting presence of pesticides in the bodies during a chronic exposure seems to complicate the process of resilience to this stressor for most individuals. This is, to our knowledge, the first study directly showing a differential effect of acute and chronic exposures to a stressor on learning performance in an animal.

For both modes of exposure, the mean cognitive performance and its variance decreased with increasing levels of pesticides. The positive correlation between the mean and the variance is consistent with this finding: the more a stressor affects the mean, the more it affects the variance. This advocates for the use of low pesticide concentrations in the field. A recent opinion piece argued that concentrations having sublethal effects on pest insects would still protect crops when pest density is low and thereby would be less damaging to non-target insects (Colin et al. 2020a).

Altogether, the results of our analyses on pesticide studies suggest that an acute exposure to low pesticide doses is the least damaging for bee populations. An unaltered variance of the learning performance following pesticide exposure means that some individuals may have maintained sufficient cognitive abilities to ensure an efficient foraging (Klein et al. 2017). Cognitive and behavioural variance is particularly important in a population as individuals with different behavioural phenotypes will have a different fitness depending on the environmental conditions (Jandt et al. 2014). Variance favours that some individuals will have an adapted behaviour in case of a change in the environment. In group-living species, such as honey bees and bumblebees, a diversity of behavioural phenotypes among individuals was also suggested to influence decision-making at the group level and to increase the flexibility of the group behaviour (Burns and Dyer 2008; Michelena et al. 2010). Unfortunately, bees often encounter pesticides over long time periods in the foraging environment, especially when colonies are located near treated crops, and in the hive due to the residues present in the honey and wax (Godfray et al. 2014, 2015; Tsvetkov et al. 2017). The consequences of such a chronic exposure to pesticides are often not a priority in risk assessment procedures. Policy regulations in the European Union and in the US regarding the commercialization of new plant protection products (PPPs) ask for acute toxicity assays on bees and other non-target animals before asking for chronic toxicity assays (EPPO 1992, 2010; U.S. Environmental Protection Agency and Code of Federal Regulations (CFR) 2010). Only when acute toxicity is significant would a chronic toxicity assay be performed. Although the European Food Safety Authority recommends the inclusion of chronic exposure assays earlier in the risk assessment procedure, such assays are not yet mandatory (EFSA 2013). The effects of PPPs that will be encountered chronically in the field might therefore be underestimated.

Beyond pesticides, our study showed that the type of stressor experienced by bees had a significant influence on the variance of the cognitive performance, but not on the mean. The mean cognitive performance assessed with various cognitive tasks was severely impacted by all types of stressors in all bee species investigated. This is consistent with a previous meta-analysis performed on olfactory appetitive learning performance in bees exposed to pesticides, but extends the effects to other cognitive tasks and stressor types (Siviter et al. 2018). On the other hand, the variance tended to decrease in bees exposed to pesticides, while other stressors seemed to increase it. This effect might be triggered by the small sample sizes found for some stressors ($N \leq 5$ for the control groups used to assess the effect of parasites, pollution, and co-exposures), but it might also reflect resilience of bee populations to some environmental stressors and to their intensity. Stressors increasing the variance of the cognitive performance while decreasing the mean probably impacted only part of the individuals of the population. Such stress-induced variation in cognitive performance may favour the diversification of cognitive abilities (Badyaev 2005). It is also likely that some mild, low intensity stressors may have had beneficial effects on the cognitive performance of a few individuals as has been observed in rodents (Hurtubise and Howland 2016). Yet, there is a need for more studies on stressors other than pesticides to confirm this trend.

For both *Bombus* and *Apis* genera, the mean and the variance of the cognitive performance of stressed bees was not different from the controls. This is consistent with previous findings suggesting that honey bees are overall suitable surrogates for non-*Apis* species in regulatory risk assessments of pesticide toxicity (Arena and Sgolastra 2014; Heard et al. 2017; Thompson and Pamminger 2019). In most cases, the honey bee risk assessment was protective of other species. Yet, the sensitivity to pesticides was variable among non-*Apis* bee species (Arena and Sgolastra 2014) and these studies focused on mortality measures. This calls for more research on the sublethal effects of human-induced stressors in non-*Apis* bees. In the meantime, risk assessments required by the European commission are focused on honey bees (EPPO 2010).

Our study of variance in cognitive performance revealed a differential effect of chronic and acute exposures to pesticides as well as an important influence of the stressor intensity on the proportion of individuals that might be impacted. Regulatory authorities should therefore consider asking for a chronic toxicity assay before deciding on the commercialization of a new PPPs. Our findings demonstrate the importance of considering phenotypic variance in future analyses of the impact of environmental stressors on animals. This will allow assessing more thoroughly the hazardous nature of the stressors and identifying the modes of exposure that might be less damaging for the population. For what regards PPPs, the results of such studies should lead to explicit guidelines for farmers on the safe use of these toxic substances.

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Supporting materials

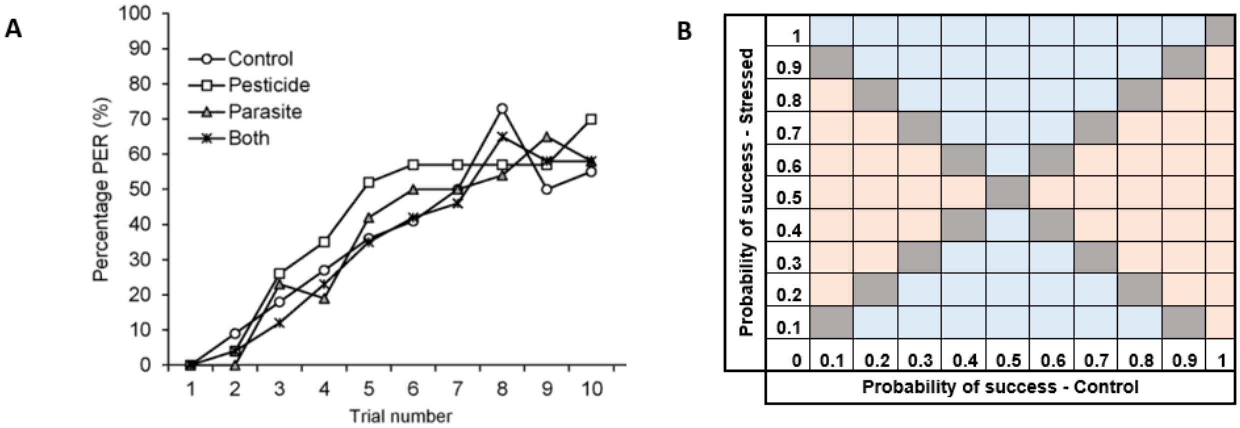

Figure S1: Analysis of variance in studies with binary data. **A)** Example of a cognitive task in which the measure of performance is binary (success/failure). The impact of a pesticide and parasite on bumblebees' learning performance is measured with a classical conditioning of the proboscis extension response (PER) (from Piiroinen et al. (2016)). The percentage of individuals that extended the proboscis in response to the conditioned stimulus (i.e percentage of successful individuals) is plotted across 10 successive learning trials. **B)** Matrix representing the impact of a stressor on the variance in cognitive performance at a given trial and for given probabilities of success. For an equal sample size in the control and stressed groups, the impact of the stressor on variance can be calculated using the probability of success of each group. An increased (orange) or decreased variance (blue) in the stressed group can be mathematically predicted.

Appendix 9: How bumblebees became model species in apidology: a brief history and perspectives

Paper under review at Apidologie

How bumblebees became model species in apidology: a brief history and perspectives

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Abstract

In recent years, bumblebees have increasingly been used to study various aspects of bee biology, ecology and evolution. They are now broadly accepted as tractable model species, complementary to the domestic honey bees, for fundamental and applied apidology. Here, we provide a brief history of how bumblebee research developed since their domestication and commercialisation for crop pollination in the 1990s. Bumblebees are large social bees that can be kept and trained in the lab year round. They are relatively easy to manipulate and track individually in their small colonies. These practical advantages have offered new possibilities for experimental bee research, leading to major breakthroughs in different fields such as cognition, navigation, nutrition, host-parasite interactions, and insect declines. Many of these findings have later been confirmed in honey bees and other pollinators. We discuss some exciting directions for future apidology research based on bumblebees.

Keywords: bumble bees, *Bombus terrestris, Bombus impatiens, Bombus ignitus*, cognition, navigation, nutrition, host-parasite interactions

Introduction

Bee research has historically been developed on, and is still largely dominated by, studies on honey bees. This is understandable given the thousands of years of domestication of honey bees and the many commercial benefits they generate for hive products and crop pollination. Beyond apidology, experimental observations on honey bees have led to discoveries of fundamental importance in animal behaviour and ecology. Over the past century or so, James Turner (Turner 1910), Karl von Frisch (von Frisch 1967), Martin Lindauer (Lindauer and Watkin 1953), Randolf Menzel (Menzel and Giurfa 2001), Tom Seeley (Seeley et al. 2012), Yves Le Conte (Le Conte and Hefetz 2008) and many others famous bee biologists, have described the impressively rich behavioural and cognitive repertoire of honey bees, supporting a sophisticated and efficient social organization in small-brained animals. This long history of research on honey bees has raised these fascinating social insects to the level of model organisms for many areas of insect science, including behaviour (Elekonich and Roberts 2005, Giurfa 2007), neurosciences (Menzel 2012), physiology (Wang et al. 2018, Zheng et al. 2018), nutrition (Wright et al. 2018), and social evolution (Woodard et al. 2015).

Yet, honey bees are quite unique among bees and insects in general. They constitute just a dozen of the 20,000 species of bees described so far (Michener 2000), and display sophisticated behaviours associated with extreme levels of social organization and division of labour that are not representative of most bee species. As a general trend in biological research, one can question the relevance of focusing all research efforts on one or two model species only (i.e. *Apis mellifera* Linnaeus, 1758 and *A. cerana* Fabricius, 1793) to study a broad group of animals (Hunter 2008). The majority of bee species live in much simpler colonies with a flexible division of labour, or are not social at all (Wilson and Hölldobler 2005). Bees are found in most terrestrial environments and thus show behavioural and physiological adaptations that cannot always be investigated using honey bees. In particular, most bee species do not actively recruit conspecifics to food resources or nest sites through a dance, but must explore and learn to exploit resources of their environment by themselves (Giurfa 2015). There is thus a need to diversify bee models in order to build a broader vision about their biology and ecology as a whole.

Over recent years, bumblebees have increasingly been used for bee research. They constitute a large group of primitively eusocial bees composed of 250 species (Goulson 2010). Bumblebees are primarily found in the Northern Hemisphere but also in South America, where a few lowland tropical species have been identified (Frison 1925). Like honey bees, bumblebees have long intrigued naturalists. Darwin explained how he was fascinated by the routing behaviour of male bumblebees looking for females (Freeman 1968) and Bennett (1883) and Christy (1883) described how individual bumblebees specialised in visiting certain flower species when foraging. However, all these observations were mainly descriptive and opportunistic due to the difficulty to raise bumblebee colonies and manipulate their behaviour and social organisation to experimentally test hypotheses.

Bumblebee research took a serious turn in the 1990s, thanks to the professional domestication and commercialization of bumblebee hives for greenhouse pollination (for a review see Velthuis and van Doorn 2006). From this moment, bee researchers began to use bumblebees in experimental programs. Bumblebees appeared particularly suitable for experimental work as they can be tested in the field and in the lab using homemade setups (e.g. mazes and experimental arenas of different shapes and sizes), followed individually during their lifetime (i.e. small colonies with short cycle), do not need intense care nor specific skills for maintenance (i.e. no beekeeping), and are relevant to address questions related to wild bee species (i.e. commercially available species are common in the wild). Their ecological and economic importance, coupled with their global declines, has led to a significantly growing interest in their behaviour, ecology and evolution. In just about three decades, a generation of talented bumblebee researchers that have all marked their own fields (e.g. James Thomson: pollination ecology; Lars Chittka: behaviour and cognition; Juliet Osborne: foraging ecology; Paul Schmidt-Hempel and Mark Brown: host-parasite interactions; Dave Goulson: pollinator conservation; Bernt Heinrich: energetics) have raised bumblebees at the level of model organisms for many areas of apidology.

This trend is illustrated by the sharp increase of published studies using bumblebees between 1990 and 2020, in research databases and the journal *Apidologie* (Figure 1).

In this short review for the 50th anniversary of *Apidologie*, we consider the growing importance of bumblebees for bee research. We explain how experimental studies became possible with the domestication and commercialization of bumblebees for crop pollination. We then describe how studies on bumblebees enabled major breakthroughs in comparative cognition, movement ecology, nutritional ecology, pollinator declines or evolutionary parasitology. We finally discuss some directions for future research in apidology and beyond.

The rise of experimental research on bumblebees

Bumblebees have long been recognised for their value as pollinators (Velthuis and van Doorn 2006). Because their proboscises are longer than those of honey bees (bumblebees: ca. 15mm, honey bees: ca. 7mm), bumblebees can pollinate flowers with deep corollas. This is why, for instance, they were imported to New Zealand at the end of the 19th century to improve the seed of a red clover used as forage for farm animals (Goulson 2010). At the beginning of the 20th century, there have been many attempts to domesticate bumblebees from wild caught queens (Sladen 1912, Plath 1923, Frison 1927). Major steps included controlling climate (i.e. temperature and humidity) in rearing rooms, storing hibernating queens, breaking the diapause, controlling mating, and delivering appropriate pollen sources (for a review see Velthuis and van Doorn 2006). But it was not until the 1980s that routine domestication became a fact, when the amateur bumblebee researcher, Roland de Jonghe, discovered the value of bumblebees for buzz pollination of tomatoes. Until then, tomato flowers in greenhouses were pollinated mechanically by vibrating the plants three times a week. In 1987, the first company for commercial rearing of bumblebees was created. Today, the largest of these companies have rearing facilities all over the world. They mainly rear colonies for the local market using native species, so that five main bumblebee species are used for crop pollination (*Bombus terrestris* Linnaeus, 1758; *B. lucorum* Krüger, 1951; *B. occidentalis* Greene, 1858*; B. ignitus* Smith, 1869 and *B. impatiens*, Cresson 1863*)* (Velthuis and van Doorn 2006).

Commercial domestication dramatically boosted experimental research with bumblebees (Figure 1). Even though research had been conducted since the beginning of the 20th century, most studies were published between 1975 and 2020. 93% of these studies were published after 1993, i.e. which was the first year when publications on bumblebees accounted for more than 1% of all publications on bees (Figure 1a). As a result of this sharp and continuous increase, 5,607 bumblebee studies were published in 2020. This is about half those published on honey bees, and more than those on solitary and stingless bees altogether. The same trend can be observed when considering only the publications in the journal *Apidologie* (Figure 1B).

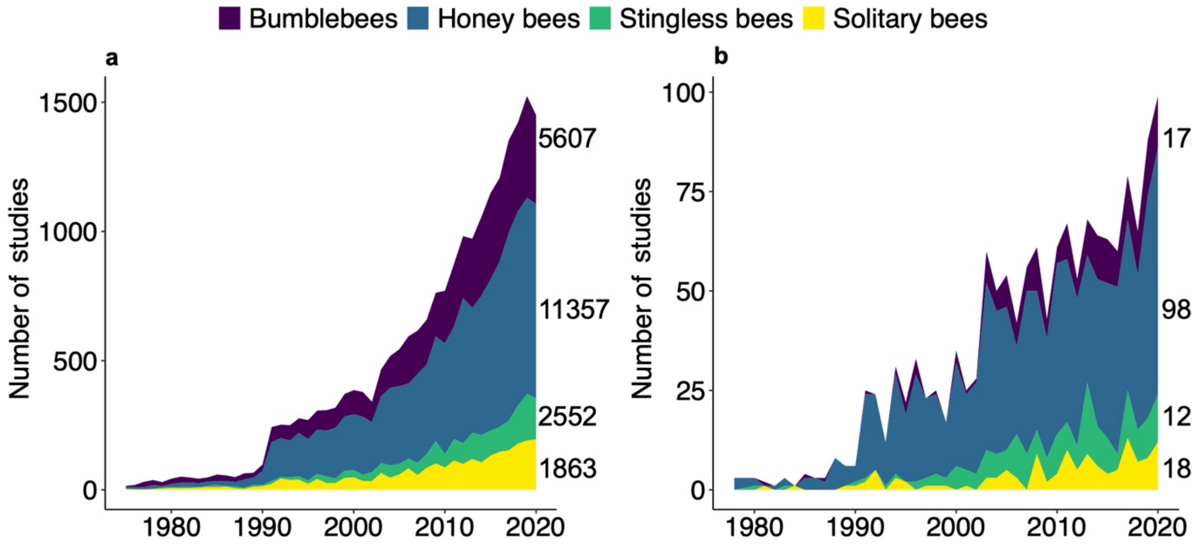

Figure 1: Numbers of studies published yearly per bee species. A) In all scientific journals. **B)** In the journal *Apidologie*. Number of studies published per bee species as of 2020. Articles in English published between 1975 and 2020 were searched on ISI Web of Knowledge database (search performed on 07/12/2020 using keywords: insect and honey bee and *Apis*; stingless bee or *Melipona*; solitary bee or leafcutter or *Osmia* or carpenter; bumblebee or *Bombus*).

Major breakthroughs involving bumblebees

There is now a rich history of bumblebee research with many influential labs across the world. *B. terrestris* is the main model species used by researchers based in Europe, while *B. impatiens* is used in North America, and *B. ignitus* in Asia. Often, the results described in one of these species were later confirmed in the others. Below we describe some research areas for which the utilisation of commercial bumblebees enabled breakthroughs in bee science and beyond (see summary in Table 1).

Table 1: Some breakthroughs made possible through experiments with commercial bumblebees. In this non extensive list, we cite key references and associated species.

Finding	**Advantage of using bumblebees**	**Bumblebee species**	**Key references**
Bees can learn new foraging techniques by observation of others (Figure 2a, 2b)	Bumblebees can be kept in colonies and trained to forage in artificial setups in the lab. Colonies are small, thus the experience of every individual can be monitored during its entire life.	*B. terrestris*	Alem et al. 2016, Loukola et al. 2017
Bees exhibit emotion-like states	Bumblebees can be kept in colonies and trained to forage in artificial setups in the lab.	*B. terrestris*	Perry et al. 2016
Bees can consider their own body size to decide to move through a gap	Bumblebees can be kept in colonies and trained to forage in artificial setups in the lab. Colonies are small, thus the experience of every individual can be monitored during its entire life.	*B. terrestris*	Ravi et al. 2020
Bees regulate their intake of protein, carbohydrates and and lipids in food	Bumblebees can be kept in artificial colonies of controlled composition (e.g. with set numbers of workers and/or brood), and fed artificial diets in the lab.	*B. impatiens* *B. terrestris*	Vaudo et al. 2016 Ruedenauer et al. 2020
Sublethal doses of pesticides affect many aspects of bee behaviour and social organisation	Bumblebee colonies can be kept and monitored in the lab. Colonies are small, thus every social interaction can be monitored.	*B. terrestris* *B. impatiens*	Gill et al. 2012 Crall et al. 2018
Bees develop optimal multi-destination	Commercial colonies can be setup in the field. Bumblebees do not	*B. impatiens* *B. terrestris*	Ohashi et al. 2007

routes (Figure 2d)	communicate locational information about food resources to nestmates. The colonies are small, thus every foraging event of an individual can be monitored.		Lihoreau et al. 2012b
Bees display sophisticated flight control (Figure 2e)	Freely moving bumblebees can be trained to forage in virtual reality flight arenas in the lab	*B. ignitus*	Frasnelli et al. 2018
Immune responses incur important fitness costs	Bumblebees are robust to injections and manipulations. They can be kept and monitored in isolation in the lab for weeks.	*B. terrestris*	Moret and Schmid-Hempel 2000
Polyandry benefits to prevent infections	The reproduction of bumblebees can be manipulated. They are easily infected in the lab.	*B. terrestris*	Baer and Schmid-Hempel 1999

Cognition

Insect cognition research is living a golden age, with an increasing number of recent studies showing how insects solve ever more impressive problems despite their miniature brains (Perry et al. 2017). In this context, bumblebees have emerged as key models to experimentally explore the frontiers of insect cognitive capacities. Bumblebees offer a unique opportunity to test individually identified insects in tightly controlled lab conditions. Foragers can be trained to collect nectar rewards over repeated trials in various kinds of mazes and arenas connected to their colony nest box, allowing to implement protocols of experimental psychology replicating those developed for humans or larger-brained vertebrates (see Figure 2). Over the past 20 years, this approach has been particularly fruitful, revealing an impressive behavioural and cognitive repertoire, that includes personalities (Chittka et al. 2003), numerosity (MaBouDi et al. 2017), social learning (Loukola et al. 2017) (Figure 2b), emotional states (Perry et al. 2016), object categorization (Solvi et al. 2020) (Figure 2c), and path planning (Lihoreau et al. 2012b) (Figure 2d), to cite some. Most of these studies were based on the use of visual information, whose neural processing begins to be understood thanks to anatomical and physiological studies of brain visual circuits (Paulk and Gronenberg 2008, Paulk et al. 2008, 2009).

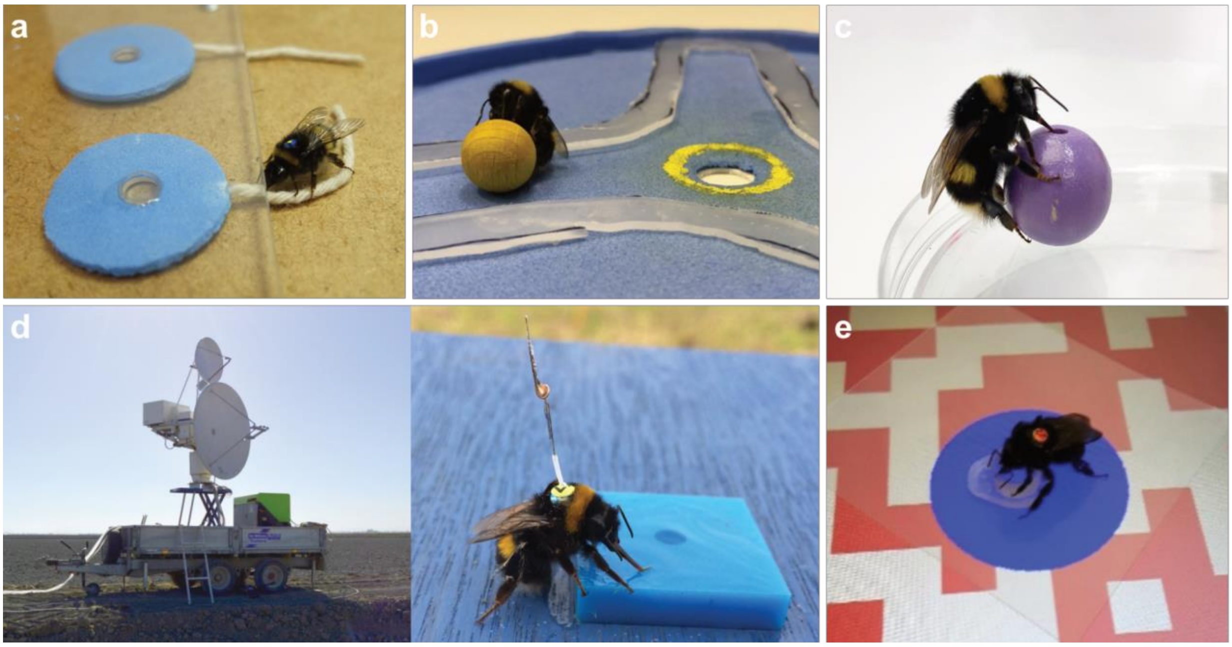

Figure 2: Bumblebees are particularly suitable for experimental research. **a)** Setup used by Alem et al. (2016) or observational learning of foraging techniques**.** The bumblebee is pulling a string to move a feeder containing a reward. Photo provided by Olli Loukola. **b)** Setup used by Loukola et al. (2017) to demonstrate innovation. The bumblebee is moving a ball to the center of a feeder**.** Photo provided by Lars Chittka **c)** Setup used by Solvi et al. (2020) to test the ability to discriminate geometric shapes visually or by touch. The bumblebee is getting a reward from a spherical object. Photo provided by Cwyn Solvi. **d)** Harmonic radar used by Lihoreau et al. (2012) to demonstrate route optimisation (left) and transponder used to track bumblebees (right). Photos provided by Tamara Gómez-Moracho (radar) and Joe Woodgate (bumblebee). **e)** Virtual reality setup used by Frasnelli et al. (2018) to show the role of visual motion cues in navigation. The bumblebee is getting a sucrose reward from a virtual feeder. Photo provided by Elisa Frasnelli.

Social learning and cultural transmissions

Bumblebees have been central for the demonstration that insects can learn behaviours through the observation of others, an ability long been thought to be specific to vertebrates. In 2005, Worden

(2005) and Leadbeater and Chittka (2005) used bumblebee colonies connected to lab arenas containing artificial flowers to show that inexperienced observers (i.e. bumblebees with no information on their foraging environment) can acquire flower preferences by looking at experienced demonstrators (bumblebees trained to visit flowers of specific colours). Social learning of flower preferences also works with resin bees placed on flowers instead of live demonstrators (Leadbeater and Chittka 2007). This ability is supported by associative learning (Dawson et al. 2013), allowing bumblebees to adjust their reliance on social information based on the context. Hence, social learning is more frequent when competition is low and the presence of other bees on flowers actually predicts a reward (Dunlap et al. 2016). In this process, bumblebees can also learn by observing foragers of other bee species that exploit the same resources, such as for instance honey bees (Dawson and Chittka 2012)**.**

These observations of social learning raised the intriguing question of longer-term cultural transmission. Alem et al. (2016) trained demonstrator bumblebees in an unnatural foraging task consisting in pulling a string with their mandibles to access a reward on an artificial flower below a screen (Figure 2a). String pulling is a classical paradigm to study mammal and bird cognition (Jacobs and Osvath 2015). Inexperienced bumblebees could acquire the technique through visual observation (Alem et al. 2016). Remarkably, these observers tended to become demonstrators themselves, suggesting learning can spread culturally in insect populations through chains of social learning, as later supported in other species (Danchin et al. 2018). This process may explain the frequent emergence of cheating behaviours in natural bumblebee populations, when foragers find easier access to nectar by making holes at the base of flowers instead of landing or entering the flowers (Leadbeater and Chittka 2008).

Perhaps even more impressive, bumblebees were recently shown to improve copied behaviours, a feat akin to innovation. Loukola et al. (2017) trained bumblebees to move a small ball into a hole in order to obtain a sucrose reward (Figure 2b). Just like flower choice or string pulling, this odd foraging technique can be transmitted to inexperienced conspecifics through visual observation, behind a screen. However, here, observers sometimes outperformed suboptimal demonstrators by choosing the closest ball to the hole when demonstrators were trained with the farthest. This suggests entirely new behaviours can emerge through social learning and innovation.

Emotions

Whether insects have emotions is another ongoing debate in comparative cognition that received important insights using bumblebees. By adapting paradigms of experimental psychology, Perry et al. (2016) demonstrated bumblebees exhibit internal states that fit with operational definitions of emotions for humans and other vertebrates (Nettle and Bateson 2012). The authors applied a well-established judgement bias paradigm in which subjects associate one stimulus with a positive event and another stimulus with a negative event, so that individuals in a positive emotional state tend to respond to an ambiguous (intermediate) stimulus as if it was predicting the positive event. In the bumblebee version of the judgment bias paradigm, insects were trained to forage in a small lan arena to obtain a sucrose reward under a vertical placard of a given colour (e.g. blue) and no reward under a placard of a different colour (e.g. green). Bumblebees that received an unexpected sucrose reward on their way out to the foraging arena, just before being presented an ambiguous colour (e.g. magenta), took less time to visit the placard than bumblebees that did not receive the reward. This suggests these individuals were in a positive emotional state. Accordingly, bumblebees trained to a feeder and given an unexpected reward on their way out to the foraging arena took less time to resume foraging after being squeezed by a sponge at the entrance of the arena (i.e. to mimic predator attack), which can be interpreted as improving their negative mood. These behavioural changes were abolished with topical application of a dopamine antagonist, suggesting common neural processing features of emotions with vertebrates (Perry et al. 2016).

Internal representations of the world

Another lively debated question is whether insects build internal representations of themselves and their world (Cheeseman et al. 2014, Cheung et al. 2014). Like many animals, bees frequently move through densely cluttered environments to forage and return home. Ravi et al. (2020) recently showed that flying bumblebees judge whether they can move through a gap based on knowledge of their body size. The authors trained small and large bumblebees to fly through a wall with a hole leading to a foraging arena. Using high-precision 3D video tracking, they found that bees successfully flew through narrow gaps, even those that were much smaller than their wingspans, by first performing lateral scanning to visually assess the aperture, and then reorienting themselves, sometimes flying sideways. This suggests insects account for their own size and shape to safely navigate through complex environments.

More than just having a sense of their body size, animals may need to develop internal images of their world and transfer these images across sensory modalities so the information can be used in different contexts. For instance, we humans build mental images allowing us to recognize an object out of view, only by touch. Solvi et al. (2020) tested this ability in bumblebees searching for sucrose rewards in objects distributed in a lab flight arena, either visually or by touch (Figure 2c). Bumblebees trained to discriminate cubic against spherical objects using vision behind a screen, could apparently also recognize by touch only in the dark, and vice versa. This suggests insects form modality-independent internal representations of object shapes, an ability that we humans are explicitly self-aware of, although other explanations cannot completely be ruled out for insects (Le Moël et al. 2020).

Navigation

Studying bee movements across landscapes is a longstanding challenge, key to understanding how insects perceive the world and pollinate plants. Since Karl von Frisch (von Frisch 1967) cracked the code of the honey bee waggle, most research on bee navigation and large-scale foraging has focused on how individual foragers learn to move between its colony nest and one or two feeders. Reading the waggle dance can tell us how distances and directions to the feeders are perceived, learnt and communicated by the bee. This research has been particularly useful to understand the basic ‘tool box’ underpinning navigation (e.g. path integration (Srinivasan et al. 2000)) and explore ways space could be encoded in a miniature brain (Dyer 1991, Riley et al. 2005, Menzel et al. 2011, Cheeseman et al. 2014, Mertes et al. 2014). However, the more recent utilisation of bumblebees in navigation studies considerably broadened the scope of investigations, with new emerging scientific questions. Unlike honey bees, bumblebees do not communicate the location of food resources to their nestmates (Dornhaus and Chittka 1999). This means bumblebees must search and exploit for multiple food resources by themselves, based on their own experience. Accordingly, the focus of bee navigation research tended to move from single-destination to multiple-destination route learning. This change was facilitated by the fact that bumblebees live in small colonies in which every individual can be distinctively tagged and tracked during their entire lifespan (Woodgate et al. 2016).

How multi-destination routes develop is a key example. For bee species exploiting patchily distributed resources, visiting multiple feedings sites in an efficient way represents a challenging routing problem analogous to the well-known Traveling Salesman Problem, in which the task is to find the shortest path to visit each location once and return to the origin (Lihoreau et al. 2013). Bumblebees, and many other wild bees, have long been reported to visit familiar feeding sites in a stable order, a routing behaviour called 'traplining' (Janzen 1971, Thomson et al. 1997). Using semi-automated feeders to remotely replenish feeding sites, it was shown that individual bumblebees spontaneously learn and remember efficient (if not the shortest possible) traplines to visit all feeders once and bring back food to their colony nest (Ohashi et al. 2007, Lihoreau et al. 2012a). This behaviour was observed in arrays of feeders with various geometries and at different spatial scales in the lab and in the field. Detailed monitoring of bumblebee flight paths with harmonic radar in commercial colonies placed in the field (Figure 2d), revealed foragers reduced overall travel distance using flight vectors to link flowers following the shortest route, while maintaining high levels of exploration to react to potential changes in resource availability (Lihoreau et al. 2012b, Woodgate et al. 2017). Recently, honey bees were also shown to learn near optimal routes between a small number of feeders (although less efficiently), suggesting this spatial behaviour is shared by most bee species (Buatois and Lihoreau 2016, Buatois et al. 2020)**.**

Nutrition

Bees have been central for the development of optimal foraging theory in the 1970s (Charnov 1976), assuming they prioritised visits to flowers delivering nectars with the highest concentrations of carbohydrates or in the largest volumes (Pyke 1979, 1980). In recent years, however, advances in nutrition research has revolutionised this vision, demonstrating the need for foragers to collect different nutrients in specific amounts and balances, in order to meet the diverging needs of all colony members, such as non-foraging workers (requiring carbohydrates as main energy source) as well as the queen and the brood (requiring proteins for reproduction and development) (Wright et al. 2018). The picture is more complex than previously thought, and bumblebees have played an important role for unravelling these regulatory processes thanks to the possibility to maintain functional microcolonies foraging on artificial diets in the lab.

Nutrient balancing

The ability of bees to balance their diet has been demonstrated using nutritional geometry, a conceptual framework to study food choice and its fitness consequences (Raubenheimer and Simpson 2020). In particular, “cafeteria” experiments, in which insects can select artificial diets of known nutritional compositions, provide a powerful approach to identify their nutritional needs and strategies to meet them (Lihoreau et al. 2018). In this approach, Stabler et al. (2015) demonstrated that bumblebee workers given access to artificial diets with different ratios of proteins and carbohydrates collected different volumes of the diets in order to reach a 1:149 w/w protein to carbohydrate ratio. This selective nutrient collection varies with the form of the nutrients in food (e.g. free amino acids or bounded in proteins) (Stabler et al. 2015). Studies using artificial pollen in food choices highlighted the importance of regulating lipid collection: as bumblebees avoid over ingesting fat contrary to protein and carbohydrates (Vaudo et al. 2016, Kraus et al. 2019). Lipid intake increases the performance on visual learning and survival (Muth et al. 2018) but can also be toxic when consumed in excess (Vaudo et al. 2016, Muth et al. 2018).

Food quality perception

Important progress has also been done with bumblebees for understanding nutrient sensing by pollinators. Bee foragers assess food quality using nutritional cues, such as the concentration of sugars in nectar (i.e. sucrose, fructose and glucose) (Mommaerts et al. 2013). Protein and lipids can also be sensed in pollen (Ruedenauer et al. 2020). For instance, bumblebees whose proboscis extension reflex was conditioned can predict the income of a reward by discriminating the presence of proteins in the conditional stimulus (Ruedenauer et al. 2015). While olfactory cues alone enable such detection, chemotactile cues through antennal contacts are necessary to sense the presence and concentration of proteins. All of these cues on flower resources help bees to select the quantity and balance of nutrients needed by bees. Note however that other food compounds can influence bee nutritional choices. In particular, foragers can be tricked by secondary metabolites such as caffeine and nicotine, that despite their relatively low amounts in nectar attract bees to flowers with nectar and/or pollen of suboptimal nutritional values (Thomson et al. 2015, Baracchi et al. 2017).

Environmental stressors

Wild bees are declining in most of the industrialized world. This decline has received considerable attention in the past decade as the loss of main pollinators can have dramatic consequences on ecosystems and food security (Goulson et al. 2015). Bumblebees are increasingly used as sentinel species to assess environmental risks for pollinators and have been key to demonstrating the impact of agrochemicals on pollinators. Firstly, their small and manageable colonies offer a unique opportunity for a comprehensive assessment of the multi-level effects of controlled exposure to stressors on individuals and colonies. Secondly, in contrast to domesticated honey bees whose populations are not declining per se, results on commercial bumblebees can realistically be extrapolated to natural populations and other declining wild bees.

Pesticides

Neonicotinoid pesticides are arguably one of the main contributors to bee declines (Goulson et al. 2015). These are widespread insecticides sprayed on plants, applied to soils or used for seed coating of flowering crops for pest control. While the first negative effects of an exposure to low concentrations of these compounds were reported on honey bees (Henry et al. 2012), detailed analyses of sublethal effects come from observations on bumblebees. For instance**,** Kessler et al. (2015) investigated whether bumblebees could detect neonicotinoids in food. Bumblebees exposed for 24h to field-realistic concentrations of three main neonicotinoids (Imidacloprid, Thiamethoxam, Clothianidin) were unable to avoid natural concentrations and increased their food consumption, even though high concentrations significantly reduced their survival. Electrophysiological recordings of gustatory neurons located on the proboscis revealed bees lack taste neurons responding to these compounds. In fact, bumblebees acquired a preference for treated food (presumably because they target neural circuits causing addiction) (Arce et al. 2018), increasing all the more the risks for the colony during prolonged pesticide exposure.

To mimic a realistic scenario of exposure and better assess the potential of colonies to buffer combinatorial effects, Gill et al. (2012) exposed bumblebee colonies to field-realistic concentrations of the neonicotinoid Imidacloprid, the pyrethroid Cyhalothrin and both. Chronic exposure to the two pesticides impaired natural foraging behaviour, significantly reduced brood development and colony success, highlighting that combined exposure to pesticides increases the propensity of colonies to fail. Crall et al. (2018) developed an automated robotic platform for continuous, multi-colony monitoring of uniquely identified workers in the long term, which would be hardly feasible with much larger honey bee colonies. They demonstrated that chronic exposure to field-realistic levels of Imidacloprid impaired the in-nest behaviour of workers, including nursing and social interactions. These results stress out the multifaceted behavioural impacts of neonicotinoids and illustrate the potential of high-throughput, automated analysis for improving the understanding of agrochemicals impacts (Marchal et al. 2019). Mechanistic approaches like brain cell cultures (Wilson et al. 2013, Moffat et al. 2015), brain volumetrics (Smith et al. 2020), pharmacology and genomic studies have also started to reveal some details of the molecular and cellular actions of these molecules that may explain their behavioural consequences in bumblebees.

The detailed studies of the sublethal effects of harmful pesticides such as neonicotinoids resulted in a more constrained utilisation of these compounds. This legislative reassessment led to the development of alternative practices and products (Colin et al. 2020). Here again bumblebees have become tractable models for a rapid risk assessment through the screening of multiple behavioural traits across several colonies. For instance, Sulfoximine-based insecticides are a priority issue since they are the most likely successors of neonicotinoids (Brown et al. 2016), already licensed for use (China, USA) or under consideration (European Union). In an attempt to pre-emptively evaluate their potential sub-lethal effects on pollinators, Siviter et al. (2018) chronically exposed bumblebee colonies to Sulfoxalor at concentrations consistent with potential post-spray field exposure. They showed this new compound had severe sub-lethal effects on colony development. The difference between life-history trajectories of treated and control colonies appeared at the eclosion of larvae, suggesting potential cumulative long-term consequences for colony fitness. These results call for caution against the use of Sulfoximine as a direct replacement for neonicotinoids.

Combinations of stressors

The study of the impact of environmental stressors on pollinators calls for a holistic approach. But disentangling the individual and interacting effects of stressors, at different scales (individuals, colonies, populations), is a considerable challenge that cannot be done by empirically testing all combinations and contexts. Mechanistic models have been used to better explore the multilevel impacts of populations under stress. To answer this need for structural realism with the incorporation of multiple stressors operating at different organizational levels, Becher et al. (2018) developed the Bumble-BEEHAVE model. In this approach, and its subsequent derivations, simulations can predict the effects of multifactorial stressors on bumblebee survival at the individual, colony and population levels. One important aspect of modelling bumblebee colonies is the ease at which model predictions can be tested using toxicological experiments and measures on actual colonies in the lab or semi-field conditions (Gill et al. 2012, Crall et al. 2018). This represents a significant step towards realistically predicting bee population dynamics under stress. It can be used by scientists and stakeholders, for instance, to explore combined effects of stressors on population success, to predict pollination services, to test the relative effects of policy recommendations, all in realistic landscapes.

Host-parasite interactions

Bees are hosts of a large number of parasites and pathogens including viruses, bacteria, fungi, protozoa, nematodes as well as other arthropods (i.e. diptera parasitoids, acarids) (Schmid-Hempel 2001). Commercial bumblebees provide many advantages to study these host-parasite interactions. Firstly, individuals of all developmental stages (larvae, adults) can be exposed and maintained in the lab, thus allowing for precise and differentiated manipulations of host-parasite interactions. Secondly, colony members are full siblings (most bumblebee species are monandrous (Estoup et al. 1995)), thereby providing optimal conditions for parasite transmission across nestmates (Baer and Schmid-Hempel 2003). Indeed, genetic diversity is thought to reduce parasite infection in insect colonies, as shown in *B. terrestris* gynes artificially mated with multiple males that produced colonies with better reproductive success and lower parasite prevalence than colonies from monandrous queens (Baer and Schmid-Hempel 1999).

Transmission dynamics

Among the numerous parasites of bumblebees, the trypanosome *Crithidia bombi* has received the most attention, both because it is highly prevalent in the field (e.g. up to 82% in North America (Gillespie 2010)) and it has moderate virulence, rarely leading to colony collapse (Schmid-Hempel 2001). *C. bombi* attaches to the wall of the epithelial cells or stays in the lumen of the gut of bumblebees (Solter et al. 2012). Within colonies, transmission occurs by ingestion of parasite cells in contaminated faeces. Using automated video-tracking to record the movements of every worker in colon**ies**, Otterstatter and Thomson (2007) showed infection spreads more or less rapidly depending on the density of individuals and the properties of contact networks. Durrer and Schmid-Hempel (1994) further showed horizontal transmission is common between colonies and between species. The authors monitored uninfected *B. lucorum* foraging on inflorescences previously visited by naturally parasitized *B. lucorum* or *B. terrestris* and evaluated their rate of contamination. Intra- and inter-specific transmission occurred, proving that shared flowers during foraging trips could act as an infection vector across wild pollinators.

Immune responses

Once parasites or pathogens enter the host, an immune response is activated. Bumblebees have recently emerged as a model system to study the collective and individual defence mechanisms in social insects. At the colony level, bumblebees display cooperative behaviours to prevent and fight infections (i.e. social immunity). For instance, workers parasitized by conopid flies tend to spend the night outside the nest, which delays the development of the parasite larvae due to lower temperatures (Müller and Schmid-Hempel 1993). At the individual level, it is possible to study the immune response of bumblebees by mimicking parasite infection with injection of immune elicitors such as lipopolysaccharides (LPS) or sterile micro-latex beads (Moret and Schmid-Hempel 2000). For instance, the injection of LPS decreases the production of an immune response which reduces the survival (Moret and Schmid-Hempel 2000) and the foraging efficiency (Mobley and Gegear 2018) of individuals. Further studies showed that individual immune responses are context-dependent, and are modulated by the social environment. Hence, bumblebee workers maintained in groups exhibited an enhanced expression of antimicrobial peptides (AMPs), compared to those kept in isolation that, on the contrary, showed a higher phenoloxidase activity (Richter et al. 2012). The immune response can also be transmitted to the next generation, even though insects do not produce antibodies that could transmit specific immunity. Indeed, bumblebee colonies challenged with LPS produced males with increased constitutive immunity, displaying high phenol oxidase (PO) activity, a protective factor against microorganisms (Moret and Schmid-Hempel 2001). Triggering the immune response with LPS can prove very useful to understand which effects are actually caused by parasites or by the immune response itself.

Microbiota

The host microbiota of insects can also be involved in defence against parasites (Koch and Schmid-Hempel 2011). This is the case of the community of bacteria inhabiting the bumblebee gut, which is mainly composed of Gammaproteobacteria, Firmicutes, Betaproteobacteria and Alphaproteobacteria (Koch and Schmid-Hempel 2012). Bumblebees raised in social isolation and later fed faeces from nestmates, developed the same microbiota community as their conspecifics, which conferred them protection against a potential infection with *C. bombi*, as they showed lower parasite loads than workers fed antibiotics or other bacteria (Koch and Schmid-Hempel 2011). More than protection against infection, microbiota can mediate host-parasite interactions. By swapping the microbiota between bumblebees from different colonies and infecting them with different *C. bombi* strains, Koch and Smith-Hempel (2012) showed the microbiota was driving host-parasite interactions more than host genotype and that some specific strains of *C. bombi* were more successful to develop and generate infection with different microbiota patterns.

Behavioural effects

Parasites and pathogens often influence the behaviour of their hosts, and sometimes even manipulate them to enhance their own chance of reproduction and dispersion (Ponton et al. 2006). Bumblebees have been important to clarify some effects of common parasites on wild bees in controlled laboratory setups. For example, bumblebees exposed to *Nosema ceranae* (Fries et al., 1996) in food, a microsporidian parasite of domestic honey bees (Higes et al. 2006), showed reduced olfactory learning (Piiroinen and Goulson 2016). Bumblebees infected with *C. bombi* showed reduced abilities to learn to discriminate between flowers of different colours and odours (Gegear et al. 2006). These effects of parasites on host cognition are likely due to the triggering of the immune response more than to the action of the parasite itself since bumblebees injected with LPS also had problems learning colours of rewarding flowers (Alghamdi et al. 2008).

Perspectives

Over the past decades, bumblebees have emerged as model species to study various aspects of apidology, sometimes opening up the possibility to tackle problems with new angles as compared to more classical models such as honey bees. This is because bumblebees can be handled in the lab all year round, and allow easier experimental manipulation of their small-size colonies. Importantly, many of the findings on bumblebees have been later confirmed in honey bees but also in other wild bee species, demonstrating their utility as model species (e.g. Riveros and Gronenberg 2009, Buatois and Lihoreau 2016). As a result, experimental research on bumblebees is developing quickly and yields considerable promises for future research on emerging topics on bee behaviour and ecology. Below we list some of these exciting avenues.

Bumblebees have been used in highly artificial experimental setups to study fundamental principles of vision underpinning various forms of learning, memory and navigation (Figure 2). Several new insights may arise from fast developing technologies to test and track insects. For instance, studies have begun to develop virtual reality setups in which freely moving individuals can be trained to fly and forage in virtual worlds and return to their colony (Frasnelli et al. 2018) (Figure 2e). Using virtual worlds enables a precise control of the visual stimuli available to insects. It therefore brings new avenues to study how flying insects learn different types of visual cues and selectively use them in different contexts of navigation, such as search, homing or route development. Virtual reality could be used for controlling the visual experience of the bee during training and implementing typical displacement experiments (i.e. the bee is caught at a familiar site and released at an unfamiliar site (Cheeseman et al. 2014)). In this approach, potential biases resulting from uncontrolled familiarity of bees with their visual environment would be totally precluded, a prerequisite for further exploration of the internal representation of space in the bee brain (Cheung et al. 2014).

Bumblebees are large and robust insects that greatly facilitate the study of spatial movements in the field. They can carry tags or transponders often required for automated movement tracking using radars (Riley et al. 1996) or telemetry (Daniel Kissling et al. 2014). Developments of such tracking technologies are necessary to study how bees navigate and interact over large spatial scales (beyond the ca. 1km catching area currently possible with an harmonic radar (Riley et al. 1996), and in three dimensions (for instance between resources at ground level and on top of hills (Dore et al. 2020)), which may require even larger equipment. Bumblebees are particularly suitable for research on social and competitive interactions as they do not exploit resources en masse, thus enabling addressing questions about resource partitioning that may face most pollinators (Pasquaretta et al. 2019). All these aspects of bee navigation are still poorly understood but may be most easily addressed with bumblebees.

Automated monitoring systems based on computer vision now allow to track in real time the behaviour of all individuals simultaneously with unprecedented details, both in and out of the hive (Marchal et al. 2019). Bumblebees greatly facilitate these approaches due to their relatively small colonies in which all individuals can be tagged for individual identification. As illustrated above, such detailed analyses of bee behaviour and interactions can inform about stress levels (Gill et al. 2012, Crall et al. 2018). Further developments of these methods will allow to better capture the sublethal effects of environmental stressors - including new molecules to be put on the market - by considering a wider diversity of fitness-related traits than the classical survival or more recent cognitive deficits. In particular, the detailed understanding of the nutritional requirements of bumblebees and their responses to environmental changes may offer powerful ways to mitigate these non-desired effects. For instance, it is easy to envision how this knowledge can be used for conservation, by offering plants with pollen and nectars providing the required nutrients for colony development and reproduction (Vaudo et al. 2020). A similar approach could be used to maximise the efficiency of plant pollination by commercial colonies, for instance by attracting bees on specific plant resources that will meet the specific nutritional needs of colonies.

The utilisation of bumblebees holds considerable promises for developing mechanistic studies thanks to the development of biotechnologies to study brains and the neuro-genetic bases of behaviour. For instance, a promising avenue is the opportunity to characterize behavioural intra- and inter-individual variability and understand their neural bases. For this, the influence of body size in division of labour (rather than age as in honey bees) offers an interesting model for studies of plastic behaviours. Indeed, for bumblebees the probability to engage into foraging tasks increases with body size, which is easily measured and varies greatly within colonies (Brian 1952). Although brain size correlates with body size, some brain centres likely involved in important cognitive functions (e.g. navigation, learning) show allometric relationships (Mares et al. 2005). In addition, their maturation appears to be partially shaped by life experiences such as early sensory stimulations (Jones et al. 2013) and foraging experience (Riveros and Gronenberg 2010). Thus, the emergence of at least some of the remarkable behaviours described in this review may depend on specific developmental trajectories and/or life histories, and may contribute to a loose but efficient task specialization within small colonies.

Finally, it is very likely that bumblebees become genetic models in the near-future. Current attempts to edit genomes of honey bees with the CRISPR/Cas9 tool face the difficulty of creating and maintaining genetic strains (queens must be genetically modified, and long-lived colonies - strains - must be maintained) (Kohno et al. 2016, Otte et al. 2018). This also raises the problem of unwanted gene flows if genetically modified gynes or males escape in the wild. As the genome of some bumblebee species is now available (Sadd et al. 2015), bumblebees constitute attractive organisms for genetic manipulations, with their short colony cycles, and the possibility to confine colonies more easily in the lab.

Conclusion

Bee research has long focused on honey bees for practical reasons and because they display a fascinating social life. However, just like for all other disciplines of biology and ecology, moving away from research based on single model organisms, by adapting the choice of species to the addressed scientific questions is a good thing that may open new opportunities and lead to breakthroughs (Laurent 2020). As we have seen above, bumblebees offer many opportunities for studying new aspects of bee behaviour in laboratory setups but also in the wild. Since their domestication for commercial pollination, bumblebees have constituted tractable species to work with and address questions that are difficult to tackle with honey bees or some other domesticated bees. An important advantage of using bumblebees is the relevance of the results for wild pollinators, although differences in behaviour and stress tolerance between domesticated and wild bumblebees should be studied in more details. Ultimately increasing the diversity of species in bee research, in particular those topics related to pollination and conservation, can only be beneficial to identify suitable solutions against population declines (Carnell et al. 2020), as these species are likely to have different nutritional requirements and responses to stressors.

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Summary

Honey bees are crucial pollinators. A plethora of environmental stressors, such as agrochemicals, have been identified as contributors to their global decline. Especially, these stressors impair cognitive processes involved in fundamental behaviours. So far however, virtually nothing is known about the impact of metal pollutants, despite their known toxicity to many organisms. Their worldwide emissions resulting from human activities have elevated their concentrations far above natural baselines in the air, soil, water and flora, exposing bees at all life stages. The aim of my thesis was to examine the effects of metallic pollution on honey bees using a multiscale approach, from brain to colonies, in laboratory and field conditions. I first observed that bees exposed to a range of concentrations of three common metals (arsenic, lead and zinc) in the laboratory were unable to perceive and avoid, low, yet harmful, field-realistic concentrations of those metals in their food. I then chronically exposed colonies to field-realistic concentrations of lead in food and demonstrated that consumption of this metal impaired bee cognition and morphological development, leading to smaller adult bees. As metal pollutants are often found in complex mixtures in the environment, I explored the effect of cocktails of metals, showing that exposure to lead, arsenic or copper alone was sufficient to slow down learning and disrupt memory retrieval, and that combinations of these metals induced additive negative effects on both cognitive processes. I finally investigated the impact of natural exposure to metal pollutants in a contaminated environment, by collecting bees in the vicinity of a former gold mine, and showed that individuals from populations most exposed to metals exhibited lower learning and memory abilities, and development impairments conducing to reduced brain size. A more systematic analysis of unexposed bees revealed a relationship between head size, brain morphometrics and learning performances in different behavioural tasks, suggesting that exposure to metal pollutants magnifies these natural variations. Hence, altogether, my results suggest that honey bees are unable to avoid exposure to field-realistic concentrations of metals that are detrimental to development and cognitive functions; and call for a revision of the environmental levels considered as ‘safe’. My thesis is the first integrated analysis of the impact of several metal pollutants on bee cognition, morphology and brain structure, and should encourage further studies on the contribution of metal pollution in the reported decline of honey bees, and more generally, of insects.

Résumé

Les abeilles sont des pollinisateurs essentiels. Une pléthore de facteurs de stress environnementaux, tels que les produits agrochimiques, a été identifiée comme contribuant à leur déclin mondial. En particulier, ces facteurs de stress altèrent les processus cognitifs impliqués dans les comportements fondamentaux. Jusqu'à présent, cependant, on ne sait pratiquement rien de l'impact de l’exposition à des métaux lourds, dont la toxicité est avérée chez de nombreux organismes. Pourtant, leurs émissions mondiales résultant des activités humaines ont élevé leurs concentrations bien au-dessus des niveaux naturels dans l'air, le sol, l'eau et la flore, exposant ainsi les abeilles à tous les stades de leur vie. Le but de ma thèse était d'examiner les effets de la pollution métallique sur l’abeille domestique en utilisant une approche multi-échelle, du cerveau à la colonie, en laboratoire et sur le terrain. J'ai d'abord observé que les abeilles exposées à une gamme de concentrations de trois métaux communs (arsenic, plomb et zinc) en laboratoire étaient incapables de percevoir et éviter des concentrations usuelles, néanmoins nocives, de ces métaux dans leur nourriture. J'ai ensuite exposé de façon chronique des colonies à des concentrations réalistes de plomb dans la nourriture et démontré que la consommation de ce métal altérait la cognition et le développement morphologique des abeilles. Comme les polluants métalliques se trouvent souvent dans des mélanges complexes dans l'environnement, j'ai exploré l'effet des cocktails de métaux, montrant que l'exposition au plomb, à l'arsenic ou au cuivre seul était suffisante pour ralentir l'apprentissage et perturber le rappel de la mémoire, et que les combinaisons de ces métaux induisaient des effets négatifs additifs sur ces deux processus cognitifs. J'ai finalement étudié l'impact de l'exposition naturelle aux polluants métalliques dans un environnement contaminé, en collectant des abeilles à proximité d'une ancienne mine d'or, et montré que les individus des populations les plus exposées aux métaux présentaient des capacités d'apprentissage et de mémoire plus faibles, et des altérations de leur développement conduisant à une réduction de la taille de leur cerveau. Une analyse plus systématique des abeilles non exposées a révélé une relation entre la taille de la tête, la morphométrie du cerveau et les performances d'apprentissage dans différentes tâches comportementales, suggérant que l'exposition aux polluants métalliques amplifie ces variations naturelles. Ainsi, mes résultats suggèrent que les abeilles domestiques sont incapables d'éviter l’exposition à des concentrations réalistes de métaux qui sont préjudiciables au développement et aux fonctions cognitives, et appellent à une révision des niveaux environnementaux considérés comme «sûrs». Ma thèse est la première analyse intégrée de l'impact de plusieurs polluants métalliques sur la cognition, la morphologie et l’organisation cérébrale chez l’abeille, et vise à encourager de nouvelles études sur la contribution de la pollution métallique dans le déclin signalé des abeilles, et plus généralement, des insectes.