

University of Mons
Faculty of Science

**When trace metals are no longer trace:
Impact of Cadmium and Copper on the
Behaviour and Physiology of a Model Pollinator
(*Bombus terrestris* L.)**

Master's thesis submitted by **Luca Dorio**, in order to
obtain the degree of **Master in Biology of Organisms and
Ecology: Research Finality**

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L'auteur, Dorio Luca, atteste avoir respecté les règles éthiques en vigueur, y compris la charte de l'Université relative à l'utilisation de l'Intelligence Artificielle

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Abstract:

The relentless human imprint on Earth has led to catastrophic failure in ecosystems, including the introduction and spread of hazardous pollutants in nature. Trace metals are of concern due to their towering and meaningful presence in the environment provided by human activities, in addition to posing a significant threat to living organisms due to their toxicity. Trace metal pollution affects many life groups including pollinators, for which the toxicity of trace metals and their impacts are still elusive. In particular, the sublethal effects caused by trace metals on the physiological systems and the behaviours of bees remain understudied. In this study, we aimed to address these knowledge gaps by testing the sublethal effects of two prevalent metal pollutants, namely copper (Cu) and cadmium (Cd), on buff-tailed bumblebee workers, as a model pollinator. Our study first assessed their ability to detect various concentrations of these metals in sucrose solutions. We found that bumblebees were unable to detect solutions laced with trace metals. Environmental concentrations of Cu and Cd, as well as a lethal concentration of Cd, were not rejected. By contrast, the lethal, field-unrealistic concentration of Cu was avoided. These results suggest that these metals are readily ingested in natural conditions. Moreover, our study assessed the impacts of environmentally relevant concentrations of Cd and Cu on bumblebees' walking behaviour, flying behaviour, oocyte size and lipidic content. We found that the behaviour and physiology of bumblebee workers were not affected by these field-realistic concentrations. Basic behaviours such as walking and flying abilities of bumblebees were not altered, nor were abdominal lipidic content or terminal oocyte size. Finally, we assessed the effects of Cd and Cu on the midgut epithelium of bumblebees. Exposure to environmental concentrations did not lead to melanisation in the midgut epithelium, yet the exposure to higher concentrations induced a strong melanisation response, highlighting sublethal effects at higher concentrations. Overall, exposure to environmental concentrations did not lead to any changes in behavioural abilities of bumblebees, nor they impacted their physiology. Compared to other studies, the environmental concentrations we used were lower, and the duration of exposure was shorter, which could explain some discrepancies with the literature. We recommend for future studies to use higher concentrations to better reflect the worst-case scenarios of the field, with multiple sources of exposition, and over longer periods of time.

Keywords: Trace metals, Pollinator, *Bombus terrestris*, Cadmium, Copper, Anthropocene

Résumé en français :

L'empreinte de l'homme sur la biosphère a mené à un état catastrophique des écosystèmes, notamment occasionné par l'introduction de polluants en grande quantité dans l'environnement. Les métaux traces font partie de ces polluants et sont préoccupants par leur présence en grande quantité dans l'environnement, due aux activités humaines, et constituent une menace importante pour la biodiversité en raison de leur toxicité. Ces métaux traces affectent de nombreuses formes de vie, y compris les pollinisateurs, pour lesquels la toxicité et l'impact de ces métaux traces sont encore difficiles à cerner. Notamment, les effets sur la physiologie et le comportement des abeilles sont encore assez flous et font toujours l'objet d'intenses discussions. Nous avons cherché à répondre à cette problématique en investiguant les effets sublétaux de deux métaux prépondérants dans la nature, à savoir le cuivre (Cu) et le cadmium (Cd), sur des ouvrières de bourdons, utilisées comme pollinisateur modèle. Notre étude a tout d'abord confirmé que les bourdons étaient incapables de détecter des métaux traces présents dans leur source de nourriture et consommait des solutions de sucrose contenant des doses environnementales de Cu et de Cd ainsi que des doses létales de Cd. Seule la dose létale mais non-environnementale de Cu a été évitée, ce qui suggère que les bourdons ingèrent ces deux métaux dans des conditions naturelles. Néanmoins, nos résultats révèlent également que ces mêmes concentrations environnementales n'affectent ni le comportement ni la physiologie des bourdons. Des comportements de base tels que la marche et le vol des bourdons n'ont pas été affectés par ces concentrations, pas plus que le contenu lipidique et la taille des ovocytes des bourdons. Enfin, l'exposition à des doses environnementales n'a pas entraîné de mélanisation dans l'épithélium du tube digestif des bourdons. Cependant, l'exposition à des concentrations plus élevées a conduit à des dégâts importants dans le tube digestif, mettant en évidence d'éventuels effets à des concentrations plus élevées. L'exposition à concentrations environnementales de Cd et Cu n'ont entraîné aucune modification des capacités cognitive des bourdons et n'ont eu aucun impact sur leur physiologie. Cependant, en comparaison à d'autres études, nos concentrations étaient assez faibles, et la durée d'exposition plus courte. Nous recommandons pour les études futures d'utiliser des concentrations plus élevées afin de mieux refléter la réalité du terrain avec des sources d'exposition multiples, sur des périodes plus longues.

Mots-clés : Métaux traces, Pollinisateur, *Bombus terrestris*, Cadmium, Cuivre, Anthropocène

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Chapter 1: Introduction

Over the past 50 years, the unstoppable loss of biodiversity across the world, mainly due to landscape transformation, climate change and environmental pollution, has led to dramatic changes in Earth's ecosystems. This regrettable observation comes as a result of the increase in human activities, driving up the demand for resource and energy production, with its associated massive industrialisation and exploitation of natural resources (Díaz et al., 2019; IPBES, 2019; Lewis & Maslin, 2015). The human imprint on the biosphere has expanded to such a rate that the 'Anthropocene' epoch – meaning that humans have influenced and modified their environment so much that it can be seen at the geological time scale – has been suggested (Lewis & Maslin, 2015; Malhi, 2017). A major consequence of these human activities has been the increasing concentration of pollutants in the environment (i.e. inorganic ions, metallic compounds, nanoparticles, industrial waste, inorganic gas, radioactive isotopes) (Briffa et al., 2020; Walker et al., 2012). Exposure to these xenobiotics represents a major threat to ecosystems and biodiversity. Among pollutants, trace metals have gained attention in these last years (Monchanin et al., 2021). Trace metals are known to naturally occur in the environment (Bradl, 2005) but a large quantity of trace metals with anthropogenic origin has been introduced in excess across all habitats (Briffa et al., 2020; El-Kady & Abdel-Wahhab, 2018; Tchounwou et al., 2012). Trace metal pollution represents a real danger for many organisms as well as for many ecosystem functions. There are carcinogenic, neurotoxic and cause severe damages on cells (Bower et al., 2005; Briffa et al., 2020; He et al., 2005). In 2011/12, trace metals and oil minerals were the main pollutants in European soils, contributing around 60% to soil contamination (Panagos et al., 2013). Even if trace metal emissions have decreased these last years in Europe (EEA, 2023), their non-biodegradable properties and bioaccumulation in organisms makes them very persistent and they continue to cause damage years after they were emitted (Bradl, 2005; Tchounwou et al., 2012).

1.1 Trace metals

1.1.1 Definition and origins

Trace metals are often related to as heavy metals, a term that has been used for decades in the literature and in the common language, both by scientists and the general public. However, this term has poor scientific support and is now considered imprecise, meaningless and inappropriate (Alloway, 2012; Duffus, 2001; Pourret, 2018). Indeed, despite the numerous articles about the origins and effects of 'heavy metals' on ecosystems, there are still a lot of misconceptions about what 'heavy metals' really are without any clear definitions. This lack of standard definition caused

a lot of misunderstanding, so there is a need to ban this term from the literature (Pourret, 2018) and the use of specific terms like 'trace metals' may be more appropriate (Alloway, 2012; Pourret, 2018). Trace metals are defined as metallic trace elements that naturally occur (so non-synthetic) in the Earth's crust (Alloway, 2012; Bradl, 2005) and are spread in the litho-, atmo-, hydro- and biosphere (Alloway, 2012; Tchounwou et al., 2012). These elements are present at low concentrations (trace amounts), <1000 mg kg⁻¹ in rocks and soil and <100 mg kg⁻¹ (depending on the organisms studied) in dry matter of living organisms (Alloway, 2012; Sabljic, 2009). They contrast with macro-elements like Si, Al, Na, C, P, S, Mg, Ca and K, that are present in ways higher concentrations (Berdanier et al., 2008; Yaroshevsky, 2006) (Fig.1).

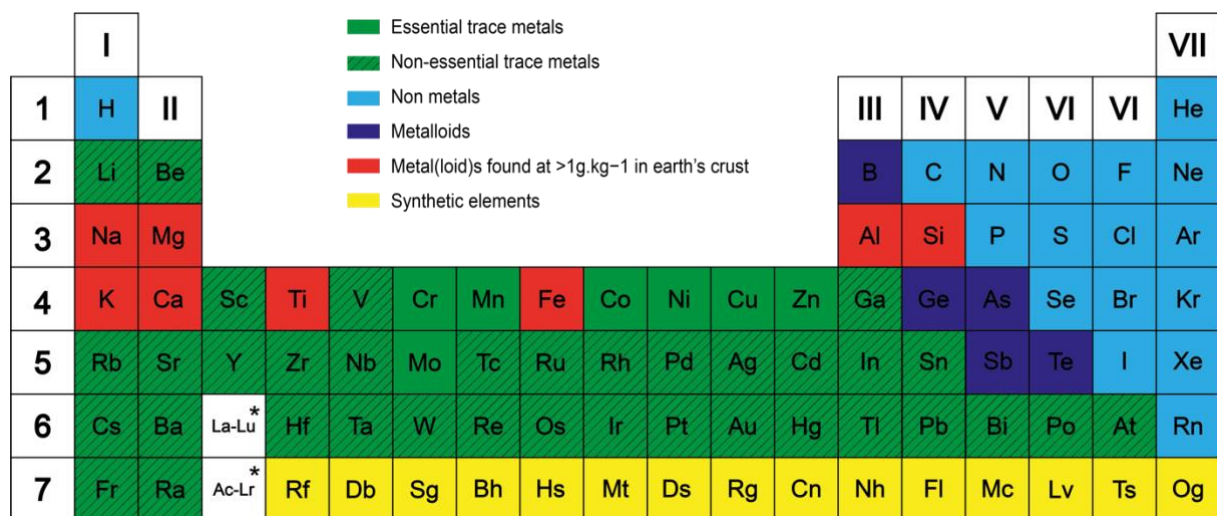


Figure 1 : **Periodic table highlighting trace metal elements:** Periodic table showing which elements are considered as trace metal according to the definition (Alloway, 2012; Bradl, 2005). Trace metals are in green with the non-essential (i.e. non-essential for biological functions) ones hatched. The elements groups of lanthanides and actinides (asterisk) are not included. Adapted from Gekière et al. (2023) and Maret et al. (2016).

Sources of trace metals have both natural and anthropogenic origins (Alloway, 2012; Bradl, 2005; El-Kady & Abdel-Wahhab, 2018; He et al., 2005; Tchounwou et al., 2012) (Fig. 2). Rocks from the Earth's crust are the main natural sources of trace metals (Bradl, 2005; Yaroshevsky, 2006). These trace metals are released and transported from rocks to environmental compartments through geochemical processes like volcanism, tectonic plate movement, soil formation, erosion, rock weathering, or fire forest (Alloway, 2012; Bradl, 2005; El-Kady & Abdel-Wahhab, 2018; He et al., 2005; Sabljic, 2009; Tchounwou et al., 2012). Trace metals are therefore naturally present in the environment and some are even essential (in small amount) for the sustainability of ecosystems and organisms' health (Maret, 2016; Sabljic, 2009). However, hazardous concentrations of trace metals are found in many locations (i.e. industrial, agricultural and urban zones) due to past or present human activities in these areas (Giglio et al., 2017; Qu et al., 2018; Zarić et al., 2016).

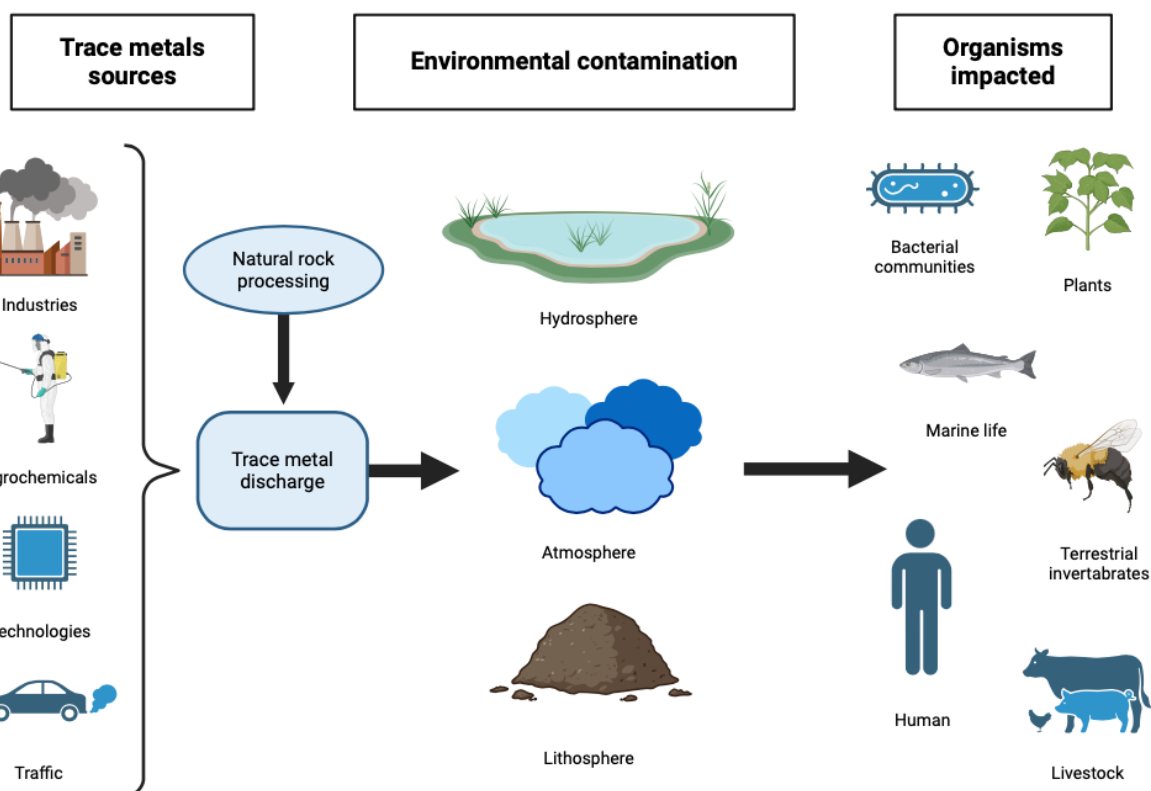


Figure 2: Summary of the transfer of trace metals in the environment: Representation of anthropogenic sources of trace metals and their spread in the different spheres of the environment, as well as organisms impacted by trace metal pollution.

The anthropogenic sources are mainly metal-based industries and agricultural activities (Fig. 2; Tab. 1) (Bradl, 2005; He et al., 2005; Nagajyoti et al., 2010; Tchounwou et al., 2012). Mining or smelting release large amount of trace metals by producing considerable quantities of mine tailings and wastes (i.e. wastewater and dust) (Bradl, 2005; Luo et al., 2023; Tchounwou et al., 2012). Metals are used as raw materials for everyday life products (Mohsen et al., 2022 ; Masindi & Muedi, 2018) like electronic devices (Lim & Schoenung, 2010; Maragkos et al., 2013), batteries (Melchor-Martínez et al., 2021), nanotechnology (Dabour et al., 2019 ; Rastogi et al., 2017), chemical products (Bernardes et al., 2022; Hooven et al., 2019), and pigments or colorants (Khan et al., 2021; Mertoglu-Elmas, 2017; Turner & Lewis, 2018). The overuse of these products and the lack of regulations for the proper storage and management of their waste led to their accumulation in open environments (Lim & Schoenung, 2010; Melchor-Martínez et al., 2021). The combustion of fossil fuel like coal, petrol and gasoline are also a non-negligible source of trace metals (Bradl, 2005). Fossil fuel contains trace metals as residues or additives which are released in atmosphere during combustion through fly ash and nanoparticles (Bradl, 2005; Briffa et al., 2020; Groysman, 2014). Agriculture is another major anthropogenic source of trace metals through the use of agrochemical products such as pesticides or fertilisers (Fig.2) (Bradl, 2005; El-Kady & Abdel-

Wahhab, 2018; He et al., 2005; Q. Zhang & Wang, 2020). It is now a common practice that fertilizers contain essential trace elements for correcting deficiencies in soil and boosting plant growth (He et al., 2005). Trace metals can be directly used as insecticides or fungicides like copper sulfate (CuSO_4) or copper hydroxide ($\text{Cu}(\text{OH})_2$) (Bernardes et al., 2022; Richardson, 1997). Trace metals are also found in non-metal-based pesticides as co-formulants like most of Glyphosate-based herbicides which contain worrying levels of trace metals (Defarge et al., 2018). Other organic materials such as farm manures, biosolids or composts also contain high concentrations of trace metals (Bradl, 2005; He et al., 2005).

1.1.2 Persistence in the environment and bioaccumulation

As metals harbour volatile (e.g. nanoparticles), solid and aqueous forms (e.g. ions), they spread easily in the lithosphere, hydrosphere and atmosphere, and are readily bioavailable and absorbed by living organisms (Bradl, 2005; Briffa et al., 2020; Tchounwou et al., 2012) (Fig.2). Trace metals are non-biodegradable compounds (Bradl, 2005; Tchounwou et al., 2012), and they have a tendency to accumulate in different compartments of the environment (e.g. water) and living organisms (bioaccumulation) like plants, marine organisms or terrestrial invertebrates (Akbar et al., 2023; Burden et al., 2019; Jakimska et al., 2011; Zwolak et al., 2019). Trace metals, through accumulation in biological materials, irremediably end up in food chains, increasing the risk of human health exposure to trace metals (Briffa et al., 2020; El-Kady & Abdel-Wahhab, 2018). Food contaminants are meat from farm animals (e.g. cattle; Waegeneers et al., 2009), plant foods (Briffa et al., 2020), animal sub-products (e.g. eggs, honey; Goretti et al., 2020; Kabeer et al., 2021; Zergui et al., 2023). Hence, metals are still present in the environment decades after their original emission (Alva 1992; Bradl, 2005; Tchounwou et al., 2012). In addition to bioaccumulation, other biochemical properties are important for trace metals mobility and assimilation like soil nature, pH, geochemical phenomena and organic matter (Akbar et al., 2023; Nnaji et al., 2023; Wang & Chen, 2009). The distance from the emission zones, the presence of other pollutants and climate parameters also play a role (Breidenbach et al., 2023; Elzwayie et al., 2017). Many remediation methods exist to remove excess of metals in the environment (C. Li et al., 2019; Tóth et al., 2016; Vareda et al., 2019). For soil, remediation methods using chemicals or biomolecules are commonly used (Vandenbossche et al., 2015; Wuana & Okieimen, 2011) but recently decontamination of polluted soils via bioaccumulator species such as metallophytes plants (i.e. phytoremediation) or microorganisms have been increasingly used (Nnaji et al., 2023).

Table 1 : Anthropogenic sources of trace metals: Table displaying anthropogenic sources of principal trace metal pollutants. Based on Alloway (2012), Bernhard et al. (2005), Bradl (2005), Briffa et al. (2020), Vithanage et al. (2022).

Sources	Be	Cd	Co	Cr	Cu	Hg	Mn	Mo	Ni	Pb	Sn	V	Zn
Batteries	○	●	○	○	○	●	●	○	●	●	○	○	●
Agrochemical products	○	●	○	●	●	●	●	○	○	●	●	○	●
Paints and pigments	○	●	●	●	●	○	●	○	●	○	○	●	●
Alloys and metal works	●	●	●	●	●	●	●	○	●	●	●	●	●
Fossil fuel	○	●	○	○	●	●	●	○	○	●	○	○	○
Steel works	○	○	○	●	○	○	●	●	●	●	●	●	●
Industrial waste	●	●	○	○	●	●	○	●	●	●	●	○	●
Ceramics	○	●	●	●	●	○	○	○	○	○	○	○	○
Catalysts	○	○	○	○	○	●	●	●	●	○	●	○	○
Electronic components	●	●	○	●	●	●	○	○	●	●	○	○	●
Chemical reaction	○	●	●	○	●	○	○	●	●	●	○	●	●
Use as treatments	○	●	●	●	○	○	●	●	○	○	○	○	●
Use or present in other products	●	●	●	●	●	●	●	●	●	●	●	●	●

1.1.3 Impacts on living organisms

The absorption of trace metals by living organisms is not necessarily problematic. On the contrary, many metals, like Zn, Mg, Cu, Co and Mo, are in fact essential in many biological processes (Fig. 1) (Maret, 2016). They are important constituents of several key enzymes, proteins and hormones. For instance, in the human proteome, one protein in ten is a zinc metalloprotein (Andreini et al., 2009). Thus they play a myriad of fundamental roles in development, metabolism, detoxification systems, neural systems, DNA synthesis and stabilisation (Andresen et al., 2018; Briffa et al., 2020; Crichton, 2020; Maret, 2016; Wright & Baccarelli, 2007). Essential trace metals play major roles not only in humans but also in other animals as well as in plants and bacteria (Andresen et al., 2018; Crichton, 2020; Nagajyoti et al., 2010). The lack of these elements can lead to deficiency (Briffa et al., 2020). For instance, deficiency in Zn affects the immune system, wound healing, the senses of taste and smell, and impairs DNA synthesis (Fraga, 2005). Essential trace metals are necessary for life but concentration matters, as high concentrations can be hazardous (Briffa et al., 2020). Notably, in humans excess of Mn lead to manganism, a neurodegenerative disease (Rybakowska et al., 2012). In addition, some trace metals have no established biological functions and are considered as non-essential like Cd, Pb, As and Hg (Fig.1), and are toxic even at low concentrations (Briffa et al., 2020; Crichton, 2020; Maret, 2016). Toxic effects of trace metals (essential and non-essential) on biological systems have been reported as alteration of organelles and cellular components, enzyme and protein degradation (loss of functions), cellular damage and nuclear protein damage leading to carcinogenesis, genotoxicity, neurotoxicity and severe damage/disease in a large range of organs and physiological systems (Fig. 3) (Andresen et al., 2018; Briffa et al., 2020; Crichton, 2020; Fraga, 2005; Maret, 2016; Wright & Baccarelli, 2007). These problems are partly caused by the production of reactive oxygen species (ROS) through Fenton-like reactions or the impairment of antioxidant activities (Bánfalvi, 2011; Pizzino et al., 2017). These generations of ROS give rise to oxidative stress damaging cells (e.g. lipid peroxidation and membrane disruption) as well as altering protein and DNA conformation (Bánfalvi, 2011; Pizzino et al., 2017; Renu et al., 2021). In addition, metals can directly interfere with protein conformation, (e.g. binding to cysteine residues or replace other metals in protein binding sites), which leads to protein misfolding, loss of function and aggregation (Lukács et al., 2021; Tamás et al., 2014; Witkowska et al., 2021). Metals also promote epigenetic alterations such as DNA methylation and histone modification leading to DNA disruption (Ijomone et al., 2020).

Trace metals affect many groups of life, from bacteria to complex metazoans, becoming a driver of populations and communities (Fig. 2). A high level of metals in the habitat causes drastic changes in bacterial communities with a decrease in total diversity (Beattie et al., 2018), bacteria

presenting metal resistance systems being overrepresented in contaminated areas at the expense of other bacteria (Besaury et al., 2013; Gillan, 2016). Besides, plant community composition and richness radically change under the influence of contaminated soil by trace metals and exerted a selective pressure leading to the domination of metallotolerant plants (Woch et al., 2016). Trace metals are potentially big drivers in invertebrate community structure. For instance, a loss of abundance, richness and diversity in terrestrial and marine invertebrates communities can be observed along a gradient of trace metals (Babin-Fenske & Anand, 2011; Costas et al., 2018; Morón et al., 2012).

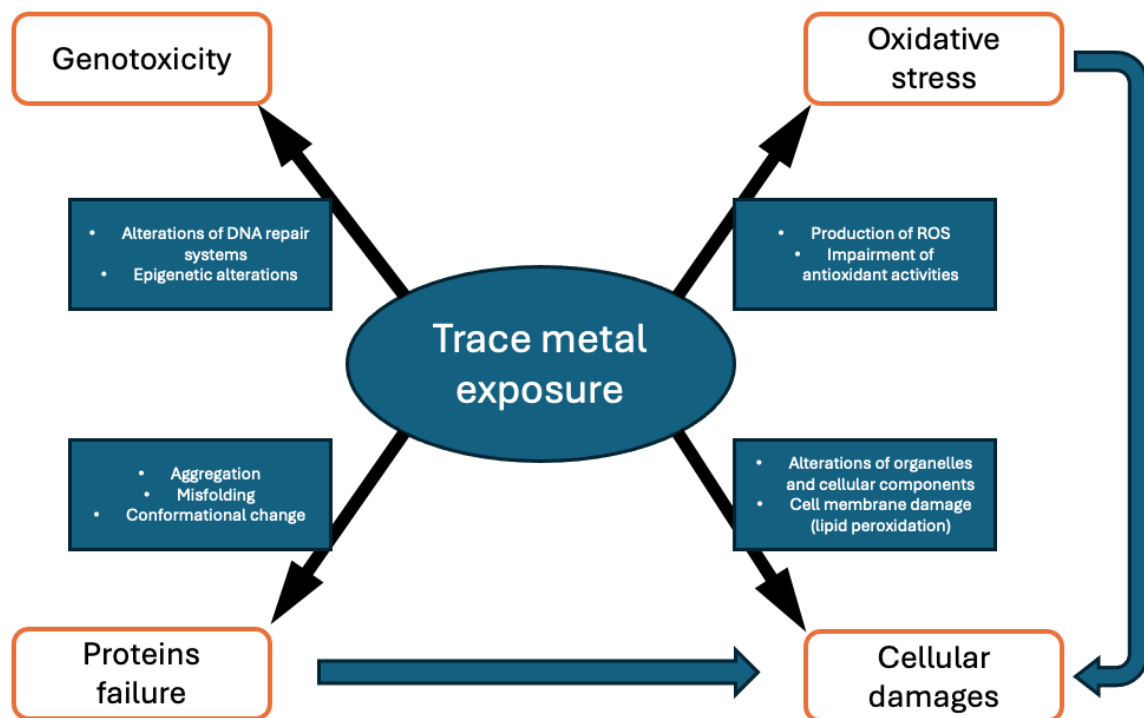


Figure 3 : Impacts of trace metal exposure on biological systems: Based on Briffa et al. (2020).

1.2 Routes of exposure

Pollination represents an undeniably essential ecosystem service for human wellness and economy (Bugin et al., 2022; Winfree et al., 2011), and negative effects on pollinators induced by metal exposure could therefore have worrying consequences for human societies and ecosystems. Pollination is mainly provided by hymenopterans, especially bees (Bugin et al., 2022; Khalifa et al., 2021). Unfortunately, bees are declining worldwide (Goulson et al., 2008; Hallmann et al., 2017; Leather, 2018). There are many factors behind this decline (e.g. habitat loss, pathogens and parasites, agrochemicals) (Wood et al., 2020). One of these potential declining factors could be trace metal exposure, as bees are more exposed to metal pollution due to their foraging activities

(van der Steen et al., 2012). Plants accumulate trace elements in their tissues including in their floral organs and more specifically in floral resources such as nectar and pollen, with concentrations varying among tissues and plant species (Nnaji et al., 2023; Subramanian et al., 2022; Xun et al., 2017, 2018). It represents a paramount threat for species depending on these floral resources for their survival and reproduction, such as pollinators (Xun et al., 2017, 2018). This is even more true since some plants hyperaccumulate metals and sequester them in their tissues at levels several orders of magnitudes higher than in soils (i.e. metallophytes) (Alford et al., 2010). For instance, metallophytes plants such as *Odontarrhena lesbiaca*, *Raphanus sativus* or *Helianthus annuus* are commonly used by bees for foraging and nesting (Hladun et al., 2015; Lange et al., 2016; Pietrelli et al., 2022; Stefanatou et al., 2020), exposing bees to hazardous concentrations of trace metals. Additionally, the increasing use of metallophytes plants for phytoremediation in polluted soils (Nnaji et al., 2023) may represent an emerging threat for bees.

Bees are not only exposed through their foraging resources, but also through polluted water and airborne fine particles (Giglio et al., 2017; van der Steen et al., 2012). The life-history of the bee (i.e. solitary or social) also influences its exposure to trace metals. Solitary bees can use contaminated elements for nesting, such as leaf-cutting bees (*Megachilidae*), which use leaf fragments to divide their nest into brood cells (Pitts-Singer & Cane, 2011). Besides, ground-nesting bees, which nest in soil, are in direct contact with xenobiotics present in the soil (Willis Chan et al., 2019). In social bees, foragers bring back xenobiotics to the colony (Johnson, 2015), exposing nestmates and larvae (Di et al., 2020). Bees bioaccumulate trace metals in their tissues (Goretti et al., 2023). Trace metals are also present in bee products like honey, wax and propolis, and can bioaccumulate in them (Goretti et al., 2020; Hladun et al., 2016). These routes of exposure (direct and indirect), from multiple sources in various compartments, make the level of trace metals accumulated in bees and their products a useful biological indicator for detecting and monitoring environmental metal pollution (Giglio et al., 2017; Goretti et al., 2020; van der Steen et al., 2012).

1.3 Response to trace metal exposure

1.3.1 Detection, avoidance and detoxification pathways

Floral resources contain a wide range of nutrients and metabolites (amino acids, fats, sugars, vitamins, micronutrients) that are essential for bees' health (Hanley et al., 2008; Somme et al., 2015). Bees do not forage randomly on every available resource but display selective foraging on specific plants according to their nutritional requirements (Hanley et al., 2008; Somme et al.,

2015). These foraging preferences are provided by a wide range of chemoreceptors composed of specialized hairs called sensilla (Bestea et al., 2021; de Brito Sanchez, 2011), mostly located on bees' antenna, tarsi and mouthpieces (Bestea et al., 2021; de Brito Sanchez, 2011). These receptors enable to detect and discriminate various concentrations of a variety of essential or toxic substances (Bestea et al., 2021; Burden et al., 2019; de Brito Sanchez, 2011; Monchanin et al., 2022). For instance, honeybees (*Apis mellifera*) have been shown to perceive and avoid the most common pesticide present in their diet (Kang & Jung, 2017). However, they could not discriminate all of them (Parkinson et al., 2023), and sometimes bees even preferred food with compounds that are toxic for them (Kessler et al., 2015). Using proboscis extension reflex (PER) assays, Burden et al. (2020) showed that honeybees can detect and avoid metal compounds in their diet, but responses were organ-, metal- and concentration-specific. Copper was rejected following antennal stimulation but was consumed following proboscis stimulation. Lead was rejected only after proboscis stimulation and Cd was not detected either by antennal or proboscis stimulation (Burden et al., 2019). Monchanin et al. (2022) showed with PER and electrophysiological analyses that As, Pb and Zn were rejected but only at high concentrations, while low, yet harmful and field-realistic concentrations, were not avoided (Monchanin et al., 2022). In presence of xenobiotics, bees change their feeding/foraging behaviour (Scheiner et al., 2004), and such behavioural plasticity can be observed on bees foraging on plants containing trace metals (Xun et al., 2018).

Recently, Borsuk et al (2021) demonstrated the capacity of honeybees to remove metals from their food resources. By analysing the residues of metals in different honeybees' body part and products, they observed lower concentrations in bees' products and a large excreted amount in faeces, showing the individual capacity of bees to excrete metals (Borsuk et al., 2021). When bees are exposed to xenobiotics such as pesticides and phytochemicals, many detoxification mechanisms take place, and one may presume close detoxification pathways for trace metals. Detoxification of organic xenobiotics occurs in three-step: (i) functionalisation (phase I), the addition of a reactive group to form a reactive site on the toxic compound, mainly performed by cytochrome P450 enzymes (CYP450), (ii) conjugation (phase II), the reactive sites will be conjugated to an endogenous hydrophilic compound resulting in a harmless molecule, more easily excreted, (iii) excretion (phase III), elimination of xenobiotics from the body in faeces by removing from the cells conjugated compounds through transporter enzymes as ATP-binding cassettes (Berenbaum & Johnson, 2015; Esteves et al., 2021; Gong & Diao, 2017; Hodges & Minich, 2015). It remains blurry whether trace metals are detoxified in this way, although the expression of detoxification gene in bees increases in polluted areas (Gizaw et al., 2020). Because exposure to metals leads to ROS production, a wide range of antioxidant enzymes are present to deal with oxidative stress, such as catalases and superoxide dismutases (Bernardes et al., 2022; Gizaw et al.,

2020; Nikolić et al., 2016). Besides, ROS-scavenging molecules or chaperone proteins have been found to be overregulated after metal exposure (Nikolić et al., 2016; Nogueira et al., 2018). The above-mentioned mechanisms are not restricted to metal detoxification and are probably only used for the detoxification of sub-product after metal exposure like ROS (Esteves et al., 2021; Jan et al., 2015; Pizzino et al., 2017; Renu et al., 2021). Other processes seem to be tightly linked with metal homeostasis. Metal-binding proteins, also known as metallothioneins, seems to be the main mechanisms to detoxify metal ions. These proteins have low molecular weight (6–10 kDa) and a high cysteine content, giving rise to a structure with a high affinity for metal ions (Carpenè et al., 2007; Ruttkay-Nedecky et al., 2013). Some genes of metallothioneins have recently been identified in the honeybee genome (Purać et al., 2019). Last but not least, it has been suggested that the bee gut symbionts could play a role in metals detoxification through their absorption and excretion (Astolfi et al., 2022), as already demonstrated in humans (Arun et al., 2021; Chen et al., 2022).

1.3.2 Impacts on behaviour and physiology

Neural systems, due to their complex structure and characteristics, are vulnerable to xenobiotics which exhibit neurotoxicity with high sublethal cognitive effects (Araújo et al., 2023; Belzunces et al., 2012; Cabirol & Haase, 2019; Caito & Aschner, 2015). Most insecticides are designed with neurotoxins as main active substances (e.g. neonicotinoids) (Araújo et al., 2023; Belzunces et al., 2012), affecting the nervous system of target organisms by acting on neurotransmission pathway (e.g. neural receptors, ionic channels, cell signalling) as agonist/antagonist (Belzunces et al., 2012; Cabirol & Haase, 2019), neural metabolism modifications (Belzunces et al., 2012) or through damage on brain cells (Araújo et al., 2023). For instance, neonicotinoids which are agonist of nicotinic acetylcholine receptors, causing disturbance in brain structure by alterations of Kenyon cells, the intrinsic neurons of the mushroom body which is fundamental to memory, guidance, learning and neuronal metabolism of insects (Catae et al., 2018; Strausfeld et al., 2000; Wilson et al., 2013). Metals are well established as neurotoxicants, inducing neuronal deficits in many organisms (Caito & Aschner, 2015; Wright & Baccarelli, 2007). Their accumulation in the nervous system increases oxidative damage to nerve cells and also leads to protein aggregation (Caito & Aschner, 2015; Molina-Holgado et al., 2007; Wright & Baccarelli, 2007). Furthermore, metals ions alter the neurometabolism of bees (i.e. synthesis and activity of neurotransmitters, synaptic enzymes and neuroreceptors) (Z. Li et al., 2022; Milivojević et al., 2015; Søvik et al., 2015). Exposure to metals cause neurodegenerative diseases, alterations of cognitive functions and neurodevelopment impairments (Lawler, 1996; Molina-Holgado et al., 2007; Wright & Baccarelli, 2007). Metals affect cognitive performance of bees through changes in behaviour, locomotion,

memory or taste perception (Bernardes et al., 2022; Burden et al., 2016; Z. Li et al., 2022; Monchanin et al., 2024). For instance, mobility functions such as flight and walking activity has been reported to be affected by trace metals exposure (Bernardes et al., 2022; Botina et al., 2023; S. Gao et al., 2024; Rodrigues et al., 2016). The reduction of cognitive abilities is probably linked to neurodevelopment issues (Z. Li et al., 2022). Recently, Monchanin et al., (2024) demonstrated that bees close to trace metals source developed smaller heads (i.e. smaller brain) resulting in cognitive function decline (Monchanin et al., 2024). However, in the case of mobility function impairments as observed in walking and flying, others factors might be involved as impairments in metabolism, thermoregulation or muscle function (Crall et al., 2018; Knoll & Cappai, 2024).

The digestive tract is a critical organ because of its role in nutrient absorption and as it is the first site of contact with xenobiotics (Dabour et al., 2019; Motta et al., 2023; Nation Sr, 2022). The midgut, between the foregut and the hindgut, is the most important section of the digestive tract as it hosts most of the digestion and detoxification processes (Motta et al., 2023; Oliveira et al., 2019; Vanderplanck et al., 2020). The midgut is lined by a laminar structure composed of chitin and proteins, also known as the peritrophic membrane, that protects cells against mechanical damage, pathogens and xenobiotics while allowing the absorption of nutrients (Nation Sr, 2022; Vanderplanck et al., 2020). Trace metals accumulate in midgut tissues (Bednarska et al., 2016; Dabour et al., 2019) and have been shown to cause severe histological and cytological damages on midgut epithelium and peritrophic matrix of honeybees (Dabour et al., 2019) and stingless bees (Bernardes et al., 2021). To deal with injuries and the entrance of foreign bodies, cells of the gut epithelium rapidly mobilise innate immune responses, including melanisation, to face pathogens (Larsen et al., 2019; Nakhleh et al., 2017; Nation Sr, 2022; Zeng et al., 2022). Melanisation is a localised blackening reaction which allows to seal off cuticular breaches at wound sites and prevent the spread of a large number of pathogens, damaged tissues and xenobiotics by sequestering them in melanotic nodules for elimination (Allam & Espinosa, 2015; Larsen et al., 2019; Nation Sr, 2022; Pham & Schneider, 2008). Melanisation is initiated by cells of the gut epithelium through secretion of pattern recognition proteins leading to activation of (pro)*phenoloxidase*, a key enzyme in the pathway leading to melanin biosynthesis (Larsen et al., 2019; Nakhleh et al., 2017; Nation Sr, 2022).

The fat bodies are the second most important tissues for detoxification in bees (L. Gao et al., 2022). Fat bodies are localised across the whole bees' body, primarily under the body surface and around organs (Skowronek et al., 2021). In bees, fat body cells are organised in layers consisting of a mix of different cell types (Skowronek et al., 2021). Fat body tissues are multifunctional and are the centre of many metabolic pathways, including hormone, detoxification enzyme and antimicrobial

peptide synthesis (i.e. immunity) as well as lipid and carbohydrate mobilisation (Nation Sr, 2022; Skowronek et al., 2021; Vanderplanck et al., 2021). Due to the multifunctional role of fat bodies, modification in lipidic content can be used to monitor bee health and used as proxy for bee immunocompetence (Vanderplanck et al., 2021). However, this proxy should be interpreted with caution, as non-published data showed that lipidic content may not be a suitable proxy to assess immunocompetence (Gekièrè et al. In prep). The relations between metals and fat bodies remain overlooked (especially in bees), although metals accumulation in insect's fat bodies has been demonstrated (J. Zhang et al., 2020), including in bees (Polykretis et al., 2016). Trace metals found in the digestive tract are transported to the fat body tissues through haemolymph, where they are immobilised/metabolised and finally chelated to be excreted with faeces (Borsuk et al., 2021). Moreover, fat metabolism can be impacted by metals has recently shown by Loskutov et al. (2023), where exposition to trace metals induced change in the fatty acid profile of black soldier fly influencing their total lipid content (Loskutov et al., 2023).

Fat bodies are also related to reproduction (i.e. sexual maturation), especially to ovarian development. Fat body cells produces Vitellogenin, an egg yolk protein precursor essential for oogenesis and egg development (Amdam et al., 2012; Nation Sr, 2022; Skowronek et al., 2021). Female bees have two ovaries, located dorsally to the alimentary tract and each consisting of four ovarioles containing numerous oocytes (Laycock et al., 2012; Nation Sr, 2022; Siviter et al., 2020). Exposure to pesticide can disrupt ovarian development. For instance, thiamethoxam, imidacloprid, and azadirachtin has been found to reduce terminal oocyte length in bumblebees (Barbosa et al., 2015; Baron et al., 2017; Laycock et al., 2012). Impact of trace metals on bee's reproductive fitness has been reported through a reduced number of brood cells in mason bees (Moroń et al., 2014) as well as through a reduced number of larvae in bumblebees (Scott et al., 2022; Sivakoff et al., 2020). However, potential impact on the female reproductive system and on oocyte production remains unknown.

Chapter 2: Objectives

The lack of knowledge about the toxicity of trace metals on bees and their ability to deal with these xenobiotics have led us to further investigate the potential sublethal impacts of trace metals on these pollinators. To this end, we investigated the sublethal effects of trace metals on the behaviour and physiology of buff-tailed bumblebee workers (*Bombus terrestris* L.), as model pollinator, using copper (Cu) and cadmium (Cd), as model metals. We also investigated their ability to detect these metals in sucrose solutions.

The study was divided in three major sections (Fig. 4): (i) assessing the capacity of bumblebees to detect trace metals in their diet through a free-moving proboscis extension reflex (free-moving PER) assay, (ii) highlighting potential impairments in basic but essential behaviours such as walking and flying abilities, (iii) investigating the impacts on physiological systems through measure of oocyte size, abdominal lipidic content and gut damages through melanisation response upon trace metal exposure. Our different assays were conducted using various metal concentrations. Environmental concentrations of copper and cadmium were retrieved from the PoshBee consortium (Durkalec et al., 2024). Concentrations related to lethal dose of both metals (i.e. LD₁₀, LD₅₀) were based on another study of ours (Gekière et al., Submitted in Environmental Toxicology and Pharmacology). We hypothesised that bumblebees would not demonstrate avoidance responses to environmental concentrations of metals and would only avoid lethal, non-realistic concentrations. For behaviour and physiology parameters, we expected to observe sublethal effects upon exposure to environmental concentrations of both metals.

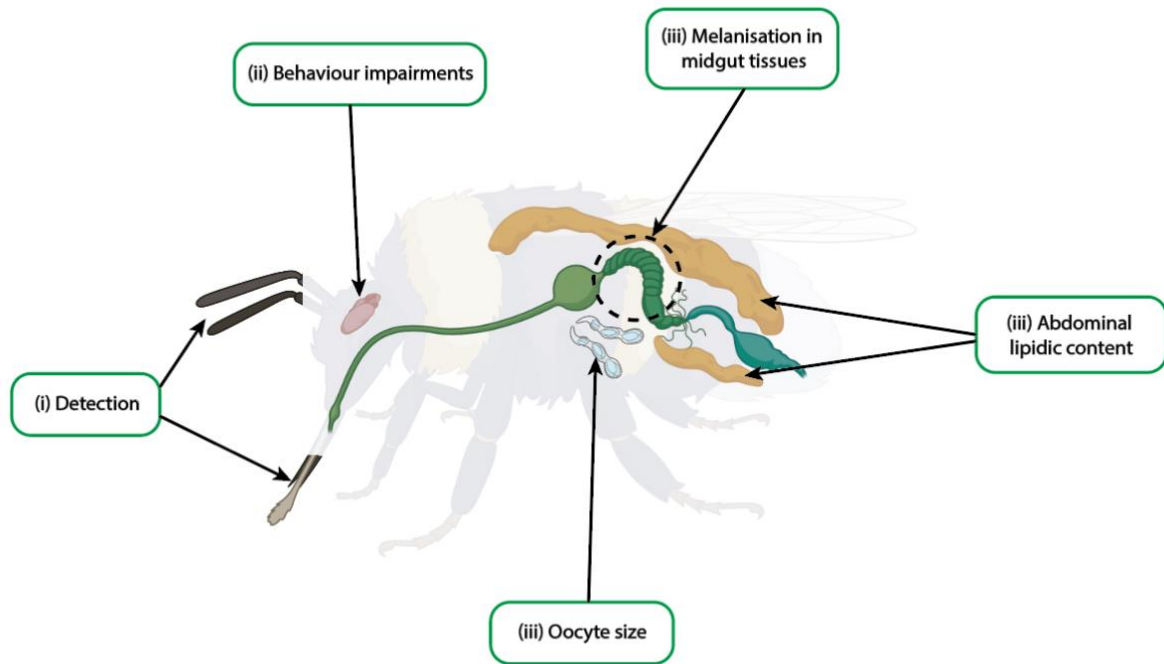


Figure 4: Schema of the bumblebee's organs investigated among different experiments: The different internal and external organs of bumblebee examined during the study are shown. The proboscis and the antennae for the detection, the brain for behaviour impairments, the midgut (encircled) for melanisation, the fat bodies for abdominal lipidic content and ovaries for oocyte size. Referred sections are indicated.

Chapter 3: Materials and methods

3.1 Pollinator model and trace metals

3.1.1 *Bombus terrestris* (L., 1758)

Bioassays were performed on the pollinator model *Bombus terrestris* (L., 1758), using workers only. Bumblebees (Hymenoptera: Apidae: *Bombus* spp.) are eusocial bees (i.e. social organisation providing division of labour through different castes in the colony) divided in several castes (i.e. queen, workers and males) (Cameron et al., 2007; Goulson, 2010). These crucial pollinators have a cosmopolitan distribution across the world, although they are missing in some Australian and African regions (Goulson, 2010; Michener, 2000; Rasmont et al., 2021). Most bumblebees, including *B. terrestris*, are generalist pollinators (i.e. polylectic) and thus provide unmatched ecosystem services through pollination (Goulson, 2010; Michener, 2000; Rasmont et al., 2021). *B. terrestris* was selected as model due to its commercial availability and easy rearing (Velthuis & Doorn, 2006). Bees have been repeatedly utilised in ecotoxicological studies as biological models to assess the toxic effects and to biomonitor many environmental pollutants (Breidenbach et al., 2023; Johnson, 2015; Rothman et al., 2020), including in our lab (Gekière et al., 2022). Furthermore, bees have been shown to bioaccumulate metals (Breidenbach et al., 2023; Giglio et al., 2017; Goretti et al., 2020) and hence have to cope with high doses of metals. All colonies were obtained from the commercial supplier *Biobest buba* (standard hive; Westerlo, Belgium), ensuring good bumblebee's health and absence of pathogens in the hive. Colonies were fed from a feeding station filled with sugar solutions (Biogluc®, Biobest). In addition, pollen candy (*Salix* sp.) mixed with bottle water (50% w/w) was given for protein and nutrient intake. Colonies were placed in a rearing room in the dark at constant temperature ($25\text{ °C} \pm 2\text{ °C}$) and relative humidity ($60\% \pm 5\%$), located on Nimy Campus at University of Mons (Belgium) throughout the experiments.

3.1.2 Copper and cadmium

We used two prevalent environmental metallic pollutants, namely copper (Cu) and cadmium (Cd) (Comber et al., 2023; Nagajyoti et al., 2010), respectively described as essential and non-essential metals (Maret, 2016). Cadmium exposure is known to cause carcinogenesis, neurotoxicity and genotoxicity through oxidative stress, epigenetic alterations or by replacing other metals (e.g. Zn, Ca) in proteins, altering their conformation (Benbrahim-Tallaa et al., 2007; Crichton, 2020; Genchi et al., 2020; Tchounwou et al., 2012). Copper, even if it is essential, causes genetic disorder, oxidative damage, neurodegenerative disease (i.e. Wilson's disease) and cytotoxicity at high

concentrations (Crichton, 2020; Gaetke & Chow, 2003; Tchounwou et al., 2008). In bees, Cd and Cu have been reported to cause cellular damage, alter colony health and immunity (Dabour et al., 2019; Hladun et al., 2016; Nikolić et al., 2016; Polykretis et al., 2016).

Field-realistic concentrations used in these experiments were based on concentrations found in the environment by the PoshBee project (www.poshbee.eu). They collected data on pollutants present in the beebread and pollen stores of different domesticated bee species (i.e. *Bombus terrestris*, *Apis mellifera*, *Osmia bicornis*), including the concentration of many metals (mg/kg) in 128 sites across Europe (Durkalec et al., 2024). We used these data to determine the mean values present for Cu and Cd for each species, then for all species together (Tab. 2). We considered these mean values (i.e. all species) as environmental concentrations in this work. The LD₅₀ of Cd and Cu on *B. terrestris* used has been determined in the lab (Gekière et al. Submitted in Environmental Toxicology and Pharmacology) (see 3.4.1; Tab. 3).

Table 2: Environmental concentrations of metals (mg/kg) found in the PoshBee project: Metal concentrations (mg/kg) found in the PoshBee project (Durkalec et al., 2024) in beebread/pollen stores of three bee species (i.e. *Bombus terrestris*, *Apis mellifera*, *Osmia bicornis*). The median and the maximum concentrations which have been found are shown. We calculated the mean values for each species and then for all species together. We used these mean values (i.e. all species) as environmental concentrations.

Bee sp.	Metal	N	Median	Mean	Max
<i>Apis mellifera</i>	Cd	123	0.0499	0.0721	0.486
<i>Apis mellifera</i>	Cu	123	7.4	8.14	48.8
<i>Bombus terrestris</i>	Cd	97	0.0329	0.0623	0.465
<i>Bombus terrestris</i>	Cu	97	7.67	9.24	47.8
<i>Osmia bicornis</i>	Cd	86	0.049	0.0727	0.343
<i>Osmia bicornis</i>	Cu	86	8.73	8.85	19.1
All sp.	Cd	306	0.0422	0.0691	0.486
All sp.	Cu	306	7.69	8.63	48.8

3.2 Detection of trace metals

Bees can detect a variety of substances, including xenobiotics, in their food (Bestea et al., 2021). In presence of xenobiotics, bees can change their feeding behaviour to avoid these toxins (Scheiner et al., 2004), but whether they have the capacity to taste and avoid metals is poorly understood. Here we tested the capacity of bumblebees to detect trace metals contained in sugar solutions using a free-moving PER assay adapted from Ma et al. (2016) and Sculfort et al. (2021).

Three concentrations (i.e. two field-realistic concentrations and the median lethal concentration LC_{50}) per metals as well as positive and negative controls were tested (i.e., eight treatments). Solutions were prepared with copper chloride ($CuCl_2$; Sigma-Aldrich, CAS 7447-39-4) and cadmium chloride ($CdCl_2$; Sigma-Aldrich, CAS 10108-64-2), which were dissolved in a 17% sucrose solution (w/w, 0.5 M). Tested concentrations for Cd were 0.07, 0.7 and 100 $mg.L^{-1}$ (i.e., 0.38, 3.82 and 545.49 μM), and tested concentrations for Cu were 0.9, 9 and 900 $mg.L^{-1}$ (i.e., 6.69, 66.94 and 6693.94 μM). Negative control was the same 0.5 M sucrose solution while the positive control was a sodium chloride solution (0.5 M sucrose solution + 15% NaCl w/w), as high concentration of NaCl in sucrose solution is known to act as a repellent in bees (Bestea et al., 2021; Lau & Nieh, 2016). Three bumblebee colonies were used. On a daily basis, eight workers were retrieved from each colony, each worker being allocated to one treatment (i.e. total of three workers per treatment per day), so twenty-four workers were tested every day. The experiment was conducted until a minimum of 20 workers per treatment tasted the test solutions (see below), for a total of minimum 160 workers tested.

Upon retrieval from the colony, workers were placed individually in Nicot® cage in the rearing room with no food for a starvation period of 2-4 hours, to increase motivation for food intake (Ma et al., 2016). Due to the number of bumblebees to test per day (twenty-four) and to avoid the risk of too much time elapsing between the first and the last tested bumblebees, the bumblebees were starved in two waves (i.e. 12 in the morning and 12 in the early afternoon.) After the starvation period, workers were transferred to a modified 15-mL centrifuge tube with a 4-mm hole drilled at the tip, so that the bees were free to move within the tube. The tube was placed in a white polystyrene holder/box to avoid every visual stimulus that can could interfere with the experiment (Fig. 5). The system was overhung by a digital microscope Dino-Lite (30 frames.sec⁻¹ and a 25× magnification rate) placed 5 cm above the tube tip (Fig. 5). This microscope was connected to a computer and the video was processed with the software DinoXcope (MacOS, Version 2.4.1) to record the feeding session. A capillary tube of 100 μL was filled with the test solution. The capillary was connected to a pumping system composed of a modified syringe and a

tip. This system allows to fill the capillary and ensures a permanent droplet of solutions at the end of the capillary throughout the recording period. The length of the liquid inside the capillary was initially measured and the quantity of the solution in the capillary was calculated ($9.1 \text{ cm} = 100 \text{ }\mu\text{L}$). The capillary was placed in front of the tube in the polystyrene support (Fig. 5). Once the experimental support ready (Fig. 5), bees were left three minutes for a habituation period. Then, a droplet of $10 \text{ }\mu\text{L}$ of sucrose 0.5 M was presented to workers to facilitate the proboscis extension (Ma et al., 2016). If the droplet was not consumed after three minutes, the worker was discarded (i.e. not motivated to eat). After droplet consumption, the capillary containing the test solution was immediately presented to the worker and the 2-min recording started right after the first proboscis contact with the solution. If there was no proboscis contact with the capillary three minutes after droplet consumption, the bee was discarded. At the end of the 2-min assay, the capillary was removed, and the amount of solution left inside the capillary was measured to calculate the amount of solution consumed. Each bumblebee was weighed just after the experiment and the volume of consumed solution was standardised by the mass of the workers. Videos were manually examined with FilmForth V2.3.18 at 0.5x speed (all videos were analysed by the same experimenter to avoid bias). The duration of the first proboscis contact, the total number of feeding bouts and the cumulative duration of feeding were recorded.

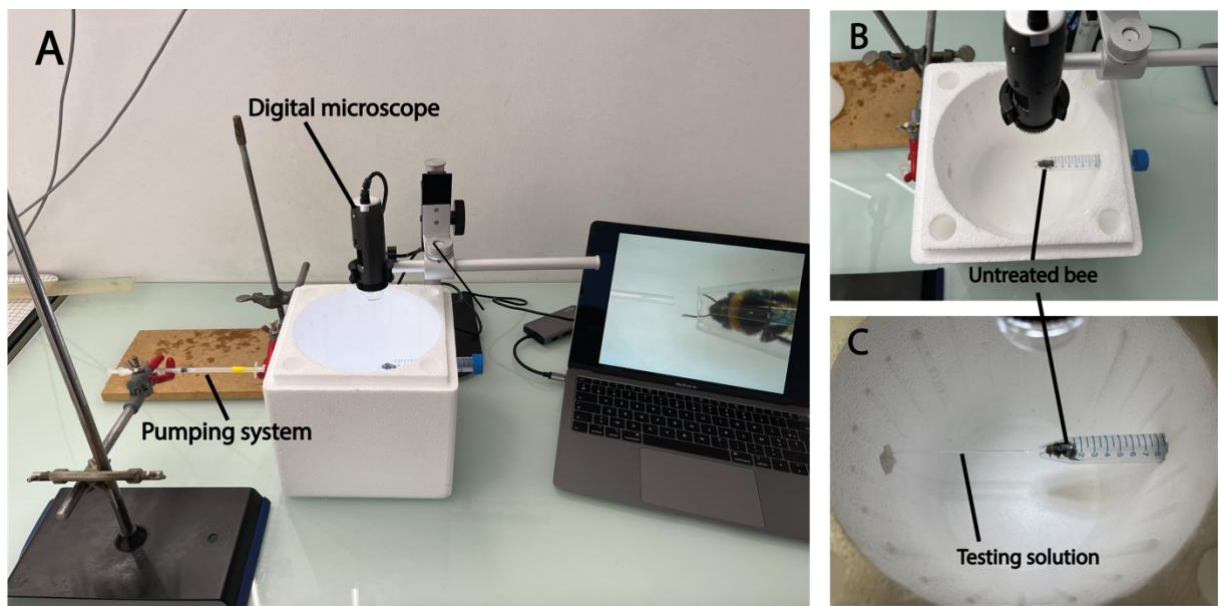


Figure 5: Experimental design for free-moving proboscis extension reflex assays: **A.** Overall design of the experiment with the camera over the system to record the bumblebee feeding. **B.** Untreated bumblebee placed in a centrifuge tube placed in a polystyrene structure. **C.** Testing solutions presented to the untreated bee through the pumping system.

All statistical analyses were performed in R 4.3.2. We compared effects among treatments using generalized linear mixed-effect models (GLMM) from the glmmTMB package (Brooks et al., 2017),

with a Gamma error structure and a log link for the duration of the first contact, the sucrose consumption and the cumulative duration of feeding, while a zero-truncated negative binomial structure with a logit link was used for the number of contacts. We considered treatment as fixed effect and the colony as random effect. The quality of fits of our models were examined using the DHARMA package (Hartig & Lohse, 2022). Custom contrast from emmeans package (Lenth et al., 2024) was used to investigate significant differences between the control (i.e. sugar solution) and treatments containing metals. Visual representations were plotted using the ggplot2 (Wickham et al., 2024) and ggpubr packages (Kassambara, 2023).

3.3 Behavioural assays

Exposure to xenobiotics like pesticides has been shown to cause several troubles in locomotive functions and behaviours in bees (Belzunces et al., 2012). The purpose of this experiment was to assess if an exposure to trace metals had sublethal effects on locomotive abilities through two behavioural assays, namely a walking behaviour assay and a flight take-off assay. These kinds of experiments have already been set with pesticides (Araújo et al., 2023; Bernardes et al., 2022; Botina et al., 2019; Rodrigues et al., 2016).

Two field-realistic concentrations and a sublethal concentration (i.e. LC₁₀) per metal were tested, all prepared from CuCl₂ or CdCl₂ dissolved in 50% sucrose solution (w/w, 2.92 M), which served as a negative control for a total of seven treatments. Tested concentrations for Cd were 0.07, 0.7 and 2 mg.L⁻¹ (i.e., 0.38, 3.82 and 10.91 µM), and tested concentrations for Cu were 0.9, 9 and 17 mg.L⁻¹ (i.e., 6.69, 66.94 and 126.44 µM). Workers came from three different colonies and were collectively exposed to their respective treatments in communal plastic boxes (Fig. 6A). These boxes consisted of three workers from the same colony. There were three boxes per treatment (one box per colony per treatment), in three replicates for a total of 63 plastic boxes (i.e. 189 workers in total). Workers were fed for three days (72h) with their respective treatment via plastic vials placed under the plastic boxes (Fig. 6A), the food coming through capillarity.

3.3.1 Walking behaviour

After three days of exposure, workers were placed individually in a Petri dish (9 cm diameter, 0.8 cm high) covered with white paper at the bottom (white) (Fig. 6B) for a two-minute acclimatisation period. After this period, the Petri dish was placed under a camera mounted on a tripod. The bumblebee activity was recorded for four minutes (~30 frames.sec⁻¹). Videos were automatically analysed with the software ToxTrac (Rodriguez et al., 2018) (version 2.98, default

settings, object size = [5,000-20,000], object colour selection = 50), to record the total walking distance (mm) into the Petri dish and the mobile average speed (i.e. velocity; mm/s).

Difference among treatments were assessed using a non-parametric aligned rank transform models from the ARTool package (Kay et al., 2021), as statistical assumptions were not met for (G)LMM models. We considered treatment as fixed effect and colony as random effect. Visual representations were obtained using ggplot2 (Wickham et al., 2024) and ggpubr packages (Kassambara, 2023).

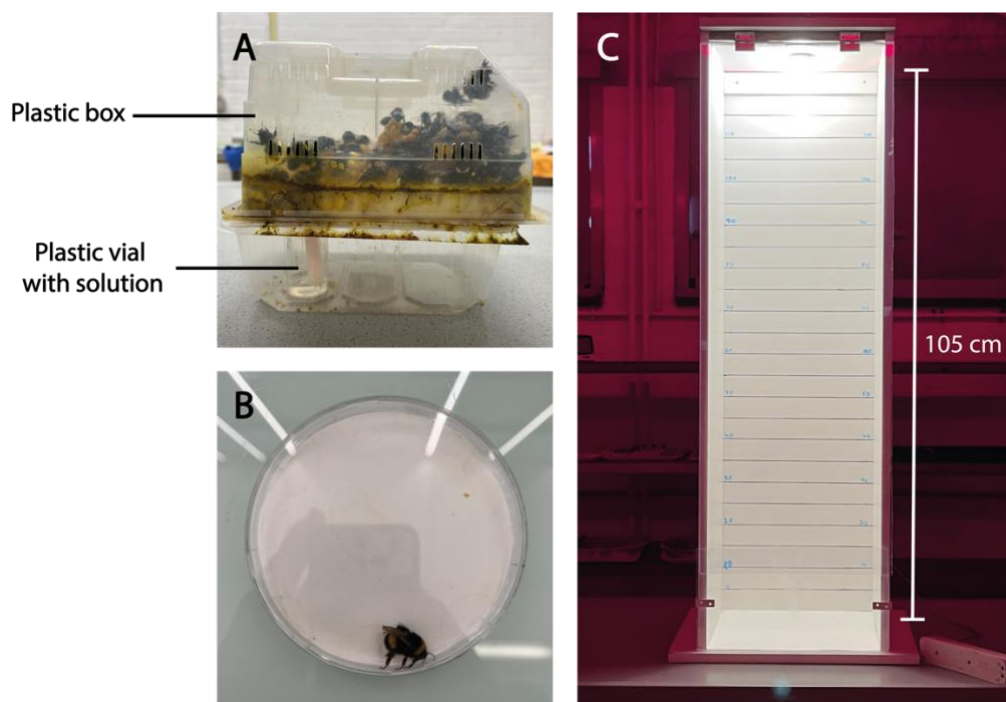


Figure 6 : Behavioural assay setup: A. Example of plastic box used for group exposure, with a plastic vial containing the solution placed under the box. *B.* Petri dish with paper place at the bottom with a treated bee inside the dish for the walking assay. *C.* Flight tower in dark with light switched on (experimental condition) for the flight take-off assay.

3.3.2 Flight take-off

Upon the end of the walking behaviour experiment, workers were directly brought to a flight cage to observe their flight take-off capacity (Botina et al., 2019). Doing so enables to reduce the numbers of animals tested during the experiments. Workers were placed at the base of a tower of 105 cm of height in a dark room (Fig. 6C). This tower was composed of white wood planks, a plastic glass in the front to observe the bumblebee flight, a lamp at the top of the tower and mark every 10 cm (Fig. 6C). Bumblebees were left for a 1-min acclimatisation in the dark at the base of the tower. After 1 min, the lamp was turned on and worker flight activity was recorded for 2 min, as workers tried to reach the lamp because they were attracted by the light (i.e. phototaxis) (Araújo

et al., 2023; Merling et al., 2020). During these two minutes, two metrics were recorded, namely the time to reach the lamp (s) and the maximum flying height, classified in five categories (ground, 1-35 cm, 36-70 cm, 71-105 cm, lamp).

As maximum flying height is an ordinal variable, difference among treatments were assessed using a cumulative link mixed effects models (CLMM) from the ordinal package (Christensen, 2023), with a logit link. We considered treatment and bee mass as fixed effect and colony as random effect. Visual representations were obtained using ggplot2 (Wickham et al., 2024) and ggpubr packages (Kassambara, 2023). The time needed to reach the lamp among treatments was not investigated because some treatments lacked replicates.

3.4 Gut melanisation

Absorption of xenobiotics, like trace metals, by oral route can cause severe damages on the gut physiology of many insects, including bees, often leading to an immune response (Bernardes et al., 2022; Dabour et al., 2019; Larsen et al., 2019; Wu et al., 2009). To study the damages that metals cause on the digestive tract of bumblebees, midgut melanisation after acute and chronic oral exposure to Cu and Cd was quantified. Gut melanisation has already been used as a proxy for describing gut damage in a previous ecotoxicological study (Straw & Brown, 2021).

3.4.1 Chronic and acute exposure

For chronic exposure, workers were exposed individually for three days in Nicot® cage at the same doses as used in behaviour experiments (see 3.3). One hundred and forty-seven workers from three colonies were tested over seven treatments (i.e. 21 workers per treatment, 7 from each colony). After exposure, bees were stored at -70 °C until gut dissection.

For acute exposure workers came from an experiment designed to determine the LD₅₀ of copper and cadmium on *Bombus terrestris* L., carried out by A.Gekière, L.Breuer and L.Dorio (Gekière et al., Submitted in Environmental Toxicology and Pharmacology). For acute exposure, five concentrations per metals were tested (Tab. 3), as well as a positive control (i.e. dimethoate, 0.1 M diluted in a 50% sucrose solution; Pestanal®, CAS 60-51-5) and a negative control (i.e. 50% sucrose solution) for a total of twelve treatments. Thirty workers from five colonies (six workers per colony) were used per treatment and exposed individually. Bees were weighted and placed in Nicot® cage and were provided ad libitum with 50% sucrose solution through a tip-less 2-mL syringe overnight in rearing room. Afterwards, bees were starved for four hours and exposed to

40 μL of their respective treatments in the tip of a 2-mL syringe. Once the 40 μL were consumed, were provided again ad libitum with 50% sucrose solution for four days. Bees that did not consume their treatment within three hours were removed. The consumption of sucrose solution of each bee was monitored by weighting the syringe every day to calculate the food intake. After 96 hours, surviving bees were weighted and stored at $-70\text{ }^{\circ}\text{C}$ until gut damage analysis. The bees dead before the end of the experiments were weighted and stored over the assay. Concentrations in solutions were measured after the experiment at the Mineral and Organic Chemical Analysis platform (MOCA; Louvain-la-Neuve, Belgium) using Inductively Coupled Plasma – Optical Emission Spectrometer (Agilent 5800 VDV ICP-OES) (Tab. 3).

Table 3: Metal concentrations for acute exposure in the melanisation experiment: Metals concentrations used for acute exposure for melanisation response in the midgut. Concentrations measured at the Mineral and Organic Chemical Analysis platform (MOCA; Louvain-la-Neuve, Belgium).

	Cu		Cd	
Conc1	96 mg/L	714.02 μM	9 mg/L	4.909 μM
Conc2	202 mg/L	1502.42 μM	19 mg/L	10.36 μM
Conc3	397 mg/L	2952.77 μM	30 mg/L	16.36 μM
Conc4	876 mg/L	6515.43 μM	68 mg/L	37.09 μM
Conc5	1618 mg/L	0.012 M	130 mg/L	70.09 μM

3.4.2 Gut analysis

Bees stored in the freezer at $-70\text{ }^{\circ}\text{C}$ were placed on ice and thawed slowly, to avoid risk of tissues destruction. Bees were dissected as follows: their thorax as well as the tip of their abdomen (the last tergite) were pinned on a wax plate (Fig. 7). Then the abdomen was open with dissecting scissors by making a horizontal incision on the last tergite and then two lateral incisions. The tergites were lifted and pinned on the thorax (Fig. 7). Fifty microliters of Ringer’s solution were added to immerse the internal organs and avoid desiccation. The digestive tract was extracted from the abdomen and the midgut part was dissected out. The Malpighian tubules were discarded to increase visibility and the midgut was placed in 50 μL of Ringer’s solution on a clean new white wax plate. Pictures of midgut were taken with a numeric microscope high resolution, Keyence

VHX-970F (Mechelen, Belgium), always using the same parameters (Brightness -> manual = 13,25), at 50x magnification. The midgut was always photographed on the side facing upwards – no effort was made to highlight gut damages. Images were further analysed using Fiji (Schindelin et al., 2012). The mean grey value of the midgut was used as proxy to quantify melanisation. In Fiji, a rectangle was drawn to cover a large area of the midgut (Edit -> Selection -> Specify), the mean grey value was measured over the entire rectangle (Analyze -> Set measurement -> mean grey scale + Analyze -> Measure). We obtained a value between 0 and 255, representing the brightness (0 = black, 255 = White). We converted the brightness to darkness (255 - brightness) to facilitate gut damage interpretation in graphs. We also ran a post-hoc experiment to make sure gut damages were not due to post-mortem decomposition (Appendix 1).

Effects of treatments on the melanisation response were compared using generalized linear models (GLM) from the MASS package (Ripley et al., 2024), using a Gamma error structure with a log link. The same model was set for the sucrose consumption 24 h after exposure. The treatments and the bee mass were considered as fixed effect. The quality of fits of our models were examined using the DHARMA package (Hartig & Lohse, 2022). The differences between each treatment were further investigating through pairwise comparisons with FRD adjustment using emmeans package (Lenth et al., 2024). Visual representations were obtained using ggplot2 (Wickham et al., 2024) and ggpubr packages (Kassambara, 2023).

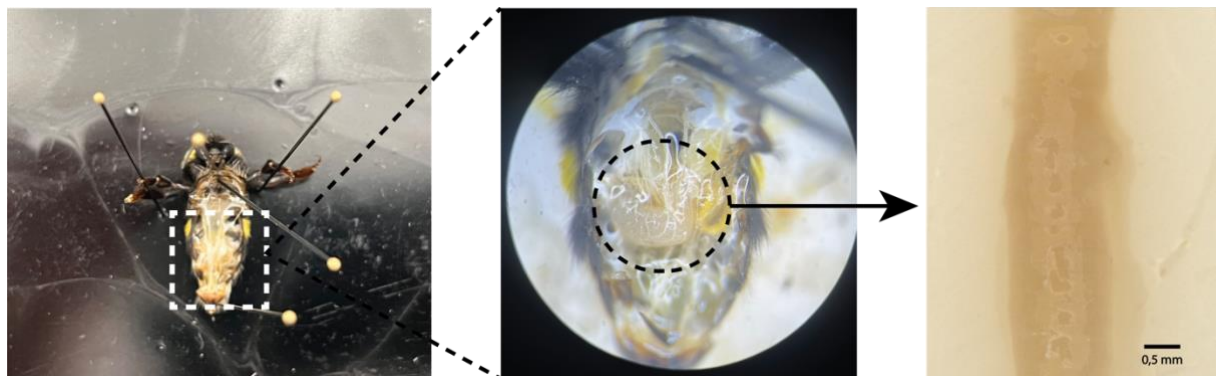


Figure 7: Dissection of a bumblebee's midgut: Dissections process of an untreated bumblebee's midgut (organ encircled on centre panel) until image capture under a numeric microscope (right image).

3.5 Impacts of trace metals on ovarian development and lipidic content

The purpose of this experiment was to highlight the sublethal effects of trace metals on the reproductive system of bumblebee workers, using as proxy the length of the terminal oocytes (Fig. 8). Previous studies already used this proxy on bumblebee workers after exposure to pesticides

(Laycock et al., 2012; Shpigler et al., 2014; Siviter et al., 2020). Abdominal lipidic content was also measured due to their importance in metabolism pathways and immunoprotein synthesis (Nation Sr, 2022; Skowronek et al., 2021), making them a good proxy for immunocompetence and bee's health (Vanderplanck et al., 2021) (but see Gekière et al. in prep), as well as their involvement in sexual maturation (i.e. oogenesis) (Amdam et al., 2012; Skowronek et al., 2021).

Two field-realistic concentrations per metal were tested and prepared from CuCl_2 or CdCl_2 dissolved in 50% sucrose solution (w/w, 2.92 M). Tested concentrations for Cd were 0.07 and 0.7 mg.L^{-1} (i.e., 0.38 and 3.82 μM), and tested concentrations for Cu were 0.9 and 9 mg.L^{-1} (i.e., 6.69 and 66.94 μM). A negative control (i.e. 50% sucrose solution) was also implemented for a total of five treatments. Fifty workers were tested per treatment (5 colony and 10 workers per colony per treatment) for a total of 250 bees. Workers were fed individually for seven days with their respective treatment in Nicot® cages through a tip-less 2-mL syringe. This syringe was weighed every three days to monitor metal intake and replaced if necessary. Each Nicot® cage was also provided *ad libitum* with pollen candy (*Salix* sp.), as pollen is necessary for ovarian and lipidic development. In social bees, workers do not develop ovaries as the queen secretes inhibiting hormones. Yet, workers shown signs of ovarian development approximately seven days after being no longer exposed to these pheromones (Laycock et al., 2012; Siviter et al., 2020). At the end of the experiment, half of the workers per treatment was chilled on ice and their ovaries were dissected. The other half was frozen (-20 °C) to measure their lipidic content.

3.5.1 Ovary dissection

Ovaries were dissected and extract from the abdomen following the same dissecting method as explained in 3.4.2. Pictures of ovaries were taken with a numeric microscope high resolution, Keyence VHX-970F (Mechelen, Belgium), always using the same parameters (Brightness -> supercharged = 35), at 50x magnification. The length (mm) of the two greater terminal oocytes was directly measured with the Keyence microscope (Fig. 8). The mean length of the two greater terminal oocytes was calculated and used as a proxy for worker ovarian development.

3.5.2 Lipidic content

Lipidic content was measured following a protocol from Ellers (1996). Worker's abdomen was isolated and weighted, before being placed in a 5-mL Eppendorf and dried for three days at 70 °C. Then, the dry abdomen was weighed. Next, 2 mL of diethyl ether ($(\text{C}_2\text{H}_5)_2\text{O}$; Merck Millipore) were added in Eppendorfs for 24 hours, so that the diethyl ether solubilised and extracted fat from the

abdomen (Thiex et al., 2003). After fat extraction, abdomens were washed twice with diethyl ether and dried again for seven days at 70 °C. They were finally weighed again and the lipidic content was defined as the abdominal mass loss during this process divided by the abdomen mass before extraction.

We compared effects of treatments using generalized linear mixed-effect models (GLMM) from the `glmmTMB` package (Brooks et al., 2017), using a Gamma error structure with a log link for the terminal oocyte size and a Beta error structure with a log link for the lipidic content, as it is a proportion variable comprised between 0 and 1. We considered treatment and bee mass as fixed effects and the colony as random effect. The quality of fits of our models were examined using the `DHARMA` package (Hartig & Lohse, 2022). Visual representations were obtained using `ggplot2` (Wickham et al., 2024) and `ggpubr` packages (Kassambara, 2023).

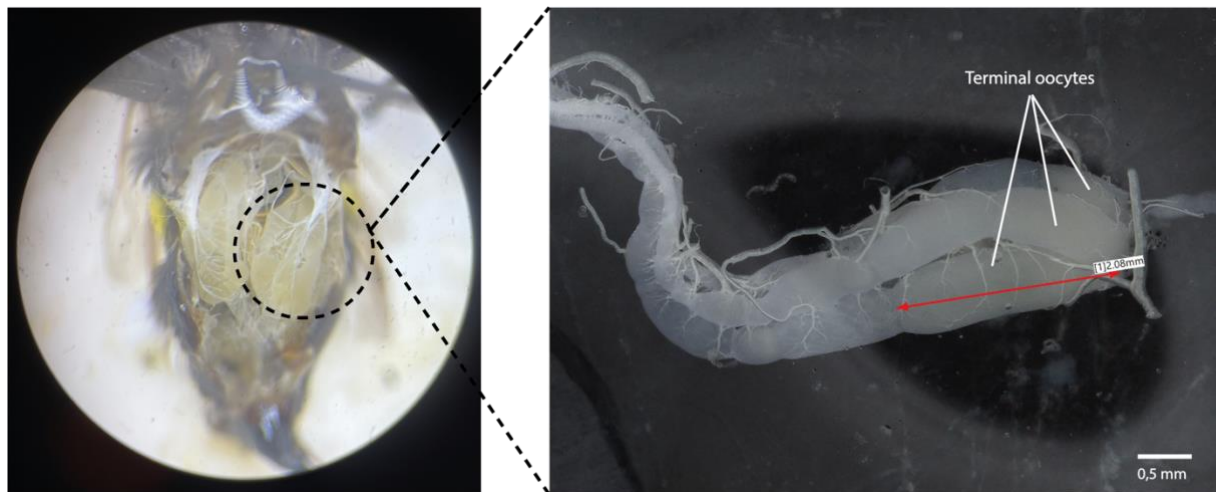


Figure 8: Measures of terminal oocyte length: Dissections of the ovary (organ encircled on the left image) and measures of the length of the terminal oocytes under a numeric microscope (right image).

Chapter 4: Results

4.1 Detection of trace metals

We found significant differences in the volume of consumed solution between the different treatments (GLMM, $\chi^2 = 43.86$, $df = 6$, $p.value < 0.001$). Custom contrast showed that this difference was only significant between the highest concentrations of copper (i.e. Cu900, LC_{50}) and the negative control (i.e. sugar solution), the Cu900 solution being significantly less consumed than the negative control solution (Fig. 9). As expected, no bees fed from the positive control (i.e. sodium chloride solution) and thus these bees are not represented in statistical analysis.

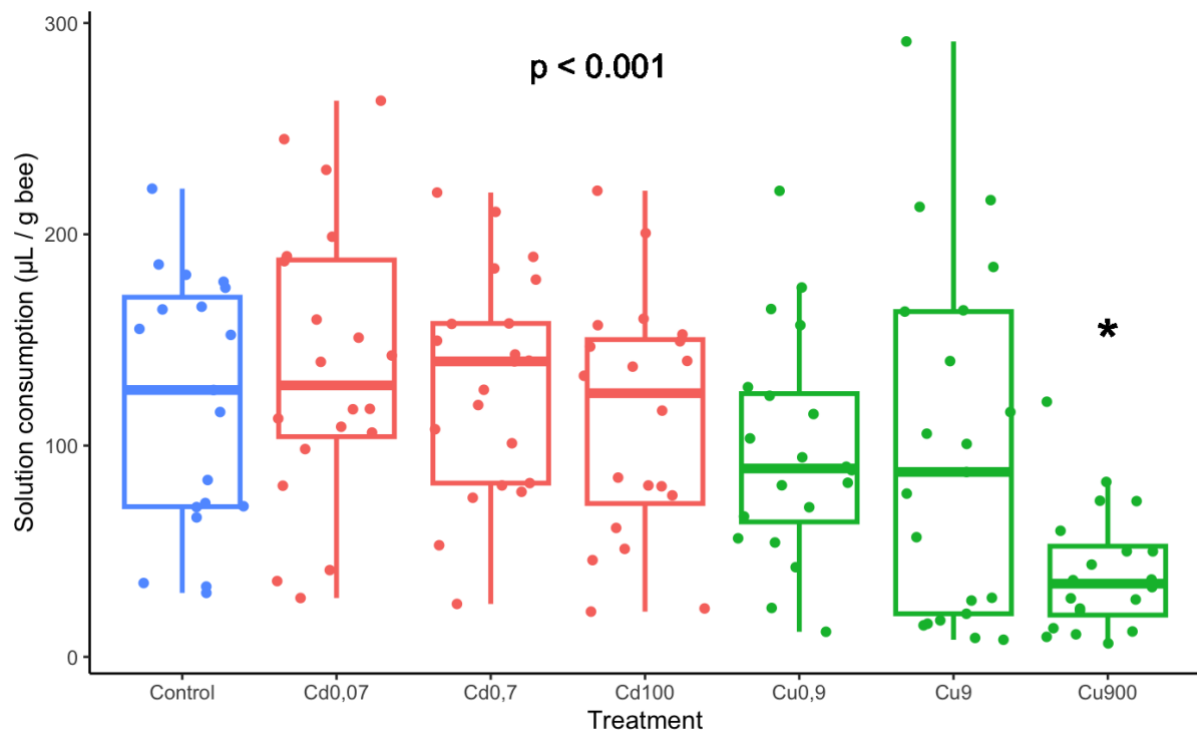


Figure 9: **Solution consumption by *Bombus terrestris* workers through free-moving PER assay:** Total volume consumed in microcapillaries filled with test solutions by individual workers. Volumes were standardised by the mass of each worker. The asterisk indicates significant difference between the treatment and the control. P-value of the GLMM is reported. $N \approx 20$ per treatment (in $mg.L^{-1}$).

The same results have been found for the duration of the first contact of the proboscis with the solution, with significant differences among treatments (GLMM, $\chi^2 = 88.71$, $df = 6$, $p.value < 0.001$). Significant differences were also found for the cumulative duration of contacts (GLMM, $\chi^2 = 72.36$, $df = 6$, $p.value < 0.001$). Through custom contrast, we saw that only the highest concentration of copper (i.e. Cu900, LC_{50}) was significantly different from the control for the two parameters, with a reduction of the duration of contact for the Cu900 solution when compared to the control

solution (Fig. 10). Conversely, the number of contacts with the solution did not differ among treatments (GLMM, $\chi^2 = 1.42$, $df = 6$, p .value = 0.98) (Fig. 11).

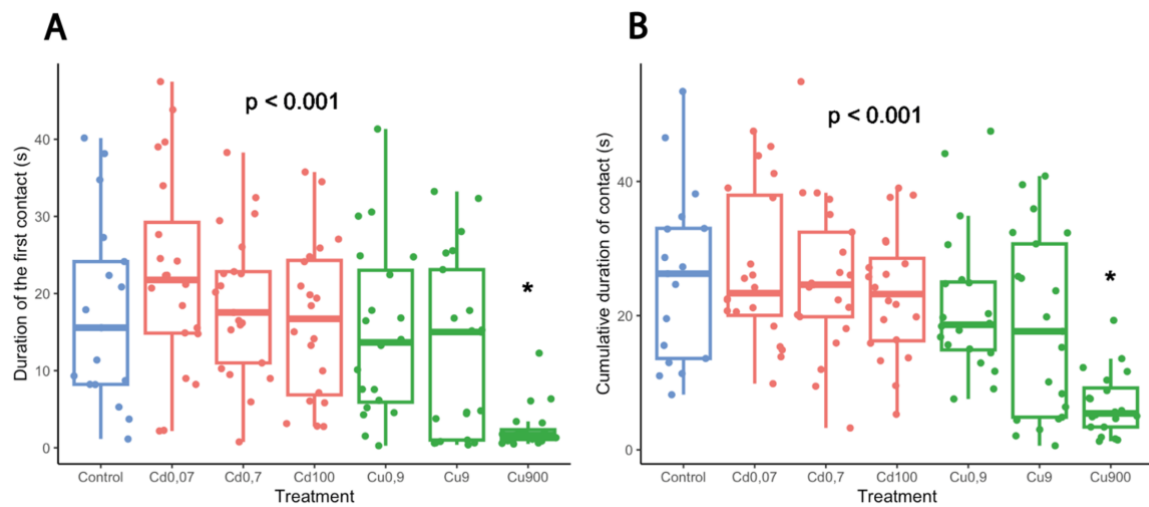


Figure 10: Duration of contacts between the proboscis of *Bombus terrestris* workers and the solutions through free-moving PER assay: A. Duration of the first contact between the proboscis and the solutions. **B.** Cumulative duration of contacts between the proboscis and the solutions. Asterisks indicate significant difference between the treatment and the control. P-values of the GLMM are reported. $N \approx 20$ per treatment (in $\text{mg}\cdot\text{L}^{-1}$).

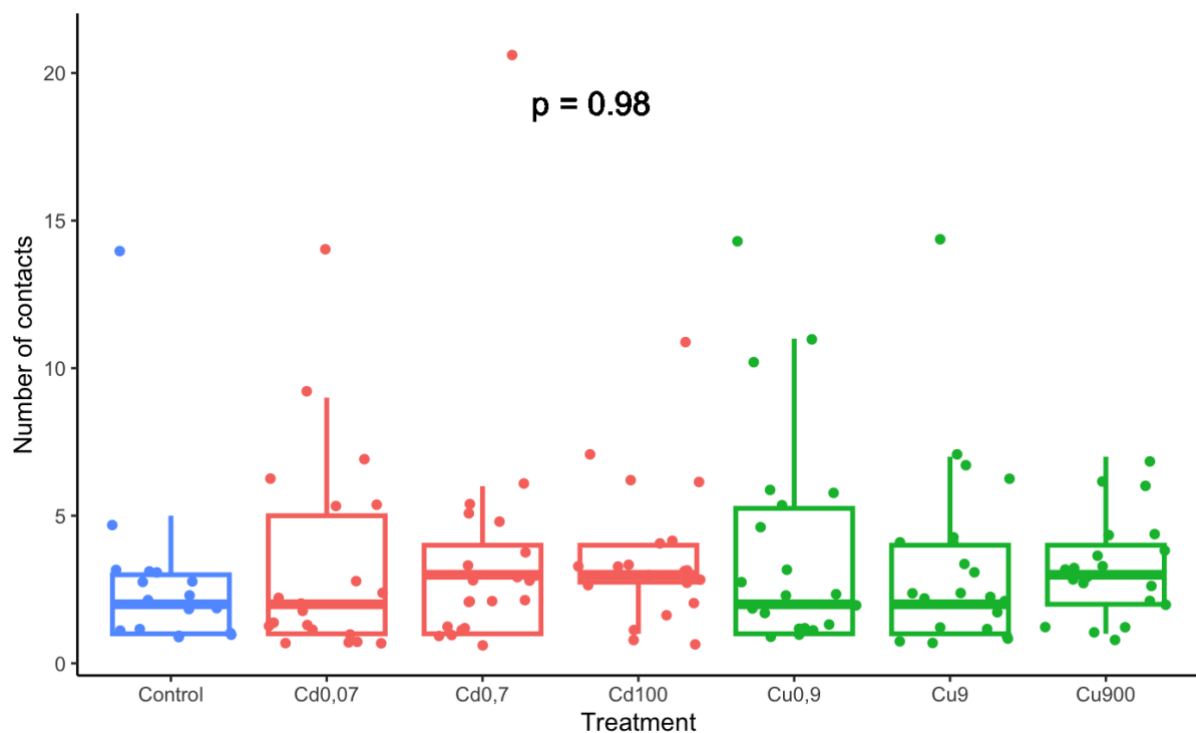


Figure 11: Number of contacts between the proboscis of *Bombus terrestris* workers and the solutions through free-moving PER assay: Total numbers of contacts between the proboscis of bees and the tested solutions. P-value of the GLMM is reported. $N \approx 20$ per treatment (in $\text{mg}\cdot\text{L}^{-1}$).

4.2 Behavioural assays

We found no significant difference among treatments in walking behavioural assays, namely regarding the total walking distance (ART, $df = 6$, p .value = 0.23) (Fig. 12A) or the mean velocity (ART, $df = 6$, p .value = 0.38) (Fig. 12B).

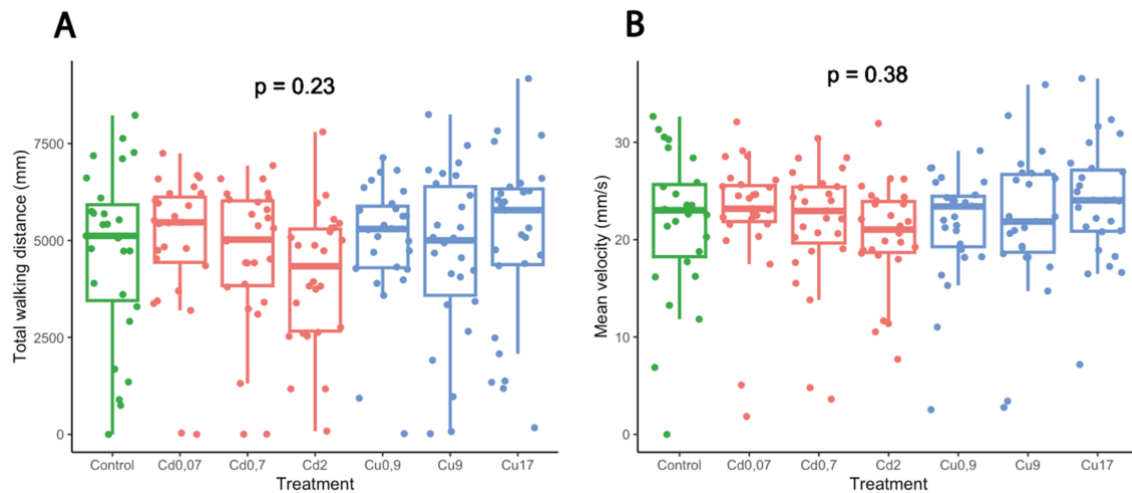


Figure 12: **Walking behaviour assays:** **A.** Total walking distance of bumblebee workers in the Petri dish. **B.** Mean walking velocity of workers in the Petri dish. P-values from ART models are reported. $N \approx 27$ per treatment (in $mg.L^{-1}$).

We found the same findings for the flight take off assays wherein there was no significant difference in flight height among treatments (CLMM, $\chi^2 = 9.85$, $df = 6$, $p = 0.13$) (Fig. 13).

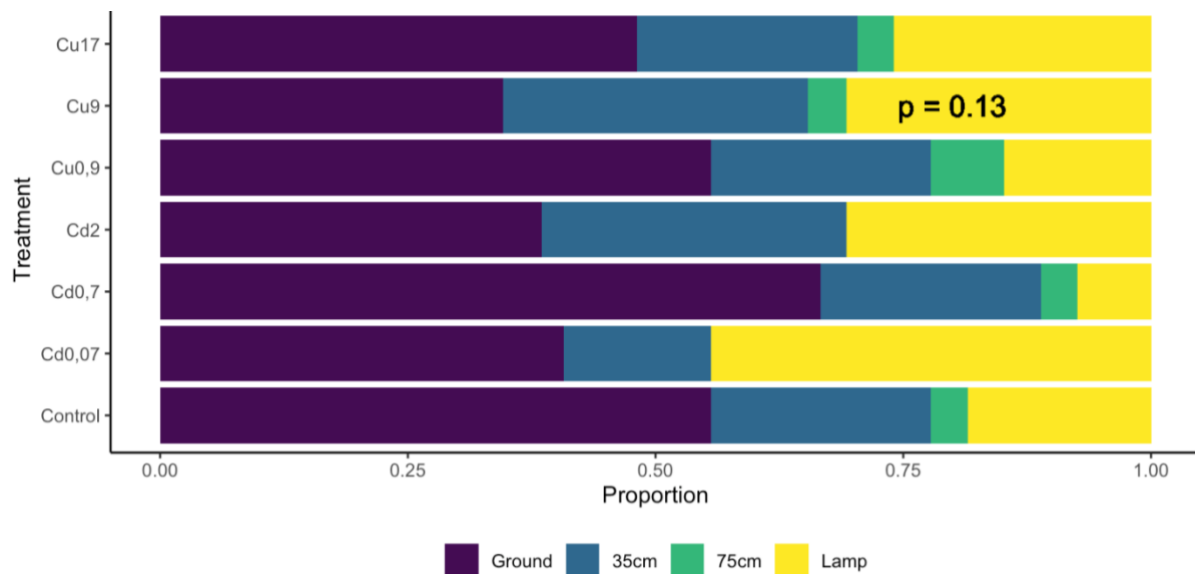


Figure 13: **Flying height reached by *Bombus terrestris* workers:** Proportion of individuals according to the flying heights reached in the tower per treatments. P-value from CLMM is reported $N \approx 27$ per treatment (in $mg.L^{-1}$).

4.3 Gut melanisation

We did not observe any melanisation in the gut of bumblebee workers following chronic exposure. Hence, chronic exposure for three days to field-realistic concentrations of copper (i.e. 0.9, 9 and 17 mg.L⁻¹) and cadmium (i.e. 0.07, 0.7 and 2 mg.L⁻¹) did not trigger melanisation in bumblebee midgut. Therefore, these results were not further analysed statistically. As far as the acute exposure is concerned, higher concentrations induced higher melanisation responses (Fig. 14).

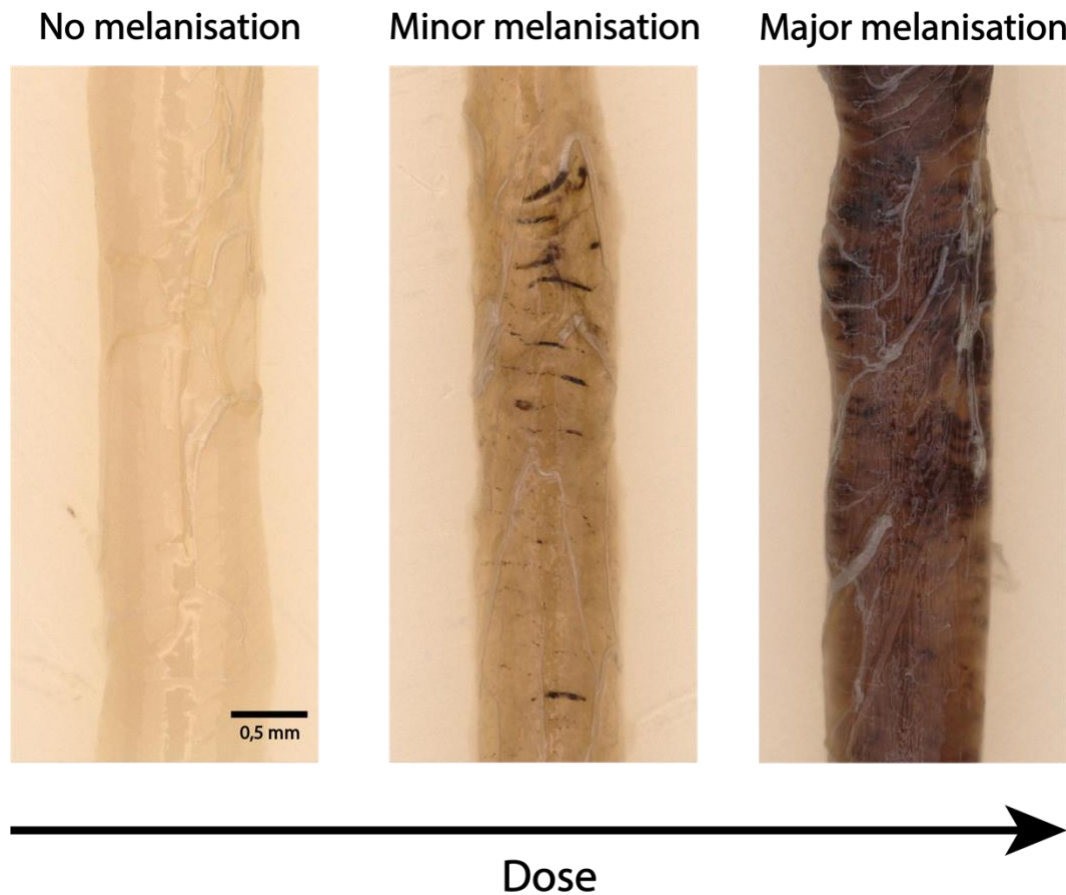


Figure 14: Melanisation of a bumblebee's midgut following acute exposure: From the lowest concentrations of Cu or Cd on the left to the highest (lethal concentrations) concentrations on the right.

We found significant differences in midgut melanisations (i.e. 'darkness' of the midgut) among treatments, both for copper (GLM, $\chi^2 = 147.60$, $df = 5$, $p.value < 0.001$) and for cadmium (GLM, $\chi^2 = 137.48$, $df = 5$, $p.value < 0.001$). Pairwise comparisons showed that only the two highest concentrations of copper and cadmium cause significant damages in the midgut (Fig. 15).

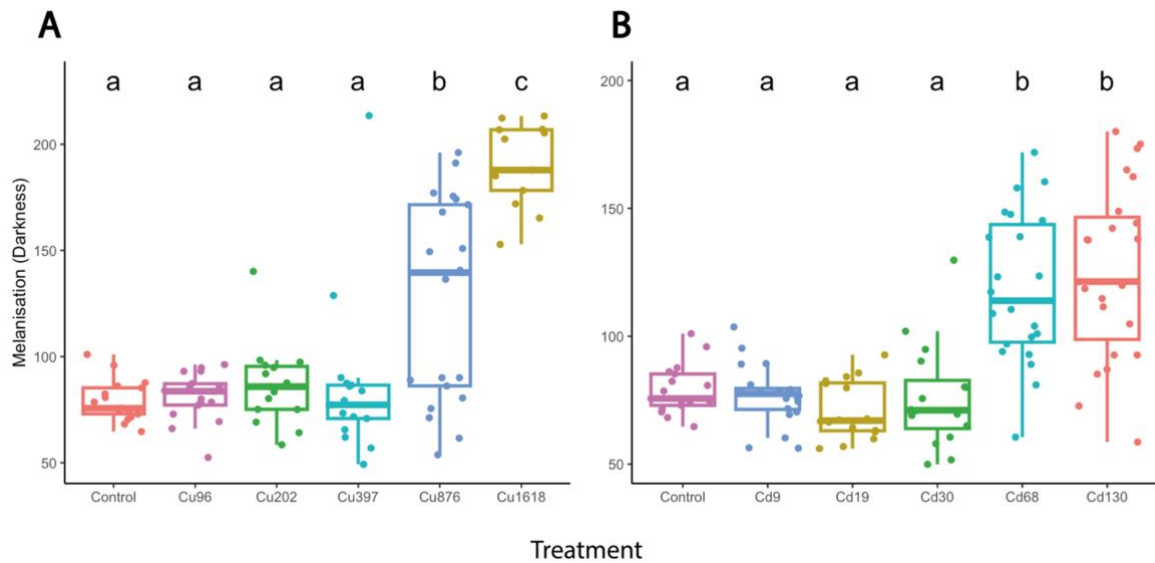


Figure 15: Melanisation in the midgut of *Bombus terrestris* workers exposed to geometric concentrations of copper (A) and cadmium (B): Darkness was used as proxy for melanisation and was calculated using the mean grey values of the midgut (darkness = 255 – mean grey value). Treatments which do not share the same letter are statistically different (pairwise comparisons with FDR adjustment after GLM). N ≈ 17 per treatment (in mg.L⁻¹).

Likewise, we found significant differences in sucrose consumption for the 24 hours following exposure, both for copper (GLM, $\chi^2 = 151.47$, df = 5, p.value < 0.001) and cadmium (GLM, $\chi^2 = 203.47$, df = 5, p.value < 0.001). Pairwise comparisons showed that the sucrose consumption decreased as concentrations of copper (Fig. 16A) and cadmium (Fig. 16B) increased. By contrast to gut damages, sucrose consumption was not only reduced at high concentrations but decreased progressively as concentrations increased.

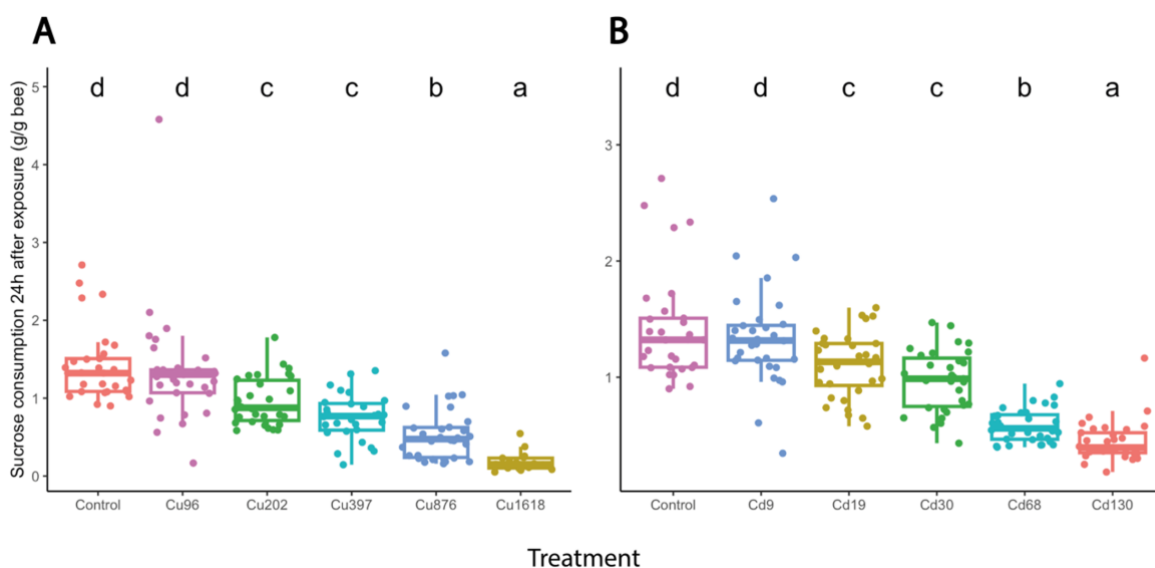


Figure 16: Sucrose consumption for 24 hours after acute exposure to geometric concentrations of cadmium (A) and copper (B). Consumptions were standardised by the mass of each worker: Treatments which do not share the same letter are statistically different (pairwise comparisons with FDR adjustment after GLM).

4.4 Oocyte length

Chronic exposures to copper and cadmium for seven days did not induce significant changes in the terminal oocyte length of bumblebee workers among treatments (GLMM, $\chi^2 = 2.64$, $df = 4$, $p = 0.64$). Moreover, bee body mass did not influence the terminal oocyte length (GLMM, $\chi^2 = 3.36$, $df = 1$, $p = 0.066$) (Fig. 17).

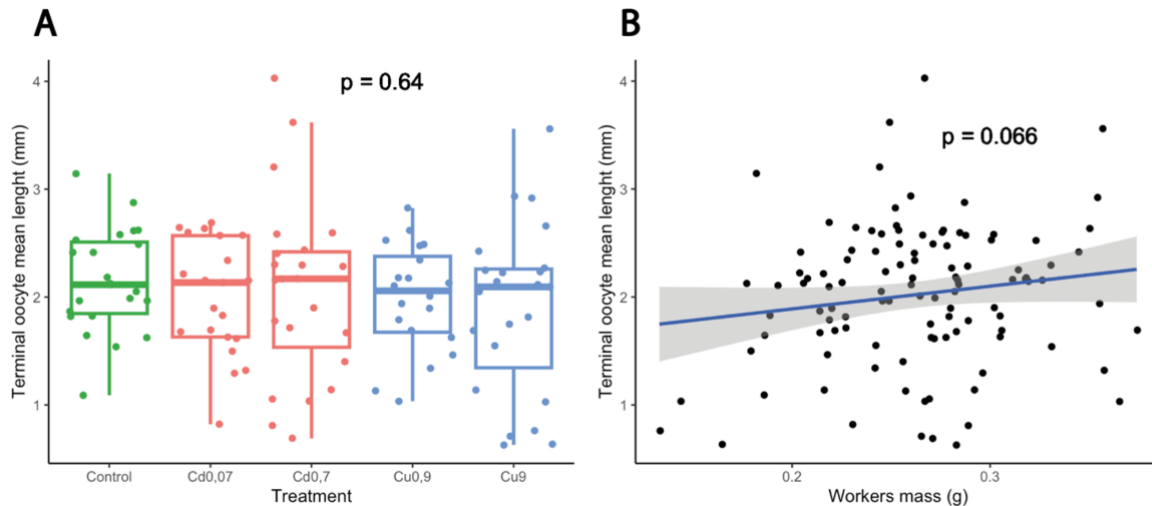


Figure 17: Mean length of the two terminal oocytes of *Bombus terrestris* workers: **A.** Effect of chronic exposure to cadmium and copper on the terminal oocyte length of *Bombus terrestris* workers. **B.** Terminal oocyte length of *Bombus terrestris* workers according to their body mass. P-values from GLMM are reported. $N \approx 25$ per treatment (in $\text{mg}\cdot\text{L}^{-1}$).

4.5 Lipidic content

Chronic exposures to copper and cadmium for seven days did not induce significant changes in the abdominal lipidic content of bumblebee workers among treatments (GLMM, $\chi^2 = 2.56$, $df = 4$, $p = 0.82$). By contrast, bee body mass was negatively correlated to the lipidic content (GLMM, $\chi^2 = 2.44$, $df = 4$, $p < 0.001$) (Fig. 18).

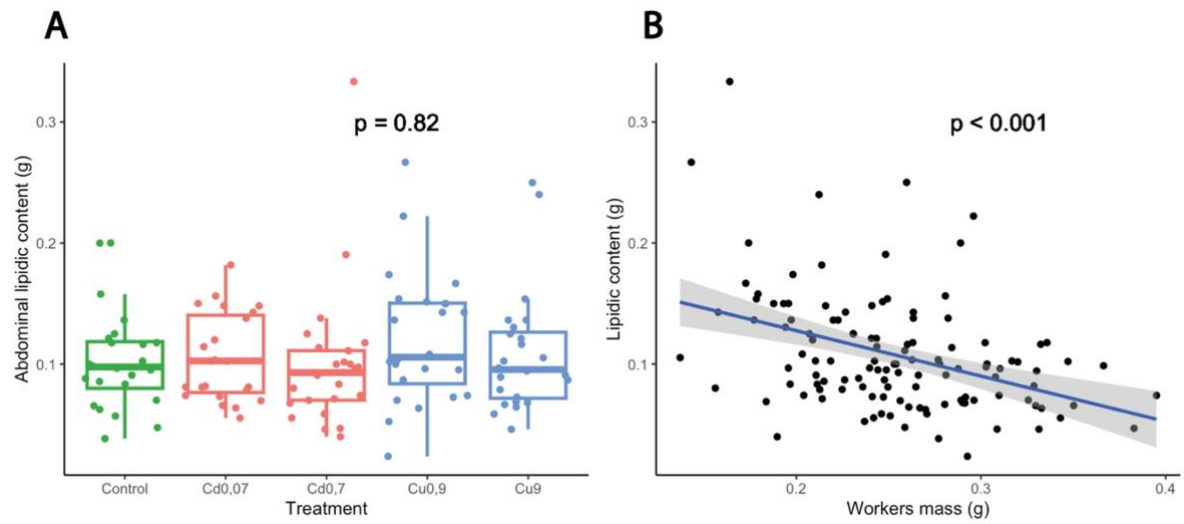


Figure 18: Abdominal lipidic content of *Bombus terrestris* workers: **A.** Effect of chronic exposure to cadmium and copper on abdominal lipidic content of *Bombus terrestris* workers. **B.** Abdominal lipidic content of *Bombus terrestris* workers according to their body mass. P-values from GLMM are reported. $N \approx 25$ per treatment (in mg.L^{-1}).

Chapter 5: Discussion

The ability of bees to discriminate a wide variety of molecules, nutrients or specialised metabolites in their food corroborates their capacity to perceive and avoid resources containing hazardous xenobiotics (Bestea et al., 2021; de Brito Sanchez, 2011; Kang & Jung, 2017). However, it has been suggested that bees were unable to detect all xenobiotics, including some pesticides, leading to their consumption and subsequent death (Parkinson et al., 2023). The ability of bees to detect trace metals remains unclear and poorly understood. Accordingly, our study focused on the capacity of bumblebee workers to detect these trace metals in a sucrose solution. Our results reveal that bumblebees were unable to detect environmental concentrations of Cu and Cd, while they did not consumed the highest, field-unrealistic concentration of Cu (i.e. 900 mg.L⁻¹/6693.94 µM). These findings are in accordance with the previous study of Burden et al. (2019) on honeybees who found no rejection of sucrose solution contaminated by Cd and by Cu through PER assay (maximum concentrations tested in the study were 20 mg.L⁻¹ for Cu and 10 mg.L⁻¹ for Cd) (Burden et al., 2019). While our results discard the existence of pre-ingestive cues regarding metal detection, the occurrence of a post-ingestive process remains an open question (e.g. post-ingestion malaise; Ayestaran et al., 2010; Liao et al., 2017) Indeed, the duration of our PER experiment (i.e. 2 min) was too short for metals to reach the haemolymph, meaning possible post-ingestive detection mechanisms could not occur (Simcock et al., 2018).

This consumption of field-realistic concentrations aligns with the fact that some trace metals, such as Cu, are essential (Maret, 2016), and their consumption in appropriate doses is vital for proper biological functions. Nevertheless, bumblebees did not detect Cd, which is a non-essential metal, and even more concerning they did not show any rejection towards the highest, lethal concentration of Cd. This discrepancy between copper and cadmium might be explained by a concentration-dependent effects as suggested by Monchanin et al. (2022) (Monchanin et al., 2022). Indeed, for the PER experiment, we based the highest concentrations on the median lethal concentrations (i.e. LC₅₀) found in a previous experiment, in which Cd was >15x more toxic than Cu for bumblebee workers (Gekièrè et al., submitted in Environmental Toxicology and Pharmacology). This is the reason why the highest concentration of Cd in the PER experiment was way lower than the highest concentration of Cu.

Exposure to xenobiotics, especially to neurotoxic pesticides, leads to sublethal effects on bees' cognitive and motor functions, which impede crucial foraging and locomotive behaviours (Araújo et al., 2023; Belzunces et al., 2012; Cabirol & Haase, 2019; Caito & Aschner, 2015). Exposure to

trace metals in stingless bees and bumblebees has been reported to affect mobility functions such as flight and walking activities (Bernardes et al., 2022; Botina et al., 2023; S. Gao et al., 2024; Rodrigues et al., 2016). Our findings did not confirm these effects, as we found no impacts on walking and flight behaviours after chronic exposure to copper and cadmium. However, our results corroborate another study by Botina et al. (2019) on *Partamona helleri* exposed to Cu (Botina et al., 2019). Discrepancies among results may be explained by the differences among the experimental designs used in these studies. In our study, we exposed bumblebees to environmental concentrations for three days. In contrast, other studies used concentrations based on mortality, as Rodrigues et al. (2016) (Rodrigues et al., 2016), which exposed bees to concentrations 250 times higher than ours. Previous studies used longer duration of exposure, as Gao et al. (2024) that exposed bumblebees for 14 days. On the other hand, Botina et al. (2019) applied concentrations six times higher than ours (i.e. 120 mg.L⁻¹) but the exposure times was three times shorter (i.e., 24h). These different outcomes between studies highlight the importance of taking concentrations and durations of exposure into account in ecotoxicological studies, since completely different conclusions could be drawn. Furthermore, our experimental design only allowed us to assess basic parameters, namely flight take-off and walking behaviours. In the wild, the behaviour of bees includes a variety of highly complex possibilities, especially during their foraging activities. Assessment of behavioural impairment by more advanced behavioural assays in the future as evaluation of foraging parameters in a free-flight room (e.g. visiting time, visiting rate, number of foraging trips; Gérard, Cariou, et al., 2022; Gérard et al., 2023) or assessment of cognitive skills (e.g. association learning task; Gérard, Amiri, et al., 2022) may reveal sublethal effects on more complex tasks.

The chronic exposure to environmental concentrations of copper and cadmium did not induce melanisation response in the midgut epithelium of bumblebee workers. By contrast, when we performed an acute exposure to higher concentrations, melanisation occurred. By measuring the mean grey values of epithelium as a proxy for melanisation, we revealed an increase in melanisation as the doses increased. Bees exposed to concentrations 200 times higher than the environmental ones already harboured complete black guts. Previous studies have already demonstrated that exposure to pesticides lead to immune response causing the melanisations in the midgut (Straw & Brown, 2021). In the case of exposure to trace metals, the formation of ROS by metal ions and the oxidative stress that it causes leads to cellular damages in gut epithelium, triggering an immune response and melanisation due to the entrance of foreign bodies (Bánfalvi, 2011; Larsen et al., 2019; Pizzino et al., 2017). Otherwise, melanisation can be induced by other factors than oxidative stress as in the case of Amistar® exposure, the melanisation response might be caused by alcohol ethoxylate present as co-formulants that disrupt the structure of the midgut

(Straw & Brown, 2021). In other hands, some agrochemicals, such as glyphosate, inhibit melanisation by inhibiting melanin production (Smith et al., 2021). These results complement previous studies showing that exposure to Cu and Cd caused histological damages in gut epithelium (Bernardes et al., 2022; Dabour et al., 2019). In addition to gut melanisation, we monitored the sucrose consumption (i.e., untreated syrup) of bumblebees for 24 h after exposure. Food intake decreased as concentrations increased, which aligns with gut melanisation. This reduction in sucrose consumption may be linked to gut damages that rendered food intake and digestion difficult for metal-exposed bees. Correspondingly, the exact cause of death in this assay remains elusive. However, we could propose that bees died due to energy depletion caused by a reduction in food intake, since caged bumblebees do not survive more than 48 hours without food (Pers. obs.).

Exposure to xenobiotics leads to many physiological impairments, including the perturbation of fat metabolism or reproductive system (Christen, 2023; Laycock et al., 2012; Siviter et al., 2020). Fat storage in bees' body is key to many metabolism pathways, in addition to playing a crucial role in the secretion of immunoproteins (Skowronek et al., 2021). The fat bodies are also involved in other physiological systems such as sexual maturation (Amdam et al., 2012; Skowronek et al., 2021). We found no changes in the abdominal lipidic content of bumblebees after chronic exposure to Cd or Cu at field-realistic concentrations for seven days. The effect of trace metals in lipid metabolism remains elusive, although trace metals have been shown to affect lipid metabolism pathways in other arthropods (e.g. black soldier fly and greater wax moth) (Loskutov et al., 2023; Shin et al., 2001) and are known to accumulate in the fat bodies of insects (J. Zhang et al., 2020), including bees (Goretti et al., 2023). Likewise, we found that the terminal oocyte size of workers did not differ after chronic exposure to Cd or Cu at field realistic concentrations for seven days, which was unexpected given previous reports with pesticides (Barbosa et al., 2015; Baron et al., 2017; Laycock et al., 2012). Despite these results, trace metals have been reported to impact the reproductive fitness of bees. For instance, lower rate of brood cells in mason bees (Moroń et al., 2014) and larvae (Scott et al., 2022; Sivakoff et al., 2020) in bumblebee colonies were found after trace metal exposure. However, an absence of effect of metals on lipidic content and oocyte size does not necessarily imply that metals have not effect on the bees' reproductive fitness. Indeed, impairment of reproductive fitness can be caused by other factors, as reproduction entails many parameters at different levels (e.g. mating behaviour, queen, male gametes, larval development). Despite our results, potential effects of trace metals on oocytes remain plausible, since the duration of exposure was relatively short compared to other studies with pesticides which used duration of exposure two times longer (i.e. 14 days; Laycock et al., 2012). Moreover, we only investigate the morphology of the oocytes, without taking in considerations the molecular

responses of the reproductive system. For instance, indirect exposure to insect growth regulators pesticides leads to reproductive impairments caused by altered expression of ovarian proteins in honeybee queens (Fine et al., 2023). Finally, our assay was conducted on workers who are not involved in colony reproduction/fitness, as worker ovaries are atrophied in a queen-right colony. It would be interesting to investigate the effects of copper and cadmium on the queen's ovaries.

In contrast with our expectation and previous results found in the literature, we found no impacts of copper and cadmium exposure on bumblebee's physiology and behaviour. However, by comparing our methodologies with the ones developed in other studies, the doses and the durations of exposure seem to be major factors underlying the discrepancies between our studies and the literature. Here, we utilised field-realistic concentrations based on data from the European Consortium PoshBee (Durkalec et al., 2024). From these data, we determined the mean values which has been considered as environmental concentrations, namely 9,000 ppb for Cu and 70 ppb for Cd, which are respectively six and five times lower than the maximum concentrations found in the same study (Durkalec et al., 2024). Furthermore, metal concentrations were only measured in colonies' beebread and pollen stores. In fact, bees are exposed to trace metals through many routes (Giglio et al., 2017; van der Steen et al., 2012), and considering only one source of contamination to define a realistic situation may not be the most relevant scenario. This is even more true as we know that metals bioaccumulate (reaching hazardous concentration) in different environmental reservoirs (Bradl, 2005; Briffa et al., 2020; Tchounwou et al., 2012). In addition, PoshBee data were only collected in agricultural areas (i.e. rape fields and apple orchards), a fairly safe place compared to highly polluted areas such as industrial or mining zones which harbour drastic amounts of trace metals (Akbar et al., 2024; Qu et al., 2018). Moreover, when we used higher concentrations as for gut damage assessment, severe damages appear, which highlight issues in physiological systems after exposure to high doses of trace metals. Future experiments need to take these factors into account to be as close as possible to worst-case, but although realistic conditions.

Chapter 6: Conclusion & perspectives

As expected, bumblebee workers did not show any ability to detect field-realistic concentrations of copper and cadmium in sucrose solutions. Only the highest concentration of Cu was avoided, suggesting that trace metal consumption readily occurs in natural environments, perhaps to match dietary requirements for essential trace elements (i.e. Cu). However, this interpretation does not align with the absence of avoidance for Cd, which is a non-essential metal. All concentrations of Cd, including the highest concentration (i.e., median lethal concentration), were not avoided, unlike Cu, suggesting that lethal concentrations of Cd can be consumed by bumblebees in the wild. Exposure to environmental concentrations of Cu and Cd did not lead to any changes in the behavioural abilities of bumblebees, namely their walking and flying abilities. The same observation was made for physiological parameters, with no effect of exposure to environmental concentrations on lipidic content, terminal oocyte length and midgut melanisation response. These results suggest no impacts of trace metal exposure on bumblebees' lipid and reproductive metabolisms at field-realistic concentrations, contrary to our expectations. However, we cannot exclude the possibility that exposure to trace metals may pose a risk to bee's health, because in natural conditions bees may encounter higher concentrations of metals, such as in polluted areas. High concentrations could lead to severe damages, as we saw through midgut melanisation. In the wild, bees are also exposed for longer periods, most likely throughout their whole lives. Future research should reconsider the durations of exposure and concentrations used to better reflect the natural environment and the multiple sources of exposure to which bees are exposed.

Despite our efforts, many questions remain. New experiments can be tested to further investigate effects and mechanisms of trace metal toxicity such as bioaccumulation, by measuring trace metal levels in different tissues and products of bees. Bees are known to bioaccumulate metals and investigating where metals are accumulated could reveal which part of the bee is the most exposed or which one involved in detoxification. Analysis of bees' proteome following exposure could be attempting to understand immune response and defence mechanisms in bees such as the production of metallothionein, which is potentially the keystone of metal detoxification in bees. Damages on vulnerable organs as mushroom bodies could be assessed by histology, as done by Dabour et al. (2019) on gut tissues. Our experimental design could be improved or used in other contexts to discover new facets of studied mechanisms. Repeating our assays under different conditions with other metals, including cocktail solutions, and investigating synergistic effects with other xenobiotics or pathogens/parasites is essential as bees in nature are exposed to

multiple hazards at the same time. We only investigated bumblebee's workers, but other castes exist (i.e. males and queens) and the effects of trace metals on them need to be investigated. Sublethal effects on larvae also need to be investigated, as trace metals such as Cd have been reported to alter development (Knoll & Cappai, 2024). We could also use other wild bees in our experiments as their relationship and exposure to xenobiotics is potentially different. Many paths need to be explored to unravel danger of these pollutants. In a world where humans have reshaped environment for their own interest and greed, we have the responsibility to work for a better future for the other species we live with.

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Chapter 8: Appendices

Appendix 1- Gut melanisation post-hoc experiment

Post-hoc experiment has been realised in order to ensure that damages on midgut were effectively melanisation from immune response and not due to post-mortem decomposition. Nine alive workers from healthy colony were directly frozen at -70 °C. Nine workers were decapitated, left 24 h in the rearing room in sterile tubes, and then frozen at -70 °C. Gut were dissected and melanisation measured. We found that melanisation (i.e. darkness) was not significantly different between the two groups (Wilcoxon rank sum test, $W = 50$, $p = 0.42$) (Fig. 19).

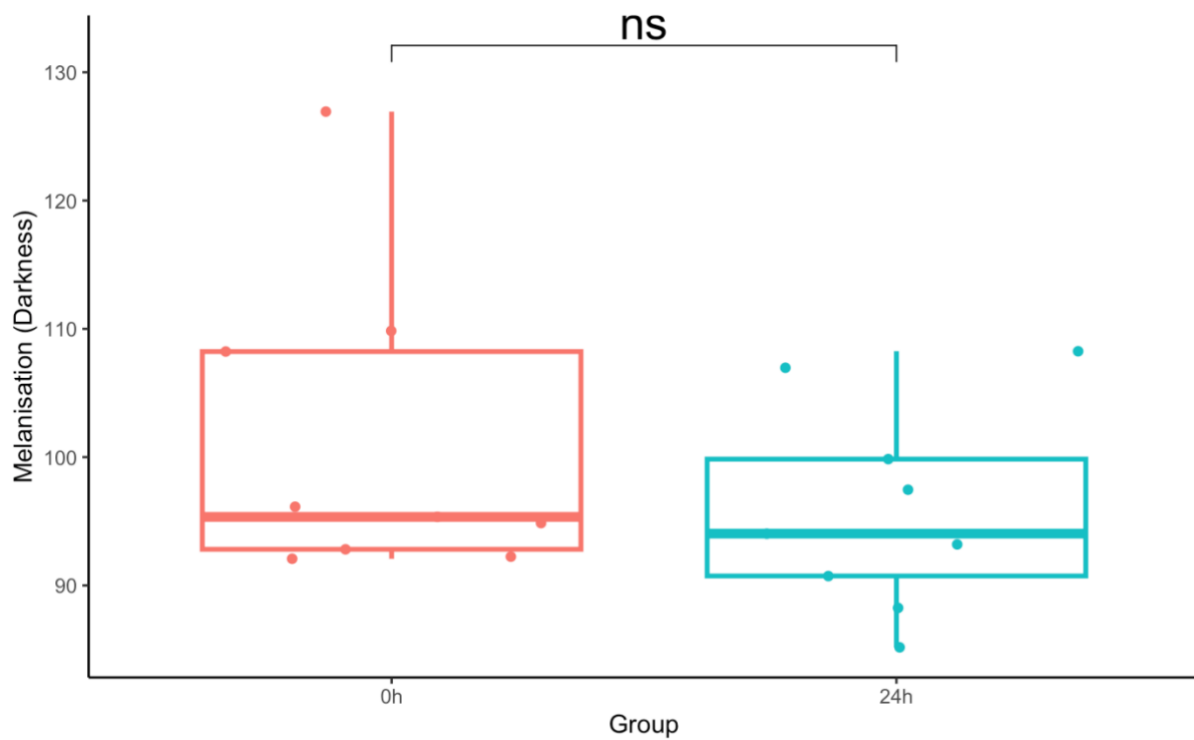


Figure 19: Melanisation post-hoc experiment: Post-hoc experiment showing that gut damages were not due to post-mortem decomposition. Darkness was used as proxy for melanisation and was calculated using the mean grey values of the midgut (darkness = 255 – mean grey value). Treatments which do not share the same letter are statistically different (Wilcoxon rank sum test). $N = 9$ per group.

