From Hormonal Pathways to Colony Development: Multiscale Investigation of Diethylhexyl and Di-n-butyl Phthalate Impact on *Bombus terrestris* (Hymenoptera: Apidae)

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Abstract – Résumé

Abstract - English Version

Phthalates are widely used as plasticisers to increase the flexibility of plastic materials or as solvents in detergents, paints, and pesticides. As they are not chemically bound to polymer matrices, they leach easily into the environment and have become ubiquitous pollutants, detected both in highly anthropogenic environments and remote locations. Identified as endocrine disruptors in vertebrates, phthalates also affect fertility, immunity, and development in aquatic invertebrates. Yet, their impact on terrestrial invertebrates, and especially insects, is understudied. Due to their lipophilic nature, phthalates are actively trapped by the lipid-rich cuticle of insects, exposing bees through flight and contact with contaminated surfaces. In Europe, two of the most common phthalates, di-(2-ethylhexyl) phthalate (DEHP) and di-n-butyl phthalate (DnBP), have been detected on honeybee and bumblebee cuticles, as well as in hive materials such as honey and wax. Bees in urban and agricultural environments, already under multiple anthropogenic pressures, are therefore additionally exposed to phthalates, which may disrupt conserved hormonal and metabolic pathways with cascading effects on health and development. During this project, we investigated for the first time the impact of DnBP and DEHP on a bee species, *Bombus terrestris*, a key European pollinator and established surrogate species in wild bee ecotoxicology. *B. terrestris* workers were exposed to environmental concentrations of phthalates, to assess multi-level impact from the molecular to the colonial scale. First, short-term exposure assays revealed a non-monotonic dose-response relationship for mortality following DnBP exposure, alongside significant antagonistic effects of DEHP combined exposure and of social contacts. At the molecular level, low-dose DEHP altered gene expression related to ecdysteroid signalling, a key developmental hormonal pathway, while surviving DnBP-exposed workers showed dysregulated expression of metabolism-related genes. Secondly, long-term repeated exposure of workers in microcolonies further highlighted transgenerational obesogenic effects. The DnBP-exposed workers displayed increased lipid storage and produced drone offspring with increased body mass. These results were associated with a dysregulation of the expression of genes involved in the insulin-signalling pathway. DEHP, alone or in combination with DnBP, caused elevated worker mortality after 35 days of repeated exposure and impacted ecdysteroid-related gene expression. These novel findings provide the first evidence of phthalate impact on bees and towards risk assessment of these pollutants in pollinators. Despite growing evidence of their disruption of conserved hormonal and metabolic pathways across taxa, existing EU legislation addresses phthalates exclusively through direct human exposure. This work underscores the need for broader environmental regulation and further research to evaluate the role of phthalates in pollinator decline.

Résumé – Version française

Les phtalates sont largement utilisés comme plastifiants afin d'augmenter la flexibilité des matériaux plastiques, ou comme solvants dans les détergents, les peintures et les pesticides. N'étant pas chimiquement liés aux matrices polymères, ils sont facilement libérés dans l'environnement et sont devenus des polluants omniprésents, détectés aussi bien dans les milieux fortement anthropisés que dans des zones reculées. Perturbateurs endocriniens reconnus chez les vertébrés, les phtalates affectent également la fertilité, l'immunité et le développement des invertébrés aquatiques. Cependant, leur impact sur les invertébrés terrestres, et plus particulièrement sur les insectes, reste peu étudié. En raison de leur nature lipophile, les phtalates sont activement piégés par la cuticule riche en lipides des insectes, exposant ainsi les abeilles lors du vol ou par contact avec des surfaces contaminées. En Europe, deux des phtalates les plus courants, le phtalate de di(2-éthylhexyle) (DEHP) et le phtalate de di-n-butyle (DnBP), ont été détectés sur la cuticule de bourdons capturés en milieu urbain, ainsi que dans certains produits de ruche d'abeilles mellifères tels que le miel et la cire. Les abeilles évoluant dans des environnements urbains et agricoles, déjà soumises à de multiples pressions anthropiques, sont donc également exposées aux phtalates, susceptibles de perturber des voies hormonales et métaboliques conservées, entraînant des effets en cascade sur leur santé et leur développement. Au cours de ce projet, nous avons étudié pour la première fois l'impact du DnBP et du DEHP sur une espèce d'abeille, *Bombus terrestris*, un pollinisateur clé en Europe et une espèce modèle établie en écotoxicologie des abeilles sauvages. Des ouvrières de *B. terrestris* ont été exposées à des concentrations environnementales de phtalates afin d'évaluer les effets à plusieurs niveaux, du moléculaire à celui de la colonie. Dans un premier temps, des tests d'exposition à court terme ont révélé une relation dose-réponse non monotone de la mortalité après exposition au DnBP, ainsi que des effets antagonistes significatifs lors d'expositions combinées avec le DEHP et lors de contacts sociaux. Au niveau moléculaire, l'expression de gènes liés à la signalisation des ecdystéroïdes, une voie hormonale clé du développement, a été dérégulée après exposition à de faibles doses de DEHP, tandis que l'exposition au DnBP a révélé une dérégulation de l'expression de gènes associés au métabolisme chez les ouvrières. Dans un second temps, une exposition répétée à long terme d'ouvrières maintenues en microcolonies a mis en évidence des effets obésogènes transgénérationnels. Les ouvrières exposées au DnBP ont montré une augmentation du stockage lipidique et ont produit une descendance de mâles présentant des masses corporelles plus élevées. Ces résultats ont pu être associés à une dérégulation de l'expression de gènes impliqués dans la voie de signalisation de l'insuline. De plus, une exposition au DEHP, seule ou combinée au DnBP, a induit une mortalité accrue des ouvrières après 35 jours d'exposition répétée et a affecté l'expression des gènes liés aux ecdystéroïdes. Ces résultats constituent les premières preuves de l'impact des phtalates sur les abeilles et contribuent à l'évaluation du risque que représentent ces polluants pour les pollinisateurs. Malgré l'accumulation de preuves concernant leur capacité à perturber des voies hormonales et métaboliques conservées chez différents taxons, la législation européenne actuelle ne traite les phtalates qu'à travers le prisme de l'exposition humaine directe. Ce travail souligne donc la nécessité d'une réglementation environnementale plus large et de recherches approfondies pour évaluer le rôle des phtalates dans le déclin des pollinisateurs.

CHAPTER I: General introduction and aim of the thesis

General introduction

Since the early 1950s, the yearly global plastic production has skyrocketed, reaching over 413 million tonnes in 2023 (PlasticsEurope, 2024), i.e., a 200-fold increase since its first production levels (fig. 1a; Geyer et al., 2017). Despite this exponential growth, global authorities have made little effort to implement effective reduction policies (Walker and Fequet, 2023). This was recently illustrated by the failure of intergovernmental negotiations at the Global Plastic Treaty conference held in August 2025 in Geneva, which ended without agreements. Yet, associated with this excessive consumption of plastic is a considerable increase in plastic pollution, which is one of the major environmental issues of our time. In 2017, Geyer et al. highlighted that 6300 Mt of plastic waste had been produced in total between 1950 and 2015, of which 79% likely ended up in natural environments because of mismanagement and fail in efficient recycling methods (fig. 1b; Geyer et al., 2017). This pollution is now visible in sedimentary records (Brandon et al., 2019). While the Anthropocene qualification of the geological period showing marks of anthropogenic activity is strongly debated, for many scientists, a new stage of this period has been reached: the Plasticene Era (Brandon et al., 2019; Rangel-Buitrago and Neal, 2023).

In the public mind, plastic pollution usually refers to the nano- and microplastic particles (NP/MP; 1µm-5mm) that result from the fragmentation of bigger polymer items. MPs and NPs come from various polymer types like polypropylene (PP), polyethene (PE), polystyrene (PS), to polyvinyl chloride (PVC; Fig. 1a), and from a wide variety of day-to-day items ranging from single-use packaging and clothing fibers to building materials and high-precision medical devices (PlasticsEurope, 2024). The generalised pollution by these particles has been detected from the ocean sediments (Brandon et al., 2019) to the atmosphere (Aeschlimann et al., 2022).

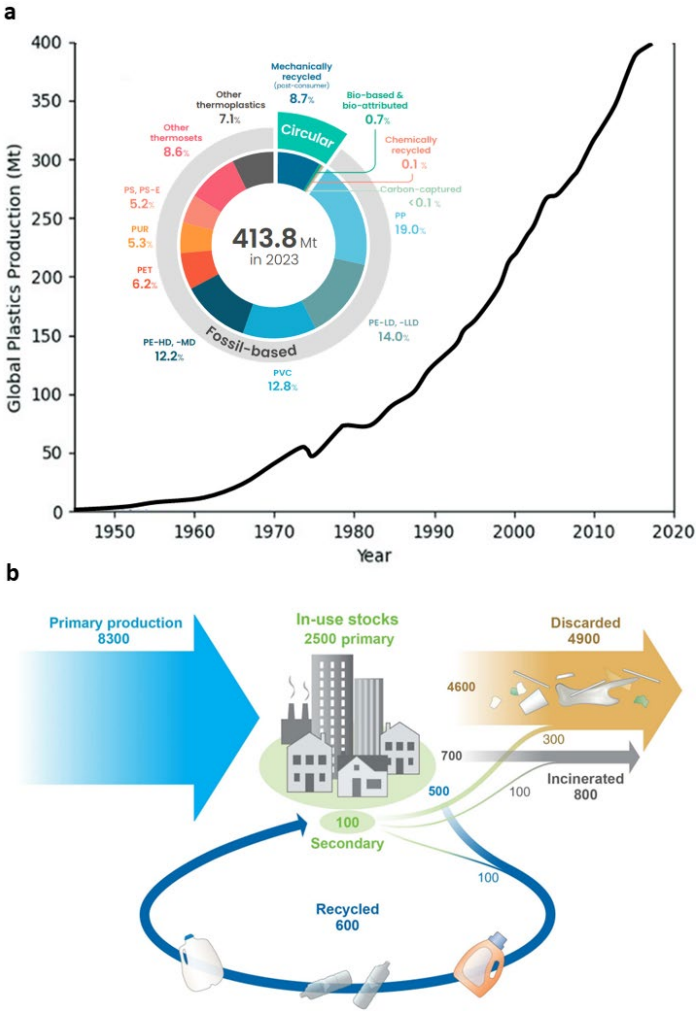


Fig. 1. Global plastic and plastic waste production. a) Global trend in yearly plastic production in Mt from 1950 to 2019 (adapted from Pires Da Mata Costa et al., 2021). The pie chart shows global plastic production in the year 2023, with the proportions occupied by the different types of plastic (from PlasticsEurope, 2024). b) Lifecycle of all plastic items ever made from primary production to fate, either discarded or recycled (from Geyer et al., 2017).

While plastic pollution in marine wildlife has been studied since the 1970s (Wright et al., 2013), investigations in terrestrial systems, including bees, are only emerging (Edo et al., 2021; Rodrigues et al., 2025). This pollution is now part of their environment, notably with some species even found nesting in plastic structures (Allasino et al., 2019). Alongside their detection in honeybee, *Apis mellifera*, hives, several studies also highlighted deleterious effects on honeybee food consumption, cognition and immunity, mainly resulting from physical obstruction and deterioration of organs and tissues (reviewed by Al Naggar et al., 2024; Bashir et al., 2024; Rodrigues et al., 2025). However, another key part of the plastic

pollution is linked to their impregnation with numerous additives such as flame retardants, colourants, UV stabilisers and plasticisers (Hahladakis et al., 2018). Indeed, these chemicals often have toxic and endocrine activity on organisms (Renault et al., 2024), and are released directly from plastic items, but also from their resulting NPs and MPs (Cao et al., 2022). However, the exposure and impact of plastic additive pollution is an even more understudied topic, notably in pollinators (Moreno-Gómez-Toledano and Jabal-Uriel, 2024). Among those widely used compounds are phthalate plasticisers, which notably account for more than 40% in weight of PVC materials, ensuring their great flexibility. Yet, these molecules are not directly bound to the polymer matrices and can therefore leach easily into the environment (Suhrhoff and Scholz-Böttcher, 2016), resulting in the widespread pollution of aquatic and terrestrial environments (Bergé et al., 2013; Billings et al., 2021). In the context of a global decline in insect populations (Wagner et al., 2021), and more particularly in pollinator populations (Dicks et al., 2021), investigating the potential impact of such widespread pollutants is crucial to correctly assess the risk, especially in already weakened populations.

Part I – "Save the [bumble]bees!"

"Save the bees", but why? The public opinion agrees with the fact that they are fascinating, important and lovely (Schönfelder and Bogner, 2017; Sumner et al., 2018). However, their actual diversity, their ecological significance, and the underlying causes of their decline are not always well understood by the wider public, despite the growing body of scientific research on the subject (Wilson et al., 2017). In recent years, numerous bee-friendly campaigns have emerged (Ellman et al., 2023). Yet, beyond this positive attention mostly focused on honeybees, wild bees, of which bumblebees, continue to face a multitude of pressures, with each species differently affected by the intensifying impacts of climate change,

ongoing habitat and floral resource loss, and the increasing diversity of chemical pollutants (Ghisbain et al., 2025; Zattara and Aizen, 2021).

1. Bumblebee generalities

1.1. Diversity and morphology

The Anthophila (Hymenoptera: Apoidea) group is a highly diverse group, including 20,000 species described worldwide (Michener, 2007), and is divided into seven families, of which six are found in Europe (Apidae, Megachilidae, Andrenidae, Halictidae, Mellitidae, and Colletidae). Bumblebees are long-tongued bees classified within the genus Bombus (Apidae: Bombini tribe), accounting for 68 species out of the 2138 wild bee species recently recorded in Europe (Ghisbain et al., 2023).

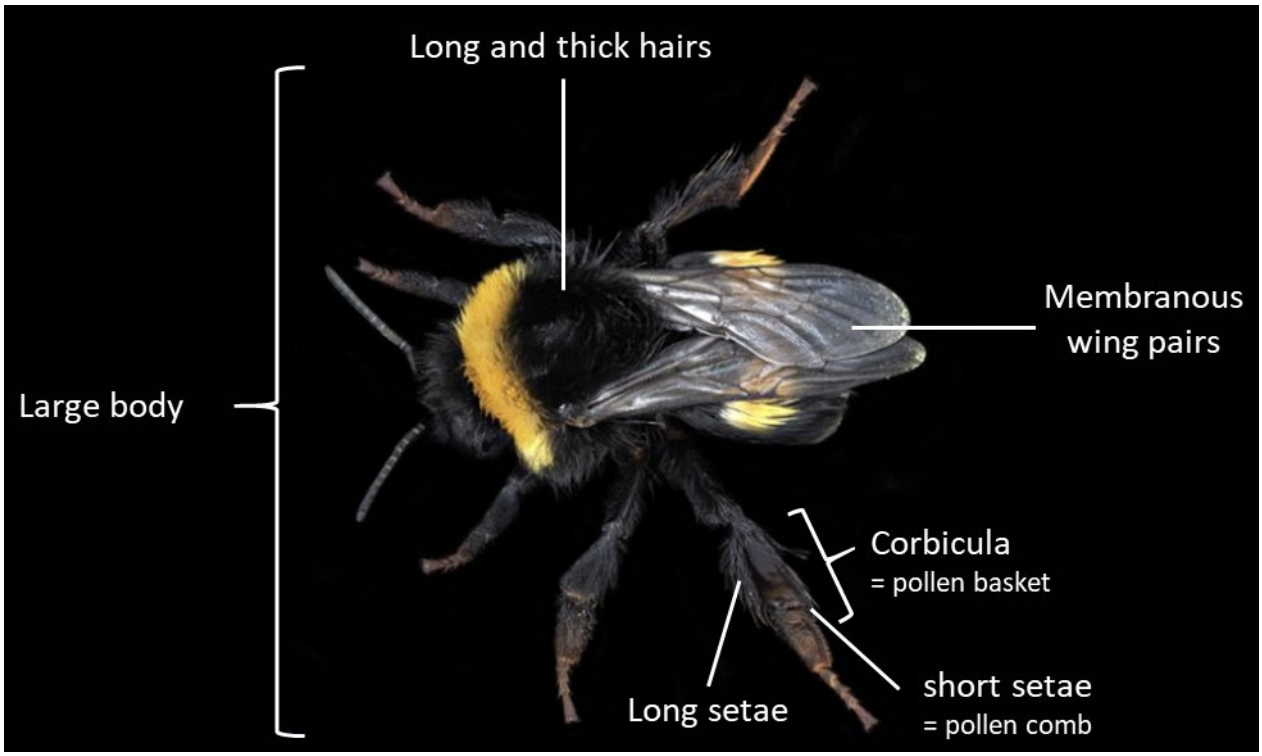


Fig. 2. General morphology of *Bombus* spp. Picture of *Bombus terrestris* by Paolo Rosa.

This group shares common morphological features with other hymenopteran species, such as a petiole and pairs of two attached wings, and with other bee species, such as branched hairs, mouthparts and body structures adapted for nectar and pollen collection,

their main food resources (Rasmont et al., 2021). However, certain morphological traits can ease the distinction of bumblebees from other bee groups. Indeed, they possess a robust, densely haired body, and their tibia bears a specialised surface known as the corbicula, bordered by stiff setae and long hairs, which facilitates highly efficient pollen collection (Fig.2; Rasmont et al., 2021).

1.2. Bumblebee sociality and lifecycle

The social behaviour of bees is highly diverse. All social complexity stages are found: from the solitary species, where reproductive females care for and provision their own progeny, to obligate eusocial colonies with several overlapping generations of non-reproductive individuals who forego their own reproduction and share tasks such as brood care and foraging, i.e. the workers, organised around a unique reproductive female, i.e. the queen (Michener, 1969; Michez et al., 2019). Within the Anthophila group, only 9.4% of species are eusocial (Danforth et al., 2019) and are included in the Apidae family. For example, species like Honeybees of genus Apis, and species like stingless bees, e.g. genera Melipona and Trigona, form perennial colonies which either survive winter by relying on resource storage or remain active throughout the year in tropical regions (Michez et al., 2019; Roubik, 2023). In contrast, the majority of *Bombus* species, especially from Arctic, boreal, and temperate regions, are characterised by an annual colony cycle that starts with the foundation by a solitary queen (Orlova, 2025).

After a winter-long diapause during which this mated queen slowed her metabolism and relied solely on body resources, she emerges and starts looking for a location to establish her nest (Fig. 3A; Alford, 1969; Rasmont et al., 2021; Rondot and Darveau, 2024). Depending on the species, queens adopt diverse nesting strategies, from tree cavities like *Bombus hypnorum* to old rodent burrows like *Bombus terrestris* (Rasmont et al., 2021). Once the nest

location is found, the colony initiation starts. During this solitary phase, the queen divides her time into foraging for herself and her progeny (Fig. 3B), as well as egg laying, with 6 to 16 eggs produced in the first brood (Fig. 3C; Duchateau and Velthuis, 1988). A second brood is produced as soon as the first larvae start their pupation (Duchateau and Velthuis, 1988). The first eggs laid are fertilised and will develop into diploid individuals, the female workers, within the first twenty days (Ribeiro et al., 1999). As soon as the first worker emerges, the second phase of the colony lifecycle starts (Fig. 3D).

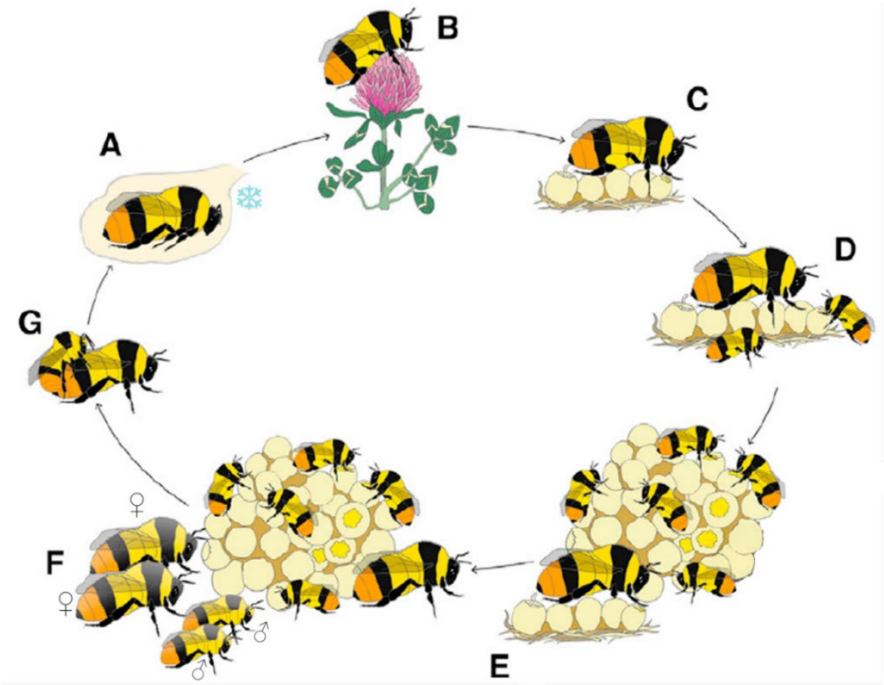


Fig. 3. Lifecycle of primitively eusocial bumblebee colonies. A) Mated diapausing queen in the hibernaculum. B) Solitary queen foraging on clover. C) Solitary queen incubating the first brood while laying the eggs of the second brood. D) First generation of workers (small individuals) emerging and helping the queen in brood care. E) Eusocial colony. F) Emergence of reproductive individuals, i.e., newborn queens (♀) and drones (♂). G) Mating of a new queen and drone (Figure adapted from Ghisbain et al., 2025, with illustrations by Olympe Tritto).

During this eusocial phase, the workers will forage and help the queen in brood care and larval feeding, allowing a linear phase of growth during which the queen lays diploid eggs without discontinuing (Fig. 3E; Duchateau and Velthuis, 1988). During this phase, tasks performed by workers encompass brood care, larval feeding, foraging and thermoregulating the nest (Jandt and Dornhaus, 2009). The queen's influence and dominance restrain the ovary

development and oviposition of unfertilised eggs by the workers. This worker sterility is partly mediated by the queen through pheromones (Van Oystaeyen et al., 2014) and aggressive behaviour (Amsalem and Hefetz, 2011), but also by workers' autoregulation by sensing of the queen's hormonal status and/or the larval type that is being laid (Alaux et al., 2004). Therefore, during the last phase of the colony lifecycle, the competition phase begins, in which workers will lay unfertilised and therefore haploid eggs that will develop into males, commonly called drones, while simultaneously taking care of the larvae destined to become queens (Amsalem and Hefetz, 2011).

It can be noted that in contrast to *A. mellifera*, *Bombus* spp. rarely perform trophallaxis (mouth-to-mouth food transfer) behaviour between adults and with the larvae. Instead, adults fetch food directly in shared pots containing either nectar and pollen regurgitated or deposited by foragers (Dornhaus and Chittka, 2005). Nectar is mostly consumed by adults either during nectar collection or from nectar stores within the colonies. Concerning larval feeding, in *Bombus* species called pocket makers, the early stages are reared together directly on freshly collected pollen forced below the brood cell. In the later stages, the larvae are still fed together, but liquid food is regurgitated onto the larval clumps (Goulson, 2010). However, in so-called pollen storer species like *B. terrestris*, larvae from the early larval stages are fed individually by regurgitation of a liquid food droplet containing pollen and nectar on their ventral part (Ribeiro et al., 1999). These larvae will weave a cocoon and form their own individual brood chambers, while workers continue to feed them individually until pupation (Ribeiro et al., 1999). At the end of the colony cycle, reproductive individuals, virgin queens and drones, emerge and mate (Fig. 3F-G). New mated queens enter a pre-diapause period during which they will forage intensively to accumulate a high amount of body resources, i.e. lipids and glycogen in the fat body tissue located in the abdomen (Votavová et al., 2015;

Woodard et al., 2019). Then, during the winter-long hibernation, diapausing queens lower their metabolic rates and rely on these body reserves, using first carbohydrates, and later proteins and lipids (Rondot and Darveau, 2024). Alford (1969), by describing the diapause of queens from several *Bombus* species, showed that a high amount of nectar was stored in their crop and used in the first months of the diapause period, but also that lipid content decreased during queen diapause, which was further confirmed by Votavová et al. (2015) in *B. terrestris*.

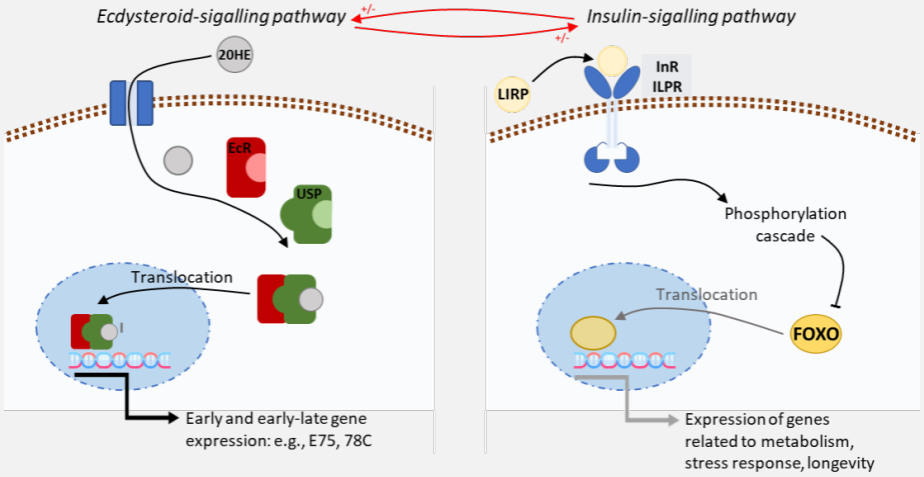
The complex social functioning of a colony, from caste-specific larval development to worker and queen behaviours, is tightly regulated by hormonal signalling as shown in Box 1.

1.3. Cold adaptation

Cold resistance of *Bombus* spp. is a key characteristic for the survival of queens to cold and freezing temperatures during their hibernation and early-spring solitary activities (Fig. 4), but also an important general adaptation of bumblebees. Indeed, bumblebees are morphologically and physiologically adapted to the temperate and cold climate of the West-Palearctic regions, where they are a common species. As highlighted by Peters et al. (2016), hairiness and body size are inversely correlated with the lower temperature at which a species remains active across several bee families, especially Apidae. Therefore, the particularly hairy and large body of bumblebees, ranging from 1 cm to 4 cm in body length, confers a better survival in cold temperatures by lowering heat loss (Maebe et al., 2021; Scriven et al., 2016).

Box 1. Bumblebee life under hormonal signalling

In insects, the insulin/insulin-like signalling (IIS) and the ecdysteroid cascade integrate nutritional and social cues to regulate developmental and reproductive processes (Antonova et al., 2012; Bonneton and Laudet, 2012). These pathways are central to the evolution of eusociality, as the decoupling of reproductive and nutritional functions between queens and workers likely arose through the complex modulation of these endocrine networks (Kocher and Kingwell, 2024).



Ecdysteroids, such as 20-hydroxyecdysone (20HE), interact with the ecdysone receptor formed by the heterodimer EcR-USP, and promote the cascading expression of genes coding for nuclear receptors (e.g. E75 and 78C), which in turn activate the expression of reproductive and development-related genes notably involved in molting, oogenesis and metamorphosis processes (Bonneton and Laudet, 2012), and in caste-specific behaviour and development. In *B. terrestris*, ecdysteroid titers differ between queen and worker-destined larvae (Hartfelder et al., 2000), while in adults, queen removal of the colony alters hemolymph ecdysteroid levels in workers, which induce ovarian development (Bloch et al., 2000). Moreover, 20HE also modulates olfactory memory acquisition in bees (Geddes et al., 2013), and peripheral olfaction regulation was shown in other insect models (Bigot et al., 2012), highlighting its role in foraging physiology. The activation of the estrogen-related receptor (ERR), notably by xenobiotics, but also by 20HE can also interact with this cascade in insects (Bonneton and Laudet, 2012).

The IIS functions, as nutrient-sensing and metabolic regulator pathway, are highly conserved among vertebrate and invertebrate groups: insulin-like peptide binding to insulin-like receptors triggers an intracellular phosphorylation cascade that ultimately inhibits the forkhead box-O transcription factor (FOXO). When active, FOXO promotes genes that enhance stress resistance, immunity, and longevity (grey arrows). IIS is also involved in growth, development duration, reproduction, diapause and ageing (Antonova et al., 2012). In *B. terrestris* workers, IIS involves one ligand, the locust insulin-related peptide-like (LIRP), and two insulin-like peptide receptors (InR and ILPR). IIS-related genes are notably downregulated in virgin and diapausing queens compared to reproducing queens and workers, and probably play a crucial role in the regulation of diapause metabolism (Jedlička et al., 2016). In *A. mellifera*, IIS is also involved in developmental caste determination (Wolschin et al., 2011) and modulates foraging behaviour in workers (Ihle et al., 2019). In bee species, IIS is also involved in stress response mechanisms, notably through the regulation of the L-LDH-mediated metabolism (Rondeau et al., 2025).

Both pathways participate in the regulation of juvenile hormone and vitellogenin production, two key hormones of social reproductive and developmental functions in bumblebees (Amsalem et al., 2014). Several crosstalks (red arrows) occur between IIS and the ecdysteroid cascade in bees, as they are known to regulate each other's components in other insects (Koyama et al., 2014), linking nutrient signalling to endocrine control of development and reproduction (Badisco et al., 2013).

Moreover, these morphological characteristics are coupled with physiological and behavioural adaptations to cold stress both at the colony (i.e. social regulation of nest temperature) and individual (i.e. endothermy) levels (Fig. 4; Heinrich, 2004; Rasmont et al., 2021).

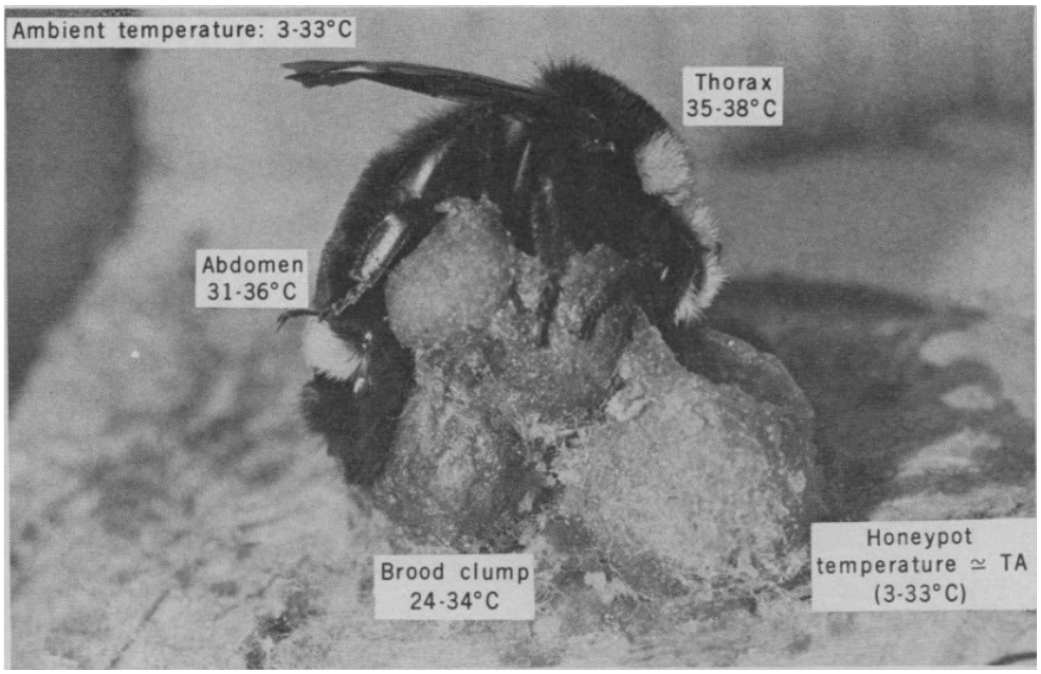


Fig. 4. Example of body and brood thermoregulation by a queen of *Bombus vosnesenskii* incubating the brood by pressing her thorax and abdomen on the first brood pupae and second brood egg cells, while simultaneously feeding in a honeypot (from Heinrich, 1974).

Bumblebees are facultatively endotherms which are notably capable of flying at relatively low temperatures and exit cold torpor states through non-flight thermogenesis that combines both muscle shivering and non-shivering metabolic mechanisms (Esch et al., 1991; Heinrich, 1975; Surholt et al., 1991). Notably, substrate cycling in flight muscles consists of an increased ATP hydrolysis producing only heat without other metabolic roles, and is achieved through the conversion of fructose-6-phosphate into fructose-1,6-bisphosphate, and inversely, by the work of phosphofructokinase and fructose-1,6-bisphosphatase enzymes (Clark et al., 1973; Surholt et al., 1991). Specific metabolic pathways related to endothermy, allowing efficient pre-warming of flight muscles, exiting cold torpor states and

thermoregulation of the nest are crucial characteristics of *Bombus* spp., tightly linked to energetic reserve and resources available (Heinrich, 1974).

1.4. Foraging behaviour and pollination

Around 130 mya, the angiosperms, a new group of plants which relied on pollen transport, commonly called pollination, for their reproduction, emerged (Le Guyader and Lecointre, 2017; Reece and Campbell, 2011). Vectors of pollination can either be abiotic, notably through wind-mediated transport, like in plants from the Poaceae family, or biotic through animal-mediated transport, like in Asteraceae, for example, but can also be a mix of both processes called ambophily, like in Salicaceae species (Culley et al., 2002). Bees evolved and differed from primary carnivorous wasps to become specialised pollen and nectar feeders. By navigating between several plants to forage for these resources, they unintentionally transfer part of their collected pollen from the anther (male part) of a flower to the stigma (female part) of a conspecific, and therefore participate in plant sexual reproduction (Michez et al., 2019). While bees collect nectar for carbohydrates, the main source of energy that notably fuels flight metabolic processes, they also collect pollen to feed their progeny (Cnaani et al., 2006). In the total amount of pollen collected on flowers, only a small part participates in the pollination process. For example, in *Aconitum septentrionale*, it has been estimated that only 1% of the collected pollen reaches the female reproductive part of a conspecific flower (Thøstesen and Olesen, 1996). The rest of the pollen will therefore partly be lost by the multiple handling, especially in bumblebees, which perform intense grooming behaviour, but the majority will serve for larval feeding (Thomson and Goodell, 2001).

Concerning foraging behaviour, in solitary species, reproductive females forage alone during their whole adult life to feed their offspring, while in higher eusocial species like *A. mellifera,* reproductive females, i.e. the queen, remain in the colony, and larval care and

feeding are performed by workers. In *Bombus* species, however, this task is first performed by the solitary queen during the first steps of colony foundation, until the first workers emerge and take this task over (Goulson, 2010). In *Bombus* spp., worker task specialisation is weaker than in other eusocial species like *A. mellifera*, and not related to age (Jandt and Dornhaus, 2009), which notably allows for resilience by quick compensation in case of a drop in colony size (Gérard et al., 2023). In the most studied species, *B. terrestris* and *Bombus impatiens,* larger workers tend to adopt forager behaviour (Goulson et al., 2002), while smaller workers are more likely to care for larval feeding and other in-nest tasks (Jandt and Dornhaus, 2009). Therefore, task-specialisation is thought to be partly determined by larval feeding behaviour, i.e. the frequency of feeding during development in those species (Couvillon and Dornhaus, 2009). A hypothesis supported by evidence that workers emerging from the centre of the nest are fed more frequently than the periphery, resulting in bigger workers potentially destined to become foragers (Couvillon and Dornhaus, 2009). As bigger workers are leaner than smaller ones in terms of body reserves, especially lipid content (Couvillon et al., 2011; Gekière et al, in prep.), increased feeding frequency could induce less body lipid storage by forager-destined larvae (Couvillon et al., 2011). The lower body storage could induce forager behaviour, as in *A. mellifera* workers, which become foragers after a quick depletion of their body reserves, though in this species, the process is regulated by age-related physiological processes not present in *B. terrestris* (Toth and Robinson, 2005).

Concerning their morphology, the large body size of *Bombus* foragers notably allows for a greater foraging range compared to their nestmates (Goulson et al., 2002), and to smaller species (Greenleaf et al., 2007). For example, the mean and maximum foraging distances estimated for *B. terrestris* are, respectively, around 550 m and 2.9km from the nest location (Redhead et al., 2016), while smaller species have smaller foraging distances, like *Andrena*

vaga (body length of 14mm; maximum foraging distance of 260m; Gathmann and Tscharntke, 2002). Their body size, therefore, makes them efficient pollinators able to operate in unfavourable weather and patchy habitats where resources are scarcer, such as arctic and boreal regions, but also in our highly fragmented environments (Rasmont et al., 2021; Theodorou et al., 2021). Moreover, the majority of *Bombus* spp are generalists in terms of floral preferences, as they forage on a wide variety of plant species and families (Rasmont et al., 2021). Indeed, they have the cognitive ability to handle flowers with highly complex structures, as well as to adapt their foraging behaviour depending on the resources available in the flowers, which other species like honeybees cannot (Laverty, 1994). Their generalist behaviour, coupled with great foraging abilities, makes them amongst the most linked pollinators in pollination networks, with their removal highly deleterious for plant diversity (Memmott et al., 2004). Moreover, in agro-ecosystems, some bumblebee domesticated species, like *B. terrestris* in Europe, have become crucial auxiliaries for the pollination of crops like greenhouse-grown tomatoes, strawberries, and blueberries (Box 2; Velthuis and van Doorn, 2006).

The high specialisation of bees and bumblebees for pollination, notably with morphological structures specifically adapted for pollen collection, makes them a major pollinator group (Ollerton et al., 2011). Notably, in Europe, insect pollination enhances crop yield by up to 71% (Bartomeus et al., 2014; Winfree et al., 2018). Therefore, to motivate protection actions towards pollinators, some studies have attempted to associate economic values with animal-mediated pollination. For example, the benefit associated with plants whose production depends on insect pollinator action in 2010 in Belgium has been estimated at 251.6 million euros (Jacquemin et al., 2017) and 153 billion euros for the world's leading food crops (Gallai et al., 2009). When not considering the human-related interest of the

pollination function, in temperate and tropical zones, animal-pollinated plants represent, respectively 78 and 94% of the plant species (Ollerton et al., 2011). Therefore, resulting from millions of years of coevolution with flowering plants towards a great diversity of shapes, colours, ecology and behaviour, bees are part of a complex network of ecological interaction, notably by their participation in plant reproduction, contributing to their diversity in general (Artamendi et al., 2024), which should suffice to aim for their protection.

2. Bee decline and the main drivers

Diverging trends with a shift in phenology and ecology in lots of insect species, including bees, have been shown over the last seventy years. Indeed, since the last century, modifications in wild bee distribution, ecology and diversity have been recorded from all around the world (Biesmeijer, 2006; Cameron et al., 2011; Duchenne et al., 2020; Zattara and Aizen, 2021). In Europe, almost ten per cent of the bee species are actually threatened, but due to the gap in data about more than 50% of the European bee species, this number could be underestimated (Fig. 5-B; Nieto et al., 2014). As a matter of fact, national trends and conservation status showed higher values in the United Kingdom and Belgium (Drossart et al., 2019; Powney et al., 2019). For example, 61% of the Belgian wild bee species are shown to be in decline since the past 70 years (Duchenne et al., 2020). Concerning bumblebees, evidence from highly documented areas shows that 23.5% of European *Bombus* species are identified as threatened (Nieto et al., 2014). Moreover, the projections for the future years are worrying for these species (Ghisbain et al., 2024). The causes of bee and bumblebee decline are mainly anthropogenic and are, like for many other insects, related to climate change, land use intensification, and chemical pollution, which act both alone and in combination on bumblebee populations (Ghisbain et al., 2025; Goulson et al., 2015, 2008).

2.1. Climate change

While *Bombus* spp. are facultative endotherms well-suited to cold conditions, with important plasticity towards positive and negative temperature variations (Kevan et al., 2024; Maebe et al., 2021), they are also ectothermic insects highly dependent on environmental temperatures. Therefore, in the face of ongoing climate change, these adaptations can help them support temperature variations, only to a certain extent. On the one hand, current climate change is characterised by a global increase in temperatures, which has shown deleterious impacts on bumblebee foraging behaviour (Gérard et al., 2024, 2022), physiology (Przybyla et al., 2025) and colony fitness (Przybyla et al., 2021). On the other hand, extreme weather events, such as unpredictable heatwaves and cold spells, will likely be more frequent and more intense in the following years (Walsh et al., 2020; Weilnhammer et al., 2021). While the impact of acute heat stress is more and more studied in bumblebees and reveals a worrying impact, especially on northern species, notably on male survival (Martinet et al., 2021) and foraging-related cognitive abilities (Muth et al., 2015). However, there are few investigations of the impact of cold stress on bumblebee workers and queens which could be exposed to longer-lasting winters and cold spells (e.g., Blasco-Lavilla et al., 2021). This kind of stress could also impact populations notably by affecting queen diapause and early-spring colony functioning, especially in combination with other anthropogenic stressors such as decreases in flower availability or insecticide use (Easton-Calabria et al., 2023; Owen et al., 2013).

Box 2. Bumblebee domestication

The domestication (without selection process) of *B. terrestris* was achieved in the 1980s, primarily driven by its efficient and cost-effective pollination, particularly in agricultural systems, notably for greenhouse tomato production (Fig. B2.1a; Lihoreau et al., 2025). Years of applied research on the management of artificial light, temperature, and humidity, as well as the manipulation of queen diapause, have enabled continuous rearing systems (Velthuis and van Doorn, 2006). This allows the year-round use of *Bombus* spp. colonies in agricultural systems (Fig. B2.1b; Velthuis and van Doorn, 2006), but also makes them widely available for scientific research (Lihoreau et al., 2025). Domestication has since been extended to other species such as *B. impatiens* in North America (Fig. B2.1c; Lihoreau et al., 2025).

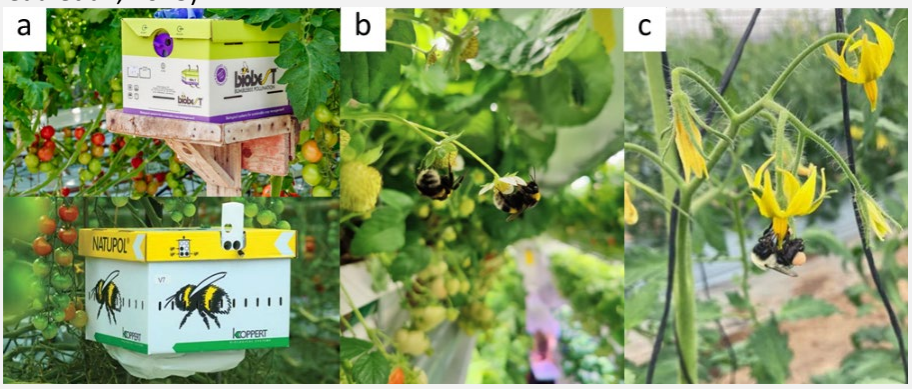


Fig. B2.1. Pollination auxiliary systems. a) bumblebee colony installed for tomato pollination, b) greenhouse pollination of strawberry by *B. terrestris* workers, and b) greenhouse pollination of tomato by *B. impatiens* workers (Pictures: Koppert, Biobest).

Besides agricultural benefits, bumblebee domestication has been pivotal for scientific progress in bee cognition, behaviour, ecology, physiology, and ecotoxicology (Lihoreau et al., 2025). Commercial queen-right colonies are easily available for researchers (Fig. B2.2a) and can be subdivided into queen-less microcolonies (5-10 workers isolated from the queen; Fig. B2.2b). This system can act as a proxy of bumblebee colonies, especially useful to observe social behaviours and reproductive outputs with great replication possibilities (Klinger et al., 2019). Indeed, isolated from the influence of their queen, worker ovaries develop and unfertilised eggs are laid. These eggs then develop into drones, comparable to the competition phase encountered at the end of queenright colony cycles (Duchateau and Velthuis, 1989).

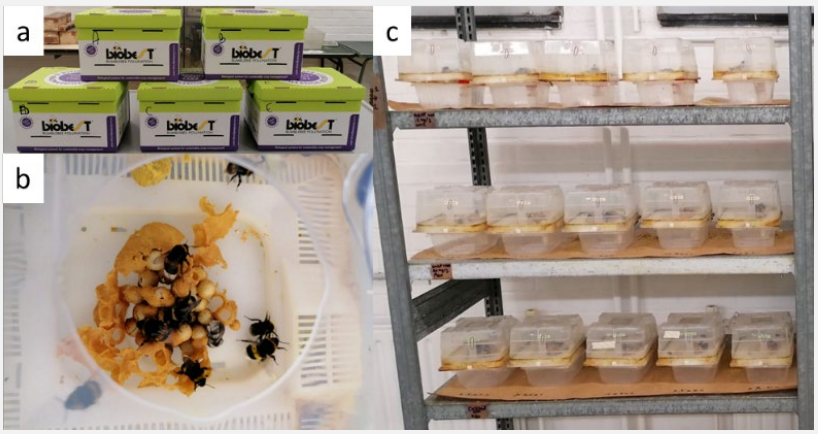


Fig. B2.2. Microcolony setup for laboratory studies using *B. terrestris* as a model. a) Commercial colonies purchased from Biobest, b) a month-old microcolony, and c) microcolony installation allowing multiple replicates (Pictures: Charlotte Terzo).

However, the resilience and global export of *B. terrestris* also have negative ecological consequences, as it has become invasive in regions such as South America, where it contributed to the decline of native species such as *Bombus dahlbomii* (Morales et al., 2013).

2.2. Land use

Habitat loss for bees is mediated by both climate change, with *Bombus* populations retreating towards the north to follow their thermal niche (Ghisbain et al., 2024; Rasmont et al., 2015), but also by direct human influence on the environment, such as the intensive land use by urbanisation and agricultural activities (Fortel et al., 2014; Vray et al., 2019)(Fortel et al., 2014; Vray et al., 2019).

First, current intensive agricultural practices are negatively affecting wild plant communities along with pollinator population sizes. Indeed, reduced floral abundance and diversity are mediated by the intensive agricultural system, which favours highly homogenised big-scale crops, including hundreds of individual plants of the same species (Hunter, 2002). In addition, the change of practices from the use of cover crops comprising diverse plant species (e.g., Fabaceae species like diverse clover species, alfalfa and common sainfoin) historically used for natural soil enrichment to the use of artificial fertilisers has led to a considerable loss in wild bee and bumblebee-adapted plants (Ghisbain et al., 2025; Goulson et al., 2008). Moreover, soil eutrophication due to artificial fertilisers decreases the diversity of wildflowers in grasslands and field margins, reshaping wild bee populations (Carvalheiro et al., 2020).

Second, with a growing human population and activities, the urban surfaces are predicted to increase in the following years (Angel et al., 2011). With the implementation of zero-phyto policies in several European cities and flowering campaigns, some urban places contrast with the rural environment by promoting the installation of wild bee populations (Baldock, 2020). Yet, urbanisation remains one of the main causes of natural habitat loss as cities present strong ecological constraints for bees (Jones and Leather, 2012). The great level of habitat fragmentation, alongside the high percentage of covered surfaces and the low floral resource availability, increases the energy dedicated to foraging and nesting site searching,

especially as more than half the wild bee species of Europe nest underground (Geslin et al., 2016; Neame et al., 2013; Theodorou et al., 2020). Moreover, the heat island phenomenon also acts as an ecological and physiological filter on communities (Hamblin et al., 2017). Some urban landscapes structure wild populations by promoting bigger and generalist species, like bumblebees (Banaszak-Cibicka and Żmihorski, 2012; Geslin et al., 2016). Yet, the parasitism rates encountered in three bumblebee species commonly found in the urban environment, i.e. *Bombus terrestris*, *B. pascuorum* and *B. lapidarius*, increase with the level of urbanisation (Theodorou et al., 2016). This suggests not only a great density of Bombus spp. individuals in urban parks and gardens, but also a modification of the immune system efficacy in individuals exposed to the urban environment. Notably, in *B. lapidarius*, the percentage of impervious surface was correlated with the activation of metabolic genes, tightly linked to the immune responses, such as AADAT and L-LDH, respectively coding for the enzymes α-aminoadipate aminotransferase and the lactate dehydrogenase (Cuvillier-Hot et al., 2024). The activation of these genes is part of the typical stress response in bees (Doublet et al., 2017; Rondeau et al., 2025).

2.3. Chemical pollution

The intensification of anthropogenic industrial, urban and agricultural activities has led to global chemical pollution with compounds currently used and marketed, but also some banned, yet still persistent in the environment (Scheringer et al., 2022).

Since restrictions were put on several neonicotinoid insecticides in Europe in the 2010s, bees are at the forefront of the pesticide regulatory decisions. This subject brought scientists from outside the standard regulatory science field to contribute to pesticide risk assessment (Demortain, 2021). Along with numerous published studies assessing pesticide toxicity on the honeybee, standardised protocols for testing the acute oral and contact toxicity

of exposure to chemicals, mainly by determining the median lethal dose (LD50%), were established for commonly used species like honeybee adults (acute: OECD, 1998a, 1998b; chronic: OECD, 2017a) and larvae (acute: OECD, 2013), as well as bumblebee adults (acute: OECD, 2017b, 2017c). Yet, these model species differ greatly from other wild bees, notably in terms of nesting habitat, floral choices or social interactions and therefore in terms of exposure routes and biological impact (Sgolastra et al., 2019). Therefore, acute and chronic exposure protocols are currently under development and standardisation process, notably to include solitary species of the genus *Osmia* spp. into risk assessment frameworks (Azpiazu et al., 2024, 2023; Kling et al., 2025), with a novel protocol for contact LD50 determination adapted for those species (OECD, 2025). It is important to note that these protocols are testing doses exceeding realistic levels to extrapolate towards lower doses using linear or monotonous models, therefore overlooking potential low and field realistic impact that would be missed by those methods (e.g. on neonicotinoids in Baines et al., 2017).

In addition, the potential effects of pesticide exposure identified on bees are multiple and not limited to direct lethal toxicity (reviewed in Basu et al., 2024). Yet, short-term lethal endpoints are currently used to extrapolate wild bee sensitivity to pesticides in regulatory procedures, a method with debated efficiency (Arena and Sgolastra, 2014; Dewaele et al., 2024; Thompson and Pamminger, 2019). Moreover, studies on sub-lethal and long-term effects of compounds such as the insecticide sulfoxaflor, the herbicide glyphosate or fungicide molecules indicate that bumblebee exposure can disrupt reproduction, thermoregulation, microbiota, and lower survival even at low doses (Motta and Moran, 2023; Potts et al., 2018; Runnion et al., 2024; Siviter et al., 2020b). Aside of laboratory experiments, the guideline document for testing the impact of pesticides, or plant protection products, has been recently revised by the European Food Safety Authority (EFSA) to include higher-tier studies like field

and semi-field studies for consideration in risk assessments (EFSA, 2013; EFSA et al., 2023). This document is one piece of evidence of the advances made in bee ecotoxicology, since the restriction of neonicotinoid use, when this field of study was only focused on honeybees and short-term lethal endpoints (Franklin and Raine, 2019). However, it also highlights that for bees in particular, but also for environmental studies in general, research and policies on chemical pollution are mainly focused on a few active pesticide molecules (Kristiansson et al., 2021), with only scarce data on plant protection product formulations, for example, and even less on other pollutants released by industrial activities, e.g. PFAS and plasticisers (Moreno-Gómez-Toledano and Jabal-Uriel, 2024).

Most of the knowledge acquired concerning bee sensitivity to chemical pollution in Europe comes from relatively few species, i.e. *A. mellifera* and *B. terrestris*. Despite their debated status as model species for risk assessment on wild bees because of their high resilience to anthropogenic stressors and their particular biology related to the eusocial lifestyle, several studies have shown their sensitivity to various pesticides and non-pesticide pollutants (Ghisbain, 2021; Thompson and Pamminger, 2019; Wood et al., 2020). These data, collected on common species, should raise an alarm on the potential impact of these compounds on wild and rarer, but also potentially more exposed or more sensitive bee species (Ghisbain, 2021; Thompson and Pamminger, 2019; Wood et al., 2020).

The existing rearing methods, behavioural and morphological metrics, as well as molecular tools available on *A. mellifera* and *B. terrestris,* allow a stress evaluation from the molecular scale to the colony structure. They have allowed the emergence of the study of non-pesticide pollutants. For example, a series of works on exposure of *B. terrestris* workers to diesel exhaust particles have highlighted negative effects on survival, flight behaviour, microbiota composition and expression of genes related to stress response mechanisms and

metabolism (Hüftlein et al., 2023; Seidenath et al., 2025, 2023). The impacts of heavy metals, cadmium and copper, have also been recently investigated in *B. terrestris*. Copper oral exposure reduced male survival and accelerated larval development without effect on reproduction and gut tissue integrity at field-realistic doses, and no effect of field-realistic exposure to cadmium was detected (Gekière et al., 2024a, 2024b; Paulet et al., 2025).

Finally, Ghisbain et al. (2024) projected European population dynamics of *Bombus* spp. highlighting that these species adapted to cold and temperate regions will likely take refuge towards northern regions. Yet, they also pointed out that these areas could become a refuge for these species as long as it is exempt from other anthropogenic pressures, such as chemical pollution (Ghisbain et al., 2024). Persistent and understudied pollutants such as several plasticisers, of which phthalates have been detected in northern regions, like in Svalbard or Russian arctic regions, probably from drift transfer (Kosyakov et al., 2021; Shaji et al., 2024). More than ever, the investigation of pollutants on key and well-known species such as bumblebees is needed both to initiate the assessment of the environmental impact of understudied pollutants and to motivate regulatory authorities to restrict the use of harmful compounds.

Part 2 – Phthalates: understudied environmental pollutants

1. Phthalate chemistry and uses

Diesters of 1,2-benzenedicarboxylic acid, better known as phthalic acid esters (PAEs) or phthalates, are synthetic compounds consisting of a benzene ring substituted with two alkyl chains (Staples, 2003). The length of these alkyl chains confers key physico-chemical properties to the variety of existing phthalate molecules (See the most commonly used molecules in Table 1). As the length of the alkyl chains increases, the lipophilicity is enhanced and the vapour pressure lowered (Cousins et al., 2003). Therefore, molecules with long alkyl

chains, such as diethylhexyl phthalate (DEHP), are adsorbed more easily to organic matrices and are less volatile than those with short alkyl chains, such as dimethyl phthalate (DMP; Table 1; Cousins et al., 2003). Moreover, their chemical structure plays a key role in their integration into plastic polymers to enhance flexibility (Staples, 2003). With those properties and the low cost of their production, phthalates have been intensively used in the chemical industry since the 1930s, with a broad range of applications (Kamrin, 2009). Historically, the high molecular weight phthalates with alkyl chains from 7 to 13 carbons were primarily used as plastic softeners and plasticisers, improving the flexibility and durability of materials, such as food packaging, toys, household furniture, clothing, and medical devices (Chang et al., 2021). For example, in polyvinyl chloride (PVC) materials, DEHP can make up to 40-50% of the weight of the final product (Clausen et al., 2004). However, as these molecules have no covalent bonds with the matrix in which they are integrated, they easily leach into the environment (Henkel et al., 2022). Moreover, mechanical actions, UV exposure and temperature variations, commonly experienced by plastic products, enhance this process (Suhrhoff and Scholz-Böttcher, 2016).

Beyond plastics, the use of phthalates has also been extended to numerous other applications. In Europe, for example, more than half of the produced plasticisers are registered for uses that are not related to plastic manufacture (Zhang et al., 2024). Indeed, low molecular weight phthalates with alkyl chains from 3 to 6 carbons, such as dibutyl phthalate (DnBP), have been used as solvents in products like detergents, paints, lacquers, varnishes, coatings, lubricants, waxes, and cleaning agents (Kamrin, 2009). These compounds have also been included as co-formulants in phytopharmaceuticals and personal care formulations (Hauser and Calafat, 2005). For example, diethyl phthalate (DEP), DMP, and DnBP have been used as

fixatives in perfumes (Kamrin, 2009), but also in insect repellents (Free et al., 1983; Sahebzadeh et al., 2009).

Once in the environment, free-form phthalates are rapidly degraded through atmospheric photodegradation, e.g. with short half-lives of approximately 9 hours for DEHP and 21 hours for DnBP (Staples, 2003). In the sediment, biodegradation also occurs, but proceeds more slowly, with half-lives of low molecular weight phthalates, such as DMP and DnBP, ranging from 2.5 to 46 days (Kickham et al., 2012). These values are higher for high molecular weight phthalates, such as DEHP, that reach 347 days, with di-iso-nonyl phthalate (DiNP) even exceeding a year (Kickham et al., 2012). However, in most environmental contexts, phthalates occur either at low concentrations (typically a few ng/L) or are adsorbed onto organic matter (Staples, 2003). In this adsorbed form, their bioavailability, along with their rates of degradation and biodegradation, are dramatically reduced, allowing these compounds to persist in the environment for extended periods (Chapon et al., 2023; Staples, 2003).

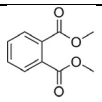
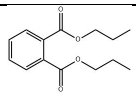
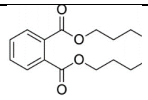
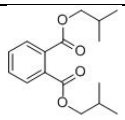
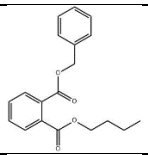
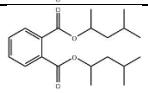
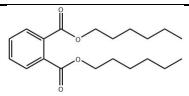
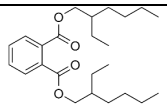
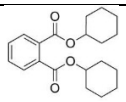
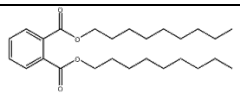
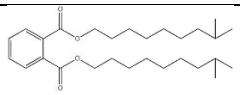
2. History of human health concerns and legal use restrictions

Early toxicological and ecotoxicological safety procedures for chemical registration solely relied on the establishment of short-term lethal endpoints, such as the calculation of the median lethal dose (LD50) using a monotonously increasing curve of mortality, comprising at least five concentration points (Morris-Schaffer and McCoy, 2021). Little to no interest was given to sub-lethal impact and long-term exposure (Perkins, 1979). This led to multiple molecules, including phthalates, being considered safe for humans and the environment, despite potential deleterious long-term, sub-lethal and low-dose effects being missed because of the low lethal toxicity data primarily obtained (Agathokleous and Calabrese, 2020; Futran Fuhrman et al., 2015). However, as evidence of increased risk of kidney failure, cancer

development, and reprotoxicity resulting from long-term exposure to DEHP and DINP in rodent models started to be highlighted in the 1980s (Ganning et al., 1984; Kluwe et al., 1982), concerns emerged notably about the presence of phthalates in toys and child care products, but also in medical material such as dialysis devices (Kamrin, 2009).

Since the 1990s, expert panels, notably in Europe, have been commissioned to evaluate the risks associated with phthalate use. Early reports were mitigated on the toxicity of these molecules, as contradictory results were found along with numerous gaps in knowledge (Koop et al., 1999). In the 2000s, however, the European Commission banned the use of phthalates DEHP, DBP and BBP in toys and child care articles at concentrations above 0.1% by mass of products, and DINP, DIDP, and DnOP in products that could be mouthed (Kamrin, 2009). As of today, the European Commission have extended the restrictions to additional phthalates and product categories, including cosmetics and electronic devices since the 2000s, as well as food contact materials since 2023 (Commission Regulation (EU) 2023/1442). Yet, most of the restrictions implemented since the early 2000s have been active only since 2020. As a result, phthalates remain present in legacy products manufactured and marketed before 2020 (Regulation (EU) 2018/2005), and before February 2025 for food contact materials (Commission Regulation (EU) 2023/1442). These compounds are therefore likely to continue entering the environment through industrial and domestic disposal. Notably, the desorption of phthalate content from PVC microplastic has been estimated in centuries depending on the environmental conditions (Henkel et al., 2022).

Table 1. Abbreviations, CAS number, chemical structure and formula, Molecular weight (MW) and recorded uses of common phthalates.

Abbreviation	CAS	Common name	Structure	Formula	MW (g/mol)	Recorded use[1]
DMP	131-11-3	Dimethyl phthalate		$C_{10}H_{10}O_4$	194.19	- Plasticiser; Solvent - Insect repellent; Ectoparasiticide
DPP	131-16-8	Dipropyl phthalate		$C_{14}H_{18}O_4$	250.30	- Plasticiser
DnBP	84-74-2	Dibutyl Phthalate		$C_{16}H_{22}O_4$	278.35	- Plasticiser; Solvent - Insect repellent; Used in insecticides
DiBP	84-69-5	Diisobutyl phthalate		$C_{16}H_{22}O_4$	278.35	- Plasticiser; Softener; Viscosity adjuster
BBP	85-68-7	Benzyl butyl phthalate		$C_{19}H_{20}O_4$	312.39	- Plasticiser; Viscosity modifier; Sealant
BMPP	84-63-9	Bis(4-methyl-2-pentyl) phthalate		$C_{20}H_{30}O_4$	334.4	- Plasticiser
DnHP	84-75-3	Dihexyl phthalate		$C_{20}H_{30}O_4$	334.50	- Plasticiser
DEHP	117-81-7	DiEthylHexyl Phthalate		$C_{24}H_{38}O_4$	390.00	- Plasticiser; Solvent - Used in pesticides (US)
DCHP	84-61-7	Dicyclohexyl phthalate		$C_{20}H_{26}O_4$	418.62	- Plasticiser; Heat sealer
DiNP	84-76-4	Dinonyl phthalate		$C_{26}H_{42}O_4$	418.62	- Plasticiser
DiDP	26761-40-0	Diisodecyl phthalate		$C_{28}H_{46}O_4$	447.00	- Plasticiser
[1]According to the Hazardous Substance Data bank and the INRS document on plastic components and adjuvants (2006)						

In addition, these recent regulations mainly prioritised human exposure. For example, outdoor use with applications that cannot reach human mucous tissues is exempted from these restrictions, such as agricultural equipment (Regulation (EU) 2018/2005). Given that phthalates are environmentally persistent pollutants, highly stable when complexed to organic matter, contamination of ecosystems remains and will remain widespread despite regulatory measures. The sources of environmental contamination and the ecotoxicological impacts of phthalates on wildlife have long been underestimated and remain insufficiently studied.

3. Sources and occurrence of phthalates in terrestrial environments

Phthalate pollution has been detected in virtually all physicochemical compartments of the environment, both in free-form and bound to particulate matter, including surface and groundwater, atmosphere, soils and sediments worldwide (Billings et al., 2021; Net et al., 2015c). The widespread contamination of environmental matrices and organisms with these synthetic molecules is mainly linked to anthropogenic activities with multiple sources, from their leaching during manufacturing to mismanagement of plastic and sewage waste disposal (Mansuri et al., 2025). Phthalate contamination may vary in terms of molecule distribution and quantities depending on the season and environment considered, and the type and distance from anthropogenic activities (Mansuri et al., 2025; Moreau-Guigon et al., 2016; Teil et al., 2016; Tran et al., 2015). Since their discovery as emerging pollutants in the marine environment in the 1970s (Giam et al., 1978), aquatic contamination by phthalates has been investigated by regional and global studies (e.g. Bergé et al., 2013; Net et al., 2015b) and thoroughly reviewed (e.g. Billings et al., 2024). Consequently, this section will rather focus on the contamination of the terrestrial (air and soil) compartments, to end with an exploration of the potential routes of exposure of bees, flying terrestrial insects, to phthalates.

3.1. Agricultural areas

The agricultural sector at the global scale consumes yearly more than six million tons of plastics (Scarascia-Mugnozza et al., 2012), 0.32Mt used in Europe over the year 2019 for vegetable production (Salama and Geyer, 2023). The great variety of plasticised items in agriculture includes agrochemical cans and containers, irrigation and drainage tubing, greenhouses and polytunnels, as well as plastic covers, notably used for bale wrapping and plastic mulching (Briassoulis et al., 2013; Salama and Geyer, 2023).

Investigating the scientific literature on phthalate occurrence and sources in the agricultural environment reveals that soil pollution is the main focus of these assessments (Kong et al., 2024). Some studies investigate air pollution in agricultural systems, but the majority of these data are only available in agricultural systems in China and mainly assess occupational exposure and vegetable crop intake in greenhouse systems (e.g., Wang et al., 2022, 2021). Because of the use of air filters and volatilisation from plastic components, these closed systems are usually more contaminated with phthalates than outdoor and open-field areas (Kong et al., 2024). Nevertheless, in the few studies that considered outdoor air of agricultural areas, the most frequently detected phthalate molecules were DEHP and DnBP, with values respectively ranging from 1400 pg/m^3 to 80ng/m^3 and from 500 pg/m^3 to 50ng/m^3 depending on the region considered (He et al., 2020; Zeng et al., 2020).

Concerning soil contamination, sources and occurrence have been more studied and have been found mostly related to agricultural practices and intense plastic use (Kong et al., 2024). First, plastic mulching is a source of phthalate contamination and consists of covering crops with sheets of plastic to increase soil temperature and moisture or to suppress weed growth. These sheets are either removed from the crops after several months or left in place and ploughed in. This process represents a major route of soil pollution by microplastics and

plasticisers (Salama and Geyer, 2023). These polymer films can notably release their plasticiser content in soil, with notably PVC films that can release around 40% of their DnBP content, which was degraded over four months, while DEHP was more slowly released but persisted longer in soil (Viljoen et al., 2023). Moreover, microplastics result from the fragmentation of plastic film and other plastic items at their disposal or during use (Salama and Geyer, 2023). Yet, these particles are known to release proportionally more plastic additives than their parent products and therefore represent a significant source of phthalate contamination in agricultural soils (Cao et al., 2022).

In addition, the spreading of sewage sludge on cultivated crops is a widespread practice, particularly in European countries such as France, where more than 200,000 tonnes of sludge were produced and over 53,000 tonnes applied to agricultural soils in 2022 (Koumoulidis et al., 2024). Sewage sludge originates from wastewater treatment processes and is commonly used as an alternative fertiliser, valued for its organic matter and nutrient content at a cheaper cost than chemical fertilisers. Yet, while current wastewater treatment techniques are effective at removing harmful compounds from effluent water, they inevitably concentrate a wide variety of contaminants within the sludge, such as pharmaceuticals, microplastics and plasticisers (Habimana and Sauvé, 2025). Current EU regulations on sludge use in agriculture only focus on heavy metal contamination, with no restriction regarding other pollutants such as phthalates (Regulation (EU) 2019/1010). As a result, sewage sludge represents a significant entry route of phthalates into agricultural soils (Billings et al., 2021). For example, in France, field applications of sewage sludge have been shown to cause an eight-fold increase in DEHP concentrations in agricultural soils, rising from 29 to 242 ng/g dry weight with an increase in phthalate content over the following month, probably attributable to the release of phthalates bound to microplastics and particulate matter (Tran et al., 2015). In the

same study, Tran and colleagues (2015) highlighted that the phthalates found in greatest quantities in French agricultural soils were DiBP (38.9ng/g), DiNP (67.4ng/g), and DEHP (242ng/g), with levels higher than in soils of rural (DEHP = 121ng/g, and other PAE<20ng/g) and forest areas (DEHP = 27.4ng/g, and other PAE<20ng/g). However, quantities of phthalate found in the urban soils of Paris, France, were more polluted than the agricultural, rural and forest soils investigated (Tran et al., 2015).

3.2. Urban and peri-urban areas

Interestingly, in urban areas, the chemical profile of air pollution has changed over the last decades, as new regulations on the emission of fossil fuel exhaust have been implemented in European and North American cities (McDonald et al., 2018). As a result, anthropogenic organic semi-volatile compounds, such as phthalates, are now revealed as the major pollutants (McDonald et al., 2018). Indeed, they are widely used in urban areas for plastic consumer products and food packaging, but also in building materials, wall and floor lining and are also released through the use of air conditioning and filter devices, with indoor participating to outdoor air pollution (Cousins et al., 2014).

While contamination of indoor air shows greater quantities than outdoor air, DiBP, DnBP and DEHP were the most common molecules found outdoors in Paris (Teil et al., 2016) with values ranging from 3.4 ng/m^3 for DnBP to 28 ng/m^3 for DEHP (Moreau-Guigon et al., 2016). These pollutants are found in the gaseous phase, but also bound to particulate matter, with which these pollutants can drift towards the environment remote from anthropogenic activity (e.g. atmospheric contamination above the North Sea: 1.06 ng/m^3 DnBP et 1.87 ng/m^3 DEHP; Xie et al., 2006).

In contrast with the agricultural environment, most investigations of phthalate pollution concerned the air compartment, with frequent comparison between outdoor and

indoor air, once again to study occupational and domestic exposure. Hence, data on urban soil contamination by phthalates are scarce in Europe. Yet, reported quantities are usually higher in urban areas than in agricultural ones. For example, Tran et al. (2015) reported high quantities for the main molecules found, i.e., DnBP (92.5ng/g), DEP (93ng/g), DEHP (310ng/g) and DiNP (500ng/g) in the city of Paris. In another European city, Novi Sad, DnBP and DEHP were also found in the greatest mean quantities, respectively 72ng/g and 729 ng/g, though DiNP was not among the monitored molecules (Škrbić et al., 2016). In this study, they highlighted that the highest quantities of phthalates were found in the surroundings of recreational facilities like children's playgrounds and skateparks (Škrbić et al., 2016), where weathering of plastic structural components, floor lining or painting can occur, associated with surface runoff of plasticiser content (Billings et al., 2021). In urban soils, contamination sources by plasticisers are multiple and notably associated with atmospheric depositions and rainfalls, pollution by the plastic litter is highly present in densely populated areas like cities, industrial activities and road traffic activities through the production of tyre-wear particles (Billings et al., 2021).

3.3. Bee exposure and potential routes

First, the insect cuticle is considered a protective barrier against environmental pollutants such as pesticides and heavy metals (Balabanidou et al., 2018; Gekière, 2025a). However, lipophilic compounds, such as phthalates, can easily enter this lipid-rich matrix (Yuan et al., 2020), such as the insect cuticle. Indeed, the insect cuticle has been identified as a significant route of exposure to atmospheric phthalate pollution (Lenoir et al., 2014, 2012). Phthalates are actively absorbed through the cuticle of live insects, a process that does not occur in dead individuals (Lenoir et al., 2014; Naz et al., 2022). It has been hypothesised that the cuticle of social insects could be particularly prone to phthalate absorption, in line with its

natural function as an exchange and metabolism site of cuticular hydrocarbons used as social communication cues (Lenoir et al., 2014).

In terms of contamination levels, DiBP, DEHP, and DnBP were detected on the cuticles of *Lasius niger* workers collected near Tours, France, with total phthalate contents averaging 2.11 ± 0.48 ng per ant (Lenoir et al., 2012). Similarly, DEHP and DEP have been identified on the cuticle of *Apis mellifera* workers (Gómez-Ramos et al., 2019), and total phthalate quantities of 28.05 ± 10.39 ng per bee were found on honeybee legs collected in France (Lenoir et al., 2012). More recently, *Bombus terrestris* workers collected across an urbanisation gradient in the European Metropole of Lille, France, were analysed for 15 different phthalate molecules (Cuvillier et al., in prep.). In this study, over 70% of the sampled workers contained two or more phthalates, with DnBP and DEHP being the most frequently detected compounds at average cuticular concentrations reaching, respectively, 108 ng/g body weight (b.w.) and 1389 ng/g b.w. (Cuvillier et al., in prep.). Together, these findings provide strong evidence that bees are exposed to phthalates in their natural environments, primarily through contact contamination of the cuticle with the contaminated atmosphere.

In insects, atmospheric contamination is one of the plausible routes of exposure, as shown in ants (Lenoir et al., 2012). In addition, the materials constituting social bee nests are rich in lipids, i.e. wax and stored pollen, and could accumulate phthalates. To date, nest material contamination by phthalates has only been investigated in honeybees, in which DEP, DEHP, and DINP have been detected in wax combs (Gómez-Ramos et al., 2016). While for honeybees, the contamination of worker cuticle and nest material could partly occur through contact with plastic items used in apiaries (Gómez-Ramos et al., 2019, 2016), the dynamics of nest contamination in wild insects have not yet been studied. On the one hand, bumblebee foragers could bring back phthalate contamination captured during foraging into their nest.

On the other hand, a great proportion of bee species nest underground (between 64 and 83% worldwide: Cane and Neff, 2011; Harmon-Threatt, 2020), including most of the bumblebee species (Liczner and Colla, 2019). Yet, soils are contaminated with significant quantities of phthalates in Europe, notably in urban and agricultural areas (Tran et al., 2015), and could therefore represent an in-nest route of exposure, especially as bumblebee nests can be found in high density in those areas (Knight et al., 2009). In addition, plant resources can be used by wild bees either as nesting material (e.g., cut alfalfa leaves in *Megachile rotundata*, or leaf hairs in *Anthidium manicatum*; Danforth et al., 2019). In alfalfa roots, a DnBP concentration of 179ng/g was also measured, but no data were found on leaf content (Ren et al., 2020). However, in other species, it has been highlighted that plant vegetative parts, such as leaves, can accumulate up to 6 µg of phthalate per gram of dry weight in corn (Fan et al., 2022). Nevertheless, plant material collection and use as nesting material could represent a supplementary route of exposure for both larvae and adult bees.

Finally, bees collect floral resources and notably pollen to feed their progeny. Yet, honeybee collected pollen stored in glass container was recently found to be contaminated with phthalates DEP, DnBP, BBP and DEHP in quantities ranging from below detection limit to 2.41 mg/kg (Martín-Gómez et al., 2024). More research would be needed to determine the contamination of pollen before collection by bees, but pollen, as a highly lipidic matrix, could also represent another potential route of exposure for both adults during collection and larvae during feeding.

While phthalate contamination is strongly linked to anthropogenic activities, ant cuticles were also found contaminated in remote regions, such as the Amazonian forest (Lenoir et al., 2016). Moreover, as mentioned earlier, phthalates have been detected in the atmosphere and soils of northern regions, like in Svalbard or Russian Arctic regions (Kosyakov

et al., 2021; Shaji et al., 2024). Therefore, wild bees, including bumblebees, are likely exposed to these compounds both in environments with intense anthropogenic activities, but probably also in the few remaining natural environments.

4. Impact of phthalates on invertebrates

The impact of phthalate exposure in invertebrates was first studied in aquatic species, e.g. crustaceans and mussels, as well as in human-related models such as *Caenorhabditis elegans* and *Drosophila melanogaster,* with the majority of the studies focused on DEHP exposure. With the evidence that insects are exposed to phthalates through cuticular contamination, as shown on ants by Lenoir et al. in 2012, investigations in insects have been extended to other species than *Drosophila* sp., such as the ant *Lasius niger*, the moths *Spodoptera littoralis* and *S. frugiperda*, and the fly *Calliphora vicina*.

Depending on the model species, the dose and the molecules tested, phthalate exposure lowered survival and shortened lifespan, activated immune response mechanisms or altered cellular immunity and affected behaviour, reproduction and larval development in aquatic and terrestrial invertebrates. As a general trend, organisms exposed to these endocrine disruptors have shown dysregulation of hormonal and metabolic pathways tightly linked with developmental and reproductive traits.

4.1. Lethal effects: survival, lifespan and longevity

Investigations into the lethal toxicity of phthalates in invertebrates primarily used short-term mortality assays to determine median lethal doses or concentrations (LD50/LC50; Staples et al., 1997). To our knowledge, these values have only been established for aquatic invertebrates, except for one collembolan species, in designs where phthalates are dissolved directly into the culture medium. A consistent trend has emerged, showing that low molecular weight phthalates are generally more toxic (i.e. lower LC_{50} values) than high molecular weight

compounds. For example, the 48h and 96h LC50s were determined for DMP, DEP, and DnBP in *Daphnia magna*, the midge *Paratanytarsus parthenogenetica* and the shrimp *Mysidopsis bahia,* ranging from 0.50 to 377 mg/L (Adams et al., 1995). In contrast, high molecular weight phthalates such as DEHP or DINP were initially considered non-toxic to invertebrates, as no mortality was observed even at the highest concentrations tested (Adams et al., 1995). Similarly, in the aquatic larvae of the midge *Chironomus riparius*, the LC50 of BBP could be established at 26.6mg/L, but not the one for DEHP (Planelló et al., 2011). Later work determined an LD50 for DEHP around 500 mg/L in another midge species, *Chironomus tentans,* and the copepod *Eurytemora affinis* (Forget-Leray et al., 2005; Lee et al., 2006). In the collembolan species *Folsomia fimetaria*, exposure through contaminated soil also followed the same pattern for toxicity exposure during three weeks: increasing doses of DEHP were not lethal, and no LC50 could be determined, while DnBP chronic LC50 was established at 305 mg/kg for adults and 19.4 mg/kg for juveniles (Jensen et al., 2001). To date, those are reference data used in the ecotoxicological section of the safety datasheet of DnBP and DEHP required by the REACH and CLP regulations (ECHA, 2017; ECHA chemical database, 2010a, 2010b). Although the tested concentrations and determined LC50s exceed those typically found in freshwater and soil samples (Net et al., 2015c), it is important to note that LD50 assays rely on exposure to a gradual, linear dose increase and rarely incorporate environmentally relevant concentrations (Morris-Schaffer and McCoy, 2021). As a result, such metrics can be misleading when assessing the ecological risk related to a pollutant, particularly for endocrine disruptors (EDs) that often exhibit low-dose effects and non-monotonic dose-response relationships (Box 3; Vandenberg et al., 2012).

Box 3. Endocrine disruption, low-dose effects and non-monotonous dose-response relationships

In regulatory frameworks, a substance is identified as endocrine disrupting compounds (EDCs) if "*(i) it shows an adverse effect in an intact organism or its progeny, which is a change in the morphology, physiology, growth, development, reproduction or life span of an organism, system or (sub)population that results in an impairment of functional capacity, an impairment of the capacity to compensate for additional stress or an increase in susceptibility to other influences; (ii) it has an endocrine mode of action, i.e. it alters the function(s) of the endocrine system; (iii) the adverse effect is a consequence of the endocrine mode of action*" (ECHA and EFSA et al., 2018). These compounds interfere with signalling pathways naturally triggered by pico/nanomolar concentrations of hormones and can therefore show low-dose effects (Vandenberg et al., 2012). Non-monotonic dose-response relationships (NMDR) are often observed after exposure to EDCs (Fig. B3.1), which challenge traditional risk assessment based on linear relationships (Futran Fuhrman et al., 2015).



Fig. B3.1. Examples of non-monotonous dose-response relationship curves.

Several hypotheses explaining low-dose effects and NMDRs have been suggested and reviewed in Lagarde et al. (2015), with the most frequent shown hereafter (Fig. B3.2).

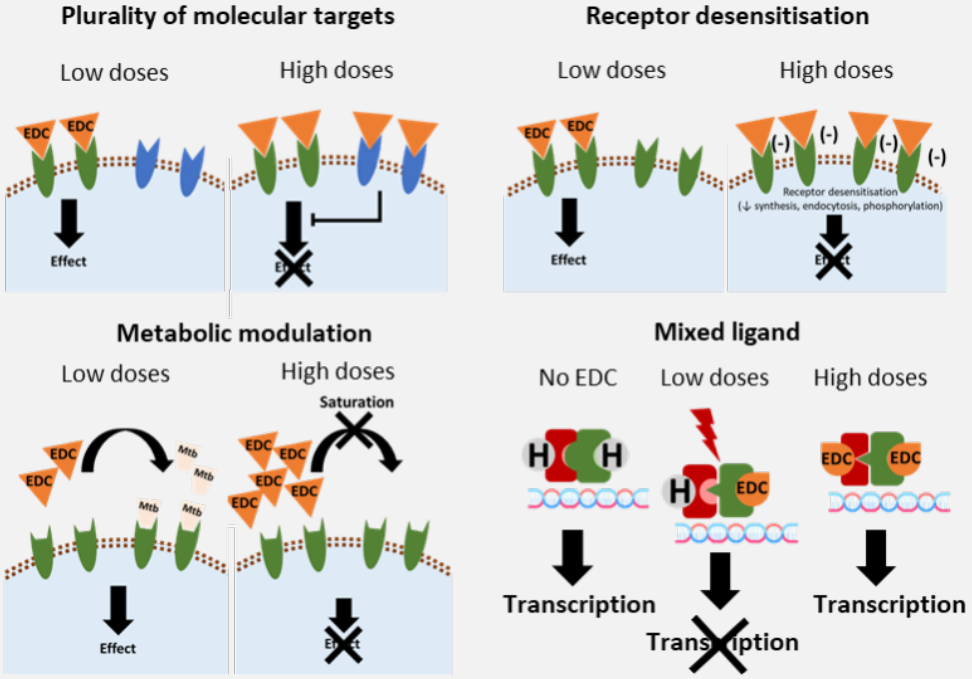


Fig. B3.2. Most frequent hypotheses explaining low-dose effects and NMDRs (adapted from Lagarde et al., 2015). The "plurality of molecular targets" hypothesis proposes that low-dose effects arise from activation of specific hormonal receptors (green), whereas at higher doses, EDCs also bind to receptors with antagonistic activities (blue), counteracting initial effects. The "receptor desensitisation" hypothesis is based on feedback regulation in hormone signalling, where receptor overactivation triggers phosphorylation, endocytosis, or reduced synthesis, ultimately lowering receptor sensitivity at high doses. According to the "metabolic modulation" hypothesis, EDC metabolites (Mtb) could be more potent than their parent compounds, but saturation of metabolic pathways at elevated doses can modify metabolite production and thus effects. Finally, the "mixed ligand" hypothesis suggests that low doses could favour the formation of mixed-ligand dimers that inhibit endogenous hormone action, while high doses promote EDC-receptor homodimers, with effects comparable to the hormone action (Lagarde et al., 2015).

Besides direct lethal effects occurring at high doses and after a short period of observation (typically 96 hours for the acute assays), sub-lethal doses of EDs can also have delayed effects. Chronic exposure to sub-lethal doses of phthalates has notably been shown to shorten the lifespan in *Caenorhabditis elegans* (DEHP at 1.5mg/L in How et al., 2019; DEP and DEHP at 0.2-0.4 mg/L in Pradhan et al., 2018) and in *Drosophila melanogaster* (DEHP and DINP at 0.4-4 mg/L; Liu et al., 2024). In *Daphnia magna*, exposure to 3.5mg/L of DnBP during a short period decreased the ultimate lifespan, even though phthalate contamination was removed from the medium (Seyoum and Pradhan, 2019). In contrast, in *Lasius niger* ant queens, no change in mortality or lifespan was reported during the 6 weeks following exposure to field-realistic doses of DEHP (2ng/ant applied onto the gaster; Cuvillier-Hot et al., 2014).

4.2. Stress response and impact on immunity

The causes underlying lifespan shortening are multiple. One of which is the physiological cost associated with an organism's general response to stress, including the activation of immune defences and detoxification mechanisms. While invertebrates lack an adaptive immune system comparable to that of vertebrates, their innate immune system plays a central role in defence against pathogens and xenobiotics through both cellular and humoral effectors (Coates et al., 2022). Several studies have reported alterations in these immune components following phthalate exposure in invertebrates.

Phthalate exposure has been shown in numerous aquatic species to induce oxidative stress, characterised by the excessive production of reactive oxygen species (ROS), either as a by-product of detoxification processes or as part of cellular defence mechanisms. However, ROS can impair cellular and tissue function and ultimately reduce lifespan (Shields et al., 2021).

Table 2. Examples of effects observed in invertebrates after phthalate exposure.

Effect type	Molecules	Model species	References
Individual traits			
Lifespan	DEP DnBP DEHP DINP	*Daphnia magna* *Caenorhabditis elegans* *Drosophila melanogaster*	Liu et al., 2024; Pradhan et al., 2018; Seyoum and Pradhan, 2019
Larval development duration	DnBP DEHP BBP	*Spodoptera littoralis* *Drosophila melanogaster* *Chironomus sancticaroli* *Calliphora Vicina*	(Atlı, 2010; Avilès et al., 2019; Dos Santos Morais et al., 2020; Šulková et al., 2022)
Starvation resistance	DnBP DEHP	*Drosophila melanogaster*	(Cao et al., 2016; Williams et al., 2016)
Lipid content	DnBP DEHP	*Caenorhabditis elegans* *Daphnia magna* *Drosophila melanogaster*	(Cao et al., 2016; Pradhan et al., 2018; Seyoum and Pradhan, 2019)
Courtship and mating behaviour	DEHP	*Spodoptera littoralis* *Drosophila melanogaster*	(Avilès et al., 2020; Chen et al., 2018)
Offspring production	DnBP DEHP	*Caenorhabditis elegans* *Daphnia magna* *Eisenia foetida* *Folsomia fimetaria* *Spodoptera littoralis* *Drosophila melanogaster* *Lasius niger*	(Atlı, 2010; Avilès et al., 2020; Chen et al., 2018; Cuvillier-Hot et al., 2014; Feng et al., 2016; Jensen et al., 2001; Pradhan et al., 2018)
Cellular effects			
Oxidative stress	DEP DnBP DEHP DINP	*Daphnia magna* *Chironomus circumdatus* *Caenorhabditis elegans* *Eisenia foetida* *Folsomia candida*	(Li et al., 2023; Shaha and Pandit, 2020; Shen et al., 2019; Tseng et al., 2013; Zheng et al., 2024)
Immune cell functions and number	DnBP DEHP	*Mytilus galloprovincialis* *Macrobrachium rosenbergii* *Eisenia foetida*	(Andreyeva et al., 2023; Ma et al., 2016; Sung et al., 2003)
Molecular effects			
Antimicrobial peptide expression	DEHP	*Caenorhabditis elegans* *Venerupis philippinarum* *Lasius niger*	(Cuvillier-Hot et al., 2014; Lu et al., 2013; Yen et al., 2023)
Insulin-signalling pathway	DnBP DEHP	*Caenorhabditis elegans* *Drosophila melanogaster*	(Cao et al., 2016; How et al., 2019; Liu et al., 2024; Williams et al., 2016)
Ecdysteroid-signalling pathway	DEP DnBP DEHP	*Daphnia magna* (homologs) *Chironomus riparius* *Spodoptera littoralis*	(Avilès et al., 2020, 2019; Seyoum and Pradhan, 2019)
Lipid metabolism	BBP DnBP DEHP	*Caenorhabditis elegans* *Physella acuta* *Daphnia magna*	(Pradhan et al., 2018; Prieto-Amador et al., 2021; Seyoum and Pradhan, 2019)
Multigenerational effects			
Body weight	DEHP	*Drosophila melanogaster*	(Chen et al., 2019)
Lipid metabolism	DnBP	*Daphnia magna*	(Wei et al., 2025)
Offspring development	DEHP	*Caenorhabditis elegans*	(Li et al., 2018)
Offspring production	DEHP DnBP	*Caenorhabditis elegans* *Daphnia magna*	(Li et al., 2018; Wei et al., 2025)

For example, *D. magna* exposed to DnBP (0.5 and 2 mg/L) for 24 hours exhibited elevated ROS levels (Shen et al., 2019). Similar increases in oxidative stress have been reported in aquatic larvae of *Chironomus circumdatus* exposed to DEP (Shaha and Pandit, 2020), as well as in *Caenorhabditis elegans* exposed for 24 hours to a wide range of concentrations (2–1000 mg/L) of DEHP, DnBP, or DINP (Tseng et al., 2013). In terrestrial organisms, comparable effects were observed in two earthworm species exposed through soil to environmentally relevant concentrations of DEHP. Indeed, ROS production increased alongside the activation of antioxidant enzymes such as superoxide dismutase (SOD; Li et al., 2023). In contrast, *L. niger* ant workers topically exposed to ecologically relevant doses of DEHP showed no lipid peroxidation and no change in SOD expression (Cuvillier-Hot et al., 2014). This absence of effect was attributed both to the low dose and short duration of exposure and observation (single drop deposition followed by observation after 24 and 48hours), as well as to the enhanced capacity of social insects to metabolise or sequester natural cuticular chemicals used as social cues (Cuvillier-Hot et al., 2014).

On the other hand, ROS inhibition following phthalate exposure has also been observed, for example, in the mussel *Mytilus galloprovincialis* and the giant freshwater prawn *Macrobrachium rosenbergii* (Andreyeva et al., 2023; Sung et al., 2003). These effects were associated with altered immune cell function. Indeed, exposure of *M. galloprovincialis* to field-realistic concentrations of DEHP (0.4 mg/L) for 48 hours modified haemocyte population composition (Andreyeva et al., 2023), while direct exposure of *M. rosenbergii* haemocytes to DEHP (100 mg/L) reduced pseudopodia formation (Sung et al., 2003). Similarly, exposure of *Eisenia foetida* to DnBP reduced the viability of circulating immune cells (coelomocyte) (Ma et al., 2016).

Another impact on innate immunity observed after phthalate exposure in invertebrates is the dysregulation of antimicrobial peptide expression, key components of the humoral immune response. In *C. elegans*, expression of the lysozyme-like 7 gene, an antimicrobial peptide regulated by the insulin-related pathway, was downregulated after exposure to DEHP (5mg/L; Yen et al., 2023). Similarly, in the clam *Venerupis philippinarum*, expression of the *lysozyme* gene was downregulated after exposure to 0.4 mg/L DEHP for 60 hours, but upregulated at a higher concentration of 4 mg/L (Lu et al., 2013). In *L. niger* workers, expression of the antimicrobial peptide *defensin* gene increased in the fat body after exposure to 2 ng of DEHP (Cuvillier-Hot et al., 2014).

Overall, these data indicate that phthalate exposure can either overstimulate or negatively affect immune activity, depending on the molecule and concentration tested. Such dysregulation could weaken invertebrate populations, either by exhausting the individual organism in the long run or by negatively affecting immune defence, thereby increasing vulnerability to natural pathogens or to other anthropogenic or natural stressors co-occurring with phthalate exposure.

4.3. Metabolic and endocrine disruption

Another cause of lifespan modification following phthalate exposure is related to their endocrine-disrupting activity. Indeed, a shortening of the lifespan was observed in *C. elegans* exposed to DEHP (1.5mg/L; How et al., 2019 and 0.4 mg/L; Pradhan et al., 2018) and in *D. melanogaster* exposed to DINP or DEHP (0.4-4 mg/L; Liu et al., 2024). This effect was found to be associated with a disruption of the insulin-related pathway (IIS), as phthalates acted notably on the serine/threonine-specific protein kinase B (AKT; member of the phosphorylation cascade) and on the forkhead box O transcription factor (FOXO, daf-16 in *C. elegans)* activities (How et al., 2019; Liu et al., 2024). The IIS components (see Box 1) are highly

conserved across species, such as FOXO, which is involved in processes related to longevity, stress response and ageing (Altintas et al., 2016). By contrast, exposure to DEHP (24-240 µg/L) and DnBP (4µg/L) in *D. melanogaster* increased lifespan under starvation stress by disrupting the expression of insulin-like peptide as well as insulin and glucagon receptors, thereby slowing lipid and carbohydrate mobilisation (Cao et al., 2016; Williams et al., 2016).

In addition to the insulin signalling pathway, phthalates are known to have obesogenic effects in vertebrates, notably by binding to the peroxisome proliferator-activated receptor (PPAR) α and γ, which are involved in lipid homeostasis (Capitão et al., 2017; De Cock and Van De Bor, 2014). In invertebrates, lipid accumulation related to the upregulation of homologs of the *PPAR* genes was notably highlighted in *C. elegans* exposed to DEHP (4mg/L; Pradhan et al., 2018). In insects, homologues of the *PPAR* genes (notably E75 and 78C; Hong and Park, 2010) are involved in the ecdysteroid signalling cascade (Box 1; Bonneton and Laudet, 2012). This cascade is notably involved in many reproduction and growth-related processes throughout the insect's life, and interacts with the insulin-related pathway to regulate nutrient-related developmental and metamorphosis processes (Bonneton and Laudet, 2012).

In the haemolymph of *Spodoptera littoralis* larvae exposed to DEHP (1.1 µg/g and 4.3mg/g), lower titres of the ecdysteroid hormone 20-hydroxy-ecdysone that binds to the ecdysone receptor were observed (Avilès et al., 2019). Moreover, the expression of the *USP* gene coding for the heterodimerisation partner of the ecdysone receptor, and the *E75* gene, a nuclear receptor activated by the cascade (see Box 1), were downregulated in the exposed larvae (DEHP 1.1 µg/g-4.3mg/g; Avilès et al., 2019). Associated with these results, the total duration of larval development was increased (Avilès et al., 2019). Similarly, in *Drosophila melanogaster*, DnBP exposure modified pupation time, either shortening or lengthening it depending on the dose tested (Atlı, 2010). Finally, in males that emerged from larvae grown

on DEHP-contaminated food (676ng/g to 4.3mg/g), the expression of the ECR, USP, E75 and 78C genes in the antennae was downregulated (Avilès et al., 2020). Some of the tested doses also affected male olfactory-related behaviours. Indeed, behavioural response to stimulation by female pheromones was negatively impacted by the lowest doses, which also altered courtship and reproductive behaviours (Avilès et al., 2020). Resulting offspring production was not impacted, but parental exposure to DEHP increased the developmental duration of the second generation (Avilès et al., 2020).

General aim of the thesis

In the current context of a widespread decline in the abundance and diversity of flying insects, particularly pollinators, addressing the knowledge gap on the effects of ubiquitous pollutants such as phthalates on these species is needed. The buff-tailed bumblebee, *Bombus terrestris*, is a well-established model species for studying anthropogenic stress on bees, and recent evidence highlighted a cuticular contamination by di-n-butyl phthalate (DnBP) and di-(2-ethylhexyl) phthalate (DEHP) in this species. In invertebrates, including insects, exposure to these molecules was associated with adverse effects on survival, reproduction, and behaviour, likely resulting from immune, metabolic and endocrine disruption. These previous findings raise the hypothesis that phthalates may similarly impair social bees, with organism-level disturbances, especially on metabolic and endocrine pathways linked to thermoregulation, caste-specific behaviour and development, but also to olfaction-related behaviour such as plant-pollinator interaction and nest-mate recognition. These potential effects could cascade into colony- and population-level effects.

The general objective of this thesis was therefore to investigate the endocrine-disrupting activity of phthalates on *B. terrestris*, especially in key processes of its social life and

metabolic particularities, by assessing the effects of two commonly detected compounds, DnBP and DEHP, at multiple levels of biological organisation. To provide a comprehensive understanding of their impact, this work was structured around two main axes:

First axis: Short-term consequences of a single contact exposure to field-realistic doses of DnBP and DEHP were described in isolated workers, using a standardised exposure protocol. Phthalate impacts were assessed at the individual level through short-term survival and respirometry assays, at the cellular level by analysing immune cell populations, and at the molecular level by quantifying the expression of genes involved in immune, metabolic, and hormonal signalling pathways (Chapter 2).

Second axis: The long-term effects of repeated exposure to field-realistic doses of DnBP and DEHP on key metabolic and hormonal pathways were investigated in workers reared in microcolonies. At the microcolony scale, offspring production in terms of number and mass was investigated. At the individual level, effects on survival, lipid body reserves and larval development were investigated. Finally, at the molecular scale, the expression of genes involved in key metabolic and endocrine pathways was analysed (Chapter 3).

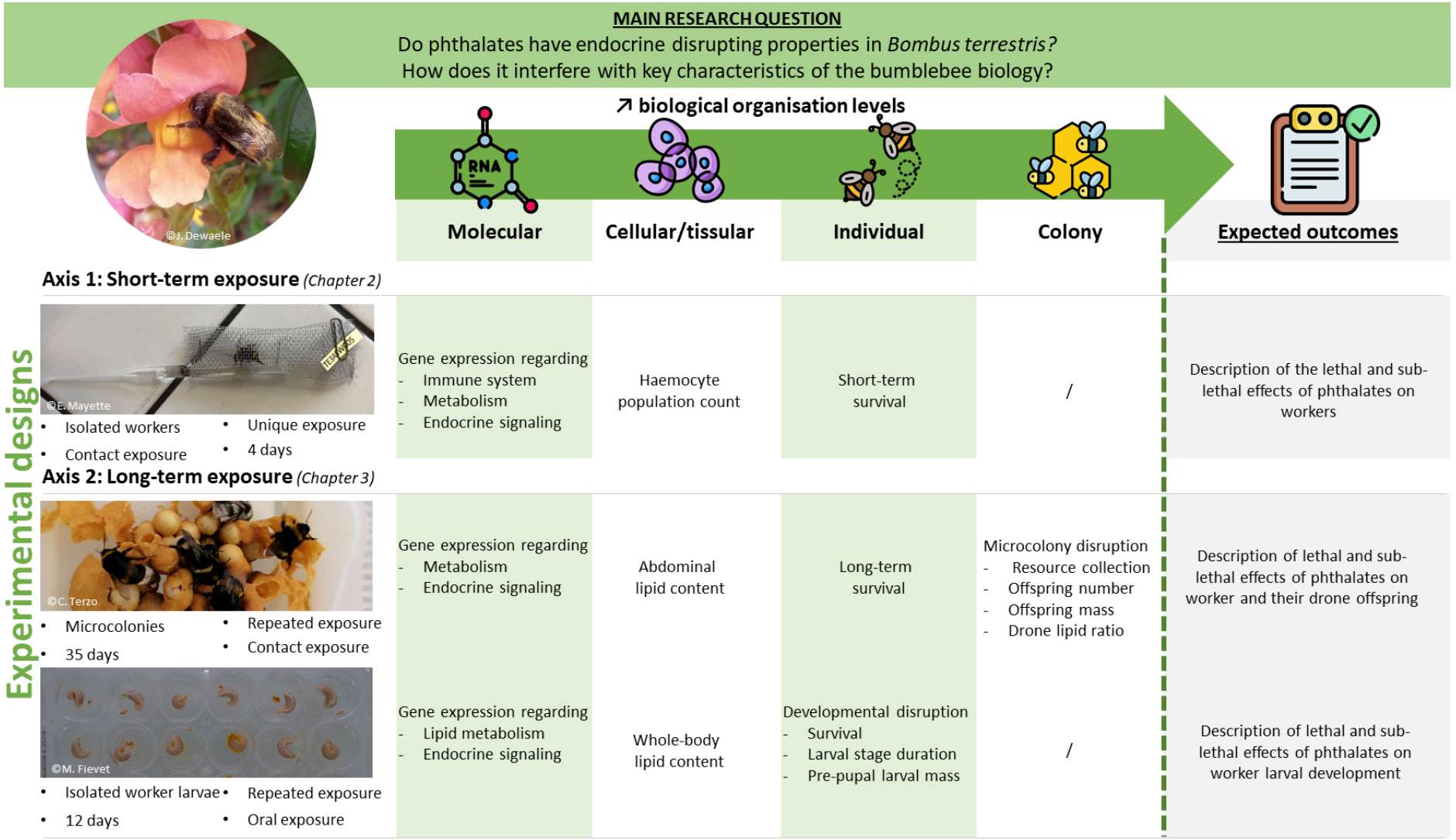


Fig. 4. Graphical summary of the general aim, experimental designs and expected outcomes of the thesis. This figure notably shows that this work is structured to investigate the effects of phthalates across multiple levels of biological organisation in *B. terrestris* using molecular, cellular and tissular, individual as well as colony-level metrics

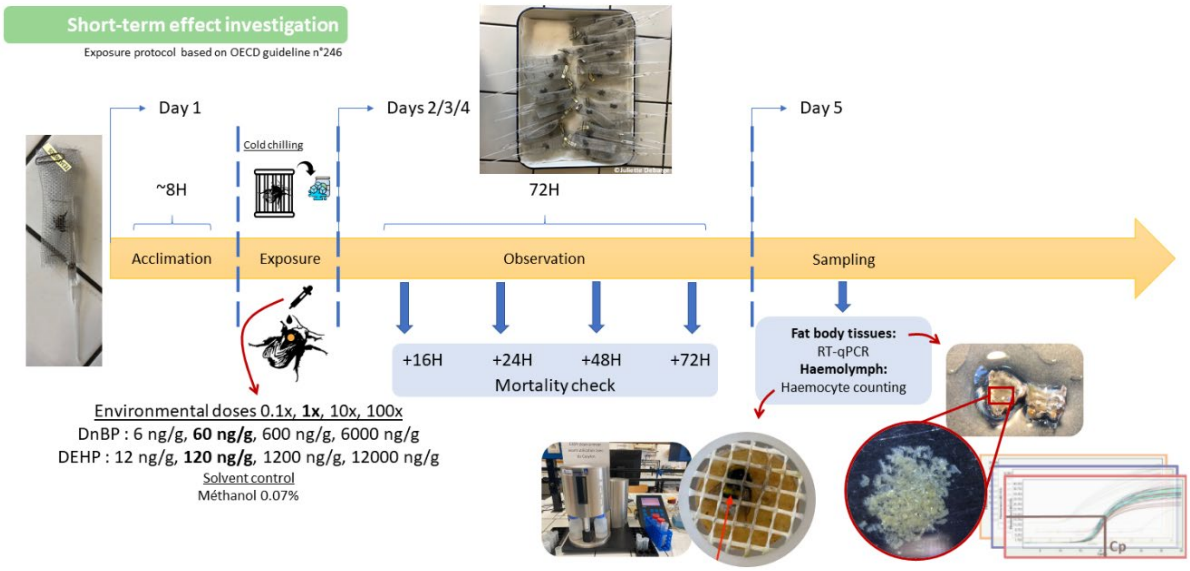
CHAPTER II: Short-term lethal and sub-lethal effects of DnBP and DEHP exposure in *Bombus terrestris* workers

This chapter investigates the impact of two common phthalates, di-n-butyl phthalate (DnBP) and di(2-ethylhexyl) phthalate (DEHP), on isolated workers of the buff-tailed bumblebee, *Bombus terrestris*. To date, assessments of phthalate toxicity in bees have been largely limited to *in silico* modelling (Arulanandam et al., 2022), with the few empirical studies focusing primarily on the repellence of these compounds when used in insect repellent formulations on honeybees (e.g., Sahebzadeh et al., 2008). However, to our knowledge, little is known about their lethal and sub-lethal effects on bumblebee workers.

To address this gap, this chapter presents an evaluation of the short-term toxicity and sub-lethal effects of DnBP and DEHP in *B. terrestris* workers. It is structured into three parts:

1. The first part of this chapter addresses the short-term toxicity of acute exposure to environmental doses of DnBP and DEHP to adult workers.
2. The second part investigates the effects of such exposure on the expression of genes involved in metabolic processes.
3. The third part explores phthalate effects on the worker immune system using haemocyte counting and gene expression analyses.

Together, these studies aim to provide a better understanding of how phthalates may disrupt key physiological pathways and affect *B. terrestris*.



└General experimental design used to investigate short-term effects of phthalate exposure on *B. terrestris* workers. The exposure protocol is based on OECD guideline n°246.

Publications :

Dewaele, J., Javal, M., Pinchon, A., Evrard, D., Hautekèete, N., Michez, D., & Cuvillier, V. (2025). Acute exposure to environmental doses of di-n-butyl phthalate but not di-2-ethylhexyl phthalate induces mortality in isolated and cold-stressed workers of *Bombus terrestris* L.(Hymenoptera: Apidae). Environmental Research, 122836. https://doi.org/10.1016/j.envres.2025.122836

Communications:

"URBAN BUMBLEBEE ECOTOXICOLOGY: Urban pollutant measures and environmental dose effects of DEHP and DnBP on *Bombus terrestris*", Oral communication, Rencontre annuelle du GDR pollinéco, 5th to 7th October 2022, Paris, France.

"Effets du DnBP et du DEHP sur la survie et l'immunité des ouvrières de *Bombus terrestris*", Oral communication, Rencontre annuelle du GDR pollinéco, 1st to 4th October 2024, Dijon, France.

"ASPI – Wild bees in the city: Urban pollutant effects on the health of insects and plant-pollinator interactions", Poster, Benelux Congress of Zoology, 2nd to 3rd September 2022, Kortrijk, Belgium.

PART I: Acute exposure to environmental doses of di-n-butyl phthalate but not di-2-ethylhexyl phthalate induces mortality in isolated and cold-stressed workers of **Bombus terrestris** *L. (Hymenoptera: Apidae)**

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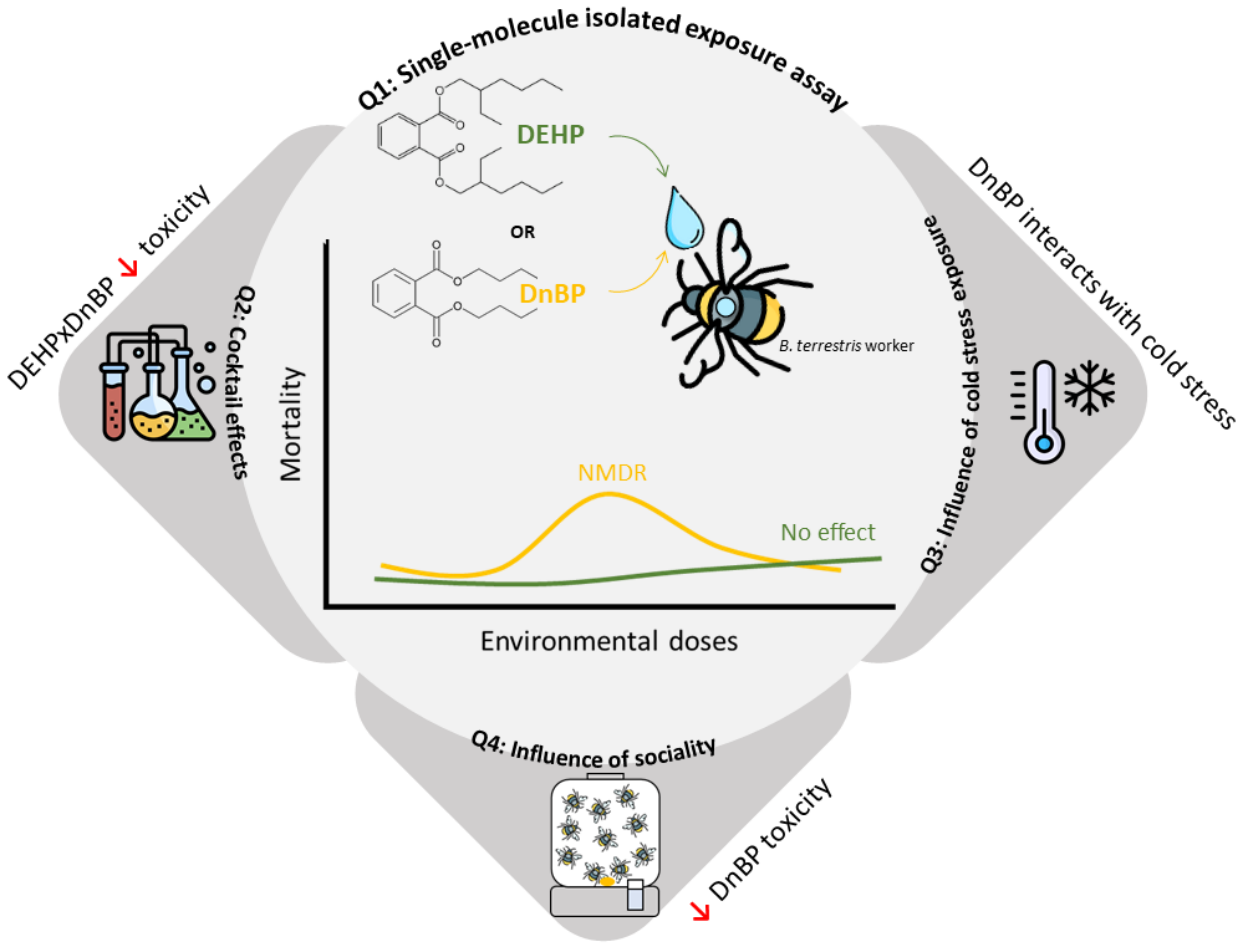
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Abstract

Since the past decades, declining trends in bee populations have been reported worldwide. Among multiple anthropogenic factors, endocrine disruptors, such as phthalates, could negatively affect bees and contribute to this decline at environmental level of exposure. Yet, no data are available on phthalate toxicity in bees. Di-2-ethylhexyl phthalate (DEHP) and di-n-butyl phthalate (DnBP), widely used as plasticisers and adjuvants, are some of the most commonly recorded phthalates in the environment, including on social bee worker cuticles. In this study, we investigated DnBP and DEHP lethal effects on B. terrestris workers after contact exposure to environmentally relevant single-molecule and mixture treatments. Interactions with cold stress and social context were additionally investigated. Firstly, we evidenced for the first time an NMDRC for DnBP toxicity in bees, with an increased mortality of 25%, 72 hours after exposure. By contrast, DEHP did not affect worker survival and even lowered DnBP toxicity when administered as a mixture. Secondly, DnBP at low environmental doses enhanced worker resistance to cold stress, while exposure to cold stress modulated DnBP toxicity, shifting the onset of adverse effects to higher concentrations. Finally, we evidenced that social contacts mitigate DnBP toxicity. Overall, this study provided the first evidence of the detrimental effects caused by these overlooked pollutants on bees, and discusses potential metabolic disruption by phthalate exposure that may affect crucial life traits in bumblebees. Finally, this study highlighted that accounting for complex realistic mixtures and the social context of the studied species seems essential for accurately assessing the toxicity of endocrine disrupting molecules.

Keywords: plasticizers; lethal effects; insects; NMDR; chemical mixture

Graphical abstract:



Highlights:

- Exposure to field-realistic doses of DnBP is lethal to ~25% of bumblebee workers

- First evidence of NMDRC in bees after exposure to phthalates

- DnBP toxicity is lowered by DEHP in bumblebees at environmental doses

- Low-dose DnBP exposure in *B. terrestris* workers increase resistance to cold stress

- DnBP toxicity is nullified in a social rearing context

1. Introduction

Phthalates (PAEs) are ubiquitous pollutants across environmental compartments, *i.e.* soils, atmosphere and water bodies (Bergé et al., 2013; Net et al., 2015c). Used as plasticisers to improve flexibility (CEFIC, 2021), they also serve as adjuvants to paints, cosmetics, pesticides or detergents (Koniecki et al., 2011). Due to the absence of covalent bonds with the polymer matrix, PAEs leach easily into the environment (Suhrhoff and Scholz-Böttcher, 2016).

Industries use numerous PAEs, but di-2-ethylhexyl phthalate (DEHP) and di-n-butyl phthalate (DnBP) are the most frequently used in European products (Fierens et al., 2012).

Both molecules co-occur in the atmosphere and soils (Net et al., 2015c) and are identified endocrine disruptors (EDs) for mammals and aquatic invertebrates (Gore et al., 2015; Regulation (EU) 2018/2005), in which they disrupt reproductive and immune functions (Liu et al., 2024; Xu et al., 2013). Only a few studies have focused on insects, revealing disruptions of immune, metabolic and hormonal pathway-related genes, some of which contribute to shortened lifespan and larval mortality (Avilès et al., 2020; Cuvillier-Hot et al., 2014; Liu et al., 2021b; Šulková et al., 2022; Williams et al., 2016). Since 2021, these molecules are listed in Annex XIV of the REACH regulation, which mandates chemical risk assessments for human and environmental hazards before marketing in the European Union (Regulation (EU) 2021/2045, 2021). However, risk assessment of EDs such as PAEs presents substantial challenges due to their low-dose effects, which result from complex interactions with hormonal receptors naturally triggered at low concentrations, and their non-monotonic dose-response curves (NMDRCs), i.e. unpredictable relationships between exposure dose and effects (Vandenberg et al., 2012). These properties challenge traditional toxicological tier-based assessments (Warner and Flaws, 2018), as these approaches rely on determining the dose or concentration lethal for 50 percent of tested individuals (LD50 or LC50) and extrapolate the effects for the lower doses (EFSA et al., 2023; OECD, 2018). These methods have been previously used to determine PAE toxicity on various aquatic species, but are not efficient to understand their effects on organisms. Investigating the lethal effects of exposure to various and environmentally relevant PAE doses is crucial given the current decline in biodiversity, and has not yet been thoroughly performed in insects.

In the context of insect decline (Goulson, 2019), wild bees get particular attention due to their role in pollinating flowering plants (Zattara and Aizen, 2021). Different drivers of decline were identified for bees, and chemical pollution ranks high in all studies (e.g., LeBuhn and Vargas Luna, 2021; Nieto et al., 2014). While the study of pesticide exposure and impact on non-targeted species is a growing field of research, terrestrial invertebrate sensitivity to other pollutants, including PAEs, remains overlooked (Benuszak et al., 2017). Yet, wild bees are likely exposed to PAEs via multiple routes throughout their life stages. Contamination might occur through contact between their lipidic cuticle and the contaminated atmosphere, vegetation or soil (Lenoir et al., 2012; Martín-Gómez et al., 2024). Recently, we quantified PAE contamination of wild *Bombus terrestris* workers sampled in the city of Lille, France: among 17 PAEs investigated, DEHP and DnBP were the most frequently detected on bumblebee cuticles, contaminating 53% and 60% of the workers, respectively (unpublished data).

Here, we investigated the impact of PAEs on the buff-tailed bumblebee, *B. terrestris*. This species is a common pollinator, well-represented in Europe (Rasmont et al., 2021) and dominant in urban and peri-urban areas (Michołap et al., 2024; Persson et al., 2020). *B. terrestris* workers navigate up to 800m from their nest (Wolf and Moritz, 2008), making them good sentinels to assess the pollution of their close environment (Moniuszko et al., 2025). Because of its domestication, commercial hives containing hundreds of workers of this species are available year-round (Velthuis and van Doorn, 2006), with standardised rearing and exposure methods available (EFSA et al., 2023). *B. terrestris* is therefore used as a surrogate species to assess acute toxicity of pesticides and other pollutants on wild pollinators (Arena and Sgolastra, 2014; Azpiazu et al., 2021; Gekière et al., 2024b; Linguadoca et al., 2022). Consequently, we chose to evaluate the short-term lethal effects of exposure to environmentally relevant doses of DnBP and DEHP on *B. terrestris* workers, maintained in

isolation according to OECD guidelines (OECD, 2017b, 2017c). Moreover, and to complete the assessment, the toxicity of DnBP and DEHP in mixture formulations was investigated, as both molecules frequently co-occur in nature (Jarošová, 2006; Net et al., 2015c; Teil et al., 2006).

Climate change is another significant driver of bee decline, especially in bumblebees (Soroye et al., 2020). Extreme weather events, such as unpredictable cold spells and longer-lasting winters, will likely be more frequent and more intense in the future (Walsh et al., 2020; Weilnhammer et al., 2021). *Bombus* spp. are well-suited to cold conditions with crucial metabolic adaptations, such as the ability to perform endothermy (Heinrich, 2004; Maebe et al., 2021). However, PAEs are known to disrupt glucose and lipid metabolism in *Drosophila melanogaster* (Williams et al., 2016) and in other model species (Prieto-Amador et al., 2021; Seyoum and Pradhan, 2019; Zhang et al., 2021a); they could thus negatively impact bumblebee cold resistance. To address this hypothesis, we combined phthalate exposure with cold stress, applied on isolated *B. terrestris* workers.

Finally, current OECD guidelines recommend testing pollutant toxicity on isolated bumblebee workers. Yet, *B. terrestris* is a eusocial species, characterised by complex hormonal interactions – that could be disrupted by PAEs – mediated by social contacts (Jedlička et al., 2016; Woodard et al., 2013). This species also benefits from social mitigation of stress (i.e. detoxification, temperature balance; Walton et al., 2024). Nevertheless, pollutant toxicity in bumblebees has never been directly compared between isolation and social rearing conditions, to our knowledge. Therefore, we considered different social contexts to evaluate the toxicity of PAEs, in order to get a broader and more biologically pertinent understanding of their effects (Camp and Lehmann, 2020; Klinger et al., 2019).

Previous pollutant toxicity assessment on bees has mainly focused on pesticide testing (Benuszak et al., 2017). Therefore, by evaluating phthalate impact on bees and their

interactions with cold stress and social context, we aim to broaden the scope of ecotoxicological testing and pollinator protection framework to such underestimated pollutants.

2. Material and methods

2.1. Study species

B. terrestris is a ubiquitous pollinator in Europe, including in urban areas (Persson et al., 2020). This species is easy to keep in laboratory conditions, making it a suitable model species for toxicity studies (e.g., Gekière et al., 2024b). Commercial colonies (about 80-120 workers) were purchased from Biobest NV (Westerlo, Belgium). Upon receipt, colonies were kept in darkness, and all handling was performed under red light to minimise worker stress.

2.2. Preventing PAE contamination in material and solutions

Phthalates are widespread pollutants, present indoors and particularly in laboratories (Feng et al., 2020). This contamination is mostly related to extensive plastic use (Reid et al., 2007). Therefore, we replaced plastic materials with glass and metal whenever possible. All metal and glass were additionally decontaminated with hexane and acetone, and when feasible, by calcination. Moreover, water marketed in glass bottles (SPA Reine, SPA NV, Bruxelles, Belgium) was used instead of tap or plastic-bottled water to prepare the treatments and feeding solutions (Luo et al., 2018).

2.3. DnBP, DEHP and control treatments

DnBP (CAS n°117-81-7) and DEHP (CAS n°84-74-2) were purchased as liquid solutions from Sigma-Aldrich. Concentrations were chosen based on data collected on wild *B. terrestris* workers during a previous field campaign in Lille, France, in June-July 2021 (unpublished data). This campaign recorded DnBP and DEHP as the most prevalent PAEs on *B. terrestris* worker cuticles at median doses around 145 ng/g for DEHP and 15 ng/g for DnBP (unpublished data).

We then considered 1X treatments of DEHP at 120ng/g of body weight (b.w.) and DnBP at 60ng/g b.w. and three other doses: 0.1X, 10X and 100X. Workers were exposed to single-molecule or mixture treatments.

All dilutions were prepared considering an averaged *B. terrestris* worker fresh weight of 0.25g (Dewaele et al., 2024). The initial dilution used methanol (CAS n°67-56-1, VWR) for good solubility, followed by further dilutions in mineral water to minimise the methanol concentration in the treatment solutions (0.07 % for 0.1X, 1X and 10X). For the 100X treatments, PAEs were diluted in acetone only (CAS n°67-64-1, Chem-Lab NV), due to PAE low water solubility.

As DnBP and DEHP frequently co-occur in the environment, mixture treatments were also tested. For the mixture preparation, initial and subsequent dilutions followed the same method as the 1X and 10X single-molecule treatments. However, at the last dilution step, both single-molecule solutions were mixed, and the volume was adjusted with mineral water to obtain either a mixture of DEHP and DnBP 1X treatments, or a mixture of the DEHP 1X and DnBP 10X treatments.

Exposure to a solvent control solution was performed using either 0.07% methanol in mineral water or 100% acetone. An additional control solution used only mineral water to assess solvent toxicity. As mortality rates in both solvent-treated groups (methanol and acetone) and the mineral water group did not differ, we considered the pooled solvent groups as control (See Table S1.1).

2.4. Experiment 1: Impact of contact exposure to single and mixed PAE on isolated workers

Here we considered 11 treatments: one control, four single exposure treatments per phthalate (0.1X, 1X, 10X, 100X), and two mixture treatments (DEHP 1X + DnBP 1X and DEHP 1X + DnBP 10X). The exposure protocol was based on OECD guideline n°246 (OECD, 2017b).

The experiment was performed during two sessions, for control and single 0.1X, 1X, 10X exposure treatments. Mixtures and single 100X treatments were only tested during the second session. Selection of exposure conditions for mixtures were established based on the results of the single exposure treatments from the first session. We used batches of 30 workers per condition in the first session and 20 in the second. Workers were sampled randomly from three colonies per batch during the first session (i.e. 10 workers from each of the three colonies), and from two during the second session (i.e. 10 workers from each of the 2 colonies), and were evenly distributed across the treatment groups.

For both sessions, workers were isolated in decontaminated, handmade metal cages similar in dimensions to Nicot cages® (for detailed worker numbers per session and protocol pictures, see fig. S1.1a and Table S1.2). Caged workers remained isolated during the whole duration of the experiment (72h). They were acclimated to the conditions for at least eight hours before exposure. They were then anaesthetised by chilling in a cold atmosphere without direct cold surface contact for 10 minutes. Two microlitres of treatment solution were deposited through the cage mesh on the dorsal hairless part of the thorax using a calcined 10 µL glass capillary (Drummond). Once recovered from anaesthesia at room temperature, caged workers were placed back on a metal tray. They were provided *ad libitum* 50% w/w sucrose-water solution via a decontaminated Pasteur pipette (removed during the anesthesia). Mortality was recorded at 16, 24, 48, and 72 hours post-exposure.

In the first session, caged workers were placed on a metal tray at room temperature under natural daylight regime (50°36'31.5"N, 3°08'39.4"E; June 2022), while they were kept under constant darkness and controlled temperature and humidity (25°C; 60% relative humidity) in the second. In the first session, anaesthesia was performed by placing workers by treatment in a freezer for 10 minutes. In the second session, anaesthesia was similarly performed in a

cold atmosphere by placing groups of two caged workers in glass jars in an ice bucket for 10 minutes (for anaesthesia parameters, see Table S1.3; fig. S1.1c).

2.5. Experiment 2: Impact of contact exposure on isolated workers in combination with cold stress

In this experiment, we partially replicated experiment 1 using a modified anaesthesia protocol mimicking cold stress to evaluate potential interactions between phthalate and cold exposure. The data set relative to the "cold stress" group was generated using the same exposure protocol as in experiment 1. However, anaesthesia was performed by direct ice contact for a longer period of 30 minutes, compared to the 10-minute cold atmosphere anaesthesia of experiment 1 (for anaesthesia parameters, see Table S1.3). Mortality was recorded 72h post-exposure. We considered seven treatment groups of ~30 workers: a control, and three single exposure treatments per phthalate (0.1X, 1X and 10X). The data set relative to the "non-cold stress" group originates from experiment 1, from which we selected the appropriate treatments, common to both experiments.

2.6. Experiment 3: Impact of DnBP and DEHP exposure in combination with social conditions

In this experiment, we partially replicated experiment 1, but we evaluated microcolonies to assess interactions between phthalate exposure and social condition. We considered five treatments: a control, two single-molecule treatments with DnBP (1X, 10X), and two with DEHP (0.1X, 1X). The data set relative to the microcolony group was generated as follows. Ten microcolonies for each treatment, i.e. 50 in total, were established in hard plastic boxes (8x16x16 cm; fig. S1.1b). Each microcolony was composed of ten workers. Workers composing a microcolony were sampled from the same colony. Five commercial colonies were used in the experiment. Microcolonies were kept under constant darkness and controlled temperature and humidity (25°C; 60% RH). They received *ad libitum* 50% mass/mass sucrose-

water solution. A pollen candi (1g), i.e., a ball made of pollen mixed with the sucrose-water solution at 0.15 mL/g of pollen, was also provided to each microcolony and replaced every other day. If not fully consumed, the initial pollen candi was not replaced. Organic frozen honeybee-collected pollen (Rosaceae: *Malus*/*Pyrus* f. 40%; Salicaceae: *Salix* 27%) was used (Abeille Heureuse, Strasbourg, France; Barraud et al., 2022). After a 24-hour acclimation period, workers were anaesthetised and exposed using the same method as described in experiment 1 (second session; fig. S1.1c). Once recovered at room temperature, all workers from one microcolony were returned to their box simultaneously. Mortality was recorded 72h post-exposure. The data set relative to the isolated group originates from experiment 1, from which we selected the appropriate treatments, common to both experiments.

2.7. Statistical analyses

Data were analysed using R v 4.4.1 (R Core Team, 2023); graphs were made with 'ggplot2' package v3.5.1 (Wickham, 2016).

Mortality rates were analysed using generalised linear mixed models (GLMMs) from 'glmmTMB' package v1.1.11 (Brooks et al., 2025) with a binomial distribution family and the commercial colony as random effect. For single-molecule exposure (experiment 1), before pooling data from both sessions, we checked that the control mortality did not differ between batches and sessions (See Table S1.4). The dose was used as a fixed effect, and we added either a cold stress (experiment 2) or social effect (experiment 3), depending on the analyses. If one of the fixed effect levels fully explained the response variable, preventing model fitting, i.e. complete separation (Heinze and Schemper, 2002), a Bayesian GLMM was used by applying the 'bglmer' function from 'blme' package v1.0-6 (Dorie et al., 2022). Weakly informative normal priors were applied for fixed effects (fixef.prior = normal). Convergence was evaluated via optimiser diagnostics in which all models terminated without warnings

(Bates et al., 2015). All model fits were further evaluated using the DHARMa package (Hartig, 2020). Contrasts using the package 'emmeans' v1.10.5 (Lenth, 2023) with the Benjamini-Hochberg's p-value adjustment method (i.e., 'fdr') were used *post-hoc*. Mortality differences between two groups exposed to different molecules were compared using two-proportion Z-tests.

For mixture exposure, a modified binomial proportion test for additivity was used to assess the potential interaction effects of environmental concentrations of DnBP and DEHP using the R code provided in Sgolastra et al. (2017), and applied similarly to Raimets et al. (2018). This test compares expected mixture mortality (based on single-molecule assays), and observed mortality (from the mixture exposure assay) by evaluating the null hypothesis of both molecules acting without interaction, *i.e.* $P_{mixture}^{observed} - P_{mixture}^{expected} = 0$. $P_{mixture}^{observed}$ is the experimentally observed mortality in the mixture-treated group, and $P_{mixture}^{expected}$ is the expected mortality for the mixture-treated group calculated under Bliss independence criterion, *i.e.* $P_{mixture}^{expected} = P_{DEHP}^{observed} + P_{DnBP}^{observed} - P_{DEHP}^{observed} \times P_{DnBP}^{observed}$. Using a lower-tailed version of the test, a significant deviation from the null hypothesis would show an antagonistic interaction.

In experiment 3, we first analysed raw mortality rates to assess the effects of phthalates and cold stress. In case of interaction, the phthalate effect was isolated by correcting the data using Sun-Shepard's control correction formula, which allows to neglect mortality solely induced by cold stress: corrected mortality = [(% treatment survival + % change in control survival) / (100 + % change in control survival)] x 100, with "% change in control survival" = [(number of alive individual in control group - total number in control group)/total number in control group] x 100 (Sun and Shepard, 1947). Negative values obtained were set to zero.

3. Results

3.1. Mortality of isolated workers induced by DnBP and DEHP (experiment 1)

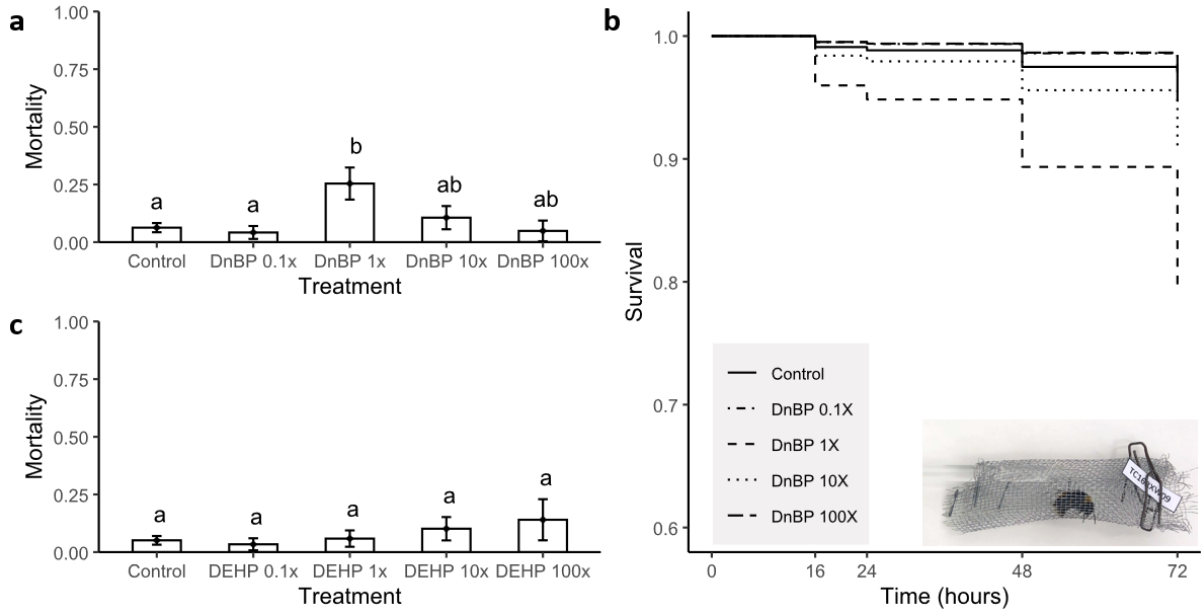


Fig. 1. Effects of single-molecule exposures to DnBP and DEHP on mortality rates of isolated workers after 72h. a) Mortality rates in control groups (N=170) and in groups exposed to DnBP at 0.1X (N=50), 1X = 60ng/g (N=49), 10X (N=50), or 100X (N=20). b) Kaplan-Meier cumulative proportion of survival during the 72h after worker exposure to DnBP 0.1X, 1X, 10X or 100X and control groups. The embedded picture shows the isolated caged setup. DnBP 0.1X and DnBP 100X curves are confounded. Please note that the Y-axis scale is cropped below 0.6. Survival curve for DEHP was not established as no treatment significantly increased mortality. c) Mortality rates in control groups (N=188) and in groups exposed to DEHP at 0.1X (N=49), 1X = 120ng/g (N=50), 10X (N=50), or 100X (N=20). In a) and b), columns show emmeans values with error-bars indicating standard errors, and groups with different letters significantly differ at $p<0.05$ (GLMMs, family binomial).

DnBP exposure significantly affected worker mortality 72h post-exposure compared to control (fig. 1a; bayesian GLMM, family binomial: chisq.=14.855, df=4, p=0.005012). The peak mortality was found in the DnBP 1X-exposed group, which showed a significant fourfold increase in mortality compared to the control group (fig. 1a; $p_{DnBP\ 1X\ vs.\ solvent}$=0.0045) and a significant sevenfold increase compared to the DnBP 0.1X-exposed group (fig. 1a; $p_{DnBP\ 1X\ vs.\ DnBP\ 0.1X}$=0.0467). However, mortality rates observed in DnBP 0.1X, 10X, and 100X-exposed groups were not significantly different from the control group, resulting in a bell-shaped curve

(fig. 1a; for detailed mortality and statistical values, see Table S1.5). Moreover, most DnBP 1X-induced mortality occurred between 48h and 72h post-exposure (fig. 1b).

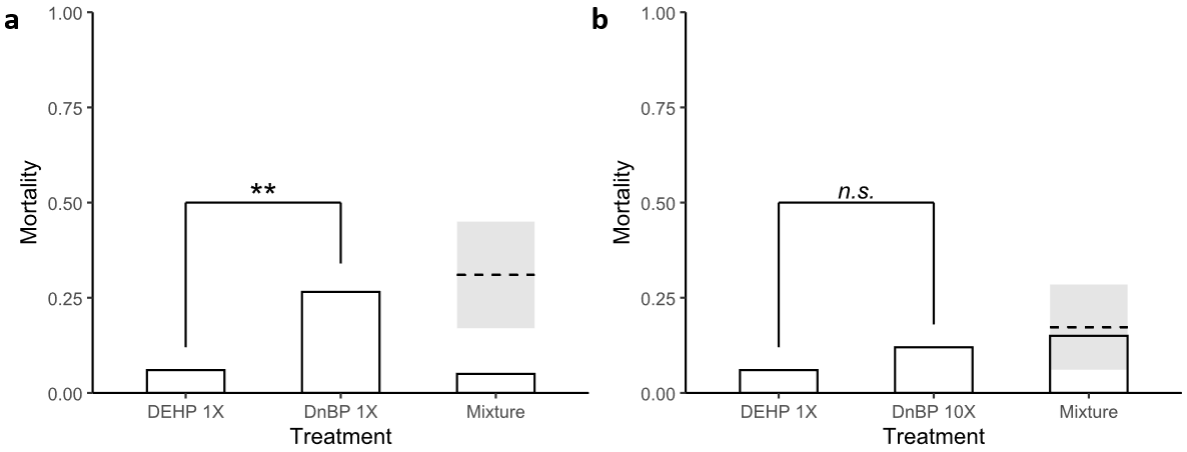


Fig. 2. Effects of mixture exposures to DnBP and DEHP on mortality rates of isolated workers after 72h. a) Mortality rates after single exposure to DEHP 1X (N=50), DnBP 1X (N=49) or their mixture (N=20). b) Mortality rates after single exposure to DEHP 1X (N=50), or DnBP 1X (N=50), or their mixture (N=20). Columns indicate observed mortality. In mixture columns, the dashed line shows the expected mortality calculated using the Bliss independence criterion. If the mixture column does not overlap with the 95%-confidence interval of the expected mortality (grey area), the expected and observed mortality differ significantly at $p<0.05$ (lower-tailed binomial proportion test). **single-molecule treatments differing at $p<0.01$; n.s. no difference between expected and observed mortality rates (Z-test).

Regarding DEHP single-molecule exposure, no significant effect on worker mortality was recorded 72h post-exposure to any of the doses (fig. 1c; for detailed mortality and statistical values, see Table S1.5; GLMM, family binomial: chisq.=2.2299, df.=4, p=0.6936).Investigating the potential cocktail effects, we observed that DEHP 1X exposure induced significantly less mortality compared to DnBP 1X (fig. 2a; for detailed mortality values, see Table S1.5 for DnBP and Table S1.6 for DEHP; Z-test: chisq.=6.26, df.=1, p=0.006). The mortality observed in the group exposed to the DEHP 1X and DnBP 1X mixture was significantly lower than expected if both molecules acted independently, suggesting an antagonistic effect at these doses (fig. 2a; for detailed mortality values, see Table S1.7; Lower-tailed binomial proportion test: p=0.001).

The mortality in the DEHP 1X-treated group was not significantly different from the mortality found in the DnBP 10X-treated group (fig. 2b; for detailed mortality values, see Table S1.5 for DnBP and Table S1.6 for DEHP; Z-test: chisq.=0.49, df.=1, p=0.2423). The mortality observed in the group treated with the mixture was not significantly different from the expected mortality (fig. 2b; for detailed mortality values, see Table S1.7; Lower-tailed binomial proportion test: p=0.408).

3.2. Worker mortality induced by DnBP and DEHP in combination with cold stress (experiment 2 vs 1)

Cold stress significantly affected raw mortality rates (bayesian GLMM, family binomial: cold stress parameter, chisq.=15.59, df.=1, p<0.001), but this effect varied across all dose groups, indicating a significant interaction between cold stress and DnBP exposure (interaction parameter, chisq.=9.62, df.=3, p=0.022). In the DnBP 0.1X group, cold stress did not significantly change mortality compared to non-cold stress group (fig. 3a, grey plots; p=0.3690). However, cold stress significantly increased raw mortality rates in the control (p=0.0002) and DnBP 10X-exposed groups (fig. 3a, grey plots; p=0.0044). Interestingly, the raw mortality rate of the cold-stressed DnBP 0.1X group was significantly lower than in the cold-stressed control group (p=0.0229), while they did not differ without cold stress (p=0.7095; fig. 3a, grey plots; for detailed mortality values, see Table S1.8)

As cold stress significantly increased control raw mortality rates (p=0.0002), we applied the Sun-Shepard's correction on the data, which enabled to adjust for mortality caused solely by cold stress, and to isolate DnBP effects. After correction, the cold stress effect on mortality was no longer significant as expected (bayesian GLMM, family binomial: for cold stress parameter, chisq.=0.17, df.=1, p=0.68), but the interaction between DnBP exposure and cold treatment remained significant (bayesian GLMM, family binomial: for interaction parameter,

chisq.=13.74, df.=2, p=0.001). Only the corrected mortality found in the cold-stressed DnBP 10X group was significantly higher than in the non-cold stressed group (fig. 3a, black plots; for detailed mortality values, see Table S1.8; p=0.0301)..

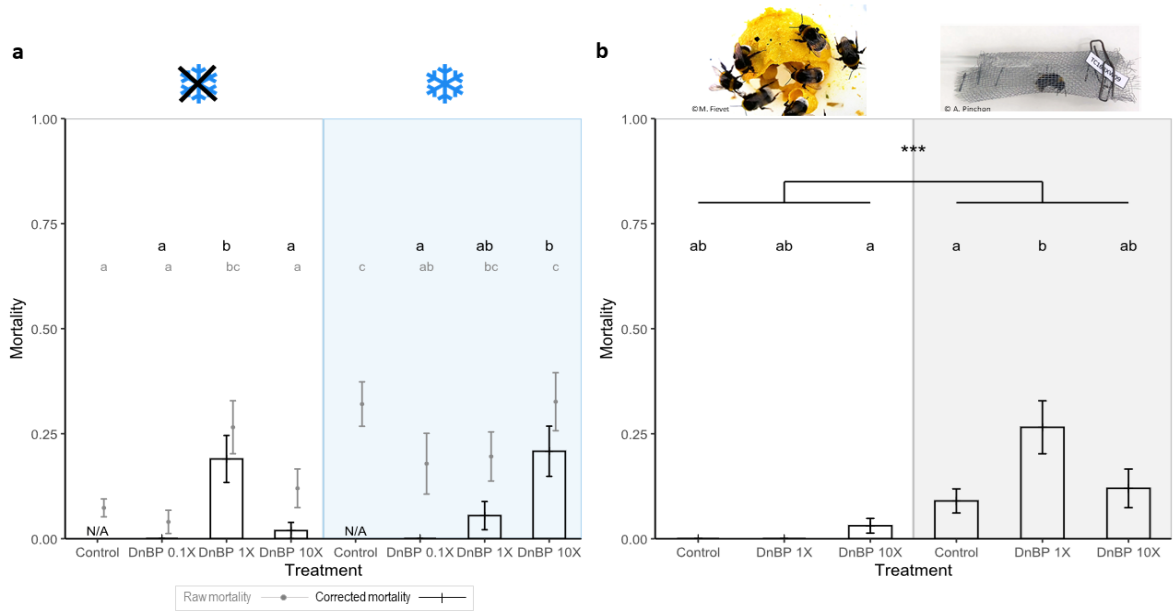


Fig. 3. a) Effects of DnBP 0.1X, 1X (=60ng/g) and 10X on mortality in non-cold stressed workers (white panel; $N_{control}$=150, $N_{DnBP\ 0.1X}$=50, $N_{DnBP\ 1X}$=49 and $N_{DnBP\ 10X}$=50) and cold-stressed workers (blue panel; $N_{control}$=78, $N_{DnBP\ 0.1X}$=28, $N_{DnBP\ 1X}$=46 and $N_{DnBP\ 10X}$=46). Grey points, error bars and letters show results for raw mortality rates. Black bars, error bars and letters show results for corrected mortality. No bars indicate no mortality, and N/A indicates no mortality data available for the control groups after correction. Groups that show different letters are significantly different at p<0.05 (bayesian GLMM, family binomial). b) Effect of DnBP 1X and 10X on mortality in microcolony (white panel; N=100 per treatment) and in isolated workers (grey panel; $N_{control}$=150, $N_{DnBP\ 1X}$=49 and $N_{DnBP\ 10X}$=50). Bars show mortality values with error bars indicating standard errors. The absence of bars indicates no mortality. Groups that show different letters are significantly different at p<0.05 for the social and treatment interaction effect and *** indicates significant effect of social conditions at p<0.001 (bayesian GLMM, family binomial).

Cold stress consistently increased mortality in all DEHP-exposed groups without interaction (for statistical and results details, see Table S1.9 and fig. S1.2a).

3.3. Mortality induced by DEHP and DnBP under different social conditions (experiment 3 vs 1)

Mortality across all DnBP dose groups was significantly higher in isolated conditions compared to the microcolony condition (bayesian GLMM, family binomial: chisq.=41.8, df.=1, p<0.001), with a significant interaction between social context and DnBP exposure (

chisq.=7.81, df.=2, p=0.0201). In isolated workers, the DnBP 1X dose significantly increased mortality compared to the control (fig. 3b; p=0.0492), whereas none of the tested doses significantly affected mortality within the microcolonies (fig. 3b; for mortality rates, see Table S1.10). Regarding DEHP exposure, mortality was also significantly higher in isolation compared to microcolonies (bayesian GLMM, family binomial: chisq.=11.6, df.=1, p<0.001; for mortality details, see Table S1.11), without significant interaction (chisq.=1.6, df.=2, p=0.451). In the microcolonies, one dead worker out of the hundred exposed to DEHP 0.1X was recorded, and no mortality was recorded in the other groups (fig. S1.2b; for mortality rates, see Table S1.10).

4. Discussion

Our study is the first to address *in vivo* PAE lethal effects on bees, specifically the social bumblebee *B. terrestris*. We report short-term mortality in workers following environmental DnBP contact exposure. We also investigated exposure to DnBP and DEHP mixtures, highlighting a dose-specific antagonistic effect. Additionally, we show that DnBP exposure modifies resistance to cold stress, notably enhancing survival at low doses. Finally, our results indicate that social conditions buffer DnBP toxicity recorded in isolated workers.

4.1. Single-exposure to DnBP and DEHP

DnBP exposure increased the mortality of *B. terrestris* workers shortly after contact exposure to environmental doses, an effect not observed with DEHP. The few acute toxicity tests performed on invertebrates highlighted that low molecular weight PAE, like DnBP, show higher toxicity, compared to high molecular weight PAEs, like DEHP (Adams et al., 1995; Staples et al., 1997). For instance, Cuvillier-Hot et al. (2014) did not report any mortality in the ant *Lasius niger* queens after exposure to DEHP environmental doses, using a protocol similar to ours (*i.e.*, drop deposition of the molecule diluted in methanol). Similarly, in the aquatic larvae of the insect *Chironomus riparius*, no mortality occurred 24 hours post-exposure to

DEHP, while the toxicity of butyl benzyl phthalate (BBP), a low molecular weight PAE, increased with dose (Planelló et al., 2011). In addition, PAE chronic exposure studies in other invertebrates align with our findings. For example, DnBP chronic exposure was highly toxic to adults and juveniles of the collembolan *Folsomia fimetaria*, while DEHP exposure did not increase mortality (Jensen et al., 2001). Overall, exposing isolated *B. terrestris* workers to PAEs provided comparable results to other models. The observed absence of DEHP effects and mortality increase caused by DnBP further confirm a toxicity pattern shared across invertebrate taxa and tested life stages, similarly in long- and short-term mortality assessments (e.g., Andreyeva et al., 2023; Green-Ojo et al., 2024; Hobson et al., 1984; Jensen et al., 2001; Li, 2020; Pradhan et al., 2018; Seyoum and Pradhan, 2019).

Interestingly, Arulanandam and colleagues (2022) approached this question *in silico* on the honey bee (*A. mellifera*) using toxicological modelling based on molecular sequences SMILES ("Simplified Molecular Input Line Entry System") and existing toxicity data (Arulanandam et al., 2022). Their results suggested strong toxicity for DnBP and low toxicity for DEHP (Arulanandam et al., 2022), which aligned with our *in vivo* results. Yet, only limited ecotoxicological data on endocrine disruptors (EDs) are available for bees, and there is a need for complementary *in vivo* testing, especially to consider NMDRCs and low-dose effects in PAE risk assessments (Crane et al., 2022). In our study, exposure to four ecologically relevant DnBP doses revealed a bell-shaped toxicity profile, with peak mortality reached at 60ng/g b.w., and negligible mortality at lower and higher tested doses. NMDRCs associated with DnBP-related mortality have not been reported so far, probably because previous studies investigated PAE toxicity in invertebrates using LD50/LC50 determination (Adams et al., 1995; Staples et al., 1997). These methods are based on linear dose-response relationship and high-to-low dose extrapolation, which overlooks NMDRC and low-dose effects (Futran Fuhrman et al., 2015;

Lagarde et al., 2015; Vandenberg, 2014). Most invertebrate risk assessment guidelines, including for bees, still rely on LD50 or LC50s (EFSA et al., 2023). In contrast, approaches testing environmental and low doses allow for revealing non-linear profiles, as previously shown for other re-evaluated xenobiotics (e.g. Baines et al., 2017) and here with DnBP. Indeed, we used the OECD guideline n°246 for toxicity testing of chemicals in *B. terrestris* (OECD, 2017b), normally used for LD50 determination, and rather tested four environmental doses, which allowed to highlight the DnBP-associated NMDRC. Therefore, these findings underline the need to include NMDRC frameworks in ecotoxicological risk assessment procedures.

This study demonstrated the lethal effect of phthalate exposure, specifically DnBP, on *B. terrestris* workers, providing the first evidence of phthalate toxic effects on bees. A more comprehensive assessment, however, requires investigation on queens and drones, which differ from workers in their sensitivity to xenobiotics (Gekière et al., 2024b; Linguadoca et al., 2022), and could also differ in their response to phthalates. In addition, protocols to test pollutant toxicity in *B. terrestris* larval stages are emerging in bee ecotoxicology (Kato et al., 2022). Such studies are particularly relevant because larvae develop within lipid-rich nest material, which can accumulate lipophilic contaminants like phthalates, as previously observed in honeybee wax combs (Gómez-Ramos et al., 2016). Nevertheless, the suitability of this species as a surrogate for wild bees in ecotoxicological assessments is debated (e.g., Dewaele et al., 2024; Ghisbain, 2021; Linguadoca et al., 2022), since morphological traits (e.g. hairiness, cuticle thickness, body mass, surface-to-volume ratio) and physiological characteristics (e.g. haemolymph pH, detoxification pathways) vary considerably among wild bees and strongly influence pollutant sensitivity (Balabanidou et al., 2018; Dewaele et al., 2024; Linguadoca et al., 2022).

Nonetheless, our findings provide a first step towards integrating underexplored pollutants into pollinator risk assessment frameworks. They also highlight the importance of extending research to other taxa, particularly emerging solitary bee models such as Osmia spp. (Megachilidae), for which standardised exposure protocols are currently under development (Azpiazu et al., 2023). Finally, current phthalate regulations insufficiently address environmental contamination, focusing mainly on direct human exposure (Billings et al., 2021; Chapon et al., 2023; Gunaalan et al., 2020; Regulation (EU) 2018/2005). By showing that DnBP exposure reduces bee survival, this study contributes to growing evidence of phthalate impacts on terrestrial insects and underlines the urgent need to strengthen regulation of plastic leachate in the environment (Crane et al., 2022), especially in agricultural and urban landscapes where bee populations are already weakened by multiple stressors (Goulson et al., 2015).

4.2. Exposure to DnBP and DEHP in mixture

We exposed workers of *B. terrestris* to mixtures of DnBP and DEHP at environmental doses to go beyond the single-molecule exposure scheme. Indeed, organisms, including bees, are simultaneously exposed in their environment to a combination of pollutants, e.g. heavy metals, pesticides, or plastic leachates (Gómez-Ramos et al., 2016), and thus to ED mixtures (Cuvillier-Hot and Lenoir, 2020). Surprisingly, we found antagonistic effects between DEHP 120ng/g of bee and DnBP 60ng/g of bee, *i.e.*, the most toxic dose when tested in single molecule solution. Similarly, in a previous study, single-molecule exposure to cadmium or DEHP caused around 15% mortality in *Spodoptera littoralis* adults that had developed on contaminated food, while mortality dropped to less than 5% in the mixture treatment (Humann-Guilleminot et al., 2024). These congruent results suggest that DEHP acts on detoxification pathways. Cadmium is another endocrine disruptor in several species groups

(Akhavan et al., 2016), that has been shown to affect ecdysteroid signalling and stress response in insects (Planelló et al., 2010). Recent LD50s calculated for cadmium in *B. terrestris* workers and males were lower than the field concentrations (Gekière et al., 2024b), revealing potential toxicity of this compound in bees. Similarly to results found in *S. littoralis*, mixture with DEHP could modify its toxicity in bees. Moreover, in rats, DEHP was shown to act on xenobiotic detoxification processes, as its mix with parathion lowered the latter's deleterious effects by disrupting the pesticide breakdown into toxic metabolites (Srivastava et al., 1976). In this study, DEHP could have similarly lowered DnBP toxicity, as PAE metabolites are often more toxic at lower doses and more potent than their parent compounds (Erkekoglu et al., 2010; Saillenfait et al., 2001). Similar results to ours were found when testing a mixture of polystyrene microparticles (MPs) and DnBP on the copepod *Tigriopus japonicus* mortality (Li et al., 2020). DnBP toxicity was lowered when mixed with MPs, and the authors hypothesised that the antagonism was caused by a lower bioavailability of the DnBP molecules adsorbed onto MPs (Li et al., 2020). Alternatively, polystyrene may have released DEHP, a common polystyrene additive (Coffin et al., 2019), which may have lowered DnBP toxicity by acting on detoxification pathways and associated enzymes. For example, exposure to DEHP has been shown to upregulate detoxification enzymes of the CYP450 family in the liver tissues of quails and rats, and in *Caenorhabditis elegans* (Eveillard et al., 2009; Yen et al., 2024; Zhang et al., 2018). Since these enzymes are involved in phthalate detoxification (Zheng et al., 2024), upregulation of the corresponding genes could enhance DnBP detoxification efficiency, thereby reducing its toxicity. In addition, this would be consistent with evidence that DnBP metabolites could be less harmful than the parent compound (reviewed in Sicińska, 2018). Bees are simultaneously exposed to diverse chemical pollutants (Gómez-Ramos et al., 2016). For example, in agricultural environments where pesticides are used, sewage sludge

application directly contributes to environmental contamination with phthalates and heavy metals, such as cadmium (Billings et al., 2021; Knoll and Cappai, 2024). Our results underline the need to investigate more deeply the effects of DEHP on detoxification-related enzymatic activity and gene expression, and to integrate mixture exposure into ecological risk assessment. Mixture of phthalates with other endocrine disrupting compounds such as cadmium (Planelló et al., 2010), or neonicotinoids (Baines et al., 2017), already known to co-occur and interact in humans (Guo et al., 2025; Lecomte et al., 2017), should especially be investigated.

4.3. Interaction between cold stress and DnBP or DEHP exposure

In this study, we demonstrated that DnBP, but not DEHP, exposure interacted with the effects of cold stress. Bumblebees are well-adapted to cold and temperate climates (Heinrich, 2004; Rasmont et al., 2021). They can exit cold torpor states using non-flight thermogenesis, which combines muscle shivering and metabolic mechanisms (Esch et al., 1991; Surholt et al., 1991). In the context of climate change, studies considering thermal stresses in addition to pollutant toxicity evaluation are emerging, but as of now, mainly focus on heat stress and pesticides (Albacete et al., 2023; Nebauer et al., 2024). Yet, acute cold stress is known to negatively affect workers (Owen et al., 2013; Poissonnier et al., 2015; Potts et al., 2018), as shown here as the cold stress increased mortality, in most treatments, including in control groups. Moreover, bee resistance mechanisms to cold can be disrupted by pollutants (Potts et al., 2018; Verheyen et al., 2022). We highlighted that low doses of DnBP counteracted the cold stress effect as worker survival was enhanced. Similarly, exposure to low doses of DnBP was found to increase survival in starved *D. melanogaster* flies. This rescueing effect was associated with a decrease in the expression of insulin-like protein related genes (Williams et al., 2016). DnBP at low doses could enhance cold stress resistance by acting on similar

endocrine and metabolic targets in *B. terrestris*. This highly conserved insulin-like signalling pathway regulates the availability of energetic resources by directly acting on glucose and lipid metabolism. It also notably modulates the juvenile hormone biosynthesis in insects (Tatar et al., 2001), a metabolic rate accelerator in *B. terrestris* which regulates the switch between energy storage and expenditure (Shpigler et al., 2021). By modifying energetic resources availability through these metabolic pathways, DnBP could therefore enhance thermogenesis ability at low dose and benefit workers by increasing cold stress resistance.

However, when neutralising the mortality solely induced by cold stress, we observed a shift of the toxicity peak from the intermediate to the highest DnBP dose. Testing higher DnBP doses combined with cold stress could provide valuable information on temperature interactions with chemical stress in bees. Stress interaction effects can depend on chemical and temperature stress types, as well as species-specific physiology (Verheyen et al., 2022). The DnBP toxicity curve under cold stress could therefore either become dose-dependent or maintained as a higher dose bell-shaped profile.

The toxicity profile observed here, compared to the results without cold stress, might highlight a hormesis effect of DnBP on thermogenesis. The hormesis phenomenon is characterised by beneficial effects at low doses that become adverse at higher doses (Cutler et al., 2022). A similar effect was previously observed after exposure of *B. terrestris* workers to the neonicotinoid imidacloprid (Potts et al., 2018). Indeed, while low imidacloprid doses enhanced cold stress survival in *B. terrestris*, higher doses increased mortality after cold stress (Potts et al., 2018). The authors hypothesised that the neonicotinoid neurotoxic effects enhanced shivering at low doses and became deleterious with increasing doses (Potts et al., 2018). Yet, Erban and colleagues (2019) highlighted a disruption of *B. terrestris* lipid metabolism by imidacloprid, which could also explain the observed hormesis. Here, we

observed a similar effect of DnBP on cold resistance, but no increased shivering has yet been reported linked to PAE neurotoxic effects (e.g. Chen et al., 2018; Ran et al., 2012; Wu et al., 2024). Therefore, the hormesis effect found here might rather be caused by a metabolism disruption resulting from DnBP exposure, already observed in other invertebrates species (Jordão et al., 2016; Liu et al., 2021b; Prieto-Amador et al., 2021; Seyoum and Pradhan, 2019; Williams et al., 2016). In the context of cold resistance, the same metabolic pathways activated by DnBP at low doses helping with thermogenesis, could be overstimulated at high doses and become deleterious. Yet, DnBP effects on insect metabolism have not been thoroughly investigated. Future research should now aim to confirm the molecular pathways disrupted by DnBP in bees, notably by investigating potential metabolic rate or metabolic-related gene expression modification in workers exposed to DnBP. While phthalates are known to disrupt metabolism in other insect models, such as *S. littoralis* (Rivas et al., 2023) and D*. melanogaster* (Williams et al., 2016), no data are yet available explaining the molecular mode of action of DnBP in bees. Moreover, DnBP disruption of molecular mechanisms specifically related to cold stress resistance in workers should be further investigated, through for example metabolic rate measurements (Bretzlaff et al., 2023) or analyses of the expression of heatshock protein, metabolism and insulin-related genes, crucial for thermal stress resistance in workers (Kim et al., 2024). Finally, during their hibernation, Bumblebee queens could be exposed to phthalate though soil contamination (Net et al., 2015c). Therefore, the impact of long-term DnBP exposure on hibernating queens should be tested, as cold resistance is a crucial characteristic of this caste (Shi et al., 2023).

4.4. Influence of sociality on the impact of DnBP and DEHP exposure

Worker mortality in the microcolonies was lower than the mortality observed in isolated workers, which highlights potential mitigation effects of social conditions. *B. terrestris*

is a eusocial species (Rasmont et al., 2008). Workers benefit from social mechanisms, such as social thermoregulation, hygienic and allogrooming behaviour, as well as social modulation of metabolic and immune pathways. These are well-studied in the context of social immunity against pathogen and parasite infections (Cremer et al., 2007; Gaubert et al., 2023). However, social mitigation of xenobiotic exposure and sensitivity is less known.

First, microcolonies thermoregulate to maintain brood temperature at higher temperature (28-32°C; Livesey et al., 2019). In contrast, isolated worker body temperature equals surrounding temperatures at rest (20-25°C in the present study; Sepúlveda-Rodríguez et al., 2024). In insects, lower body temperatures can thus lower chemical detoxification and excretion rates (Verheyen et al., 2022), and could similarly strengthen DnBP effects.

Moreover, social grooming effects on xenobiotic exposure and sensitivity have not yet been investigated, but the removal of parasites by intensified allogrooming is an efficient mechanism against *Varroa* mite infections in *Apis* species (Mondet et al., 2020). This behaviour could modify cuticle contaminant quantities and lower their impact on workers, as we observed. Though *B. terrestris* perform less allogrooming behaviour than honeybee, self-grooming and brood rubbing are part of the behavioural array performed by workers in healthy colonies (Dronnet et al., 2005). Investigating the effects of grooming on cuticle chemical contamination could help understand contamination dynamics in social systems.

The bee microbiota could also play a crucial role in xenobiotic detoxification. It was shown in *A. mellifera* that gut microbiota depletion increases sensitivity to neonicotinoid insecticides (Wu et al., 2020). In social bees, the microbiota is maintained by grooming and use of shared honeypots (Motta and Moran, 2024). A quick drop in relative abundance of several bacterial groups, of which *Lactobacillus*, was highlighted after isolation of adult *B. terrestris* workers from their colony, with a timescale comparable to ours (72 hours; Billiet et

al., 2017). Interestingly, *Lactobacillus* were also shown to mitigate DEHP and DnBP reprotoxic effects in rodents (Chen et al., 2022; Shi et al., 2020; Tian et al., 2019). The increased vulnerability of isolated workers to DnBP observed here could result from a modification of microbiota composition following social isolation, but this hypothesis would benefit from further investigation.

Finally, when kept in queenless microcolonies, workers undergo deep endocrine changes. In the first days after microcolony installation, reproductive competition, with aggressive behaviour and ovary development, occurs among workers (van Doorn, 1989). In this context, multiple hormonal and metabolic pathways are activated and represent as many new targets for potential ED-induced sub-lethal effects. Notably, in *B. terrestris*, variation in ecdysteroid titres in the haemolymph and vitellogenin gene expression were found to influence aggressivity, dominance and oviposition behaviour at queen-less microcolony establishment (Amsalem et al., 2014; Bloch et al., 2000). Therefore, PAE effects on microcolony and isolated workers might differ. While mortality was buffered in the microcolony setup, DnBP could differentially affect individuals depending on reproductive and metabolic status, especially as DnBP and DEHP were shown to affect the ecdysteroid pathway in *S. littoralis* (Avilès et al., 2020) and vitellogenin gene expression in *L. niger* and *Daphnia magna* (Cuvillier-Hot et al., 2014; Seyoum and Pradhan, 2019). Investigating the effects of PAEs on the fitness and health of *B. terrestris* in microcolonies would provide a better understanding of their effects on hormonal pathways and the consequences of such disruptions.

4.5. Perspectives

The current bee population decline is the result of multiple anthropogenic factors probably acting in synergy. Characterising the effects of PAE alone and in combination with other stressors on bee fitness seems important as these pollutants are ubiquitous and will

continue to contaminate environments for long (Chapon et al., 2023). In this context, *B. terrestris* would be a relevant and useful model for PAE toxicity evaluation. Commercial colonies readily available give access to all life stages, castes and sexes (e.g., Gekière et al., 2024b; Kato et al., 2022; Linguadoca et al., 2022), and would allow a comprehensive evaluation of PAE effects on several levels of biological complexity, from molecular pathway to social behaviour disruption. Given the ED characteristics of PAEs (e.g. NMDRCs and low-dose sub-lethal effects), research must focus on evaluating the risk PAEs pose to both bee larval development and adult health at environmental doses. Finally, while laboratory experiments overly simplify exposure schemes, they allow control over non-investigated external stressors (e.g., floral resources shortage, bad weather, pollutant combined exposure, etc.), which can be detrimental to bee populations in field studies (Straw and Stanley, 2025). Yet, further field study would also provide complementary insights on phthalate risk.

Aligning with the current shift towards a multiple stressor paradigm in bee ecotoxicology, this study provided new evidence of PAE toxic effects on bees. Adding to the existing data on bee phthalate exposure, it should motivate further multiscale investigation from the molecular assay to field and semi-field experiments that would allow a better understanding of the impact of phthalates on wild populations of bees.

PART II: DnBP and DEHP short-term effects on metabolism and ecdysteroid-related gene expression of isolated workers of **Bombus terrestris****

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Abstract

Bees are essential pollinators which not only maintain wild flowering plant diversity but also agricultural productivity. Yet, they are increasingly threatened by anthropogenic stressors, of chemical pollution. While the impact of pesticide exposure on bees is well documented, less is known about the potential effects of other pollutants, including phthalates. Phthalates, such as di-n-butyl phthalate (DnBP) and di-2-ethylhexyl phthalate (DEHP), are ubiquitous synthetic chemicals with endocrine-disrupting properties identified in many models, including invertebrates. These compounds have been detected on insect cuticles, including *Bombus terrestris* bumblebees. Exposure to field-realistic doses of DnBP was notably found to affect worker survival, and it also enhanced worker resistance to cold potential, potentially by affecting metabolism. However, the physiological consequences of phthalate exposure in bees remain largely unexplored. Here, we examined the short-term effects of environmentally relevant phthalate exposure on *Bombus terrestris* workers. Using respirometry assays, we assessed metabolic activity under single and combined exposures to DnBP and DEHP. We also measured expression levels of key genes involved in metabolism (ATP synthase, L-LDH, AADAT), insulin signalling (InR, LIRP), and the ecdysteroid pathway (ERR, ECR, USP, E75, 78C). Our findings reveal that field-realistic exposure to DnBP altered metabolic activity as the respiratory rate was increased and expression of the ATP synthase gene was downregulated in exposed workers. Moreover, DEHP at low doses downregulated the expression of *L-LDH* gene as well as genes of nuclear receptors involved in the ecdysteroid cascade (*E75* and *78C*). These results highlight that phthalates affect crucial components of metabolic and endocrine signalling pathways with potential effects on development, olfaction and foraging behaviour. Phthalates could therefore represent overlooked drivers of bee decline.

Keywords: plasticisers; respirometry; endocrine disruption; bumblebee; RT-qPCR

1. Introduction

Insect pollinators are vital for maintaining biodiversity, as well as agricultural productivity and resilience (Elizalde et al., 2020; Klein et al., 2007; Ollerton, 2017). Bees in particular are crucial pollinators of a wide range of crops and wild plants, making them essential to ecosystems worldwide (Klein et al., 2018). However, these species are increasingly facing environmental disturbances, including habitat loss, pollution, climate change, and intensive agricultural practices. These stressors can have significant impacts on pollinator populations (Gekière et al., 2025a; Potts et al., 2010; Zattara and Aizen, 2021), potentially leading to a decline in pollination services (Vanbergen and The Insect Pollinators Initiative, 2013).

Numerous studies have explored how anthropogenic activities and land use influence the population dynamics of pollinators in various habitats (Carvell et al., 2017; Goulson et al., 2010; Gunnarsson and Federsel, 2014; Osborne et al., 2008). However, investigations of associated pollution impact on their health have been limited and largely focused on pesticide active ingredients (Basu et al., 2024). However, pollinators are exposed to many chemical pollutants such as heavy metals (Gekière et al., 2023), microplastics (Rodrigues et al., 2025), and plastic leachates (Martín-Gómez et al., 2024; Ouyang et al., 2023). These pollutants can disrupt key physiological pathways, thereby altering survival, development, and metabolism (Dewaele et al., 2025; Gekière et al., 2025b; Xue et al., 2025).

A growing concern in this context is the exposure to plastic-related chemicals, such as phthalates (Moreno-Gómez-Toledano and Jabal-Uriel, 2024). These compounds are widely used as plasticisers and are known to leach easily from plastic matrices into the environment due to the absence of covalent bonds (Suhrhoff and Scholz-Böttcher, 2016). Phthalates have contaminated all ecosystem compartments: they have been detected in waters, sediments and soils (Net et al., 2015a; 2015c), but also air and dust (Bergé et al., 2013). Additionally,

phthalates are rarely encountered in isolation and can co-occur with other phthalates or other pollutants, such as pesticides, in which they are often used as adjuvants to facilitate the passage of the solution through physiological barriers (Seralini, 2024). Indeed, insect lipid-rich cuticles actively retain these lipophilic compounds (Lenoir et al., 2014).

More recently, evidence of cuticular contamination of honeybee and bumblebee workers by phthalates has been reported (Gómez-Ramos et al., 2019; Cuvillier et al., in prep.). Preliminary studies near Lille (France) confirmed widespread phthalate contamination, particularly di-n-butyl phthalate (DnBP) and di-2-ethylhexyl phthalate (DEHP), on buff-tailed bumblebee, *Bombus terrestris*, cuticles (Cuvillier et al., in prep.). Due to their endocrine activity, phthalate effects can appear at low doses and follow non-monotonic dose-response (NMDR) patterns, making traditional toxicological assessments unreliable (Futran Fuhrman et al., 2015). Hence, previous results showed that mortality in *B. terrestris* workers exposed to field-realistic doses of DnBP followed an NMDR, with up to a 25% increase in mortality within 72 hours after exposure to 60 ng/g DnBP (Dewaele et al., 2025). Worker exposure to DnBP at low environmental doses also enhanced cold stress resistance, with potential effects on metabolic pathways regulating thermoregulation, a crucial ability of *Bombus* species (Dewaele et al., 2025). In addition, repeated exposure to field-realistic doses of DnBP in workers reared in microcolonies has shown obesogenic effects in adult workers, with an increase in abdominal lipid content, and in the drone progeny, with an increase in weight (Dewaele et al., In prep., Chapter 3, Part I). In contrast, previous results showed that DEHP did not impact worker survival in the short term and even reduced the toxicity of DnBP in a mixture (Dewaele et al., 2025). Nevertheless, it lowered worker survival after repeated exposure in microcolonies (Dewaele et al., In prep., Chapter 3, Part I).

Experimental evidence in invertebrate models suggests that DnBP and DEHP interfere with key metabolic and hormonal pathways (Avilès et al., 2019; Cao et al., 2016; Rivas et al., 2023; Williams et al., 2016). Notably, in *Drosophila melanogaster*, exposure to low-dose DnBP and DEHP enhanced starvation stress resistance by slowing lipid mobilisation mechanisms through disruption of the expression of insulin-like peptide and insulin-like receptor genes, involved in the highly conserved insulin-signalling pathway (Cao et al., 2016; Williams et al., 2016). Moreover, in *Spodoptera littoralis*, DEHP was found to affect larval development duration and adult sexual behaviours, through the disruption of nuclear receptor gene expression involved in the ecdysteroid-signalling cascade (Avilès et al., 2020, 2019). Since both these pathways regulate longevity, development and reproduction through metabolic processes in insects (Altintas et al., 2016; Antonova et al., 2012; Bonneton and Laudet, 2012), similar disruption could explain the previously observed effect and be deleterious for bee population health.

Monitoring the expression of genes involved in the insulin-signalling pathway in B. terrestris, such as the insulin receptor (*InR*) and its ligand, the locust insulin-related peptide (*LIRP*), as well as genes of the ecdysteroid-signalling pathway (ECR, USP, E75 and 78C), would provide insights into potentially conserved endocrine activity of DEHP and DnBP observed in previous insect models (Avilès et al., 2019; Cao et al., 2016; Williams et al., 2016). The *ECR* and *USP* genes code for heterodimers forming the ecdysone receptor. Upon binding to 20-hydroxyecdysone, it activates downstream nuclear receptors such as E75 and 78C, which play key roles in metabolic processes regulating development and reproduction (Bonneton and Laudet, 2012). In addition, DEHP acts as an estrogen-mimicking compound capable of binding to the estrogen-related receptor (ERR), an orphan receptor known to interact with xenoestrogens, as demonstrated in honeybees exposed to bisphenol A (Ouyang et al., 2023).

Activation of ERR can interfere with normal ecdysteroid signalling (Bonneton and Laudet, 2012). These hormonal pathways regulate the expression of numerous metabolic and stress-response genes, including those encoding ATP synthase (*ATPs*), responsible for ATP production during mitochondrial respiration (Chang et al., 2022), and lactate dehydrogenase (*LLDH*), which converts lactate into pyruvate to sustain metabolism under anaerobic conditions (Strachecka et al., 2019). In bees, upregulation of LLDH reflects enhanced energy production to meet increased metabolic demands, for example, during detoxification processes (Rondeau et al., 2025). Finally, the gene encoding α-aminoadipate aminotransferase (*AADAT*) is used as a biomarker of immune and metabolic stress in bees (Cuvillier-Hot et al., 2024; Doublet et al., 2017). While the AADAT enzyme role in insects remains unclear, kynurenine aminotransferase, its vertebrate equivalent, is activated in cases of human obesity (Favennec et al., 2015).

In this study, we investigated the short-term impacts of exposure to environmental doses of DnBP and DEHP on the metabolism of isolated *B. terrestris* workers in two experiments. Firstly, a respirometry assay was performed on *B. terrestris* workers exposed to phthalates as single molecules and in mixtures. This approach enabled us to assess the metabolism and cellular respiration of bumblebees exposed to different phthalate treatments. Secondly, doses of interest were investigated for potential disruption of the expression of genes related to metabolism (*ATP synthase*, *LLDH*, *AADAT*), as well as genes involved in the ecdysteroid (*ECR*, *USP*, *ERR*, *E75* and *78C*) and insulin signalling pathways (*InR* and *LIRP*).

2. Material and methods

2.1. Model species

We used the buff-tailed bumblebee *Bombus terrestris* (L.) (Hymenoptera, Apidae), which is the most prevalent and widely distributed bumblebee species in the Western Palearctic (Rasmont et al., 2021). *Bombus terrestris* plays a crucial role as a pollinator in both natural and cultivated plant communities (Klein et al., 2007; Rasmont et al., 2008). Commercial colonies of *B. terrestris* are available and easy to rear under controlled laboratory conditions. Colonies purchased from Biobest (Westerloo, Belgium) were not used for longer than 3 weeks after receipt, as suggested by standardised procedures (OECD, 2017b). The colonies were fed ad libitum with sugar syrup (BIOGLUC®, Biobest) and crushed multifloral honeybee-collected pollen (provided by Ruchers de Lorraine). They were kept in a darkroom at 27°C and 76% relative humidity. Syrup and pollen provisions, as well as worker manipulation, were carried out in a darkroom using red light to avoid disturbing colonies (OECD, 2017b).

2.2. Treatment preparation

For the respiratory assay, we selected doses used in Dewaele et al. (2025), i.e. DEHP 12ng/g, 120ng/g and 1200ng/g as well as DnBP 6ng/g, 60ng/g and 600ng/g. DEHP at 120ng/g of body weight (b.w.) and DnBP at 60ng/g b.w. were considered as field-realistic concentrations, following chemical dosages performed on wild *B. terrestris* populations (Cuvillier et al. in prep). We also tested a mixture treatment at these doses, as an antagonistic effect was previously highlighted between both molecules on isolated worker mortality (Dewaele et al., 2025). For gene expression analyses, we selected DnBP 60ng/g, which increased mortality in isolated workers, as well as DnBP 600ng/g for which metabolic disruption was suspected (Dewaele et al., 2025). As no mortality occurred in workers exposed

to DEHP from 12ng/g to 12µg, we selected two doses from Dewaele et al. (2025), i.e., 12ng/g and 1200ng/g.

DnBP (CAS n°117-81-7) and DEHP (CAS n°84-74-2) were purchased as liquid solutions from Sigma-Aldrich, and all treatment and control solutions were prepared as detailed in Dewaele et al. (2025).

2.3. Preventing uncontrolled PAE contamination

Before the start of the exposure process and between each contaminant transition, all equipment underwent a meticulous decontamination protocol. This involved a thorough cleansing in a hexane bath or rinse, followed by an acetone rinse, to eliminate any potential phthalate residues. Rigorous measures were implemented to avoid any introduction of phthalates from the surrounding environment. Specifically, the water used was exclusively purchased and stored in glass containers, and glass syringes were employed for all procedures in order to remove contact with plastic instruments (Luo et al., 2018; Reid et al., 2007).

2.4. Experiment 1 – Respiratory assay

Phthalate exposure. Exposure protocol was designed based on the OECD guideline n°246 for the testing of chemicals (OECD, 2017b). Workers dedicated to the respiratory assay were distributed in eight treatment groups: a control group exposed to methanol 0.07%, three DnBP-treated groups exposed to DnBP at concentrations of 6ng/g, 60ng/g and 600 ng/g, and three DEHP-treated groups exposed to DEHP at concentrations of 12ng/g, 120 ng/g and 1200 ng/g. Workers were taken from their colony and placed individually in handmade metal cages with similar dimensions to Nicot cages. They were then placed in a polystyrene container with ice for about 30 minutes to reduce their activity and facilitate exposure. Exposure itself was performed using a glass calibrated capillary, cleaned of all phthalate contamination before starting the exposure process using a hexane bath or rinse followed by acetone. Exposure was

made topically by applying a 2 µL drop of solution on the hairless part of the insect's thorax. Workers were then kept in a darkroom at 27°C and 76% relative humidity for 72 hours before undergoing the respiratory assay experiment. The wide end of a glass Pasteur pipette was used to feed the isolated individuals *ad libitum* with 50% sugar syrup for the duration of the experiment.

Respiration assay. Stop-flow respirometry is based on the difference between the initial and the final O2 percentages in a fixed volume of air after an individual has been left to breathe for a given time. A multiplexer combined with a gas chromatograph (GC) was used to measure this parameter. The bees were placed individually in hermetic glass jars (125mL) fitted with a rubber stopper and sealed with a metal ring to ensure airtightness. A needle placed in the stopper was used to withdraw 2.5 mL of air at regular intervals, which was analysed by the GC programmed to quantify the level of CO2 in ppm. Bumblebees do not fly when placed in the dark. The jars used for the measurements were therefore covered with opaque adhesive tape to ensure almost total darkness and to reduce bumblebee activity to a minimum.

All the measurements were obtained per batch, one batch corresponding to 4 jars, 3 of which contained a bee (exposed to a phthalate treatment or to the control treatment). The last jar was left empty to serve as a control, making it possible to determine the initial CO2 concentrations in the jars and also to monitor any fluctuations in CO2 during each measurement due to the setup itself. For each batch, the 3 measured bees were placed in the jars with a 3-minute interval between each individual, to standardise the time spent by each individual in the jar before the first measurement. Subsequently, the air in the jars, including the control jar, was analysed 4 times with a sampling interval of 12 minutes for each jar, for a total of 51 minutes. A cycle corresponded to the 4 jars being analysed, and the total measurement for a batch included 4 cycles.

The duration of the tests was adapted so that the oxygen level in the jars remained sufficiently high not to place the bumblebees in a hypoxic situation, and the maximum detection threshold for CO2 was adjusted to the size and assumed metabolism of the study model. To obtain the actual CO2 emissions, it was necessary to subtract the values obtained in the control (empty jar). For each cycle, the value obtained in the control (empty jar) was therefore subtracted from the 3 values obtained from the jars containing bumblebees. As metabolic activity is strongly linked to weight, each individual was weighed following the test. The CO2 emission was divided by the weight (in mg) of each individual. The corrected values formed 4-point curves for each individual, which made it possible to define the CO2/mg emission as a function of time for each individual.

2.4. Experiment 2 – Gene expression analysis

Phthalate exposure. Workers dedicated to the gene expression analysis were sampled in five treatment groups used in Dewaele et al. (2025): a control group exposed to methanol 0.07%, two DnBP-treated groups exposed to DnBP at concentrations of 60ng/g and 600 ng/g, and two DEHP-treated groups exposed to DEHP at concentrations of 12ng/g and 1200ng/g. As detailed in Dewaele et al. (2025), the exposure was organised in two batches (first batch: DnBP 60ng/g & DEHP 12ng/g; second batch: DnBP 600ng/g & DEHP 1200ng/g). Briefly, a solvent control group was set up in both batches. In each batch, thirty isolated workers were sampled from three commercial queen-right colonies (N total per batch = 90 workers; Biobest NV, Westerlo, Belgium), and installed in decontaminated handmade metal cages with similar dimensions to Nicot cages. They were acclimated to the lab conditions for eight hours. Then, the exposure protocol took place as detailed in Dewaele et al. (2025) and for the respiratory assay, except that workers were anaesthetised by chilling for 10 min at -20°C without direct contact with the ice.

Fat body tissue sampling. After 72hours, six to eight workers per treatment (equally distributed between initial colonies) were anaesthetised by chilling for 10 min at -20°C. Their abdomens were dissected on ice and under a binocular microscope. Fat body tissues were carefully separated from the other abdominal tissues (respiratory and digestive tract, reproductive system and venom gland), using RNase Zap (Sigma-Aldrich) rinsed precision tweezers. The collected fat body samples were individually flash frozen and stored at -80°C until extraction.

RNA extraction and RT-qPCR. The fat body samples were gently crushed using a pestle in the lysis buffer from Nucleospin RNA Plus kit (Macherey-Nagel; Germany). RNA extraction was performed following kit instructions. RNA quantity after extraction was measured using a Qubit 4 fluorometer from Thermofisher. For each sample, 650 ng of total RNA were used as template for cDNA synthesis using RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific) following the manufacturer's protocol. Real-time PCR were performed on a Lightcycler 480 from Roche Applied Science. Amplifications were performed in a final volume of 10 µL containing 2 µL of cDNA (diluted 1:3), 1 µM of each specific primer, and 5 µL of BioRad SYBR Green master mix. Analyses were run in technical duplicates for each sample and each gene. The amplification conditions were 95 °C for 30 s, followed by 40 cycles of 95 °C for 15 s and T_m for 30s with a single read taken at the end of each cycle (See Table S2.1 for primer sequences and specific T_m). A melting curve analysis was carried out at the end of the PCR by heating the plate to 95 °C at a ramp rate 0.11°C/s and continuous fluorescence reading. Relative gene expression data were produced using Roche Applied Science LightCycler 480 software v1.5.1. First, the concentration thresholds (Ct) of target and reference genes were produced using the Second Derivative Maximum method. Then, Ct ratios between each target gene and the two reference genes, RPL13 and PLA2, were calculated using the E-method,

which enables correction for specific primer efficiency and normalisation using a standard sample (calibrator) identical between plates and for all genes (Tellmann, 2006).

2.5. Statistical analyses.

All statistical analyses and calculations were performed in R v4.4.1 (R Core Team, 2023). For the first experiment, we analysed CO2 emissions per mg of insect body weight using linear mixed-effects models. First, we modelled total CO2 emission and slope of emission over time as a function of treatment, with commercial colony identity included as a random factor to account for non-independence among individuals from the same colony. Second, we assessed individual CO2 trajectories over four time points by calculating the CO2 emission per milligram at each time point. We then fit a mixed model including a Treatment × Time interaction, with random effects for commercial colony and individual, to test whether treatments influenced the dynamic pattern of metabolic rate. Post-hoc pairwise comparisons were conducted using estimated marginal means (emmeans function) with Tukey adjustment. All models were implemented using the lme4, lmerTest, and emmeans packages (Bates et al., 2015; Lenth, 2023).

To compare relative gene expression between the treatment and control groups, ANOVA on Aligned Rank Transformed data (ART ANOVA) using package 'ARTool' v0.11.1 (Kay and Wobbrock, 2019) was performed using the treatment as fixed effect and the commercial colony as a random effect. As sample sizes were small, effect sizes as Cohen's coefficients with Hedge's correction for small sample size (i.e., "*g*"), were also calculated to help with the interpretation of the results. Its magnitude was interpreted as follows: g = 0.2 indicates a small effect, 0.5 a moderate effect and 0.8 a large effect (Schumacker and Tomek, 2013). For each treatment group, an Integrated Biomarker Response index (IBRv2) was calculated following the method described by Sanchez et al. (2013) to estimate the global molecular disruption

occurring in each treatment. Briefly, for each gene "*e*", the mean relative expression "X_i" of the treatment group "*i*" was divided by the mean expression in the corresponding control group "X_0", and log-transformed: $Y_e = \log(X_i/X_0)$. For each gene, Ye values were standardised as $Z_e = (Y_i-\mu)/\sigma$, where "μ" is the general mean and "σ" the standard deviation of Z_e across all treatment and control groups. For each treatment, deviation indices A_e were then computed as the difference between Z_e and the standardised reference value Z_0: $A_e = Z_e – Z_0$. A_e values therefore reflect relative deviation from the control, accounting for gene-specific variability, which allows for investigating global disruption patterns. The IBRv2 index was finally obtained by summing the absolute values of A_e across all genes. Higher IBRv2 values calculated with an equal number of genes indicate greater overall molecular disruption in the treatment groups. Spiderchart showing A_e were made using radarchart function from package 'fmsb' v0.7.6 (Nakazawa, 2024) and all the other graphs were made with 'ggplot2' package v3.5.1. (Wickham, 2016).

3. Results

3.1. Experiment 1 – Respiratory assay

Respiratory activity was unevenly affected by exposure to DEHP and DnBP. The analysis of slopes showed a marginal effect of phthalate exposure (treatment effect: p = 0.074), with treatments DEHP 120 ng/g and 1200ng/g, DnBP 60ng/g, and 600ng/g having higher estimated slopes compared to the control (Fig.1), though these differences were not significant (post-hoc analysis: p>0.05). We also revealed a significant Treatment × Time interaction (p = 0.035), suggesting that the shape or rate of this increase varied between treatments.

Post-hoc comparisons showed no significant differences between treatments at early time points (0, 12, 24 min), but at 36 minutes, the group treated with DnBP 60ng/g had a

significantly higher CO_2/mg than the control group (p = 0.038), with similar trends in other comparisons that did not reach statistical significance (mixture vs control, DEHP 120ng/g vs control, and DnBP 600ng/g vs control; p≤ 0.2; fig 2).

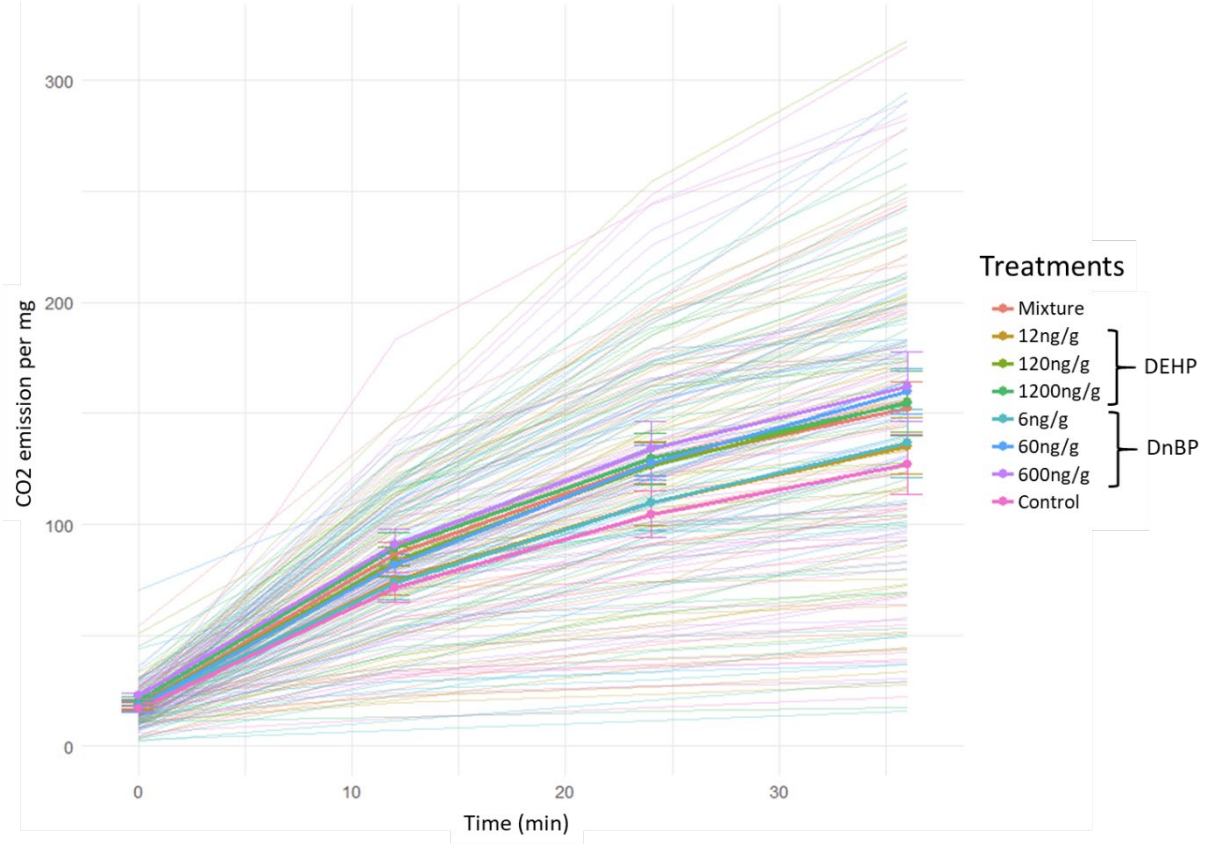


Fig. 1. Emission of CO2 per mg of body weight measured at each time point during the respiratory assay. Thin curves show individual values and thick curves show averaged values for each treatment group with bars showing standard error.

3.2. Experiment 2 – Gene expression analysis

In the group of workers exposed to DEHP 12 ng/g, a significant 4.5-fold decrease in LLDH relative expression was observed compared to the control group (ART ANOVA: F = 6.2, d.f. = 1, p-value = 0.0315, Hedge's g = 1.25; Fig. 3b; Table S2.2). Moreover, in this group, a significant down-regulation of ecdysteroid-induced genes was observed compared to the control group, with a 5.3-fold decrease in E75 relative expression (ART ANOVA: F = 8.4, d.f. = 1, p-value = 0.0157, Hedge's g = 1.46) and a 4.3-fold decrease for 78C (ART ANOVA: F = 5.2, d.f. = 1, p-value = 0.0456, Hedge's g = 1.14; Table S2.2; Fig. 3i,j). While LIRP and ECR gene

expression were not significantly downregulated compared to the control group, both were associated with large effect sizes of 0.84 and 1.05, respectively (ART ANOVAs with both p-values>0.05; Table S2.2; Fig. 3d,h).

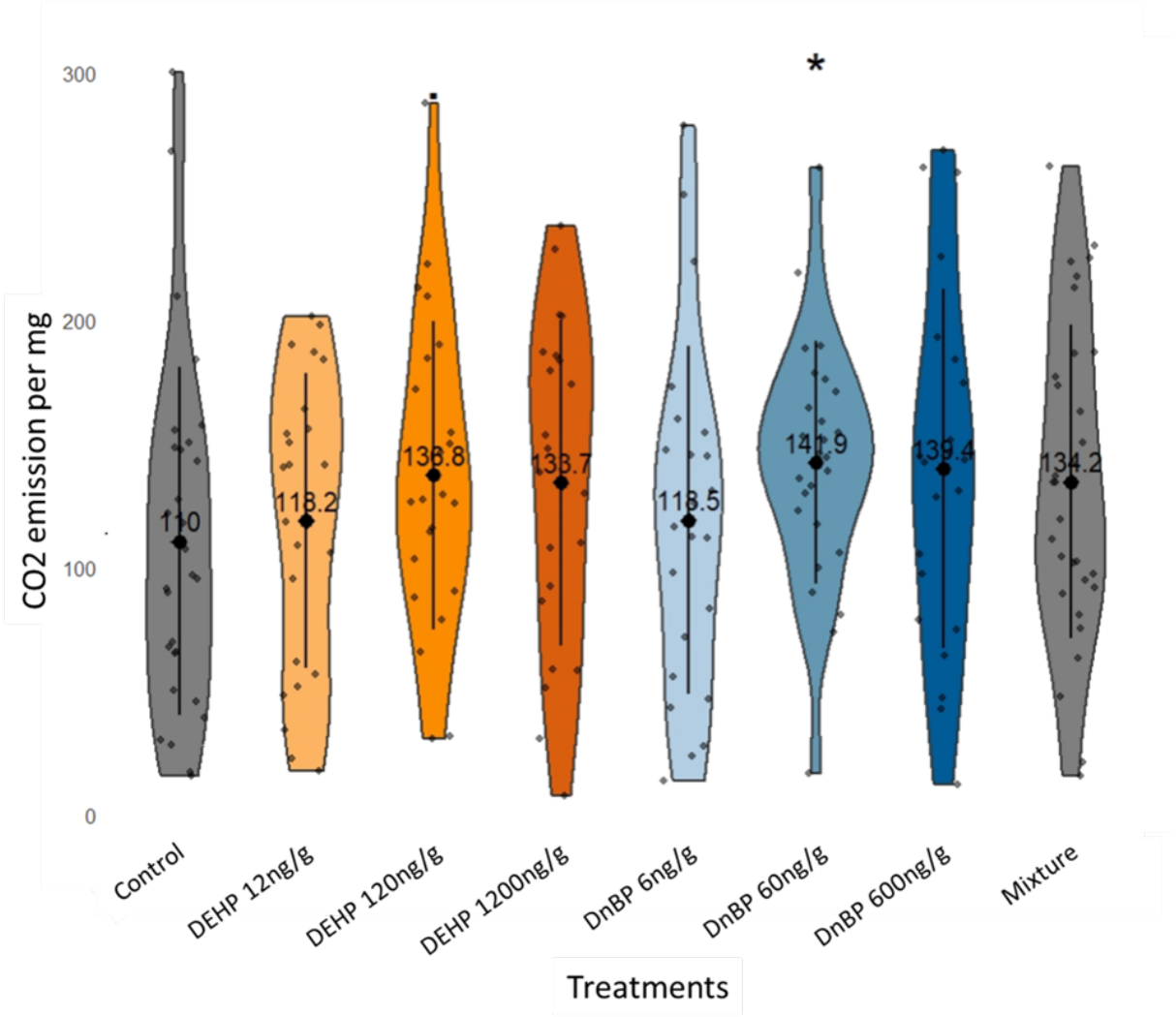


Fig. 2. Emission of CO2 per mg of body weight measured at 36 minutes of the respiratory assay. Dots show individual values while the black points show mean value for each treatment with bars showing confidence interval 95%. *shows significant difference with control at p<0.05

In contrast, exposure to a higher concentration of DEHP, i.e., 1200 ng/g, did not significantly affect any of the gene relative expressions (Table S2.2). We nevertheless observed non-significant effects with an up-regulation of InR and a downregulation of ECR,

associated with large and moderate effect sizes of respectively 0.87 and 0.67 (ART ANOVAs with all p-values>0.05; Table S2.2; Fig. 3e,h).

Concerning exposure to DnBP, no significant effect of the lowest tested dose, i.e., 60 ng/g, was detected on any of the measured genes (Table S2.2). Yet, several genes related to metabolism (LLDH and ATP synthase) and ecdysteroid pathways (ECR, E75, and 78C) were slightly down-regulated in this group, with moderate to large effect sizes between 0.59 to 1.05 (ART ANOVAs with all p-values>0.05; Table S2.2; Fig. 3b,c,h,i and j).

When increasing the dose, exposure to DnBP 600 ng/g significantly down-regulated ATP synthase relative expression with a four-fold decrease compared to the control (ART ANOVA: F = 10.4, d.f. = 1, p-value = 0.0177, Hedge's g = 1.86; Fig. 3c; Table S2.2). A non-significant 1.6-fold increase in LLDH relative expression was also observed (ART ANOVA: F = 1.1, d.f. = 1, p-value = 0.3409), with a moderate effect size of 0.59 (Fig. 3b; Table S2.2).

.The IBRv2 calculated in the group exposed to DnBP 60 ng/g (IBRv2 = 10.1, Figure 4a) was slightly higher than in the group exposed to DnBP 600ng/g (IBRv2 = 3.7, Figure 4b), indicating a higher overall deviation from the control situation at the lowest DnBP dose. The genes contributing the most to the index of the DnBP 60ng/g-treated group were distributed between metabolism-related genes and genes involved in the ecdysteroid-related pathway, with the highest deviation values found for InR (A = 1.09) and 78C (A = -3.11). In the group exposed to DnBP 600 ng/g, ecdysteroid pathway-related genes did not deviate much, with the highest value found for E75 at A = 0.58, in contrast to metabolism- and insulin-related genes with the highest deviation found for LIRP (A = -0.97), InR (A = 0.87) and ATPs (A=-0.70) (Figure 4b; Table S2.2).

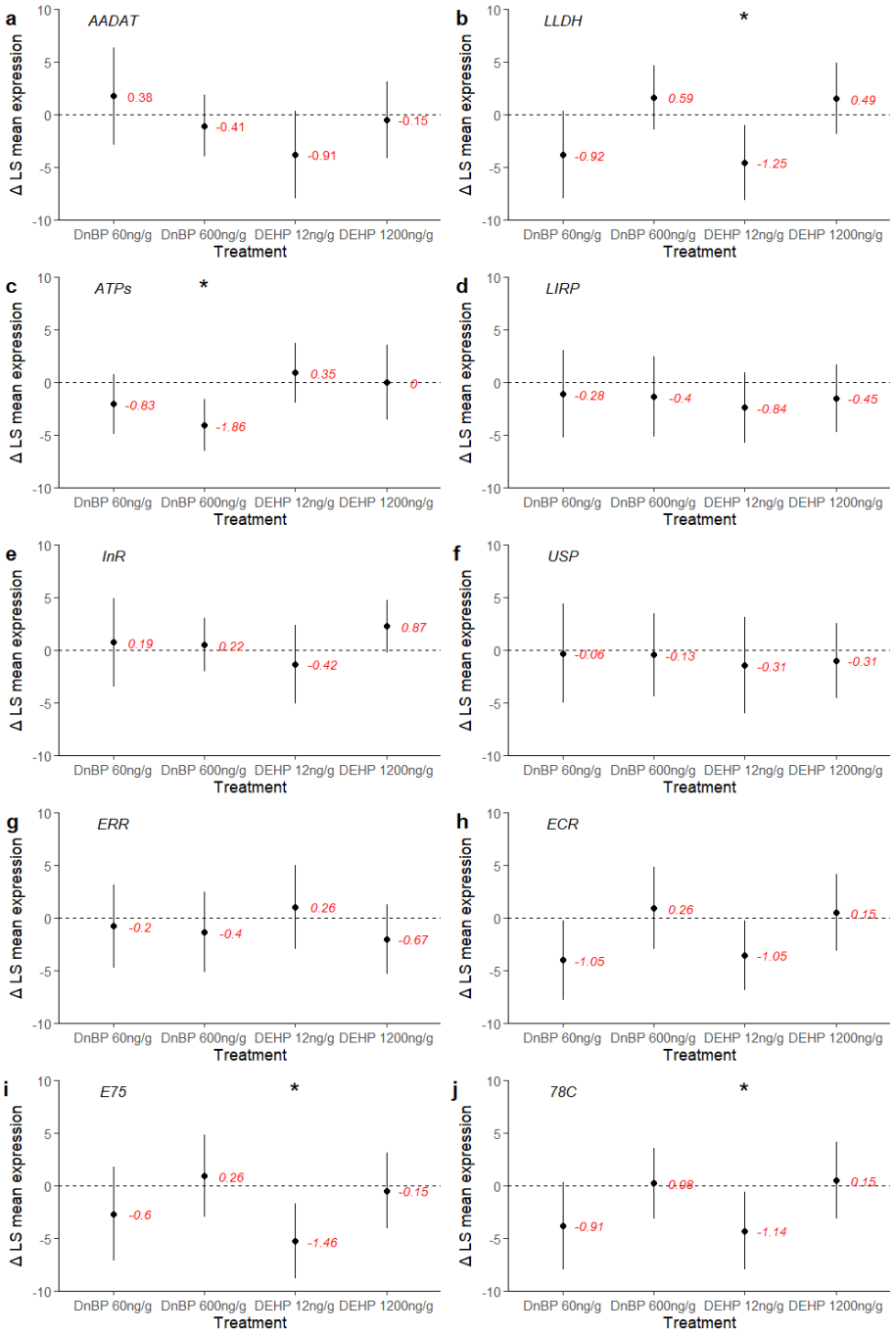


Fig. 3. Model-adjusted mean relative gene expression difference between the treatment and control groups with Hedges's *g* coefficient indicating effect size in red related to change in expression of genes related to metabolism, i.e., a) AADAT and b) LLDH (for both genes, N first batch: control = 8, DnBP 60ng/g = 6, DEHP 12ng/g = 6; N second batch: control = 4, DnBP 600ng/g = 4, DEHP 1200ng/g = 4); c) in expression of ATPs gene and in expression of genes related to the insulin-signalling pathway, i.e., d) LIRP, and e) InR (for the three genes, N first batch: control = 4, DnBP 60ng/g = 6, DEHP 12ng/g = 3; N second batch: control = 4, DnBP 600ng/g = 5, DEHP 1200ng/g = 2); and ecdysteroid-related genes, i.e., f) USP, g) ERR, h) ECR, i) E75, and j) 78C (for the four genes, N first batch: control = 8, DnBP 60ng/g = 6, DEHP 12ng/g = 6; N second batch: control = 4, DnBP 600ng/g = 4, DEHP 1200ng/g = 4). Black dots indicate the difference in Least Square Means of relative gene expression (Δ LS mean expression) between treatment and control groups, with bars showing the 95% confidence interval. *rpl13* and *pla2* are used as reference genes. *indicates significant change at p-value<0.05 (ART ANOVA).

Concerning DEHP, exposure to the lowest concentration, i.e., 12 ng/g, resulted in a higher index value (IBRv2 = 15.5) compared to the group exposed to DEHP 1200ng/g (IBRv2 = 3.8; Figure 4c). For the calculation of the index associated with the DnBP 12 ng/g-treated group, the genes contributing the most were those related to the ecdysteroid pathway, with "A" values ranging from -3.95 for 78C to -2.86 for E75 (Table S2.2). On the contrary, after exposure to DEHP 1200 ng/g, the two genes deviating the most were related to the insulin-signalling pathway, i.e., InR (A = 1.48) and LIRP (A = -0.97).

4. Discussion

In this study, two experiments were conducted on isolated *B. terrestris* workers exposed to environmentally relevant doses of DnBP and DEHP. Exposure to DnBP at 60 ng/g led to an increased respiratory rate, whereas a higher dose (600 ng/g) downregulated the expression of the ATP synthase gene, involved in aerobic energy metabolism. Overall, the IBRv2 values for DnBP-treated groups were close, but decreased with increasing dose (60ng/g = 10.1 and 600ng/g = 3.7). In contrast, DEHP exposure did not alter worker respiratory rate, but at the lower dose (12 ng/g), it downregulated the expression of the nuclear receptor genes E75 and 78C, both induced by ecdysteroid signalling, as well as LLDH, involved in anaerobic energy metabolism. For DEHP, the IBRv2 values showed a large divergence between both dose groups compared to DnBP groups, but also showed a lower value at the highest tested concentration (12ng/g = 15.5 and 1200 ng/g = 3.8).

4.1. Exposure to DnBP impacts respiratory rate and energy production

Firstly, during the respiratory assay, exposure to DnBP 60ng/g increased the metabolic rate of isolated workers, measured as CO2 emission. Research focusing on DnBP metabolic disruption is scarce in invertebrates.

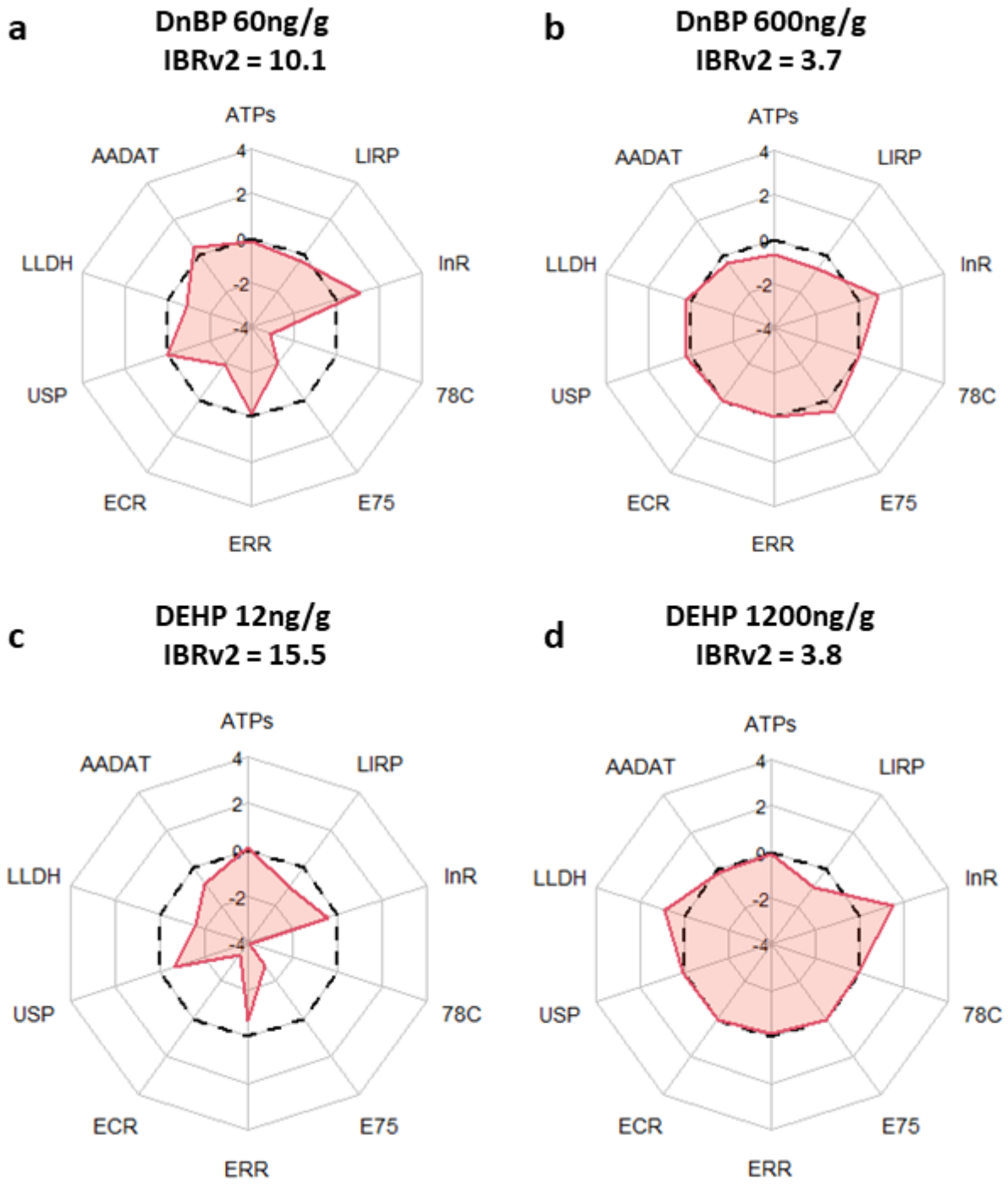


Fig. 4. Spiderchart showing deviation indices *"A"* calculated for each gene in groups exposed to a) DnBP at 60ng/g or b) at 600ng/g, and c) DEHP at 12ng/g or d) at 1200ng/g compared to control, with IBRv2 index of exposure. Dashed lines show the reference at 0 (i.e. no change in gene expression relative to the expression of the reference genes), and on all plots, the center corresponds to a value of -3.

In the swimming crab, DnBP exposure increased the expression of xenobiotic detoxification enzymes such as cytochrome P450 and glutathione-S-transferase (He et al., 2022). The activity of such detoxification enzymes can generate metabolic and oxidative stress, ultimately damaging cells and tissues and reducing survival (Murataliev et al., 2008). In a previous experiment, exposure of isolated workers to DnBP at 60ng/g increased mortality by up to 25% (Dewaele et al., 2025). Thus, the elevated respiratory rate observed here may indicate a metabolic stress response to DnBP exposure, potentially underlying the toxicity previously reported in isolated *Bombus terrestris* workers (Dewaele et al., 2025). In addition, when workers were simultaneously exposed to cold stress, individuals treated with DnBP 6ng/g and 60 ng/g survived better than untreated controls under the same cold stress. This suggests that the metabolic increase may have had a protective effect under cold stress, although this benefit disappeared at higher DnBP concentrations (Dewaele et al., 2025). Such a response is consistent with a potential hormesis effect, as discussed in Dewaele et al. (2025), and is similar to findings in *Bombus terrestris* exposed to the insecticide imidacloprid (Potts et al., 2018). Indeed, in the study of Potts et al. (2018), they highlighted that recovery from cold stress was improved through increased shivering movements triggered by the neurotoxic insecticide. Here, a similar effect could have happened but linked to metabolic disruption rather than neurotoxic impact.

Secondly, no significant effect of the mixture treatment was detected on the respiratory rate of workers, indicating a lower impact of mixture exposure compared to DnBP 60 ng/g alone. This result tends to confirm an antagonistic effect of DEHP on DnBP both at the individual level, as previously observed in isolated *B. terrestris* workers, but also at the physiological level (Dewaele et al., 2025). Indeed, a mortality increase was previously observed in the group exposed to DnBP 60 ng/g, while in the group exposed to a mixture with

DEHP 120ng/g, no significant increase in mortality occurred (Dewaele et al., 2025). Yet, more research would be needed to understand the molecular pathway disrupted by DnBP and how DEHP can lower these effects.

Thirdly, while the expression of metabolic genes was investigated in the second experiment, no significant change in expression was associated with exposure to DnBP 60ng/g. However, exposure to DnBP 600ng/g downregulated the ATP synthase expression. A similar result was observed after exposure of adult zebrafish, *Danio rerio*, to elevated concentrations of mono-butyl phthalate (MBP) (from 0.5 to 10 mg/L), a metabolite of DnBP (Tao et al., 2020). MBP was shown to inhibit aerobic ATP synthesis pathways and strengthen the anaerobic pathway, notably by increasing LLDH activity (Tao et al., 2020), which could explain the observed results here. However, we did not find strong evidence of increased anaerobic metabolism, as we only observed a slight non-significant increase in LLDH expression associated with a medium effect size ($g>0.5$) in the same group.

After honeybee exposure to pesticide pyraclostrobin, fipronil and imidacloprid, ATP production was inhibited, and a decrease in the mitochondrial respiratory rate, measured as oxygen consumption, was observed (Nicodemo et al., 2020, 2014). However, in the present study, the observed inhibition of ATP synthase in the DnBP 600ng/g-treated group occurred without significant changes in respiratory rate (non-significant increase). This could indicate the activation of a compensatory mechanism to maintain ATP production. A similar effect was also observed in honeybee workers exposed to the clomazone herbicide, an identified uncoupling agent (Fidelis et al., 2024). Uncouplers are molecules that affect the coupling between the respiratory chain and ATP synthesis by compromising mitochondrial membrane integrity and dissipating the proton gradient (Song and Villeneuve, 2021). DnBP and MBP have also been identified as uncoupler agents in the rat, as they notably impair succinate oxidation

and increase membrane permeability to H+ ions (Inouye et al., 1978; Melnick and Schiller, 1985). These potential effects of DnBP have not yet been highlighted in invertebrates. Therefore, while more data are needed to assess the effect of DnBP on mitochondrial activity in insects, our results confirm that DnBP negatively affect the energy metabolism of bees, with potential impact on thermoregulation as previously shown by the interaction between cold stress and DnBP at 600ng/g in isolated workers (Dewaele et al., 2025).

Finally, the IBRv2 approach allowed us to highlight that exposure to DnBP globally altered gene expression in both endocrine and metabolic pathways. There was a stronger overall response at the lower dose (60 ng/g, IBRv2 = 10.1) than at the higher dose (600 ng/g, IBRv2 = 3.7), which suggests an inverse dose-response relationship, which is likely to occur after exposure to endocrine disruptors (Vandenberg et al., 2012). At 60 ng/g, most genes involved in the ecdysteroid pathway (E75, ECR, 78C) were consistently repressed along with LLDH, while the ATP synthase expression only slightly deviated and AADAT was upregulated. This might indicate a shift induced by a stress response to DnBP exposure, leading to the prioritisation of metabolic pathways over endocrine signalling of growth-related pathways such as ecdysteroid signalling. At 600 ng/g, these responses were less pronounced, with only slight disruptions observed in insulin- related genes, LIRP and InR, as well as slight dysregulations of the ATP synthase gene, suggesting a general metabolic disruption by DnBP.

Moreover, in both dose groups, a positive A deviation value was calculated for the insulin receptor gene (fig. 4a,b). Dysregulation of this gene is associated with modification of body lipid storage in insects (DiAngelo and Birnbaum, 2009). Interestingly, lipid content of workers repeatedly exposed to DnBP 600ng/g was observed alongside an increase in the weight of their offspring (Dewaele et al., In prep., Chapter 3, Part I). Moreover, crosstalk between the ecdysteroid signalling and the insulin signalling pathway occurs in insects, linking

nutrient sensing to growth and development (Koyama et al., 2014; Lin et al., 2018), and the positive deviation observed for the InR gene in the group treated with DnBP 60ng/g might also explain the negative deviation observed for ECR, E75 and 78C (Li et al., 2021). Therefore, further research should aim at identifying potential sub-lethal effects on individual and colonial traits linked with those intertwined molecular pathways in bees, such as reproduction and development for the ecdysteroid-related pathway, as well as feeding behaviour for the insulin signalling pathway.

4.2. DEHP exposure impacts the expression of genes related to metabolism and ecdysteroid signalling

While no impact of DEHP on the respiratory rate of *B. terrestris* workers was detected, downregulation of all the measured genes except ATP synthase and InR occurred in *B. terrestris* workers exposed to DEHP 12ng/g, as shown by the IBRv2 value, which was the highest of all treatment groups. This downregulation was significant for the expression of the L-LDH, E75, and 78C genes.

First, exposure to DnBP 12ng/g led to significant downregulation of the expression of the L-LDH gene in isolated workers. The expression of the L-LDH gene is a commonly used marker of cell injury and tissue damage (Lemos, 2021). In bees, upregulation of this gene is also part of the typical stress response of the organism (Rondeau et al., 2025). However, in this study, exposure to DnBP 12ng/g led to downregulation of the expression of the L-LDH gene in isolated workers. Similarly, a decrease in lactate dehydrogenase enzyme activity was found after chronic exposure to environmental doses of Bisphenol A (BPA) and other endocrine-disrupting compounds like 17β-estradiol in planarians (Li, 2020). 17β-estradiol, BPA, and DEHP are known ligands of the human estrogen-related receptor (ERR; Okada et al., 2008), which modulates anaerobic glycolysis in human tumour cells, notably by inhibiting L-LDH activity (Mirebeau-Prunier et al., 2013). While the natural ligand of the insect homologue

of this receptor is unknown (Bonneton and Laudet, 2012), expression of the ERR gene was upregulated in *C. riparius* exposed to low doses (50mg/L) of DEHP, BPA, and other endocrine-disrupting compounds (Park and Kwak, 2010). In this study, only a non-significant downregulation of the *ERR* gene was observed, but associated with a large effect size (g>0.8) was observed. A hypothesis could be associated with a negative feedback effect on the activity of this receptor. Indeed, overactivation of hormonal receptors can lead to a decrease in receptor activity either through internalisation or metabolic processes, but also by inducing a downregulation of the associated genes (Lagarde et al., 2015). However, no evidence supporting such a feedback mechanism after DEHP exposure has been described in other models. Nevertheless, in *B. terrestris* workers, DEHP or its metabolites could interact with ERR and inhibit the expression of the *LLDH* gene. Through inhibition of LLDH activity, DEHP could disrupt the anaerobic energy production activated in case of chemical and thermal stress in bees (Rondeau et al., 2025), and therefore weaken bee resistance to exposure to multiple-stressors.

Secondly, we observed significant downregulation of the E75 and 78C genes in the group treated with DEHP 12ng/g. These genes are involved in the ecdysteroid pathway, a crucial pathway for insect larval development as it notably regulates metamorphosis and molting rates, but also reproduction-related processes, such as vitellogenesis and oogenesis in reproductive females (Bonneton and Laudet, 2012). In invertebrates, DEHP exposure has been shown to alter ecdysteroid production and expression of genes involved in this crucial pathway. For example, in *Spodoptera littoralis*, development of males on food contaminated with DEHP at all tested doses (from 443 ng/g to 4.3 mg/g of food) resulted in a downregulation of the ECR, USP, E75, and E78 (78C homolog) genes in the adult antennal tissues (Avilès et al., 2020), which echoes with our results. However, here the expression of EcR and USP was not

significantly disrupted. Nevertheless, a non-significant downregulation of the EcR gene associated with a strong effect size exceeding 0.8 was observed in workers exposed to DEHP at 12ng/g. In fact, the IBRv2 approach highlighted a marked biological disturbance in this group (Fig. 4c). Indeed, the downregulation of all genes involved in endocrine signalling, notably related to growth and nutrition, i.e., ecdysteroid and insulin signalling pathways, observed here, could indicate a global stress response with the prioritisation of the ATP-related energy production over endocrine signalling involved in energetically costly processes like growth, reproduction and development. At the higher dose of 1200ng/g of DEHP, while no effect was observed on genes involved in the ecdysteroid-related pathway, a dysregulation of the insulin-signalling pathway and the LLDH-related metabolism was highlighted. These genes are part of a typical response to thermal and chemical stress in bees (Rondeau et al., 2025). This might therefore indicate that high field realistic doses of DEHP generated a stress response without dysregulating the ecdysteroid-related response.

Finally, inhibition of the nuclear receptors involved in the ecdysteroid-signalling pathway, such as that observed here, notably E75, could directly affect crucial caste specific and olfaction-related behaviour, such as foraging. Indeed, changes in the expression of genes involved in the ecdysteroid cascade in honeybee adult worker fat body can delay the age at which nurse transition to forager workers through cross-talk with the juvenile hormone signalling pathway (Ament et al., 2012). Moreover, the ecdysteroid-signalling pathway is also involved in the regulation of insect olfaction (Bigot et al., 2012). For example, in honeybees, ecdysteroid signalling is involved in the regulation of olfactory memory acquisition, especially in forager bees (Geddes et al., 2013). Bumblebees rely on odour cues for plant recognition as they are the more frequently encountered while foraging (Sprayberry, 2018), but also for nest recognition (Eckel et al., 2023; Rottler et al., 2013). In social species, olfaction is also crucial

for in-nest social relationships such as nestmate recognition (Breed et al., 2004) and larval feeding (Boer and Duchateau, 2006). In this study, we measured the expression of ecdysteroid-related genes in the fat body tissues of adult workers, while olfaction-related molecular pathways have been mainly studied in insect antennae and brain tissues (Avilès et al., 2020). In another study, antennal response to floral compound detection was altered in *B. terrestris* workers exposed to DEHP at 1200ng/g, but not at lower doses (Javal et al., In prep.). While analyses targeting antennal expression would allow a better understanding of the underlying processes, these results indicate that phthalates may disrupt plant pollinator interactions by acting on olfaction-related processes.

4.3. Conclusion

While exposure to environmental doses of DEHP has previously shown no lethal effects on isolated *B. terrestris* workers (Dewaele et al., 2025), the present study indicates that it could cause a wide range of sub-lethal effects from developmental disorders to colony disruption by dysregulating associated gene expression. Moreover, this study also confirms metabolic disruption occurring in workers exposed to DnBP at field-realistic doses. Therefore, future studies should focus on determining the impact of DEHP exposure, life traits associated with the disrupted genes, such as colony fitness, pollination efficiency, thermoregulation and reproduction

PART III: No evidence of the short-term effect of exposure to environmental doses of DEHP and DnBP on the immunity of isolated workers

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1. Introduction

Phthalates are semi-volatile compounds widely used as plasticisers, plastic softeners or as solvents in detergents, paints, cosmetics and pesticides (CEFIC, 2021; Koniecki et al., 2011). They leach easily into the environment as they do not form covalent bonds with the matrix (Suhrhoff and Scholz-Böttcher, 2016). Therefore, they are now widespread pollutants detected in highly anthropised environments such as farmland (Tran et al., 2015) and urban areas (Teil et al., 2006). The study of the impact of phthalates on wildlife has mainly focused on aquatic invertebrates, with less knowledge of their effects on terrestrial species (Cuvillier-Hot and Lenoir, 2020). Yet, in aquatic invertebrates, they are notably known to impact the immune system by negatively affecting haemocyte populations and functions (Burgos-Aceves et al., 2021). For example, in mussels *Unio* spp and *Mytilus galloprovencialis*, exposure to phthalates increased the total number of circulating haemocytes (Kuzukiran et al., 2022), and altered the composition of haemocyte populations as well as their function (Andreyeva et al., 2023). In the giant freshwater prawn, *Macrobrachium rosenbergii*, exposure to diethyl-hexyl phthalate (DEHP) and di-n-butyl phthalate (DnBP), two of the most common phthalates, negatively affected haemocyte functions by reducing pseudopodia formation and phenoloxidase activity (Sung et al., 2003). Aside from impacting cellular immunity, phthalates have also been shown to impact the expression of several immune-related genes in invertebrates. Exposure to DEHP notably downregulated the expression of antimicrobial

peptide genes, such as *lysozyme* genes in *Caenorhabditis elegans* (Yen et al., 2023), or *big defensin* in the clam *Venerupis philippinarum* (Lu et al., 2013). Though data on the effects of phthalates on terrestrial invertebrates is scarce, some studies have evidenced that these molecules can also trigger immune responses. For instance, in the ant *Lasius niger*, an upregulation of the gene expression of the antimicrobial peptide *defensin*, as well as genes involved in immune signalling, such as the receptor PGRP, has been highlighted after exposure to environmental concentrations of DEHP (Cuvillier-Hot et al., 2014).

Recently, we quantified phthalate contamination of wild *Bombus terrestris* workers sampled in the city of Lille, France: among the 15 PAEs investigated, DEHP and DnBP were the most frequently detected on bumblebee cuticles, contaminating 60% and 54% of the workers, respectively (Cuvillier et al., In prep). Quantities found on their cuticles were at median doses of around 145 ng/g for DEHP and 15 ng/g for DnBP (Cuvillier et al., In prep). Anthropogenic organic volatile compounds, including phthalates, are now the major pollutants in terms of quantities in cities (McDonald et al., 2018). Though no data on the direct effect of phthalates on bees are yet available, investigations on the health of managed and wild bee populations in urban areas have evidenced a weakened immune state in this pollinator group. Notably, in the Giant Asian Honeybee, *Apis dorsata*, arrhythmia, haemocyte depletion, increase in cellular stress and induction of immune gene expression were highlighted in individuals exposed to atmospheric pollution from cities, with negative effects on survival (Thimmegowda et al., 2020). Moreover, parasitism rates encountered in three bumblebee species commonly found in the urban environment, notably in France, i.e., *B. terrestris*, *B. pascuorum* and *B. lapidarius*, were shown to increase with the level of urbanisation (Theodorou et al., 2016). Moreover, an activation of the immune system of *B. lapidarius* was highlighted in the urban area that was not related to the urbanisation level, bee density or the prevalence of parasites (Cuvillier-Hot

et al., 2024). Exposure to chemical pollutants such as phthalates could therefore negatively affect bee populations by weakening their immune system, making them more vulnerable to parasites and other naturally occurring immune challenges (Ferrari et al., 2025).

As no data are available on the direct effect of phthalates on the bee immune system, in the final part of this second chapter, we explored the impact of environmental doses of DnBP and DEHP on the haemocyte population and antimicrobial peptide expression of *B. terrestris* workers in the first 96 hours after exposure. Then, we investigated possible correlations between the expression of AMP genes and of genes involved in the insulin-signalling pathway measured in Chapter 2, Part II. Indeed, in insects, this pathway is tightly linked to the regulation of antimicrobial peptide production (Zhang et al., 2018). In a second experiment, we investigated changes in haemocyte populations potentially occurring on a shorter time scale, i.e., 8 and 24 hours after phthalate exposure.

2. Experiment 1 – Phthalate effect on immunity 96 hours after exposure

2.1. Material and methods

Sample origin. The hemolymph and fat body samples used in this experiment were collected at the end of the first experiment of the acute exposure mortality assay from *Bombus terrestris* workers exposed during the first session of the experiment presented in Dewaele et al. (2025), i.e. four days after exposure to either solvent control or one of the six phthalate treatments (DnBP 0.1X, 1X, 10X or DEHP 0.1X, 1X, 10X).

Haemocyte count. Seven to ten workers per treatment group were isolated and anaesthetised by chilling (max. 10min at -20°C) in glass jars. 2x2.5 µL haemolymph were collected from each individual by inserting a pulled calibrated 3µL capillary connected to a suction hose under the second tergite (Przybyla et al., 2025). The capillary was rinsed using distilled water between each individual.

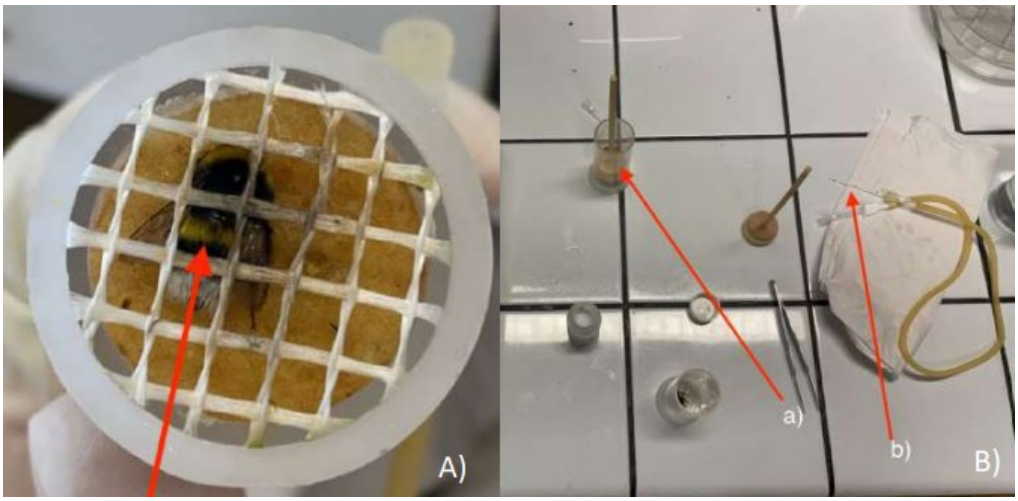


Fig. 1. Haemolymph sampling. A) Second tergite of a worker in a marking cage and B) sampling material with the marking cage (a) and the capillary linked to a suction hose. (Picture: Lilou Théret-Désiré)

Cell counting was performed using a CASY cell counter & analyser (OLS Omni Life, science) on fresh haemolymph. To do so, the 2x2.5 µL samples of haemolymph were placed in a CASYcup filled with 5mL of CASYton directly after collection. This preparation was gently mixed by repeated inversion and placed under the 150µm sampling capillary of the CASY cell counter. For each sample, particle counting was performed on three volumes of 400µL of the diluted sample, so that the particle number per microliter was calculated in a total volume of 1200µL. Each particle measuring between 3 to 50 µm was counted.

Based on cell morphology, five living haemocyte types have been described in *Bombus terrestris*: prohemocytes, plasmatocytes, granulocytes, oenocytoids, and spherulocytes (Çakıcı et al., 2023). Prohemocytes are the smallest haemocytes (approximately 4–6 µm). They possess a nucleus that occupies nearly the entire cell, leaving only a thin layer of cytoplasm (Çakıcı et al., 2023). These stem-like cells have the potential to differentiate into other haemocyte types (Lavine and Strand, 2002). Plasmatocytes (8-12 µm) contain a centrally located nucleus and a relatively larger cytoplasmic volume (Çakıcı et al., 2023). They occur in various forms (oval, spindle-shaped, or round) and are primarily involved in phagocytic activity (Lavine and Strand, 2002). Granulocytes (9-15 µm) are generally round cells with a small

nucleus and numerous large granules distributed throughout the cytoplasm (Çakıcı et al., 2023). They serve as major effectors in phagocytosis (Lavine and Strand, 2002). Oenocytoids (10-20 µm) are mostly oval-shaped and characterised by non-central nuclei. These cells are mainly responsible for phenoloxidase production in the melanisation process (Lavine and Strand, 2002). Spherulocytes contain distinct, large, round, membrane-bound inclusions of varying composition (Çakıcı et al., 2023). However, their specific function remains unclear (Lavine and Strand, 2002).

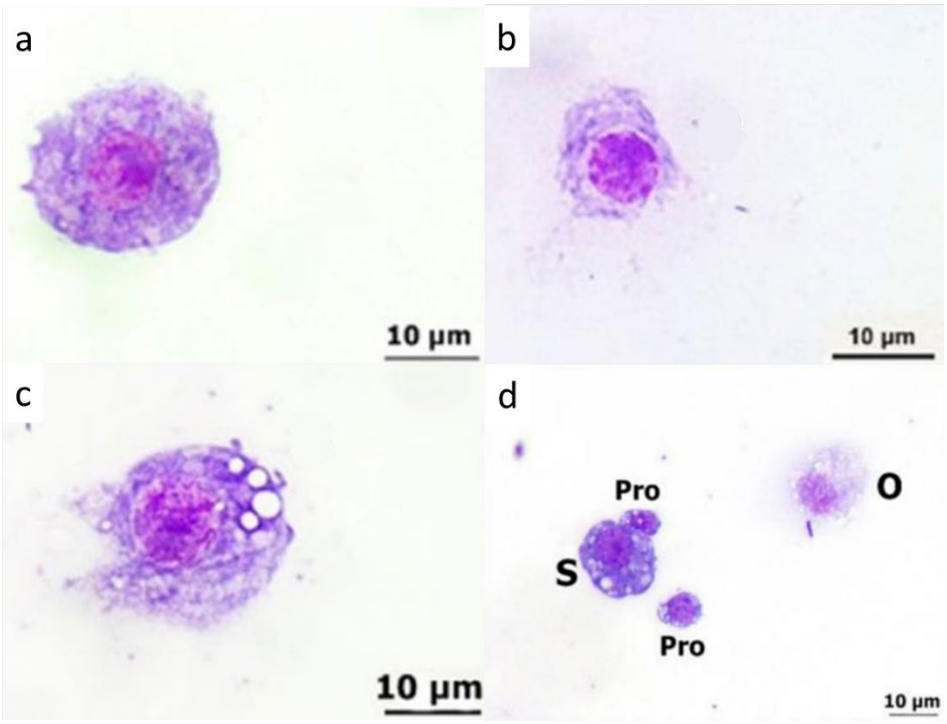


Fig. 2. Haemocyte cell types in *Bombus terrestris* haemolymph coloured with Wright's stain. a) Granulocyte, b) Plasmatocyte, c) Oenocytoid, d) Prohemocyte (Pro) and Spherulocyte (S) with an Oenocytoid (O) (adapted from Çakıcı et al., 2023)

In the present study, haemocytes were detected at sizes smaller than those previously reported using light microscopy (Çakıcı et al., 2023). The classification of bee haemocyte populations is complex and highly influenced by the measurement and observation technique used. For example, Negri et al. (2016) observed three haemocyte type groups with smaller sizes by using *in vivo* differential interference contrast on *Apis mellifera* haemocytes. By visual confirmation of the cells detected by the cell counter, we also observed three size-based

haemocyte populations: cells measuring 4.5–5.5 µm were classified as Population 1, those measuring 5.5–6.5 µm as Population 2, and those measuring 6.5–14 µm as Population 3. Using the CASY blue solution on a healthy worker haemolymph following fabricant instructions, we determined that dead cells and debris found in the haemolymph measured less than 4.5µm. We also considered the total number of haemocytes (THC) by summing the number found in each population.

AMP gene expression. Antimicrobial peptide gene expression was measured on the fat body of the exposed workers using RT-qPCR. The fat body tissues of workers were sampled and extracted for RNA as explained in Chapter 2, Part II). Real-time PCR were performed on a Lightcycler 480 from Roche Applied Science. Amplifications were performed in a final volume of 10 µL containing 2 µL of cDNA (diluted 1:3), 1 µM of each specific primer, and 5 µL of BioRad SYBR Green master mix. Analyses were run in technical duplicates for each sample and each gene. Amplification conditions were 95 °C for 30 s, followed by 40 cycles of 95 °C for 15 s and T_m for 30s with a single read taken at the end of each cycle. A melting curve analysis was carried out at the end of the PCR by heating the plate to 95 °C at a ramp rate 0.11°C/s and continuous fluorescence reading.

Table 1. Primer characteristics and efficiency. Size, sequences and Tm (°C) with associated NCBI Gene ID.

Gene name (abbreviate)	Type	NCBI ID	Primer sequences	efficiency	T_m
Ribosomal protein L13 (rpl13)	reference	XM_012316284.1	Fw TTGGAATTGCTGTAGATCCTAGAAG Rv CATTCAGTGGGAAGAGAATCAA	1.943	60
Phospholipase A2 (pla2)	reference	FN_391388	Fw TATCTTTCAATGCCCAGGAG Rv GTCGTAACAAATGTCATGCG	1.932	60
Hymenoptecin	target	KX146600.1	Fw TCCTCGTTTTCGACGACAC Rv TAATCGACGTCCAAGGATGG	1.90	60
Abaecin	target	KC662132.1	Fw GCGCATCTTTTCTTTTCGTC Rv CACGAGTTCTCTATTAAAGGCAAA	1.90	60
Apidaecin	target	XM_012389042.1	Fw TCTACCACCACAATCCCAAATACA Rv AATTGGTGGGAGACTTATAGGTCG	1.94	60
Defensin1	target	KC662144.1	Fw CTTCTCTCCATCAAAGGAGTCG Rv TCACGTACTTGCGACAAAGG	1.91	60

The relative expression values of target genes, i.e., Defensin1, Hymenoptaecin, Abaecin, Apidaecin, were calculated using rpl13 and pla2 as reference genes and the E-method from Roche Applied Science LightCycler 480 software (v 1.5.1; Table 1 for primer sequences, specific T_m and efficiency)

Statistical analyses. Data were analysed using R v 4.4.1 (R Core Team, 2023). graphs were made with 'ggplot2' package v3.5.1 (Wickham, 2016). The number of particles counted in each category was analysed using a generalised mixed model (GLMM) and family negative binomial with package glmmTMB v. 1.1.10 (Brooks et al., 2025). Model fits were checked using DHARMa package v0.4.7 (Hartig, 2020). As the number of dead and live cells differed between exposure batches in the solvent group, we added a nested random effect of commercial colony nested within session. Customs contrasts using the package 'emmeans' v1.10.5 (Lenth, 2023) with Benjamini-Hochberg's p-value adjustment method (i.e., 'fdr') were used *post-hoc* to compare the treatment and solvent control. The relative gene expression was compared between the treatment and solvent groups using ANOVA on Aligned Rank Transformed data (ART ANOVA) with package 'ARTool' v0.11.1 (Kay and Wobbrock, 2019) using the treatment as fixed effect and the initial colony as random effect. The difference in Least-Squares means of each treatment group between each treatment and the corresponding solvent control group was reported. Effect sizes were also calculated using Cohen's coefficient with Hedge's correction for small sample size (i.e., "*g*"), and their magnitude was interpreted as in Chapter 2, Part II. Correlations among expression values for AMP genes, but also of insulin-signalling genes analysed in Chapter 2, Part II, were examined using Spearman's correlation with the Hmisc package (Harrell and Dupont, 2025).

2.2. Results

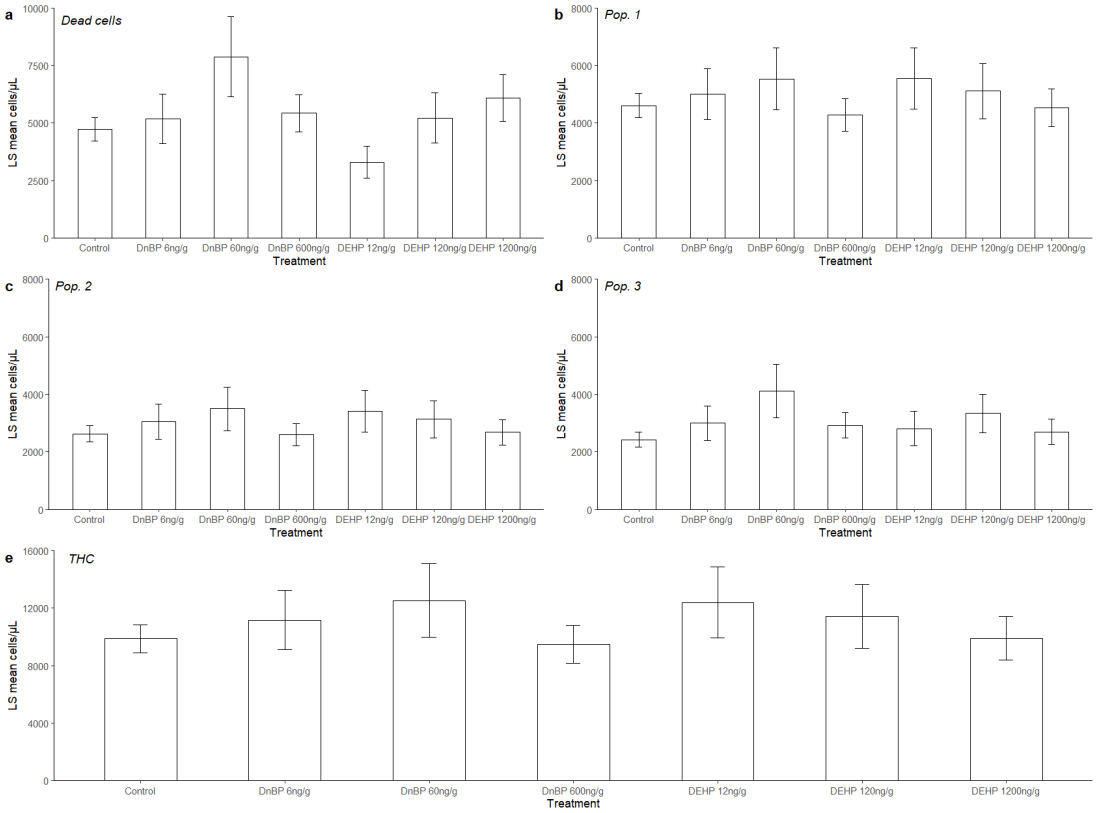


Fig. 2. Dead and live haemocyte numbers measured per µL of haemolymph of isolated workers 96 hours after exposure to DEHP, DnBP or solvent control. a) Dead cell numbers, b) numbers of haemocytes in Population 1, c) in Population 2, d) in Population 3 and e) Total live haemocyte (THC) numbers. Columns show Least-square mean numbers calculated for each treatment group with error bars showing the associated 95% confidence interval.

Haemocyte count. DnBP and DEHP exposure had a marginal effect on dead cell number (GLMM, family negative binomial: chisq. = 12.41, d.f. = 6, p-value = 0.0535). The highest number of dead cells was found in the haemolymph of workers exposed to DnBP 60ng/g, while the lowest was found in the group exposed to DEHP 12ng/g (Fig. 2a). The number of dead cells found in the group exposed to DnBP 60ng/g only tended to be higher than the solvent control (p-value $_{\text{vs solvent}}$: uncorrected = 0.0281, fdr corrected = 0.1687; Fig. 2a). The number of dead cells found in the group exposed to DEHP 12ng/g tended to be lower than the solvent control, but this effect was even more marginal (p-value $_{\text{vs solvent}}$: uncorrected = 0.1010, fdr corrected = 0.3030; Fig. 2a).

Table 2. Statistical comparison between dead and live cell numbers found in the haemolymph of workers 96 hours after exposure to DnBP, DEHP or control solvent (GLMM, family negative binomial). *Post-hoc* comparisons were made against solvent control, and uncorrected and "fdr" corrected p-values are reported. Symbols: *p<0.05.

Cell population	Chisq.	d.f.	p-value	Treatment	$p\text{-value}_{vs.\ solvent}$
Dead cells	12.41	6	0.0535	DnBP 6ng/g	0.6950
				DnBP 60ng/g	0.1687
				DnBP 600ng/g	0.6360
				DEHP 12ng/g	0.3030
				DEHP 120g/g	0.6950
				DEHP 1200g/g	0.3522
Population 1	2.31	6	0.8889	DnBP 6ng/g	0.8076
				DnBP 60ng/g	0.8076
				DnBP 600ng/g	0.8076
				DEHP 12ng/g	0.8076
				DEHP 120g/g	0.8076
				DEHP 1200g/g	0.9110
Population 2	3.05	6	0.8025	DnBP 6ng/g	0.7325
				DnBP 60ng/g	0.7302
				DnBP 600ng/g	0.9465
				DEHP 12ng/g	0.7302
				DEHP 120g/g	0.7325
				DEHP 1200g/g	0.9465
Population 3	6.77	6	0.3431	DnBP 6ng/g	0.5016
				DnBP 60ng/g	0.1622
				DnBP 600ng/g	0.5016
				DEHP 12ng/g	0.5604
				DEHP 120g/g	0.4394
				DEHP 1200g/g	0.5604
Total	2.80	6	0.8341	DnBP 6ng/g	0.8178
				DnBP 60ng/g	0.8178
				DnBP 600ng/g	0.9511
				DEHP 12ng/g	0.8178
				DEHP 120g/g	0.8178
				DEHP 1200g/g	0.9851

Concerning the other haemocyte populations, no significant effect, even marginal, was detected in any of the treatments (See statistical tests details in Table 2; Fig. 2b, c, d). The total haemocyte number did not differ between treatments either (GLMM, family negative binomial: chisq. = 2.80, d.f. = 6, p-value = 0.8341; Fig. 2e).

AMP gene expression. No significant effect of phthalate exposure was detected on the expression of abaecin and apidaecin (ART ANOVA: $p\text{-values}_{vs.\ solvent} > 0.05$), and the effect sizes calculated for each group were very low, ranging from 0.03 and 0.35 for both genes (Fig. 3b,c).

A marginal effect, associated with high effect sizes, was detected on hymenoptecin (ART ANOVA for DnBP 60ng/g vs. solvent: F = 4.3, df. = 1, p = 0.0669; g = -1.07), and defensin1 (ART ANOVA for DnBP 60ng/g vs. solvent: F = 4.7, df. = 1, p = 0.0545; g = -1.1), as DnBP 60ng/g tended to decrease the expression of both genes compared to solvent control (Fig. 3a,d).

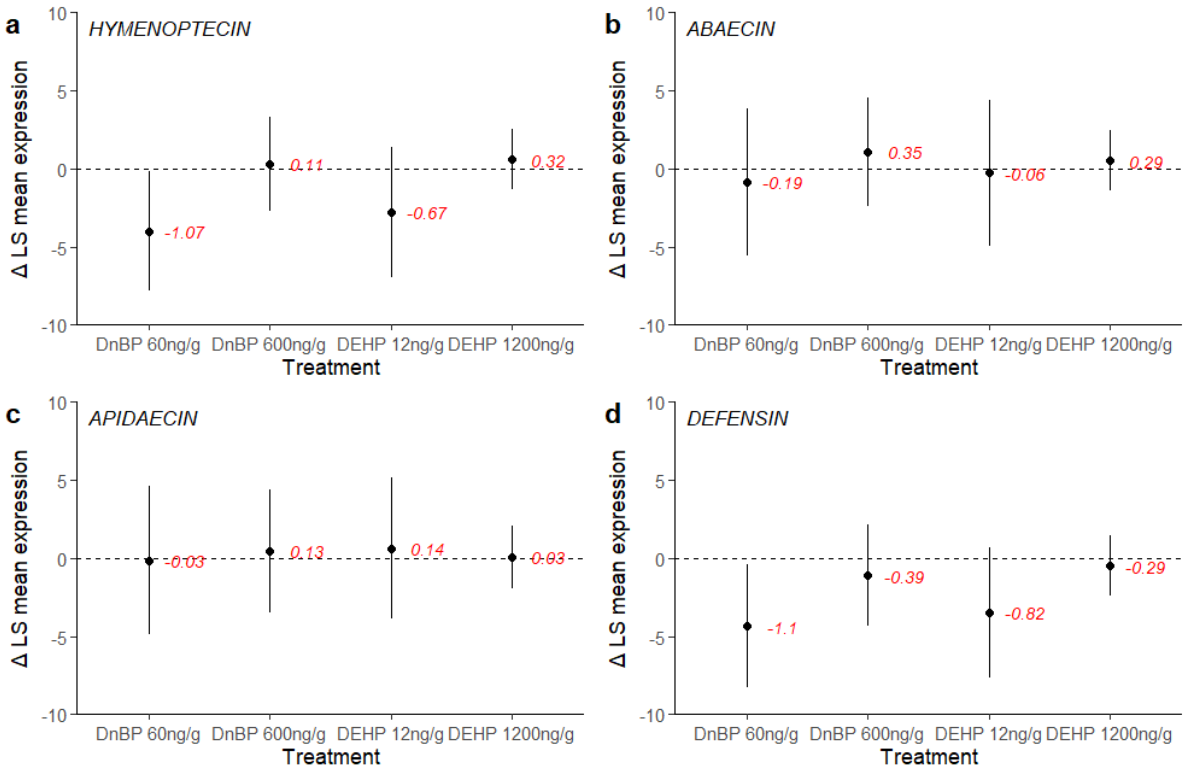


Fig. 3. Model-adjusted mean relative gene expression difference between the treatment and control groups with Hedge *g* coefficient indicating effect size in red related to change in expression of antimicrobial peptide genes, i.e., a) Hymenoptecin, b) Abaecin, c) Apidaecin, d) Defensin1. Black dots indicate the difference in Least Square Means of relative gene expression (Δ LS mean expression) between treatment and control groups, with bars showing the 95% confidence interval.

Significant positive correlations were highlighted between gene expression values of the different AMP (p-value<0.001), except for Apidaecin, for which only the correlation with Abaecin was positive and significant (p-value<0.001; Fig.4). We also highlighted a significant negative correlation between the expression values of the defensin gene and the expression of the Insulin-like receptor, InR (Fig. 4; data from Chapter 2, Part II).

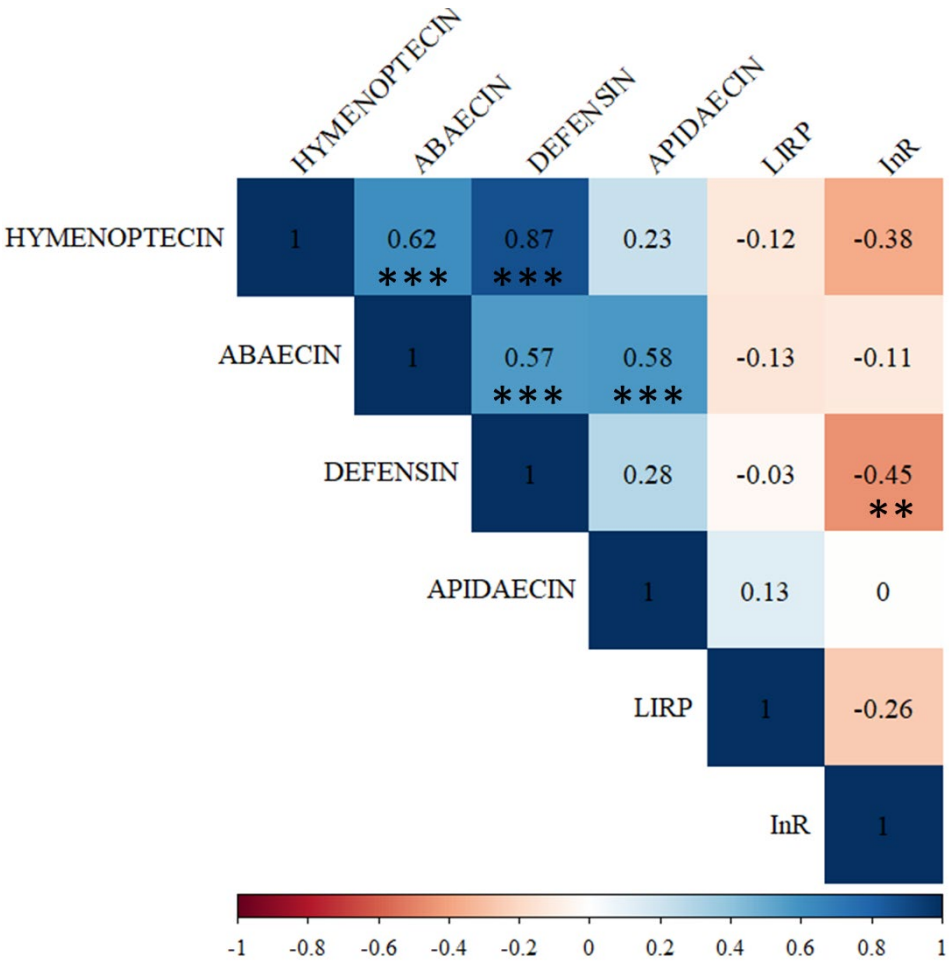


Fig. 4. Spearman correlation index among AMP genes and gene included in the insulin-signaling pathway. The colour scale indicated the value and sign of the correlation index. Significant correlations are shown by *p<0.05, **p<0.01, ***p<0.001.

3. Experiment 2 – Phthalate effect on haemocyte populations 8 and 24 hours after exposure

3.1. Material and method

Phthalate treatment. Six treatment groups were installed during this experiment. Workers were either exposed to a solvent control (Methanol 0.07% diluted in mineral water), or to a phthalate treatment. Two DnBP doses (60 ng/g and 600 ng/g) were tested, as lethal effects had been previously detected in Dewaele et al. (2025). Two DEHP doses (12ng/g and 120ng/g) were also tested. DEHP had no lethal effect on workers. Therefore, we chose to use the dose for which the IBRv2 value was the highest in Chapter 2, Part II, and the environmental concentration. All the treatment solutions were prepared as in Dewaele et al. (2025).

Phthalate exposure. Two commercial colonies (about 80-120 workers) were purchased from Biobest NV (Westerlo, Belgium). Upon receipt, colonies were kept in darkness, and all handling was performed under red light to minimise worker stress. 12 workers per treatment group were randomly sampled from each of both colonies (2x12, i.e., 24 workers per treatment group). The exposure took place following the same protocol as in Dewaele et al. (2025). Briefly, workers were isolated in decontaminated, handmade metal cages similar in dimensions to Nicot cages®. Caged workers remained isolated during the whole duration of the experiment (24h). They were acclimated to the conditions for at least eight hours before exposure. They were then anaesthetised by placing groups of two caged workers in glass jars in an ice bucket for 10 minutes. Two microlitres of treatment solution were then deposited through the cage mesh on the dorsal hairless part of the thorax using a calcined 10 µL glass capillary (Drummond). Once recovered from anaesthesia at room temperature, caged workers were placed back on a metal tray. They were provided *ad libitum* 50% w/w sucrose-water solution via a decontaminated Pasteur pipette (removed during the anaesthesia). No mortality occurred during the 24hours of experiment.

Haemocyte count. Haemolymph was sampled from 12 exposed workers equally distributed between commercial colonies eight hours after the exposure, then another 12 workers were sampled 24 hours after the exposure. Haemolymph was sampled and analysed using CASY cell counter & analyzer as explained for the first experiment, with the exception that the pulled capillary was rinsed between each individual with distilled water and Phenylthiourea (CAS: 103-85-5) at 1 mg/mL to prevent cell clumping.

Statistical analysis. Data were analysed using R v 4.4.1 (R Core Team, 2023); graphs were made with 'ggplot2' package v3.5.1 (Wickham, 2016). A random effect on the commercial colony was applied, and the counts were analysed separately for both time points. Customs contrasts

using the package 'emmeans' v1.10.5 (Lenth, 2023) with Benjamini-Hochberg's p-value adjustment method (i.e., 'fdr') were used *post-hoc* to compare the treatment and solvent control.

3.2. Results

No effect of phthalate exposure was detected on any of the dead and live cell groups at both timings (See statistical tests details in Table 3; Fig. 4).

Table 3. Statistical comparison between dead and live cell numbers found in the haemolymph of workers 8 and 24 hours after exposure to DnBP, DEHP or control solvent (GLMM, family negative binomial).

Cell population	Measure timing	Chisq.	d.f.	p-value
Dead cells	+8H	2.98	4	0.2250
	+24H	1.35	4	0.8530
Population 1	+8H	1.44	4	0.8379
	+24H	1.82	4	0.7689
Population 2	+8H	3.94	4	0.4137
	+24H	2.29	4	0.6820
Population 3	+8H	7.75	4	0.1013
	+24H	0.95	4	0.9174
Total	+8H	4.96	4	0.2920
	+24H	1.66	4	0.7989

4. Discussion

In this part of the chapter, we investigated the effects of phthalates on isolated *B. terrestris* workers through analysis of their haemocyte population and antimicrobial genes expression. In contrast to previous results on aquatic invertebrates, DEHP and DnBP at environmental doses did not significantly affect any of the immune metrics measured.

In *Apis mellifera*, adult bees across all castes and sexes have haemocyte number and nodulation abilities that reduce with age, and are more likely to rely on molecular effectors such as antimicrobial peptides and the pro-phenoloxidase/phenoloxidase system to fight immune challenges (Negri et al., 2016). This has been interpreted as a trade-off between the energy allocated to colony performance compared to individual immunity (Schmid et al.,

2008). Therefore, haemocyte number in response to an immune challenge might show less variation in eusocial species, such as *B. terrestris*, compared to other models.

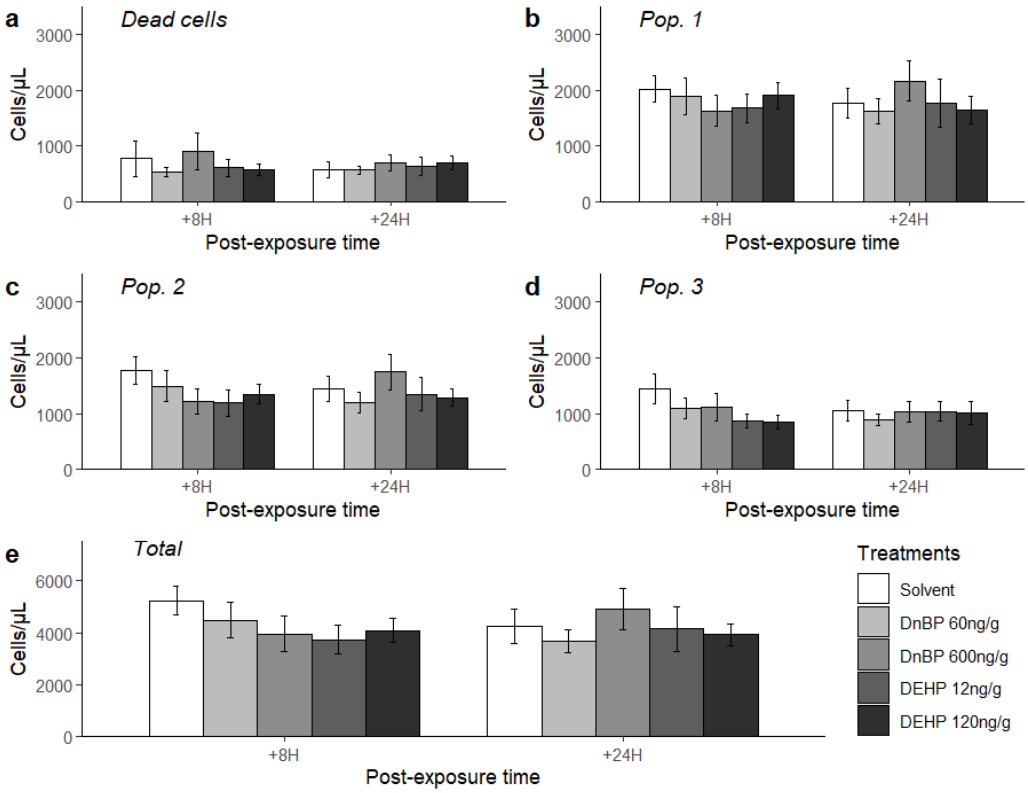


Fig. 5. Dead and live haemocyte numbers measured in 1µL of haemolymph of isolated workers 8 and 24 hours after exposure to DEHP, DnBP or solvent. a) Dead cell numbers, b) numbers of haemocytes in Population 1, c) in Population 2, d) in Population 3 and e) Total live haemocyte numbers. Columns show mean numbers calculated for each treatment group with error bars showing the associated standard error. Grey shades indicate the treatment group.

In Mussels *Unio* spp and *Mytilus galloprovencialis*, the impact of phthalate exposure affected haemocyte number and population composition (Andreyeva et al., 2023; Kuzukiran et al., 2022). However, in this study, no significant effect was observed at any time point measured. We nevertheless detected a non-significant increase in dead cell number found in the haemolymph of workers 96 hours after exposure to DnBP 60ng/g. This trend might be linked to the increase in mortality found previously with this treatment (Dewaele et al., 2025), but this effect did not appear strong or homogeneous enough across individuals to become significant at the times we studied.

Another trend at the same time-point in the group exposed to DnBP 60ng/g was also observed with a non-significant downregulation of the expression of *defensin* and *hymenoptecin* genes. In insects, the expression of AMP genes is induced by the transcription factor relish after activation of the Imd/Toll signalling pathways following bacterial molecular pattern detection (Brutscher et al., 2015). Therefore, expression of AMP genes has been used as an immune biomarker in bees (e.g. Pervez et al., 2025). In *L. niger* exposed to environmental doses of phthalates DEHP and BBP, the upregulation of *defensin* gene expression indicated an activation of the immune system 24 hours after exposure (Cuvillier-Hot et al., 2014), a result that contrasts with the trend observed in this study. However, in aquatic invertebrate models, DEHP exposure often induced a downregulation of antimicrobial gene expression (Lu et al., 2013; Yen et al., 2023), which echoes the trend observed here. No data on the effect of DnBP on AMP gene expression are available, but the downregulation of the AMP gene *lysozyme-like-7* in *C. elegans* has been linked with DEHP-induced senescence through the effect of the molecule on the Insulin/Insulin-like growth factor signalling (IIS) pathway (How et al., 2019; Yen et al., 2023). The trend observed here could arise from a similar mechanism, as the IIS pathway is closely linked to the regulation of AMP gene expression in insects like the silkworm *Bombyx mori* (Zhang et al., 2018), and in male drosophila developmentally fed with DnBP showed disrupted expression of crucial components of this pathway in the first week after eclosion (Williams et al., 2016). Moreover, in the second part of this chapter, we analysed the same individuals for the relative expression of genes involved in the insulin-signalling pathway, and we observed a non-significant upregulation of the insulin receptor-related gene InR in workers exposed to DnBP 60ng/g. This might have led to the trends observed here, as demonstrated by the inverse correlation highlighted between AMP expression defensin and insulin-like receptor (Fig. 4).

While no significant effect of phthalate exposure on haemocyte population and AMP gene expression was detected at the dose tested in these two experiments, these data, coupled with the previous results obtained in this chapter, might point towards a negative impact of phthalate exposure on hormonal signalling and metabolism, rather than a direct effect on immunity. Therefore, this stresses the need to assess the effect of phthalate exposure on biological traits and functions directly associated with these pathways, such as larval development and lipid metabolism, notably associated with developmental caste-determination processes.

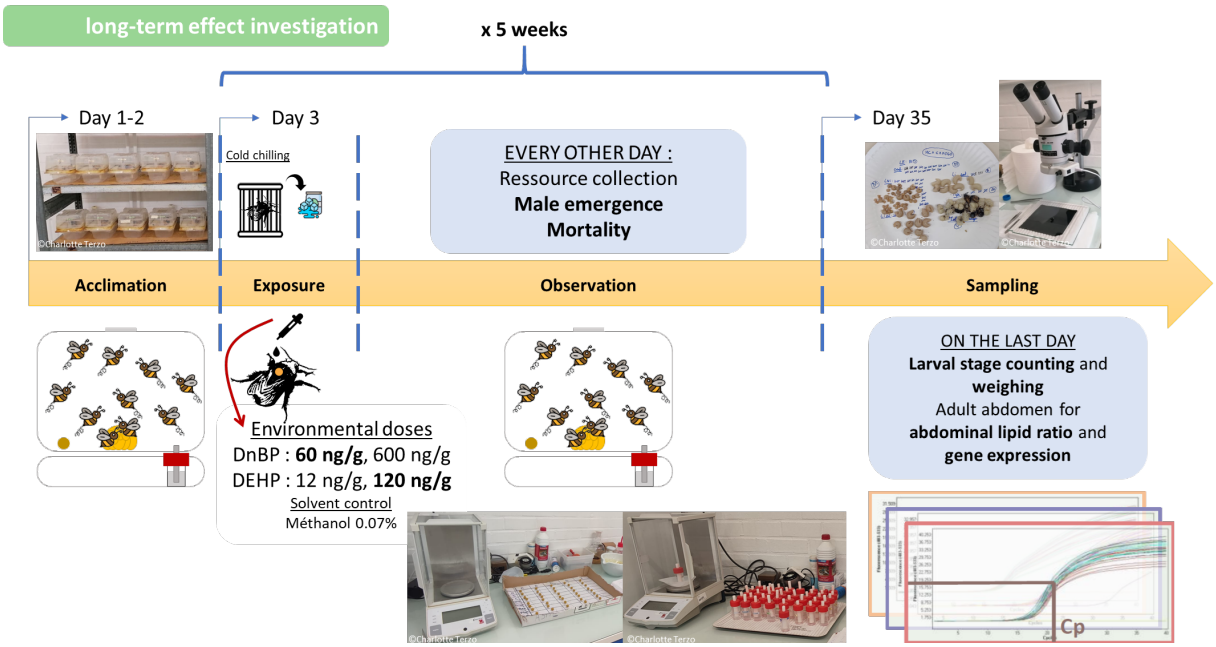
CHAPTER III: Impact of DnBP and DEHP long-term exposure on social and individual development of *B. terrestris*

In the previous chapter, we examined the short-term effects of phthalate exposure from the molecular to the organismal level. In natural conditions, however, adults are likely to experience repeated exposure, while larvae may be subjected to chronic exposure throughout development. Existing research on phthalate impacts on invertebrate development and behaviour largely concerns solitary species without parental care, such as *Daphnia magna*, *Spodoptera littoralis*, and *Drosophila melanogaster*.

This chapter investigates long-term effects of phthalates in bees, with a focus on hormonal disruption and colony-level outcomes, and is organised into three parts:

1. The first part of this chapter presents an assessment of the impacts of worker exposure to environmentally relevant concentrations of DnBP and DEHP on queenless microcolony development, including offspring production, adult survival, and fat storage.
2. The second part describes an exploratory experiment in which *in vitro*-reared larvae were exposed to DnBP and DEHP to elucidate the potential intergenerational effects observed in the first part.
3. The third part analyses the expression of genes involved in key metabolic and hormonal pathways of workers after repeated exposure to phthalates.

Together, these experiments provide a broader perspective on the potential long-term impacts of phthalates under social conditions.



└Microcolony design and timeline of the experiment investigating long-term effects of phthalate exposure on *B. terrestris* workers.

Publications:

Dewaele, J., Terzo, C., Duterne, S., Verdy, L., Hautekèete, N., Cuvillier, V. and Michez, M. Repeated exposure to field-realistic concentrations of di-2-ethylhexyl phthalate and di-n-butyl phthalate in bumblebees impacts adult survival and lipid reserves with intergenerational obesogenic effects. (Submitted)

Dewaele, J., Holl, A.-C., Hautekèete, N., Michez, D., Cuvillier, V. Expression of genes involved in crucial hormonal signalling pathways in *Bombus terrestris* workers is disrupted after repeated exposure to di-2-ethylhexyl phthalate and di-n-butyl phthalate. (In prep.)

Communications:

"**Effets des phtalates DnBP et DEHP sur la mortalité, le développement et la santé du pollinisateur urbain, *Bombus terrestris*.”**, Oral communication, 3rd to 6th October 2023, Bordeaux, France.

“Effects of DnBP and DEHP on the survival and immunity of *Bombus terrestris* workers”, Oral communication, Benelux Congress of Zoology, 12th to 13th September 2024, Mons, Belgium.

“Effects of Di-Butyl Phthalate and Di-Ethyl-Hexyl Phthalate at Environmental Doses on Health and Development of B. terrestris Microcolonies”, Oral communication, SETAC Europe 34th Annual Meeting, 5th to 9th May 2024, Sevilla, Spain.

*Part. I: Repeated exposure to field-realistic concentrations of di-2-ethylhexyl phthalate and di-n-butyl phthalate in bumblebees impacts adult survival and lipid reserves with intergenerational obesogenic effects**

*Submitted manuscript

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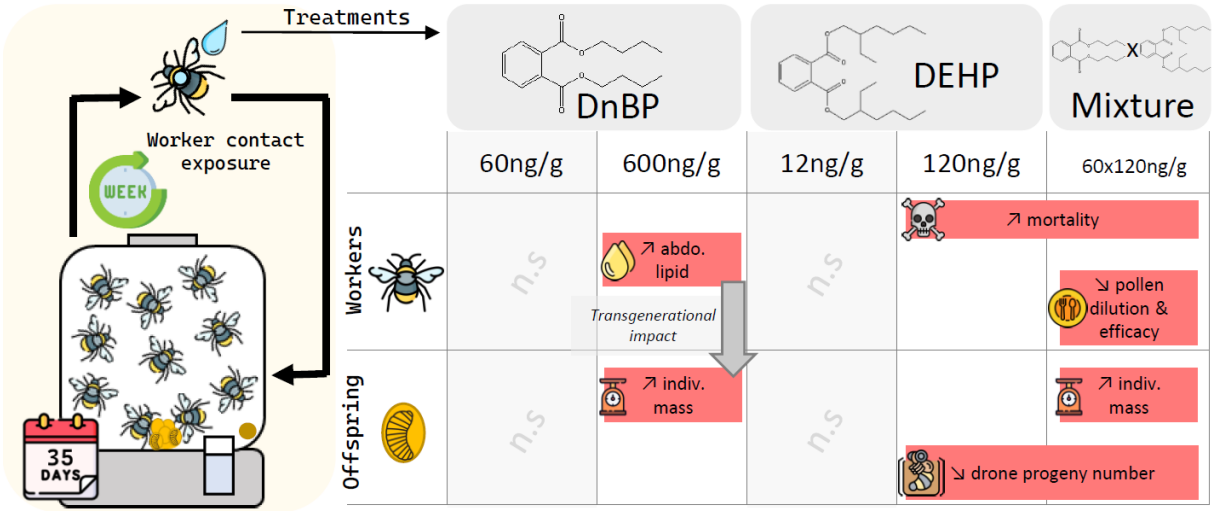
Data statement: Data are available at https://doi.org/10.5281/zenodo.18486280

Abstract

Wild bees, like many other insects, are declining. Multiple drivers have been identified, but the potential effects of endocrine disruptors, like phthalates, have been little studied. Yet, di-2-ethylhexyl phthalate (DEHP) and di-n-butyl phthalate (DnBP), two of the most common phthalates that pervade the environment, are both known to affect crucial hormonal and metabolic pathways in invertebrates even at environmental doses. These ubiquitous pollutants are semi-volatile molecules used to increase the flexibility of plastic matrices, or as solvents for certain detergents, pesticides, etc. Bees are notably exposed through their cuticle to these lipophilic molecules, in varying quantities and chemical profiles. To investigate the potential impacts of phthalate exposure, *Bombus terrestris* workers were repeatedly exposed (1x/week) to field-realistic concentrations of DEHP and DnBP and maintained in microcolonies during 35 days. We recorded worker mortality and male emergence throughout the experiment. Offspring production was measured in number and mass, and abdominal lipid ratio was assessed in adults. The results showed that repeated exposure to DEHP increased worker mortality up to 50%, while DnBP increased worker lipid stores and offspring mass, suggesting potential metabolic disruptions. These results suggest that the two most common phthalates in the environment can have lethal and sublethal effects on bees, probably by disrupting crucial physiological pathways with cascading effects on individual health and colony development at environmental concentrations.

Keywords: bees; plasticizers; endocrine disruptors; lipid metabolism; mortality

Graphical abstract:



Highlights:

-Exposure to DEHP increased bumblebee worker mortality in the long run

-Exposure to DnBP increased worker abdominal lipid ratio and offspring mass

-Exposure to DEHP and DnBP in mixture decreased pollen-syrup collection ratio

-Exposure to DEHP and DnBP in mixture decreased pollen-offspring mass ratio

-Microcolonies exposed to DEHP and DnBP in mixture produced less but heavier drones

1. Introduction

The current global decline in insect populations has been well-documented, notably in North America and Europe (van Klink et al., 2020; Wagner et al., 2021). The identified causes are anthropogenic, e.g. land use intensification, climate change and chemical pollution (Wagner et al., 2021). Bee species are no exception, and chemical pollution is frequently reported as one of the multiple anthropogenic pressures weakening their populations (Michez et al., 2026). Given their essential role in pollinating wild and cultivated plants (Klein et al., 2007; Ollerton et al., 2011), bees have become model organisms for assessing environmental risk and measuring impact of pollutants on pollinators (EFSA et al., 2023). Current methods for

evaluating these effects are mainly related to pesticide risk assessments (EFSA et al., 2023). However, the impacts of other pollutants remain poorly understood.

Phthalates are used as plasticisers and solvents, commonly found in agricultural and urban environments (Billings et al., 2021; McDonald et al., 2018), but also far from the original area of use including regions remote from anthropogenic activity (Lenoir et al., 2016). They have been detected in varying concentrations in soil, water and atmosphere (Kashyap and Agarwal, 2018; Net et al., 2015c; Tran et al., 2015). In these matrices, while free phthalate molecules are quickly degraded, they can persist for years when complexed to organic matter (Kickham et al., 2012). Moreover, these lipophilic pollutants can be adsorbed onto insect cuticles (Lenoir et al., 2012), absorbed by the insect body (Lenoir et al., 2014), transported into the nest by foragers (Gómez-Ramos et al., 2019; Martín-Gómez et al., 2024), or introduced via contact between contaminated soil or air and lipid-rich nest structures (e.g. wax). The chemical composition of their cuticle and of their nest structures make bees particularly susceptible to daily exposure to these contaminants (Gómez-Ramos et al., 2016). Di-2-ethylhexyl phthalate (DEHP) and di-n-butyl phthalate (DnBP) are among the most widely used and frequently detected phthalates in the environment (Kashyap and Agarwal, 2018; Net et al., 2015c). DEHP was notably found on honeybee worker cuticles, pollen loads and wax combs (Gómez-Ramos et al., 2019, 2016; Lenoir et al., 2012; Martín-Gómez et al., 2024), along with DnBP on the cuticle of wild *Bombus terrestris* foragers sampled in a French metropolis (unpublished results). DnBP and DEHP are identified as endocrine disruptor compounds (EDCs) in vertebrates and aquatic invertebrates (Gore et al., 2015; Regulation (EU) 2018/2005), but for terrestrial insects, only limited data are available. Investigation of the long-term impact of phthalate exposure on insects has mainly been performed on the non-social species *Drosophila melanogaster* and *Spodoptera littoralis* (Avilès et al., 2019; Williams et al., 2016).

In these models, DnBP altered the lipid metabolism notably via the disruption of the insulin-signalling pathway (Williams et al., 2016), and DEHP impacted olfaction-related behaviour and developmental duration through alteration of the ecdysteroid-related cascade (Avilès et al., 2019). In solitary insects, both pathways are involved in reproduction, development, and metabolism (Antonova et al., 2012; Bonneton and Laudet, 2012). The regulatory scheme is more complex for eusocial bee species, especially because the insulin-signalling pathway is involved in key processes of developmental caste determination and caste-specific behaviour regulation (Ament et al., 2012; De Azevedo and Hartfelder, 2008; Jedlička et al., 2016). Differential activation of the ecdysteroid-related pathway during larval development is also involved in caste determination (Pinto et al., 2002). Moreover, 20-hydroxyecdysone notably influences the olfactory memory of pollen foragers in the honeybee *Apis mellifera* (Geddes et al., 2013), as well as nest-mate recognition and aggressive behaviours (Pandey and Bloch, 2015). Finally, these pathways, by directly influencing juvenile hormone production (i.e. metabolic rate accelerator in *B. terrestris*, Shpigler et al., 2021), could be indirectly involved in thermoregulation processes, a crucial ability of *Bombus* colonies (Livesey et al., 2019).

We previously reported lethal effects of DnBP, which caused 25% mortality in isolated *B. terrestris* workers, four days after a single exposure (Dewaele et al., 2025). This previous approach followed OECD guidelines for testing chemical impacts on *B. terrestris* (short-term tests, i.e. 72h, on isolated workers; OECD, 2017), but is limited by its lack of ecological realism when applied to social insects. Indeed, these methods do not allow the evaluation of pollutant impact after repeated or chronic exposure to pollutants in a context of normal social interactions (i.e. multiple workers interacting in the same nest). Moreover, assessments performed on isolated workers limit the evaluation of sub-lethal effects to non-reproductive individuals and ignore potential effects on alloparental care and offspring production that may

alter colony development and fitness (Klinger et al., 2019). Hence, phthalates could alter a wide range of traits, through the disruption of key hormonal pathways as observed in solitary species, but with cascading impact on social organisation, alongside development, reproduction and foraging behavior (Moreno-Gómez-Toledano and Jabal-Uriel, 2024).

The present study aims to investigate the lethal and sublethal impact of long-term exposure to field-realistic concentrations of phthalates on a social species, *B. terrestris*. Here, we monitored the development of microcolonies composed of ten exposed workers and measured (i) worker mortality and (ii) adult abdominal lipid content, (iii) worker resource collection (i.e. syrup and pollen), and (iv) offspring production (i.e. number and mass)

2. Material and methods

2.1. Model species

We selected the buff-tailed bumblebee, *B. terrestris*, for our study. This species is counted among the most abundant bumblebee species in Europe (Rasmont et al., 2021) and is a well-represented species in urban (Persson et al., 2020) and agricultural environments (Kleijn et al., 2015). This species forms colonies with a queen, which inhibits the reproductive abilities of workers (Ghisbain et al., 2025). When the influence of the queen is weakened, workers develop ovaries and can lay unfertilised eggs that will develop into drones (Amsalem et al., 2015). In this context, queenless microcolonies of ten workers were used to assess sub-lethal effects on the health of workers and their offspring under controlled conditions (Klinger et al., 2019). For this study, five queenright colonies of *B. terrestris* (*ca.* 80-120 workers) were purchased from Biobest NV (Westerlo, Belgium). Upon receipt, they were placed in a dark room with controlled temperature and humidity (25°C, 60% relative humidity).

2.2. Phthalate treatments

We evaluated the sub-lethal effects of phthalate exposure considering DEHP at 120ng/g of body weight (b.w.) and DnBP at 60ng/g b.w. as field-realistic concentrations, as in Dewaele et al. (2025). We also tested a mixture treatment for these two doses, as an antagonistic effect was highlighted between both molecules on isolated worker mortality (Dewaele et al., 2025). In addition, as potential metabolic disruption was suspected in isolated workers after exposure to DnBP at 600 ng/g (Dewaele et al., 2025), we also investigated exposure to this dose in the present experiment. Finally, as DEHP at doses ranging from 12ng/g to 12µg/g did not affect worker mortality in Dewaele et al. (2025), we selected for the present experiment the lowest dose previously used, i.e. 12 ng/g, in order to deepen current knowledge about DEHP low-dose effects.

DnBP (CAS No. 84-74-2) and DEHP (CAS No. 117-81-7) were purchased from Sigma-Aldrich as liquid solutions (both >99%). All dilutions were prepared assuming an average *B. terrestris* worker fresh weight of 0.25g (Dewaele et al., 2024). The initial dilution used methanol (CAS No. 67-56-1, VWR) to ensure good solubility of PAEs, while subsequent dilutions used mineral water to ensure the lowest possible methanol concentration in the solutions (0.07%). For the mixture preparation, initial and subsequent dilutions followed the same method. However, at the final dilution step, both single-molecule solutions were mixed, and the volume was adjusted with mineral water to obtain a mixture containing DEHP at 120ng/g and DnBP at 60ng/g. Additionally, two control solutions were used: a first control containing 0.07% methanol diluted in mineral water, and a second control containing only mineral water to account for any solvent effect.

2.3. Preventing uncontrolled PAE contamination

Phthalates can be found in laboratory environments from plastic material use (Feng et al., 2020) and in both tap and bottled water (Bach et al., 2020; Luo et al., 2018). To minimise unintended phthalate contamination of treatment and feeding solutions, we used water marketed in glass bottles (SPA Reine, SPA NV, Bruxelles, Belgium). Moreover, glass and metal materials were decontaminated with hexane and acetone and, when possible, by calcination. When plastic materials could not be avoided, we preferably used hard plastic materials (Cao et al., 2022).

2.4. Experimental design

2.4.1. Microcolony preparation. Five queenless microcolonies were established for each of the seven treatments (i.e., thirty-five microcolonies prepared in total) in boxes (10×16×16 cm). Each microcolony contained 10 workers randomly sampled from a single queenright colony. Each treatment group contained one microcolony from each commercial colony origin. The microcolonies were maintained under constant darkness and controlled temperature and humidity conditions (25°C; 60% relative humidity) for 35 days. Each microcolony had *ad libitum* access to a syrup solution of 50% w/w sucrose in mineral water (SPA Reine, SPA NV, Bruxelles, Belgium), and to pollen, provided as pollen candi, i.e., a ball of pollen mixed with syrup at 0.15 mL/g of pollen. Organic frozen honeybee-collected pollen (Rosaceae: *Malus*/*Pyrus* sp. 40%; Salicaceae: *Salix* 27%) was used (Abeille Heureuse, Strasbourg, France; Barraud et al., 2022). Every other day, syrup and pollen were renewed and weighed, and dead workers were recorded and weighed. An empty microcolony box containing only syrup and pollen, but no workers, was also installed to account for evaporation. At the end of the experiment, all remaining alive workers were weighed. Syrup and pollen collection were standardised by the mass of alive workers in the microcolony at the time of recording, and

cumulated. Pollen dilution (i.e., ratio between cumulated pollen and syrup collection) and pollen efficacy (i.e., ratio between total offspring mass and cumulated pollen collection) were also calculated as a stress response metric for each microcolony (Barraud et al., 2020; Moerman et al., 2016).

2.4.2. Exposure protocol. Once a week, all workers were exposed to their assigned treatment using an exposure protocol based on OECD guideline No. 246 (OECD, 2017b) and similar to Dewaele et al. (2025). For each microcolony, all workers were simultaneously placed inside glass jars in groups of two. The glass jars were plunged into ice for anaesthesia by chilling (maximum ten minutes). Then, each worker was exposed by depositing a 2 µL droplet of either one of the control or one of the treatment solutions on the hairless dorsal part of the thorax. Once recovered at room temperature, all workers from a microcolony were simultaneously placed back in their box. The first exposure occurred three days after microcolony installation, and was then repeated every seven days for a total of 35 days of follow-up (5 exposure sessions).

2.5. Offspring monitoring

Thirty-two days after first exposure, microcolonies were dissected to count the number of eggs and larvae at each life stage (i.e., non-isolated, pre-defecation, post-defecation, pupae and non-emerged drones; see Figure 1). All hatched larvae were weighed individually, except for non-isolated larvae, which were weighed together for each microcolony (their mass was considered total and per larvae in the analyses). Every day, male emergence in the microcolonies was recorded. Emerged males were weighed and stored at -20°C.

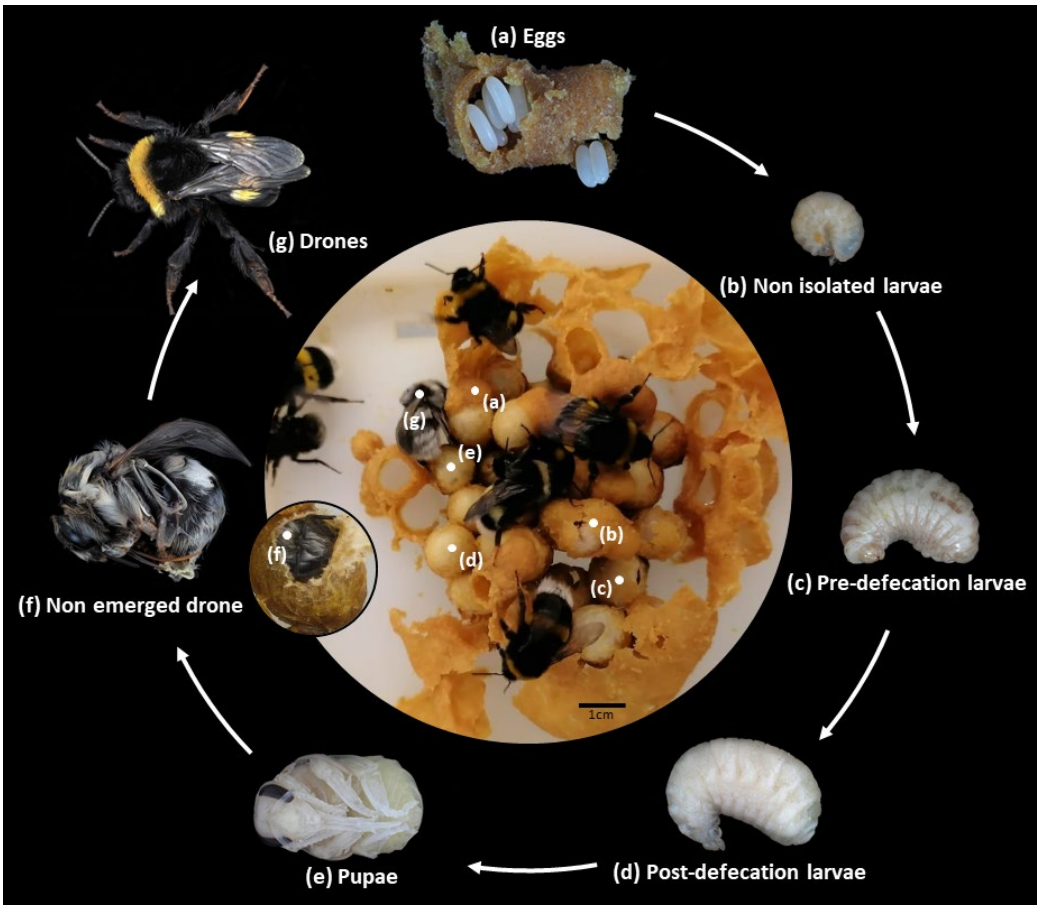


Fig. 1. Drone developmental stages monitored in the microcolonies, with associated locations in the brood. a) Brood cells containing several eggs are located on top of the brood. b) Non-isolated larvae develop together in brood cells. c) Pre-defecation larvae are isolated in individual brood cells. Brood chambers containing non-isolated and pre-defecation larvae bear small feeding holes. d) Post-defecation larvae, e) pupae and f) non-emerged drones develop in closed, isolated brood cells, located under the brood. g) Newly emerged drones are primarily white, and their colours develop in the first 24 hours. (Picture credits: individual larvae and drones by Paolo Rosa; brood and emerging male by Charlotte Terzo).

2.6. Worker and drone abdominal lipid ratio

The fat storage of adults was estimated by calculating the abdominal lipid ratio following Ellers (1995). To do so, the fresh abdomen of each individual was excised and weighed. Then, it was dried for three days at 70°C and weighed again. Abdomens were then placed in 2mL of diethyl ether for 24 hours for lipid extraction. Diethyl ether was discarded, and abdomens were dried again at 70°C for seven days. Abdominal lipid ratio was calculated as the difference between the dry mass of the abdomen before and after lipid extraction,

divided by the fresh abdominal mass. Two workers per microcolony and all the emerged drones were analysed.

2.7. Statistical analyses

All analyses were performed in R v 4.3.0 (R Core Team, 2023), and all graphs were made using "ggplot2" package v 3.5.1 (Wickham, 2016).

The survival analysis was performed using a mixed Cox regression with solvent control as the reference group and the initial queen-right colony as random factor using the package "coxme" v2.2-22 (Therneau, 2022). The hazard ratios (HR) compared to the solvent control are reported.

Using the package "glmmTMB" v1.1.11 (Brooks et al., 2025), generalised linear mixed models (GLMM) were used to analyse the treatment effects on the metrics. The initial colony was considered as a random effect and the treatment as a fixed effect. The distribution families used were chosen depending on the metric considered, and visual inspection of model fits using the "DHARMa" package v0.4.7 (Hartig, 2020) was performed to validate the models used. The beta family with "logit" link function was used for abdominal lipid ratio analysis and pollen dilution, as these metrics were ratios between 0 and 1. The Gaussian family with "identity" link function was used to analyse pollen and syrup collection, pollen efficacy as well as larval and drone mass when normality was respected. Otherwise, when data were right-skewed and as masses are strictly positive, the gamma family with "inverse" link function was used instead. Finally, the Poisson family with "log" link function was used to analyse larval and drone numbers. In case of overdispersion, the negative binomial family with "log" link function was used (Brooks et al., 2025).

As solvent and mineral water control did not differ in any of the performed analyses, we considered only the solvent group as the control group (Table S3.1). Custom contrasts using

package emmeans v1.10.5 (Lenth, 2023) with solvent as control group and Benjamini-Hochberg's p-value adjustment method (i.e., "fdr") were used on the models as *post-hoc* analyses.

3. Results

3.1. Impact on worker mortality

Exposure to phthalates significantly affected worker survival (Mixed Cox regression: Chisq. = 18.30, d.f. = 6, p-value = 0.006). Survival of workers exposed to single-molecule DEHP at 120ng/g and to the mixture (DEHP 120 ng/g + DnBP 60 ng/g) was half that of the control group at the end of the experiment (for DEHP at 120ng/g, HR = 2.25, 95% CI = [1.15, 4.41], z = 2.37, p-value = 0.018; for the mixture, HR = 1.99, 95% CI = [1.00, 3.94], z = 1.98, p-value = 0.048; Fig. 2). Survival in both these groups began to decline after the mid-point of the experiment (Fig. 2).

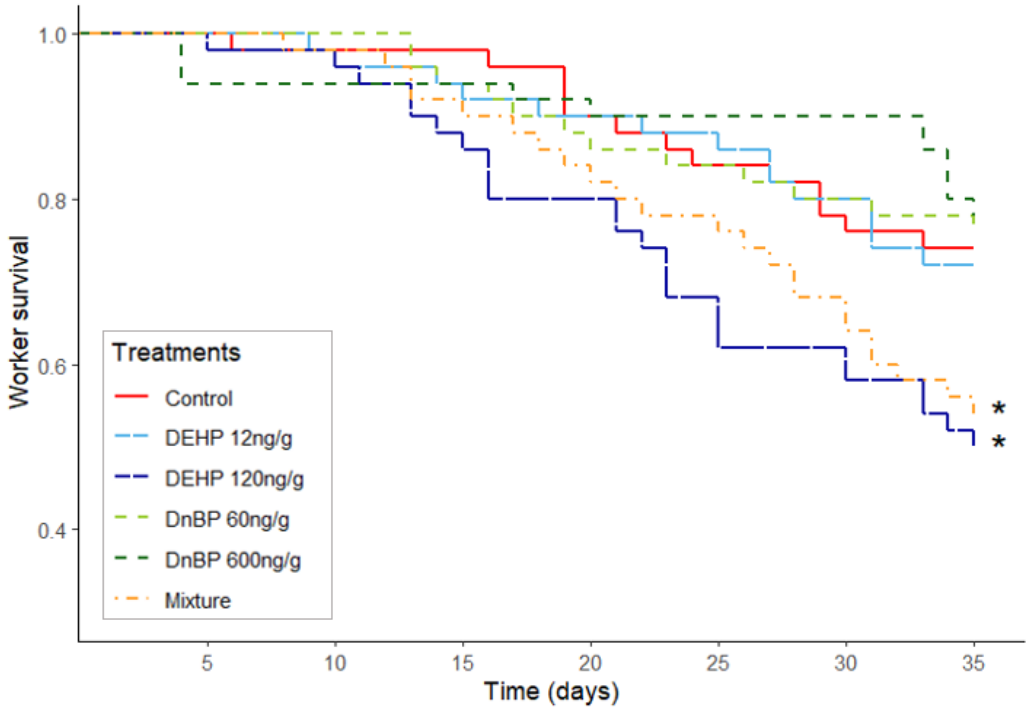


Fig. 2. Worker survival in each of the five treatment groups and control (N = 50 workers per treatment). The solid red line shows the control group. *indicates a significant difference at p<0.05 compared to the control group.

3.2. Impact on worker resource collection

Cumulative syrup collection slightly differed between treatment groups (GLMM, family gaussian: Chisq. = 11.61, d.f. = 5, p-value = 0.0405; Table 1), with the highest mean quantity found in the microcolonies exposed to DEHP at 120ng/g b.w. (105.3±7.8g) and the lowest in those exposed to DEHP at 12ng/g b.w. (84.8±6.6g; Fig. 3a). However, these values did not differ significantly from the control group (95.8±2.2g; p-values>0.05). Cumulative pollen collection was not affected by phthalate exposure (GLMM, family gaussian: Chisq. = 8.29, d.f. = 5, p-value = 0.1407; Table 1), but a non-significant decrease was observed in the microcolonies exposed to the mixture (p-value>0.05; Fig. 3b).

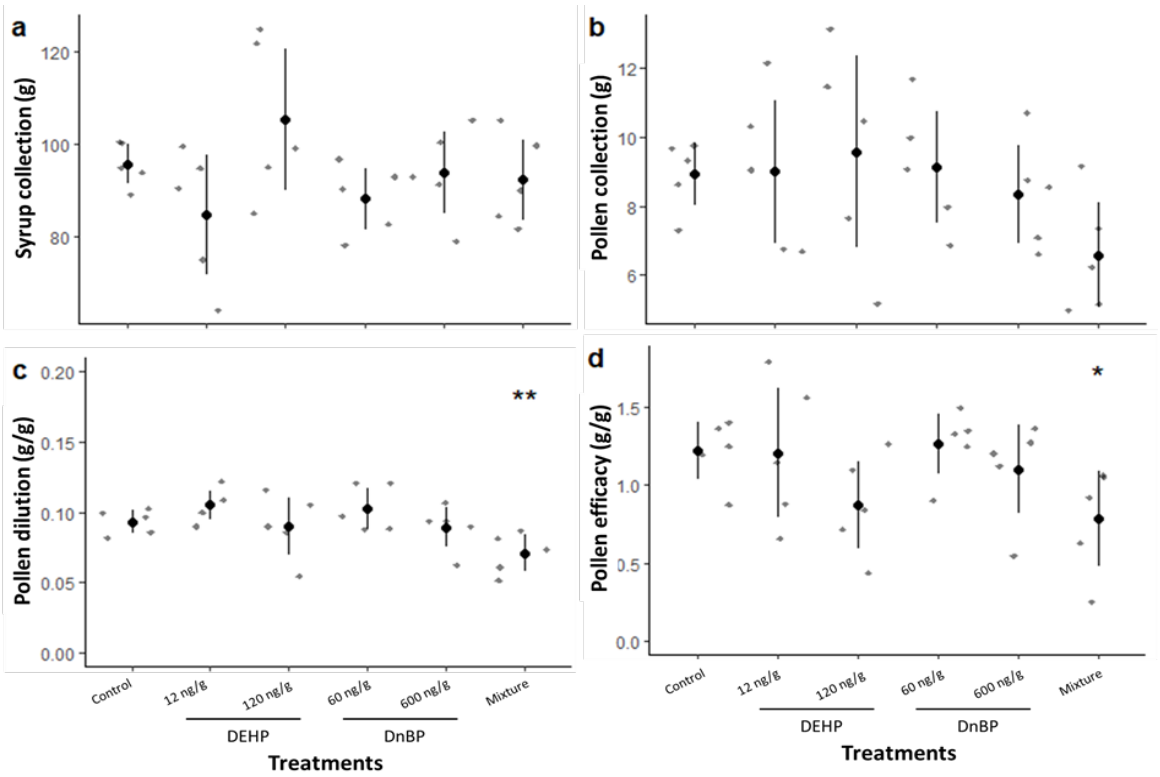


Fig. 3. Resource collection in microcolonies exposed to DnBP and DEHP treatments as single molecules and as a mixture compared to the control group. a) Cumulative standardised syrup collection (g), b) cumulative standardised pollen collection (g), c) pollen dilution (g/g; i.e., ratio between collected pollen and syrup) and d) pollen efficacy (g/g; i.e., ratio between total offspring mass and cumulated pollen collection). Grey dots represent individual microcolonies (N=5 per treatment). Black points show mean values for each treatment group, with error bars indicating the 95% confidence interval. *indicates a significant difference compared to the control group at p<0.05, and **p<0.01.

The exposure to the phthalate mixture significantly decreased pollen dilution (p-value $_{vs.control}$ = 0.0072; Table 1) and pollen efficacy (p-value$_{vs.control}$ = 0.0240; Table 1), compared to the control (Fig. 3c,d).

3.3. Impact on offspring number and mass

Phthalate exposure did not significantly affect the number of eggs, non-isolated larvae, post-defecation larvae, or pupae in the microcolonies at the end of the 35 days (see Table S3.1 and S2 for values and statistical details). However, pre-defecation larval production was negatively affected by phthalate exposure (GLMM, family Poisson: Chisq. = 19.14, d.f. = 5, p-value = 0.0018; Table 1), with a significant decrease observed in microcolonies exposed to the mixture compared to the control (p-value=0.0002; Table S3.2; Fig. 3a). Additionally, non-emerged drone production differed between treatments (GLMM, family Poisson: Chisq. = 14.2, d.f. = 5, p-value = 0.0142), with the lowest mean number of individuals found in the microcolonies exposed to DnBP at 60ng/g and the highest in those exposed to DnBP at 600ng/g (Fig. 4a; Table S3.2). However, these values did not differ from the control group (p-values>0.05; Fig. 4a; Table S3.2).

The first drones emerged on day 25 from microcolonies exposed to DnBP at 60 and 600 ng/g, while in control microcolonies, the first drones emerged on the following day (Fig. 4b). Their final numbers differed between treatments (GLMM, family Poisson: Chisq. = 26.1, d.f. = 5, p-value<0.001; Table 1), and significantly fewer drones emerged from microcolonies exposed to DnBP at 600ng/g and to the mixture compared to the control group (p-value $_{vs.\ DnBP\ 600ng/g}$ =0.0411 and p-value$_{vs.\ mixture}$ =0.0001; Fig. 4b).

Overall, the total number of hatched individuals (i.e., all post-egg stages) produced by each microcolony did not differ between treatments (GLMM, family Poisson: Chisq. = 8.4, d.f. = 5, p-value = 0.1338; Table 1).

Regarding mass, non-isolated larvae total mass did not significantly differ between treatments (GLMM, family gaussian: Chisq. = 10.8, d.f. = 5, p-value =0.0547; Fig. S3.1; Table 1). However, an effect of phthalate exposure was highlighted for the mass per non-isolated larvae (GLMM, family gaussian: Chisq. = 15.6, d.f. = 5, p-value =0.0080; Table 1), with the highest mean mass found in microcolonies exposed to DnBP at 60ng/g b.w. (0.042±0.005g) and the lowest in those exposed to the mixture (0.013±0.006g), but no significant difference with the control group was observed (0.032±0.009g; p-values>0.05; Fig. S3.1b).

Regarding the isolated larval stages, we found no effect of phthalate exposure on pre-defecation larval mass (GLMM, family gaussian: Chisq. = 8.2, d.f. = 5, p-value =0.1474; Fig. S3.1c; Table 1) or non-emerged drone mass (GLMM, family gaussian: Chisq. = 5.4, d.f. = 5, p-value =0.3711; Fig. S3.1e; Table 1).

However, post-defecation larval mass differed between treatments (GLMM, family gaussian: Chisq. = 11.3, d.f. = 5, p-value =0.04595; Table 1), with an increase for DnBP treatment at 600ng/g compared to control (p-value =0.0464; Fig. 4c). Pupal mass also differed between treatments (GLMM, family gaussian: Chisq. = 17.8, d.f. = 5, p-value =0.0032; Table 1), with the highest mean observed for pupae produced by microcolonies exposed to DnBP at 600ng/g b.w. (0.339±0.010) and the lowest in those exposed to DEHP at 120ng/g b.w. (0.295±0.010). However, these values did not differ significantly from the control (0.308±0.010; p-value $_{\text{vs. DnBP 600ng/g}}$ =0.1362 and p-value $_{\text{vs. DEHP 120ng/g}}$ =0.2489).

Concerning the emerged drones, we did not observe any significant effects of the treatment on their mass (GLMM, family gaussian: Chisq. = 6.7, d.f. = 5, p-value =0.2464; Table 1). However, the mass of drones that emerged during the last two days of the experiment (i.e., from eggs laid by workers exposed more than once) differed significantly between treatments (GLMM, family gamma: Chisq. = 20.1, d.f. = 5, p-value =0.0012; Table 1). A significant increase

in lastly-emerged drone mass was observed in microcolonies exposed to DnBP at 600ng/g b.w. and to the mixture compared to the control group (p-values=0.0150; Fig. 4d).

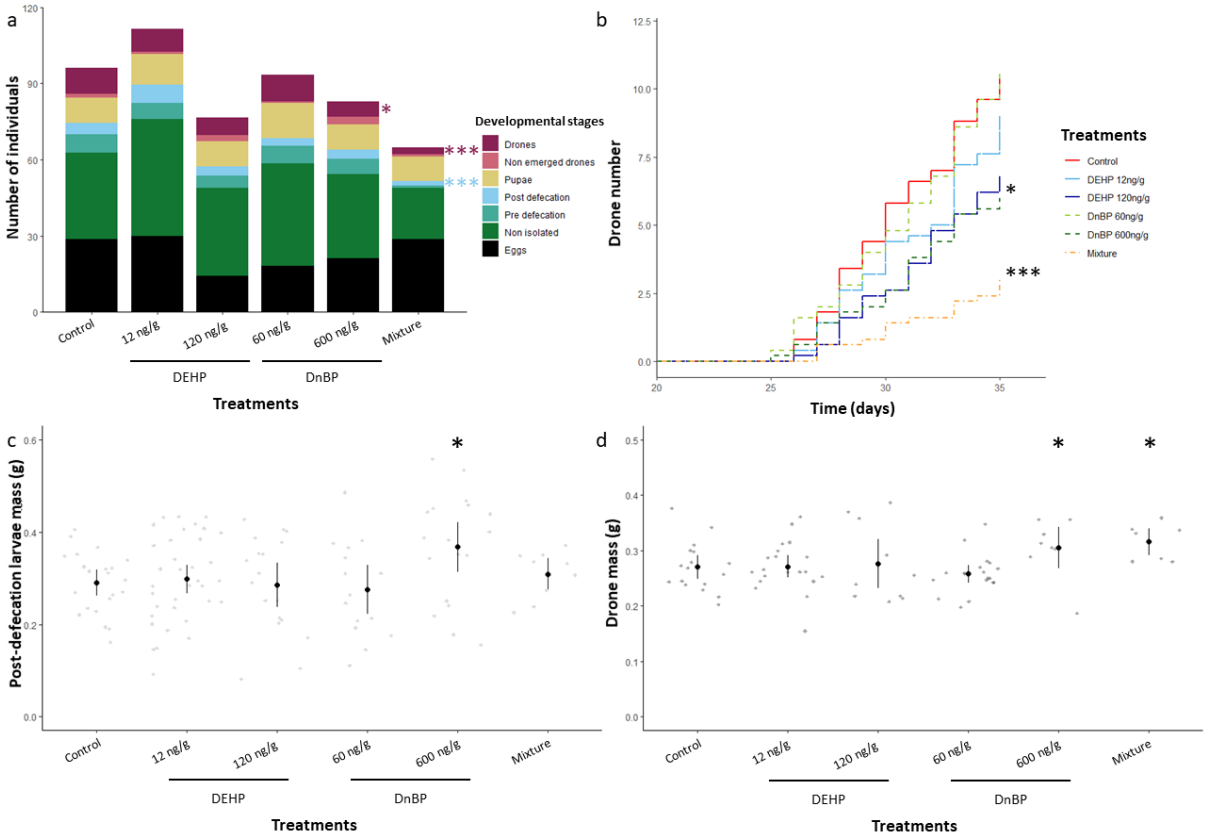


Fig. 4. Offspring production in microcolonies. a) Number of individuals found in each developmental stage for the six treatments. Each block shows the mean number of individuals found in the stage indicated by the colour (N=5 microcolonies per treatment). b) Dynamics of drone emergence in each of the five phthalate exposure groups and control over the 35-day experiment. The lines show the mean number of emerged drones at each time point (N = 5 microcolonies per treatment), with the solid red line showing the control group. c) Individual mass of post-defecation larvae from the microcolonies exposed to DEHP at 12ng/g (N=35) or 120ng/g (N=18), DnBP at 60ng/g (N=15) or 600ng/g (N=18), or the mixture (N=9) compared to the control group (N=22). d) Individual mass of drones emerged during the last two days, from the microcolonies exposed to DEHP at 12ng/g (N=20) or 120ng/g (N=10), DnBP at 60ng/g (N=19) or 600ng/g (N=8), or the mixture (N=7) compared to the control group (N=17). Black points show mean values with error bars indicating 95% confidence intervals. Coloured dots show values of individual mass in graphs “c”, and “d”. * and *** indicate a significant difference compared to the control at $p<0.05$ and $p<0.001$, respectively.

Overall, despite a slight decrease observed in the microcolonies exposed to the mixture, total hatched offspring mass only tends to differ between treatments (GLMM, family

gaussian: Chisq. = 10.2, d.f. = 5, p-value =0.0696; Table 1), with the lowest mass found in microcolonies exposed to the mixture (Fig. S3.1f).

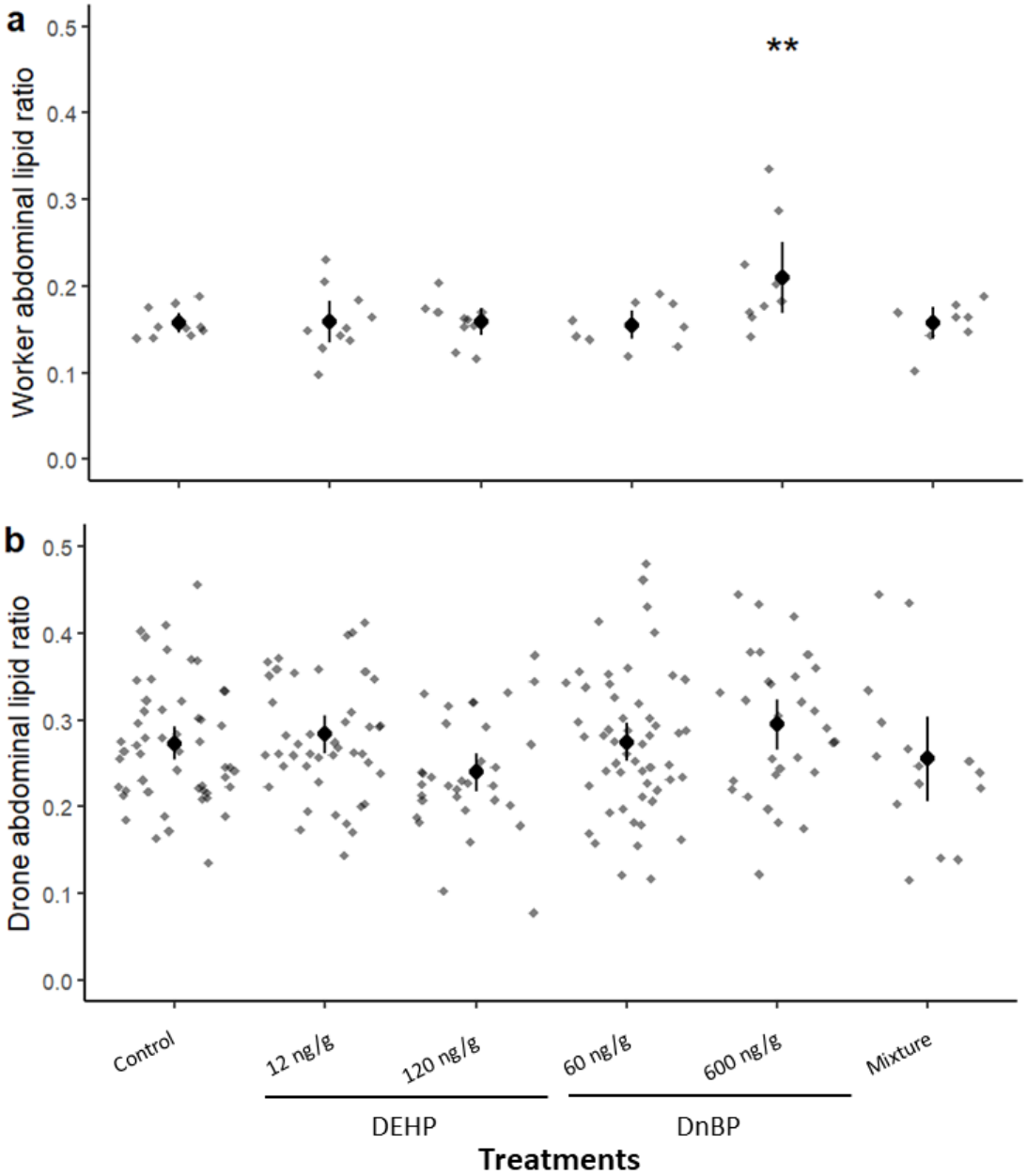


Fig. 5. Abdominal lipid ratio of a) workers, and b) emerged drones from microcolonies exposed to DEHP at 12ng/g ($N_{workers}$=10; N_{males}=45) or 120ng/g ($N_{workers}$=10; N_{males}=34), DnBP at 60ng/g ($N_{workers}$=9; N_{males}=53) or 600ng/g ($N_{workers}$=9; N_{males}=30), or the mixture ($N_{workers}$=8; N_{males}=15) compared to the control group ($N_{workers}$=10; N_{males}=52). Black points show mean values with error bars indicating 95% confidence intervals. **indicates a significant difference at p<0.01 compared to the control group.

3.4. Worker and drone abdominal lipid ratio

Phthalate exposure significantly affected worker abdominal lipid content (GLMM, family beta: Chisq. = 18.65, d.f. = 5, p-value = 0.002; Table 1). Abdominal lipid content of workers exposed to DnBP at 600ng/g b.w. increased significantly by 4.8% compared to the control group (p-value =0.0012; Fig. 5a).

No effect of worker phthalate exposure was observed on the abdominal lipid content of drones when considering all emerged individuals (GLMM, family beta: Chisq. = 8.32, d.f. = 5, p-value = 0.1392; Fig. 5b; Table 1), nor only those that emerged during the last two days of the experiment (GLMM, family beta: Chisq. = 3.7, d.f. = 5, p-value = 0.5895; Table 1).

4. Discussion

In this study, we investigated the impact of repeated environmental exposure to phthalates DnBP and DEHP, single or mixed, on the development of *B. terrestris* microcolonies over 35 days. Our results revealed an increase in the mortality of workers exposed to DEHP 120ng/g, whether administered alone or in a mixture solution with DnBP 60ng/g. We also observed a reduction in the number of drones emerging from microcolonies treated with the mixture, as well as from those exposed to DnBP 600 ng/g b.w. Moreover, under this latter condition, we reported an increase in the individual mass of the progeny, observed at the post-defecation stage and of the last emerged drones, along with an elevated abdominal lipid ratio in exposed workers.

4.1. Increase in worker mortality

While in a previous study isolated *B. terrestris* workers exhibited a four-fold increase in mortality 72 hours post-exposure to DnBP at an environmental dose (60 ng/g; Dewaele et al., 2025), here, we did not observe any increase in worker mortality in microcolony conditions. This is consistent with previous findings that sociality reduces DnBP toxicity

(Dewaele et al., 2025). DnBP-exposed microcolonies maintained control-level mortality throughout the 35 days, suggesting that the buffer effect of sociality on phthalate toxicity (mediated by social behaviours, such as grooming or thermoregulation, or microbiota composition; see Dewaele et al., 2025 for a thorough discussion) persists during long-term exposure.

Table 1. Summary of statistical results for all metrics. Significant difference with control is reported when the model is significant and is indicated in bold with associated symbols: *p<0.05, **p<0.01, ***p<0.001. "None" means that no treatment group differed significantly from the control.

Metrics	GLMM family	Chisq.	d.f.	p-values	Effect vs. control
Feeding behaviour					
Pollen collection	Gaussian	8.29	5	0.1407	
Syrup collection	Gaussian	11.6	5	**0.0405***	none
Pollen dilution	Gaussian	19.4	5	**0.0017****	↓ Mixture
Pollen efficacy	Gaussian	12.5	5	**0.0281***	↓ Mixture
Offspring number					
Eggs	Negative binomial	4.9	5	0.4247	
Non-isolated	Negative binomial	7.2	5	0.2067	
Pre-defecation	Poisson	19.1	5	**0.0018****	↓ Mixture
Post-defecation	Negative binomial	6.1	5	0.2933	
Pupae	Negative binomial	3.5	5	0.6270	
Non-emerged drones	Poisson	14.2	5	**0.0142***	none
Emerged drones	Poisson	23.2	5	**<0.001*****	↓ DnBP 600 ng/g ↓ Mixture
Total hatched	Poisson	8.4	5	0.1338	
Offspring mass					
Non-isolated total	Gaussian	10.8	5	0.0547	
Non-isolated per larvae	Gaussian	15.6	5	**0.0080****	none
Pre-defecation	Gaussian	8.2	5	0.1474	
Post-defecation	Gaussian	11.3	5	**0.0460***	↑ DnBP 600 ng/g
Pupae	Gaussian	5.4	5	0.3711	
Non-emerged drones	Gaussian	17.8	5	**0.0032****	none
Emerged drones	Gaussian	6.7	5	0.2464	
Lastly emerged drones	Gamma	20.1	5	**0.0012****	↑ DnBP 600 ng/g ↑ Mixture
Total hatched	Gaussian	10.2	5	0.0696	
Abdominal lipid content					
Workers	beta	18.7	5	**0.0022****	↑ DnBP 600 ng/g
Drones	beta	8.3	5	0.1392	
Lastly emerged drones	beta	3.7	5	0.5895	

In contrast, we observed an increase in worker mortality in microcolonies repeatedly exposed to DEHP 120ng/g, both alone and in mixture. Our results contradict the general pattern observed in invertebrates, where high molecular weight phthalates like DEHP are typically less toxic than low molecular weight ones like DnBP (e.g., Andreyeva et al., 2023; Green-Ojo et al., 2024; Hobson et al., 1984; Jensen et al., 2001; Planelló et al., 2011; Pradhan et al., 2018; Seyoum and Pradhan, 2019). This discrepancy could be explained by our original protocol where the bumblebees are repeatedly exposed to the phthalates for a relatively long period. While no increased mortality was previously reported in isolated workers 72 hours post-exposure to environmental doses of DEHP ranging from 12 ng/g to 12 µg/g (Dewaele et al., 2025), in this study, we measured a negative effect of DEHP on survival, not at the beginning of the experiment but from the middle time of the experiment. Such delayed toxicity is characteristic of endocrine disruptors (reviewed in Crews et al., 2000), and has been observed in other invertebrates. For example, exposure to high concentrations of DEHP (>3µg/L) during development reduced lifespan in *Caenorhabditis elegans* and *D. melanogaster* (How et al., 2019; Liu et al., 2024). This effect was linked to the disruption of longevity and ageing-related genes in both species (How et al., 2019; Liu et al., 2024). More specifically, DEHP disrupted the expression of genes involved in the insulin-related pathway in *C. elegans* and of genes involved in the AKT-FOXO pathway in *D. melanogaster*, inducing accelerated ageing and reducing lifespan in exposed individuals (How et al., 2019; Liu et al., 2024). A similar disruption might have occurred in *B. terrestris*, which could explain the decreased survival observed here.

Moreover, DEHP detoxification is energetically costly (Rivas et al., 2023). In rats, it involves cytochrome P450 enzymes (Liu et al., 2021a). In insects, these enzymes are also crucial for xenobiotic detoxification, but their activity causes oxidative stress associated with

cellular damage and lowered survival (Murataliev et al., 2008). Therefore, the increased mortality observed in this study might also result from oxidative stress caused by DEHP detoxification. As DEHP is known to specifically increase xenobiotic detoxification processes (Srivastava et al., 1976), repeated exposure might have further activated these pathways, increasing oxidative stress over time, and lowering the survival of workers in the long run.

It is worth noting that DEHP effects on survival in previous studies occurred at higher doses than the ones we used here (e.g., for *C. elegans* in How et al., 2019 and *D. melanogaster* in Liu et al., 2024: > 3µg/L DEHP in development medium). This indicates that *B. terrestris* could be more sensitive than other invertebrate models, perhaps because their number of cytochrome P450-related genes is reduced compared to other groups (Claudianos et al., 2006). This also raises concerns about the vulnerability of some wild bee species, such as those included in the Megachilidae family, which lack specific cytochrome P450-related genes (Hayward et al., 2024). To fully understand the impact and mode of action of phthalates in bees in general, further research at the physiological and molecular levels is therefore needed.

Alternatively, the increased mortality observed here could originate from disrupted social behaviour due to the repellent properties of DEHP against terrestrial insects, as observed in ants (Chen, 2005; Cuvillier-Hot et al., 2014). In the present study, workers were exposed via their cuticle, potentially interfering with cuticular chemical cues essential for nestmate recognition in insects (van Zweden and D'Ettore, 2010). In *Apis mellifera*, altered cuticular scents have been shown to trigger aggression towards nestmates (Cappa et al., 2019). A similar mechanism could explain the increase in mortality in DEHP-exposed microcolonies. However, DnBP has also been reported as repellent in *A. mellifera* (Sahebzadeh et al., 2009), yet it did not increase mortality in the DnBP-exposed microcolonies. A thorough

investigation of phthalate effects on intra- and inter-species recognition could clarify how these pollutants could impair bumblebee social behaviours.

An increase in mortality comparable to the DEHP-exposed group was also observed in the group exposed to the mixture. This contrasts with our previous study, which showed that exposure to DEHP-DnBP mixture at comparable doses in isolated workers reduced DnBP toxicity at 72h post-exposure (Dewaele et al., 2025). Here, we did not observe such an antagonistic effect, showing that DEHP toxic effects not only appear in the long run, but also that combined exposure with DnBP does not mitigate these effects. Indeed, while the antagonism between DEHP and DnBP observed in the short-term could result from an activation of DnBP detoxification pathways by DEHP (Dewaele et al., 2025), our results suggest that DnBP might not activate such pathways for DEHP. Yet, more research is needed to identify the molecular targets of phthalates in bees.

4.2. Decrease in offspring production

We observed a decrease in the number of post-defecation larvae and emerged drones in the DEHP-DnBP mixture treatment along with an increase in worker mortality. In microcolonies, lowered offspring production often results from increased worker mortality, as observed for example when microcolonies are treated with dimethoate in standardised microcolony tests using *B. impatiens* (Krueger et al., 2021). However, while comparable mortality was observed in microcolonies exposed to DEHP alone, no reduction in larval number was observed, suggesting a negative combined effect of DEHP and DnBP on brood survival. To our knowledge, this impact has not yet been highlighted in invertebrates.

Additionally, while the mixture treatment had no clear impact on total pollen and syrup collection, we observed a reduction in pollen dilution (i.e., collected pollen relative to syrup), suggesting altered resource collection behaviours among workers. Although workers

in the mixture treatment collected slightly less pollen than controls, this effect was not significant. This suggests a shift toward syrup collection that may reflect an energetic trade-off favouring detoxification over brood care in the workers (Fischer et al., 2024). Indeed, sugar found in syrup and nectar serves as an energy source mainly in adult bees (Suarez et al., 2005), whereas pollen provides proteins and lipids needed for larval development (Pereboom, 2000).

Pollen efficacy (i.e., brood mass per unit of collected pollen), used to assess microcolony performance (e.g., Barraud et al., 2020), was also significantly reduced in the mixture treatment, associated with the non-significant reduction in pollen collection. This again probably suggests a shift in priorities of workers from brood care to detoxification, as previously observed with pesticide exposure on *B. terrestris* (e.g., O'Reilly and Stanley, 2023; Stanley et al., 2016).

Microcolonies exposed to DnBP 600 ng/g or to the mixture produced fewer emerged drones, despite no significant changes in worker mortality or resource intake. This suggests that DnBP may also affect offspring production, at environmental doses and in combination with DEHP. On the one hand, both molecules were elsewhere shown to disrupt egg-laying, as observed in ant queens exposed to DEHP (Cuvillier-Hot et al., 2014), and in *D. melanogaster* exposed to DnBP (Atlı, 2010). Here, egg number did not vary between treatments, but counting was only performed at the end of the experiment; monitoring egg-laying dynamics could provide deeper insight into this aspect (e.g., Siviter et al., 2020b). On the other hand, exposure to the mixture of DnBP 600 ng/g may have delayed larval development, reducing drone emergence by the end of the experiment. Both DnBP and DEHP affect post-embryonic development in insects. For example, in *S. littoralis*, exposure to high concentrations of DEHP shortened larval stage duration, but prolonged pupation (Avilès et al., 2019). In *D. melanogaster*, exposure to DnBP extended pupation (Atlı, 2010). Direct larval exposure (e.g.,

Kato et al., 2022) to DEHP and DnBP alone and in mixture could help clarify phthalate effects on larval development.

4.3. Increase in worker abdominal lipid ratio and offspring mass

In this study, microcolonies exposed to environmental doses of DEHP showed no difference in larvae and drone body mass or adult abdominal lipid ratio compared to controls. Similarly, in *S. littoralis*, high DEHP levels (19.5 mg/g of food) affected larval development and mass, but environmental concentrations (e.g., 1.1 ng/g of food) had no impact on these metrics (Avilès et al., 2019). Effects on body mass and lipid content in invertebrates generally occur at concentrations exceeding environmental levels, as seen in *Daphnia magna* and *Caenorhabditis elegans* exposed to > 3µg/L DEHP (Pradhan et al., 2018; Seyoum and Pradhan, 2019).

In contrast, workers treated with DnBP 600ng/g showed increased lipid content, without any corresponding rise in resource collection, alongside increased mass in post-defecation larvae and last-emerged drones. DnBP is known to have obesogenic effects in vertebrates (Dalamaga et al., 2024), including at low doses (Majeed et al., 2017); and similar effects were shown in aquatic invertebrates (Lee et al., 2018). For instance, increased lipid content in *D. magna* reared in DnBP-contaminated media was linked to a disruption of fatty acid catabolism (Seyoum and Pradhan, 2019). Unlike other hymenopterans, such as parasitoid wasps, in which adults lack enzymes performing *de novo* lipogenesis and lipid reserves are accumulated during larval development, adult bumblebees rely on both dietary intake and *de novo* lipogenesis for lipid storage (Furse et al., 2023). Thus, increased lipid content without greater dietary intake, as observed here, may arise from reduced lipid catabolism and/or enhanced lipogenesis, suggesting disruption of lipid metabolism pathways following DnBP exposure. Such disruption might have happened in the insulin-related pathway, which

regulates fat body cell production and lipogenesis (Skowronek et al., 2021), and which was shown to be disrupted by low concentrations of DnBP in *D. melanogaster*, leading to increased starvation resistance through diminished lipid mobilisation (Williams et al., 2016).

Abdominal lipid content, often used as a proxy for fat body status (e.g., Moret and Schmid-Hempel, 2009; Vanderplanck et al., 2021), is critical for storing energy reserves (e.g., glycogen and triglycerides) essential for flight, diapause, and development (Arrese and Soulages, 2010; Furse et al., 2023; Toprak, 2020).

Alongside the results observed in workers, post-defecation larvae and lastly emerged drones from microcolonies exposed to DnBP 600ng/g had a higher body mass than the control. Pre-pupal stages such as post-defecation larvae are sensitive to juvenile hormone signalisation (Rachinsky et al., 1990), which is notably influenced by the insulin-related pathway (Zhang et al., 2022), and affects lipid metabolism and lipid mobilisation (Shpigler et al., 2021). Therefore, a disruption of this specific pathway could have led to the observed obesogenic effect on larvae, through a similar disruption of lipid metabolism and insulin-signalling pathway as potentially occurring in workers. Additionally, it is important to note that microcolonies exposed to DnBP 600 ng/g produced fewer emerged drones despite collecting similar resources, suggesting larvae might have received proportionally more food. Larval food intake strongly affects adult body size in bumblebees (Couvillon and Dornhaus, 2009), a parameter positively correlated to body mass (Cane, 1987; Maebe et al., 2021). Such a change in food intake could therefore explain the increased weight observed in emerged drones, but we observed no significant change in pollen collection and efficacy (i.e., offspring produced per gram of pollen) in the microcolonies exposed to DnBP 600ng/g. This indicates that a change in larval weight might not result from a change in larval feeding behaviour. However, DnBP could have disrupted larval development duration, as observed in *D. melanogaster* (Atlı, 2010). An

increase in larval development duration could have led to the emergence of fewer drones while having the same number of bigger larvae in the microcolonies, explaining the absence of pollen efficacy modification, but allowing the larvae more time to feed and grow. Considering that, in *Bombus*, development duration and food intake influence caste determination (Ribeiro, 1994), phthalate exposure may potentially lead to an imbalance in production of workers and queens, which would strongly impact colony fitness. To date, such effects have only been reported in *B. terrestris* queenright colonies exposed to elevated temperatures (Guiraud et al., 2021), not pollutants. Future research should investigate phthalate impacts on caste-specific development and related molecular pathways in queenright colonies.

4.4. Transgenerational impact of phthalates

In this study, larvae were not directly exposed to phthalates. Yet, in microcolonies treated with DnBP 600ng/g, the drones emerging late in the experiment and the post-defecation larvae weighed at the end of the experiment showed an increased mass, suggesting an effect of DnBP after repeated exposure of the mother workers. As phthalates and other endocrine disruptors are known for their delayed effects, these results reinforce the need for long-term and multi-generational studies in risk assessments (Futran Fuhrman et al., 2015). Moreover, several mechanisms could explain the observed effects in the offspring of the exposed workers.

First, larvae could have been indirectly exposed via transgenerational transfer, through contact with contaminated workers and nest structures. In nature, foragers bring environmental pollutants, including plasticisers, back to the nest, as highlighted by studies using honeybee and bumblebee material for biomonitoring of environmental pollution (AL-Alam et al., 2019; Moniuszko et al., 2025). Those pollutants can accumulate in lipid-rich nests

(Gómez-Ramos et al., 2016). Similarly, *B. terrestris* larvae may experience chronic exposure due to accumulation in nest materials by contact with exposed workers. While nest contamination has been documented in honeybees (e.g., Gómez-Ramos et al., 2019, 2016; Martín-Gómez et al., 2024), phthalate transfer dynamics within social bee colonies remain largely unexplored.

Second, in microcolonies, queenless workers develop ovaries and some will start laying unfertilised eggs in the first few days after isolation from queenright colony (Klinger et al., 2019). Therefore, exposure may have occurred during oogenesis in contaminated workers. In terrestrial invertebrates, like wolf spiders and soil mites, the entire burden of inorganic pollutants (e.g. mercury and cadmium) can transfer to eggs (Saxton et al., 2013). However, data on organic pollutant transfer, particularly in bees, remain scarce. Notably, *Spodoptera frugiperda* individuals reared on DnBP-contaminated media (1mg/L) produced second-generation individuals with approximately 100 times more DnBP than their parents, suggesting contamination transfer and bioaccumulation, despite the absence of parental care (Filho et al., 2013). However, the DnBP content of the eggs was not assessed in these studies.

Third, larval mass gain in DnBP-exposed microcolonies could reflect transgenerational effects mediated by parental hormonal status, as highlighted in species lacking parental care. In *D. melanogaster*, long-term parental exposure to high-dose DEHP altered offspring body mass, with maternal exposure reducing, and paternal exposure increasing body weight (Chen et al., 2019), highlighting parental endocrine influence. In our study, only workers were exposed to DnBP, and transgenerational effects were observed only for drones. Given that bumblebees are haplodiploid, i.e. males develop from unfertilised eggs and are haploid, while females develop from fertilised ones and are diploid (Goulson, 2010), potential transgenerational phthalate impacts in bumblebees could vary from those observed in

Drosophila. Indeed, drone traits reflect only maternal factors, while female development is influenced by both maternal and paternal contributions. Thus, assessing the potential transgenerational effects of phthalate exposure in queens and drones is essential for understanding risks to colony development and population health.

4.5. Conclusion

Overall, our findings suggest that environmental exposure to the ubiquitous phthalates DEHP and DnBP can negatively affect *B. terrestris* worker survival and larval development, likely through potential disruptions of lipid metabolism and hormonal signalling. The contrast between short- and long-term effects highlights the need for chronic and repeated exposure assessments in chemical risk evaluations. Current frameworks, which rely heavily on short-term toxicity tests (EFSA et al., 2023; Gross et al., 2017), often fail to capture delayed impacts typical of endocrine disruptors (Futran Fuhrman et al., 2015). These overlooked effects could have long-term and colony-level consequences, posing serious risks to bumblebee populations.

PART II: Investigating the effects of repeated exposure to di-2-ethylhexyl phthalate and di-n-butyl phthalate on larval development of in vitro reared **Bombus terrestris** *workers larvae**

*Adapted from Fievet, M. (2023). Set up of an *in vitro* method to test the effects of phthalates on the health and development of *Bombus terrestris* larvae. Master's thesis. University of Lille, France

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Abstract

Phthalates are plasticisers used to enhance the flexibility of plastic polymers and as solvents in household products, paints and pesticides. As they are not covalently bound to plastic matrices, these lipophilic compounds easily migrate into the environment, where they adsorb to organic particles and persist for extended periods. In honeybee hives, di-n-butyl phthalate (DnBP) and diethylhexyl phthalate (DEHP) have been detected in wax combs, on worker cuticle and in pollen loads, suggesting contamination from apiary practices, but also from environmental pollution. Given the lipid-rich composition of bumblebee nests, developing brood could be chronically exposed to these pollutants. Yet, DnBP exposure of workers in microcolonies highlighted altered brood production, adult lipid content and larval weight. Yet, the mechanism of larval development disruption was not identified. The present study adapts *in vitro* larval rearing protocols recently developed for *B. terrestris* to assess the direct effects of environmentally relevant DnBP and DEHP concentrations on larval survival, developmental duration, and physiological traits such as weight and lipid content. In addition, the expression of genes associated with metabolism and ecdysteroid signalling was analysed in larvae. No impact of direct exposure to phthalates was found. However, the solvent (methanol) used in the treatment solutions might have affected metabolism gene expression, with DEHP exposure buffering this effect. We therefore discuss the absence of deleterious effects on lipid content and larval weight in regards to previous results obtained in microcolonies and give future recommendations for in vitro exposure methodologies in bumblebees.

Keywords: larval growth; plasticisers; lipid; bumblebee

1. Introduction

Phthalates are plasticisers commonly used in plastic products to increase flexibility, as well as in detergents, paints, pesticides, and other products as solvents (CEFIC, 2021; Koniecki et al., 2011). These lipophilic molecules are not chemically bound to the matrices to which they are added, and leach easily into the environment (Suhrhoff and Scholz-Böttcher, 2016). As they can be absorbed onto lipidic particulate matter in water and soil (Billings et al., 2021), or insect cuticles (Lenoir et al., 2012), their half-lives can be extended from a few days or hours in their free form to months or years once complexed (Kickham et al., 2012). However, those ubiquitous pollutants have been classified as endocrine disruptors, with evidence of obesogenic and reprotoxic effects in mammals (Regulation (EU) 2018/2005).

From a public health perspective, the frequent use of plastic in apiaries and hive products packaging has led to investigations into plasticiser contamination of hives and hives products (e.g., Gómez-Ramos et al., 2019, 2016; Martín-Gómez et al., 2024). In this context, DnBP and DEHP were among the most commonly detected plasticisers in honeybee workers, pollen, and combs (Gómez-Ramos et al., 2019, 2016; Martín-Gómez et al., 2024). While phthalate contamination of honey has primarily been attributed to apiary management and product processing practices (Zhou et al., 2014), these molecules were also frequently detected in honeybee workers and collected pollens (Gómez-Ramos et al., 2019; Martín-Gómez et al., 2024) as well as on wild workers of the buff-tailed bumblebee, *Bombus terrestris*, collected in the City of Lille, France (Cuvillier et al., In prep.). Bee foragers, by flying varying distances to collect floral resources outside the nest, can capture environmental pollution in the surroundings of the nest (Moniuszko et al., 2025). Moreover, nests are composed of highly lipidic materials, such as pollen stores and waxy structures, which can accumulate organic pollutants, including phthalates, either transported back by foragers or directly infiltrating

from nearby matrices like contaminated soil (AL-Alam et al., 2019; Gómez-Ramos et al., 2019). Therefore, the brood, comprising the developing progeny cared for by the workers, could be chronically exposed to phthalate concentrations equal to those experienced by the foragers or even higher in case of accumulation.

In this context, phthalates could affect bee development as shown in other invertebrate models, notably through the disruption of the ecdysteroid-signalling pathway. For example, in the moth, *Spodoptera littoralis*, a downregulation of genes coding for the ecdysone receptor was observed alongside larval duration and weight alteration in larvae reared on DEHP-contaminated food (Avilès et al., 2019). In aquatic larvae of the midge *Chironomus riparius*, expression of the ecdysone receptor gene (ECR) was disrupted after exposure to DEHP and BBP (Herrero et al., 2017, 2015). Moreover, developmental exposure to DnBP in *D. melanogaster* was shown to increase pupation duration while other developmental stage durations decreased (Atlı, 2010). Extension of larval stage duration in insects can notably be observed when the ecdysteroid-signalling pathway is disrupted (Zhang et al., 2021b), as this hormonal cascade tightly regulates development-related processes in insects, including embryogenesis, metamorphosis, and moulting (Bonneton and Laudet, 2012).

Previously, DnBP exposure of workers in microcolonies led to the production of fewer larvae but with increased weights (Chapter 3, Part. I). Although microcolony assays are recommended by EFSA to assess sub-lethal developmental effects in *B. terrestris* (EFSA et al., 2023), they do not allow precise monitoring of larval feeding, nor clear identification of the origin of the effects. Indeed, the larvae are not directly exposed in such designs and the results observed may result from indirect exposure or transgenerational impact (Chapter 3, Part. I). While standardised methods have been developed for *in vitro* rearing of honeybee workers

(Crailsheim et al., 2013), the same methods are still under development in *B. terrestris* (Kato et al., 2022; Siviter et al., 2020a), and could overcome the drawbacks mentioned above and greatly help identify the mechanisms behind larval development alteration in this species.

In the experiment described below, we adapted the protocols developed by Kato et al. (2022) for *B. terrestris* larval rearing to investigate the effects of direct exposure of larvae to environmental DnBP and DEHP concentrations on post-embryonic development. We monitored survival, instar duration, and pre-pupal morphological and physiological traits, such as weight and lipid content, to detect possible obesogenic effects and metabolic alteration of phthalate exposure, considering the results described in the first part of this chapter. In addition, we investigated potential disruption of the expression of genes related to metabolism and the ecdysteroid-signalling pathway. Finally, we discuss future ameliorations and recommendations for *Bombus* larvae *in vitro* rearing protocols.

2. Material and methods

2.1. Model species

B. terrestris worker development lasts around 22 days, with approximately 11 to 14 days of larval development (Duchateau and Velthuis, 1988). To our knowledge, no detailed *B. terrestris* development sequence is available for *Bombus* species. Therefore, the following description is constructed based on colony observation and information gathered in the literature. First, a fertilised queen lays the eggs (Fig. 1) in batches of around eight (Cnaani et al., 1997). Eggs hatch within the first four days, and the first-instar larvae (L1) emerge, entirely white and almost transparent, with the first striae already visible (Fig. 1). They develop in wax cells containing multiple L1 (Fig. 2; Duchateau and Velthuis, 1988), and workers start to feed them individually by regurgitating a droplet of food directly onto their abdomen through a hole pierced in the wax (Ribeiro et al., 1999). This hole is immediately repaired after feeding

(Pendrel and Plowright, 1981; Ribeiro et al., 1999). Feeding frequency at this stage is very low, with intervals that can reach up to four hours (Ribeiro et al., 1999). Two days after the hatch, the larva undergoes its first moult and enters the second instar (L2), which grows and acquires brownish tones (Fig. 1). Feeding frequency increases considerably at this stage, but remains highly variable, as intervals range from a few seconds up to three hours, with the majority of larvae being fed in average every 20 minutes (Ribeiro et al., 1999). This stage lasts for about two days, after which the larva moults again and enters the third instar (L3; Fig. 1). Towards the end of this stage, the larva begins spinning its cocoon and isolates itself from the others (Fig. 2), although a permanent feeding hole is maintained in the cocoon wall (Ribeiro et al., 1999). Just before defecation, the larva undergoes its third moult to become a fourth-instar larva (L4), which feeding frequency decreases (Ribeiro et al., 1999). After emptying its gut, the post-defecation larva turns white and stops feeding in preparation for pupation (Fig. 1). Pupation lasts for about nine days, during which the insect acquires its adult morphological features, including pigmented eyes, body melanisation, setae, legs, wings, mouthparts, and antennae (Fig. 1; Tian and Hines, 2018). Feeding does not occur during this period, in which up to 20 distinct pupal stages have been described in Bombus species, including *B. impatiens*, but is not yet documented in *B. terrestris* (Tian and Hines, 2018).

In this experiment, the larvae were sampled from three commercial queenright *B. terrestris* colonies (±80-120 workers), purchased from Biobest NV (Westerlo, Belgium). The commercial colonies were kept at room temperature (±20°C), and were provided ad libitum 50% w./w. sucrose-water solution.

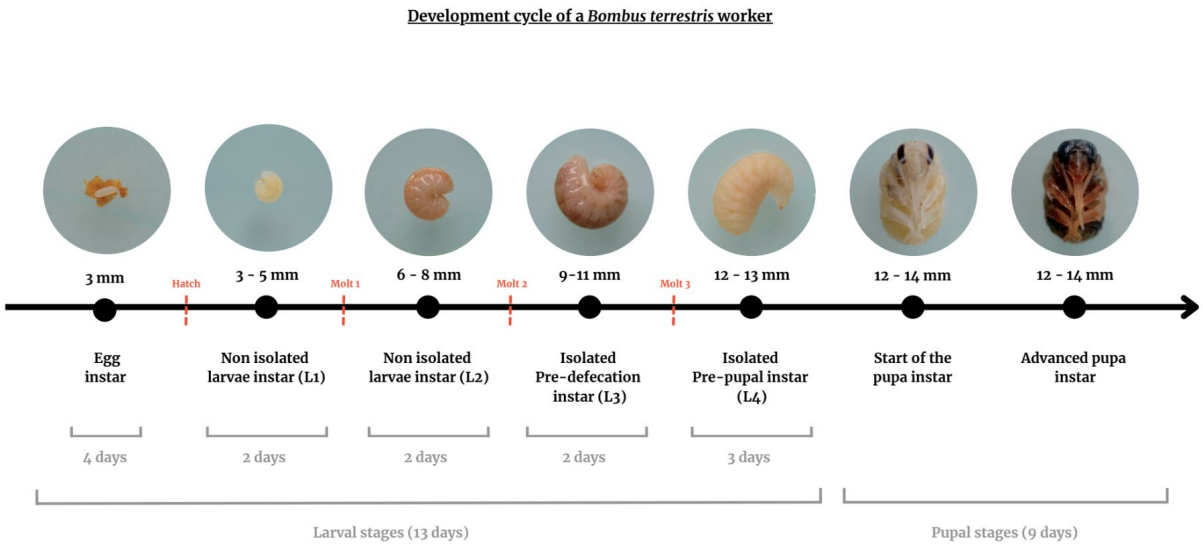


Fig. 1. *In vivo* development of worker larvae. Pictures and credits: Manon Fievet.

2.2. Feeding solution and treatment preparation

A stock feeding solution was prepared using mineral water marketed in glass bottles (SPA, Belgium) mixed with 50% sucrose, 40% pollen (Heather pollen purchased from Abeille Heureuse, Strasbourg, France), 10% yeast extract and 1% sodium caseinate (Kato et al., 2022). All ingredients except the pollen were first autoclaved. Once prepared, the feeding solution was aliquoted in glass jars and stored at -20°C until further use. A new aliquot was used every two days.

DnBP (CAS n° 84-74-2) and DEHP (CAS n° 117-81-7) were purchased from Sigma-Aldrich in liquid form (Both >99%). The treatment concentrations were based on previous results. We chose to use DnBP at a concentration of 600 ng/g b.w., as it was shown to increase the weight of late larval stages, and abdominal lipid content of workers in the microcolony experiment (See Chapter 3, Part I). To calculate the final working concentration, we considered the total amount of phthalate received by the workers of the microcolony experiment during the 35 days of experiments (e.g. for DnBP 600ng/g was multiplied by five exposure sessions). Then, we adapted it to the duration of larval exposure and to the fact that the treatment would be administered once a day. Finally, as the larvae would change in weight throughout their

development, we decided to calculate the final concentration needed in the feeding solution based on the amount of pollen in the solution (0.004g/µL), more practical to control than larval body weight. As DEHP showed no effect on larval weight or number at the concentrations tested in the microcolony experiment (12, 120, and 1200 ng/g b.w.), we chose to increase the tested concentration of DEHP to 1200 ng/g b.w., and we followed the same calculation process as for DnBP 600ng/g.

To prepare the contaminated feeding solutions, the initial dilution used methanol (CAS n°67-56-1, VWR) as a solvent for better solubility, as phthalates are lipophilic molecules. The subsequent dilutions were prepared in mineral water to ensure the lowest methanol concentration in the solutions (0.1%). The water control group was fed the uncontaminated feeding solution, the solvent control group the feeding solution containing 0.1% methanol, the DnBP group the feeding solution contaminated with DnBP 600ng/g of pollen and the DEHP group was fed the feeding solution contaminated with DEHP 1200 ng/g of pollen.

The contaminated feeding solutions were aliquoted and stored at -20°C. The aliquots were thawed at least two hours before use.

2.3. Preventing PAE contamination of materials and solutions

Because phthalates are commonly present in indoor air, particularly in laboratories (Feng et al., 2020), it was important to minimise uncontrolled contamination, mostly linked to extensive plastic use (Reid et al., 2007). Therefore, plastic items were replaced with glass and metal whenever possible, which were additionally decontaminated with hexane and acetone, and when feasible, by calcination. We chose to use hard plastic material with lower phthalate content (Cao et al., 2022) for the 24-well plates, as plastic could not be avoided. In addition, treatments and feeding solutions were prepared using water marketed in glass bottles (SPA

Reine, SPA NV, Bruxelles, Belgium) to minimise phthalate contamination from plastic bottles and tap water (Bach et al., 2020; Luo et al., 2018).

2.4. Experimental protocol

2.4.1. Larvae selection. Shortly after reception, commercial colonies were emptied of their queen and workers, which were transferred into empty boxes under red light. Pictures of the broods were taken to identify the egg and larval brood cells that were already present at hive reception (Fig. 2). Queen and workers were kept together by nestmate groups. They were then transferred back into the corresponding initial hive.

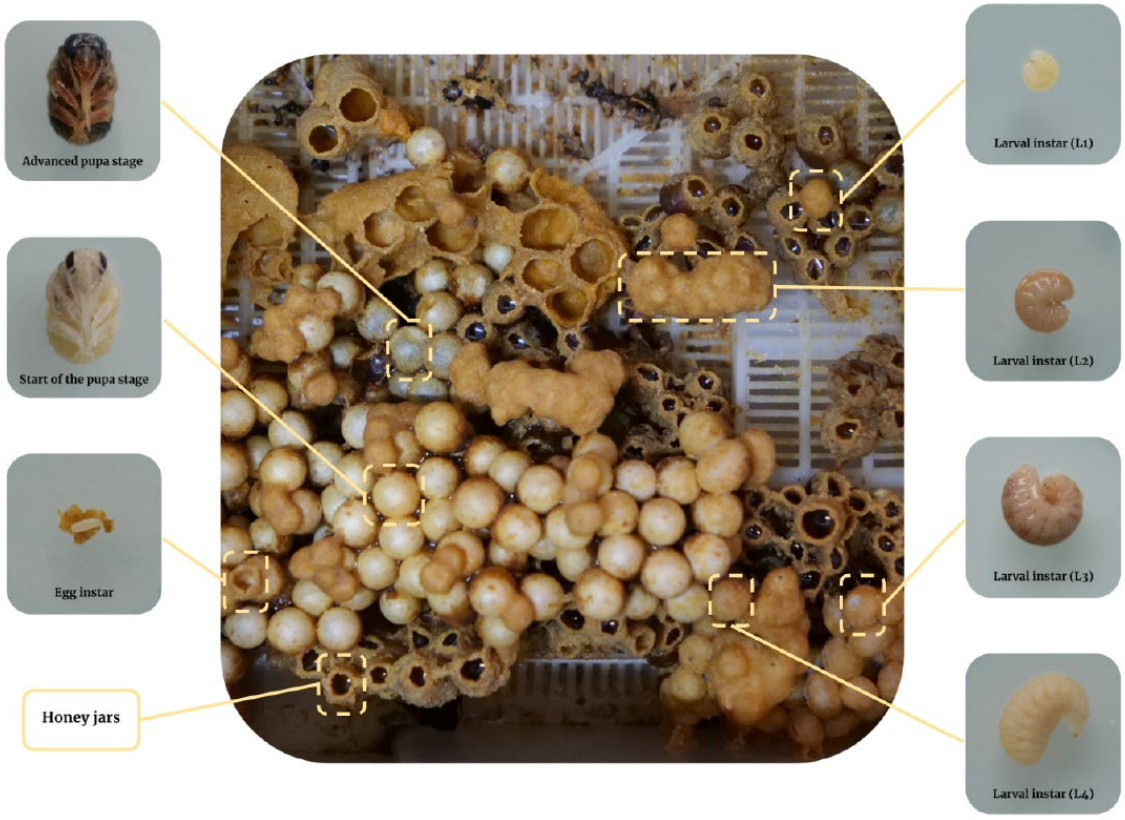


Fig. 2. Example of brood composition of a queenright commercial colony upon reception. On the right: larvae for L1 to L4 are localised in the brood. During the L1 and L2 stages, the larvae of the same cohort are grouped in the same cell. During the transition from L2 to L3, the larvae individualise in a single cocoon until emergence. At the beginning of the L4 stage, the larva is in the defecation phase and has the same aspect as the pre-defecation larva (i.e. L3), but its cocoon resembles the L4 one. On the left: Pupae, egg cells and honey jars are localised in the brood. Pupae are found below the brood and can be identified with cocoons that thin during transition throughout the pupal phases, allowing eye identification. Eggs are laid by batch in cells localised atop the brood. Pictures: Manon Fievet.

This process was repeated the following day to identify new egg cells using manual marking on pictures (Fig. 3). The colonies were not further disturbed until the transplant. After several unsuccessful trials of handling eggs and young hatched larvae, we decided not to select the youngest larvae (less than 6 days old) because they were too immature to be handled, grafted and to survive long in the plates. Moreover, we did not use the older larvae to avoid missing the first peak of ecdysteroids, appearing at the L2 stage (Hartfelder et al., 2000). This method, while limiting the number of larvae available for the experiment compared to previous studies (Kato et al., 2022; Richardson et al., 2025; Siviter et al., 2020a), allows a more accurate control of the stage of the selected larvae.

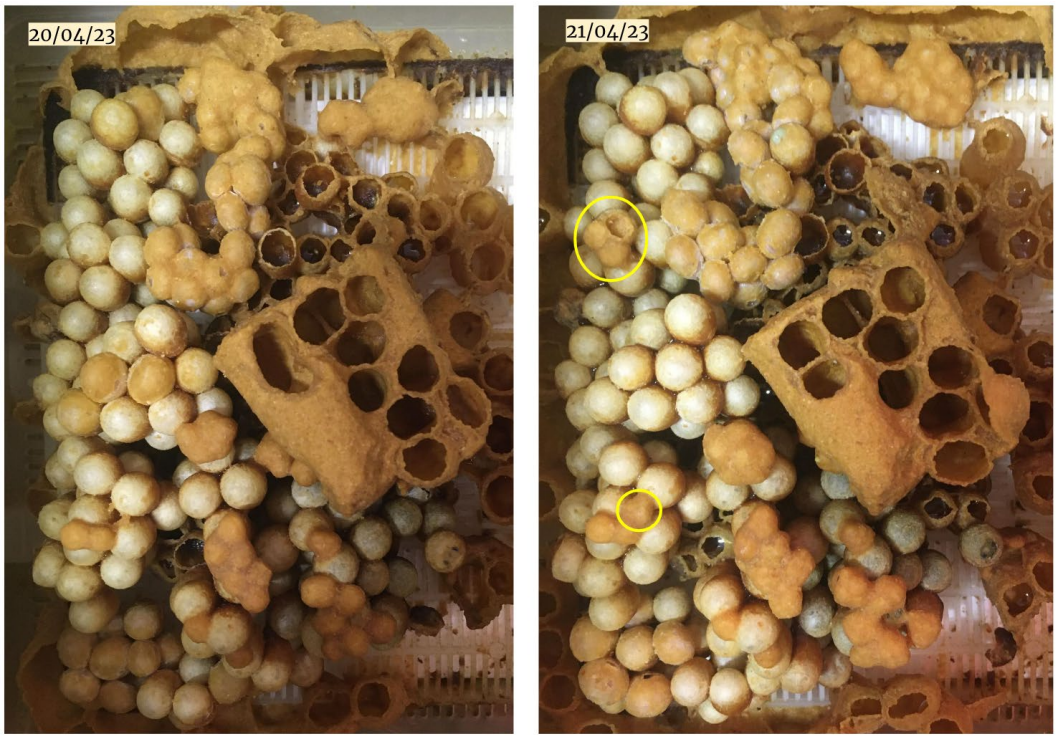


Fig. 3. Egg cell labelling method. The photo from 04/20/23 shows the state of the brood at reception. The photo from 04/21/23 shows the new egg cell (yellow circles), dedicated to the experiment. Pictures: Manon Fievet.

2.4.2. Grafting process. On the sixth day, the colonies were ultimately emptied under red light to allow safe access to the broods. Then, the brood cells that had been identified six days prior were carefully detached from the brood using surgical tweezers. They were placed on a glass dish to be transported for further handling under an extraction hood.

Once under the hood, all the available larvae were carefully collected and randomly arranged (one larva per well) in sterile 24-well culture plates. Throughout the graft process, the petri dish and multi-well plates were placed on heating plates to limit thermal stress (35°C; Kato et al., 2022) under the extraction hood. Once the grafting was completed, the plates were placed individually in wet chambers, i.e. hermetic boxes with paper towels soaked in a sterilesaturated NaCl solution (relative humidity: 75% to 80%), within an incubator (temperature: 34°C). The soaked paper towels were renewed every 2 days to maintain the relative humidity. Temperature and humidity were controlled throughout the experiment. Finally, following installation, the larvae were deprived of food for one hour for acclimation to rearing conditions (Siviter et al., 2020a).

2.4.3. Feeding of the larvae. The feeding process was mainly based on Kato et al. (2022). Throughout the experiment, the larvae were fed thrice a day, as Kato et al. (2022) determined that the adult emergence rate was higher at this frequency compared to twice a day. The feeding was done at fixed times, i.e. 8:00 am, 1:00 pm and 6:00 pm (Kato et al., 2022), under a hood with the 24-well plates and feeding solutions placed on heating plates at 35°C. This allowed for limiting stress from temperature variations (Fig. 4; Kato et al., 2022).

The feeding solutions were administered using a glass micropipette. After several attempts, we saw that the L1 and early L2 larvae easily drowned in the feeding solution. Therefore, for the early stages (late L1 and L2), a line of feeding solution was put at the bottom of the well near the larvae (Fig. 5a). For older larvae, a droplet of feeding solution was placed on the abdomen (Fig. 5b).

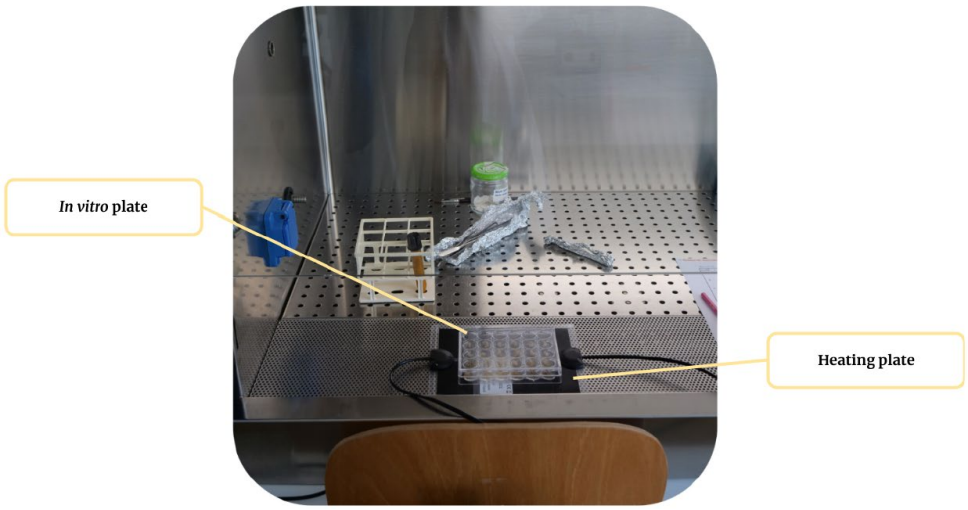


Fig. 4. Feeding session installation under the hood. The 24-well plate (*in vitro* plate) was placed on heating plates at 35°C during feeding sessions. Pictures: Manon Fievet.

2.5. Larval endpoints

2.5.1. Monitoring of larval stage duration and mortality. During the feeding process, mortality and molting were recorded. Larvae that were immobile and did not readily start to feed after the drop deposition were considered dead. Molting was recorded during the feeding sessions to monitor transition and between larval stages, and therefore larval stage duration. It was identified as a distinct dandruff on the hind part of the larvae, corresponding to the insect's exuvia. We ended the experiment after the third molt, when most (>70%) of the L4 larvae had defecated. The duration of L1 to L4 instars were thus monitored during this experiment.

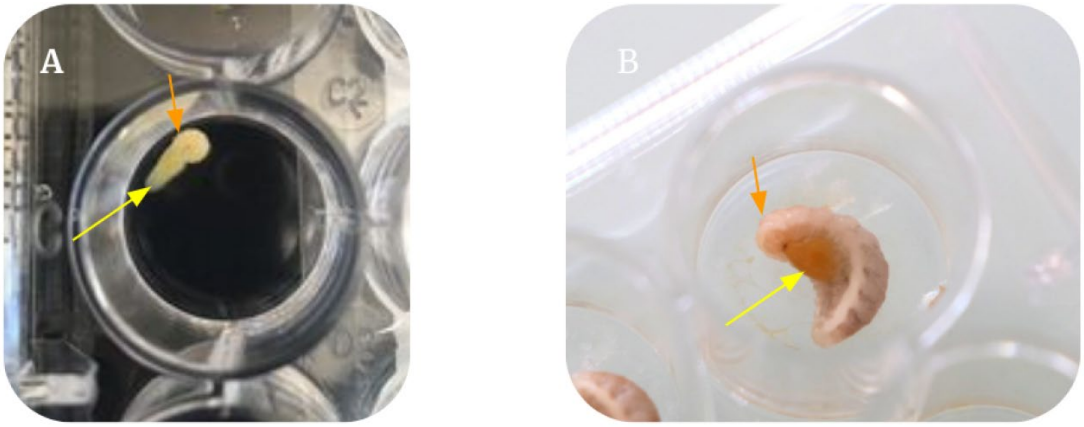


Fig. 5. Larval feeding method. A) For young larvae (L1-L2), a line of solution was placed at the bottom of the well to avoid drowning. B) For older larvae, the drop of feeding solution was placed on the abdomen. Orange arrows show the larvae head, and yellow arrows show the feeding solution. Pictures: Manon Fievet.

2.5.2. Larval weight and lipid ratio. After 12 days, the final mass of each living larva was recorded, and the larvae were flash-frozen using liquid nitrogen. A sagittal section of each frozen post-defecation L4 larva was performed using a clean razor blade. We measured the larval lipid ratio individually in each half larvae. To do so, the samples were first dried at 60°C for eight days, and the dry weight was recorded. An extraction using 700µL of Dichloromethane-methanol 2:1 bath was performed on the samples. Each sample was crushed for 60 seconds using pestles and homogenised using a vortex for 15 minutes. A centrifuge at 14000 rpm for four minutes was performed. After allowing to settle for four minutes, the supernatant was collected and deposited in a new tube. The Dichloromethane-methanol extraction was repeated twice per sample, and both supernatants were combined in the same tube. 540µL NaCl 0.9% was added to the supernatant, and the tubes were agitated for 30 seconds, then allowed to settle for five minutes. The aqueous layer was discarded, and the lipidic layer was left under an extracting hood for 24 hours. The dried lipidic content was then weighed, and the lipidic ratio was calculated by dividing the weight of the lipidic content by the mass of the half larvae recorded before the drying process (Couvillon et al., 2011).

2.5.3. Gene expression analyses. The second half of the post-defecation L4 larvae was allocated to gene expression analyses and stored at -80°C until RNA extraction. To extract RNA, the samples were individually and gently crushed using a pestle in the lysis buffer from Nucleospin RNA Plus kit (Macherey-Nagel; Germany). We removed the hard cuticle before proceeding to the RNA extraction, following the kit instructions, with the final elution performed in 30µL of pure water. RNA quantity after extraction was measured using a Qbit 4 fluorometer from Thermofisher. cDNA synthesis was performed on 650ng of RNA using RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific) following the manufacturer's protocol. Briefly, each 10 µL reaction contained 2 µL of cDNA (diluted 1:3), 1 µM of each gene-

specific primer, and 5 µL of Bio-Rad SYBR Green Master Mix. Amplification was carried out on a LightCycler 480 system (Roche Applied Science) with an initial denaturation at 95 °C for 30 s, followed by 40 cycles of 95 °C for 15 s and the primer-specific annealing temperature (Tm) for 45 s. Fluorescence was recorded at the end of each cycle, and a melting curve analysis was performed by heating to 95 °C at a ramp rate of 0.11 °C/s with continuous fluorescence acquisition. Data were analysed using LightCycler 480 Software v1.5.1, with threshold cycle (Ct) values obtained via the Second Derivative Maximum method. Ct ratios between each target gene and two reference genes were calculated using the E-method, correcting for primer-specific efficiency and normalising against a standard calibrator identical across plates and genes (Tellmann, 2006).

The Ribosomal protein L13 gene, *RPL13*, and the Phospholipase A2 gene, *PLA2*, were used as reference genes as they are amongst the most stable housekeeping genes in *B. terrestris larvae* (Qin et al., 2021). Genes selected as targets included the Ecdysone receptor gene, EcR and the early and early-late genes *E75* and *78C* coding for nuclear receptors, as previous work has shown that DEHP can alter the expression of genes within the ecdysteroid-signalling cascade in *S. littoralis* larvae (Avilès et al., 2019). As increased worker lipid content and larval weight were highlighted after exposure to DnBP in the previous microcolony experiment (Chapter 3, Part I), we also included genes coding lipid synthesis enzymes, i.e. Fatty acid synthase and Acetyl-CoA carboxylase enzymes, *FAS* and *ACC*. In addition, as in isolated adults, exposure to DnBP showed metabolic disruption, genes coding for enzymes involved in the metabolic stress response, i.e. Kynurenine/alpha-aminoadipate aminotransferase, *AADAT*, and the L-lactate dehydrogenase, *L-LDH*, were also added to the selection. A differential expression of these genes is often associated with metabolic stress, as these genes are metabolic regulators that interact with multiple immune and hormonal

pathways (Cuvillier-Hot et al., 2024; Doublet et al., 2017; Rondeau et al., 2025; Vasamsetti et al., 2024). Primer characteristics, specific T_m and efficiency can be found in Table S2.1 (Chapter 2 , Part II).

2.6. Statistical analyses

All statistical analyses were performed using R v 4.4.1 (R Core Team, 2023), and all graphs were made using "ggplot2" (Wickham, 2016).

The global survival analysis was performed using a mixed Cox regression with solvent control as the reference group and the initial colony as random factor using the package "coxme" v2.2-22 (Therneau, 2022). The hazard ratios (HR) compared to the solvent control are reported.

Using the package "glmmTMB" v1.1.11 (Brooks et al., 2025), generalised linear mixed models (GLMM) were used to analyse the treatment effects on the metrics. The initial colony was considered as random effect and the treatment as fixed effect. The distribution families used were chosen depending on the metric considered, and visual inspection of model fits using the "DHARMa" package v0.4.7 (Hartig, 2020) was performed to validate the models used. The binomial family with "logit" link was used to compare the mortality occurring at each stage as well as the defecation success of L4 larvae between treatments. The Gaussian family with "identity" link function was used to analyse post-defecation L4 larvae mass. The gamma family with "log" link function was used to analyse the larval stage duration and relative gene expression (with Ct ratio as response variable). The beta family with "logit" link function was used for the lipid ratio analysis. Custom contrasts using package emmeans v1.10.5 (Lenth, 2023) with solvent as control group and Benjamini-Hochberg's p-value adjustment method (i.e., "fdr") were used on the models as post-hoc analyses.

3. Results

3.1. Larval survival

Six days after egg-cell marking, we sampled 17 two-day-old L1 larvae from the first colony, 42 from the second and 37 from the third. They were randomly allocated to the four treatment groups. During the first 24 hours (pre-treatment period), 13 larvae died, but the mortality did not differ between the initial colonies (GLMM, family binomial: chisq. 1.97, df = 2, p-value = 0.3730), and these larvae were discarded from further analyses.

At the end of the experiment, the survival did not differ between groups (Mixed Cox regression: Chisq. = 2.9, df = 4, p-value = 0.5747). 50% of the larvae survived in the solvent group, while the lowest value was found in the mineral water control group with 33% (HR = 1.63, 95% CI = [0.73, 3.59], z = 1.20, p-value = 0.2300) and the highest in the group exposed to DnBP 600 ng/g with 57% (HR = 0.84, 95% CI = [0.35, 2.03], z = -0.39, p-value = 0.7000).

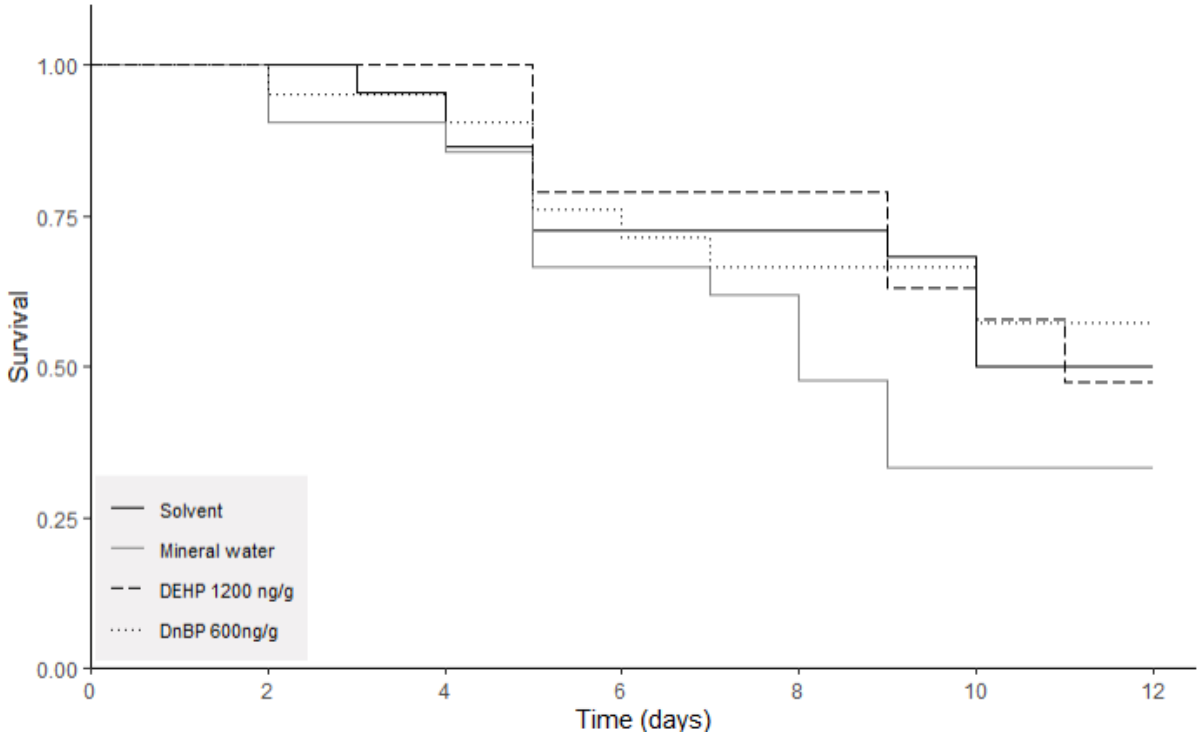


Fig. 6. Larval survival in solvent (N = 22) and water control groups (N = 21), as well as groups treated with DEHP 1200 ng/g (N = 19) or DEHP 600 ng/g (N = 21) over the 12 days. The black solid line shows the solvent control group, and the grey one the water control group.

3.2. Duration of larval stages

In vitro, the mean duration between grafting (6-day-old larvae) and defecation was a minimum of 9.94 days in the group exposed to DEHP 1200ng/g, and a maximum of 10.08 days in the water control group (Fig.7). No significant effect of the solvent, nor DnBP 600ng/g and DEHP 1200ng/g on the larval development duration was highlighted (GLMM: p-values>0.05 for all stages duration and total; Fig. 7, Table S4.1).

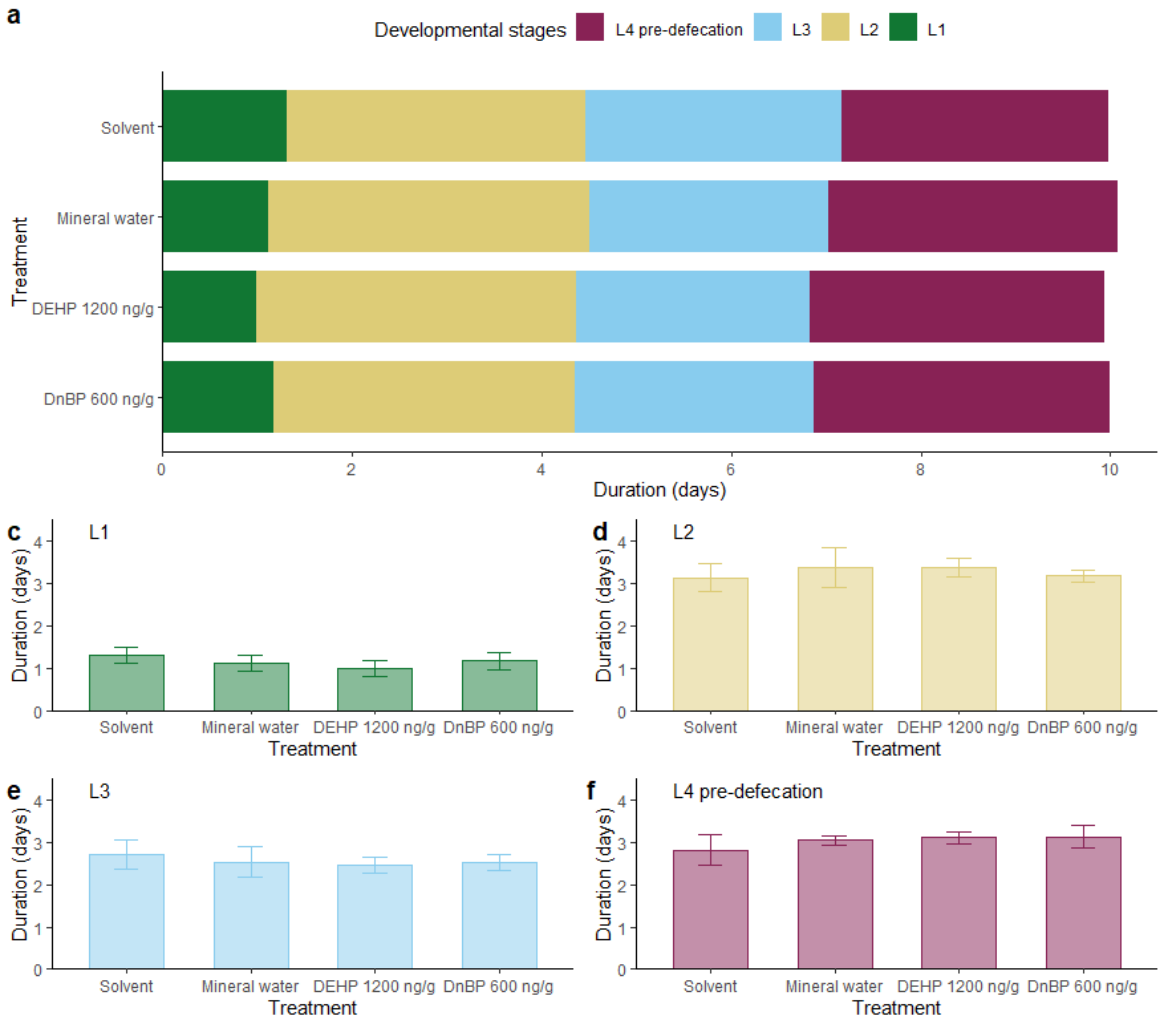


Fig. 7. Duration of larval development in the control and treatment groups in days. a) Total duration of the development in the four groups, b) L1 stage duration in control solvent (N=22), control water (N=21), DEHP 1200ng/g (N=19) and DnBP 600ng/g (N=21) groups, c) L2 stage duration in control solvent (N=16), control water (N=15), DEHP 1200ng/g (N=16) and DnBP 600ng/g (N=15), d) L3 stage duration in control solvent (N=16), control water (N=10), DEHP 1200ng/g (N=13) and DnBP 600ng/g (N=14), e) L4 stage duration in control solvent (N=11), control water(N=6), DEHP 1200ng/g (N=9) and DnBP 600ng/g (N=10). Columns show the mean value of larval stage duration with error bars indicating standard error. Colours indicate the larval stage considered.

3.3. Larval mass and lipidic content

The final stage larval mass did not differ between the treatment groups (GLMM: Chisq. = 1.66, d.f. = 3, p-value = 0.6449; Fig. 8a), nor did the lipid ratio (GLMM: Chisq. = 2.46, d.f. = 3, p-value = 0.4823; Fig. 8b).

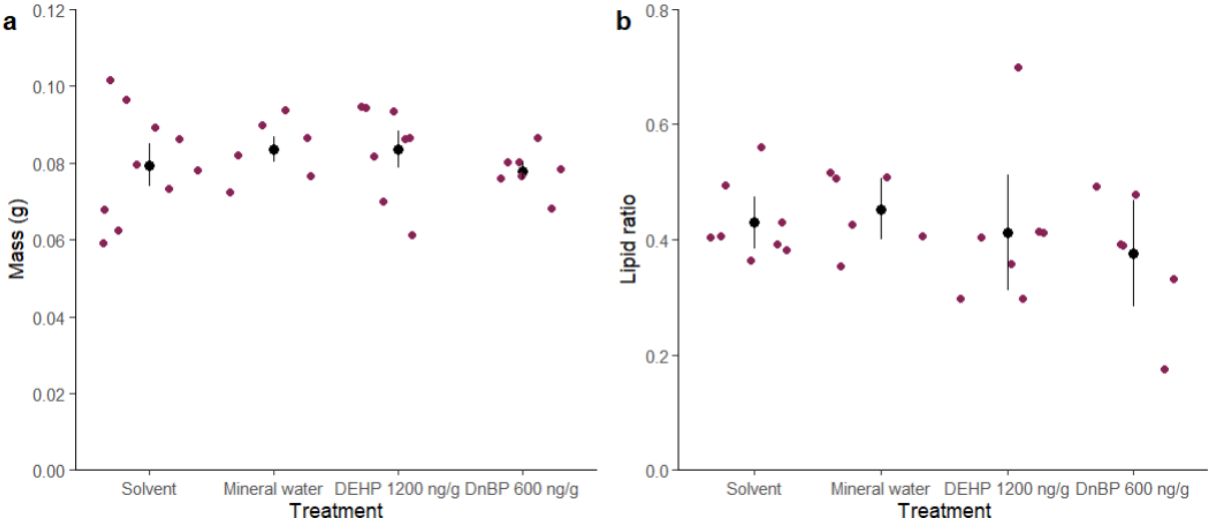


Fig. 8. Larval traits measured at the end of the experiment in group exposed to the control solvent (N=11), control water (N=6), DEHP 1200ng/g (N=9) and DnBP 600ng/g (N=10). a) Mass and b) the lipid ratio of L4 larvae measured at the end of the experiment. Black dots indicate mean values for each treatment, with data points showing individual values and error bars showing the 95% confidence interval.

3.4. Gene expression

AADAT relative gene expression significantly differed between groups (GLMM: Chisq. = 19.282, d.f. = 3, p-value = 0.0002). Indeed, relative expression of this gene was lower in the water control and DEHP 1200ng/g groups compared to solvent control and DnBP 600ng/g groups (p-values<0.05; Fig. 9a). The relative expression of *78C* also differed between groups (GLMM: Chisq. = 9.18, d.f. = 3, p-value = 0.0270), with the highest values found in the water control group compared to all others (p-values<0.05). However, the relative expression of *LLDH*, *ECR*, *E75*, *FAS* and *ACC* did not differ between groups (Fig. 9; Table S4.2).

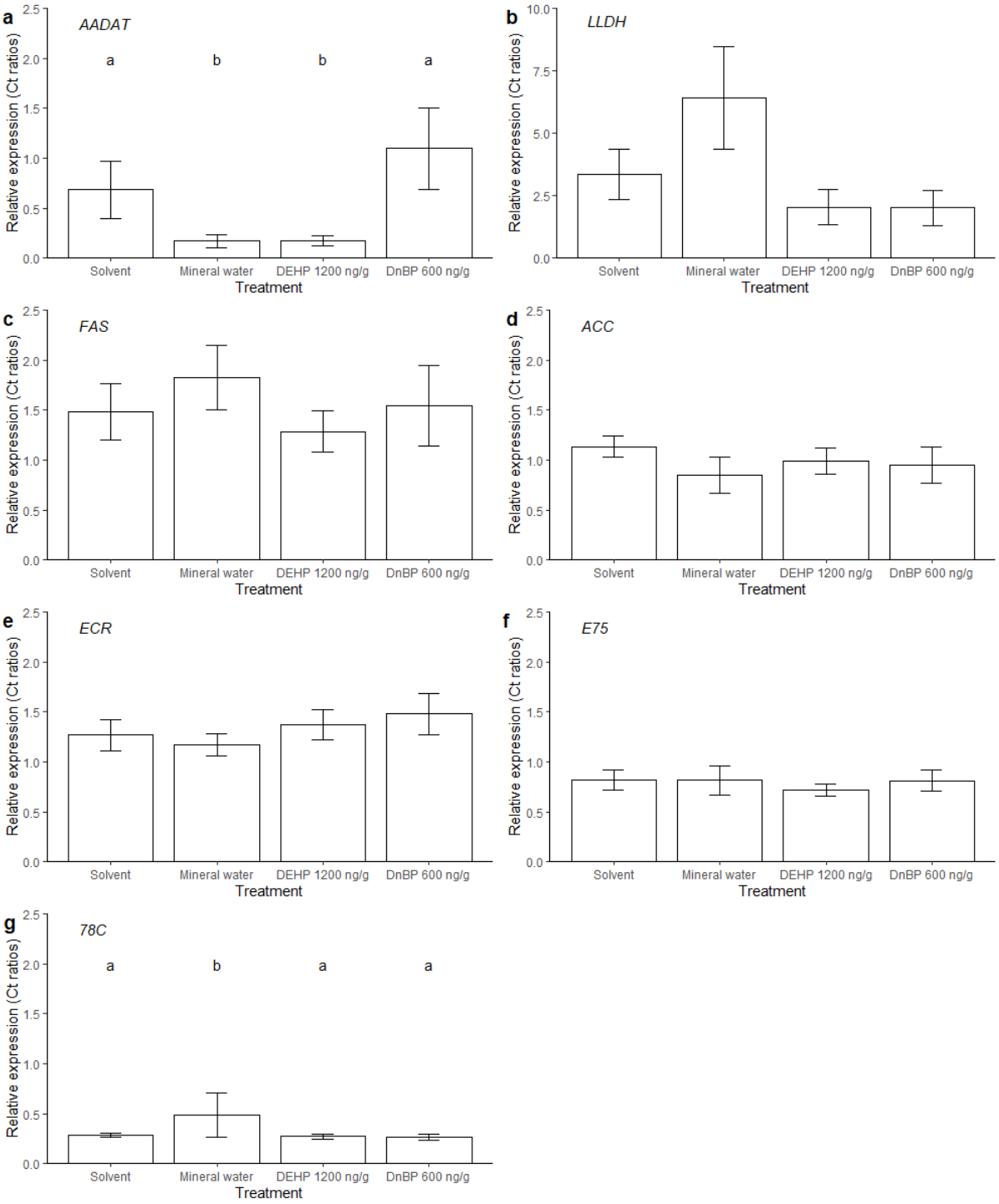


Fig. 9. Normalised relative expression of the analysed genes, i.e., a) *AADAT*, b) *LLDH*, c) *ECR*, d) *E75* and e) *78C* in abdominal tissues of workers exposed to control solvent, control water, DnBP 600ng/g, DEHP 1200ng/g or the mixture. Columns indicate mean normalised relative expression with bars showing standard error. *RPL13* and *PLA2* are used as reference genes. Groups not sharing the same letters are significantly different at p-value<0.05 (GLMM, gamma family, log-link function). No letter indicates no difference between groups.

4. Discussion

In this experiment, an *in vitro B. terrestris* larval rearing method was adapted from protocols by Kato et al. (2022) and Siviter et al. (2020a) to investigate the impact of phthalate DnBP and DEHP direct exposure on worker larval development. Overall, the results were negative with no impact on survival, instar duration, final stage mass and lipid content. However, we observed an increase in the relative expression of *AADAT*, likely caused by the solvent with a buffering effect of DEHP 1200ng/g. The relative expression of the nuclear receptor, *78C* gene, was downregulated in all groups except the mineral water control, an effect likely caused also by the solvent.

4.1. Absence of DnBP impact on larval traits

In this experiment, phthalate exposure had no detectable effect on any of the larval traits measured, including larval mass and development duration. These negative results contrast with previous findings, where exposure of workers to DnBP 600 ng/g in microcolonies led to a reduced drone emergence but increased larval and emerged drone mass (Chapter 3, Part I). In that earlier study, we hypothesised that the effects observed on the offspring could result either from indirect brood exposure via contaminated workers, or from transgenerational impacts inherited through disrupted hormonal or metabolic pathways in exposed mother (Chapter 3, Part I), as observed in *Caenorhabditis elegans* (Li et al., 2018) and in *D. melanogaster* (Chen et al., 2019) exposed to DEHP. Phthalate exposure has also been shown to increase larval development duration in *Drosophila melanogaster* (DnBP) and *Spodoptera littoralis* (DEHP) (Atlı, 2010; Avilès et al., 2019). Such an effect could indirectly result in a longer larval food intake period and, consequently, increased adult body mass, since larval food consumption is tightly correlated with final size, and therefore mass, in bees (Couvillon and Dornhaus, 2009). However, in the present experiment, where larvae were

directly exposed to phthalates through food, no changes in larval duration or mass were observed. While this absence of effects might support the transgenerational impact hypothesis explaining the microcolony results, several methodological differences between experiments are more likely responsible for the discrepancies observed.

First, the larval exposure routes likely differed between the present study and the previous microcolony experiment. In the earlier experiment, only the workers caring for the brood were topically exposed to phthalates, meaning if those effects resulted from indirect larval exposure, this exposure was topical rather than oral (Chapter 3, Part I). In contrast, in the present experiment, larvae were directly exposed via food contaminated with phthalates. However, as shown by Avilès et al., (2020, 2019) in *S. littoralis*, spiking food with phthalates presents technical challenges that can cause substantial discrepancies between theoretical and actual concentrations. These authors suggested that part of these inconsistencies might result from contamination of the feeding medium coming from the lab environmental pollution. Similarly, in our case, the preparation of the larval feeding solutions involved multiple manipulation steps and a greater number of ingredients (water, methanol, sucrose, pollen, yeast extract and sodium caseinate) than those used for topical exposure (water, methanol and the phthalate molecule) in the previous assays, increasing the risk of contamination from manipulation. Moreover, the pollen used, although sourced from certified organic apiaries, was not analysed for phthalate contamination before the experiment. Various levels of DnBP and DEHP have been reported in *Apis mellifera* pollen loads (Martín-Gómez et al., 2024), suggesting that background contamination of the pollen matrix cannot be excluded and may have contributed to the variability in exposure, and therefore of the observed effects. Considering that DnBP exhibited a non-linear dose-

response pattern in effects observed on adult workers (Chapter 2), variations in the actual concentrations of DnBP could have masked potential effects in larvae too.

In addition, in the microcolony exposure experiment, we did not monitor the fraction of phthalate transferred from the exposed worker to the brood, and larvae could have been exposed to quantities that varied greatly from the quantities administered to the workers. For example, in *Spodoptera frugiperda* larvae produced by parents exposed to DnBP, the molecule accumulated and was almost 100X higher than in the parents (Filho et al., 2013). Generally speaking, contaminant transfer dynamics, especially transgenerational transfer and through contact, have not been thoroughly studied in social and eusocial systems, and pollutant risk assessment would greatly benefit from such investigation, as already suggested for pesticides in the honeybee model (Sponsler and Johnson, 2016). On the other hand, as phthalates are highly lipophilic, they could be trapped by and accumulate in the waxy structures enclosing the larvae (Gómez-Ramos et al., 2016) and expose the progeny through contact. In contrast, in the present study, the larvae were exposed through food by depositing a droplet on their abdomen, which likely resulted in a combined contact and oral exposure. The toxicity of chemicals can greatly vary depending on the exposure route in bees (Linguadoca et al., 2022). As phthalates are lipophilic, they tend to be trapped in the cuticle of insects (Lenoir et al., 2014) and could directly affect them once absorbed. When ingested, phthalates could first be detoxified before entering the body through the intestinal tract. For example, the lowering of phthalate impacts by gut microbiome, especially *Lactobacillus* sp., has been previously highlighted in vertebrates (Shi et al., 2020; Tian et al., 2019; Zhao et al., 2021), and could also play an important role in phthalate detoxification in bees. Moreover, in the honeybee, microbiome composition was shown to enhance the expression of the enzymes of the Cytochrome P450 family (Wu et al., 2020), notably involved in the detoxification of phthalates

in vertebrates (Liu et al., 2021a). Moreover, larval microbiota in bumblebees is socially transmitted (Zhang and Zheng, 2022). However, in the larval plate, the sampled larvae had only short access to alloparental care from workers and might not have acquired the same microbiota as larvae from social setups like microcolonies, which could also explain differences in results obtained in both experiments. Yet, only a few studies have explored the role of microbiome composition in pollutant detoxification and sensitivity in bees (reviewed in Peterson, 2024; e.g. in bumblebees: Rothman et al., 2019).

Second, in the microcolony design, the effects observed occurred in male larvae (Chapter 3, Part I), as workers reared in microcolonies can only lay unfertilised drone-destined eggs (Duchateau and Velthuis, 1989). Here, the larvae exposed were destined for worker production as laid by the queen in commercial colonies that were purchased young, and used in the first days upon reception. The impact of anthropogenic pollutants on invertebrates can affect individuals differently depending on their sex (McClellan-Green et al., 2007). For example, in *S. littoralis*, larvae orally exposed to DEHP, accumulation of the pollutant differed between female and male chrysalis and adults (Avilès et al., 2019). Sex-biased impact of phthalates and other endocrine disruptors has also been highlighted in rodent models and humans (Palanza et al., 2021). They are likely caused by sex-specific differences in contaminants uptake, energetic metabolism or differential activation of hormonal pathways throughout the lifecycle of males and females, both in invertebrates (McClellan-Green et al., 2007) and vertebrates (Palanza et al., 2021). Transgenerational impact also depends on the sex of the exposed parent and the sex of the progeny (Chen et al., 2019). For example, in *D. melanogaster*, transgenerational effects on progeny body weight caused by DEHP depended on the sex of the parents exposed (Chen et al., 2019). In *S. littoralis* N+1 females, which came from eggs laid by a contaminated mother, a decrease in longevity was observed along with a

lengthening of pupation time, particularly pronounced in N+1 females, compared to N+1 males of *S. littoralis* (Avilès et al., 2020). No data were available on eggs resulting from the mating of a contaminated male with an uncontaminated female. The difference in results observed between the experiments could be caused by variations effects of DnBP exposure in worker and drone-destined larvae.

In the relative gene expression analyses, a significant downregulation of *AADAT* was observed in the solvent control group and the DnBP-treated group compared to the water control and DEHP-treated group. The *AADAT* gene codes for a metabolic enzyme involved in multiple hormonal and immune pathways in bees (Doublet et al., 2017), suggesting a metabolic stress caused by exposure to methanol. Yet, this impact seemed counteracted by DEHP 1200ng/g, as *AADAT* relative expression in this group was maintained at the water control level. Therefore, DEHP exposure in larvae might have lowered the impact of methanol on these genes through a similar mechanism. However, this result contrasts with other work on early-life exposure to DEHP, which was recently found to upregulate the expression of kynurenine aminotransferase, a homologue enzyme to AADAT, in *Drosophila melanogaster* (Cai et al., 2025). In addition, the expression of 78C was also downregulated in all groups compared to the water control. 78C is a gene involved in the late steps of the ecdysteroid signalling cascade (Bonneton and Laudet, 2012), but this gene is also tightly linked to metabolic regulation (Li et al., 2025). This suggests that, although low (0.1%), developmental exposure to methanol might have induced metabolic stress in the larvae or might have been used as a resource by the larvae. We also observed a lower mortality rate in groups exposed to diets containing methanol, but this effect was not significant. Interestingly, no effect of long-term repeated exposure to the solvent had been found on workers and their progeny (Chapter 3, Part I), as well as on metabolic gene expression (Chapter 3, Part III).

4.2. In vitro *larval rearing methods*

After the twelve days needed to reach the L4 stage, 80% of mortality was reached in the water control group. The method used here was adapted from protocols suggested by Siviter et al. (2020a) and Kato et al. (2022), who respectively obtained lower mortality rates of around 40% and 75%, over 10 days of larval development. In Kato et al. (2022), all living larvae from cells containing multiple individuals (from L1 to L3) were sampled from commercial colonies of *B. terrestris* to experiment in a single sampling session, and the stages were identified *a posteriori*, based on the molt number. The larvae were fed three times a day with a diet supplemented with yeast extract and sodium caseinate, and the volume of solution provided increased with larval instar (Kato et al., 2022). In Siviter et al. (2020a), a sampling method similar to Kato et al. (2022) was used. In their protocol, larvae were fed four times a day with 2µL of pollen suspended in sucrose solution (Siviter et al., 2020a).

In the present study, we specifically selected larvae at known stages to allow a more accurate identification of the stages. Yet, the previous protocols of Kato et al. (2022) suggested a sampling of larvae comprised within multiple larval stages and identified instar *a posteriori*, which might have increased survival probabilities. In colonies of *B. terrestris*, survival in egg cells is rarely higher than 50% (Duchateau and Velthuis, 1988). Therefore, by selecting only larvae with an identified instar, we might have artificially decreased survival probabilities compared to Kato et al. (2022). However, Siviter et al. (2020a) used the same technique as Kato et al. (2022), but the mortality was comparable to our results, i.e. ~75%. In Siviter et al. (2020a), a cause of mortality might be the low feeding frequency. In *B. terrestris* colonies, the intervals between larval feeding by the workers can last up to four hours, but each larva is fed every 20 minutes on average (Ribeiro et al., 1999). The low frequency of feeding used in hand-feeding methods imposed by technical possibilities could contribute to the increased mortality

observed in the plates. The supplementation of the diet used by Kato et al. (2022), but not by Siviter et al. (2020a), might have helped maintain more larvae alive during the experiment. Nevertheless, we used this supplemented diet in this study, and the survival rate obtained remained low.

Another explanation of the high mortality observed in the present work could be related to the sampling method. We first emptied the colonies to identify new egg cells, before emptying them a second time to sample the larvae four days later. This might have caused stress during egg incubation, as workers maintain a stable temperature of 32 to 34°C in the colonies (Wynants et al., 2021). A short drop in temperatures in the brood caused by the removal of all workers and the queen during egg incubation might have weakened the future larvae. In Kato et al. (2022), they anaesthetised the whole colony using CO2 before sampling the larvae in a single sampling session. With the aim of investigated possible endocrine disruption due to phthalate exposure in larvae, we decided to avoid CO2 anaesthesia as it is known to disrupt endocrine-regulated behaviour and metabolic processes, by decreasing egg production and increasing larval ejection by the workers in *B. terrestris* (Poissonnier et al., 2015), as well as lipid body storage in queens of *B. impatiens* (Amsalem and Grozinger, 2017). More recently, Richardson et al. (2025) managed to maintain control group survival above 50% in *B. impatiens* larvae while using a four-feeding method of Siviter et al. (2020a) with a similar pollen-syrup diet. However, to sample the larvae in the colonies, they again used a single sampling session and sorted the larvae into instar groups based on size as described in Rozen et al. (2018). While this technique could be useful for *B. terrestris* to avoid disturbing the colony, to our knowledge, no study has yet described the development of *B. terrestris* in terms of larval morphological traits. Such information would allow an accurate selection of the stage without stressing the colony.

Finally, microbiota composition plays a key role in larval survival and development, and in social insects, is mainly acquired through contact with nest material and with the nurse workers during feeding (Zhang and Zheng, 2022). While in a colony, the microbiota composition changes and the diversity increases throughout the development (Guo et al., 2023), the *in vitro* larvae rearing does not allow social transmission of this microbiota. Therefore, supplementation of the larval feeding diet with a beneficial microbial community might enhance survival in *in vitro* protocols, as shown in full colony supplemented with *Lactobacillus melliventris* extracted from *B. terrestris* worker gut (Yu et al., 2024).

4.3. Conclusion

In vitro rearing method adapted for *B. terrestris* would allow a better understanding of the effects of various stress exposure on the larvae by separating the brood-care dimension from larval physiology. While these methods need further adjustments, they could provide great insights into *B. terrestris* development and pollutant risk assessment. In this study, while we did not find any effects of developmental larval exposure to phthalates, we highlighted that solvent use in treatment preparation might affect the larvae, stressing the need for proper control groups in future standardised procedures.

PART III: Expression of genes involved in crucial hormonal signalling pathways in Bombus terrestris *workers is disrupted after repeated exposure to di-2-ethylhexyl phthalate and di-n-butyl phthalate**

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Abstract

Di-2-ethylhexyl phthalate (DEHP) and di-n-butyl phthalate (DnBP) are ubiquitous environmental contaminants, used as plasticisers and adjuvants in products like paint, pesticides and detergents. Both compounds have been detected on the cuticle of social insects, including ants, honeybees, and bumblebees. Evidence is emerging that these endocrine disruptors affect invertebrate metabolism and development through the disruption of conserved metabolic and hormonal pathways, including the insulin-related and ecdysteroid signalling cascades. Previous results showed that repeated exposure to field-realistic doses of DEHP, alone or in mixture with DnBP, reduced survival in microcolonies. While environmental DnBP exposure induced mortality in isolated workers, it did not affect survival in the long run in the microcolonies. However, worker exposure to DnBP 600ng/g increased the abdominal lipid ratio in workers and the body weight of their male offspring. To investigate the causes of organism-level impact at the molecular scale, the expression of genes involved in key endocrine pathways was quantified in *B. terrestris* workers repeatedly exposed to DEHP and DnBP in queenless microcolonies over 35 days. Overall, DEHP 12ng/g, DnBP 600ng/g and the mixture were the groups in which the greatest molecular disruption happened. DEHP downregulated the *USP* gene, encoding the heterodimerisation partner of the ecdysone receptor, and upregulated the insulin receptor gene (*ILPR*). DnBP at 600 ng/g significantly downregulated the expression of the *LIRP* gene, an insulin-like peptide of B. terrestris. These findings provide molecular evidence that phthalates disrupt key endocrine pathways in social insects, with cascading effects at the individual level that could ultimately affect the social lifestyle and the pollination services.

Keywords: plasticizers; lipid metabolism; insulin-like; endocrine disruption; bumblebee; gene expression

1. Introduction

Di-2-ethylhexyl phthalate (DEHP) and di-n-butyl phthalate (DnBP) have been widely used as adjuvants in household and cosmetic products, and are used as plasticisers, often in high proportions (Fierens et al., 2012; Koniecki et al., 2011). For example, DEHP can make up over 40% of the weight of polyvinyl chloride (PVC) medical devices (Tickner et al., 2001). As these molecules are not chemically bound to the polymer matrix, they readily leach into the environment (Suhrhoff and Scholz-Böttcher, 2016). Their extensive use has led to global pollution of soils, water bodies, and the atmosphere (Net et al., 2015a; 2015c). While free molecules of phthalates can be easily broken up by ultraviolet rays or high temperatures, their half-lives are counted in months once bound to particulate matter or lipidic materials (Kickham et al., 2012). Phthalates can therefore contaminate the lipid-rich cuticle of social insects (Lenoir et al., 2012). For example, DEHP and DnBP have been detected on honeybees and their collected pollen, as well as in wax combs (Gómez-Ramos et al., 2019, 2016; Martín-Gómez et al., 2024). More recently, they have also been measured on the cuticle of wild *Bombus terrestris* sampled in the urban environment (Cuvillier et al., in prep).

Previous results showed that repeated exposure to field-realistic doses of DEHP, either alone or in combination with DnBP, reduced worker survival in queenless *B. terrestris* microcolonies (Chapter 3, Part. I). Regarding DnBP, short-term exposure in isolated workers decreased survival but enhanced cold stress resistance, likely through metabolic modulation (Dewaele et al., 2025; Chapter 2, Part II). Under social conditions, repeated exposure to DnBP did not affect survival, but increased worker abdominal lipid content and resulted in the production of male offspring with higher body weight (Chapter 3, Part. I). These observations suggest that phthalate exposure may disrupt key endocrine and metabolic pathways in bees. Both DEHP and DnBP are recognised endocrine-disrupting compounds (Gore et al., 2015), and

increasing evidence indicates that phthalates can similarly affect invertebrates by targeting conserved hormonal and metabolic systems (Crane et al., 2022).

In vertebrates, DEHP alters the expression of the peroxisome proliferator-activated receptor (PPAR) and its dimerisation partner, the retinoid X receptor (RXR), central regulators of lipid homeostasis and energy metabolism (Desvergne et al., 2009; Lee et al., 2016). Indeed, foetal and postnatal DEHP exposure has been linked to metabolic disorders such as childhood obesity, type II diabetes, and metabolic syndrome (Kagawa, 2024; Kim and Park, 2014; Perez-Diaz et al., 2024). Although insects lack the vertebrate PPARγ/RXR pathway, homologous receptors are involved within the ecdysteroid signalling cascade (Bridgham et al., 2010; Hong and Park, 2010; Jones and Jones, 2000). Hence, DEHP has also been shown to disrupt this pathway by altering the expression of genes coding for the ecdysone receptor heterodimer (ECR/USP) and its downstream nuclear receptor E75 in *Spodoptera littoralis* (Avilès et al., 2020, 2019) and *Chironomus riparius* (Herrero et al., 2017; Planelló et al., 2011). Similarly, in *B. terrestris* workers exposed to low environmental doses of DEHP, the gene expression of nuclear receptor of this cascade, i.e. E75 and 78C, was downregulated 72 hours post-exposure (Chapter 2, Part II). In invertebrates, the ecdysteroid signalling pathway is known to regulate metabolic processes linked to reproduction and development (Bonneton and Laudet, 2012). In addition, ECR inactivation has been associated with lifespan modulation in *Drosophila melanogaster* (Simon et al., 2003; Tricoire et al., 2009). Therefore, a disruption of the ecdysteroid signalling cascade by DEHP could underlie the long-term reduction in worker survival observed in the previous study (Chapter 3, Part I).

DnBP has been highlighted as a potent disruptor of the insulin-related signalling pathway, notably contributing to insulin resistance and the aggravation of type II diabetes (Deng et al., 2018), and also increasing the risk of metabolic syndrome (Perez-Diaz et al., 2024)

in humans. In invertebrates, similar molecular disruptions have been reported, suggesting a conserved mode of action across taxa. For instance, DnBP exposure altered lipid storage and metabolism in *Daphnia magna* (Seyoum and Pradhan, 2019) and significantly reduced lifespan in *Caenorhabditis elegans* and *Drosophila melanogaster* (How et al., 2019; Liu et al., 2024). These effects were directly linked to disruptions of the insulin-related signalling pathway (How et al., 2019; Liu et al., 2024). In addition, in *Drosophila melanogaster*, both DnBP and DEHP were found to increase starvation resistance by impairing lipid mobilisation processes, an effect associated with altered expression of insulin pathway genes (Cao et al., 2016; Williams et al., 2016). Furthermore, multigenerational effects on lipid metabolism have been documented in *D. magna* following chronic exposure to DnBP, suggesting potential heritable or epigenetic consequences of this disruption (Wei et al., 2025). In social insects such as bees, the insulin signalling pathway is deeply integrated into multiple physiological and behavioural processes beyond metabolic regulation (Kocher and Kingwell, 2024). It is involved not only in maintaining lipid storage homeostasis but also in regulating key social life-history traits such as reproductive division of labour, foraging behaviour, and developmental plasticity (Kocher and Kingwell, 2024). In *Bombus* and *Apis* species, this pathway modulates worker foraging onset (Ament et al., 2008), regulates queen diapause preparation (Jedlička et al., 2016), and influences caste-specific larval development (De Azevedo and Hartfelder, 2008). Therefore, the obesogenic effects previously observed in *B. terrestris* workers and their male offspring (Chapter 3, Part I) may result from a conserved endocrine disruption of the insulin signalling pathway by DnBP. Such interference could have cascading consequences for individual physiology, social organisation, and colony functioning.

In this study, we aimed to investigate the potential disruption of two highly conserved hormonal signalling pathways, i.e., the ecdysteroid and insulin signalling cascades, in *Bombus*

terrestris, and to assess whether phthalates induce molecular effects similar to those previously described in other invertebrates. To do so, we analysed the expression of genes involved in these pathways, as well as genes associated with metabolic processes and stress responses, in abdominal tissues of workers from the microcolony assay described in Chapter 2, Part I. Given that DEHP has been shown to affect the expression of genes involved in the ecdysteroid signalling cascade in insects (e.g., Avilès et al., 2019), we focused on key components of this hormonal cascade, including the genes coding for the Ecdysone Receptor complex (*ECR* and *USP*), and the early and early-late nuclear receptor genes *E75* and *78C*. To explore potential effects on the insulin-signalling pathway, we selected *B. terrestris* genes coding for the locust insulin-related peptide-like gene (*LIRP*), two insulin receptor genes (*ILPR* and *InR-1*), and the forkhead box protein O transcription factor (*FOXO*), which acts as a major downstream regulator of this pathway (Jedlička et al., 2016). To further assess potential impacts on energy metabolism, we included genes coding for enzymes central to *de novo* lipid synthesis in social bees, i.e. the Fatty acid synthase (*FAS*) and Acetyl-CoA carboxylase (*ACC*) (Furse et al., 2023). Additionally, we examined the expression of genes encoding the Kynurenine/α-aminoadipate aminotransferase (AADAT) and the L-lactate dehydrogenase (LLDH), two metabolic regulators whose expression reflects metabolic stress and interconnections with immune and endocrine signalling pathways in bees (Cuvillier-Hot et al., 2024; Doublet et al., 2017; Rondeau et al., 2025; Vasamsetti et al., 2024).

Finally, because these metabolic and endocrine pathways are tightly interconnected (Antonova et al., 2012; Bonneton and Laudet, 2012), we also used an integrated biomarker response approach to allow a more comprehensive description of the effects. Specifically, we calculated the IBRv2 index (Sanchez et al., 2013) for each treatment to integrate molecular

responses across pathways and exposure doses, providing a comprehensive view of the overall disruption patterns induced by phthalates.

2. Material and methods

2.1. Model species and microcolony design

The Buff-tailed bumblebee, *Bombus terrestris*, is a eusocial bee species forming annual colonies of more than one hundred workers organised around a single queen. The queen mates in autumn and enters a winter-long diapause, before starting its primary active life in early spring during a solitary phase, after which the first emerged workers take over foraging and brood care until late summer (Ghisbain et al., 2025; Rasmont et al., 2021). This species is commonly found across Europe and is important for the pollination of wild and managed plants (Rasmont et al., 2021). As a domesticated species, colonies are commercially available year-round (Velthuis and van Doorn, 2006), allowing for controlled laboratory experiments, particularly in assessing the effects of pollutants (Klinger et al., 2019).

Five queenright colonies were obtained from Biobest (Westerlo, Belgium). Upon arrival, 70 workers were randomly selected from each colony and split in 7 groups of 10 workers. We thus established 35 queenless microcolonies, each consisting of ten workers housed in boxes (10×16×16 cm) as described in Chapter 3, Part I. Microcolonies were maintained in constant darkness and controlled temperature and humidity conditions (25 °C; 60% relative humidity). They were supplied ad libitum syrup (sugar water 50% w/w) and pollen (Composition: Rosaceae, Malus/Pyrus sp. 40% and Salicaceae, Salix 27%; from Abeille Heureuse, Strasbourg, France; Barraud et al., 2022), which were replenished every other day. In this experiment, all workers were exposed to phthalates once a week, monitored during 35 days, as described in Chapter 3, Part I, and sampled at the end of the experiment.

2.2. Treatment preparation

As detailed in Chapter 3, Part I, the 35 queenless microcolonies were evenly assigned to seven treatment groups with colony origins balanced across treatment groups (five microcolonies per treatment): two control groups (one exposed to solvent diluted in mineral water, and one exposed only to mineral water), two di-n-butyl phthalate (DnBP) treatment groups (60 and 600ng/g of body weight, i.e. b.w.), two di-(2-ethylhexyl) phthalate (DEHP) treatment groups (12 and 120ng/g b.w.), and one mixture (DnBP 60ng/g b.w. and DEHP 120 ng/g b.w.). As the solvent control group did not differ significantly from mineral water control for the metric previously, i.e., resource collection, offspring production, offspring weight and abdominal lipid ratio of adults tested in Chapter 3, Part I, only the solvent group was used as the control in the present study.

DnBP (CAS No. 84-74-2) and DEHP (CAS No. 117-81-7) were purchased in liquid form (both >99%) from Sigma-Aldrich. Method for treatment solutions preparation can be found in Chapter 3, Part I. Briefly, all dilutions were prepared assuming an average B. terrestris worker fresh weight of 0.25g (Dewaele et al., 2024). The chemicals were first diluted in methanol (CAS No. 67-56-1, VWR) to ensure complete solubility, and then further diluted in mineral water to ensure low methanol concentration in the treatment solutions (0.07%). For the mixture preparation, both single-molecule solutions underwent the same initial dilution steps; in the final steps, the two were combined, and the volume was adjusted with mineral water to achieve a mixture containing DEHP at 120ng/g b.w. and DnBP at 60ng/g b.w. The control solution consisted of 0.07% methanol diluted in mineral water.

As phthalates are present in laboratory atmosphere and soft plastic materials (Cao et al., 2022; Feng et al., 2020; Reid et al., 2020), as well as in both tap and bottled water (Bach et al., 2020; Luo et al., 2018). We therefore chose to use water marketed in glass bottles (SPA

Reine, SPA NV, Bruxelles, Belgium) to avoid unintended contamination. Moreover, we decontaminated glass and metal materials by successive hexane and acetone baths and, when possible, by calcination. When plastic materials could not be avoided, we preferably used hard plastic materials (Cao et al., 2022).

2.3. Exposure protocol

Once a week, all workers were exposed to their assigned treatment solution using a protocol adapted from OECD guideline No. 246 (OECD, 2017b), as detailed in Chapter 3, Part I. Briefly, workers were first anaesthetised by chilling: groups of two were placed in glass jars and plunged in ice (maximum ten minutes). A 2 µL droplet of either the control or one of the treatment solutions was then applied to the hairless dorsal part of the thorax. Once recovered at room temperature, all workers from a microcolony were returned to their box simultaneously. The final exposure took place five days before tissue sampling.

2.4. Abdominal tissue sampling and RNA extraction

At the end of the experiment, four workers from each microcolony were anaesthetised by chilling. All dissections were performed on ice under a binocular microscope using precision tweezers and scissors rinsed with RNase Zap (Sigma-Aldrich). Once the worker was anaesthetised, the abdomens were carefully separated from the head and thorax using precision scissors. Head and thorax were immediately discarded into 100% ethanol. The digestive tract, reproductive system and venom gland were then removed by gently pulling the sting from the abdominal cavity with precision tweezers, and these tissues were also discarded. The collected abdomens were then individually flash-frozen and stored at -80°C until extraction.

For RNA extraction, each abdomen was opened, and gently crushed using a pestle in the lysis buffer from Nucleospin RNA Plus kit (Macherey-Nagel; Germany). As cuticle

fragments remained after crushing, the lysate was centrifuged (30s at 11000g) before being transferred onto the gDNA removal columns. The remaining steps of the total RNA extraction followed the kit protocol. The final elution was performed in 30µL of pure water, and was repeated. RNA concentration of both elutions was measured using a Qbit 4 fluorometer (Thermofisher). The most concentrated elution was kept, and only samples yielding more than 60ng/µL of total RNA were retained for subsequent analyses. The samples were stored at -80°C until further use.

2.5. cDNA production and qPCR

650 ng of total RNA was used as a template for cDNA synthesis with the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific), following the manufacturer's instructions. Quantitative Real-time PCR (qPCR) was performed on a Lightcycler 480 (Roche Applied Science). Each reaction was run in a final volume of 10 µL containing 2 µL of cDNA (diluted 1:3), 1 µM of each specific primer, and 5 µL of SYBR Green master mix (BioRad). All reactions were carried out in technical duplicates for each sample and each gene. The cycling conditions were as follows: initial denaturation at 95 °C for 30 s, followed by 40 cycles of 95 °C for 15 s and the primer-specific annealing temperature (Tm) for 30s, with a single fluorescence read taken at the end of each cycle. A melting curve analysis was performed at the end of the run by heating to 95 °C at a ramp rate 0.11°C/s with continuous fluorescence reading. Relative gene expression data were produced using Roche Applied Science LightCycler 480 software v 1.5.1. First, the concentration thresholds (Ct) of target and reference genes were produced using the Second Derivative Maximum method. Then, Ct ratios between each target gene and the two reference genes were calculated using the E-method, which enables correction for specific primer efficiency and normalisation using a standard sample (calibrator) identical between plates and for all genes (Tellmann, 2006). Two reference genes, the Ribosomal

protein L13 gene, *RPL13*, and the Phospholipase A2 gene, *PLA2*, were selected because they are amongst the most stable housekeeping genes expressed in the fat body tissues of *B. terrestris* (Horňáková et al., 2010). Working T_m (°C) were confirmed by PCR on standard samples using the same cycling conditions as described for the qPCR (See Table S5.1 for reference and target primer characteristics).

2.6. Statistical analyses

All statistical analyses were performed in R v4.4.1 (R Core Team, 2023). Relative gene expressions between treatment and control groups were compared using generalised mixed models (gamma distribution, log-link), with normalised C_t ratios as the response variable, treatment as the fixed effect and initial colony as a random effect using package "glmmTMB" v1.1.11 (Brooks et al., 2025). Post-hoc contrasts were performed between the control group and each treatment group with the package "emmeans" v1.10.5 (Lenth, 2023), and p-values were adjusted for multiple comparisons using Benjamini-Hochberg's method (i.e., "fdr"). To complete result interpretations, effect sizes "g" were calculated following Cohen's method with Hedge's correction for sample size, using package "effsize" v0.8.1 (Torchiano, 2020). The magnitude was interpreted as follows: g = 0.2 indicates a small effect, g = 0.5 a moderate effect and g = 0.8 a large effect (Nakagawa and Cuthill, 2007).

To assess global molecular disruption, the Integrated Biomarker Response index (IBRv2) was calculated following the method of Sanchez et al. (2013), and applied exactly as explained in Chapter 2, Part II. Briefly, for each gene with an effect size > 0.5 in at least one treatment group, normalised C_t ratios for each treatment were log-transformed, standardised across treatment groups, and expressed as deviations from the control (A). The IBRv2 score for each treatment was then obtained by summing the absolute deviations (|A|) across all selected genes, with higher values indicating greater overall disruption (see step-by-step

algebraic details in Chapter 2, Part II). Spiderchart showing A values were made using radarchart function from package 'fmsb' v0.7.6 (Nakazawa, 2024) and all the other graphs were made with 'ggplot2' package v3.5.1 (Wickham, 2016).

3. Results

Exposure to DEHP significantly affected USP expression in worker abdominal tissues (Chisq.=14.2, df. =3, p-value = 0.0026). This gene was significantly downregulated in workers exposed to DEHP 12ng/g compared to control (z ratio=3.63; Hedge's g = -0.83; $p_{vs.\ control}$=0.0008; Fig. 1b).

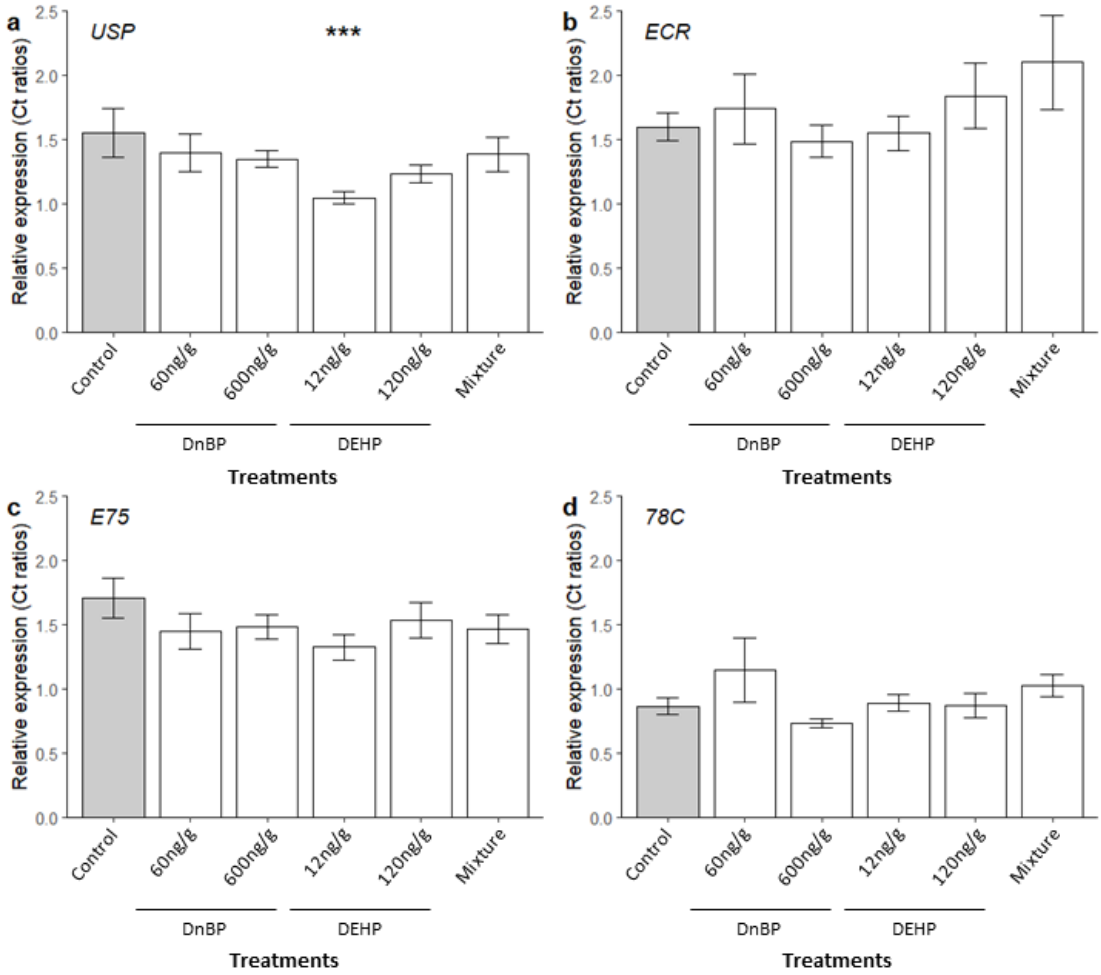


Fig. 1. Normalised relative expression of genes involved in the ecdysteroid-signalling pathway i.e., a) USP, b) ECR, c) E75, and d) 78C in abdominal tissues of workers exposed to control solution or DnBP 60ng/g, 600ng/g, DEHP 12ng/g, DEHP 120ng/g or the mixture (DnBP 60ng/g and DEHP 120ng/g). Columns indicate mean normalised relative expression with bars showing standard error. Significant differences with the control group (grey column) are indicated by ***p-value<0.001 (GLMM, gamma family, log-link function).

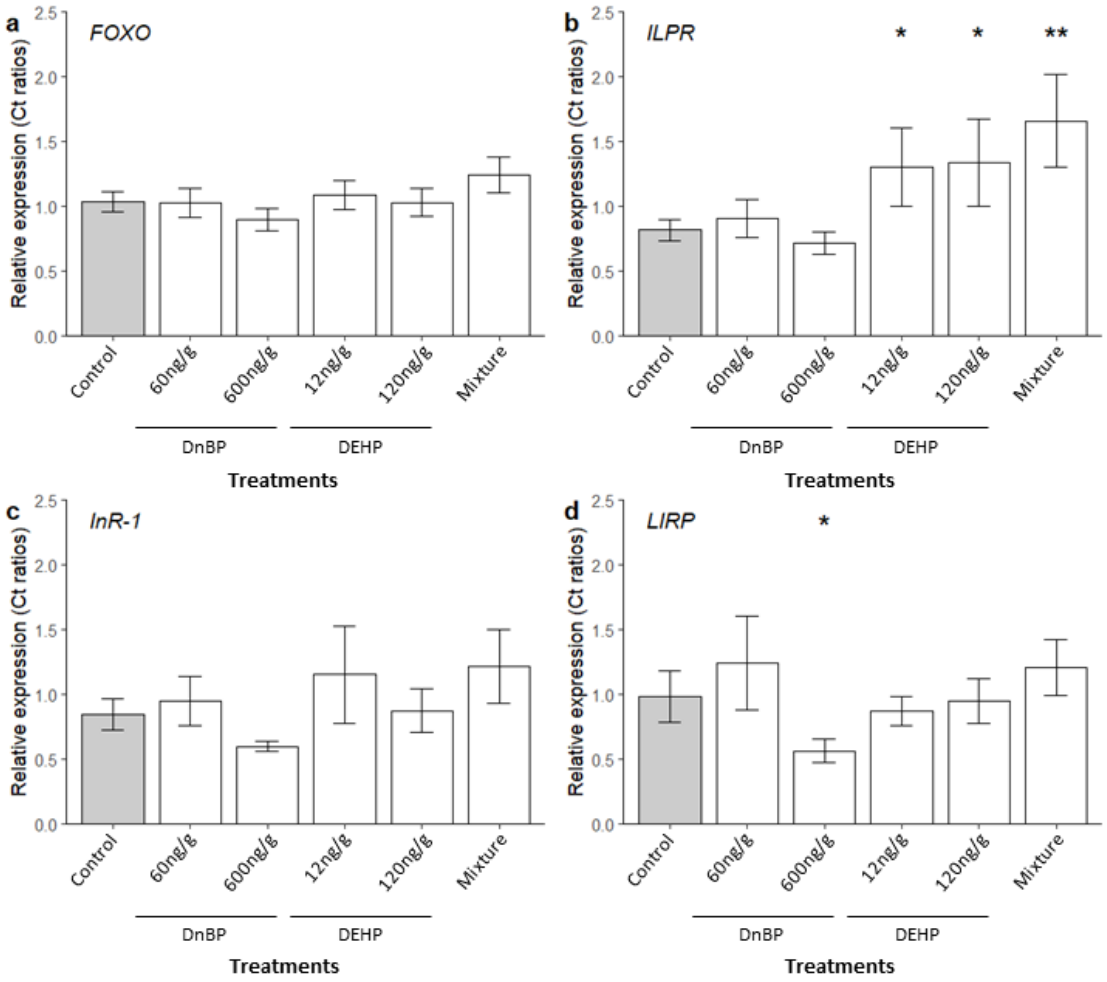


Fig. 2. Normalised relative expression of genes involved in the insulin-signalling pathway i.e., a) FOXO, b) ILPR, c) InR , and d) LIRP in abdominal tissues of workers exposed to control solution or to DnBP 60ng/g, 600ng/g, DEHP 12ng/g, DEHP 120ng/g or the mixture (DnBP 60ng/g and DEHP 120ng/g). Columns indicate mean normalised relative expression, with bars showing standard error. Significant differences with the control group (grey column) are indicated by *p-value<0.05 and **p-value<0.01 (GLMM, gamma family, log-link function).

Moreover, a non-significant downregulation of USP expression associated with a moderate effect size was also observed in the group exposed to DEHP 120ng/g (z ratio=1.68; Hedge's g = -0.50; $p_{vs.\ control}$=0.0584; Fig. 1b). DEHP exposure did not affect the expression of any of the other genes involved in the ecdysteroid-signalling pathway that were tested here (Fig. 1). Both DEHP doses significantly dysregulated the expression of ILPR (Chisq.=11.02, df.=3, p-value = 0.0116). ILPR expression was similarly up-regulated by DEHP 12 ng/g and DEHP 120 ng/g compared to the control (z ratio: DEHP 12ng/g = -2.11 and DEHP 120ng/g = -2.06; Hedge's g: DEHP 12ng/g = 0.55 and DEHP 120ng/g = 0.64; both pvs. control<0.05; Fig. 2b). The

expression of the other genes involved in the insulin-signalling pathway tested here was not affected by DEHP exposure, nor was the expression of genes related to metabolism (Fig. 2 and 3).

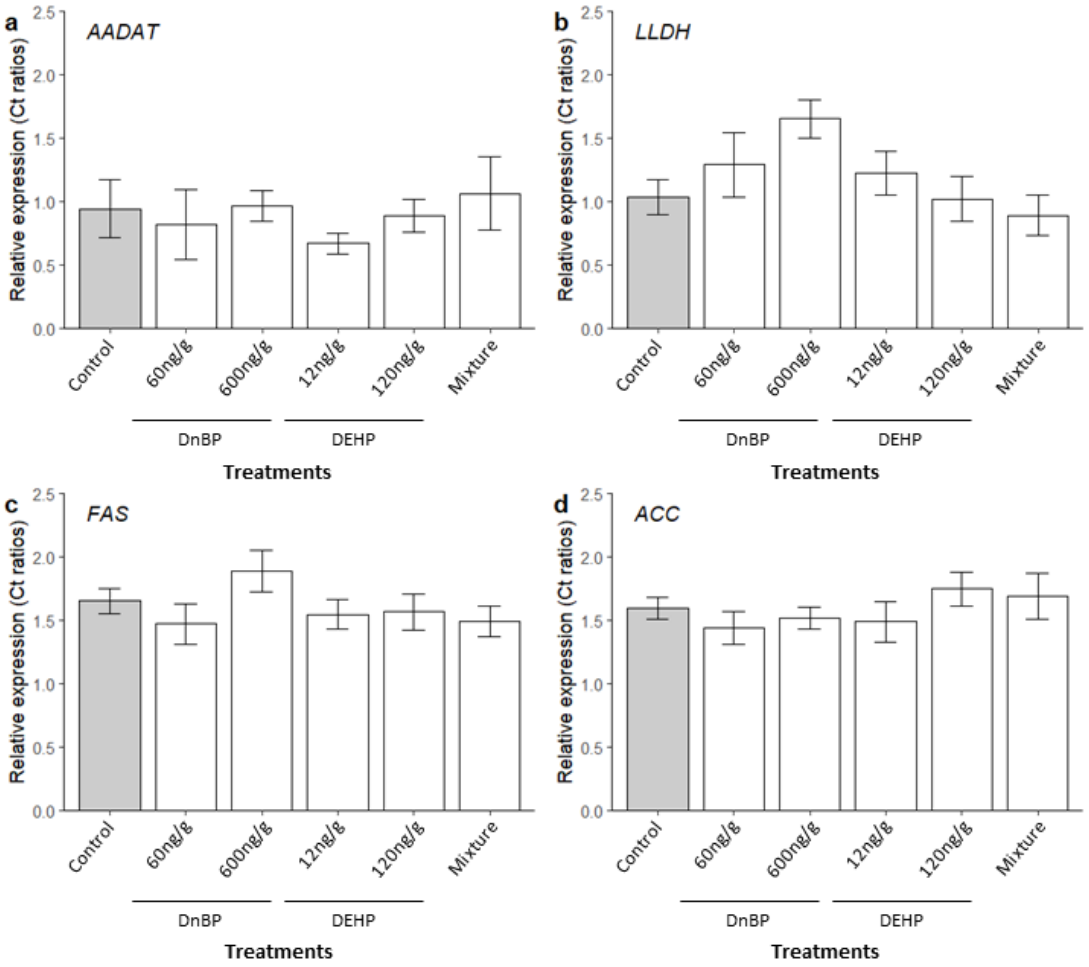


Fig. 3. Normalised relative expression of genes involved in metabolic pathways i.e., a) AADAT, b) LLDH, c) FAS, and d) ACC in abdominal tissues of workers exposed to control solution or to DnBP 60ng/g, 600ng/g, DEHP 12ng/g, DEHP 120ng/g or the mixture (DnBP 60ng/g and DEHP 120ng/g). Columns indicate mean normalised relative expression with bars showing standard error (control group is shown in grey).

DnBP 60ng/g did not significantly affect the expression of any of the tested genes (all p-values>0.05; Fig. 1 to 3). We nonetheless observed a non-significant dysregulation of the expression of the nuclear receptor 78C, with the highest mean relative expression found in workers exposed to DnBP 60ng/g (z ratio = -2.02; Hedge's g=0.37; $p\text{-value}_{vs.control}$=0.1288; Fig. 1d). DnBP 600ng/g downregulated the expression of all tested genes involved in the Insulin-

signalling pathway, i.e. FOXO, ILPR, InR-1 and LIRP (Fig. 2). However, this effect was only significant for LIRP gene expression (z ratio=2.60; Hedge's g = -0.66; $p_{vs.\ control}$=0.0281; Fig. 2d). In workers exposed to DnBP 600ng/g, a non-significant downregulation associated with a moderate effect size was also observed for InR-1 (z ratio=1.54; Hedge's g = -0.64; $p_{vs.\ control}$=0.1834; Fig. 2b), as well as a non-significant up-regulation associated with a large effect size for LLDH (z ratio=-2.20; Hedge's g=1.08; $p_{vs.\ control}$=0.0833; Fig. 3b).

For all genes, the effect size associated with the mixture (DEHP 120ng/g and DnBP 60ng/g) never exceeded the effect size associated with its single-molecule counterparts, except for ILPR expression (Fig. 4). Not only did mixture exposure significantly up-regulate ILPR (z ratio=-3.19; $p_{vs.\ control}$=0.0043; Fig. 2b), but the effect size associated (g=0.88) was greater than for DnBP 60ng/g (g=0.19) and DnBP 600 ng/g (g=0.64; Fig. 4) administered as single-molecule solutions.

The IBRv2 were calculated with all genes except AADAT and ACC, for which the effect sizes did not exceed 0.4 in any treatment groups (Fig. 4). The group expoed to the lowest DEHP concentration, i.e., 12 ng/g, showed the higher index value (IBRv2 = 8.7) compared to the group exposed to DEHP 120ng/g (IBRv2 = 6.8; Fig. 5a,b). The genes that deviated the most in both groups were ILPR ($A_{DEHP\ 12ng/g}$= 0.63; $A_{DEHP\ 120ng/g}$=0.71), USP ($A_{DEHP\ 12ng/g}$= -1.42; $A_{DEHP\ 120ng/g}$=-0.85) and E75 ($A_{DEHP\ 12ng/g}$= -1.11; $A_{DEHP\ 120ng/g}$=-0.59; Fig. 5a,b). However, the IBRv2 calculated for the group exposed to DnBP increased with the dose (Fig. 5c,d). In the group exposed to DnBP 60ng/g (IBRv2 = 7.7), the gene that deviated the most was 78C (A= 1.01; Fig. 5c). In the group exposed to DnBP 600ng/g (IBRv2 = 12.2), all genes involved in the insulin-signalling pathway were negatively deviated (A for FOXO = -0.65; A for ILPR = -1.47; A for InR-1 = -1.74; A for LIRP = -1.82), as well as the E75 gene (A=-0.70), while the LLDH and FAS genes were positively deviated (A for LLDH = 1.08 and A for FAS = 0.53; Fig. 5d).

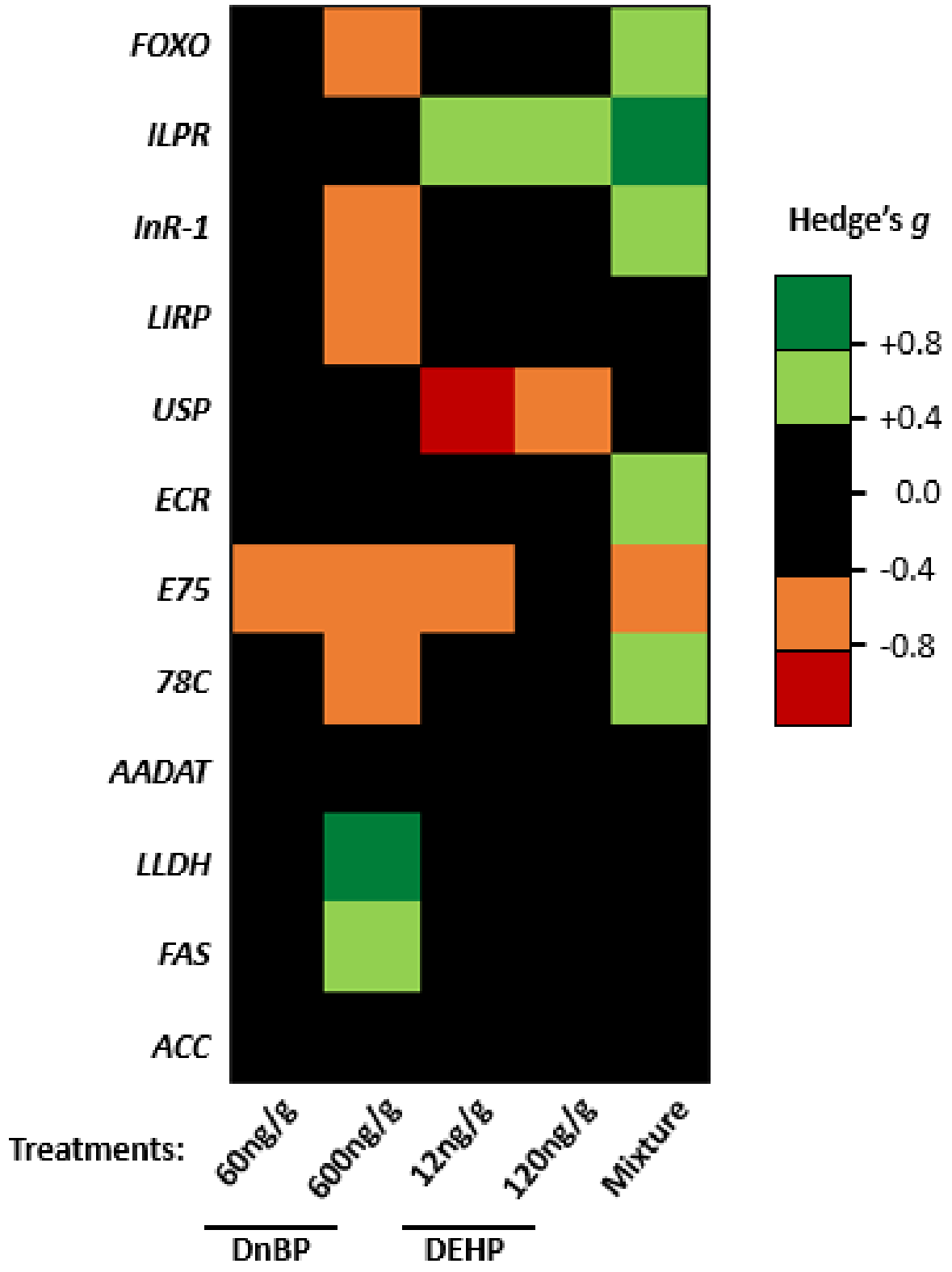


Fig. 4. Heatmap of the Hedge's g effect size calculated for all tested genes (in rows) in the treatment groups (in columns). Colour indicates the magnitude of the effect size, with red scale showing downregulation and green scale upregulation compared to the control.

The IBRv2 calculated for the mixture group (IBRv2 = 9.7) was higher than for the DEHP 120ng/g and DnBP 60ng/g single-molecule groups (Fig. 5b,c,e). The deviation profile showed a positive deviation of the expression of all genes involved in the insulin-signalling pathway (A for FOXO = 0.47; A for ILPR = 1.47; A for InR-1 = 0.73; A for LIRP = 0.85) and the ECR gene (A=0.83), but a negative deviation of the expression of LLDH (A=-1.09), 78C (A=-0.62) and E75 (A=-0.76) in this group.

4. Discussion

The expression of genes related to key hormonal pathways and metabolic functions was analysed in workers maintained in microcolonies and weekly exposed to environmentally relevant doses of phthalates during 35 days. Selected genes were involved in the ecdysteroid-signalling (*ECR*, *USP*, *E75*, *E78*), the insulin-signalling pathways (*LIRP*, *ILPR*, *InR-1*, *FOXO*), immunometabolism (*AADAT*, *LLDH*), and lipid metabolism (*FAS*, *ACC*). DEHP caused the highest level of disturbance at the lowest tested dose (12ng/g), downregulating *USP* and upregulating *ILPR*. *ILPR* was also upregulated at 120ng/g DEHP, both alone and in mixture with DnBP 60ng/g. In contrast, DnBP only affected the expression of some genes at the highest dose tested. Exposure to DnBP 60ng/g had no impact on the expression of the tested genes, while exposure to DnBP 600ng/g downregulated all insulin-signalling related genes, with a significant reduction in *LIRP* expression. The mixture-treated group had the highest disturbance level. The tested genes that were involved in lipid metabolism were not impacted by phthalate exposure.

4.1. *DEHP affect key hormonal signalling pathways and associated cross-play*

Single gene expression levels and the IBRv2 calculation showed that global molecular disruption levels caused by DEHP decreased with increasing doses, and the expression of two genes, ILPR and USP, was significantly disrupted.

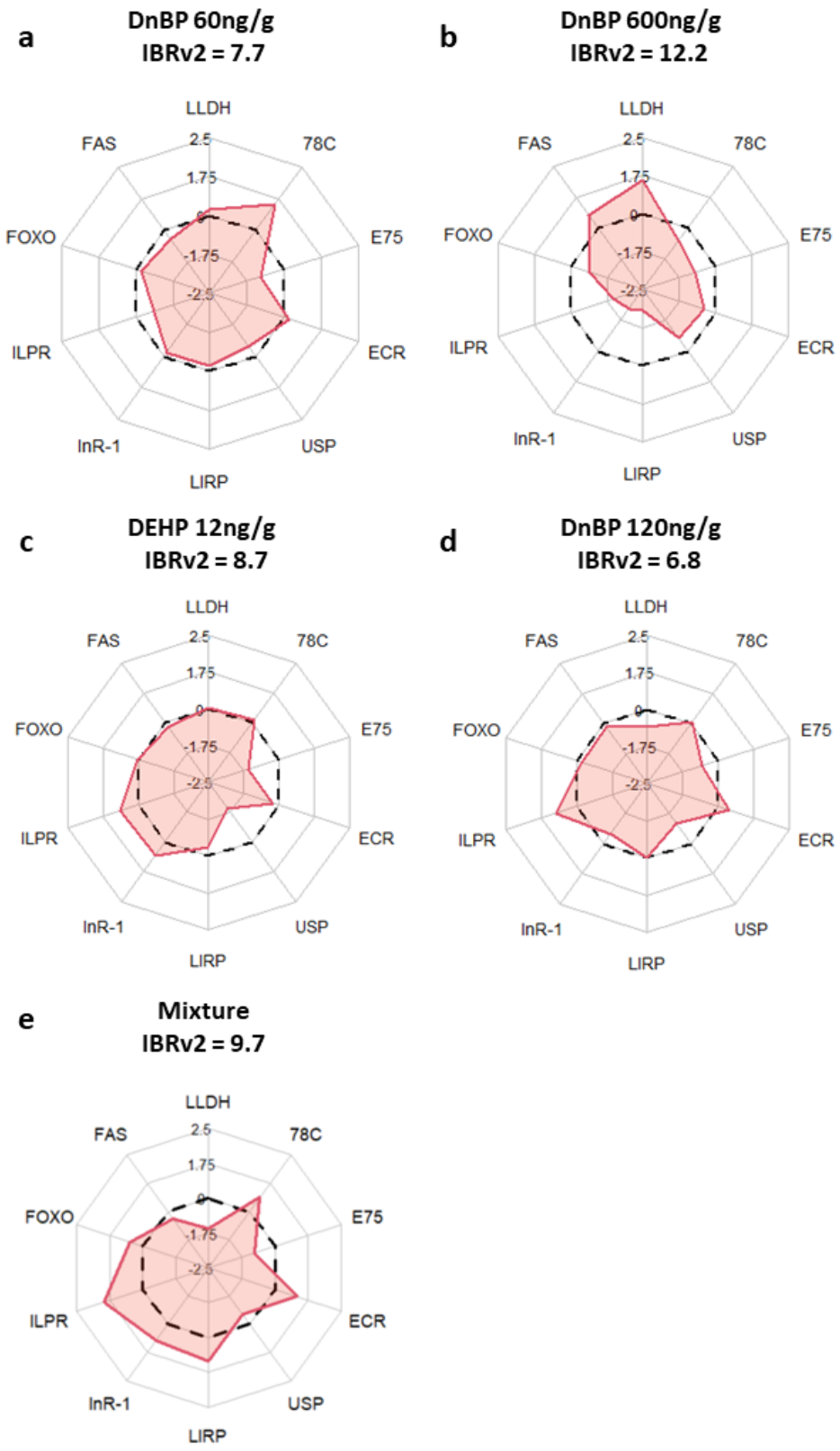


Fig. 5. Spiderchart showing deviation indices "A" calculated for each gene in groups exposed to a) DnBP at 60ng/g or b) at 600ng/g, and c) DEHP at 12ng/g or d) at 120ng/g, as well as e) the mixture of DnBP 60ng/g and DEHP 120ng/g compared to control, with IBRv2 index of exposure. Dashed lines show the reference at 0 (i.e. no change in gene expression relative to reference genes expression), and on all plots, the center is at -5.

Repeated contact exposure of workers to low-doses of DEHP downregulated *USP* gene. A similar downregulation of this gene was found in *Spodoptera littoralis* larvae exposed to food contaminated with DEHP (19g.7µg/g and 4.3mg/g) for at least 48 hours (Avilès et al., 2019). This supports the hypothesis that DEHP directly affect the components of the ecdysone receptor within the ecdysteroid-signalling cascade. However, the expression of the first component of the ecdysone receptor, *ECR*, was unaffected in this study. This contrasts with previous findings in *C. riparius* (Herrero et al., 2017; Planelló et al., 2011), yet in *S. littoralis* larvae, only the highest dose of DEHP (4.3mg/g) affected ECR expression (Avilès et al., 2019). Indeed, while previous results were obtained at higher doses, only the lowest dose of DEHP affected USP gene expression in this study. Similarly, in the rat, *in-utero* exposure to low-dose DEHP was shown to affect the expression of the *RXR* genes (Lee et al., 2016), the vertebrate ortholog of *USP* (Jones and Jones, 2000). In mammals, this gene is involved in regulating lipid homeostasis through lipid transport and metabolism, depending on its dimerisation partner (He et al., 2013). In insects, the EcR-USP complex, when bound to 20-hydroxyecdysone, is indirectly involved in lipolysis (Wang et al., 2010). Although no change in abdominal lipid proportion was previously detected in workers from the same microcolonies exposed to DEHP 12ng/g, the technique used does not differentiate the different types of lipids (Chapter 3, Part I), and more precise investigations might be needed to assess the impact.

On the other hand, USP within the ecdysteroid-signalling cascade is primarily linked to reproduction and development-related processes (Henrich and Beatty, 2010). For example, changes in *USP* expression influence juvenile hormone production, which controls the production of vitellogenin, the main yolk protein, in female insects (Elgendy et al., 2014). In honeybees, *USP* downregulation in larvae also led to delayed pupation (Barchuk et al., 2008). Alterations of USP expression as observed here could affect development and egg production

in *B. terrestris*. However, no impact on offspring production was detected in the exposed microcolonies (Chapter 3, Part I). Moreover, in previous experiments, we directly exposed *B. terrestris* worker larvae to DEHP-contaminated food, but observed no significant changes in larval development duration (Chapter 3, Part II). Yet, this experiment was performed with a higher dose (DEHP 1200ng/g of food), which might explain the discrepancies. Exposure of larvae during their development to low-DEHP doses could provide further insight into the long-term consequences of USP downregulation caused by this pollutant in bees (Chapter 3, Part II).

On the other hand, all worker groups exposed to DEHP treatments (12ng/g, 120ng/g and the mixture) upregulated the expression of *ILPR*. A similar upregulation of an insulin-like peptide receptor was observed on *D. melanogaster* exposed to DEHP, associated with a decrease in circulating sugar levels without observed changes in fat stores (Cao et al., 2016). Cao et al. (2016) suggested that long-term chronic exposure to low-dose DEHP either directly altered insulin-signalling-related gene expression, which in turn changed the sugar levels, or decreased carbohydrate metabolism, which could have indirectly influenced gene expression in *D. melanogaster* (Cao et al., 2016). Although circulating sugar levels were not measured, no change in fat stores was observed in DEHP-exposed workers from the same microcolonies (Chapter 3, Part II), and here, we observed that genes directly involved in lipid metabolism were not impacted. This supports the hypothesis of a direct effect on the insulin-signalling pathway.

We reported previously that workers exposed to DEHP 120ng/g alone and in mixture with DnBP 60ng/g had a lower survival (Chapter 3, Part 1). The insulin-signalling pathway is notably involved in the regulation of lifespan-related genes, as the inhibition of this cascade activates the transcription FOXO (Dillin et al., 2002), which regulates the expression of stress

resistance genes, a mechanism conserved across invertebrate species (Altintas et al., 2016). Considering that lifespan reduction caused by DEHP is dependent on FOXO activity in *D. melanogaster* (Liu et al., 2024), the increased mortality previously observed in bumblebee microcolonies might result from the same processes (Chapter 3, Part I). Here, we did not observe any change in FOXO gene expression, but the upregulation of *ILPR* in DEHP-treatment groups could have led to a phosphorylation of FOXO, inhibiting it without changing its expression (Antonova et al., 2012). However, in the group exposed to DEHP 12ng/g, an upregulation of *ILPR* expression was observed without a corresponding increase in mortality (Chapter 3, Part I). A hypothesis explaining this absence of increased mortality could involve the combined disruption of USP and ILPR, as USP protein interacts with FOXO in insects (Koyama et al., 2014; Lin et al., 2018). This interaction has been described in larval stages as they play a key role in regulating ecdysteroid biosynthesis and larval metamorphosis through the detection of critical weight (Koyama et al., 2014; Lin et al., 2018). In adult insects, the impact of this interaction has not been described, but in the *Drosophila* fly, alteration of the ecdysone signalling system has been notably linked to extended lifespan and increased stress resistance (Uryu et al., 2015). Therefore, a combination of the effects on *ILPR* and *USP* could have lowered ecdysone biosynthesis and helped maintain control-level survival throughout the experiment in the group exposed to DEHP 12ng/g.

4.2. DnBP specifically target the insulin signalling pathway with metabolic consequences

For workers exposed to DnBP, the greatest disruption level (the highest IBRv2) was observed at the highest dose tested (600 ng/g). In this group, genes coding for both insulin receptors, *ILPR* and *InR-1*, and the insulin-like ligand, *LIRP*, were downregulated, with a significant reduction in *LIRP* expression. These results support that the disruption of the insulin-signalling pathway by DnBP is conserved across species groups, from vertebrates,

including humans (Deng et al., 2018), to invertebrates, such as *D. melanogaster*. In the latter species, dietary exposure to DnBP during development downregulated insulin-like peptide expression at concentrations of 15–150 nM (Williams et al., 2016), consistent with the downregulation observed here. In that species, transcriptional changes were associated with increased starvation resistance and lipid accumulation (Williams et al., 2016). Similarly, our previous work on the same *B. terrestris* microcolonies reported that DnBP 600 ng/g increased the abdominal lipid content of workers (Chapter 3, Part II). A similar increase in fat storage has also previously been observed in *Daphnia magna* which was associated with a disruption of genes related to fatty acid metabolism (Seyoum and Pradhan, 2019). However, in the present study, although FAS was slightly upregulated, no significant changes were observed in *FAS* or *ACC* expressions, two key genes involved in d*e novo* fatty acid synthesis (Furse et al., 2023). Taken together, these findings point to a disruption of the insulin-signalling pathway by DnBP, linked with increased lipid storage in *B. terrestris* workers that was not related to modification of the lipid synthesis processes. A hypothesis could rather be related to a slowed catabolism, for which we did not test related genes.

In eusocial bees, the insulin pathway not only regulates lipid metabolism but is also involved in caste-specific development, physiology and behaviour (Kocher and Kingwell, 2024). For example, the knockdown of genes involved in the insulin-signalling pathway in honeybee queen-destined larvae produced queens with worker-like traits, i.e., smaller size throughout development, and emerging as adults with fewer ovarioles, worker-specific morphology with a corbicula on the hind leg and lacking mandibular notch, a queen-specific morphological trait lacking mandibular notch (Wolschin et al., 2011). In pre-diapausing queens of *B. terrestris and B. ignitus,* the expression of insulin-like peptides and receptors is upregulated, which is likely helping in the preparation for the long starvation of the

hibernation period (Jedlička et al., 2016; Sasaki et al., 2021). Moreover, insulin signalling functions as a nutrient-sensing mechanism, influencing foraging behaviour, but also the ageing process and lifespan in honeybees (Ihle et al., 2019). Although further work is needed to establish direct links between insulin-pathway disruption and social performance in *B. terrestris* and other social bee species, our results suggest that DnBP exposure could affect traits central to the eusocial system, such as caste differentiation and task-related behaviour, with potential consequences for colony performance and pollination services.

4.3. Repeated exposure to a mixture of phthalates results in combined effects

In this study, workers exposed to the mixture of DnBP (60ng/g) and DEHP (120ng/g) showed greater overall disruption (IBRv2 = 9.7) than those exposed to each molecule alone (IBRv2 = 7.7 and 6.8, respectively). The disruption profile of the mixture combined mild dysregulation of both insulin- and ecdysteroid-related genes, with a significant upregulation of *ILPR*, consistent with the DEHP 120 ng/g group. Previous work showed that exposure to the same mixture dysregulated resource collection towards a greater syrup collection and lowered offspring production (Chapter 3, Part II). One possible explanation of this result was that lower survival reduced brood care, as often highlighted after exposure to pesticides (Ceuppens et al., 2015). Nonetheless, the lower survival observed in the DEHP 120 ng/g group did not affect offspring production, despite comparable mortality to the mixture group (Chapter 3, Part II). An alternative explanation is a stronger toxic effect when both molecules act in combination. The present results therefore support this hypothesis. In this case, workers may have prioritised detoxification over brood care, collecting syrup for energy rather than pollen for larvae (Chapter 3, Part II). Interestingly, short-term exposure of isolated workers to the same mixture treatment has shown contrasting results, with DEHP lowering DnBP toxicity (Dewaele et al., 2025). In the rat, parathion toxicity was lowered when administered in

combination with DEHP exposure (Srivastava et al., 1976). Similarly, development on food contaminated with mixture of Cadmium and DEHP caused around three times less mortality in *S. littoralis* adults than in groups exposed to Cadmium alone (Humann-Guilleminot et al., 2024). These short-term observations could have led to the conclusion that DEHP could enhance detoxification processes. However, here, DEHP in combination with DnBP appeared to exacerbate molecular disruption and impact offspring after repeated exposure rather than mitigate phthalate impact.

Overall, these results further highlight the importance of testing pollutant mixtures under long-term, more realistic exposure (Jatkowska et al., 2021). As bees can be exposed to phthalates in combination with pesticides and other anthropogenic pollutants (Gómez-Ramos et al., 2016), chronic exposure to mixtures could induce a trade-off between detoxification and reproduction, ultimately weakening bee population sustainability.

CHAPTER IV: General discussion

General discussion

The past decades have seen a global decline in the terrestrial insect populations in terms of diversity but also abundance, with strong evidence in North America and Europe (Hallmann et al., 2017; van Klink et al., 2020). Particularly, insect pollinators such as butterflies (Lepidoptera: Warren et al., 2021), syrphids (Syrphidae: Powney et al., 2019) and bees (Apoidea: Zattara and Aizen, 2021) are affected by this decline, which is also impacting their functional importance in natural ecosystems (IPBES, 2016). The causes seem plural, but are mainly anthropogenic (climate change, urbanisation, water and air pollution, pesticide use, etc; Wagner et al., 2021). Among these drivers of decline, chemical pollution is often cited as an important factor. Yet, most of the studies investigating its effects on invertebrates focused on pesticide exposure, and other pollutants have received less interest.

Plastic additives like phthalates, which were described as emerging pollutants some decades ago, are now ubiquitously found in water bodies, agricultural and urban soils (Bergé et al., 2013; Mansuri et al., 2025; Net et al., 2015b). Moreover, insects such as ants and honeybees were found to be contaminated by phthalates (Lenoir et al., 2012), as their cuticle can trap these lipophilic compounds (Lenoir et al., 2014). To our knowledge, only three studies using the honeybee as a model have been published. The first two assessed the repellent effects of fixatives Dimethyl phthalate and di-n-butyl phthalate (DnBP), and found no sign of detection by the honeybee (Free et al., 1983; Sahebzadeh et al., 2009). The third evaluated the toxicity of DnBP and dietylHexyl phthalate (DEHP) on bees, using an *in-silico* approach on the honeybee, predicting that DnBP could be more toxic than DEHP (Arulanandam et al., 2022). In this context, the evidence of phthalate contamination of hive material and workers in the honeybee (Gómez-Ramos et al., 2019, 2016; Martín-Gómez et al., 2024) and of *Bombus terrestris* worker cuticles (Cuvillier et al., In prep.) prompted us to initiate an assessment of

phthalate impacts on bees, and more specifically on *B. terrestris*, a common model in bee ecotoxicology.

First, using a short-term toxicity standardised protocol, we highlighted negative effects on the survival of *B. terrestris* workers within the first 72 hours following a single exposure to environmental doses of DnBP, that was buffered by social context. DnBP toxicity also interacted with cold stress with low-doses lowering cold-stress induced mortality but not high doses. Associated with these results, we observed a metabolic disruption likely caused by DnBP, while it did not affect the immune response of the isolated workers. In contrast, DEHP exposure did not impact survival but reduced DnBP toxicity when administered in a mixture. While exposure to DEHP alone did not affect immunity or metabolism, low-dose exposure disrupted the expression of genes linked to the ecdysteroid-signalling pathway, a key hormonal cascade regulating insect development, reproduction and olfaction.

Secondly, we investigated the impact of long-term repeated exposure to the same environmental doses in queenless microcolonies. This experiment showed that DEHP alone, or in mixture with DnBP, increased worker mortality in the long run. The mixture further impaired offspring production and resource collection, while DnBP alone caused transgenerational obesogenic effects in workers and their drone progeny. At the molecular level, both molecules altered the expression of genes involved in the highly conserved insulin-signalling pathway, while DEHP disrupted again ecysteroid-signalling related genes.

Part. I – When **Bombus terrestris** *meets phthalates: the factors that matter*

Across experiments, the effects of phthalates were contrasted, depending on experimental design and key biological features of the species. Three main factors appeared to potentially influence the effects observed, namely (i) the duration and timing of exposure (short-term versus long-term, single versus repeated), (ii) the route of exposure (topical versus

oral), and (iii) the physiological or hormonal status of individuals, likely influenced by the social context, the lifestage and the reproductive status.

1.1. The timing of exposure

During this study, the exposure protocol varied in exposure frequency across experiments. In the first chapter, a standardised protocol for assessing the short-term effect of acute contact exposure to chemicals (OECD, 2017b) was adapted to expose workers in isolation and in microcolonies to environmental doses of phthalates. This method involved a single exposure event with observations of lethal and sub-lethal effects during the following 96 hours post-exposure. In the second chapter, workers reared in microcolonies were exposed similarly to the first chapter, but with repeated exposures once a week over 35 days, with lethal effects monitored throughout and sub-lethal effects assessed at the end. Interestingly, DEHP, which did not increase mortality in the short-term assay, nearly doubled worker mortality by the end of the microcolony experiment. Conversely, DnBP increased mortality in the short-term assay but did not affect survival in the long-term experiment. Pulse-like exposure is common in nature, and resulting effects can differ from those observed both after unique exposure and chronic exposure (i.e. long-term but constant; Reinert et al., 2002), as frequently assessed in phthalate studies. Indeed, a first exposure to those molecules can weaken or strengthen an organism, which may therefore influence its responses to subsequent exposures (Reinert et al., 2002). For example, DEHP acts on xenobiotic detoxification mechanisms by increasing their activity (as in Srivastava et al., 1976 already discussed in Chapter 2, Part I), but also by repressing it, and could therefore have modified the sensitivity to exposure that follows. Indeed, in vertebrates and invertebrates, DEHP notably impairs Glutathione S-transferase (GST) activity, a key enzyme involved in xenobiotic conjugation (Aydemir et al., 2025; Herrero et al., 2015). In larvae of the insect *Chironomus*

riparius exposed to DEHP, a decrease in Glutathione S-transferase (GST) activity was highlighted, which remained 24 hours after the end of exposure (Herrero et al., 2015). As this enzyme might play a central role in the detoxification of DEHP (Liu et al., 2021a), the first exposure could have compromised detoxification mechanisms involved in its own detoxification. In contrast, concerning DnBP, aside from the potential social mitigation mechanisms, repeated exposure could have activated detoxification pathways and promoted better worker survival (Reinert et al., 2002). Yet, the detoxification enhancement hypothesis was not supported by the results observed in the group treated with the mixture of DnBP and DEHP. Indeed, the mixture-treated group mortality remained at the level of the DEHP single molecule treated group. Hence, social mitigation remains the most probable hypothesis for the mitigation of DnBP toxicity.

In bees, xenobiotic exposure can also occur in pulses due to anthropogenic activities, weather conditions and foraging habits, making the investigation of the effects of repeated and chronic exposures crucial for comprehensive and realistic risk assessments (Raine and Rundlöf, 2024). To provide an overarching perspective, future research should focus on long exposure periods and could, for example, evaluate the impact of repeated exposure to phthalates during a whole queen-right colony lifecycle.

1.2. The route of exposure

In the experiments conducted during this thesis, workers were only exposed through contact with the cuticle, a likely route of exposure in insects (Lenoir et al., 2012), but no experiment has yet considered oral exposure to phthalates in adult bees. Although phthalate exposure in bees is likely to occur through atmospheric contamination (Lenoir et al., 2012), nectar and pollen could also represent potential exposure routes. While this has not been extensively studied, some plants can accumulate phthalates in their vegetative parts (Fan et

al., 2022; Sun et al., 2015), and honeybee-collected pollen was found to be contaminated by phthalates (Martín-Gómez et al., 2024). The route of exposure can strongly influence the impact of xenobiotics. For example, *Osmia bicornis* and *B. terrestris* were shown to be more sensitive when exposed orally than topically to various agrochemicals (e.g., Linguadoca et al., 2022; Marletto et al., 2003; Mokkapati et al., 2021). Indeed, depending on the exposure route, xenobiotic penetration, but also detoxification rates, can be modified along with their impact. While the cuticle may act as a barrier against topical exposure (Yahouédo et al., 2017; Yang et al., 2024), oral exposure allows more direct absorption through the digestive tract (Conner et al., 1978). For phthalates, this subject remains largely unexplored, but in the second chapter of this thesis, we scratch the surface of this topic. Indeed, one of our hypotheses concerning the increased weight observed in the drone offspring of microcolonies exposed to DnBP was the potential indirect larval exposure through contact of contaminated workers with brood material. However, when the exposure occurred orally in the *in vitro* rearing plate assay, these effects were absent in worker larvae. While sex-dependent differences, maternal exposure effects or solvent effects (discussed in Chapter 2) may explain these discrepancies, the impact of phthalates on *B. terrestris* might also differ depending on the route of exposure. Oral exposure could permit partial phthalate detoxification by the gut microbiome before their uptake (Gupta et al., 2025). However, this argument could also apply with the cuticular microbiota (Yang et al., 2024). Investigation of the impact of insect cuticular microbiota on xenobiotic detoxification is an emerging topic, with cuticular fungi found to increase resistance to insecticides in mosquitoes (Hu et al., 2025). Yet, highly lipophilic compounds are actively trapped by the insect cuticle (Lenoir et al., 2012). Therefore, phthalates may enter the cuticle more easily or more rapidly than the epithelial barrier of the digestive tract allowing more time for detoxification by the gut microbiota. As these are only hypotheses, further

research should therefore disentangle the effects of phthalates depending on the different routes of exposure for a better understanding of their impact on bees.

1.3. The hormonal status

The impact of endocrine-disruptors (EDs) obviously depends on the endocrine system status of the organism considered (Lister and Van Der Kraak, 2001). In this study, short-term DnBP toxicity was compared between isolated workers and workers maintained in queenless microcolonies. As discussed in Chapter 2, the hormonal dynamics during the competition phase at microcolony establishment could explain the absence of short-term DnBP toxicity in microcolonies. Hormonal status may also explain that the alteration by DnBP and DEHP exposure of the expression of genes related to ecdysteroid and insulin signalling pathways was observed in the adult workers from the microcolony experiment, but not in the one from the isolated setup. Indeed, workers in queenless microcolonies undergo a profound modification of their hormonal system, notably as they develop their ovaries once isolated from the queen, and have associated increased ecdysteroid titers (Bloch et al., 2000; Duchateau and Velthuis, 1989). Moreover, recent evidence shows that reproductive and non-reproductive workers have highly distinct gene expression patterns, notably in terms of vitellogenin and lipid metabolism-related genes in the brain and fat body (Prince et al., 2024), while the transcriptomic profile of reproductive workers is shared with that of reproductive queens (Harrison et al., 2015). Therefore, not only could hormonal status explain the discrepancies observed in our results, but these data obtained in queenless workers may be relevant to *B. terrestris* queens, or at least, might underline the need to evaluate phthalate impact on queens. Indeed, the transgenerational obesogenic effects of DnBP, coupled with insulin pathway dysregulation observed in queenless workers, could also affect queen fitness as this pathway plays a crucial role in diapause regulation (Jedlička et al., 2016). Yet, even

among queens, hormonal status must be considered as this pathway is likely involved in pre-diapause body resource storage, diapause and reproduction metabolism regulation (Jedlička et al., 2016). Therefore, while drones and non-reproductive workers have similar expression profiles for the insulin-related genes, it varies greatly between reproductive queens and non-reproductive workers, but also between queens of different stages (virgin, diapausing, and reproducing; Jedlička et al., 2016). Disruption of these pathways at different stages of a queen's lifecycle could lead to divergent outcomes, including obesogenic impact on the progeny (Chapter 3, part I), but also on the pre-diapausing queens with deleterious impact on diapause survival (Treanore and Amsalem, 2020). Therefore, endocrine disruptors (EDs) testing on bees highlights the importance of considering the endocrine status of the individual exposed to understand the impact of a xenobiotic.

As of today, *B. terrestris* and *Apis mellifera* workers are widely used as surrogates in pesticide ecotoxicology because of their commercial availability and the existence of standardised protocols that ease the experiments (EFSA et al., 2023). Yet, their role as surrogate is increasingly discussed. Indeed, interspecific variations in sensitivity have been documented (Arena and Sgolastra, 2014; Dewaele et al., 2024; Uhl et al., 2016). The role of bee body mass (Dewaele et al., 2024) and body surface-to-volume ratio (Pamminger, 2021), specific differences in detoxification mechanisms, body size, cuticle thickness and haemolymph pH have been thoroughly discussed as factors that might explain caste-, sex-, and species-specific sensitivity towards pesticides in bees (Dewaele et al., 2024; Gekière et al., 2024b; Hayward et al., 2019; Linguadoca et al., 2022). As these studies usually compared isolated workers of a eusocial species with reproductive females of solitary bee species, accounting for hormonal state (e.g., through hormonal titers or related gene expression) would add a critical parameter to pollinator ecotoxicology and seems crucial to thoroughly

describe EDC impact on managed and wild bees. While the use of traditional surrogate species is increasingly debated, the long-term results presented in this thesis on workers of *B. terrestris*, particularly queenless ones, should serve as an early warning for other bee groups. They emphasise the urgent need to test multiple species and castes, while explicitly incorporating hormonal status as a determinant of sensitivity to EDCs, and potentially other xenobiotics.

Part 2 – Disrupted and conserved hormonal pathways seek caring environmental legislation

The hormone-signalling pathways are complex and tightly coordinated to ensure a transfer of information between the external environment and the organism's physiological and behavioural responses (Kuppusamy et al., 2009; Tata, 2017). Many hormonal pathways present components and functions that are conserved across animal species, demonstrating their stability and importance throughout evolution (Kleine and Rossmanith, 2016). These pathways notably involve hormone receptors, which can be triggered by low and precise concentrations of hormones produced in more or less distant tissues (Tata, 2017; Vandenberg et al., 2012). A cell response is then activated or inhibited, and can be both tissue-specific and time-dependent (Tata, 2017). However, these tightly controlled systems can be easily disrupted by various anthropogenic chemicals known as endocrine disruptor compounds (EDCs), through multiple mechanisms. For example, EDCs can bind receptors by either mimicking hormones or blocking natural hormone binding, which results in over- or under-stimulation of cell or tissue responses. They can also affect hormone production, transport and metabolism or receptor expression and localisation (Combarnous and Nguyen, 2019). The presence of conserved characteristics and components in endocrine systems likely implies

potential disruptions that could similarly affect a wide variety of animal species, from the smallest insect to the largest mammal.

2.1. Conserved hormonal pathways are similarly disrupted

Research on the impact of EDCs on insects, and bees in particular, has largely focused on plant protection products (PPPs), such as insect growth disruptors (reviewed for honeybees in Christen et al., 2018, and studied in a wild bee species in Giejdasz et al., 2021) and, more recently, neonicotinoid pesticides (Baines et al., 2017; Christen et al., 2018). By contrast, the effects of non-pesticide anthropogenic EDCs, such as pharmaceuticals or plastic additives, have mainly been investigated in humans and human-related models (e.g., rodents, zebrafish, and, more recently, *Drosophila melanogaster*), leaving a major knowledge gap regarding their effects on invertebrates that had already been highlighted since 2003 (Oehlmann and Schulte-Oehlmann, 2003). As of today, this lack of data, particularly for terrestrial species, likely prevents the development of effective regulations addressing environmental contamination with respect to the risks they may pose (Crane et al., 2022). In bees, information on non-pesticide EDCs remains especially scarce, particularly on xenoestrogen compounds, with a single study of Bisphenol A (BPA) impact on honeybee queens (Ouyang et al., 2023). Because of this important gap of knowledge, most of the discussion in their study revolved around comparison with other non-bee species (Ouyang et al., 2023). Similarly, the lack of study of phthalate impact on bees led us to discuss our results in light of data obtained on other species groups and to draw comparisons across invertebrates and vertebrate groups in the discussions of the different parts.

In this thesis, the expression of genes coding for components of two hormonal signalling pathways that exemplify the evolutionary conservation of components, functions and disruptions was studied in *B. terrestris* exposed to phthalates. Firstly, we found short- and

long-term effects of low-dose DEHP exposure on the expression of components of the ecdysteroid-signalling pathway. This hormonal cascade regulates embryogenesis, post-embryonic development, reproduction and metabolism (Nijhout, 1994), but also olfactory response and memory in insects (Bigot et al., 2012; Geddes et al., 2013). Though similar across invertebrate species, this pathway presents diverse species-specific roles and timing of signalling in insects (Kocher and Kingwell, 2024), and is not present in vertebrates (Bonneton and Laudet, 2012). Yet, some nuclear receptors of this hormonal cascade have vertebrate homologues (Bonneton and Laudet, 2012). These are effectively bound and activated by DEHP in mammals (Sarath-Josh et al., 2014). On the first hand, the ultraspiracle (USP) is the heterodimerisation partner which forms the ecdysone nuclear receptor once associated with the protein ECR, but is also an insect homologue of the retinoid X receptor (RXR). In mammals, RXR is a steroid and thyroid hormone receptor involved in lipid metabolism, growth and development as well as male spermatogenesis (Mark et al., 2006), and is also a heterodimerisation partner of the peroxisome proliferator-activated receptor (PPAR), a major regulator of cell metabolism and lipid storage (Michalik et al., 2002). In vertebrates, the dysregulation of the RXR-PPAR pathway by DEHP has notably revealed obesogenic effects (Hao, 2013) and male reprotoxicity (Lee et al., 2016, 2009). In invertebrates, however, PPAR is closely related to the insect nuclear receptors E75 and E78, both expressed as early primary and early late responses of the ecdysteroid signalling cascade activation (Bonneton and Laudet, 2012; Hong and Park, 2010). In *Spodoptera littoralis*, DEHP developmental exposure induced a downregulation of *EcR*, *USP, and E75* genes that was associated with longer development duration and lower male mating olfactory responsiveness (Avilès et al., 2019). In the present thesis, a downregulation of the same genes was observed in *B. terrestris* adult workers exposed to low-dose DEHP. However, no adverse outcome on development, survival

or physiology has yet been associated with this disruption in bees. Yet, we only measured adult traits, and further work would be needed on larval development and reproductive individual fitness, as we only focused on workers. Nevertheless, a disruption of elements included in this first pathway seems conserved across species.

Secondly, the expression of genes involved in the insulin signalling pathway was also analysed after exposure to DEHP and DnBP in adult *B. terrestris* workers. This pathway is conserved across eukaryotic species from unicellular yeast to humans, and including insects. The diversity of components included in this pathway and their specific roles vary among species groups (notably between social and solitary species). Yet, the nutrient-sensing role of insulin-related pathway which acts as a regulator of glucose and lipid homeostasis, and its associated involvement in lifespan, growth, and developmental processes, is highly conserved (Barbieri et al., 2003; Pertseva and Shpakov, 2002), as well as its disruption by EDCs. At a human safety scale, studies have shown a link between prenatal exposure to phthalates and childhood obesity (Harley et al., 2017), and between adolescent exposure and increased insulin resistance (Trasande et al., 2013). By the disruption of endocrine signalling pathways such as both the insulin- and PPAR-related pathways, phthalates could be linked to the increased obesity, diabetes, and cardiovascular disease occurrence (Dalamaga et al., 2024). These diseases are considered among the greatest public health problems and economic burdens of the twenty-first century in occidental regions (Mohajan and Mohajan, 2023). In this thesis, repeated exposure to environmentally high doses of DnBP and low doses of DEHP led to dysregulation of the *B. terrestris* insulin-related pathway in workers. Moreover, DnBP exposure was associated with an increase in worker fat content and offspring body weight. Similar patterns have been reported in invertebrates, with studies in *Drosophila melanogaster* and *Caenorhabditis elegans* exposed to phthalates demonstrating dysregulations of insulin

signalling, increases in lipid content (Pradhan et al., 2018; Williams et al., 2016), and obesogenic effects on their progeny (Chen et al., 2019). Comparable results have been obtained in vertebrate models, such as rodents, in which prenatal DEHP and DnBP exposure caused the downregulation of insulin-signalling related genes, increased offspring body and fat weight, and were linked to heightened risk of type II diabetes in humans (Deng et al., 2018; Rajesh and Balasubramanian, 2014). Deriving from the evolutionary conservation of its nutrient sensing role, the insulin signalling pathway shows species-specific variability with particularities associated with social and solitary lifestyles (Kocher and Kingwell, 2024). For example, in social bees, previous works have revealed its role in shaping the honeybee caste-specific development (De Azevedo and Hartfelder, 2008; Wolschin et al., 2011) and behaviour (Ament et al., 2008). In the less-studied solitary bees such as *Megachile rotundata* and *Osmia bicornis*, it is at least partially implicated in overwintering and diapause regulation, also through sensing of body reserves throughout the hibernation period (Cambron et al., 2019; Wasielewski et al., 2014). In addition, in both *A. mellifera* and *M. rotundata*, genes activated by the insulin-signalling pathway can be dysregulated in response to stress (Rondeau et al., 2025). Indeed, in many species, insulin-signalling is involved in the stress response of organisms through the activation of detoxification, oxidative stress protection and longevity genes (Braeckman, 2001). Therefore, the findings of this thesis on *B. terrestris* reinforce evidence of the conserved disruption of homologue components of the insect ecdysteroid-signalling pathway and the shared insulin-signalling pathways in invertebrate (*D. melanogaster*, *C. elegans*) and vertebrate (rodents, humans) models, with potential transgenerational consequences.

2.2. Disruption of conserved hormonal pathways are overlooked by regulatory authorities

While phthalates exemplify pollutants that could be affecting entire ecosystems, including bee and human populations, existing regulations are strongly limited as they only target direct human exposure routes (Crane et al., 2022). In 1962, Rachel Carson published her book 'Silent Spring', along with years of ecotoxicological research and the personal experiences of farmers and rural communities with agrochemical use (Carson, 1962). With this knowledge accessible to a wider public, her book profoundly reshaped public opinion, now informed that agrochemical pollution could damage wildlife and, directly or indirectly, human health. While many agrochemicals, such as the organochloride insecticide dichloro-diphényle-trichloro-éthane (DDT) were at first considered safe to humans, their use was regulated and prohibited in the decade following this public opinion change, when people and politics became aware of the link between environmental impact and public safety (Eskenazi et al., 2009). Interestingly, the thinking around the regulation made for non-pesticide pollutants, and especially EDCs such as phthalates, bisphenols, and polyfluoroalkyl substances (PFAS), seems to follow the opposite logic. European phthalates regulation, while being one of the strictest in the world, remains limited to human everyday use plastics (Regulation (EU) 2018/2005). Restrictions began in 2000 with their prohibition in children's toys, and by 2021, all consumer and medical products were required not to contain more than 0.1% phthalates by weight (Regulation (EU) 2021/2045). However, these rules notably exclude *"articles exclusively for industrial or agricultural use, or for use exclusively in the open air, provided that no plasticised material comes into contact with human mucous membranes or into prolonged contact with human skin"* (Regulation (EU) 2018/2005). As semi-volatile molecules, not chemically bound to the plastic matrix, they leach easily into the environment from both plastic waste and everyday products such as detergents and cosmetics (Suhrhoff and Scholz-Böttcher, 2016). In

addition, wastewater treatment cannot prevent their release into water systems, and they also accumulate in sewage sludge, which is still used as crop fertiliser in many countries (Anne and Paulauskiene, 2021; Billings et al., 2021). Despite recent public health cases over other persistent organic pollutants, such as the endocrine disruptors PFAS, current regulations of sewage sludge use only include information on heavy metals (see Regulation (EU) 2019/1010 based on 1986 regulation). As highlighted earlier, these endocrine disrupting compounds target highly conserved pathways across species groups. Effective regulations should therefore address these pollutants not only in terms of direct human exposure but also regarding their environmental release and ecological consequences. In this context, this first study of phthalate impacts on *B. terrestris* highlights their potential impacts on pollinators and stresses the need for risk assessment and potentially global regulations. Regulatory modifications are slow-paced mainly because of the lack of data (Crane et al., 2022). Therefore, "*scientia potentia est*", and studying such conserved disruptions in *B. terrestris* and other bee models could allow a first step towards phthalate risk assessments for pollinators. In addition, the use of this species also provides an exploration playground to understand the importance of hormonal pathways in insects and how EDC could affect gene expression and physiology at the individual scale, and cascade towards colony and population-level effects.

Part. 3 – Current ecological risk assessments for bees do not take EDC effects into account

In Europe, several authority agencies regulate the use of industrial and agrochemicals, including phthalates. On the one hand, the European Chemical Agency (ECHA) regulates the use, import and production of all chemicals in the European Union (EU), and requires companies to provide minimal safety data for each compound before marketing (Oltmanns et al., 2023). Under the regulation on Classification, Labelling and Packaging (CLP) and the one on Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH),

manufacturers and importers must collect all toxicological and ecotoxicological information available before registration of a compound (ECHA, 2025; Oltmanns et al., 2023). Yet, these data are limited to endpoint values based on linear dose-response determination methods, such as the lethal doses/concentrations (LD10, LD50 or LC10, LC50), the effect concentrations (EC10, EC50) and the no effect/lowest effect concentrations (NOEC/LOEC; Tarazona et al., 2014; Versonnen et al., 2014). These values for invertebrates are mostly collected on aquatic species and predominantly on *Daphnia magna* (Tarazona et al., 2014). Concerning terrestrial invertebrates, ECHA only requires ecotoxicological data for compounds with tonnage exceeding 100 tonnes per year in the European Union, and less information is available about endpoints limited to acute toxicity values (Versonnen et al., 2014). Phthalates such as DEHP and DnBP are respectively registered as exceeding 10,000 and 1000 tonnes per year in the European Union (ECHA chemical database, 2010a, 2010b). As they are not registered as plant protection products (PPPs), no data on bees is explicitly required. Yet, should it be required, current active regulations only recommend honeybee testing, i.e. using standardised guidelines, for registration (Regulation (EC) 1107/2009). Therefore, the only ecotoxicological information available for these molecules on terrestrial invertebrates is solely based on the results of Jensen et al. (2001) on the collembolan *Folsomia fimetaria* (ECHA chemical database, 2010a, 2010b). In this study, they applied linear dose-response determination protocols, which failed to determine an LC50, but calculated EC10 and EC50 for growth and development (Jensen et al., 2001). Yet, these linear methods do not test low or field-realistic concentrations, and can therefore not capture potential low-dose effects and NMDRCs (Futran Fuhrman et al., 2015), which could notably explain the absence of LC50 in their study.

In case of suspicion of endocrine disrupting activity, guidance documents of ECHA (ECHA, 2024) redirect towards OECD guidelines for testing endocrine disrupting compounds,

in which described standardised procedures do not yet include any terrestrial insect models (OECD, 2018). The ED-related frameworks are still young, and more importantly, ED activity testing of compounds is not required from the importers and producers to include in the safety data requested for all compounds at registration. Instead, they are used for risk assessment by scientific and regulatory communities, once the chemical is already registered. This highlights an imbalance between the current and slow scientific shift in paradigm from "the dose makes the poison", which has long ruled the field of ecotoxicology and is still used by the regulatory sector to implement public health legislations, and the consideration of NMDRC and field-realistic dose investigation, a rationale that led us to highlight the impact of realistic exposure to phthalates on bees during this thesis.

On the other hand, the European Food Safety Authority (EFSA) advises risk managers and the European Commission on the use and release of chemicals that can enter the food chain or expose humans and the environment through feed and food, such as pesticides (Oltmanns et al., 2023). To perform valid peer-reviewed assessments of the impact of these molecules on humans and wildlife, the EFSA develops guidelines, such as the guiding document for ecotoxicological risk assessment of plant protection products on bees established in 2013 (EFSA, 2013), recently revised (EFSA et al., 2023). This document is the only one indicating an ecotoxicological framework for assessing a risk linked to xenobiotic exposure in bees, and has therefore quickly become a reference not only for PPP testing, but also in bee ecotoxicology in general. In this document, the risk assessment framework is detailed as a tier-based procedure on bee model species, i.e., *A. mellifera* and *B. terrestris,* for which standardised first-tier toxicity assessment protocols exist (OECD, 2017a, 2017b, 2017c, 1998a, 1998b), as well as *Osmia* spp., for which short-term protocols have recently been published (OECD, 2025). Dose-response curves are studied using standardised laboratory

protocols that, as in the context of ECHA regulation, assume monotonic relationships (e.g. OECD, 2017b). In the bee guidance document, while NMDRCs are acknowledged, there is yet no requirement to investigate them (EFSA et al., 2023). In addition, standardised methods do not evaluate sub-lethal effects. While common experimental designs, such as *B. terrestris* microcolonies (Klinger et al., 2019), are suggested in the guidance document (EFSA et al., 2023), no standardised procedures yet exist, which hampers their systematic inclusion in peer-reviewed risk assessments of molecules only including sub-lethal impact on honeybee hives (e.g., EFSA et al., 2025). There is growing evidence of the importance of sub-lethal effects of pollutants on managed and wild bee populations (Barascou et al., 2021; Sgolastra et al., 2020), and endocrine-disrupting activity highlighted for insecticides, such as neonicotinoids, that cannot be captured by existing bee-related guidelines (Baines et al., 2017). In the event of suspicion of endocrine-disrupting activity, assessment of EFSA-regulated compounds is performed under a separate framework, following the guidance document on assessing the endocrine-disrupting properties of plant protection products and biocides (ECHA and EFSA et al., 2018). Yet, this document does not consider invertebrate species, and as discussed by Crane et al. (2022), the main reason is probably linked to a huge knowledge gap around the endocrine pathways in invertebrates.

Most ED-related regulatory frameworks suggest the use of novel approaches to study endocrine-disrupting effects, such as the Adverse Outcome Pathway (AOP), which aims to link a molecular initiating event (MIE) to an adverse outcome (AO), and allows a realistic description of an endocrine disruptor activity (Crane et al., 2022; ECHA and EFSA et al., 2018; Liszewska et al., 2024). In this context, we propose a tentative AOP for the effects of DEHP and DnBP on *Bombus terrestris* (Fig. 1), constructed based on the discussions of this thesis.

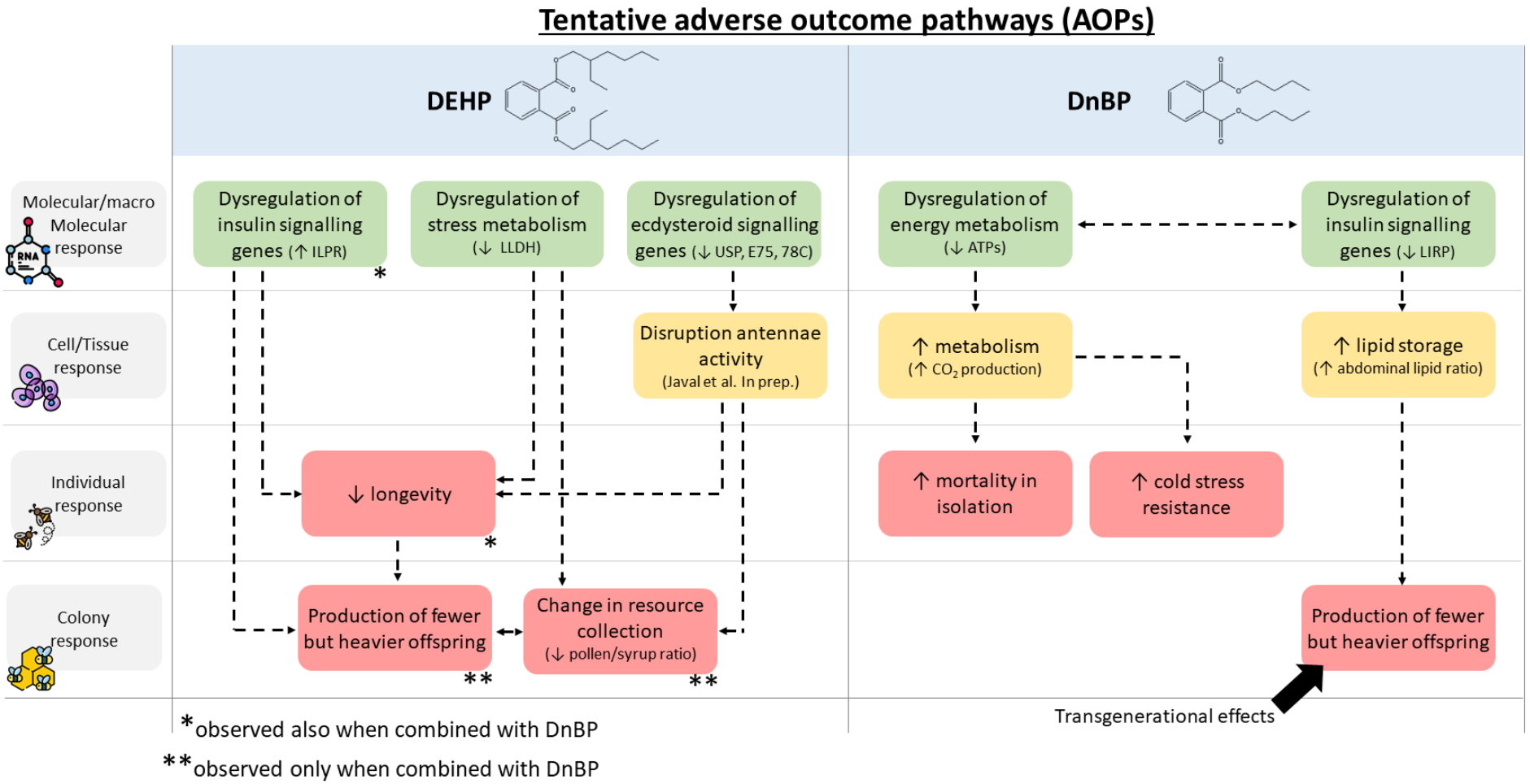


Fig. 1. Tentative adverse outcome pathways for DEHP and DnBP with hypothetical link between the effects observed throughout the three experiments comprised in this project based on AOP wiki templates (https://aopwiki.org/aops).

The present AOP connects the adverse outcomes observed at both the individual and colony levels to molecular and tissue-level disruptions occurring in bumblebee systems after exposure to phthalates. Notably, it highlights the consistent effects of DnBP on lipid storage, metabolism and insulin signalling across biological levels, leading to the observed transgenerational obesogenic effects in the microcolony experiment (Fig. 1). It also underscores the broader disruption of endocrine pathways and stress metabolism in workers exposed to DEHP, which likely contributes to the observed decrease in longevity (Fig. 1). Although more research would be needed to fill the knowledge gaps, such as the effect of DEHP exposure on bee detoxification pathways, and to confirm these tentative links, this method of visualisation primarily offers a comprehensive overview of the phthalate impact scheme at a glance (Fig. 1).

When the literature on a stressor is beginning to grow, the AOP can also be used to confirm the hypotheses and identify knowledge gaps. For example, the AOP approach has been applied to evaluate neonicotinoid impact on honeybees and bumblebees by Camp and Lehmann (2020), highlighting that this kind of framework could be implemented in bee risk assessment. Indeed, such inclusion would allow an emancipation of the frameworks that only rely on dose and concentration-based toxicity evaluation with mortality as the main considered metric, and include realistic evaluation of the risk for both ED and non-ED compounds on bees. However, the existing regulatory frameworks for ED testing focus mainly on estrogenic, androgenic, thyroid, and steroidogenic (EATS) modalities, omitting potential disruption of other insect specific pathways such as the juvenile hormone signalling and other generally crucial pathways such as the insulin pathway (ECHA and EFSA et al., 2018; Liszewska et al., 2024). This type of disruption is likely to occur after exposure to phthalates in many species, including bees, as highlighted in this thesis. The present thesis not only highlights the

potential impact of phthalates on bees, but also underlines that existing frameworks still need further adjustments to fully evaluate the potential impact of ED and non-ED xenobiotics on bees.

To conclude, EU chemical regulation authorities have banned or restricted the use of phthalates, but only for specific purposes related to human exposure. ECHA classified phthalates as reproductive toxicants under CLP regulation (Regulation (EC) 1272/2008) which led to restrictions to 0.1% w/w of any plasticised material under REACH regulation (Regulation (EU) 2018/2005, 2018), medical device and *in vitro* diagnostic regulations (Regulation (EU) 2017/745; Regulation (EU) 2017/746), as well as the RoHS (Restriction of Hazardous Substances in electric and electronic equipment) directive (Commission Delegated Directive (EU) 2015/863), and more recently to the establishment of specific migration limits in food contact materials (Commission Regulation (EU) 2023/1442; EFSA et al., 2022a, 2022b). However, several studies have highlighted phthalate contamination of many environments, including agricultural areas. Their presence is mediated by the application of sewage sludge and the use of plastic materials, including farming vehicle parts such as air filters and tyres, mulching techniques, and greenhouse plastic covers (Billings et al., 2021). Additionally, the accumulation of phthalates in the vegetative parts of edible and non-edible plants has also been highlighted (Barroso et al., 2019; Sun et al., 2015). This evidence underscores that chemical authorities in Europe should consider these environmental pollutants for their potential impact on wildlife, in addition to human exposure risk and provide the tools necessary to establish thorough risk assessments. With the existing EDC framework so isolated from existing bee ecotoxicological guidelines, it is certain that ED effects might still be undermined in bee ecotoxicology.

With the societal impact emerging from “Silent Spring”, Rachel Carson paved the way towards the implementation of multidisciplinary approaches such as the One Health framework considering humans as part of their environment and equally affected by the current biodiversity decline. More than sixty years later, unfortunately, and despite relentless efforts to implement more ecologically relevant risk assessment, the case of phthalates only reminds us that legal frameworks supposedly protecting the environment while also human health, is still mostly focused on the human part of the equation.

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Supplementary material

Supplementary material 1*

*Chapter 2 – Part I: Acute exposure to environmental doses of di-n-butyl phthalate but not di-2-ethylhexyl phthalate induces mortality in isolated and cold-stressed workers of *Bombus terrestris* L. (Hymenoptera: Apidae)

Table S1.1. Comparison between mortality rates found 72 hours post exposure across all exposure sessions in mineral water and both solvent groups, using glmm with family binomial and session as random factor.

Treatment groups	Mortality rate (%)	Chisq.	df.	p-value
Mineral water	8	0.2875	2	0.8661
Methanol 0.07%	7			
Acetone 100%	3			

Table S1.2. Worker number details of experiment 1.

Dataset	Exposure batches	Treatment		N
		Molecule	Concentration	
Session 1	1	Solvent control (Methanol)	0.07%	30
		Water control		30
		DnBP	0.1X	30
		DEHP	1X	30
	2	Solvent control (Methanol)	0.07%	30
		Water control		30
		DnBP	1X	30
		DEHP	0.1X	29
	3	Solvent control (Methanol)	0.07%	30
		Water control		30
		DnBP	10X	30
		DEHP	10X	30
Session 2	1	Solvent control (Methanol)	0.07 %	20
		Water Control		20
		DnBP	1X	20
	2	Solvent control (Methanol)	0.07%	20
		Water Control		20
		DnBP	10X	20
	3	Solvent control (Methanol)	0.07%	20
		Water Control		20
		DEHP	10X	20
	4	Solvent control (Methanol)	0.07%	20
		Water Control		20
		DEHP	0.1X	20
	5	Solvent control (Methanol)	0.07%	20
		Water Control		20
		DEHP	1X	12
	6	Solvent control (Methanol)	0.07%	20
		Water Control		20
		DnBP	0.1X	20

Table S1.2. continuation.

Session 2 (continuation)	7	Solvent control (Acetone)		20
		DEHP	100X	20
		DnBP	100X	20
	8	Solvent control (Methanol)	0.07%	8
		Water Control		8
		DEHP	1X	8
	9	Solvent control (Methanol)	0.07%	20
		Water Control		20
		DEHP + DnBP	1X : 1X	20
	10	Solvent control (Methanol)	0.07%	20
		Water Control		20
		DEHP + DnBP	1X : 10X	20

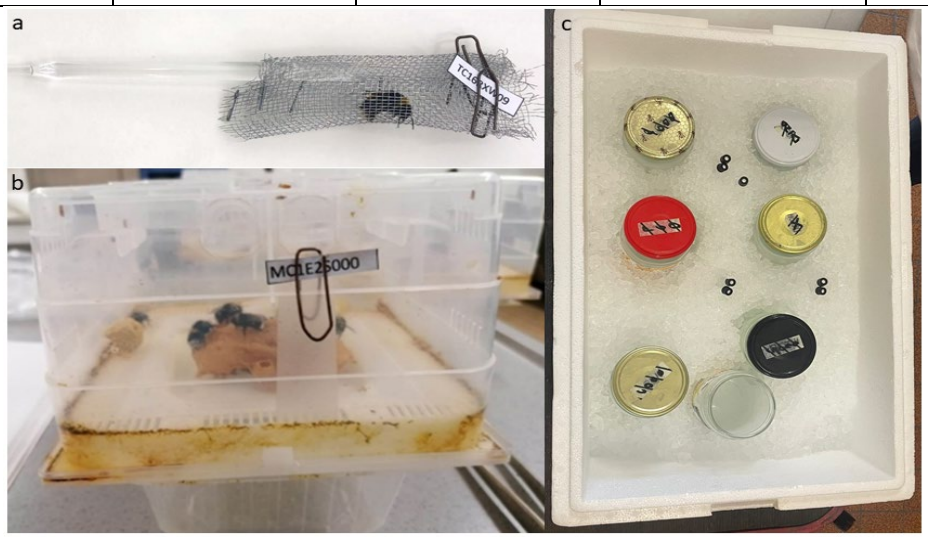


Fig. S1.1. Protocol pictures showing a) worker caged in the isolated setup, b) groups of ten workers installed in a microcolony box and c) ice bucket with glass jars used for anaesthesia.

Table S1.3. Anaesthesia parameters.

Experiment	Session	Cooling rate	Duration (min.)
Experiment 1 – single exposure	Session 1	-0.03°C/s (from 23 to 3°C)	10
	Session 2	-0.02°C/s (from 23°C to 11°C)	10
Experiment 2 – Cold stress	Session 1	-0.01°C/s (from 21°C to 3°C)	30

Table S1.4. Comparison between mortality rates at 72h post-exposure in solvent groups across experimental batches of both exposure sessions, using glmm with family binomial and batch as random factor.

Response variable	Chisq	Df.	P-value
Trtmt ctrl	0.1199	1	0.7292
Batches	0.1485	1	0.7000

Trtmt ctrl: batch	0.2117	1	0.6454

Table S1.5. Mortality values (%) ± se induced by DnBP exposure in isolated workers. N is the number of exposed workers in each control and dose group. P-values were obtained using an emmeans post-hoc analysis against the solvent treatment following the Bayesian generalised linear model with family binomial. Symbols: *p<0.01.

Dose	Mortality ± se	N	P-value
Solvent	6.5 ± 1.9 %	170	
DnBP 0.1X	4.0 ± 2.8 %	50	0.7160
DnBP 1X	26.5 ± 6.3 %	49	0.0045*
DnBP 10X	12.0 ± 4.6%	50	0.5064
DnBP 100X	5.0 ± 4.9 %	20	0.8714

Table S1.6. Mortality values (%) ± se induced by DEHP exposure in isolated workers. N is the number of exposed workers in each control and dose. The Bayesian generalised linear model with family binomial did not show any significant effect of the treatment. Therefore, no p-value is shown.

Dose	Mortality ± se	N
solvent	5.9 ± 1.7 %	188
DEHP 0.1X	4.1 ± 2.8 %	49
DEHP 1X	6.0 ± 3.4%	50
DEHP 10X	12.0 ± 4.6 %	50
DEHP 100X	15.0 ± 8.0 %	20

Table S1.7. Mortality (%) and expected mortality (%) ± se induced by DnBP and DEHP, in single and mixture exposure treatments, in isolated workers. N is the number of workers exposed to each mixture.

Mixture	Mortality ± se	Expected mortality ± se	N
DEHP 1X – DnBP 1X	5.0 ± 4.9 %	30.9 ± 7.2 %	20
DEHP 1X – DnBP 10X	15.0 ± 8.0 %	17.3 ± 5.7 %	20

Table S1.8. Corrected and uncorrected mortality (%) values ± se induced by DnBP and DEHP in isolated workers subjected or not to cold stress. N is the number of exposed workers in each control and dose group.

Molecule	Dose	Mortality ± se without cold stress			Mortality ± se with cold stress		
		corrected	uncorrected	N	corrected	uncorrected	N
DnBP	Solvent	-	7.5 ± 2.2 %	150	-	36.5 ± 5.5 %	78
	0.1X	0.0 ± 0.0 %	4.0 ± 2.8 %	50	0.0 ± 0.0 %	17.9 ± 7.2 %	28
	1X	19.0 ± 5.6 %	26.5 ± 6.3 %	49	5.5 ± 3.4 %	19.6 ± 5.9 %	46
	10X	1.9 ± 1.9 %	12.0 ± 4.6%	50	20.8 ± 6.0 %	32.6 ± 6.9 %	46
DEHP	Solvent	-	5.9 ± 1.7 %	188	-	36.5 ± 4.3 %	125
	0.1X	0.0 ± 0.0 %	4.1 ± 2.8 %	49	4.4 ± 3.1 %	18.6 ± 5.9 %	43
	1X	2.3 ± 2.1 %	6.0 ± 3.4%	50	12.5 ± 4.8 %	25.5 ± 6.4 %	47
	10X	10 ± 4.2 %	12.0 ± 4.6 %	50	0.0 ± 0.0 %	34.4 ± 8.4 %	32

Table S1.9. Comparison between corrected and uncorrected mortality rates found 72h post-exposure to DnBP and DEHP combined with a cold stress using a Bayesian generalised linear model, family binomial. Corrected mortality values were calculated using Sun-Shepard's formula, i.e., corrected mortality = (treatment survival - control survival) / control survival x 100, when solvent mortality differed between cold treatment. Symbols: *p<0.05; **p<0.01; ***p<0.001.

Molecule	Sun-shepard's correction	Response variable	Chisq	Df.	P-value
DnBP	No	Dose	9.27	3	0.0259*
		Cold stress	15.59	1	7.852e-05***
		Dose:Cold stress	9.62	3	0.0221*
	Yes	Dose	18.16	2	0.0001***
		Cold stress	0.1667	1	0.6830
		Dose:Cold stress	13.74	2	0.0010**
DEHP	No	Dose	1.14	3	0.7664
		Cold stress	11.96	1	0.0005***
		Dose:Cold stress	3.01	3	0.3900
	Yes	Dose	4.74	2	0.0932
		Cold stress	0.38	1	0.5381
		Dose:Cold stress	5.54	2	0.06274

Table S1.10. Mortality (%) values ± se induced by DnBP and DEHP in isolated and non-isolated workers.

Molecule	Dose	Isolated workers		Non-isolated workers	
		Mortality ± se	N	Mortality ± se	N
DnBP	Solvent	9 ± 2.9 %	100	0.0 ± 0.0 %	50
	1X	26.5 ± 6.3 %	49	0.0 ± 0.0 %	50
	10X	12.0 ± 4.6%	50	6.4 ± 3.6 %	50
DEHP	Solvent	5.6 ± 2.2 %	108	0.0 ± 0.0 %	50
	0.1X	4.1 ± 2.8 %	49	0.0 ± 0.0 %	50
	1X	6.0 ± 3.4%	50	2.1 ± 2.1 %	50

Table S1.11. Comparison between mortality rates found 72h post-exposure to DnBP and DEHP in isolated and microcolony workers using a Bayesian generalised linear model, family binomial. Symbols: *p<0.05; **p<0.01; ***p<0.001.

Molecule	Response variable	Chisq.	Df.	P-value
DnBP	Treatment	6.66	2	0.0358*
	Social conditions	20.95	1	4.722e-06***
	Treatment:Social conditions	8.10	2	0.0174*
DEHP	Treatment	0.69	2	0.7088
	Social conditions	6.64	1	0.0100**
	Treatment:Social conditions	1.76	2	0.4085

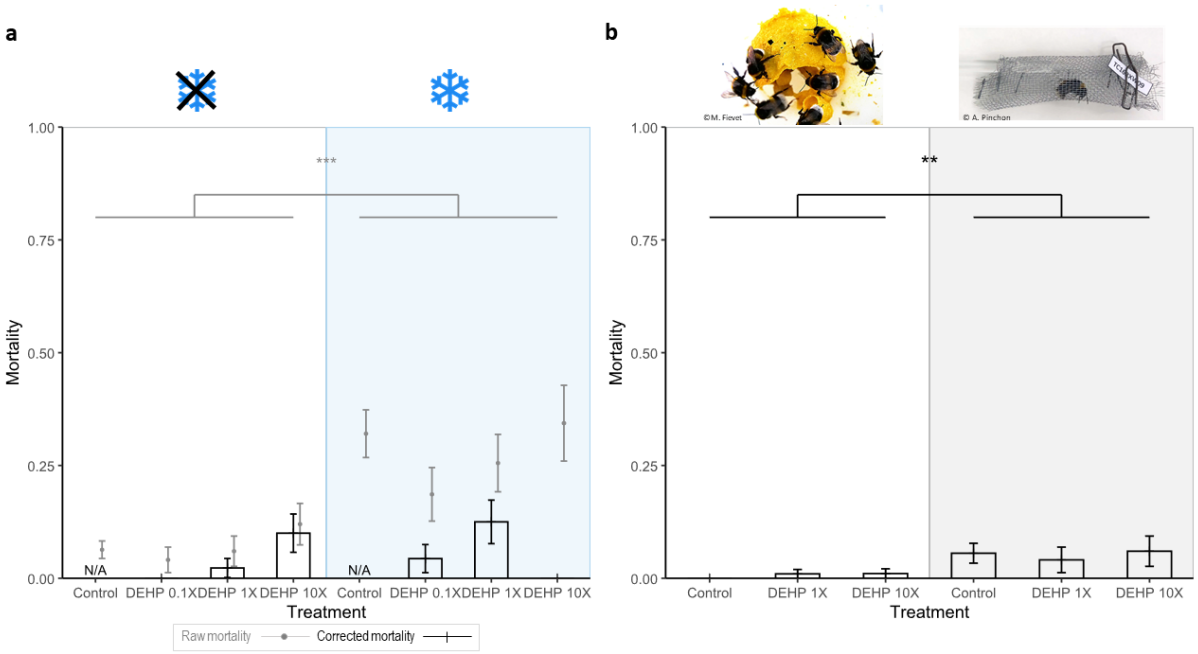


Fig. S1.2. a) Effects of DEHP 0.1X, 1X and 10X mortality in non-cold stressed workers (white panel; $N_{control}$=188, $N_{DEHP\ 0.1X}$=49, $N_{DEHP\ 1X}$=50 and $N_{DEHP\ 10X}$=50) and cold stressed workers (blue panel; $N_{control}$=125, $N_{DEHP\ 0.1X}$=43, $N_{DEHP\ 1X}$=47 and $N_{DEHP\ 10X}$=32). Grey points, error bars and letters show results for raw mortality rates. Black points, bars, error bars and letters show results for corrected mortality. No bars indicate no mortality, and N/A indicates no mortality data available for the solvent groups after correction. ***indicate the significant effect of the cold stress at p<0.001 (Bayesian generalised linear model, family binomial). Grey points and error bars show results for uncorrected mortality values including for the control group. b) Effect of DEHP 1X and 10X on mortality in microcolony (white panel; N=100 per treatment) and in isolated workers (grey panel; $N_{solvent}$=150, $N_{DEHP\ 1X}$=49 and $N_{DEHP\ 10X}$=50). Bars show mortality values with error bars indicating standard errors. No bars indicate no mortality. **indicate the significant effect of social conditions at p<0.01 (Bayesian generalised linear model, family binomial).

Supplementary material 2*

*Chapter 2 – Part II: DnBP and DEHP short-term effects on metabolism and ecdysteroid-related gene expression of isolated workers of *Bombus terrestris*

Table S2.1. Primer characteristics and efficiency. Sequences and Tm with associated NCBI Gene ID.

Gene name (abbreviate)	Type	NCBI ID	Primer sequences	efficiency	T_m (°C)
Ribosomal protein L13 (rpl13)	reference	XM_012316284.1	Fw TTGGAATTGCTGTAGATCCTAGAAG Rv CATTCAGTGGGAAGAGAATCAA	1.943	55/60
Phospholipase A2 (pla2)	reference	FN_391388	Fw TATCTTTCAATGCCCAGGAG Rv GTCGTAACAAATGTCATGCG	1.932	55/60
Kynurenine/alpha-aminoadipate aminotransferase (AADAT)	target	XM_003393468.2	Fw AATTGGGTTCAGCTTTGCAG Rv GAGACGATCACGATGTCACG	2.107	60
L-lactate Dehydrogenase (LLDH)	target	XP_003396770.1	Fw GACGTTCAATCTCTGCGACA Rv CTGCCAAAGAACCGAGTGAT	1.932	60
ATP synthase (ATPs) beta subunit	target	XM_003400566.4	Fw ACGAATCAGAGACCATTCCG Rv CCTTGGATCCTGCAGCCTTT	1.925	60
Insulin receptor (InR)	target	XM_003397898.2	Fw GAAGCCTCTTGCCCTGGATG Rv CTCGTGCCGATAATGCTTGC	1.964	60
Locust insulin-related peptide-like (LIRP)	target	XM_003400730.2	Fw GCGGTAGTGTTTACAATAGCCG Rv ATTCCCCGTCCATTTCTTCG	1.833	60
Oestrogen-related receptor (ERR)	target	XM_012308778.2 XM_012308777.2	Fw GGACTTCATGGAATCGCTGT Rv CCAAAGCTCTACGCTCCAAT	1.842	60
Ecdysteroid receptor (EcR)	target	XM_020867166.1 XM_012317565.2	Fw TATCACTACAACGCACTGAC Rv CCGCATGTACATATCGATCT	1.914	55
Ultraspiracle (USP)	target	XM_020864351.1 XM_012312252.1	Fw CAGTGGCAAGCATTATGGTG Rv TCTCTCTTCATGCCCATCGC	1.900	60
E75	target	NM_001080110.1 XM_00339894	Fw GCACGGACATCATTCACAAC Rv TTGAGATCCTTGGGGTGAAC	1.982	55
78C	target	XM_003395889.3 XM_012309181.2	Fw ACGACGTGAAAACAGTGCAG Rv TTGTGAGACGCTCGAGACAC	1.919	55

Table S2.2. Comparison of relative gene expression between treatment and control groups and effect size. "Δ LS means" indicates model-adjusted differences in mean relative gene expression between the indicated treatment group and its relative control group, "*g*" indicates the effect size calculated using Hedge's coefficient, and the reported p-values were calculated using ART ANOVA statistical test. Bold values indicate significant change in treatment group relative gene expression compared to control group at p-value<0.05.

Genes	DnBP 60 ng/g				DnBP 600 ng/g				DEHP 12ng/g				DEHP 1200 ng/g			
	Δ LS means ± SE	*g*	p-value	A	Δ LS means ± SE	*g*	p-value	A	Δ LS means ± SE	*g*	p-value	A	Δ LS means ± SE	*g*	p-value	A
Metabolism																
AADAT	1.75 ± 2.35	0.38	0.4725	+0.41	-1.06 ± 1.49	-0.41	0.5016	-0.40	-3.79 ± 2.1	-0.91	0.1005	-0.85	-0.50 ± 1.86	-0.15	0.7988	-0.16
LLDH	-3.82 ± 2.11	-0.92	0.0987	-0.95	1.61 ± 1.56	0.59	0.3409	+0.21	**-4.52 ± 1.81**	**-1.25**	**0.0315**	**-1.61**	1.50 ± 1.72	0.49	0.4224	+0.87
ATPs	-2.06 ± 1.45	-0.82	0.2058	-0.16	**-4.05 ± 1.26**	**-1.86**	**0.0177**	**-0.70**	0.90 ± 1.45	0.35	0.5784	+0.11	0.00 ± 1.81	0.00	1.000	-0.06
LIRP	-1.10 ± 2.12	-0.28	0.6195	-0.32	-1.35 ± 1.94	-0.40	0.5122	-0.97	-2.40 ± 1.70	-0.84	0.2286	-1.00	-1.50 ± 1.65	-0.45	0.4300	-0.97
InR	0.73 ± 2.14	0.19	0.7415	+1.09	0.50 ± 1.30	0.22	0.7171	+0.87	-1.33 ± 1.90	-0.42	0.5192	-0.41	2.25 ± 1.28	0.87	0.1777	+1.48
Ecdysteroid pathway																
USP	-0.29 ± 2.40	-0.06	0.9057	-0.04	-0.45 ± 2.00	-0.13	0.8295	+0.20	-1.46 ± 2.33	-0.31	0.5457	-0.63	-1.00 ± 1.83	-0.31	0.6074	+0.02
ECR	-4.00 ± 1.92	-1.05	0.0634	-1.93	0.90 ± 1.98	0.26	0.6650	+0.02	-3.53 ± 1.68	-1.05	0.0621	-3.41	0.50 ± 1.86	0.15	0.7988	+0.00
E75	-2.66 ± 2.24	-0.6	0.2628	-1.99	0.90 ± 1.98	0.26	0.6650	+0.58	**-5.25 ± 1.81**	**-1.46**	**0.0157**	**-2.86**	-0.50 ± 1.83	-0.15	0.7956	-0.01
78C	-3.79 ± 2.13	-0.91	0.1038	-3.11	0.23 ± 1.7	0.08	0.8975	-0.05	**-4.26 ± 1.87**	**-1.14**	**0.0456**	**-3.95**	0.50 ± 1.86	0.15	0.7988	-0.08
ERR	-0.783 ± 2.00	-0.2	0.7039	-0.13	-1.35 ± 1.94	-0.40	0.5122	+0.01	1.03 ± 2.02	0.26	0.6193	-0.66	-2.00 ± 1.68	-0.67	0.2881	-0.14

Supplementary material 3*

*Chapter 3 – Part I: Repeated exposure to field-realistic concentrations of di-2-ethylhexyl phthalate and di-n-butyl phthalate in bumblebees impacts adult survival and lipid reserves with intergenerational obesogenic effects

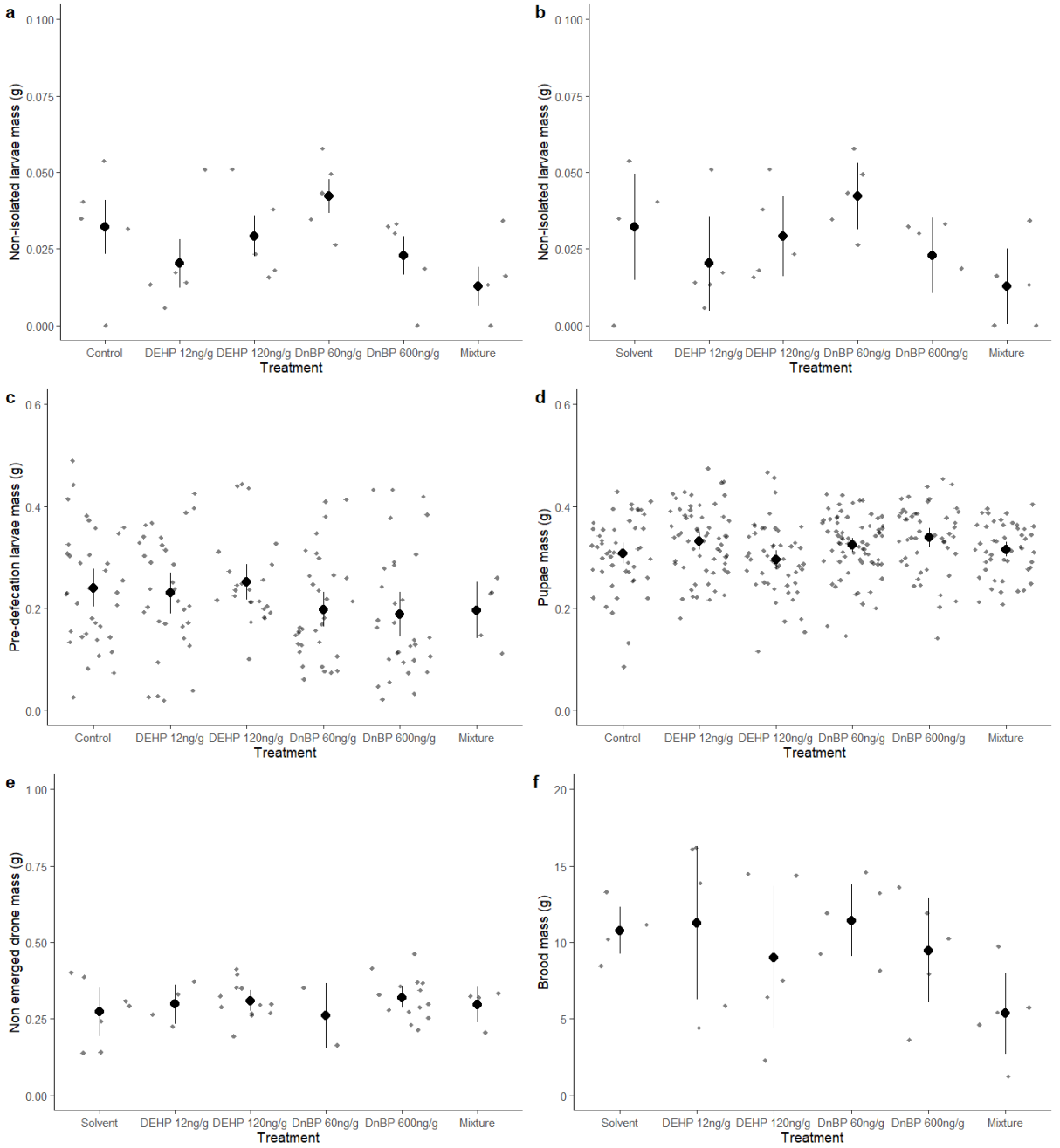


Fig. S3.1. Offspring mass in grams (g) in the microcolonies. a) Mass of non-isolated larvae. b) Standardised mass of non-isolated larvae (N=5 microcolonies per treatment). c) Individual mass of pre-defecation larvae. d) Individual mass of pupae. e) Individual mass of non-emerged drones. f) Total hatched offspring mass. Black points show mean values calculated per treatment with error bars indicating 95% confidence intervals. Grey dots show mean mass calculated for each microcolony (N=5 microcolonies per treatment) in graphs "a", "b", and "f", and individual mass (N=numbers reported in Table S3.2) in graphs "c", "d" and "e".

Table S3.1. Comparison between mineral water control and solvent control for general measured metrics. Statistical tests used are reported with the distribution family when needed.

Metrics	**Statistical test**	**Stat.**	**d.f.**	**p-value**
Worker mortality	Mixed Cox regression	z=1.03	1	0.3000
Pollen collection	GLMM (gaussian)	χ=0.16	1	0.6919
Syrup collection	GLMM (gaussian)	χ=1.91	1	0.1668
Total offspring number	GLMM (negative binomial)	χ=0.73	1	0.3926
Total offspring mass	GLMM (gaussian)	χ=0.65	1	0.4189
Worker abdominal lipid content	GLMM	χ=3.5	1	0.0610

Table S3.2. Offspring production. Mean number of individuals ± standard error [95%CI] found in each stage from eggs to drone emergence. Significant differences from control are indicated in bold with associated symbols: *p<0.05; **p<0.01; ***p<0.001.

Treatments	Eggs	Non-isolated	Pre-defecation	Post-defecation	Pupae	Non-emerged drones	Emerged drones	Total hatched
Control	28.8±9.2 *[10.7,46.9]*	34.0±11.0 *[12.4,55.6]*	7.2±1.9 *[3.5,10.9]*	4.4±1.0 *[2.4,6.4]*	10.0±2.0 *[6.1,13.9]*	1.4±0.4 *[0.6,2.2]*	10.4±1.5 *[7.5,13.3]*	67.6±11.1 *[45.9,89.3]*
DEHP 12ng/g	29.8±7.7 *[14.7,44.9]*	46.2±11.7 *[23.2,69.2]*	6.4±3.1 *[0.3,12.5]*	7.0±2.6 *[1.9,12.1]*	12.2±3.0 *[6.4,18.0]*	0.8±0.4 *[0.1, 1.5]*	9.0±2.9 *[3.4,14.6]*	81.6±18.3 *[45.8,117.4]*
DEHP 120ng/g	14.2±6.3 *[1.9,26.5]*	34.6±14.8 *[5.6,63.6]*	4.8±1.9 *[1.1,8.5]*	3.6±1.8 *[0.2,7.0]*	10.2±2.1 *[6.1,14.3]*	2.4±0.5 *[1.4,3.4]*	6.8±1.8 *[3.3,10.3]*	62.4±20.9 *[21.5,103.3]*
DnBP 60ng/g	18.2±7.9 *[2.7,33.7]*	40.4±9.0 *[22.73,58.1]*	6.8±1.7 *[3.6,10.0]*	3.0±1.1 *[0.9,5.2]*	14.0±2.4 *[9.3,18.7]*	0.6±0.4 *[-0.2,1.4]*	10.6±1.0 *[8.7,12.5]*	75.4±10.3 *[55.2,95.6]*
DnBP 600ng/g	21.2±9.1 *[3.4,39.0]*	33.0±10.8 *[11.8,54.2]*	6.2±2.2 *[1.9,10.5]*	3.6±2.0 *[-0.3,7.5]*	9.8±1.4 *[6.9,12.7]*	3.0±0.7 *[1.6,4.4]*	**6.0±1.7*** ***[2.7,9.3]***	61.8±14.9 *[32.7,90.9]*
Mixture	28.8±7.5 *[14.1,43.5]*	20.0±13.0 *[-5.6,45.6]*	**1.0±0.6*** ***[-0.1,2.1]***	1.8±0.5 *[0.8,2.8]*	9.6±2.5 *[4.8,14.4]*	0.8±0.4 *[0.1,1.5]*	**3.0±1.2*** ***[0.6,5.4]***	36.2±13.9 *[9.0,63.4]*

Supplementary information 4*

*Chapter 3 – Part II: Investigating the effects of repeated exposure to di-2-ethylhexyl phthalate and di-n-butyl phthalate on larval development of in vitro reared *Bombus terrestris* workers larvae

Table S4.1. Statistical comparison in larval development duration between groups exposed to controls or DnBP 600ng/g, DEHP 1200ng/g (GLMM, family gamma).

Larval stage	Chisq.	d.f.	p-value
L1	5.28	3	0.1526
L2	2.21	3	0.5292
L3	1.49	3	0.6842
L4 pre-defecation	1.51	3	0.6790
Total	1.90	3	0.5931

Table S4.2. Statistical comparison in gene expression between groups exposed to controls or DnBP 600ng/g, DEHP 1200ng/g (GLMM, family gamma).

Gene	Chisq.	Df.	p-value
AADAT	19.3	3	0.0002***
LLDH	4.95	3	0.1753
ECR	2.57	3	0.4637
E75	1.11	3	0.7746
78C	9.18	3	0.0270*
FAS	1.73	3	0.6295
ACC	2.09	3	0.5548

Supplementary material 5*

*Chapter 3 – Part III: Expression of genes involved in crucial hormonal signalling pathways in *Bombus terrestris* workers is disrupted after repeated exposure to di-2-ethylhexyl phthalate and di-n-butyl phthalate.

Table S5.1. Primer characteristics and efficiency. Sequences and Tm with associated NCBI Gene ID.

Gene name (abbreviate)	Type	NCBI ID	Primer sequences	efficiency	Working T_m (°C)
Ribosomal protein L13 (rpl13)	reference	XM_012316284.1	Fw TTGGAATTGCTGTAGATCCTAGAAG Rv CATTCAGTGGGAAGAGAATCAA	1.943	55-60
Phospholipase A2 (pla2)	reference	FN_391388	Fw TATCTTTCAATGCCCAGGAG Rv GTCGTAACAAATGTCATGCG	1.932	55-60
Kynurenine/alpha-aminoadipate aminotransferase (AADAT)	target	XM_003393468.2	Fw AATTGGGTTCAGCTTTGCAG Rv GAGACGATCACGATGTCACG	2.107	60
L-lactate Dehydrogenase (LLDH)	target	XP_003396770.1	Fw GACGTTCAATCTCTGCGACA Rv CTGCCAAAGAACCGAGTGAT	1.932	60
Insulin receptor (InR)	target	XM_003397898.2	Fw GAAGCCTCTTGCCCTGGATG Rv CTCGTGCCGATAATGCTTGC	1.964	60
Locust insulin-related peptide-like (LIRP)	target	XM_003400730.2	Fw GCGGTAGTGTTTACAATAGCCG Rv ATTCCCCGTCCATTTCTTCG	1.833	60
Oestrogen-related receptor (ERR)	target	XM_012308778.2 XM_012308777.2	Fw GGACTTCATGGAATCGCTGT Rv CCAAAGCTCTACGCTCCAAT	1.842	60
Ecdysteroid receptor (EcR)	target	XM_020867166.1 XM_012317565.2	Fw TATCACTACAACGCACTGAC Rv CCGCATGTACATATCGATCT	1.914	55
Ultraspiracle (USP)	target	XM_020864351.1 XM_012312252.1	Fw CAGTGGCAAGCATTATGGTG Rv TCTCTCTTCATGCCCATCGC	1.900	60
E75	target	NM_001080110.1 XM_00339894	Fw GCACGGACATCATTCACAAC Rv TTGAGATCCTTGGGGTGAAC	1.982	55
78C	target	XM_003395889.3 XM_012309181.2	Fw ACGACGTGAAAACAGTGCAG Rv TTGTGAGACGCTCGAGACAC	1.919	55