Effects of traffic-derived airborne particulate matter on social Hymenoptera

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“I am, somehow, less interested in the weight and convolutions of Einstein’s brain than in the near certainty that people of equal talent have lived and died in cotton fields and sweatshops.”

Stephen Jay Gould (1941-2002)

Content

Summary

Biodiversity loss is threatening the functionality of ecosystems worldwide. The decline in insect biomass and diversity is of special concern as they provide many vital ecosystem services, including pollination, nutrient cycling, and pest control. The reasons for this decline are multifactorial, with the main drivers being habitat destruction, land use intensification, climate change, invasive species, and pollution. Among pollutants, research focused on the effects of pesticides and fertilisers due to their extensive application in the environment. Another group of anthropogenic pollutants is airborne particulate matter, such as diesel exhaust particles. It could be dangerous as it is ubiquitous and may contain harmful substances. While negative effects on human health have been reported, the impact on insects is still largely unknown. As airborne particulate matter is very small, it may enter an insect's body via the tracheae or by the ingestion of contaminated food.

Social Hymenoptera, such as ants, social wasps, and social bees, are an ecologically important and widespread group of insects. They have a reproductive division of labour, breed cooperatively, and generations overlap. Encounters with an array of pollutants may happen in their typically large foraging areas from where they transport food into their colonies. Therein, pollutants might accumulate in the food storage and affect the different life stages.

In my thesis, I investigated the effects of airborne particulate matter, primarily diesel exhaust particles, on the buff-tailed bumblebee *Bombus terrestris* and the black garden ant *Lasius niger*. First, I assessed the lethal and sublethal effect of diesel exhaust particles on *B. terrestris* in the laboratory after oral exposure. I could show that chronic exposure to high doses leads to increased mortality, while single exposure and lower concentrations did not affect the bumblebee's survival (Article 1). Chronic oral exposure to diesel exhaust particles caused shifts in the composition of the workers' gut microbiome and gene expression. I found a significantly lower abundance of the common bacterium *Snodgrasella*, which is associated with protection against gut parasites. Exposed workers showed changes in the gene expression associated with metabolism and stress, also indicating potential health issues (Article 2). In a field experiment, I tracked the homing and foraging behaviour of bumblebees after exposure via air to evaluate the effects of diesel exhaust particles under natural conditions. While a one-time exposure did not affect the homing flight duration and subsequent foraging, it significantly delayed the take-

off to start the homing flight. The delay was mainly caused by a struggle to take off vertically out of the exposure box, which may indicate underlying physiological constraints (Article 3). Colony founding is one of the most important and, at the same time, most vulnerable stages in the life cycle of social insects. I regularly exposed bumblebee colonies at the early founding stage to diesel exhaust particles. However, the development of the treated colonies did not differ from the control colonies, indicating no harmful effects (Article 4). To compare single and multiple stressor effects on the ant *L. niger*, we exposed wild-caught queens at the colony-founding stage to soil containing different combinations and concentrations. Diesel exhaust particles, microplastic particles and fibres, or brake abrasion did not affect any of the investigated colony founding parameters. In contrast, manure application caused prolonged egg development and a smaller number of pupae and workers. This highlights the potential harm of manure application to soil-dwelling insects (Article 5).

When trying to generalize the results from my thesis, I need to be aware of some limitations that I had to accept. In my thesis, choosing field-realistic doses was one of the biggest challenges for me as there is a lack of reliable data on environmental concentrations. Additionally, I mostly studied single stressor effects, even though insects encounter various other stressors, such as parasites or limited food availability, in the wild. The slight changes caused in my studies might indicate problems to the organisms if encountering multiple stressors. Insects may be able to compensate for the impacts of one stressor but will eventually be overstrained by multiple stressors. It also must be considered that *B. terrestris* and *L. niger* are very abundant species, especially in urban areas. Thus, they could be more tolerant towards anthropogenic pollution or have already adapted to higher levels of air pollution than other species.

Nevertheless, the novel approaches and results from my thesis lay an important foundation for future research on the effects of airborne particulate matter on insects. My thesis adds to the understanding of the role these pollutants play in the global insect decline. I am looking forward to future studies that build on this work to investigate these pollutants in multiple stressor setups and look at the effects on other, less common species.

Zusammenfassung

Der Biodiversitätsverlust bedroht die Funktionalität von Ökosystemen weltweit. Der Rückgang der Insektenbiomasse und -diversität ist besonders bedenklich, da Insekten viele wichtige Ökosystemfunktion haben, unter anderem Bestäubung, die Kontrolle von Schädlingen und das Aufrechterhalten von Nährstoffkreisläufen. Die Gründe für diesen Rückgang sind multifaktoriell, wobei die Haupttreiber Lebensraumzerstörung, Landnutzungsintensivierung, Klimawandel, invasive Arten und Umweltverschmutzung sind. Bei Schadstoffen hat sich die Forschung hauptsächlich auf Pestizide und Dünger fokussiert, da diese in großen Mengen in die Umwelt ausgebracht werden. Eine weitere Schadstoffgruppe, die sehr gefährlich sein könnte, ist Feinstaub, wie beispielsweise Dieselrußpartikel, da dieser allgegenwärtig in unserer Umwelt ist und mitunter schädliche Substanzen beinhaltet. Während die schädlichen Auswirkungen auf die menschliche Gesundheit gut belegt sind, sind die Auswirkungen auf Insekten noch größtenteils unbekannt. Aufgrund seiner kleinen Partikelgröße könnte Feinstaub über die Tracheen oder kontaminierte Nahrung in den Insektenkörper gelangen.

Eine ökologisch wichtige und weit verbreitete Insektengruppe sind soziale Hymenopteren. Zu ihnen gehören Ameisen und viele Wespen- und Bienenarten. Sie zeichnen sich durch reproduktive Arbeitsteilung, gemeinsame Brutpflege und überlappende Generationen aus. Sie können einer Vielzahl von Schadstoffen begegnen, da sie normalerweise sehr große Gebiete zur Nahrungssuche haben, von denen sie Futter in ihre Kolonie bringen und dort lagern. Dort könnten alle Lebensstadien der Insekten von den Schadstoffen betroffen sein.

In meiner Dissertation untersuchte ich die Effekte von verkehrsbedingtem Feinstaub, insbesondere Dieselrußpartikeln, auf die Dunkle Erdhummel *Bombus terrestris* und die Schwarze Wegameise *Lasius niger*. Zunächst ermittelte ich die lethalen und sublethalen Effekte von Dieselrußpartikeln auf *B. terrestris* nach oraler Aufnahme. Ich konnte zeigen, dass eine chronische Aufnahme von hohen Dosen zu einer erhöhten Mortalität führt. Geringe Dosen und Einzelapplikationen hatten keinen Einfluss auf das Überleben der Hummeln (Artikel 1). Chronische Aufnahme von Dieselrußpartikel führte zu Veränderungen im Darmmikrobiom der Hummeln, insbesondere die Häufigkeit des Bakteriums *Snodgrassella* war deutlich verringert. Dieselbe Behandlung führte auch zu Veränderungen in der Genexpression, welche in Verbindung mit dem Metabolismus und Stress steht. Diese Erkenntnisse deuten auf

gesundheitliche Probleme der Hummeln nach Partikelaufnahme hin (Artikel 2). Um Effekte unter natürlichen Bedingungen zu testen, führte ich ein Feldexperiment durch, bei dem ich den Heimflug und die Sammelflüge von Hummeln beobachtete, nachdem diese Dieselrußpartikeln über die Luft ausgesetzt waren. Die einmalige Exposition hatte keinen Einfluss auf den Heimflug und anschließende Sammelflüge. Es wurde jedoch beobachtet, dass die behandelten Hummeln deutlich länger brauchen, um aus der Expositionsbox zu fliegen. Diese Probleme beim vertikalen Flug aus der Box könnten Hinweise auf tiefergehende physiologische Einschränkungen geben (Artikel 3). Ich führte zudem Experimente während der Koloniegründung durch, da diese eine der wichtigsten und zugleich empfindlichstes Phasen im Lebenszyklus von sozialen Insekten ist. Ich behandelte Hummelkolonien in der Frühphase regelmäßig mit Dieselrußpartikeln. Die Folgeentwicklung unterschied sich jedoch nicht von den Kontrollkolonien, weshalb keine Hinweise auf schädliche Effekte gefunden wurden (Artikel 4). Um die Effekte von einzelnen und multiplen Stressoren auf die Ameise *L. niger* zu testen, fingen wir wilde Königinnen kurz vor der Koloniegründung und setzten diese auf Erde, welche verschieden Kombinationen und Konzentrationen von Schadstoffen beinhaltete. Dieselrußpartikel, Mikroplastikpartikel und -fasern sowie Bremsstaub hatten keinen Effekt auf die Koloniegründung. Dünger führte jedoch zu einer verlangsamten Eientwicklung sowie weniger Puppen und Arbeiterinnen. Dies unterstreicht die möglichen Folgen von Düngeranwendung auf bodenlebende Insekten (Artikel 5).

Es gibt jedoch auch Unsicherheiten und Beschränkungen zu beachten, wenn man generelle Aussagen über die Ergebnisse meiner Arbeit ziehen möchte. Es war schwierig feldrealistische Dosen zu wählen, da die Datenlage bezüglich der Stoffkonzentrationen in der Umwelt ungenau ist. Zudem habe ich hauptsächlich die Effekte von Einzelstressoren untersucht, was außer Acht lässt, dass Insekten in der Natur einer Vielzahl an Stressoren gleichzeitig ausgesetzt sind, wie beispielsweise Parasiten oder Nahrungsknappheit. Die kleinen Effekte, die ich in meinen Studien beobachtete, könnten darauf hinweisen, dass die Insekten in der Lage sind, einen Stressor weitestgehend zu kompensieren, dann jedoch mit mehreren Stressoren überfordert sind. Außerdem muss beachtet werden, dass *B. terrestris* und *L. niger* sehr häufige Arten sind, insbesondere in urbanen Gebieten. Es könnte sein, dass sie toleranter gegenüber anthropogenen Schadstoffen sind oder sich bereits an höhere Luftverschmutzung angepasst haben.

Nichtsdestotrotz legen die neuen Ansätze und Ergebnisse meiner Arbeit eine wichtige Grundlage bei der Erforschung der Effekte von verkehrsbedingtem Feinstaub auf Insekten. Meine Arbeit verbessert das Verständnis darüber, welche Rolle diese Schadstoffe beim globalen Insektenrückgang spielen. Ich freue mich auf zukünftige Studien welche aufbauend auf meiner Arbeit diese Schadstoffe mit zusätzlichen Stressoren kombinieren und an anderen, weniger häufigen Insekten testen.

Abbreviations

DNA = Deoxyribonucleic acid

LD50 = Lethal dose, 50%

OECD = Organisation for Economic Co-operation and Development

PAH = Polycyclic aromatic hydrocarbons

PCR = Polymerase chain reaction

PM2.5 = Particulate matter with a diameter of 2.5 µm or less

ROS = Reactive oxygen species

RNA = Ribonucleic acid

Figures

Figure 1: Factors affecting the health of social Hymenoptera on the individual, colony and population level including suggested biomarkers to measure the impact of these factors on insect health.

Introduction

Global insect decline

Global biodiversity loss is threatening the functionality of ecosystems and consequently human well-being in the Twenty-first century (Diaz et al. 2006, Dirzo et al. 2014). Ongoing declines in the diversity and abundance of organisms will result in an uncertain scenario in which essential goods and services provided by nature might not be given anymore (Cardinale et al. 2012). While vertebrate extinctions and losses have been documented for decades, global insect decline has shifted more strongly into the focus of the scientific community and the general public in recent years (Cardoso et al. 2020, Ceballos & Ehrlich 2002, Wagner et al. 2021). With more than a million described species and about 5.5 million species estimated, insects are the richest taxon on earth (Stork 2018). Insects are essential for ecosystems by providing and contributing to various functions such as pollination, nutrient cycling by decomposition of dead wood and leaf litter, regulating herbivores and plants, linking trophic levels, or pest control (Cardoso et al. 2020, Noriega et al. 2018). However, there is increasing evidence that the abundance and richness of insects are decreasing, globally. A long-term study in protected areas in Germany revealed a decline of 75% in the biomass of flying insects (Hallmann et al. 2017). Similarly, in the tropical rainforest of Puerto Rico, arthropod biomass has fallen by 10 to 60 times compared to the 1970s (Lister & Garcia 2018). In German grasslands and forests, substantial declines in insect species diversity have been observed (Seibold et al. 2019) and in Great Britain, pollinators have declined in similar habitats (Powney et al. 2019). Even though not all taxa are affected in the same way and the numbers differ between regions, several meta-analyses agree on an unprecedented global loss of insect biomass and diversity (Crossley et al. 2020, Dirzo et al. 2014, Sánchez-Bayo & Wyckhuys 2019, Van Klink et al. 2020). The reasons for this decline are multifactorial with the main drivers being habitat destruction, intensification of land use, climate change, invasive species, and pollution (Miličić et al. 2021, Müller et al. 2023, Sanchez-Bayo & Wyckhuys 2019).

Pollution – a main driver of insect decline

Anthropogenic pollutants mostly originate from traffic, industrial production, and agriculture (Cachada et al. 2018). They enter the environment via deliberate application or leakage and poor waste management (Briggs 2003). Due to its extensive application in agriculture, research focused on the effects of pesticides and fertilisers on insects (Sanchez-Bayo & Wyckhuys 2019). The wide range of negative effects of pesticides include changes in behaviour, learning performance, immunity, microbiome, development, reproduction, and increased mortality (Desneux et al. 2007, Serrão et al. 2022, Uhl & Brühl 2019). Next to effects on individual insects, fertilizers cause the homogenisation of plant and insect communities on the landscape level resulting in a loss of many specialist insect species due to the lack of resources (Buhk et al. 2017, Haddad et al. 2000, Kleijn et al. 2009, Harvey & MacDougall 2015). Other pollutants, such as heavy metals or airborne particulate matter have received less attention (Feldhaar & Otti 2020). Heavy metals can accumulate in insects, which can cause negative effects such as impaired development reducing body size (Skaldina et al. 2018, Skaldina & Sorvari 2019, Szentgyörgyi et al. 2011). Due to its small particle size and ubiquity in the environment, particulate matter poses a potential risk to insects, which needs investigation.

Particulate matter – Definition, composition, and its effects on organisms

In general, particulate matter is defined by its particle size. Depending on the diameter it is referred to as coarse particulate matter (< 10 µm), fine particulate matter (< 2.5 µm), or ultrafine particulate matter (< 0.1 µm) (Brook et al. 2010, Harrison 2020, Kelly & Fussell 2012). The physicochemical composition of the particles differs depending on their source. Natural sources of particulate matter include sea salt, soil dust, and wildfires (Harrison 2020, Mazzei 2008). Early anthropogenic sources of particulate matter were dominated by burning wood and later coal. However, in recent decades, road traffic has become one of the main contributors to particulate matter emissions (Harrison 2020). Non-exhaust emissions from road traffic include brake dust deriving from the attrition of the disc and the pad, and tyre wear (Harrison 2020). Brake dust particles consist of various metals and phenolic compounds, depending on the brake lining used (Iijima et al. 2007, Thorpe & Harrison 2008). Originally made from natural rubber, tyres nowadays consist of a mixture of natural and synthetic rubbers, which are polymers made from petroleum, and some additives (Kole et al. 2017). While driving, the contact between road and tyre leads to the generation of many small particles which are released to the environment. Due to its ubiquitous use, tyre wear is a major contributor to global microplastic pollution (Kole et al. 2017). However, among the biggest concerns of traffic emissions are diesel-exhaust particles from industrial vehicles and passenger cars. They are mainly generated by incomplete combustion and consist of an elemental carbon core with adsorbed organic compounds, metals, and trace elements (Greim 2019, Wichmann 2007). The organic fraction includes problematic substances, such as polycyclic aromatic hydrocarbons (PAHs), which are toxic, mutagenic, and genotoxic to various life forms, such as microorganisms or animals (Douben 2003, Patel et al. 2020, Sun et al. 2021).

There are efforts to limit and lower the emissions of airborne particulate matter due to its well-documented negative effects on human health, which include cardiopulmonary diseases and lung cancer (Kim et al. 2015, Valavanidis et al. 2008). The latest air quality guidelines by the World Health Organization (WHO) recommend annual mean concentrations of PM2.5 not exceeding 5 µg/m^3 (WHO 2021). Despite constant improvements, exceedances of air quality standards well above recommendations are common in cities around the globe, including the European Union (European Environment Agency 2024, Hammer et al. 2020).

Insects may encounter these pollutants in various ways, e.g. by foraging in contaminated areas, consuming contaminated food or direct deposition on the insect’s cuticle (Azpiazu et al. 2023, Feldhaar & Otti 2020, Lukowski et al. 2018, Negri et al. 2015). The particles might enter an insect´s body via oral ingestion or the tracheal system where they could impair physiology or interfere with biochemical processes (Feldhaar & Otti 2020, Negri et al. 2015). In contrast to the findings from human medical studies, research investigating the effects of airborne particulate matter on other organisms, such as insects, is still scarce and often of a correlative nature. This lack of a mechanistic understanding of the findings makes them difficult to interpret, as there are confounding factors, often associated with urbanisation, such as other pollutants, limited food sources, or increased heat (Ferrari 2024, Polidori et al. 2023). A field study on the Giant Asian honeybee *Apis dorsata* in Bangalore, India observed significant correlations between airborne particulate matter deposition and changes in bee survival, heart rate, haemocyte levels, and flower visitation (Thimmegowda et al. 2020). Laboratory experiments showed increased mortality of cotton bollworm larvae *Helicoverpa armigera* when fed with leaves laden with coal dust (Vanderstock et al. 2018). These results indicate the potential risk of particulate matter exposure to various insect groups and the need for further investigation.

Social Hymenoptera – Widespread, important, and at risk

An ecologically important and widespread group of insects are social Hymenoptera, such as ants, social wasps, and social bees. Social insects live in colonies and are characterized by the reproductive division of labour, cooperative breeding, and overlapping generations (Wilson 1971). They provide many important ecosystem services, such as pollination, nutrient cycling, and linking of trophic levels, which are vital for a functioning ecosphere but also to us humans (Cardoso et al. 2020, Elizalde et al. 2020, Noriega et al. 2018). Due to their role in soil perturbation, seed dispersal, and pest control, some insect species, like ants, are referred to as ecosystem engineers (De Almeida et al. 2020, Farji-Brener & Werenkraut 2017, Philpott & Armbrecht 2006, Wills & Landis 2018). The economic value of crop pollination by insects is estimated to be several hundred billion dollars each year which highlights their importance for human food production (Porto et al. 2020). Social Hymenoptera inhabit virtually every terrestrial habitat around the world (Hölldobler & Wilson 1990, Schultheiss et al. 2022). They often occur in very high numbers that lead to an enormous biomass. For ants alone, a study estimates around 12×10^{15} individuals worldwide which translates to around 12 megatons of dry carbon. This is equivalent to about 20% of human biomass and exceeds the combined biomass of wild birds and mammals by far (Bar-On et al. 2018, Schultheiss et al. 2022). The conservation of social Hymenoptera is crucial for the ecosphere considering their pervasive abundance and important services they provide.

Consistent with the general insect decline, social insect species are threatened by the same factors, such as land-use change, climate change and pollution. On different continents bumblebees (*Bombus*), one of the most important and intensively studied pollinator group, have declined in numbers and distribution (Cameron & Sadd 2020). Global warming seems to be the major threat to these cold-adapted insects. The increasing frequency of hot temperatures is linked to local extinction risk, colonisation and changes in species richness of *Bombus* species in North America and Europe, partly explaining the observed declines (Soroye et al. 2020). The North American western bumblebee (*B. occidentalis)* is a good example of how a formerly widespread species can become increasingly rare due to the accumulation and interaction of various negative factors. A spillover of the pathogen *Vairimorpha (*previously *Nosema) bombi* in the late 20th century from commercially reared *B. occidentalis* colonies to wild specimens probably weakened wild populations (Janousek et al. 2023). Between 1998 and 2020, increased temperatures and droughts best predict the decline of the species after the pathogen invasion (Janousek et al. 2023). Additionally, declining populations of *B. occidentalis* seem to be under stress due to the application of neonicotinoid pesticides, which have negative effects on bees (Alkassab & Kirchner 2017, Camp & Lehmann 2020, Czerwinski & Sadd 2017, Janousek et al. 2023). What ultimately leads to the observed decline is often hard to identify but the list of examples highlights that insect species often face multiple stressors simultaneously. Thus, the challenge is to disentangle the individual effects of each stressor and describe potential interactions between them.

The evidence for global declines of other social Hymenoptera, such as wasps and ants, is less profound (Sanchez-Bayo & Wyckhuys 2019). However, in certain areas studies show the loss of diversity and abundance in ants. With increasing land-use intensity in temperate grasslands, including mowing or fertilization, ant species richness and abundance decreased (Dahms et al. 2005, Heuss et al. 2019). In strongly human-impacted habitats, such as agricultural fields, roadside habitats, or surroundings of industrial sites, declines observed in ant diversity and abundance are indirect evidence for habitat fragmentation, habitat loss and soil pollution as driving forces (Eeva et al. 2004, Philpott et al. 2010). A comparative field study on *Camponotus japonicus* showed that that labial gland disease was only present in polluted areas, which indicates that pollution stress makes ants more vulnerable to infections (Zhang et al. 2024). In addition, neonicotinoid insecticides which are widely used in agriculture have been shown to have negative effects on the colony growth rate of ants (Schläppi et al. 2020).

Due to their way of living, social Hymenoptera are at an increased risk of encountering different pollutants. Large foraging areas and colonies comprising a few dozen to many thousand workers lead to the potential transfer of contaminated food or nesting material to the central nest areas where it may accumulate (Feldhaar & Otti 2020). Therein, conspecifics and the brood could be chronically exposed to a mixture of pollutants (Hladun et al. 2016, Morales et al. 2020). In different compartments of honeybee hives, such as wax and honey, heavy metals and pesticides were identified and resulted in negative effects on individual bees and colony development, primarily affecting brood stages negatively (Chauzat & Faucon 2007, Conti & Botrè 2001, Hladun et al. 2016). Despite the potentially increased encounter of pollutants, social Hymenoptera have different strategies to cope with this risk. In many eusocial species, extensive cleaning behaviour has evolved to prevent the entrance or spread of diseases, parasites, or contamination (Cremer et al. 2007, Cremer et al. 2018. Otti et al. 2014). This collective action is referred to as social immunity and comprises other physiological, behavioural, and organisational adaptations, such as the removal of infested conspecifics or allogrooming (Cremer et al. 2007, Cremer et al. 2018). Additionally, a large colony size may buffer the negative effect of pollutants and large foraging areas enable the avoidance of contaminated patches (Crall et al. 2019, Easton-Calabria et al. 2023, Feldhaar & Otti 2020, Straub et al. 2015). If those avoidance behaviours fail, there are a few potential ways how pollutants interact with the insect's body. Deposition of pollutant particles on the body surface might impair important sensory organs, such as the antennae, causing problems in chemoreception (Wang 2023). After oral ingestion of pollutants, toxic substances may negatively affect insect physiology or immunity leading to problems in development, reproduction, and longevity (Milivojevic et al. 2015, Tan 2018). If pollutants are small enough to enter the tracheae, they might interfere with the respiratory system causing subsequent health issues, such as inflammation reactions in tissues (Feldhaar & Otti 2020, Reich et al. 2023).

The life cycle of social Hymenoptera

Social Hymenoptera colonies typically comprise one or more queens, many workers, brood at different stages and – in parts of the colony life cycle - new reproductive individuals. During a colony cycle, the relative abundance of the different sexes, castes and life stages changes (Starr 2006). Different types of colony cycles exist. Some species have annual life cycles (e.g. *Bombus*), while others are perennial and last for several years (e.g. ants and honeybees) (Goulson 2003, Hölldobler & Wilson 1990). New colonies are founded by queens, either on their own (independent-founding, e.g. *Bombus*) or together with conspecifics (swarm-founding, e.g. *Apis*) (Starr 2006). During independent colony-founding, queens must raise their first offspring on their own. In species that evolved semi-claustral foundation, queens are required to forage during that period to feed their first brood (e.g. *Bombus*). In contrast, during claustral foundation, the nutrition of offspring is provided from the depletion of stored energy reserves of the queen (e.g. *Lasius*) (Brown & Bonhoeffer 2003). After successfully raising the first batch of workers, the queen's task shifts to reproduction (i.e. egg laying) for the rest of her life and the workers provide food and brood care. The colony founding is the most vulnerable stage of the colony cycle, as the future of the colony solely depends on the survival of one or few individuals (Helanterä 2016). As the colony grows bigger, the risk of colony failure due to the death of single individuals decreases, leading to more resilience against harmful events (Crall et al. 2019). The maximum age of castes varies tremendously between species. In annual species, the queen's maximum lifespan is typically about a year, while the workers only live for a few weeks or months. Reproductive males arising later in the colony stages live only during mating season (Stubblefield & Seger 1994). In perennial colonies, the maximum queen life span can be extraordinarily high. They can live up to 30 years exceeding their workers' maximum age by a multiple (Keller & Genoud 1997).

Assessing the impact of stressors on social Hymenoptera health – a framework

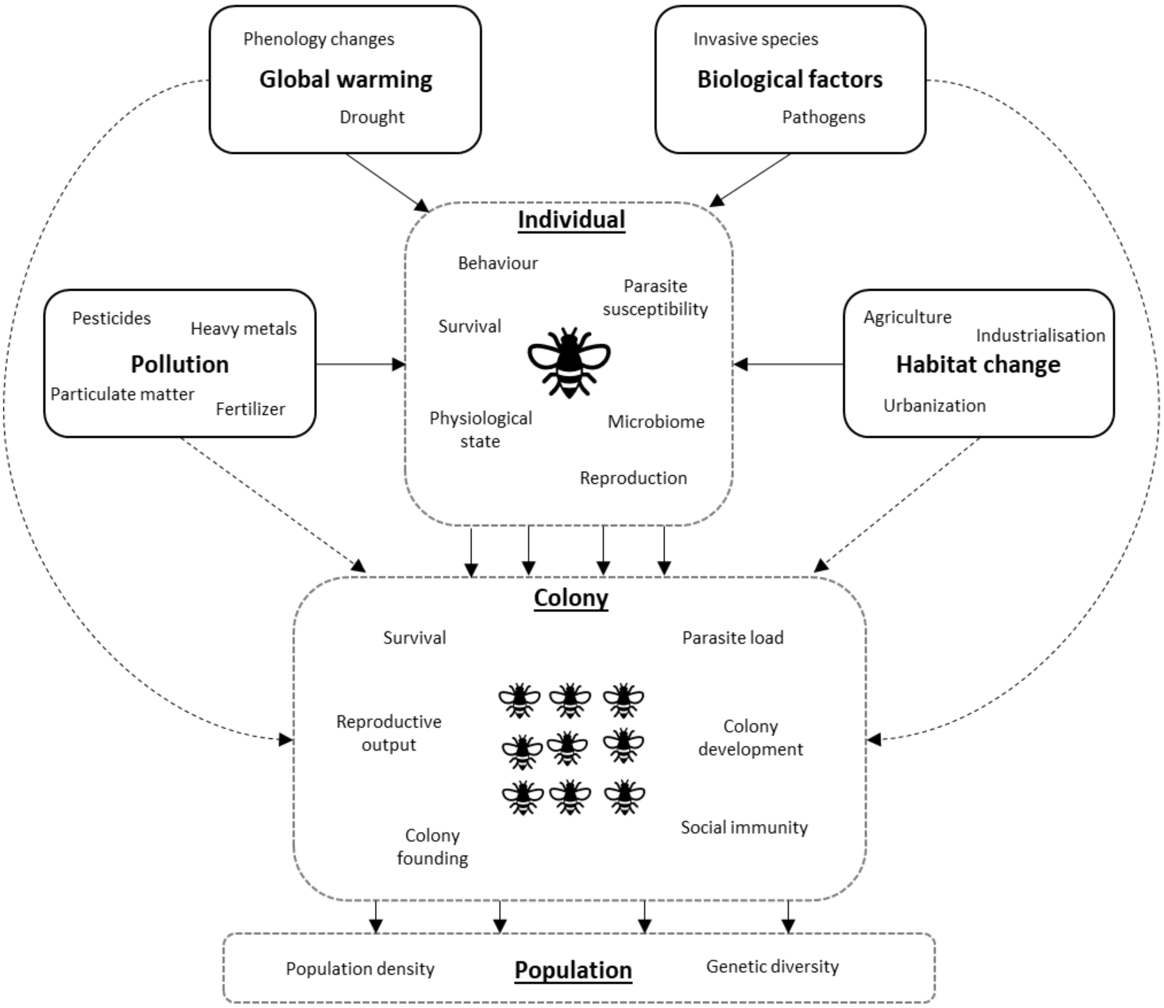
As social Hymenoptera live in colonies, there are different levels of organization a potential stressor could affect. Health must be considered at the individual, the colony and the population level when trying to assess the impact of a stressor on a social Hymenoptera species. Generally, health can be defined as the state of well-being that allows the optimal acquisition, allocation, and utilization of resources to increase fitness (Lopez-Uribe et al. 2020). In social Hymenoptera, optimal health would lead to healthy individuals contributing to prosperous, growing colonies that produce lots of offspring. However, health is affected by the multiple stressors occurring in environment. These stressors are a natural phenomenon as species interact and coevolve, resulting in competition or predation, and there have always been abiotic factors, such as weather, shaping the environment. However, human activity added several stressors, such as pollution, invasive species, or habitat destruction (Kaunisto et al. 2016).

Research assessing the impact of stressors on social Hymenoptera needs suitable biomarkers to identify the health of the different organisational levels. Biomarkers should be unbiased indicators which give qualitative or quantitative measures to assess the health of individuals, colonies, or populations (Burger & Gochfeld 2001, Lopez-Uribe et al. 2020). The most general health assessment is the survival of individuals in toxicity tests, best suited for pollutant stressors. By standardised methods, such as tests in accordance with OECD Test Guidelines for Chemicals, the negative effects of pollutants can be measured and compared (e.g. LD50), even across studies (Morris-Schaffer & McCoy 2020). However, stressors often do not have a lethal toxic effect on individuals. Here, biomarkers that uncover sublethal effects are necessary. On the individual level, there are many established markers for this purpose in social Hymenoptera. Physiological markers, such as reactive oxygen species (ROS), immune parameters, fat content, body size and weight, are used to evaluate nutritional state or stress levels. Worker body size and weight are known indicators of larval nutritional status and development (Daly et al. 1995, Goulson & Sparrow 2009). Increased levels of ROS leading to oxidative damage are associated with accelerated ageing in insects and could indicate underlying health concerns (Carvalho et al. 2013, Kramer et al. 2021). The immune response plays an important role in maintaining the health of insects. Measuring the quality of innate and inducible immune responses, such as melanisation or antimicrobial peptides, provides insight into the capability of individuals to defend against pathogens or parasites (Gonzalez-Santoyo & Cordoba-Aguilar 2012, Schmid-Hempel & Schmid-Hempel 1998, Wu et al. 2018). Vice versa, the presence and load of parasites or pathogens, such as *Nosema* or *Varroa*, raises individual health concerns and might indicate underlying problems in immunocompetence (Doums & Schmid-Hempel 2000, Rosenkranz et al. 2010). Behavioural changes, such as decreased foraging activity, are also biomarkers that may indicate negative effects of stressors, such as sublethal doses of insecticides or parasites (Colin et al. 2014, Goblirsch et al. 2013). A long-underestimated trait of insects is their associated microbiome. Especially the gut microbial community provides many important functions that include protection from pathogens, detoxification, digestion, and the production of essential nutrients (Engel & Moran, 2013). Stressors disrupting the gut microbiome may cause constraints in these microbial functions resulting in health issues (Koch & Schmid-Hempel 2011). For social Hymenoptera queens, reproductive output is an important individual marker, as it is affected by different stressors, such as pesticides or parasites, and is ultimately responsible for the evolutionary fitness of the whole colony (Amiri et al. 2017, Walsh et al. 2020, Whitehorn et al. 2012). Hence, queen reproduction is often measured, not only as an individual biomarker but as a proxy of colony health.

To assess health on the colony level, often averages of individual biomarkers are used (Lopez-Uribe et al. 2020). In bumblebees, mean worker size predicts colony performance, as a higher average size leads to greater colony mass and gyne production (Herrmann et al. 2018). Typical colony traits that can be measured throughout colony development and are strong health indicators include the number of workers, the amount of brood, gyne and drone production, food storage, or colony mass (Delaplane et al. 2013). A minimum number of workers is important for colony health, as it assures a reliable food provision or enables thermoregulation (Bretzlaff et al. 2024). Due to the increased risk of disease transmission and parasites associated with living in colonies, social Hymenoptera have evolved a collective immune defence against parasites, called social immunity (Cremer et al. 2007). The defence mechanism can be prophylactic or inducible and consists of behavioural, physiological, and organizational adaptions, such as cleaning, allogrooming, or social fever (Cremer et al. 2007, Van Meyel et al. 2018). Measuring social immunity is difficult, but proteomic markers for hygienic behaviour were identified and used for selection in honeybees (Guarna et al. 2015, Guarna et al. 2017). Another important marker for colony health is the abundance of parasites or pathogens. In the honeybee *Apis mellifera*, levels of *Varroa* mite infection are the best predictor of colony survival often causing colony failure during winter (Dainat et al. 2012, Kielmanowicz et al. 2015). In the ant, *Camponotus castaneus* infection with the fungus *Beauveria bassiana* can cause the failure of small colonies (Loreto & Hughes 2016).

On the population level, a species' health status is typically measured with abundance data across a specific geographic range (Lebuhn et al. 2012). To evaluate observed population densities, comparisons to longitudinal data from monitoring programs or historical collections are necessary (Burns et al. 2020, Mathiasson & Rehan 2019). A more detailed approach to population health is the assessment of genetic diversity. It is key to long-term resilience against various stressors, like parasites (Whitehorn et al. 2011). There are different methodological approaches to estimating genetic diversity and relatedness, such as the use of microsatellites or single-nucleotide polymorphisms (Coates et al. 2009). Genetic fingerprinting can be used to evaluate connectivity within and between populations, inbreeding status, or dispersal ability, all of which are important for the long-term health of social Hymenoptera populations (Pamilo et al. 1997). However, the genetic population structure also depends on the social system and colony structure of the species investigated. For example, invasive ants, like the Argentine ant *Linepithema humile*, may form supercolonies with a very low genetic diversity, but are still capable of spreading over hundreds of square kilometres (Suhr et al. 2010, Vogel et al. 2010).

The organisational levels of hymenopteran sociality are highly connected and so are their biomarkers and measures. Effects detected on one organizational level, typically also affect the other levels. To disentangle the effect of a single stressor on insect health in the multiple-stressor environment of social Hymenoptera is difficult and complicated (Kaunisto et al. 2016, Figure 1) but a logical step in the process of identifying the main factors driving observed insect declines to advise action plans.



Inspired by Lopez-Uribe et al. 2020

Figure 1: Factors affecting the health of social Hymenoptera on the individual, colony and population level including suggested biomarkers to measure the impact of these factors on insect health.

Nature as a multiple stressor environment – approaches in research

In their natural habitat, most organisms are exposed to numerous physical, chemical, or biotic factors that move them out of their normal operating range, so-called stressors (Segner et al. 2004, Pirotta et al. 2022). Due to the rapidly expanding human activity, the diversity and intensity of stressors have increased tremendously with the growing human population (Geldmann et al. 2014, Halpern et al. 2015). These stressors often do not only act individually but rather often interact with each other. The combined effect of multiple stressors can be greater, i.e. synergistic, smaller, i.e. antagonistic, or equal, i.e. additive, than predicted based on their individual effects (Folt et al. 1999, Piggott 2015). Despite the difficulty of predicting and understanding the interaction network among stressors and their importance in nature, the field of multiple stressor research is expanding rapidly (Pirotta et al. 2022). Although observational studies are very much constrained, they can inform the design of sophisticated experiments in ecological stressor research. Also, observational studies give important insight into ecological patterns and processes, often on a large scale, by gathering data from natural surroundings that have not been purposefully manipulated (Sagarin & Pauchard 2009). By extensive data analysis, the effects of a single stressor can be extracted with a certain degree of confidence. However, correlating and confounding factors commonly add uncertainty and drawing conclusions from observations will never provide a causal explanation for the observed effect (Sugihara et al. 2012). On the other hand, experimental research in controlled, randomized settings actively manipulates only one or few factors, making it easier to study the effect of a single stressor on different response variables. Like this, causal relationships can be determined with a high degree of confidence (Larsen et al. 2019). However, one must be careful when interpreting the results of experiments in a broader context. In a natural environment, a variety of other factors might interact with the manipulated stressor leading to potentially different outcomes (Haag & Matschonat 2001). Observational and experimental approaches should complement each other to achieve a comprehensive understanding of how and what effects stressors have on organisms (Clements et al. 2002). Intermediate approaches, such as field experiments or mesocosm studies, also add important information (Boyle & Fairchild 1997).

Research on the effects of stressors on insects must incorporate their social organization and potential interactions among stressors, in the study design and interpretation of the results. A variety of potential stressors affect individual insects and thus colonies, populations and whole ecosystems (Elizalde et al. 2020). Global warming will increase the severity and frequency of droughts (Dai 2012). Moreover, changes in plant phenology will be challenging for plant-associated insects (Renner & Zohner 2018). Biological factors, such as invasive species or pathogens, affect insect health and are often vectored by human activity (Sanchez-Bayo & Wyckhuys 2019). Pollutant stressors, such pesticides or heavy metals, can negatively affect insect physiology and cause a wide range of negative effects (Sanchez-Bayo & Wyckhuys 2019). Adding to these factors, massive changes in habitat quality and quantity due to urbanization, industrialization, and agricultural intensification may fail to provide enough resources and breeding sites (Tscharntke et al. 2002, Vanbergen 2014). Even though nature *per se* is a multiple-stressor environment, human activity has increased the intensity and quantity of stressors, ultimately leading to the observed global declines of insects. To effectively change and reduce the pressure induced by these stressors, research must disentangle the effects of single stressors to uncover causal relationships but also evaluate their effects and interactions in realistic multiple stressor scenarios. There is a need for mechanistic understanding and analytical tools to predict the effects of single and combined exposure to stressors (Pirotta et al. 2022).

The ecology of *Bombus terrestris* and *Lasius niger*

The buff-tailed bumblebee *Bombus terrestris* is one of the most abundant and widespread European wild bee species, especially in human-altered landscapes, such as urban areas (Herbertsson et al. 2021, Whitehorn et al. 2022). It is bred for commercial farming, as buzz-pollination increases the fruit yield for crops like strawberries or tomatoes (De Luca & Vallejo-Marin 2013, Nayak et al. 2020). Due to the introduction and the escape from captivity that followed, *B. terrestris* has established several populations outside its natural European range, such as Japan, Chile, and Tasmania, where it is considered invasive (Fontúrbel et al. 2021, Hingston et al. 2002, Inoue et al. 2008). It is a primitive eusocial pollinator and a model organism in ecotoxicological research serving as a proxy for other bumblebee species, many of which are threatened by anthropogenic disturbance (Cameron & Sadd 2020, OECD 2017). Like most bumblebees, *B. terrestris* has an annual life cycle with only hibernating queens surviving the winter (Alford 1975). Nests are formed underground and can contain up to 400 individuals or more (Duchateau & Velthuis 1988).

The black garden ant *Lasius niger* is a prevalent species across Europe with a wide range of habitats including urban areas and agricultural fields (Seifert 2018). *L. niger* is monogynous, meaning each colony only has a single queen. In contrast, the worker number can be several thousand individuals (Collingwood 1979). *L. niger* has a perennial life cycle with queens living up to 29 years (Hölldobler & Wilson 1990), whereas workers typically only survive one or two years under laboratory conditions (Dussotour & Simpson 2012). Colony foundation is claustral and independent, as queens do not forage during that period and raise their brood with energy from their body reserves (Keller & Passera 1989). Both, *B. terrestris* and *L. niger* are well-established model organisms in different research areas. Their commonness and robustness in captivity make them a suitable system in behavioural, ecological, genetic, or ecotoxicological studies.

Objectives

Environmental pollution is one of the main drivers of insect decline. Research focused mainly on the effects of pesticides or fertilizers on insects while other potential pollutants lack detailed risk analysis. A potentially impactful pollutant is traffic-derived airborne particulate matter, such as diesel exhaust particles or brake dust, as it is ubiquitous in the environment and its negative effects on vertebrates are profound. To understand the potential role of these pollutants in observed insect declines, it is important to assess the impact on different insect species under laboratory conditions. To get a detailed picture of the mode of action, different approaches are needed to uncover sublethal effects and effects on different organisational levels.

In this thesis I used two common social Hymenoptera species, *B. terrestris* and *L. niger*, to test the impact of diesel exhaust particles and other pollutants in experimental setups. I looked at individual and colony-level effects and assessed toxicity as well as various sublethal effects.

1. Assessment of lethal and sublethal effects of diesel exhaust particles on *Bombus terrestris* in the laboratory

Standard OECD protocols exist to evaluate the acute and chronic effects of potentially harmful substances on honeybees. I applied those methods to test the effects of diesel exhaust particles on workers of the bumblebee *B. terrestris* to see toxic effects. To uncover potentially hidden, sublethal effects, I examined further parameters. After oral exposure to diesel exhaust particles, I measured changes in fat body content, microbiome composition and gene expression. This provides valuable insights into the biochemical processes and functions that are potentially affected by the pollutant.

2. Evaluation of the effect of diesel exhaust particles on the flight activity of *B. terrestris* in the field

Hidden effects of pollutants may only emerge when complex tasks have to be performed. Measuring the homing flight duration and success is a method to evaluate the sublethal effects of substances on bees. By releasing bumblebees away from their nest, they need to remember their surrounding and navigate back to their colony. I looked at the flight activity of *B. terrestris* workers exposed to a single dose of diesel exhaust particles in comparison to a control group to uncover potential interference with flight performance or navigation.

3. The impact of diesel exhaust particles and other pollutants on colony founding – as a single or combined stressor

Colony founding is one of the most important and at the same time most vulnerable stages in the life cycle of social insects. The survival of the whole colony depends on one or few individuals and even small effects of pollutants may translate into big ones in the long-term. I assessed the effects of diesel exhaust particle exposure during the colony founding stage for *B. terrestris* and *L. niger*. In the *L. niger* setup, I also added other stressors to compare single versus multiple stressor effects, as these may be present in a natural environment.

Summary of the Articles

Article 1: Effects of diesel exhaust particles on the health and survival of the buff-tailed bumblebee *Bombus terrestris* after acute and chronic oral exposure

In Article 1, we assessed the effect of diesel exhaust particles on *B. terrestris* by performing acute and chronic exposure experiments according to OECD guidelines. Diesel exhaust particles were collected from a four-cylinder diesel engine and subsequently analysed with several methods. Thermogravimetric analysis was done to measure the proportion of organic components. We measured sub-micron particle size distributions with a fast response differential mobility particulate spectrometer. Polycyclic aromatic hydrocarbons were analysed via gas chromatography-mass spectrometry. The measurements revealed a median particle diameter between 52.1 nm and 101.9 nm with an organic fraction of 23.2 %. Characterization of PAHs revealed the presence of Pyrene with a concentration of 444 ppm. We fed different concentrations of the collected diesel exhaust particles to bumblebee workers, observed survival and measured fat body content as a proxy of bumblebee health. There was no effect of a single, acute exposure on bumblebee survival or fat body content. In contrast, chronic exposure to diesel exhaust particles significantly reduced survival with a concentration of 1 g/l or higher. Fat body content was only reduced in the chronic exposure with 0.5 g/l, which indicates the negative effects of the diesel exhaust particles on bumblebee health. There was no effect on fat body content in the higher concentrations which could be explained by the increased mortality in these treatments potentially selecting healthier, fitter individuals. Our results show the negative effects of diesel exhaust particle exposure on bumblebee workers when fed in high concentrations and over an extended period. In their natural environment, bumblebees may not encounter dosages as high as in this artificial setup. However, they must face many different abiotic and biotic stressors simultaneously. The additional stress by diesel exhaust particles could then cause negative effects also in lower concentrations.

Article 2: Diesel exhaust particles alter gut microbiome and gene expression in the bumblebee *Bombus terrestris*

In Article 2, I regularly exposed workers of the bumblebee *B. terrestris* to sublethal doses of diesel exhaust particles and brake dust, orally or via air. After seven days, we analysed the composition of the gut microbiome and tracked changes in gene expression. Diesel exhaust particles were collected from a four-cylinder diesel engine and brake dust was generated by milling brake pads in a vibrating cup mill with a tungsten carbide grinding set. After the seven-day exposure, bumblebees were dissected and further processed. Metagenomic DNA of gut samples were examined using PCR amplification and sequencing of 16S rDNA fragments. Microbial data were then analysed by calculating different diversity indices, multivariate analyses, generalized linear mixed models and principal component analysis. Changes in gene expression of bee abdomens were investigated by RNA-sequencing followed by differential expression analysis. Oral exposure to diesel exhaust particles changes the gut microbiome and gene expression of bumblebee workers, while exposure via air does not. Brake dust, the second pollutant we tested via oral exposure, did not induce changes in the gut microbiome or gene expression in the bumblebee workers. I detected major shifts in microbial composition after oral exposure to diesel exhaust particles. For example, *Snodgrassella*, one of the core bacteria with important functions, was nearly absent in exposed bumblebees. The microbial community is essential for various functions including immunocompetence, detoxification, or digestion. Thus, the observed gut dysbiosis may be harmful to bumblebee health. The transcriptome analysis revealed significant changes in gene expression after oral exposure of bumblebees to diesel exhaust particles. Upregulated genes indicate that these changes could be related to a general stress response against pollutants. In this article, we could show how molecular methods can be used to reveal hidden, sublethal effects of pollutants, in this case, diesel exhaust particles. The results indicate potential consequences for insect health after oral exposure, highlighting the potential role of airborne particulate matter as a driver of insect decline.

Article 3: Do diesel exhaust particles affect the flight activity of the buff-tailed bumblebee *Bombus terrestris* in the field?

In Article 3, we investigated the effect of short-time exposure to diesel exhaust particles via air on the flight activity of *B. terrestris* workers in the field. A colony box was placed in a meadow on the campus of the University of Bayreuth. After two weeks of acclimatization, we intercepted bumblebee workers leaving their colony. They were tagged with individual square tags for automatic identification. We transported them to two different locations, 380 meters and 1100 meters away from their colonies, respectively. Before release, the treatment group was exposed to diesel exhaust particles in small plastic boxes. We then measured take-off time and return time to their colonies and observed subsequent foraging behaviour via cameras at the colony entrance. The take-off time was drastically prolonged when the bumblebees were exposed to diesel exhaust particles caused by the inability of some workers to take off vertically from the exposure box and intensified grooming behaviour. However, there was no effect on the subsequent homing flight to the colony, which should be the more challenging task. Also, the foraging activity after return to their colonies did not differ between treatments, as the number of foraging flights and mean duration were similar. Our findings provide new insights into the potential role of airborne particulate matter in the insect decline by impairing flight activity. However, it remains unclear why bumblebees struggle to take off vertically after exposure to diesel exhaust particles. This observation needs further investigation to elucidate, for example, if this behaviour may indicate underlying physiological constraints.

Article 4: Do diesel exhaust particles in pollen affect colony founding in the bumble bee *Bombus terrestris*?

In Article 4, we investigated the effect of diesel exhaust particles on the colony founding of *B. terrestris* in a laboratory setup. Particles were collected from a four-cylinder diesel engine. We created artificial early-stage colonies by placing the bumblebee queen together with ten randomly selected workers in fresh nesting boxes. They were fed pollen spiked with diesel exhaust particles and the colony development was compared to control colonies for ten weeks. At the end of the experiment, we measured individual and colony-level traits. We did not see any effects of pollen spiked with diesel exhaust particles on worker count, brood count, brood weight, worker size, and relative fat body weight. These results indicate no harmful effects of diesel exhaust particles on colony founding in *B. terrestris* in my single-stressor setup. This study is the first to evaluate the effects of diesel exhaust particles on whole bumblebee colonies in a laboratory setup and adds to the understanding of how such particles may affect insect colonies. The next step is to repeat my approach in a more natural setup adding different stressors such as heat stress or food shortages which could uncover hidden effects of diesel exhaust particles.

Article 5: Individual vs. Combined Short-Term Effects of Soil Pollutants on Colony Founding in a Common Ant Species

In Article 5, we investigated the effects of different pollutants, as single exposure or combination, on the colony founding of the black garden ant *L. niger*. We collected wild queens at the colony founding stage and put each in a small container with soil to initiate digging and egg-laying. The soil contained specific concentrations and combinations of pollutants (brake dust, soot, polystyrene microplastic particles and fibres, manure) to determine dose-dependent effects and interactions between stressors. Colony establishment and development were regularly observed until the first worker hatched. As proxies for colony founding success, we measured queen survival, the development time of the different life stages, the brood weight, and the number of offspring. Throughout the experiment, queen mortality was very low and similar across treatments. Brake dust particles, soot, microplastic particles and fibres did not affect any of the investigated colony foundation parameters, independent of concentration or combination. This indicates no negative effects of these pollutants on the queen or the offspring. During their claustral colony founding ant queens do not forage but consume their energy resources to feed their offspring. Therefore, there was probably nearly no uptake of pollutants which could explain the missing effects. In contrast, a high concentration of manure applied to the soil led to a prolonged egg development time and a smaller number of pupae and workers at the end of the experiment. This could be explained by a temporary reduction of the oxygen levels in the soil due to manure application as insect development can be slower under such conditions. Low oxygen levels could also have caused losses in the first egg batch leading to the observed smaller number of pupae and first workers.

As we did not measure oxygen levels in my experiment these hypotheses remain speculative. Altogether, our results could show no effects of different combinations and concentrations of soil pollutants on the early stage of colony founding in *L. niger*. However, they highlight the potential harm of manure application to soil-dwelling insects.

General discussion

This thesis aimed to gain a wider understanding of the effects of traffic-derived airborne particulate matter on social Hymenoptera. By conducting laboratory and field experiments with bumblebees and ants, I investigated the impact on different organisational levels and endpoints.

Ecotoxicological assessment

As a first step, I conducted an ecotoxicological study on the effects of diesel exhaust particles on the buff-tailed bumblebee *B. terrestris* according to OECD guidelines (Article 1). We identified the physicochemical properties of the diesel exhaust particles. They showed a count median diameter between 52.1 nm and 101.9 nm. This small size increases the risk of unintended uptake by organisms via food or air, potentially damaging inner tissues and metabolism. The diesel exhaust particles are composed of 72% elemental carbon, 23.2 % organic substances, and 4.6% inorganic components. Especially the organic fraction is of ecotoxicological interest. We identified and quantified the PAHs pyrene, phenanthrene, and fluoranthene with concentrations of 444 ppm, 220 ppm, and 107 ppm, respectively. PAHs induce the formation of reactive oxygen species causing mutagenic and genotoxic effects on various lifeforms, including microorganisms and animals (Patel et al. 2020, Sun et al. 2021). In my study, I exposed bumblebee workers to different concentrations of diesel exhaust particles via spiked sugar water uptake. A single dose of up to 16 g/l did not increase the mortality of the workers. In contrast, extended exposure over 10 days led to a significantly increased mortality in workers fed with concentrations of 1 g/l and higher. Bumblebees seem to be capable of tolerating an acute, single exposure to high dosages of diesel exhaust particles but are prone to chronic exposure. The constant uptake may lead to an accumulation of the particles in the bumblebees' tissues resulting in an enrichment of PAHs which could explain the observed toxic effects. Our data also show a decreased uptake of sugar water with increasing concentrations of diesel exhaust particles. This could be due to a deterrent effect resulting in avoidance behaviour, which was also observed for bumblebees exposed to neonicotinoid pesticides (Thompson et al. 2015). Another explanation is the increased viscosity of the sugar water when spiked with high concentrations of diesel exhaust particles, potentially hindering the uptake via the proboscis. However, the decreased uptake of sugar water does not explain the increase in mortality, as the energy uptake would still be enough to enable survival. Instead, it could rather indicate a behavioural change due to the toxification resulting in apathetic behaviour with less energy demand. Such sublethal effects often appear at low dosages and can be overlooked in classical toxicological studies. As a proxy for insect health, we therefore also measured the relative fat body weight of the bumblebee workers. The fat body is an insect's central storage and biosynthesis organ that is responsible for many metabolic processes, including detoxification (Cohen 2009). While there was no effect in the single exposure experiment, chronic exposure led to reduced fat body weight in the intermediate treatment, with 0.5 g/l diesel exhaust particles, which indicates sublethal effects. This result is intriguing as one would expect the fat body weight to be low in the high concentrations, too. This may be due to a selective effect caused by the increased mortality in these treatments. Animals with a lower amount of initial fat body could have a lower likelihood of survival. The data set would then be biased towards individuals with a larger initial fat body, as they may constitute a significant portion of the group of survivors. The potentially reduced energy requirements due to apathetic behaviour, as discussed earlier, could partly explain the observed phenomenon, too. Relative fat body weight is a generalist, easy to measure, indicator for insect health, that has been used in ecological research for several decades (Knapp & Knappová 2013, Hulse et al. 2025).

Genetic methods show sublethal effects

Nowadays, there are more detailed approaches, for example by using molecular methods, to uncover sublethal effects and the processes involved. Therefore, in article 2, I investigated the effects of airborne particulate matter on the microbiome and transcriptome of the bumblebee *B. terrestris.* I regularly exposed workers to sublethal concentrations of diesel exhaust particles and brake dust, orally or via air. After 7 days, the metagenomic DNA of bumblebee gut samples were amplified and sequenced to analyse the bacterial composition of the gut microbiome. Abdominal RNA was sequenced to track changes in gene expression. The results show that oral exposure to diesel exhaust particles changed the gut microbiome and gene expression of bumblebee workers, while exposure via air did not. Brake dust, the second pollutant we tested via oral exposure, did not induce changes in the gut microbiome or gene expression. The observed shifts in microbiome composition after oral exposure could be caused by the toxic PAHs contained in the diesel exhaust particles. Studies reported changes in the microbial gut community after PAH exposure for different animal groups, including fish, sea cucumbers, or potworms (Enchytraeidae) (DeBofsky et al. 2020, Ding et al. 2020, Zhao et al. 2019). However, the large amount of elemental carbon in diesel exhaust particles may itself provide another explanation. The particles may function like activated carbon with its large surface-area-to-volume ratio and could adsorb microbes that are then discharged by excretion (Naka et al. 2001, Rivera-Utrilla et al. 2001, Wichmann 2007). Even though activated carbon typically has no direct negative impact, constant adsorption and discharge might disrupt the bacterial community resulting in compositional and quantitative changes. The bacterium *Snodgrassella*, one of the dominant core bacteria in undisturbed gut microbiomes of bumblebees (Hammer et al. 2021), is nearly absent after exposure to diesel exhaust particles. *Snodgrassella*, together with *Gilliamella*, forms a biofilm coating the inner wall of the ileum (Hammer et al. 2021, Martinson et al. 2012), which prevents bacteria from washout, enables the formation of a syntrophic network, and protects the host from gut parasites (Koch et al. 2019, Kwong et al. 2014, Näpflin & Schmid-Hempel 2018, Powell et al. 2016, Zhang & Zheng 2022). This mutualistic relationship between the microbes and the host seems to be disrupted as *Snodgrassella* abundance is extremely diminished. In contrast, *Gilliamella* increases in relative abundance after exposure to diesel exhaust particles, which could indicate the ability of *Gilliamella* to form a biofilm independently from *Snodgrassella.* However, it could also just be the consequence of *Gilliamella* being the leftover dominant bacteria in the gut. *Snodgrassella* seems especially prone to pollutants, as a decrease in its relative abundance was also observed after exposure of bees to copper, selenate, or glyphosate (Rothman et al. 2020). Another explanation could be that *Snodrassella* is poor in recolonizing the gut tissue after being discharged due to adsorption to the diesel exhaust particles. However, this remains speculative.

The changes in microbial gut composition may negatively affect the health of the bumblebees, as such a dysbiosis can negatively affect reproductive fitness, immunity, and resistance to pathogens in insects (Ami et al. 2010, Daisley et al. 2020, Raymann et al. 2017). For bumblebees, the abundance of *Gilliamella*, *Lactobacillus*, and *Snodgrassella* is negatively correlated with the parasites *Crithidia* and *Nosema*, while non-core bacteria are more abundant in infected bumblebees (Cariveau et al. 2014, Koch et al. 2012, Koch & Schmid-Hempel 2012, Mockler et al. 2018).

The transcriptome analysis revealed significant changes in gene expression after oral exposure of bumblebees to a sublethal dose of diesel exhaust particles. Enrichment and network analysis indicate that these changes could be related to a general stress response against pollutants. Upregulated gene ontology terms involve many metabolic and catabolic processes, while terms related to metabolic and biosynthetic processes are downregulated. This supports the idea of increased energy demand caused by pollutants (Beyers et al. 1999, Calow 1991). Changes in metabolic processes seem to be a typical reaction in insect species, like bees, mosquitos, or moths, against pollutants such as pesticides, heavy metals, or PAHs (Bebane et al. 2019, Chen et al. 2021, Colgan et al. 2019, David et al. 2010). Even though the affected gene terms are quite general, they show that there are processes going on inside the insect's body which may come to light in the long term or when facing additional stressors.

The results of my experiment highlight the sublethal effects of oral exposure to diesel exhaust particles in bumblebees as gut dysbiosis may increase the susceptibility to pathogens, while a general stress response may lower available energetic resources.

In contrast to the oral exposure, I did not find any changes in gene expression after exposure to diesel exhaust particles via air. To cause effects the particles need to enter the tracheal system or attach to sensory organs, such as the antennae. The exposure of bumblebees for three minutes per day may not have been enough to affect them. Particles on the antennae may have been removed quickly by cleaning behaviour and the spiracles seem to be an effective protective barrier against the uptake of particles into the tracheae (Harrison 2009, Schönitzer 1986). Thus, our results should be taken with care as we cannot be sure if any particles entered the tracheal system of the bumblebees. Similarly, we must be careful with the results of our brake dust treatment, where we did not see any effect on the microbiome or gene expression. As we used artificially milled brake pads resulting in a relatively big particle size (10 µm) and weight, there might have been an inadequate uptake of the particles in our study which may explain the lack of effects.

Effects on flight activity in the field

The laboratory experiments from articles 1 and 2 are important steps to gain insight into how airborne particulate matter affects insect health. The controlled conditions ensure that only the single stressor effect is measured, with no or few confounding factors adding uncertainty. The next logical step was to apply this stressor in a field experiment, where the scenario is more realistic and natural stressors may arise. Thus, in article 3, I captured bumblebee workers from an outdoor colony, exposed them to diesel exhaust particles via air, and released them away from their colony. Contrary to our expectations, neither the cognitively challenging homing flight to their colony nor the subsequent foraging activity was affected by the exposure to diesel exhaust particles, no matter from which distance the bumblebees were released. Also, the proportion of bumblebees that returned to the colonies did not depend on diesel exhaust particle exposure or flight distance. This indicates that a one-time exposure prior to release does not impair cognitive abilities and thus negatively affects spatial orientation and navigation. Also, diesel exhaust particles do not decrease the motivation for subsequent foraging flights, contrary to other anthropogenic pollutants such as pesticides (Lämsä et al. 2018, Muth and Leonard 2019). We did, however, discover a significant effect of diesel exhaust particle exposure on the bumblebees' vertical take-off. Many bumblebees struggled to overcome the only a few centimetres high wall of the exposure box, from which they were released. This resulted in a notably increased take-off time. The reason why some fail to take off vertically from the box but are still capable of navigating back to their colony is unclear. It could indicate underlying physiological malfunctions. Diesel exhaust particles might affect the sensory systems, especially on the antennae that carry different types of sensilla with functions in sensing chemical, thermal, mechanical and water stimuli (Fialho et al. 2014, Rands et al. 2023). In addition, the visual perception of the bumblebees may be affected which could impede the take-off by failing to identify the walls of the box as a barrier and thus flying straight into them. Diesel exhaust particles deposition on mechanosensory hairs of the bumblebees may also impair the perception of electric fields leading to motoric struggles to overcome the barrier (Sutton et al. 2016). To test these hypotheses, it would be important to do further studies on the vertical take-off of bumblebees.

Effects on colony founding

As social Hymenoptera live in colonies, there are different levels of organization a potential stressor could affect. Health must be considered at the individual, the colony and the population level when trying to assess the holistic impact of a stressor. While articles 1-3 focused on the effect on individual workers, articles 4 and 5 investigated the effects of pollutants on the early colony founding stage. This stage is very important in the life cycle of social Hymenoptera, as the survival of the whole colony is dependent on only one or very few individuals (Helanterä 2016). Small effects of stressors here could have serious consequences in the long term.

In article 4 I provided queens and workers of the bumblebee *B. terrestris* with pollen spiked with diesel exhaust particles at the early colony founding stage and compared the colony development to control colonies. After 10 weeks, I did not find any effect on individual-level, i.e. worker size and relative fat body weight, and colony-level life history traits, i.e. worker and brood count. These results indicate no harmful effects of diesel exhaust particles on colony founding in *B. terrestris* in my single-stressor setup. Hence, we have no reason to expect effects at later colony stages. However, as I did not measure the production and development of sexual offspring for the next generation, we should be careful with further speculations. Hidden effects that were not investigated in this study might come to light once a complete colony cycle is investigated, or when multiple stressors are added, such as heat stress or food shortages.

A different species of social Hymenoptera, the black garden ant *L. niger*, was the subject of article 5. Here, I exposed wild-caught queens shortly after their nuptial flight to different concentrations and combinations of pollutants (brake dust, soot, microplastic particles and fibres, manure) to determine dose-dependent effects and interactions between stressors. The pollutants were added to the soil where the ant queens established their new colony by raising the first clutch of workers. I did not find any effects of the particles used in this study on any of the life-history parameters, independently of the concentration and combination. This lack of effects could be explained by the way this ant species behaves. During claustral colony founding, ant queens do not consume any food as they meet their energy demands by using internal resources, for example by degenerating their flight muscles (Hölldobler & Wilson 1990, Wheeler & Buck 1996). For a pollutant to be harmful at this stage, it would have to be toxic at a very low dose or capable of entering via the cuticle or the trachea. Consequently, most pollutants at field-realistic doses, such as insecticides and fungicides, do not increase mortality in founding ant queens but may impact other life-history parameters (Schläppi et al. 2020, Pech & Heneberg 2015, Wang et al. 2015). What I did see in my study were the negative effects of manure application on the colony founding of *L. niger*. Eggs from queens exposed to high manure concentrations took longer to hatch, which resulted in a delayed emergence of workers. Also, fewer pupae and workers were raised by those queens. These results may be explained by manure-induced changes in oxygen levels in the early days of the experiment. The application of manure reduces oxygen levels in the soil, sometimes locally even leading to anoxic areas (Hossain et al. 2005, Zhu et al. 2014). Peak oxygen deficits in soil occur 16 h after the manure application and go back to near-normal within the following days (Hossain et al. 2005). Insect egg development is dependent on surrounding oxygen levels as they have diffusion holes in the shell (Hinton 1969, Woods & Hill 2004). *Tenebrio molitor* (Greenberg & Ar 1996) and *Drosophila melanogaster* (Frazier et al. 2001) show slower development under low oxygen levels in the immediate environment. The manure application at the beginning of my experiment may have caused problematic oxygen conditions leading to the observed delay in egg development. Additionally, it may also have led to losses in the first egg clutch. Those early losses would explain the observed fewer pupae and workers at the time of the first worker emergence. However, as I did not measure oxygen levels during my study, this explanation remains hypothetical. Still, the findings from my study underline the issue of excessive manure application in our environment.

Implications and limitations of this thesis

Taken together, my thesis provides a good framework for the evaluation of the effects of airborne particulate matter on social Hymenoptera, a topic which has not been studied in detail yet. I identified which concentrations are toxic and uncovered sublethal effects. However, I often did not see any or only small effects of certain pollutants on a measured parameter, which is also important information on how the pollutants act. However, there are also some limitations and uncertainties, especially when trying to generalize the results from my thesis.

It was challenging to choose field-realistic doses of the tested pollutants because identifying and quantifying airborne particulate matter, such as diesel exhaust particles, in terrestrial environments is very difficult. Hence, we lack reliable data on the level of exposure to insects. Isotopic analyses revealed that certain Arizona soils contain up to 0.54% (w/w) of soot black carbon, presumably produced by burning fossil fuels (Hamilton & Hartnett 2013). While reports exist of contaminated bees in the wild (Negri et al. 2015; Thimmegowda et al. 2020), we still lack realistic modelling on the uptake of these particles. The doses I used in this study are presumably higher than those encountered naturally.

In addition, my experimental setups did not include other stressors insects have to face in the wild, such as parasites, limited food availability, or abiotic factors such as drought or heat stress (Cameron & Sadd 2020; Holmstrup et al. 2010; Padda & Stahlschmidt 2022; Raine & Rundlöf 2024). Insects may be able to compensate the impacts of one stressor but will eventually be overstrained by multiple stressors. Often, these multiple stressors interact and may even reinforce their negative impacts synergistically (Goulson et al. 2015). In honeybees the combination of the fungicide difenoconazole and the insecticide acetamiprid reduced the number of foraging trips per day in comparison to the control, while each pesticide applied on its own did not cause any significant changes in behaviour (Schuhmann & Scheiner 2025). Thus, one must be careful when interpreting the results from my single stressor, laboratory studies.

In my thesis, I focused on social Hymenoptera, bumblebees and ants. As they live in colonies, their exposure and susceptibility to pollutants may differ from other insects. On one hand, social insects tend to have large foraging areas where they can encounter an array of pollutants, and transfer them to their nests, which could result in early exposure of their offspring (Morales et al. 2020). As there are so many individuals living in a small space, the general risk of exposure increases. On the other hand, the large colony sizes may also buffer the negative effect of pollutants, as the individual workers do not matter that much for the survival of the colony. Moreover, contaminated patches can be avoided when larger foraging areas are available (Crall et al. 2019, Easton-Calabria et al. 2023, Feldhaar & Otti 2020, Straub et al. 2015). The risk and effects of pollution could be very different in other, non-social insects.

Another aspect, which must be considered, is that I used two very common species in this thesis that are established in laboratory research. *B. terrestris* is one of the most abundant European wild bees, especially in urban areas (Herbertsson et al. 2021, Whitehorn et al. 2022). Similarly, *L. niger* is a very prevalent ant species across Europe and has a wide range of habitats, including urban areas and agricultural fields (Seifert 2018). It was identified as one of the most tolerant ant species in a study on land-use intensification in temperate grasslands (Heuss et al. 2019). Consequently, those two species could be more tolerant towards anthropogenic pollution, such as diesel exhaust particles, or have already adapted to higher levels of air pollution than other species. Higher resilience to disturbance and pollutants forms an important trait to tolerate and survive in human-altered landscapes. A meta-analysis comparing the sensitivity of bees to pesticides showed that *B. terrestris* is relatively tolerant to most pesticides. Other bee species, like honeybees or solitary species, are more sensitive towards neonicotinoids or carbamates (Arena & Sgolastra 2014). Also, a trait-based vulnerability concept suggests *B. terrestris* to be less vulnerable than other wild bee species

(Schmolke et al 2021). For *L. niger*, genomic analysis revealed a higher number of cytochrome P450 genes which could increase the detoxification abilities of anthropogenic pollutants (Konorov et al. 2017). In general, common species tend to be more tolerant towards stressors than rarer, often more specialized species (Vincent et al. 2020). We thus need to be careful when trying to extrapolate the results of my thesis to other species with more specific dietary needs, narrower niches, or non-social lifecycles. Future research should also incorporate rarer species to evaluate the impact of potentially harmful substances in a comparative approach.

Nevertheless, the results from this thesis lay an important foundation for the research on the effects of airborne particulate matter on insects. I could show at which doses diesel exhaust particles become lethal and uncover different sublethal effects. However, I did not see any effect on the colony foundation. My novel approaches add to the understanding of the role that airborne particulate matter plays in the global insect decline. I am looking forward to future studies that build on this work to investigate these pollutants in multiple stressor or semi-natural setups to see potential interactive effects under realistic scenarios. Moreover, it is crucial to conduct experiments with other, less common insect species to identify general patterns and see how the effects of pollutants differ between species.

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Declaration of author contributions

Article 1

Authors: Frederic Hüftlein, Dimitri Seidenath, Andreas Mittereder, Thomas Hillenbrand, Dieter Brüggemann, Oliver Otti, Heike Feldhaar, Christian Laforsch, Matthias Schott

Title: Effects of diesel exhaust particles on the health and survival of the buff-tailed bumblebee *Bombus terrestris* after acute and chronic oral exposure

Journal and Status: Published in Journal of Hazardous Materials, 458. 131905 (2023)

Own contribution: Concept and study design: 30%, Data acquisition: 30%, Data analysis: 30%, Interpretation of results: 30%, Manuscript writing: 30%.

FH, DS, OO, MS, HF and CL designed the study. AM, TH and DB provided and characterized the particles. FH and DS carried out the experiment. FH and DS analysed the data. FH, DS, OO, MS, HF and CL interpreted the results. FH and DS wrote the first draft of the manuscript. All authors read and commented on the manuscript.

Article 2

Authors: Dimitri Seidenath, Alfons R. Weig, Andreas Mittereder, Thomas Hillenbrand, Dieter Brüggemann, Thorsten Opel, Nico Langhof, Marcel Riedl, Heike Feldhaar, Oliver Otti

Title: Diesel exhaust particles alter gut microbiome and gene expression in the bumblebee *Bombus terrestris*

Journal and Status: Published in Ecology and Evolution, 13(6), e10180 (2023)

Own contribution: Concept and study design: 60%, Data acquisition: 30%, Data analysis: 60%, Interpretation of results: 60%, Manuscript writing: 80%

DS, OO and HF designed the study. AM, TH, DB, TO and NL provided and characterized the particles. DS and MR carried out the experiment. AW analysed samples. DS and AW analysed the data. DS, AW, OO and HF interpreted the results. DS, AW and AM wrote the first draft of the manuscript. All authors read and commented on the manuscript.

Article 3

Authors: Dimitri Seidenath, Sara Pölloth, Andreas Mittereder, Thomas Hillenbrand, Dieter Brüggemann, Matthias Schott, Christian Laforsch, Oliver Otti, Heike Feldhaar

Title: Exposure to diesel exhaust particles impairs takeoff but not subsequent homing and foraging behavior of workers of the buff-tailed bumblebee *Bombus terrestris*

Journal and Status: Published in Insectes Sociaux, 1-13 (2025)

Own contribution: Concept and study design: 50%, Data acquisition: 40 %, Data analysis: 50%, Interpretation of results: 50%, Manuscript writing: 60 %

DS, SP, MS, OO, and HF designed the study. AM, TH and DB provided and characterized the particles. DS and SP carried out the experiment. DS and SP analysed the data. DS, SP, MS, OO, and HF interpreted the results. DS and SP wrote the first draft manuscript. All authors read and commented on the manuscript.

Article 4

Authors: Dimitri Seidenath, Andreas Mittereder, Thomas Hillenbrand, Dieter Brüggemann, Oliver Otti, Heike Feldhaar

Title: Do diesel exhaust particles in pollen affect colony founding in the bumble bee *Bombus terrestris*?

Journal and Status: Published in Insectes Sociaux 71, 157-163 (2024)

Own contribution: Concept and study design: 70%, Data acquisition: 60 %, Data analysis: 80%, Interpretation of results: 70%, Manuscript writing: 80 %

DS, OO and HF designed the study. DS carried out the experiment. AM, TH and DB provided and characterized the particles. DS analysed the data. DS, OO, and HF interpreted the results. DS wrote the first draft manuscript. All authors read and commented on the manuscript.

Article 5

Authors: Dimitri Seidenath, Anja Holzinger, Klara Kemnitz, Nico Langhof, Darleen Lücker, Thorsten Opel, Oliver Otti, Heike Feldhaar

Title: Individual vs. Combined Short-Term Effects of Soil Pollutants on Colony Founding in a Common Ant Species

Journal and Status: Published in Frontiers in Insect Science, 1, 761881 (2021)

Own contribution: Concept and study design: 50%, Data acquisition: 40 %, Data analysis: 80%, Interpretation of results: 70%, Manuscript writing: 80 %

DS, AH, OO and HF designed the study. NL and TO provided and characterized the particles. DS, KK and DL carried out the experiment. DS analysed the data. DS, OO, and HF interpreted the results. DS wrote the first draft manuscript. All authors read and commented on the manuscript.

Article 1

Effects of diesel exhaust particles on the health and survival of the buff-tailed bumblebee *Bombus terrestris* after acute and chronic oral exposure

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Effects of diesel exhaust particles on the health and survival of the buff-tailed bumblebee *Bombus terrestris* after acute and chronic oral exposure

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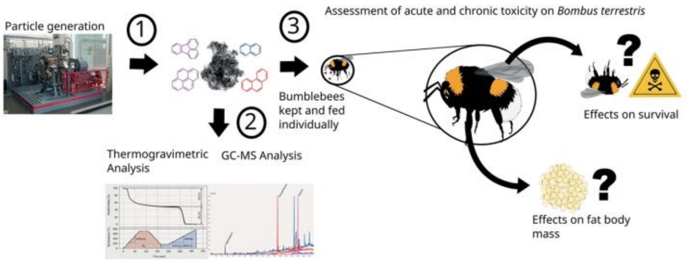
HIGHLIGHTS

- Increased mortality in *Bombus terrestris* workers after chronic oral exposure to diesel exhaust particles
- Workers showed significantly reduced food consumption when DEPs were present in food suspensions
- Diesel exhaust particles have no adverse effect on the survival of *Bombus terrestris* workers after acute oral exposure
- Fat body weight of *B. terrestris* is not affected after acute or chronic oral exposure

GRAPHICAL ABSTRACT



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ABSTRACT

The diesel-powered transportation sector is a major producer of environmental pollution in the form of micro- and nanoscale diesel exhaust particles (DEP). Pollinators, such as wild bees, may inhale DEP or ingest it orally through plant nectar. However, if these insects are adversely affected by DEP is largely unknown. To investigate potential health threats of DEP to pollinators, we exposed individuals of *Bombus terrestris* to different concentrations of DEP. We analysed the polycyclic aromatic hydrocarbons (PAH) content of DEP since these are known

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Adsorbed pollutants
Insects
Fine particulate matter

to elicit adverse effects on invertebrates. We investigated the dose-dependent effects of those well-characterized DEP on survival and fat body content, as a proxy for the insects' health condition, in acute and chronic oral exposure experiments. Acute oral exposure to DEP showed no dose-dependent effects on survival or fat body content of *B. terrestris*. However, we could show dose-dependent effects after chronic oral exposure with high doses of DEP where significantly increased mortality was observed. Further, there was no dose-dependent effect of DEP on the fat body content after exposure. Our results give insights into how the accumulation of high concentrations of DEP e.g., near heavily trafficked sites, can influence insect pollinators' health and survival.

1. Introduction

Biodiversity loss is one of the main concerns of human impact on the environment in the twenty-first century [14]. Without identifying its causes, we risk an exponential increase in biodiversity loss until the end of the century [33,60]. Invertebrate taxa, especially insects, are particularly at risk and have already suffered drastic decreases, both in abundance and diversity [14,24,85]. Depending on the habitat, biomass losses in terrestrial ecosystems have been estimated up to 78% of arthropods over the last three decades [24,40,72]. Insects, such as pollinators, are essential to maintain ecosystem functioning and services and, therefore, human well-being [73,89]. Approximately 75% of the leading global crops depend on insect pollination [39]. The causes for the decline of insect species are manifold. However, a common feature is that most of those causes are anthropogenic, such as intensive agriculture, habitat fragmentation and loss as well as urbanization and pollution [22,68,85].

Pollution comprises several stressors, such as pesticides, fertilizers, industrial chemicals and air pollution [68]. Currently, most studies on the negative effects of pollution on insects and especially pollinators focus on the effects of pesticides and fertilizers, both of which are widely used in agriculture. Other pollutants, such as fine particulate matter from industrialisation, urbanisation and increased motorised traffic, have been shown to have adverse effects on human health, but their potential effects on insects have been little studied [16].

In western Europe, 20% of airborne particulate matter originates from traffic [30]. Traffic-related airborne particulate matter can be divided into non-exhaust and exhaust particles. The most important sources of exhaust particles are diesel-powered heavy- and light-duty vehicles and passenger cars (Statistisches [75,88]). Particle number weighted size distributions from diesel engines are usually bimodal and range from 5 to 50 nm and 50–1000 nm, the latter contributing most of the particle mass but the minority of particle number [38]. Diesel exhaust particles (DEP) are mainly generated during incomplete combustion and are composed of an elemental carbon core with adsorbed organic compounds, metals, and trace elements [23,88]. Depending on the driving cycle (representation of different speeds of a vehicle as a function of time e.g. inner city cycles, highways etc.), the organic fraction of exhaust particles can make up to 36% of the total particle mass and may be the predominant factor for potential adverse effects [1].

To that organic fraction, up to 40% of the components can be attributed to polycyclic aromatic hydrocarbons (PAH) [34]. PAHs are important organic pollutants, as DEP belong to the most prevalent components in urban atmospheres [18,3,83]. Especially vehicles driven in short journeys, in urban areas promote the emission of PAH [63]. Exposure of animals to PAH leads to the activation of detoxification (Cytochromes, Glutathione-S-transferase (GST), bioactivation (Cytochrome P450 - CYP1) enzymes and biotransformation processes and can affect the regulation of proteasome related genes, toxicity and tumour promotion [50,61,84,9]. PAHs and their metabolites are further known for producing reactive oxygen species and may thereby induce cell death, influence cell proliferation, and disrupt cell-to-cell communication [84].

Evidence on how DEP and their associated chemicals can affect other invertebrates, e.g., pollinators, is scarce. Diesel exhaust causes detrimental damage to the learning ability and lower their tolerance against additional stressors of honey bees [64]. Further, diesel exhaust induce the degradation of floral volatiles, thereby disrupting chemical communication between pollinator and flowers making it difficult for insects to locate suitable foraging sites [20,43]. For pollinators relying strongly on scent rather than on visual cues, such as bumblebees, floral scent degradation might negatively affect foraging success [51]. Bumblebees or wild bees in general are supposedly more sensitive to stressors than domesticated honeybees, as the latter have the capabilities to buffer toxic effects caused by the stressors with their high number of individuals per hive [70]. Among those is the common buff-tailed bumblebee, *Bombus terrestris*, a primitive eusocial pollinator and a well-established model in ecotoxicological research serving as a proxy for other bumblebee species, many of which are threatened by anthropogenic disturbance [21,54,8]. However, effects of DEP on pollinating insects, such as *B. terrestris*, after oral exposure have not been investigated yet.

We, therefore, exposed *B. terrestris* workers to different concentrations of well-characterized and in-house produced DEP introduced into their food to study if oral exposure to DEP has a dose-dependent effect on the survival and health condition of the bumblebees. We expected to see a reduction in the fat body content of the bumblebees due to detoxification processes, which would lead to increased energy costs.

To assess if DEP can negatively affect the survival of *B. terrestris* workers after oral intake we performed an acute toxicity test according to OECD standards ([53] and [54]). Subsequently, we performed a chronic oral exposure test to assess the toxicity of DEP over ten days following the OECD guidelines for honey bees [53]. Given the lack of data on the toxicity of DEP to pollinators, we performed limit tests in both the acute and chronic exposure experiments according to OECD standards [52].

The relative fat body content of the exposed and non-exposed bumblebees was determined as a proxy for the insects' health condition [5,19].

2. Methods

2.1. Production and characterization of diesel exhaust particles (DEP)

The engine used in this study was a four-cylinder diesel engine (OM 651, Daimler AG, Stuttgart, Germany) as representative of a passenger car equipped with a light-duty diesel engine, operated on a test bench with a water-cooled eddy-current brake. The aggregate is equipped with a two-stage supercharging system and a common rail-system with direct-acting piezoelectric injectors from Delphi (Dublin, Ireland). The maximum injection pressure is 2000 bar, the rated power is 150 kW at maximum torque of 500 Nm [41,90].

The engine was operated with a repeating cycle of transient and stationary operating points, resembling an inner-city driving scenario with stop-and-go traffic after standardized NEDC-UDC guidelines [15]. Its engine speed and torque setpoints consist of acceleration phases at partial load ("go") and phases of deceleration and idle speed ("stop") which are shown in Fig. 1. We repeated the engine cycle of the inner-city scenario at least 15 times a day and within three days of measurement to offset the impact of environmental conditions and varying engine temperatures. Diesel oxidation catalyst and particle filters were removed while collecting soot samples. Soot samples were collected through an electrostatic filtering system (OekoTube Inside, Mels-Plons, Switzerland). We adapted the exhaust train with a high voltage electrode (30 kV) to ionize particulate matter and divert soot samples towards the ground surface, where we collected samples after each run.

We measured the proportion of organic components of the DEP by thermogravimetric analysis (TGA). For this, we conducted measurements consisting of three replicates for each cycle for which we filled 85 µl aluminium oxide crucibles with 2.9 ± 0.2 mg soot sample. Using a thermogravimetric analyzer STA 449 F5 Jupiter (Netzsch-Gerätebau GmbH, Selb, Germany), we exposed the samples to two controlled atmosphere and temperature programs (see Fig. 2). We evacuated the sample chamber before each analysis and refilled it with nitrogen at normal pressure. The desorption of volatile fractions was analysed at heating rates of 10 K/min at 50 ml/min N_2 purge gas flow. The weight loss up to about 200 °C is attributable to water evaporation-desorption, between 200 °C and 400 °C to hydrocarbon desorption or decomposition of labile functional groups, up to 450 °C to oil fractions [17,4,57]. Subsequently the samples are exposed to an oxidative environment (5% O_2, 95% N_2) introduced at a flow rate of 50 ml/min to the sample chamber. Here weight loss between 400 ° and 900 °C at a heating rate of 10 K/min is related to the oxidation of the carbonaceous core [4,57].

Sub-micron particle size distributions were measured by a fast response differential mobility particulate spectrometer DMS500 (Combustion, Cambridge, England) at 10 Hz data rate [77]. The raw exhaust was continuously sampled and diluted in two stages with compressed air. The primary stage (1:5 vol sample gas/volume compressed air) aimed to avoid condensation of water vapour in the instrument, the secondary stage by rotating disc diluter (1:150 v/v) to avoid agglomeration and pollution of the classifier [77].

After the measuring campaign, we monitored the size calibration by polystyrene latex spheres with a certified mean diameter traceable to the National Institute of Standards and Technology (NIST, Gaithersburg, USA). The count median diameter measured by the particulate spectrometer deviated by a maximum of 0.1% from nanosphere size standards with 221 ± 6 nm and 496 ± 8 nm mean diameter (3320 A/3495 A, Thermo Scientific, Waltham, USA).

2.2. Polycyclic aromatic hydrocarbon (PAH) characterization via GC-MS

For comparison and validation of the method, a deuterated PAH standard containing multiple analytes was used (Table 1) (M-8272 Deuterated Analogs, Accustandard Inc., New Haven, USA). Calibration curves were generated, acting as external standard, by injecting increasing concentrations from 100 pg to 10 ng Naphthalene/l of the PAH standard in triplicates. The deuterated PAH standard was also used as internal standard with a concentration of 1 ng Naphthalene/µl for quantification of the PAH concentration in the DEP. For the analysis of the PAH content, DEP were solved in 1:1 cyclohexane: toluene (as keeper) to a concentration of 1 µg DEP/µl solvent to extract PAH from the DEP. Subsequently we centrifuged the suspension for 20 min at 14 000 turns/min (Centrifuge 5415 C, Eppendorf SE, Hamburg, Germany). The supernatant was removed with a glass pipette and transferred in a conical 1 ml glass vial (Macherey-Nagel GmbH & Co.KG, Düren, Germany). Then the supernatant was constricted with nitrogen until approximately 100 µl remained. This concentrated extract was injected by an AOC-20 s Plus Auto Sampler (Shimadzu, Kyoto, Japan) in a 280 °C hot splitless injector of a Shimadzu Nexis GC2030/GCMS-QP2020 NX (Shimadzu) with Rtx-5MS fused silica (30 m * 0.25 mm* 0.25 µm, Restek, Bellefonte, Pennsylvania). The column oven started with a temperature of 80 °C which was held for 5 min, then increased by 20 °C per minute until 280 °C which was also held for 5 min. The MS measured in single ion mode. See supplement Table 1 for quantifier and qualifier ions of the target molecules. A total of six replicates per driving cycle were analysed.

2.3. Husbandry of Bombus terrestris

We kept eight queenright colonies of the buff-tailed bumblebee *B. terrestris* (Biobest Group NV, Westerlo, Belgium) in a climate chamber under constant conditions with a temperature of 26 °C, a humidity of 70%, and a 12 h/12 h light/dark cycle. Each colony was provided ad libitum with sugar water (1:1 ratio of H_2O and inverted sugar solution from ApiInvert, Südzucker AG, Mannheim, Germany) and pollen (Imkerpur, Osnabrück, Germany). We replaced the sugar water every three days to prevent the formation of mold.

2.4. Acute toxicity test

For assessing the acute toxicity, we used five concentrations (1, 2, 4, 8 and 16 g particles/l inverted sugar solution) of DEP obtained from the diesel engine described in 2.1. As there was no mortality in preliminary experiments, we chose these five concentrations for a dose-response experiment with increasing concentrations by the factor of two, until the limit of solubility. To feed the particles to the bumblebees, we suspended the DEP in sugar water (1:1 ratio of H_2O and inverted sugar solution) with 2% (v/v) Tween20 (Carl Roth GmbH + Co. KG, Karlsruhe, Germany). We used 16 g/l carbon black particles PRINTEX 30 Furnace Black (Degussa AG, Frankfurt, Germany) as a negative particle control (control for toxicity of pure carbon particles, without adsorbed organic fraction). We chose to expose bumblebee workers only to the equivalent

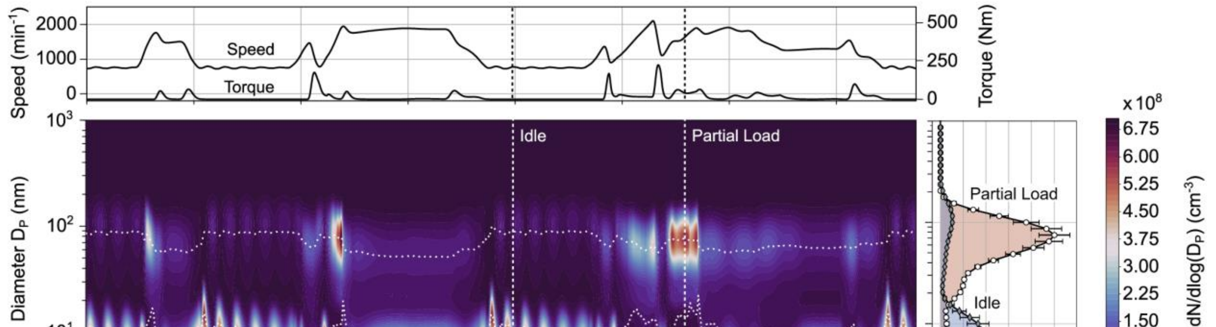

Fig. 1. Particle size-distribution within the inner-city scenario as a function of time and engine parameters. Upper graph depicting speed and torque values of four-cylinder diesel engine, lower graph depicting particle load in respective time points.

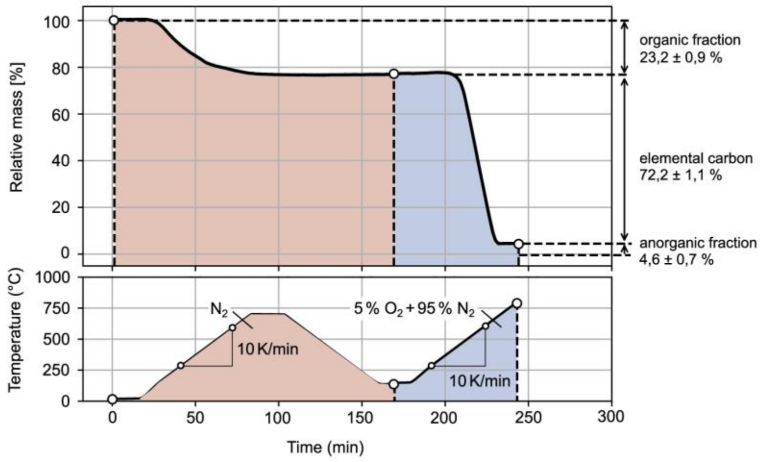

Fig. 2. : Mass reduction of diesel exhaust particles derived from the simulated inner-city-scenario as a function of temperature and atmosphere. Resulting mass reduction (upper graph) under nitrogen atmosphere (see red area in lower temperature profile graph) and oxygenated Atmosphere. In nitrogen atmosphere up to 200 °C weight loss is attributable to water evaporation-desorption, between 200 °C and 400 °C to hydrocarbon desorption or decomposition of labile functional groups, up to 450 °C to oil fractions, in the oxygenated atmosphere weight loss between 400° and 900 °C is attributable to the oxidation of the carbonaceous core of the diesel exhaust particles.

Table 1
Overview of polycyclic aromatic hydrocarbon (PAH) and their concentration present in the deuterated PAH-Standard. Standard is solved in Methanol.

Analyte	CAS Number	Target Concentration
Naphthalene-d8	1146–65–2	5 mg/ml
1-Methylnaphthalene-d10	38072–94–5	6 mg/ml
Acenaphthene-d10	15067–26–2	1.2 mg/ml
Fluorene-d10	81103–79–9	1.2 mg/ml
Phenanthrene-d10	1517–22–2	0.96 mg/ml
Fluoranthene-d10	93951–69–0	0.93 mg/ml
Pyrene-d10	1718–52–1	0.84 mg/ml
Chrysene-d12	1719–03–5	0.033 mg/ml

of the highest DEP concentration (16 g/l), as we did not expect mortality in the particle control. In contrast to the motor produced DEP containing 23% organic compounds, carbon black particles are generated under very controlled conditions and consist of more than 97% elemental carbon and only < 1% organic compounds (Supplemental Figure 1 for comparison). As a control, we fed sugar water (1:1 ratio of H_2O and inverted sugar solution). The solvent control consisted of sugar water with 2% (v/v) Tween20. To ensure the validity of the test system, we used dimethoate (10 µg active ingredient per dose, degree of purity: 98.2%, Sigma-Aldrich, St. Louis, Missouri) in sugar water as positive control according to OECD guideline 247 [52]. Dimethoate is an organophosphate insecticide, inhibiting acetylcholinesterase and highly toxic towards bees [74,81]. From each colony we collected adult bumblebee workers and randomly assigned seven individuals to each treatment, resulting in 7 workers x 8 colonies = 56 replicates per treatment (N = 56). For the dimethoate treatment we randomly assigned four workers per colony resulting in 32 replicates (N = 32) as the OECD guideline 247 suggest a lower number of replicates for toxic reference substances [53]. Each worker was fed 40 µl of the respective solution/suspension via the tip of a 10 ml syringe (B. Braun SE, Melsungen, Germany) at the beginning of the experiment and then starved until the experiment was terminated after 48 h. Every worker consumed all presented food. The syringes fit exactly into a Nicot®-Queen cage (Nicotplast SAS, Maisod, France), in which we kept the workers for the complete period of the experiment (48 h). Survival was recorded 4, 24 and 48 h after ingesting the treatment solution.

2.5. *Chronic toxicity test*

Concentrations used were based on the findings of [25] who detected up to 0.54% (wt/wt) soot in soils in Arizona. For the assessment of chronic toxicity of DEP, we chose 0.5 g/l as the lowest concentration and the two lowest concentrations from the acute toxicity test 1 g/l and 2 g/l as high concentrations to generate dose-dependent effects after long-term exposure. We reduced the dose of the positive control to 4 µg dimethoate per bumblebee to avoid high direct mortality as in the acute test. The control and the solvent control remained the same. We did not include the negative particle control, as no toxicity could be detected in preliminary experiments (Supplemental Fig. 21). We again exposed seven workers per treatment and colony (N = 56). Food provisioning had to be adapted for chronic exposure. The bumblebee workers were fed ad libitum with the respective feeding solutions/suspensions for 10 days. We changed the feeding syringes every two days to prevent bacteria and mould contamination in the solutions/suspensions. The tips of the syringes had to be cut off, to ensure feeding. Because we provided the solutions/suspensions ad libitum, we measured the amount of solution ingested by each worker, to account for differences in food uptake due to possible repellent effects of the particles, as the amount of ingested food could also have an influence on the bumblebees' health. We weighed each syringe before and after the two-day exposure. The resulting weights were subtracted to determine the amount of food ingested by the bumblebees over the two-day period. To assess evaporation of the treatment solutions/suspensions, three syringes without queen cages were placed in the climate chamber for each treatment solution. The evaporation rates were less than 1% of the daily presented sugar solution. Evaporation rates did not differ between treatments (see supplement Fig. 23). Survival was recorded daily for the 10-day period. At the end of the experiment, dead and surviving bumblebees were frozen at − 20 °C for subsequent analysis of the fat body.

2.6. *Fat body assay*

In the first step, the abdomens of the workers from the chronic toxicity test were separated from the head and thorax. Then we made three cuts to open the sternites, one cut along the abdominal sternites from top to bottom and two lateral cuts from the middle to the side to enhance the drying of the tissue and enabling the access of chloroform to all tissue parts (following [6]). Immediately after cutting, the abdomens were transferred to a 5 ml glass vial and incubated in a drying oven (UFE 600, Memmert GmbH + Co. KG, Schwabach, Germany) at 70 °C for 72 h. After the first incubation, the dry weight of each abdomen was weighed on a fine scale PLE 420–3 N (Kern & Sohn GmbH, Balingen-Frommern, Germany). To remove the fat body, we transferred the abdomens to glass reaction tubes containing 2 ml of chloroform and incubated them at 70 °C for 24 h. This step was repeated with fresh chloroform three times to ensure the complete removal of fat body tissue. Finally, the chloroform was removed from the vials and the abdomens were dried at 70 °C for 72 h. Then we weighed the abdomens again and calculated the fat body content by subtracting the weight after fat body removal from the initial dry weight of the abdomens. We corrected for body size differences by measuring the length of the radial cell of the right wing as it is positively correlated with body size [47]. Relative fat body content was calculated by dividing the fat body content in mg by the radial cell length of the right forewing in mm [47]. Dimethoate individuals were excluded in the statistical analysis of the fat body content, because only one survived.

2.7. Statistical analysis

All statistical analyses were performed using R version 4.1.0 (R [62]). Residual plots of response variables were used to test for the homoscedasticity and normality of residuals using the R package *DHARMa* [26]. The survival analysis for the concentrations of DEP of the acute and the chronic exposure was carried out using a Kaplan-Meier estimator through the *survfit* function of the R package *survival* [78]. Survival curves were drawn using the *ggsurvplot* function from the R package *survminer* [35]. The survival data were analyzed with a Cox proportional hazard regression (COXPH) with treatment as a predictor from the R package *survival* [78]. Survival model assumptions were tested using Schoenfeld residuals from the R package *survminer* [35]. We then produced F-statistics with the function anova() to calculate p-values for differences between treatments. For significant treatment effects, we ran pairwise comparisons using Tukey-HSD *post-hoc* test with Benjamin-Hochberg correction with the R package *multcomp* [31]. Data was plotted using the *ggbetweenstats* function from the R package *ggstatsplot* [58]. General linear mixed models were created with the R package *glmmTMB* [44]. We excluded two negative consumption values from the analysis (i.e. residual amount of food was higher than initial weight), likely caused by measurement deviations of the scale. For the fat body analysis, three data points were defined as outliers when surpassing the three-fold standard deviation around the mean or when falling below zero [55]. However, we report the results including outliers (except negative values) in the supplement as suggested by Aguinis et al. [2]. We excluded the dead individuals from the fat body analysis, which is why there is only one individual left in the dimethoate treatment in the fat body analysis. Differences in the statistical analyses were considered significant when the p-value was < 0.05.

3. Results

3.1. Characterization of diesel exhaust particles (DEP)

Fig. 1 illustrates the particle number weighted size distribution as a function of time and engine speed/torque. Both, concentration and particle diameter varied depending on the "stop" and "go" scenario. Highest concentrations of accumulation mode particles were emitted during acceleration and partial load periods ("go"). Solid particulates showed a count median diameter between 52.1 ± 1.8 nm and 101.9 ± 1.7 nm within the simulated inner-city scenario. By contrast, concentrations of mainly volatile, nucleation mode aerosols peaked during deceleration and idle conditions ("stop"). Aerosols showed a count median diameter between 7.4 ± 1.6 nm and 22.7 ± 1.6 nm. One characteristic size distribution of either condition is depicted at the right. A fraction of 23.2 ± 0.9% of the DEP mass collected during the inner-city scenario can be ascribed to organic fractions, 72.2 ± 1.1% to elemental carbon and 4.6 ± 0.7% to inorganic components (Fig. 2).

3.2. Polycyclic aromatic hydrocarbon (PAH) characterization via GC-MS

As described in 2.2, we searched for the presence of the seven PAHs, belonging to the EPA list of high priority PAHs [37]: Naphthalene, 1-methylnaphthalene, acenaphthene, fluorene, phenanthrene, fluoranthene and pyrene. Of those seven PAHs only, pyrene was above the limit of quantification with a concentration of 444.05 µg/g (+/- 75.18 SE) pyrene in DEP equal to 444 ppm. Fluoranthene and phenanthrene concentrations were above the limit of detection (~10 µg/g) but were below the limit of quantification (~200 µg/g, see Table 2).

3.3. Acute toxicity testing

The survival analysis revealed no toxic effects of acute exposure with DEP to individuals of *B. terrestris* in concentrations of 1–16 g/l. Only dimethoate, as the positive control, induced nearly 100% mortality (pairwise log-rank test comparisons, $p < 0.001$ compared to every other treatment). There was no increased mortality in the DEP treatments compared to the control; pairwise log-rank test comparisons, $p > 0.05$ for every DEP treatment compared to control, see Supplement Fig. 2). Also, we found no effect of acute exposure of DEP to the relative fat body content of bumblebees in any treatment (GLM with gamma distribution, $F_{(8410)} = 1.296$, $p = 0.34$) (Supplement Fig. 3).

3.4. Chronic toxicity testing

The chronic exposure of sublethal doses of DEP to bumblebees over a 10-day period significantly reduces survival in comparison to the control and the solvent control (COXPH overall LR-test: $X^2 = 14$, $df = 5$, $p < 0.001$) (Fig. 3).

The Cox proportional hazard model revealed that mortality was significantly elevated for exposure of bumblebees with 1 g/l and 2 g/l DEP (Tukey comparisons: $p = 0.002$ (1 g/l) and $p < 0.001$ (2 g/l) (Fig. 4).

The survival of individuals exposed to 1 g/l and 2 g/l DEP was also significantly reduced compared to the solvent control and the 0.5 g/l DEP (Tukey comparisons: $p < 0.01$ for every treatment combination, for details see supplement). There was no significant difference in survival between 1 g/l and 2 g/l DEP ($p = 0.087$). There was no significant effect on survival of the bumblebees in the control, the solvent control or in the DEP 0.5 g/l treatment. Exposure to DEP had a significant effect on the relative fat body content of the bumblebees (GLMM with gamma distribution, $X^2 = 32.136$, $df = 4$, $p < 0.001$, for details see supplement).

Pairwise comparisons revealed that the exposure to 0.5 g/l DEP reduced the relative fat body content significantly when compared to the control (Tukey comparison: $p = 0.0115$), but also in comparison to the higher concentrations 1 and 2 g/l DEP (Tukey comparisons: $p = 0.0115$ and $p = 0.038$ respectively). In contrast, there were no significant differences between the DEP exposure treatments and the solvent control (Tukey comparisons: $p = 0.43$ for 0.5 g/l DEP; $p = 0.06$ for 1 g/l and $p = 0.17$ for 2 g/l DEP, Fig. 5).

Furthermore, we tested for differences in sugar water consumption between treatments. We could observe that the higher the concentration of DEP the lower the consumed sugar water in the experiment (GLM with gamma distribution, $F_{(5316)}= 52.71$, $p < 0.001$, for details see supplement). Pairwise comparisons revealed significant differences between the control and all other treatments (Tukey comparisons: $p < 0.01$ for every combination) except DEP 0.5 g/l and control (Fig. 6).

We further tested for differences in survival between colonies in a Kaplan-Meier plot but could not find significant differences between the survival of the workers from single bumblebee colonies (COXPH overall LR-test: $X^2 = 6.914$, $df = 7$, $p = 0.44$, see Supplement Figure 16). However there were significant differences in sugar water consumption between the colonies (GLM with gamma distribution, $F_{(7314)}= 4.429$, $p < 0.001$). There was no correlation between mean consumption per bee and the relative fat body weight present (Pearson correlation coefficient, df =163, $r = 0.053$, $p = 0.44$). Nonetheless, there was a negative correlation between the consumed food and the concentration of DEP present in the food with the solvent control and control (pooled) as status quo (Pearson correlation coefficient, df = 215, $r = -0.54$,

Table 2
Concentration of the analysed PAHs found in the DEP samples. A total of three replicates were analysed via GC-MS. Naphthalene and 1-methylnaphthalene were below the limit of detection. (LOD = Limit of detection, LOQ = Limit of quantification).

PAH	n	Mean [µg/g]	Standard deviation	Standard error	95% - CI
1-Methylnaphthalene	3	<LOD	0	0	0
Naphthalene	3	<LOD	0	0	0
Fluoranthene	3	107.03 (<LOQ)	39.16	22.61	97.28
Phenanthrene	3	220.47	49.04	28.32	121.84
Pyrene	3	444.05	130.22	75.18	323.49

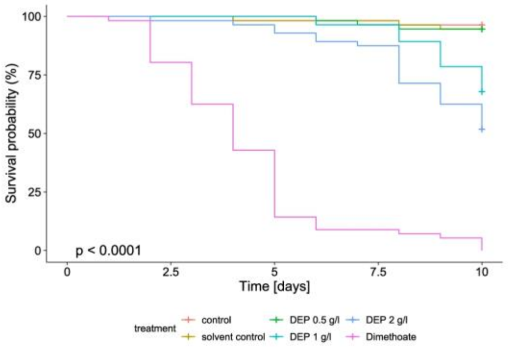

Fig. 3. : Survival probability across the 10 day period for the bumblebees exposed to the control, solvent control, DEP concentrations 0.5, 1 and 2 g/l and dimethoate. The p-value indicates significant differences between the Kaplan-Meier curves of the different treatment.

$p < 0.01$, Fig. 7).

4. Discussion

Our experiments, simulating an exposure with DEP deposition on nectar sources, showed no significant differences in mortality or changes in the fat body content of *B. terrestris* individuals after acute exposure to concentrations ranging from 1 g/l to 16 g/l DEP in sugar solution over 48 h. Components that are discussed to be one cause of DEP toxicity are PAHs, which are formed during incomplete combustion processes [48]. Pyrene was the highest concentrated PAH we found in the utilized DEP with 444.05 µg/g. This concentration may be still too low to cause significant mortality in *B. terrestris* after acute oral exposure after 48 h as indicated by previous studies performed with mosquito larvae [7]. In nauplius larvae of the brine shrimp *A. salina*, an acute toxic effect of DEP in terms of increased mortality could be shown after the exposure to 250 mg/l exhaust particles in medium over 96 h [59]. Nevertheless, these results are barely comparable to ours, as the DEP were not characterized and, therefore, the PAH content cannot be compared. Further, the sensitivity of juvenile stages might be higher and exposure conditions in aquatic environments are more complex than pure oral ingestion, as they are additionally exposed over their respiratory surface [29,32,36,49].

In contrast, the survival of bumblebees exposed to 1 g/l and 2 g/l DEP in sugar solution in a 10-day chronic test was significantly reduced by 32.1% and 48.2%, respectively, compared to the control. The accumulation of DEP in tissues of organisms and resulting enrichment of PAHs is believed to be the reason for unfolding their toxicity, but only after prolonged exposure periods [13,46]. Further, food consumption was significantly reduced in bumblebees of these two treatment groups. This reduction led to a negative correlation between the concentration

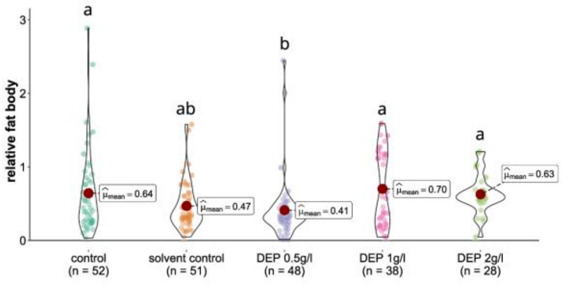

Fig. 5. Effect of DEP exposure on the relative fat body weight corrected for body size of the surviving bumblebees after 10-day exposure. Number of replicates per treatment are represented by n. The values in the boxes represent the mean relative fat body of the treatment. Relative fat body values represent the fat body weight of the individual bumble in mg divided by the length of the radial cell in mm. Letters indicate significance between treatments.

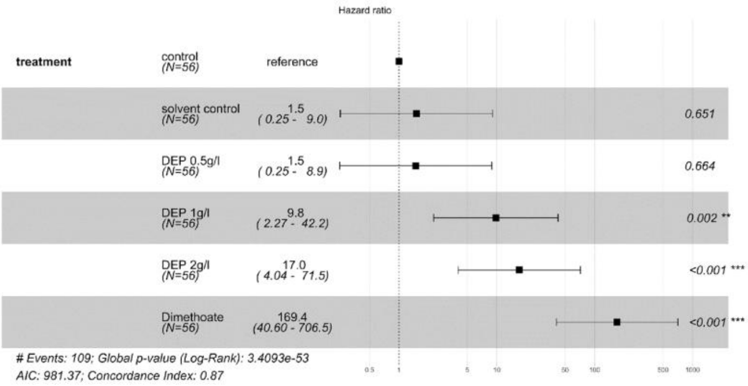

Fig. 4. : Forest plot of hazard ratio from multivariable Cox proportional hazard regression model on survival for DEP exposure in comparison with the control. Bars represent 95% confidence intervals.The p-values with asterisks indicate significantly elevated mortality risk compared to control organisms.

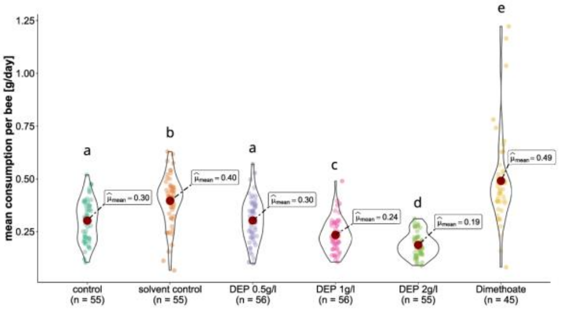

Fig. 6. : Effect of 0.5, 1 and 2 g/l DEP exposure in the diet on the daily sugar water consumption of the bumblebees after the 10- day period. Number of replicates per treatment are represented by n. The values in the boxes represent the mean relative fat body weight of the treatment. Letters indicate significance between treatments.

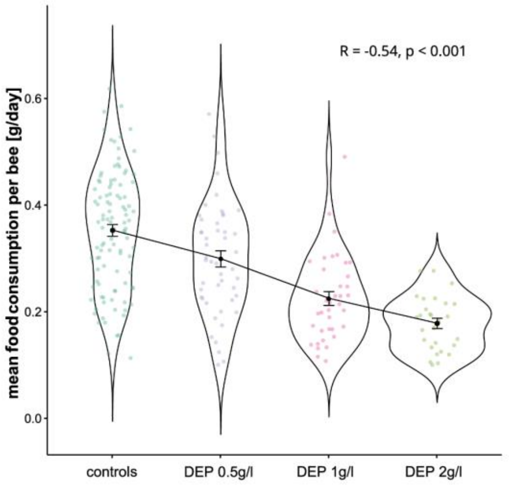

Fig. 7. : Effect of the dose of DEP on the food consumption per bumblebee per day. The graph shows a significant negative correlation between the two variables. The black point with whiskers represents the mean with standard error for every dose respectively. For the dose 0 g/l we pooled the food consumption values from the control and solvent control individuals.

of DEP in the provided food and the amount of food consumed by the exposed individuals. A deterrent effect of the high DEP content could be the reason, as reported in previous studies on pesticide exposure [79]. Nonetheless, the net ingested dose per bumblebee increased with increasing DEP concentration (Supplemental Figure 22).

Additionally, the sugar water solution with the high DEP concentration (2 g/l) was more viscous than the control solution or the low concentration of DEP, possibly impairing the nutritional uptake. However, a possible explanation for reduced food intake is that the intoxication in the higher DEP treatments led to lower activity in the bumblebees and thereby reduced the energy requirements. On the other hand, the food consumption in the dimethoate treatment was significantly higher compared to controls, possibly due to physiological requirements facilitating detoxification, immune responses or increased energy expenditure due to overstimulation of muscles [71,80].

Insects, in general, are prone to ingest sedimented atmospheric particulate matter via nectar or plant surfaces. This fine particulate matter can negatively impact polyphagous herbivores by reducing long-term larval survival or performance [42,82]. The concentrations used in these experiments were deliberately chosen high to generate a dose-response curve for acute and chronic oral exposure. The fat body is a good indicator of the bumblebees' health and to assess sublethal effects, it is one of the main insect organs responsible for innate immunity and detoxification [5,6]. However, we could find no sublethal effect on the relative fat body weight after dietary exposure of DEP compared to the solvent control of *B. terrestris* individuals. There was a significant reduction of fat body weight in animals exposed to 0.5 g/l compared to the control with no significant difference in consumption rates in this treatment group. However, no significant effects on the fat body content of bumblebee workers were observed when chronically exposed to 1 and 2 g DEP/l in sugar water in spite of the relatively high total mortality (see above). The potentially reduced energy requirements, as discussed earlier, could partly explain the observed phenomenon. Animals with a lower amount of initial fat body could have a higher likelihood of mortality. The data set could be biased towards individuals with a larger initial fat body, as they may constitute a significant portion of the group of survivors. Another explanation for the absence of treatment effects on the fat body could be that the fat body is an unreliable proxy when analysing the effects of DEP on insects, as previously described for other stressors like pesticides or mercury [11,12]. It is possible that immune cells and free floating pro-phenoloxidase in the haemolymph are sufficient for detoxifying the effects of particulate stressors [10,69]. Nevertheless, it is to mention, that the observed effects in our study were relatively low, considering the high concentrations we used for the acute and chronic toxicity tests.

With *B. terrestris* we chose one of the most abundant wild bee species in Europe, especially in anthropogenic dominated and modified habitats, like urban areas [27,67,87]. Theoretically *B. terrestris* is supposed to be more tolerant towards stressors of any kind than other wild bee species, due to its large body and colony size, thereby granting them access to resources from more distant patches [86] and the buffering capacity on the colony level [76]. For a better understanding of the effects of DEP on pollinators, a greater set of species should be tested. In addition, our setup represents a highly controlled in-vivo experiment, where animals were fed ad libitum, which is not comparable to a multi-stressor environment with i.e. limited food resources in parallel to parasite pressure or heat stress. Under natural conditions, organisms often face more than one stressor at a time. If a pollinator, such as *B. terrestris*, faces another stressor simultaneously, for example, pesticides, the ability to tolerate this other stressor might add to the hardship of an organism. Holzinger et al. [28] have already shown that chronic exposure to DEP in combination with brake dust and microplastic particles negatively reduces fertility in *Eisenia fetida*, i.e. highlighting an increased toxicity in multi stressor scenarios. The effect of DEP on oxidative stress with a possible subsequent change in lipid profiles would be favourable in future studies to characterise the mechanisms underlying DEP toxicity in insects, as DEP have been shown to induce lipid changes in mice after ingestion and exposure via the trachea [45,56,65,66].

5. Conclusion

This study shows the negative impact DEP can have on the survival, the consumption rate and the fat body content of the buff-tailed bumblebee *Bombus terrestris* and demonstrates the dose-dependent effects of DEP. Further, it is to mention, that the effects we observed were low in relation to the concentration we used. Our experimental setup combines the controlled and standardised generation of DEP, their physicochemical analysis and the subsequent exposure of a model organism for wild bees to those particles and, therefore, highlights the importance to characterize the properties of particulate stressors. Future studies on DEP should include varying particle parameters like different concentrations of PAH, metals and other adsorbed components, to be able to identify significant parameters for the toxicity. In conclusion, this study provides a basis for understanding the adverse effects of DEP on

B. terrestris. It highlights the need for further research to assess the sublethal impact of environmentally relevant concentrations and to identify key parameters of DEP toxicity on pollinators' health.

Environmental implication

In Western Europe, 20% of airborne particulate matter originates from traffic, with diesel exhaust particles posing the most prevalent particle type. Diesel exhaust particles can have adverse effects on organisms, as they are known to induce tumours and inflammation. There is an astonishing lack of data on how these particles affect the fitness and survival of insects. Our study provides insight into how fully characterised diesel exhaust particles can impair the survival of the most abundant wild bee pollinator, Bombus terrestris, after acute and chronic exposure.

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CRediT authorship contribution statement

Frederic Hüftlein: Methodology, Validation, Formal analysis, Investigation, Data curation, Writing – original draft, Visualization, Writing – review & editing. **Dimitri Seidenath**: Methodology, Validation, Formal analysis, Investigation, Writing – original draft, Visualization. **Andreas Mittereder:** Methodology, Formal analysis, Investigation. **Thomas Hillenbrand:** Conceptualization, Resources, Supervision. **Dieter Brüggemann:** Conceptualization, Resources, Supervision, Funding acquisition. **Oliver Otti:** Conceptualization, Methodology, Writing – original draft, Supervision, Project administration. **Heike Feldhaar:** Conceptualization, Methodology, Resources, Writing – original draft, Writing – review & editing, Supervision, Funding acquisition. **Christian Laforsch:** Conceptualization, Methodology, Resources, Writing – original draft, Writing – review & editing, Supervision, Funding acquisition. **Matthias Schott:** Methodology, Validation, Formal analysis, Writing – original draft, Writing – review & editing, Visualization, Supervision, Project administration.

Declaration of Competing Interest

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

Data Availability

Data is available on zenodo: https://doi.org/10.5281/zenodo.8060262.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jhazmat.2023.131905.

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Effects of diesel exhaust particles on the health and survival of the buff-tailed bumblebee *Bombus terrestris* after acute and chronic exposure.

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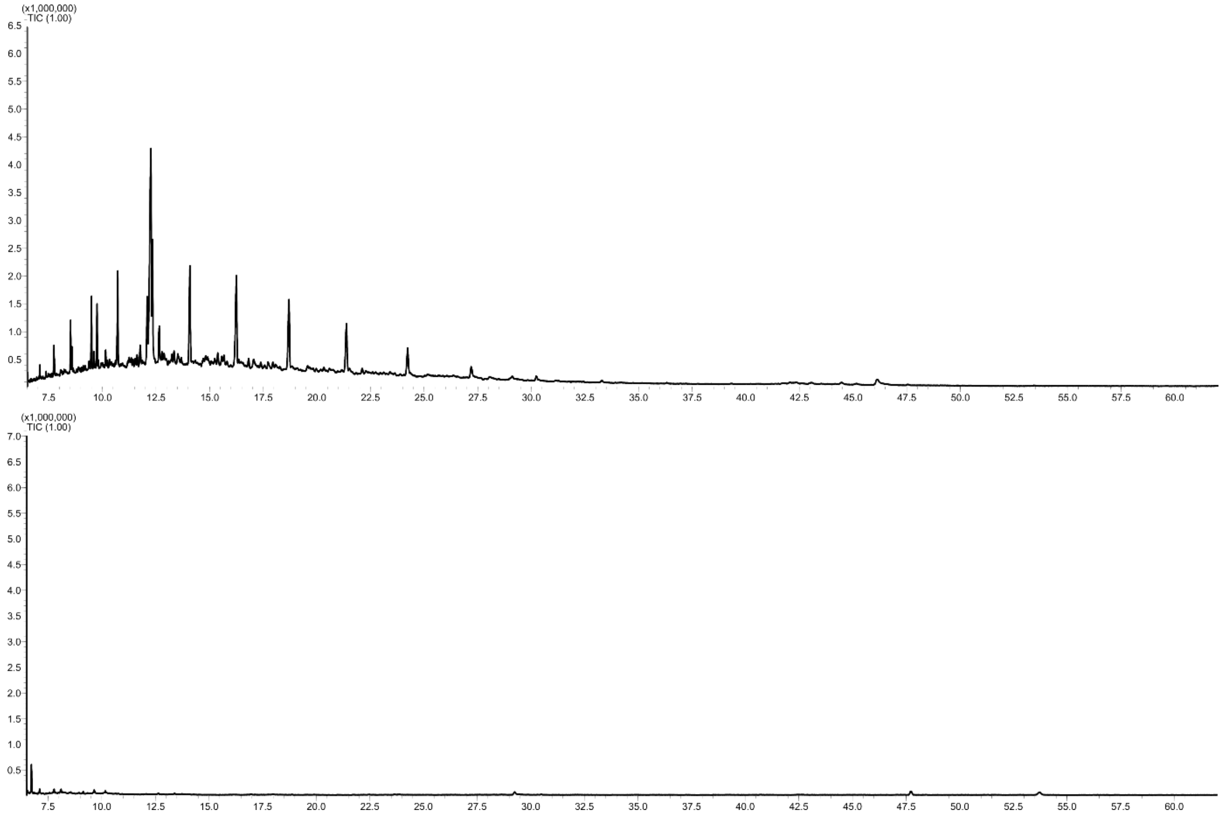
Supplemental material

GCMS- of DEP

As described in the manuscript, we used carbon black particles from the PRINTEX 30 Furnace Black type as a negative particle control. In the following we demonstrate the total ion chromatogram from the carbon black particles (lower graph) and the diesel exhaust particles generated from our experimental setup (top graph) (Fig. 1).



Supplemental Figure 1: total ion chromatogram from diesel exhaust particles generated with our experimental setup (top graph) and total ion chromatogram from the negative control particles (PRINTEX 30 Furnace Black)

Supplemental Table 1: Qualifier and Quantifier for the used EPA- PAH

PAH	Quantifier [m/z]	Qualifier 1 [m/z]	Qualifier 2 [m/z]	Qualifier 3 [m/z]
Naphthalene	128	102	64	
1-Methylnaphthalene	142	141	115	
Acenaphthene	154	153	76	
Fluoren	166	165	139	82
Phenanthrene	178	152	89	
Fluoranthene	202	101	88	
Pyrene	202	200	101	
Chrysene	228	226	113	

Preparation of R and loading of data

```
r = getOption("repos")
r["CRAN"] = "http://cran.us.r-project.org"
options(repos = r)

#install.packages("survival")

#install.packages("survminer")
#install.packages("dplyr")
#install.packages("DHARMa")
#install.packages("tidyverse")
#install.packages("plotly")
#install.packages("ggstatsplot")

library(survival)
library(survminer)
library(dplyr)
library(DHARMa)

## Warning: Paket 'DHARMa' wurde unter R Version 4.2.3 erstellt

library(tidyverse)
library(plotly)
library(ggstatsplot)
library(lme4)
citation("DHARMa")

##
## Um Paket 'DHARMa' in Publikationen zu zitieren, nutzen Sie bitte:
##
##   Hartig F (2022). _DHARMa: Residual Diagnostics for Hierarchical
##   (Multi-Level / Mixed) Regression Models_. R package version 0.4.6,
##   <https://CRAN.R-project.org/package=DHARMa>.
##
## Ein BibTeX-Eintrag für LaTeX-Benutzer ist
##
##   @Manual{,
##     title = {DHARMa: Residual Diagnostics for Hierarchical (Multi-Level / Mixed)
## Regression Models},
##     author = {Florian Hartig},
##     year = {2022},
##     note = {R package version 0.4.6},
##     url = {https://CRAN.R-project.org/package=DHARMa},
##   }

#install.packages('Rcpp')
library(Rcpp)
library(multcomp)
library(coxme)
library(glmmTMB)
library(car)
library(ggpubr)
```

Read the data and set treatment and colony to factors. Sort the treatment column.

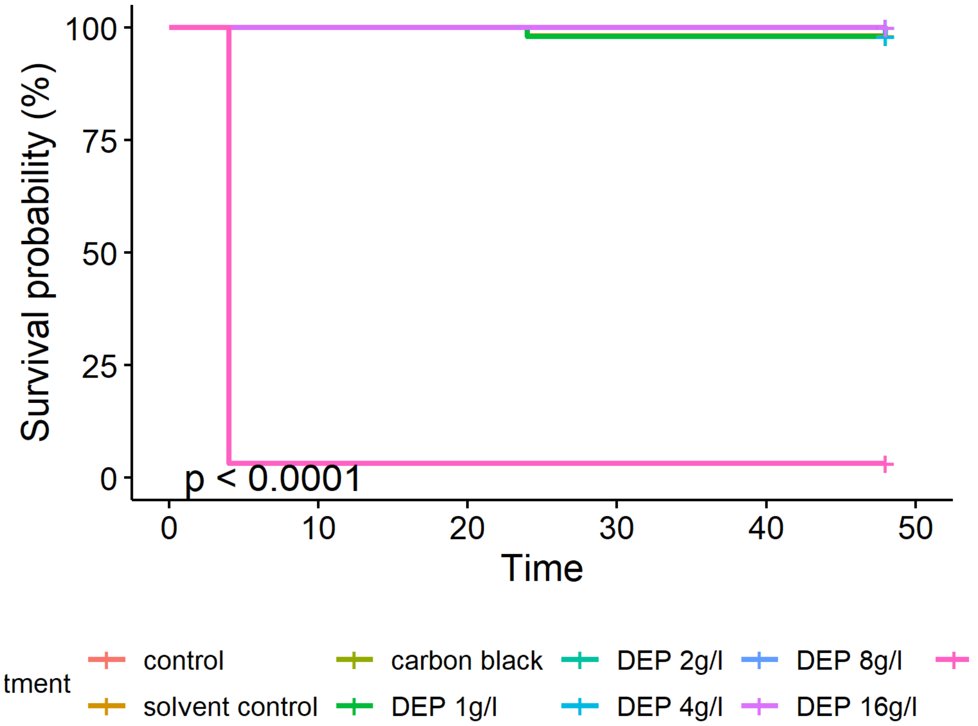
```
setwd("C:/Users/Frederic/Desktop/Phd/Manuskript_DEPtoxicity")

acute<-read.csv2("data_acute_toxicity_soot.csv")

acute<-na.omit(acute)
acute$treatment<-as.factor(acute$treatment)
acute$relative_fatbody<-as.numeric(acute$relative_fatbody)
```

```
acute2 <- acute %>%
  dplyr::mutate(treatment = factor(treatment,
                                   levels = c("control", "solvent control",
                                              "carbon black","DEP 1g/l","DEP 2g/l",
                                              "DEP 4g/l","DEP 8g/l","DEP 16g/l","Dimethoate
")))
str(acute2)

## 'data.frame':    452 obs. of  13 variables:
##  $ ID                : int  2 3 4 5 6 7 8 9 11 12 ...
##  $ treatment         : Factor w/ 9 levels "control","solvent control",..: 1 1 1 1 1 1 1
1 1 1 ...
##  $ colony            : int  23 23 23 23 23 23 24 24 24 24 ...
##  $ fed..1..yes..0.no.: int  1 1 1 1 1 1 1 1 1 1 ...
##  $ death             : int  0 0 0 0 0 0 0 0 0 0 ...
##  $ survival_hour     : int  48 48 48 48 48 48 48 48 48 48 ...
##  $ weight_before..mg.: chr  "51.55" "29.08" "36.72" "60.93" ...
##  $ weight_after..mg. : chr  "50.1" "27.12" "34.37" "57.96" ...
##  $ fatbody..mg.      : chr  "1.45" "1.96" "2.35" "2.97" ...
##  $ preparation_date  : chr  "21.01.2022" "14.01.2022" "14.01.2022" "21.01.2022" ...
##  $ radial_cell       : chr  "2.858" "2.572" "2.742" "3.091" ...
##  $ relative_fatbody  : num  0.507 0.762 0.857 0.961 0.836 ...
##  $ comment           : chr  "" "" "" "" ...
##  - attr(*, "na.action")= 'omit' Named int [1:28] 1 10 16 30 35 37 91 112 143 150 ...
##   ..- attr(*, "names")= chr [1:28] "1" "10" "16" "30" ...
```

Survival analysis of acute exposure

###Effect of treatment on survival, no random effect.

```
surv_object <- Surv(time = acute2$survival_hour, event = acute2$death)
head(surv_object)

## [1] 48+ 48+ 48+ 48+ 48+ 48+

fit1 <- survfit(surv_object ~ treatment, data = acute2)

ggsurvplot(fit1, data = acute2,
           pval = TRUE,
           fun = "pct",
           risk.table = FALSE,
           size = 1,
           legend = "bottom",
           legend.title = "treatment",
           legend.labs = c("control", "solvent control","carbon black","DEP 1g/l",
                           "DEP 2g/l", "DEP 4g/l","DEP 8g/l","DEP 16g/l","Dimethoate"))
```



Supplemental Figure 2: survival plot after acute exposure

```
res.cox <- coxph(Surv(survival_hour, death) ~ treatment, data = acute2)

## Warning in coxph.fit(X, Y, istrat, offset, init, control, weights = weights, :
## Ran out of iterations and did not converge

res.cox

## Call:
## coxph(formula = Surv(survival_hour, death) ~ treatment, data = acute2)
##
##                                 coef  exp(coef)   se(coef)     z     p
## treatmentsolvent control  2.835e+00  1.703e+01  9.649e+03 0.000 1.000
## treatmentcarbon black    -4.465e-16  1.000e+00  3.973e+04 0.000 1.000
## treatmentDEP 1g/l         2.963e+00  1.936e+01  9.314e+03 0.000 1.000
## treatmentDEP 2g/l        -1.335e-15  1.000e+00  3.936e+04 0.000 1.000
## treatmentDEP 4g/l         2.952e+00  1.914e+01  9.344e+03 0.000 1.000
## treatmentDEP 8g/l        -6.686e-16  1.000e+00  4.048e+04 0.000 1.000
## treatmentDEP 16g/l       -6.686e-16  1.000e+00  3.973e+04 0.000 1.000
## treatmentDimethoate       2.696e+01  5.137e+11  5.211e+03 0.005 0.996
##
## Likelihood ratio test=100.6  on 8 df, p=< 2.2e-16
## n= 452, number of events= 33

anova(res.cox)

## Analysis of Deviance Table
##  Cox model: response is Surv(survival_hour, death)
## Terms added sequentially (first to last)
##
##             loglik  Chisq Df Pr(>|Chi|)
## NULL      -200.55
## treatment -150.24 100.63  8  < 2.2e-16 ***
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
```

```
pairwise_survdiff(Surv(survival_hour,death) ~ treatment, data=acute2, p.adjust.method = "BH
", rho = 0)

## Warning in pchisq(chi, df, lower.tail = FALSE): NaNs wurden erzeugt

## Warning in pchisq(chi, df, lower.tail = FALSE): NaNs wurden erzeugt

## Warning in pchisq(chi, df, lower.tail = FALSE): NaNs wurden erzeugt

## Warning in pchisq(chi, df, lower.tail = FALSE): NaNs wurden erzeugt

## Warning in pchisq(chi, df, lower.tail = FALSE): NaNs wurden erzeugt

## Warning in pchisq(chi, df, lower.tail = FALSE): NaNs wurden erzeugt

## Warning in pchisq(chi, df, lower.tail = FALSE): NaNs wurden erzeugt

## Warning in pchisq(chi, df, lower.tail = FALSE): NaNs wurden erzeugt

## Warning in pchisq(chi, df, lower.tail = FALSE): NaNs wurden erzeugt

## Warning in pchisq(chi, df, lower.tail = FALSE): NaNs wurden erzeugt

##
##  Pairwise comparisons using Log-Rank test
##
## data:  acute2 and treatment
##
##                 control solvent control carbon black DEP 1g/l DEP 2g/l DEP 4g/l
## solvent control 0.53    -               -            -        -        -
## carbon black    1.00    0.53            -            -        -        -
## DEP 1g/l        0.53    1.00            0.53         -        -        -
## DEP 2g/l        1.00    0.53            1.00         0.53     -        -
## DEP 4g/l        0.53    1.00            0.53         1.00     0.53     -
## DEP 8g/l        1.00    0.53            1.00         0.53     1.00     0.53
## DEP 16g/l       1.00    0.53            1.00         0.53     1.00     0.53
## Dimethoate      <2e-16  <2e-16          <2e-16       <2e-16   <2e-16   <2e-16
##                 DEP 8g/l DEP 16g/l
## solvent control -        -
## carbon black    -        -
## DEP 1g/l        -        -
## DEP 2g/l        -        -
## DEP 4g/l        -        -
## DEP 8g/l        -        -
## DEP 16g/l       1.00     -
## Dimethoate      <2e-16   <2e-16
##
## P value adjustment method: BH
```

Lowest survival in dimethoate ($p < 0.001$ compared to every other treatment). No differences among other treatments ($p >= 0.52$).

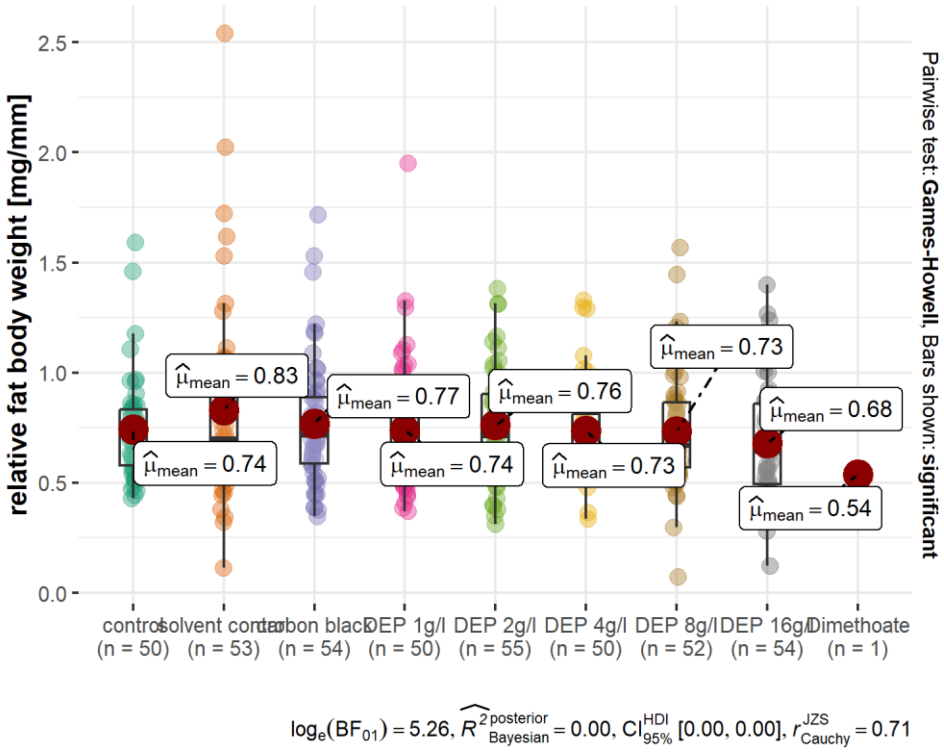
Effect of treatment on relative fat body after acute exposure

Only chose bumblebees that were alive until the end of the experiment.

```
alive<-subset(acute2,death=="0")

ggstatsplot::ggbetweenstats(
  data = alive,
  x = treatment, xlab = "",
  y = relative_fatbody,
  ylab = "relative fat body weight [mg/mm]",
  plot.type = "box",
```

```
  mean.plotting = FALSE,
  type = "p",
  conf.level = 0.95,
  ggplot.component =  list(theme(text = element_text(size = 10),
                               plot.subtitle = element_text(size = 10, face = "bold")))

)
```

```
## Warning: Number of labels is greater than default palette color count.Select
## another color `palette` (and/or `package`).
```

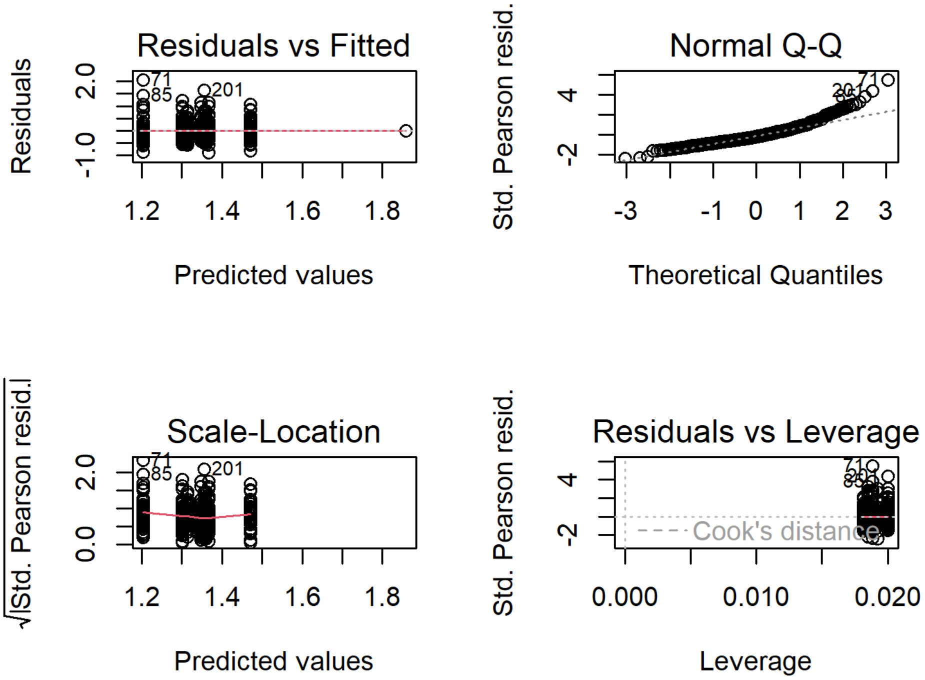


Supplemental Figure 3: relative fat body of the bumblebees after acute exposure.

Generalized Linear Model (GLM) for the fat body data after acute exposure

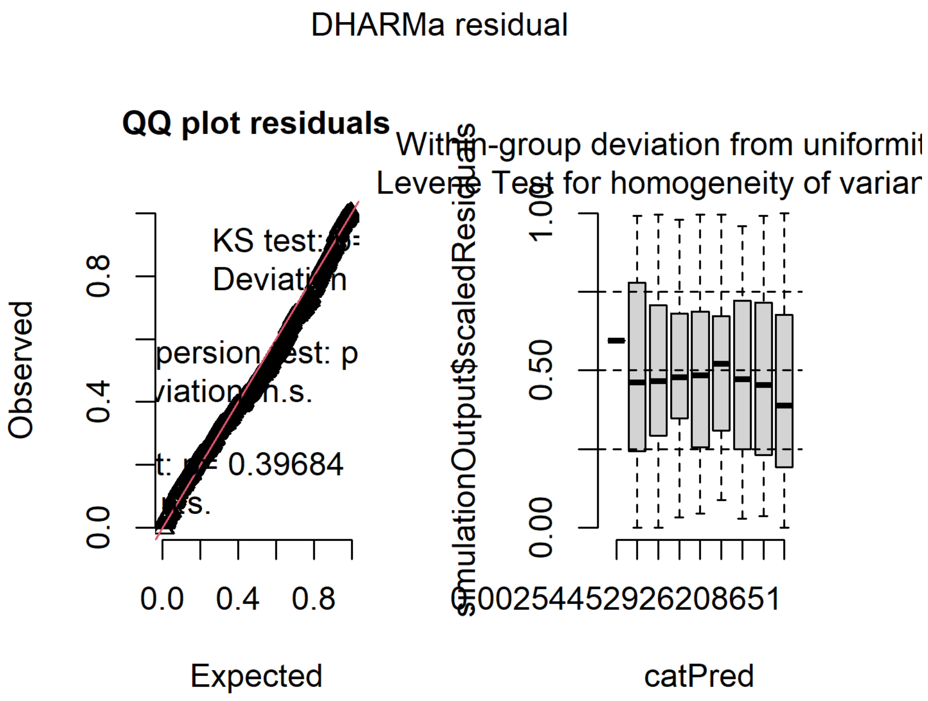
```
my_glm <- glm(relative_fatbody ~ treatment, data = alive, family = "Gamma")
par(mfrow=c(2,2))
plot(my_glm)
```

```
## Warning: kein Plot der Beobachtungen mit Leverage 1:
##   419
```



Supplemental Figure 4: plot of the residuals of the glm of the fat body data from the acute exposure experiment

```
summary(my_glm)

##
## Call:
## glm(formula = relative_fatbody ~ treatment, family = "Gamma",
##     data = alive)
##
## Deviance Residuals:
##      Min        1Q    Median        3Q       Max
## -1.67241  -0.26928  -0.07358   0.15275   1.37210
##
## Coefficients:
##                           Estimate Std. Error t value Pr(>|t|)
## (Intercept)               1.347456   0.072158  18.674   <2e-16 ***
## treatmentsolvent control -0.144251   0.095517  -1.510    0.132
## treatmentcarbon black    -0.047034   0.098475  -0.478    0.633
## treatmentDEP 1g/l         0.007378   0.102327   0.072    0.943
## treatmentDEP 2g/l        -0.033812   0.098518  -0.343    0.732
## treatmentDEP 4g/l         0.013145   0.102546   0.128    0.898
## treatmentDEP 8g/l         0.018771   0.101754   0.184    0.854
## treatmentDEP 16g/l        0.123647   0.104658   1.181    0.238
## treatmentDimethoate       0.510713   0.707318   0.722    0.471
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
##
## (Dispersion parameter for Gamma family taken to be 0.1433886)
##
##     Null deviance: 57.603  on 418  degrees of freedom
## Residual deviance: 56.307  on 410  degrees of freedom
## AIC: 76.838
##
## Number of Fisher Scoring iterations: 5

sim_my_glm<-simulateResiduals(my_glm)
plot(sim_my_glm)
```



Supplemental Figure 5: DHARMa plots of the residuals of the fat body data glm after acute exposure

```
anova(my_glm,test="F")

## Analysis of Deviance Table
##
## Model: Gamma, link: inverse
##
## Response: relative_fatbody
##
## Terms added sequentially (first to last)
##
##
##           Df Deviance Resid. Df Resid. Dev      F Pr(>F)
## NULL                        418     57.603
## treatment  8   1.2961       410     56.307 1.1299 0.3418
```

Good fit. No differences between treatments

Survival analysis of chronic exposure

```
chronic<- read.csv2("data_chronic_Bumblebee.csv")
```

Set values below zero in consumption columns to NA, as negative consumption is not possible. Calculate mean consumption per day.

```
str(chronic)

## 'data.frame':    336 obs. of  14 variables:
##  $ ID              : int  114 236 140 143 150 111 54 141 160 55 ...
##  $ treatment       : chr  "DEP 0.5g/l" "DEP 2g/l" "DEP 0.5g/l" "DEP 0.5g/l" ...
##  $ colony          : int  37 38 40 41 42 44 44 41 43 44 ...
##  $ consumption1    : num  0.884 -0.196 0.407 0.935 0.574 0.992 0.958 0.691 0.778 0.67 ..
.
##  $ consumption2    : num  0.775 0.398 1.156 0.749 0.45 ...
##  $ consumption3    : num  0.45 0.276 0.585 0.665 0.223 0.912 0.567 NA -0.394 0.898 ...
```

```
##  $ consumption4    : num  0.452 0.232 1.302 0.485 0.259 ...
##  $ consumption5    : num  0.381 0.195 0.764 0.886 0.197 ...
##  $ survival        : int  10 10 10 10 10 10 10 6 10 10 ...
##  $ death           : int  0 0 0 0 0 0 0 1 0 0 ...
##  $ measurement..mm.: chr  "Fl\xfcgel zu verklebt " "Fl\xfcgel zu verklebt " "2.75" "3.09
7" ...
##  $ fat.body..g.    : chr  "0.0012" "0.00143" "0.0201" "0.02027" ...
##  $ fat.body..mg.   : chr  "1.2" "1.43" "20.1" "20.27" ...
##  $ rel..Fat.body   : chr  "#WERT!" "#WERT!" "7.309090909" "6.545043591" ...
```

```
chronic<-chronic %>%
  mutate(max_dose = case_when(
    endsWith(treatment, "control") ~ "0",
    endsWith(treatment, "0.5g/l") ~ "1.1",
    endsWith(treatment, "1g/l") ~ "2.2",
    endsWith(treatment, "2g/l") ~ "4.4"
    ))

chronic$treatment<-as.factor(chronic$treatment)
chronic$survival<- as.numeric(chronic$survival)
chronic$death<- as.numeric(chronic$death)
chronic$treatment <- factor(chronic$treatment, levels = c("control","solvent control", "DEP
 0.5g/l","DEP 1g/l", "DEP 2g/l","Dimethoate"))
chronic$colony<-as.factor(chronic$colony)



chronic$consumption_mean <- rowMeans(chronic[,4:8],na.rm = TRUE)
chronic$consumption_mean <- chronic$consumption_mean/2
chronic$max_dose<-as.numeric(chronic$max_dose)

chronic$dose_ingested<- chronic$max_dose*chronic$consumption_mean
str(chronic)
```

```
## 'data.frame':    336 obs. of  17 variables:
##  $ ID              : int  114 236 140 143 150 111 54 141 160 55 ...
##  $ treatment       : Factor w/ 6 levels "control","solvent control",..: 3 5 3 3 3 2 1 3
3 1 ...
##  $ colony          : Factor w/ 8 levels "37","38","39",..: 1 2 4 5 6 8 8 5 7 8 ...
##  $ consumption1    : num  0.884 -0.196 0.407 0.935 0.574 0.992 0.958 0.691 0.778 0.67 ..
.
##  $ consumption2    : num  0.775 0.398 1.156 0.749 0.45 ...
##  $ consumption3    : num  0.45 0.276 0.585 0.665 0.223 0.912 0.567 NA -0.394 0.898 ...
##  $ consumption4    : num  0.452 0.232 1.302 0.485 0.259 ...
##  $ consumption5    : num  0.381 0.195 0.764 0.886 0.197 ...
##  $ survival        : num  10 10 10 10 10 10 10 6 10 10 ...
##  $ death           : num  0 0 0 0 0 0 0 1 0 0 ...
##  $ measurement..mm.: chr  "Fl\xfcgel zu verklebt " "Fl\xfcgel zu verklebt " "2.75" "3.09
7" ...
##  $ fat.body..g.    : chr  "0.0012" "0.00143" "0.0201" "0.02027" ...
##  $ fat.body..mg.   : chr  "1.2" "1.43" "20.1" "20.27" ...
##  $ rel..Fat.body   : chr  "#WERT!" "#WERT!" "7.309090909" "6.545043591" ...
##  $ max_dose        : num  1.1 4.4 1.1 1.1 1.1 0 0 1.1 1.1 0 ...
##  $ consumption_mean: num  0.2942 0.0905 0.4214 0.372 0.1703 ...
##  $ dose_ingested   : num  0.324 0.398 0.464 0.409 0.187 ...
```

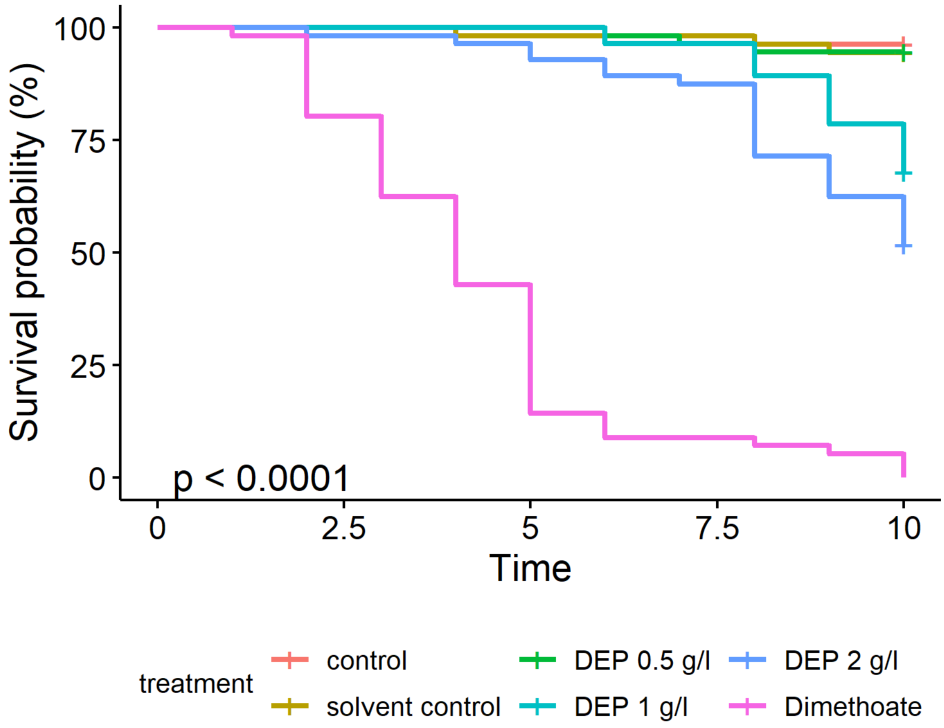
Effect of treatment on survival, no random effect.

```
fit_chronic <- survfit(Surv(survival, death)~treatment, data = chronic)
summary(fit_chronic)
```

```
## Call: survfit(formula = Surv(survival, death) ~ treatment, data = chronic)
##
## 2 Beobachtungen als fehlend gelöscht
##                 treatment=control
##  time n.risk n.event survival std.err lower 95% CI upper 95% CI
```

```
##     4     55       1    0.982  0.0180        0.947            1
##     8     54       1    0.964  0.0252        0.915            1
##
##                 treatment=solvent control
##  time n.risk n.event survival std.err lower 95% CI upper 95% CI
##     4     55       1    0.982  0.0180        0.947            1
##     8     54       1    0.964  0.0252        0.915            1
##     9     53       1    0.945  0.0306        0.887            1
##
##                 treatment=DEP 0.5g/l
##  time n.risk n.event survival std.err lower 95% CI upper 95% CI
##     6     56       1    0.982  0.0177        0.948            1
##     7     55       1    0.964  0.0248        0.917            1
##     8     54       1    0.946  0.0301        0.889            1
##
##                 treatment=DEP 1g/l
##  time n.risk n.event survival std.err lower 95% CI upper 95% CI
##     6     56       2    0.964  0.0248        0.917        1.000
##     8     54       4    0.893  0.0413        0.815        0.978
##     9     50       6    0.786  0.0548        0.685        0.901
##    10     44       6    0.679  0.0624        0.567        0.813
##
##                 treatment=DEP 2g/l
##  time n.risk n.event survival std.err lower 95% CI upper 95% CI
##     2     56       1    0.982  0.0177        0.948        1.000
##     4     55       1    0.964  0.0248        0.917        1.000
##     5     54       2    0.929  0.0344        0.864        0.999
##     6     52       2    0.893  0.0413        0.815        0.978
##     7     50       1    0.875  0.0442        0.793        0.966
##     8     49       9    0.714  0.0604        0.605        0.843
##     9     40       5    0.625  0.0647        0.510        0.766
##    10     35       6    0.518  0.0668        0.402        0.667
##
##                 treatment=Dimethoate
##  time n.risk n.event survival std.err lower 95% CI upper 95% CI
##     1     56       1   0.9821  0.0177       0.9481        1.000
##     2     55      10   0.8036  0.0531       0.7060        0.915
##     3     45      10   0.6250  0.0647       0.5102        0.766
##     4     35      11   0.4286  0.0661       0.3167        0.580
##     5     24      16   0.1429  0.0468       0.0752        0.271
##     6      8       3   0.0893  0.0381       0.0387        0.206
##     8      5       1   0.0714  0.0344       0.0278        0.184
##     9      4       1   0.0536  0.0301       0.0178        0.161
##    10      3       3   0.0000     NaN           NA           NA
```

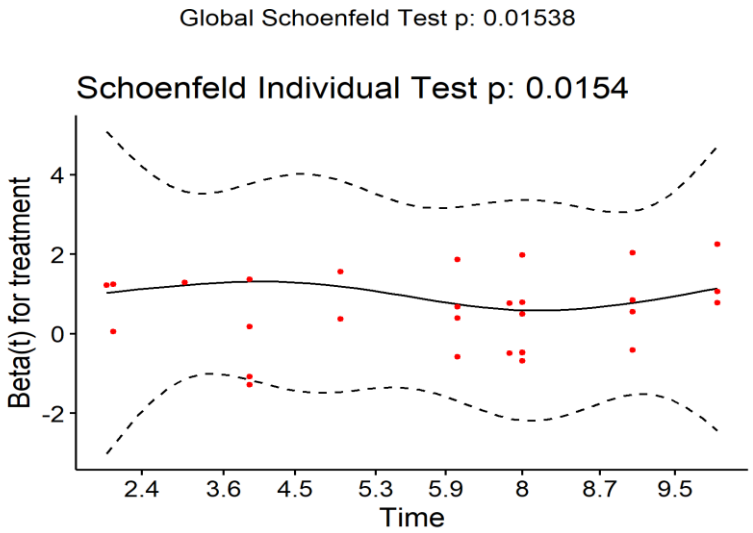
```
ggsurvplot(fit_chronic, data = chronic,
           pval = TRUE,
           fun = "pct",
           risk.table = FALSE,
           size = 1,
           legend = "bottom",
           legend.title = "treatment",
           legend.labs = c("control","solvent control","DEP 0.5 g/l","DEP 1 g/l","DEP 2 g/l
","Dimethoate" ))
```



Supplemental Figure 6: Survival probability across the 10 day period for the bumblebees exposed to the control, solvent control,DEP concentrations 0.5, 1 and 2 g/l and dimethoate. The p-value indicates significant differences between the Kaplan-Meier curves of the different treatment.

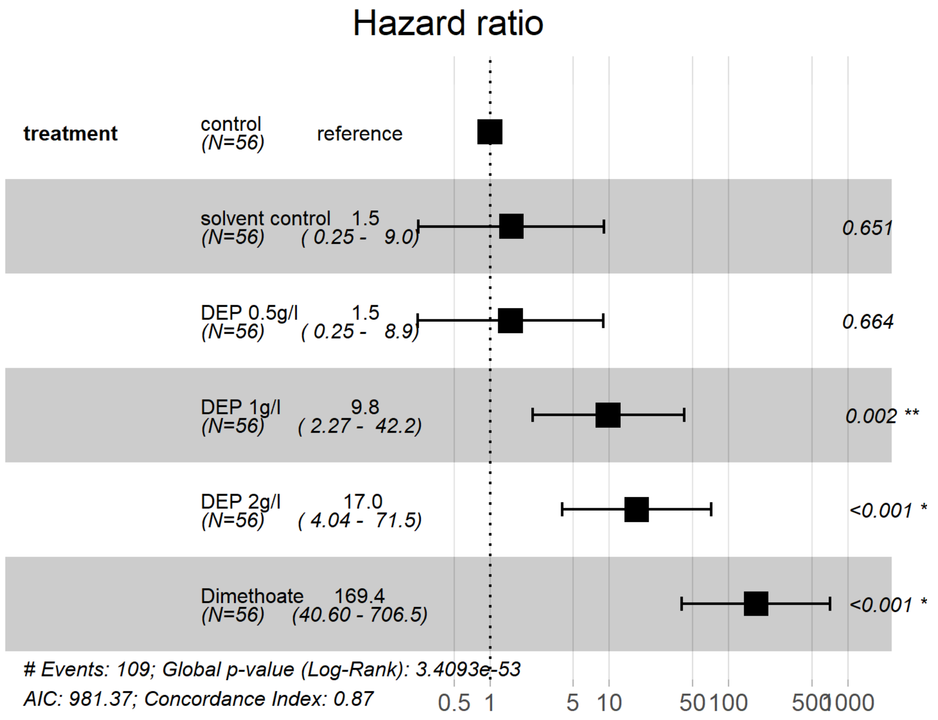
```
fit.model <- coxph(Surv(survival, death)~treatment, data = chronic)
summary(fit.model)

## Call:
## coxph(formula = Surv(survival, death) ~ treatment, data = chronic)
##
##   n= 334, number of events= 109
##    (2 Beobachtungen als fehlend gelöscht)
##
##                                coef exp(coef) se(coef)     z Pr(>|z|)
## treatmentsolvent control   0.4124    1.5105   0.9129 0.452 0.651408
## treatmentDEP 0.5g/l        0.3961    1.4860   0.9129 0.434 0.664389
## treatmentDEP 1g/l          2.2818    9.7947   0.7455 3.061 0.002207 **
## treatmentDEP 2g/l          2.8333   17.0013   0.7332 3.864 0.000111 ***
## treatmentDimethoate        5.1320  169.3631   0.7287 7.043 1.89e-12 ***
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
##
##                          exp(coef) exp(-coef) lower .95 upper .95
## treatmentsolvent control     1.511   0.662032    0.2524     9.040
## treatmentDEP 0.5g/l          1.486   0.672967    0.2483     8.893
## treatmentDEP 1g/l            9.795   0.102096    2.2721    42.224
## treatmentDEP 2g/l           17.001   0.058819    4.0400    71.546
## treatmentDimethoate        169.363   0.005904   40.6012   706.478
##
## Concordance= 0.87  (se = 0.017 )
## Likelihood ratio test= 255.6  on 5 df,   p=<2e-16
## Wald test            = 210.7  on 5 df,   p=<2e-16
## Score (logrank) test = 467  on 5 df,   p=<2e-16

ftest <- cox.zph(fit.model)
ftest
```

```
##             chisq df     p
## treatment     14  5 0.015
## GLOBAL        14  5 0.015

ggcoxzph(ftest)
```



Supplemental Figure 7: schoenfeld model assumptions for the cox model

```
ggforest(fit.model)

## Warning in .get_data(model, data = data): The `data` argument is not provided.
## Data will be extracted from model fit.
```



Supplemental Figure 8: Forest plot of hazard ratio from multivariable Cox proportional hazard regression model on survival for DEP exposure in comparison with the control. Bars represent 95% confidence intervals.The p-values with asterisks indicate significantly elevated mortality risk compared to control organisms.

```
anova(fit.model)

## Analysis of Deviance Table
##  Cox model: response is Surv(survival, death)
## Terms added sequentially (first to last)
##
##            loglik  Chisq Df Pr(>|Chi|)
## NULL      -613.50
## treatment -485.68 255.63  5  < 2.2e-16 ***
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

multi.fit <- glht(fit.model, linfct = mcp (treatment = "Tukey"))
summary(multi.fit, test = adjusted("BH"))

##
##   Simultaneous Tests for General Linear Hypotheses
##
## Multiple Comparisons of Means: Tukey Contrasts
##
##
## Fit: coxph(formula = Surv(survival, death) ~ treatment, data = chronic)
##
## Linear Hypotheses:
##                                   Estimate Std. Error z value Pr(>|z|)
## solvent control - control == 0     0.41244    0.91287   0.452 0.711846
## DEP 0.5g/l - control == 0          0.39606    0.91287   0.434 0.711846
## DEP 1g/l - control == 0            2.28184    0.74550   3.061 0.003679 **
## DEP 2g/l - control == 0            2.83329    0.73320   3.864 0.000209 ***
## Dimethoate - control == 0          5.13205    0.72871   7.043 5.66e-12 ***
## DEP 0.5g/l - solvent control == 0 -0.01638    0.81650  -0.020 0.983993
## DEP 1g/l - solvent control == 0    1.86940    0.62376   2.997 0.003718 **
## DEP 2g/l - solvent control == 0    2.42085    0.60899   3.975 0.000151 ***
## Dimethoate - solvent control == 0  4.71960    0.60349   7.820 2.00e-14 ***
## DEP 1g/l - DEP 0.5g/l == 0         1.88578    0.62376   3.023 0.003718 **
## DEP 2g/l - DEP 0.5g/l == 0         2.43723    0.60899   4.002 0.000151 ***
## Dimethoate - DEP 0.5g/l == 0       4.73599    0.60342   7.849 2.00e-14 ***
## DEP 2g/l - DEP 1g/l == 0           0.55145    0.30444   1.811 0.087606 .
## Dimethoate - DEP 1g/l == 0         2.85021    0.29065   9.806  < 2e-16 ***
## Dimethoate - DEP 2g/l == 0         2.29876    0.25386   9.055  < 2e-16 ***
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
## (Adjusted p values reported -- BH method)
```

Survival differed significantly between treatments (Cox proportional hazards with colony as a random effect: χ2=266.059, df=5, P<0.001).Lowest survival in dimethoate 4.286 ± 1.347 days (mean±s.d.). No differences between individuals exposed to control, solvent control and DEP 0.5g/l. Bumblebees exposed to 1 and 2g/l DEP have lower survival than those exposed tp control, solvent control and DEP 0.5g/l. No significant difference between DEP 1g/l and DEP 2g/l (p = 0.087).

Effect of treatment with colony as random effect after chronic exposure

```
fit_random <- coxme(Surv(survival,death) ~ treatment + (1|colony), data = chronic)
summary(fit_random)

## Cox mixed-effects model fit by maximum likelihood
##   Data: chronic
##   events, n = 109, 334 (2 Beobachtungen als fehlend gelöscht)
##   Iterations= 16 132
##                     NULL Integrated    Fitted
## Log-likelihood -613.4959  -480.4662 -472.8352
##
##                    Chisq    df p    AIC    BIC
## Integrated loglik 266.06  6.00 0 254.06 237.91
```

```
##  Penalized loglik 281.32 10.26 0 260.80 233.18
##
## Model:  Surv(survival, death) ~ treatment + (1 | colony)
## Fixed coefficients
##                               coef  exp(coef)  se(coef)    z       p
## treatmentsolvent control 0.4241324   1.528264 0.9128880 0.46 6.4e-01
## treatmentDEP 0.5g/l      0.4259052   1.530976 0.9129108 0.47 6.4e-01
## treatmentDEP 1g/l        2.3206453  10.182243 0.7456824 3.11 1.9e-03
## treatmentDEP 2g/l        2.9153744  18.455720 0.7336463 3.97 7.1e-05
## treatmentDimethoate      5.5080147 246.660941 0.7387404 7.46 8.9e-14
##
## Random effects
##  Group  Variable  Std Dev   Variance
##  colony Intercept 0.4862948 0.2364826

anova_fit<-anova(fit_random)
anova_fit#to be able to put the anova fit in the text, you can give the fit a name and then
 refer to it in the text below. You could also directly call the fit in the text, but this
make the text very long

## Analysis of Deviance Table
##  Cox model: response is Surv(survival, death)
## Terms added sequentially (first to last)
##
##            loglik  Chisq Df Pr(>|Chi|)
## NULL      -613.50
## treatment -480.47 266.06  5  < 2.2e-16 ***
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

mult.fit_random <- glht(fit_random, linfct = mcp (treatment = "Tukey"))
summary(mult.fit_random, test = adjusted ("BH"))

##
##   Simultaneous Tests for General Linear Hypotheses
##
## Multiple Comparisons of Means: Tukey Contrasts
##
##
## Fit: coxme(formula = Surv(survival, death) ~ treatment + (1 | colony),
##     data = chronic)
##
## Linear Hypotheses:
##                                  Estimate Std. Error z value Pr(>|z|)
## solvent control - control == 0    0.424132   0.912888   0.465 0.688087
## DEP 0.5g/l - control == 0         0.425905   0.912911   0.467 0.688087
## DEP 1g/l - control == 0           2.320645   0.745682   3.112 0.003096 **
## DEP 2g/l - control == 0           2.915374   0.733646   3.974 0.000133 ***
## Dimethoate - control == 0         5.508015   0.738740   7.456 2.68e-13 ***
## DEP 0.5g/l - solvent control == 0 0.001773   0.816558   0.002 0.998268
## DEP 1g/l - solvent control == 0   1.896513   0.623968   3.039 0.003257 **
## DEP 2g/l - solvent control == 0   2.491242   0.609456   4.088 9.42e-05 ***
## Dimethoate - solvent control == 0 5.083882   0.615409   8.261 8.33e-16 ***
## DEP 1g/l - DEP 0.5g/l == 0        1.894740   0.623851   3.037 0.003257 **
## DEP 2g/l - DEP 0.5g/l == 0        2.489469   0.609343   4.085 9.42e-05 ***
## Dimethoate - DEP 0.5g/l == 0      5.082110   0.614204   8.274 8.33e-16 ***
## DEP 2g/l - DEP 1g/l == 0          0.594729   0.305019   1.950 0.063999 .
## Dimethoate - DEP 1g/l == 0        3.187369   0.311940  10.218  < 2e-16 ***
## Dimethoate - DEP 2g/l == 0        2.592640   0.274750   9.436  < 2e-16 ***
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
## (Adjusted p values reported -- BH method)

chronic_means<-chronic %>% group_by(treatment, colony) %>% summarise(colony_mean=mean(survival,na
.rm=T)) %>% group_by(treatment) %>% summarise(surv_mean=mean(colony_mean, na.rm=T),surv_sd=sd(col
ony_mean, na.rm=T))#make means of means using the pipe from dplyr which is also implemented in ti
dyverse
```

```
## `summarise()` has grouped output by 'treatment'. You can override using the
## `.groups` argument.
```

Survival differed significantly between treatments (Cox proportional hazards with colony as a random effect: χ^2=266.059, df=5, P<0.001).

Lowest survival in dimethoate 4.286 ± 1.347 days (mean±s.d.). No differences between control, solvent control and soot 0.5. Soot 1 and soot 2 have lower survival than control, solvent control and soot 0.5. No significant difference between soot 1 and soot 2

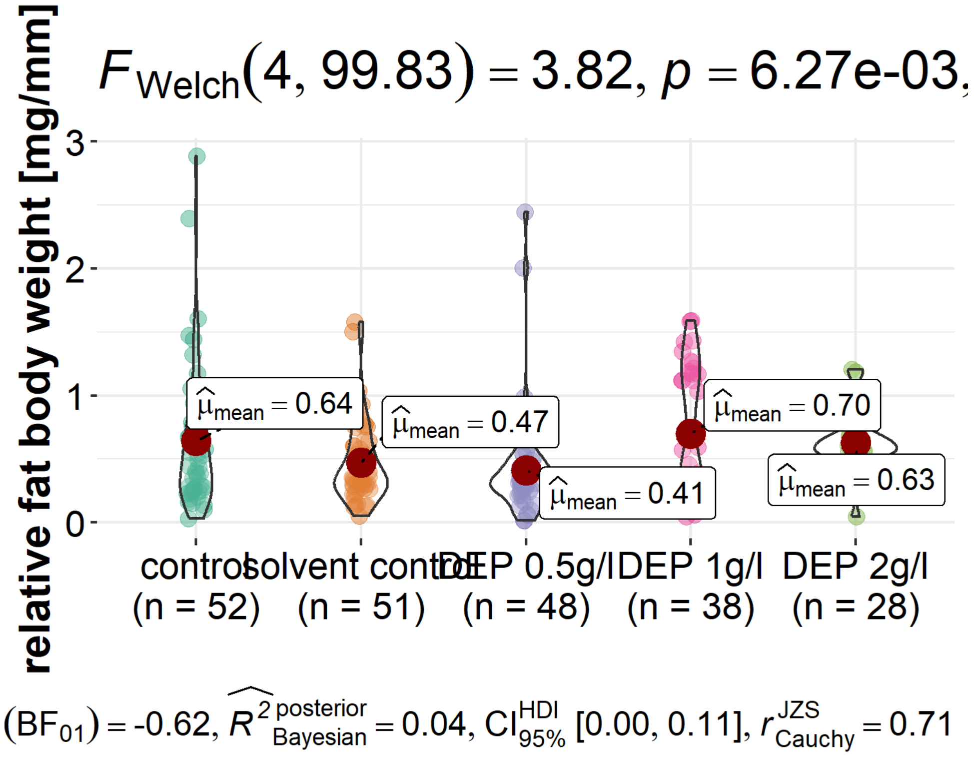
Effect of treatment on relative fat body weight after chronic exposure

Removed dead bumblebees and outliers (below zero or heavier than mean + 3*s.d.) prior to analysis as suggested by Aguinis et al. (2013).

```
str(chronic)

## 'data.frame':    336 obs. of  17 variables:
##  $ ID              : int  114 236 140 143 150 111 54 141 160 55 ...
##  $ treatment       : Factor w/ 6 levels "control","solvent control",..: 3 5 3 3 3 2 1 3
3 1 ...
##  $ colony          : Factor w/ 8 levels "37","38","39",..: 1 2 4 5 6 8 8 5 7 8 ...
##  $ consumption1    : num  0.884 -0.196 0.407 0.935 0.574 0.992 0.958 0.691 0.778 0.67 ..
.
##  $ consumption2    : num  0.775 0.398 1.156 0.749 0.45 ...
##  $ consumption3    : num  0.45 0.276 0.585 0.665 0.223 0.912 0.567 NA -0.394 0.898 ...
##  $ consumption4    : num  0.452 0.232 1.302 0.485 0.259 ...
##  $ consumption5    : num  0.381 0.195 0.764 0.886 0.197 ...
##  $ survival        : num  10 10 10 10 10 10 10 6 10 10 ...
##  $ death           : num  0 0 0 0 0 0 0 1 0 0 ...
##  $ measurement..mm.: chr  "Fl\xfcgel zu verklebt " "Fl\xfcgel zu verklebt " "2.75" "3.09
7" ...
##  $ fat.body..g.    : chr  "0.0012" "0.00143" "0.0201" "0.02027" ...
##  $ fat.body..mg.   : chr  "1.2" "1.43" "20.1" "20.27" ...
##  $ rel..Fat.body   : chr  "#WERT!" "#WERT!" "7.309090909" "6.545043591" ...
##  $ max_dose        : num  1.1 4.4 1.1 1.1 1.1 0 0 1.1 1.1 0 ...
##  $ consumption_mean: num  0.2942 0.0905 0.4214 0.372 0.1703 ...
##  $ dose_ingested   : num  0.324 0.398 0.464 0.409 0.187 ...

chronic$measurement..mm.<-as.numeric(chronic$measurement..mm.)

## Warning: NAs durch Umwandlung erzeugt

chronic$ fat.body..g.<-as.numeric(chronic$ fat.body..g.)
chronic$ fat.body..mg. <-as.numeric(chronic$ fat.body..mg.)
chronic$ rel..Fat.body   <-as.numeric(chronic$ rel..Fat.body)

## Warning: NAs durch Umwandlung erzeugt

chronic_alive <- subset(chronic,chronic$death =="0")
upper_limit <-mean(chronic_alive$rel..Fat.body , na.rm=T)+3*sd(chronic_alive$rel..Fat.body
, na.rm=T)
lower_limit <-0
chronic_no_outliers <-subset(chronic_alive,chronic_alive$rel..Fat.body  > 0 & chronic_alive
$rel..Fat.body  < upper_limit)

str(chronic_alive)

## 'data.frame':    225 obs. of  17 variables:
##  $ ID              : int  114 236 140 143 150 111 54 160 55 147 ...
##  $ treatment       : Factor w/ 6 levels "control","solvent control",..: 3 5 3 3 3 2 1 3
1 3 ...
##  $ colony          : Factor w/ 8 levels "37","38","39",..: 1 2 4 5 6 8 8 7 8 5 ...
##  $ consumption1    : num  0.884 -0.196 0.407 0.935 0.574 0.992 0.958 0.778 0.67 0.925 ..
```

```
.
##  $ consumption2    : num  0.775 0.398 1.156 0.749 0.45 ...
##  $ consumption3    : num  0.45 0.276 0.585 0.665 0.223 0.912 0.567 -0.394 0.898 0.552 ..
.
##  $ consumption4    : num  0.452 0.232 1.302 0.485 0.259 ...
##  $ consumption5    : num  0.381 0.195 0.764 0.886 0.197 ...
##  $ survival        : num  10 10 10 10 10 10 10 10 10 10 ...
##  $ death           : num  0 0 0 0 0 0 0 0 0 0 ...
##  $ measurement..mm.: num  NA NA 2.75 3.1 2.91 ...
##  $ fat.body..g.    : num  0.0012 0.00143 0.0201 0.02027 0.01643 ...
##  $ fat.body..mg.   : num  1.2 1.43 20.1 20.27 16.43 ...
##  $ rel..Fat.body   : num  NA NA 7.31 6.55 5.64 ...
##  $ max_dose        : num  1.1 4.4 1.1 1.1 1.1 0 0 1.1 0 1.1 ...
##  $ consumption_mean: num  0.2942 0.0905 0.4214 0.372 0.1703 ...
##  $ dose_ingested   : num  0.324 0.398 0.464 0.409 0.187 ...

chronic_no_neg<-subset(chronic_alive,chronic_alive$rel..Fat.body  > 0)

str(chronic_no_outliers)

## 'data.frame':    217 obs. of  17 variables:
##  $ ID              : int  54 160 55 147 7 205 221 79 103 5 ...
##  $ treatment       : Factor w/ 6 levels "control","solvent control",..: 1 3 1 3 1 4 4 2
2 1 ...
##  $ colony          : Factor w/ 8 levels "37","38","39",..: 8 7 8 5 1 6 8 4 7 1 ...
##  $ consumption1    : num  0.958 0.778 0.67 0.925 0.807 ...
##  $ consumption2    : num  1.11 0.587 0.911 1.014 0.729 ...
##  $ consumption3    : num  0.567 -0.394 0.898 0.552 1.307 ...
##  $ consumption4    : num  0.827 0.66 0.75 0.426 0.887 ...
##  $ consumption5    : num  0.558 1.072 0.684 0.455 0.648 ...
##  $ survival        : num  10 10 10 10 10 10 10 10 10 10 ...
##  $ death           : num  0 0 0 0 0 0 0 0 0 0 ...
##  $ measurement..mm.: num  3.03 2.88 2.99 3.02 2.98 ...
##  $ fat.body..g.    : num  0.00873 0.00705 0.00715 0.00606 0.00478 0.00527 0.00505 0.0047
 0.00352 0.00466 ...
##  $ fat.body..mg.   : num  8.73 7.05 7.15 6.06 4.78 5.27 5.05 4.7 3.52 4.66 ...
##  $ rel..Fat.body   : num  2.88 2.45 2.39 2.01 1.6 ...
##  $ max_dose        : num  0 1.1 0 1.1 0 2.2 2.2 0 0 0 ...
##  $ consumption_mean: num  0.402 0.27 0.391 0.337 0.438 ...
##  $ dose_ingested   : num  0 0.297 0 0.371 0 ...

chronic_nocontrol<- subset(chronic_no_outliers,chronic_no_outliers$max_dose>0)
str(chronic_nocontrol)

## 'data.frame':    114 obs. of  17 variables:
##  $ ID              : int  160 147 205 221 204 222 210 224 220 208 ...
##  $ treatment       : Factor w/ 6 levels "control","solvent control",..: 3 3 4 4 4 4 4 4
4 4 ...
##  $ colony          : Factor w/ 8 levels "37","38","39",..: 7 5 6 8 6 8 6 8 8 6 ...
##  $ consumption1    : num  0.778 0.925 0.897 0.861 0.458 ...
##  $ consumption2    : num  0.587 1.014 0.949 0.748 0.762 ...
##  $ consumption3    : num  -0.394 0.552 1.029 0.578 0.384 ...
##  $ consumption4    : num  0.66 0.426 0.539 0.412 0.574 0.77 0.53 0.215 0.392 0.191 ...
##  $ consumption5    : num  1.072 0.455 0.421 0.411 0.768 ...
##  $ survival        : num  10 10 10 10 10 10 10 10 10 10 ...
##  $ death           : num  0 0 0 0 0 0 0 0 0 0 ...
##  $ measurement..mm.: num  2.88 3.02 3.32 3.19 3.46 ...
##  $ fat.body..g.    : num  0.00705 0.00606 0.00527 0.00505 0.00496 0.0048 0.00396 0.00366
 0.00404 0.00369 ...
##  $ fat.body..mg.   : num  7.05 6.06 5.27 5.05 4.96 4.8 3.96 3.66 4.04 3.69 ...
##  $ rel..Fat.body   : num  2.45 2.01 1.59 1.58 1.43 ...
##  $ max_dose        : num  1.1 1.1 2.2 2.2 2.2 2.2 2.2 2.2 2.2 2.2 ...
##  $ consumption_mean: num  0.27 0.337 0.384 0.301 0.295 ...
##  $ dose_ingested   : num  0.297 0.371 0.844 0.662 0.648 ...

str(chronic_no_outliers)
```

```
## 'data.frame':    217 obs. of  17 variables:
##  $ ID               : int  54 160 55 147 7 205 221 79 103 5 ...
##  $ treatment        : Factor w/ 6 levels "control","solvent control",..: 1 3 1 3 1 4 4 2
2 1 ...
##  $ colony           : Factor w/ 8 levels "37","38","39",..: 8 7 8 5 1 6 8 4 7 1 ...
##  $ consumption1     : num  0.958 0.778 0.67 0.925 0.807 ...
##  $ consumption2     : num  1.11 0.587 0.911 1.014 0.729 ...
##  $ consumption3     : num  0.567 -0.394 0.898 0.552 1.307 ...
##  $ consumption4     : num  0.827 0.66 0.75 0.426 0.887 ...
##  $ consumption5     : num  0.558 1.072 0.684 0.455 0.648 ...
##  $ survival         : num  10 10 10 10 10 10 10 10 10 10 ...
##  $ death            : num  0 0 0 0 0 0 0 0 0 0 ...
##  $ measurement..mm.: num  3.03 2.88 2.99 3.02 2.98 ...
##  $ fat.body..g.     : num  0.00873 0.00705 0.00715 0.00606 0.00478 0.00527 0.00505 0.0047
 0.00352 0.00466 ...
##  $ fat.body..mg.    : num  8.73 7.05 7.15 6.06 4.78 5.27 5.05 4.7 3.52 4.66 ...
##  $ rel..Fat.body    : num  2.88 2.45 2.39 2.01 1.6 ...
##  $ max_dose         : num  0 1.1 0 1.1 0 2.2 2.2 0 0 0 ...
##  $ consumption_mean: num  0.402 0.27 0.391 0.337 0.438 ...
##  $ dose_ingested    : num  0 0.297 0 0.371 0 ...

ggstatsplot::ggbetweenstats(
  data = chronic_no_outliers,
  x = treatment, xlab = "",
  y = rel..Fat.body,
  ylab = "relative fat body weight [mg/mm]",
  plot.type = "violin",
  pairwise.comparisons = FALSE,
  breaks = seq(0, 1.25, 0.25),
  type = "p",
  centrality.label.args = list(size = 4, nudge_x = 0.4, segment.linetype = 4,
                             min.segment.length = 0),
  conf.level = 0.95,
  ggplot.component =  list(theme(text = element_text(size = 16),
                               plot.subtitle = element_text(size = 20, face = "bold"),
                               axis.text  = element_text(color = "black",size = 14)))
)
```



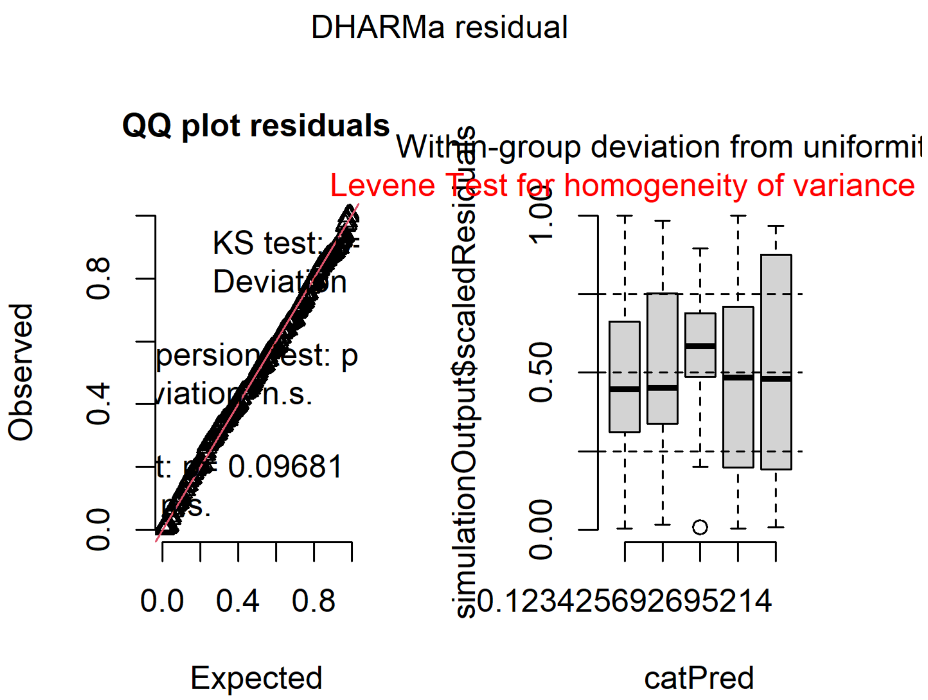
Supplemental Figure 9: *Effect of DEP exposure on the relative fat body weight (corrected for body size) of the living bumblebees after 10-day exposure. Number of replicates per treatment are represented by n. The values in the boxes represent the mean relative fat body of the treatment. Relative fat body values represent the fat body weight of the individual bumble in mg divided by the length of the radial cell in mm. Letters indicate significance between treatments.*

Table with Tukey tests is provided on page 23 with highlighted significant results.

Generalized Liner Mixed Model (GLMM) Family = “Gamma”, as DHARMa reported problems with “gaussian”.

```
my_glmm <- glmmTMB(rel..Fat.body ~treatment + (1|colony), data = chronic_no_outliers, famil
y="Gamma")
summary(my_glmm)

##  Family: Gamma  ( inverse )
## Formula:          rel..Fat.body ~ treatment + (1 | colony)
## Data: chronic_no_outliers
##
##      AIC      BIC   logLik deviance df.resid
##    133.3    157.0    -59.7    119.3      210
##
## Random effects:
##
## Conditional model:
##  Groups Name        Variance Std.Dev.
##  colony (Intercept) 0.1098   0.3313
## Number of obs: 217, groups:  colony, 8
##
## Dispersion estimate for Gamma family (sigma^2): 0.484
##
## Conditional model:
##                          Estimate Std. Error z value Pr(>|z|)
## (Intercept)               1.61087    0.19078   8.444  < 2e-16 ***
## treatmentsolvent control  0.53107    0.25239   2.104  0.03536 *
## treatmentDEP 0.5g/l       0.85985    0.28215   3.047  0.00231 **
## treatmentDEP 1g/l        -0.05744    0.21635  -0.265  0.79064
## treatmentDEP 2g/l         0.05589    0.25322   0.221  0.82531
```

```
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

sim_my_glmm<-simulateResiduals(my_glmm)
plot(sim_my_glmm)
```



Supplemental Figure 10: DHARMa plots of the residuals of the fat body data glm after chronic exposure

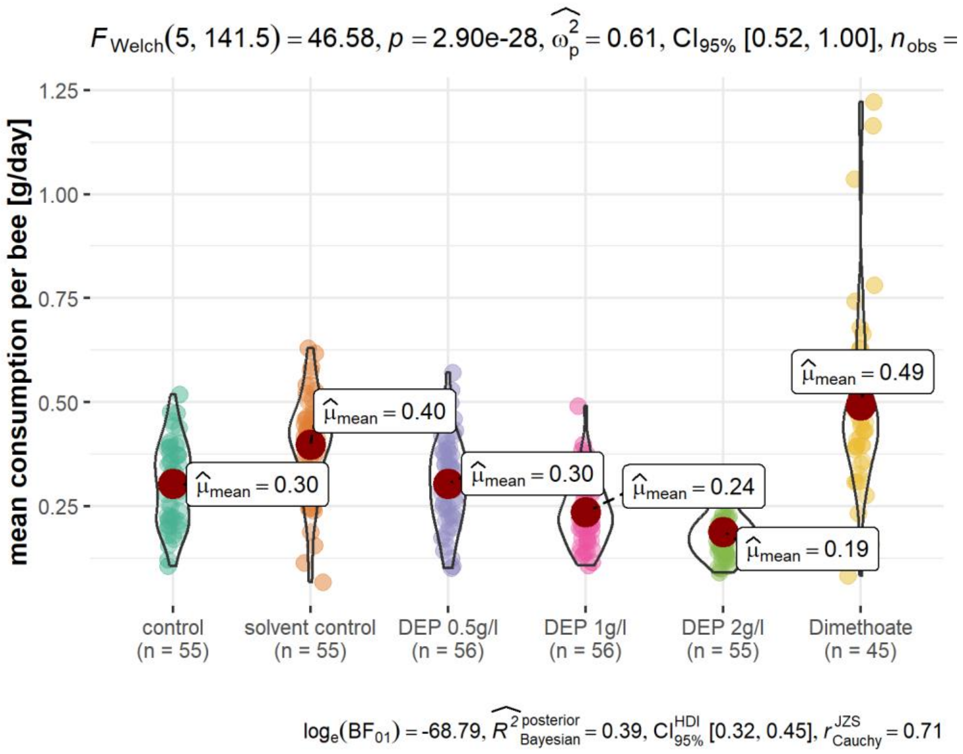
```
Anova(my_glmm, Test="F")

## Analysis of Deviance Table (Type II Wald chisquare tests)
##
## Response: rel..Fat.body
##            Chisq Df Pr(>Chisq)
## treatment 14.815  4   0.005101 **
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

mult.fatbody_random <- glht(my_glmm, linfct = mcp(treatment = "Tukey"))
summary(mult.fatbody_random,test=adjusted("BH"))

##
##   Simultaneous Tests for General Linear Hypotheses
##
## Multiple Comparisons of Means: Tukey Contrasts
##
##
## Fit: glmmTMB(formula = rel..Fat.body ~ treatment + (1 | colony), data = chronic_no_outli
ers,
##     family = "Gamma", ziformula = ~0, dispformula = ~1)
##
## Linear Hypotheses:
##                                      Estimate Std. Error z value Pr(>|z|)
## solvent control - control == 0        0.53107    0.25239   2.104   0.0707 .
## DEP 0.5g/l - control == 0             0.85985    0.28215   3.047   0.0115 *
## DEP 1g/l - control == 0              -0.05744    0.21635  -0.265   0.8253
## DEP 2g/l - control == 0               0.05589    0.25322   0.221   0.8253
```

```
## DEP 0.5g/l - solvent control == 0  0.32878    0.31641   1.039   0.4268
## DEP 1g/l - solvent control == 0   -0.58851    0.26160  -2.250   0.0612 .
## DEP 2g/l - solvent control == 0   -0.47518    0.29148  -1.630   0.1718
## DEP 1g/l - DEP 0.5g/l == 0        -0.91728    0.28953  -3.168   0.0115 *
## DEP 2g/l - DEP 0.5g/l == 0        -0.80396    0.31776  -2.530   0.0380 *
## DEP 2g/l - DEP 1g/l == 0           0.11333    0.25999   0.436   0.8253
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
## (Adjusted p values reported -- BH method)
```

No variance homogeneity (Levene Test < 0.05). Soot 0.5 with lower relative fat body weight than the other treatments (p < 0.05). 1 and 2 g/l DEP with lower relative fat body weight than the solvent control. Dimethoate not included in analysis as all animals died before the end of the experiment.

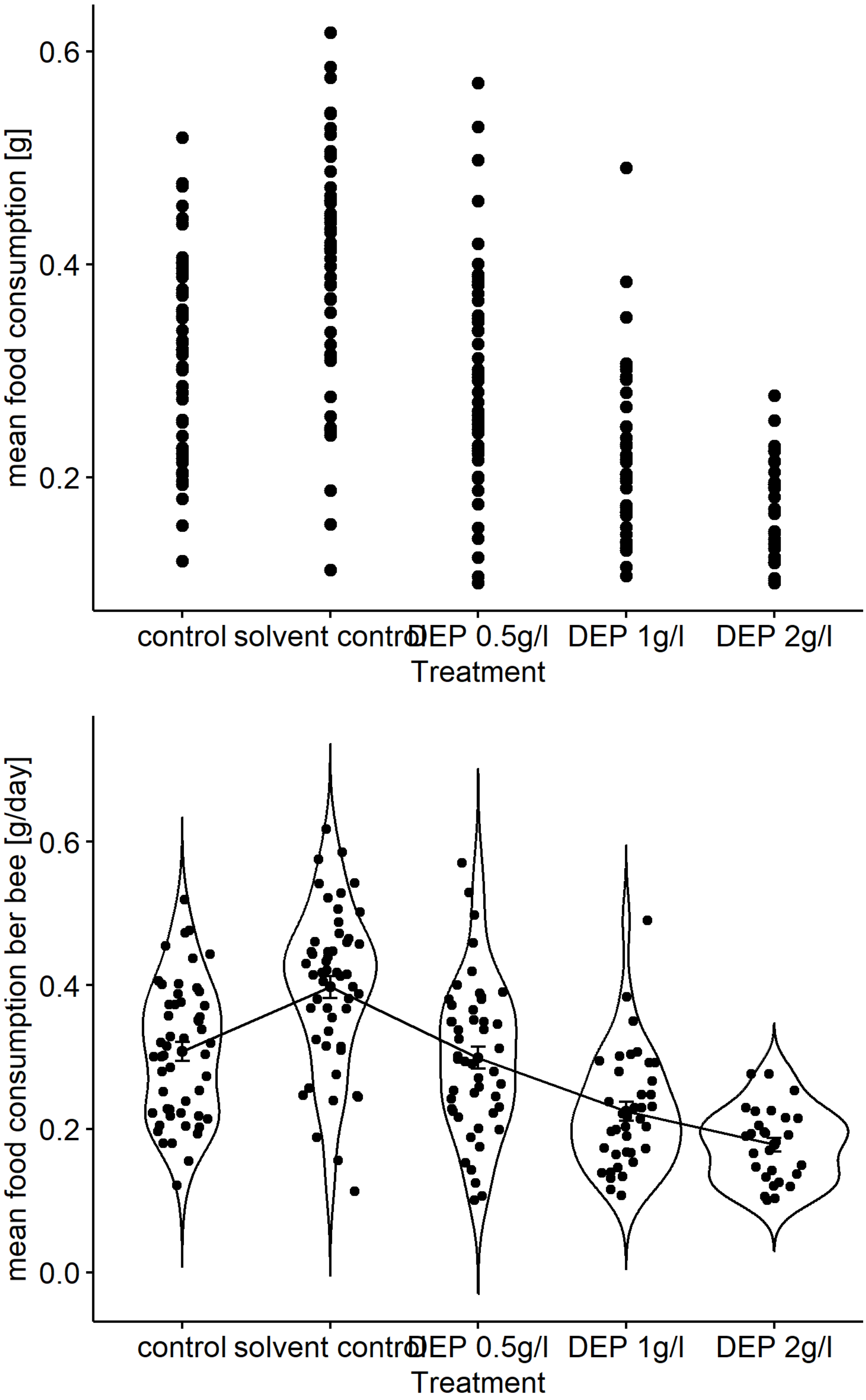
Effect of treatment on sugar water consumption after chronic exposure

```
## 'data.frame':    336 obs. of  17 variables:
##  $ ID              : int  114 236 140 143 150 111 54 141 160 55 ...
##  $ treatment       : Factor w/ 6 levels "control","solvent control",..: 3 5 3 3 3 2 1 3
3 1 ...
##  $ colony          : Factor w/ 8 levels "37","38","39",..: 1 2 4 5 6 8 8 5 7 8 ...
##  $ consumption1    : num  0.884 -0.196 0.407 0.935 0.574 0.992 0.958 0.691 0.778 0.67 ..
.
##  $ consumption2    : num  0.775 0.398 1.156 0.749 0.45 ...
##  $ consumption3    : num  0.45 0.276 0.585 0.665 0.223 0.912 0.567 NA -0.394 0.898 ...
##  $ consumption4    : num  0.452 0.232 1.302 0.485 0.259 ...
##  $ consumption5    : num  0.381 0.195 0.764 0.886 0.197 ...
##  $ survival        : num  10 10 10 10 10 10 10 6 10 10 ...
##  $ death           : num  0 0 0 0 0 0 0 1 0 0 ...
##  $ measurement..mm.: num  NA NA 2.75 3.1 2.91 ...
##  $ fat.body..g.    : num  0.0012 0.00143 0.0201 0.02027 0.01643 ...
##  $ fat.body..mg.   : num  1.2 1.43 20.1 20.27 16.43 ...
##  $ rel..Fat.body   : num  NA NA 7.31 6.55 5.64 ...
##  $ max_dose        : num  1.1 4.4 1.1 1.1 1.1 0 0 1.1 1.1 0 ...
##  $ consumption_mean: num  0.2942 0.0905 0.4214 0.372 0.1703 ...
##  $ dose_ingested   : num  0.324 0.398 0.464 0.409 0.187 ...
```



Supplemental Figure 11: Effect of 0.5, 1 and 2 g/l DEP exposure in the diet on the daily sugar water consumption of the bumblebees after the 10- day period. Number of replicates per treatment are represented by n. The values in the boxes represent the mean relative fat body weight of the treatment. Letters indicate significance between treatments.

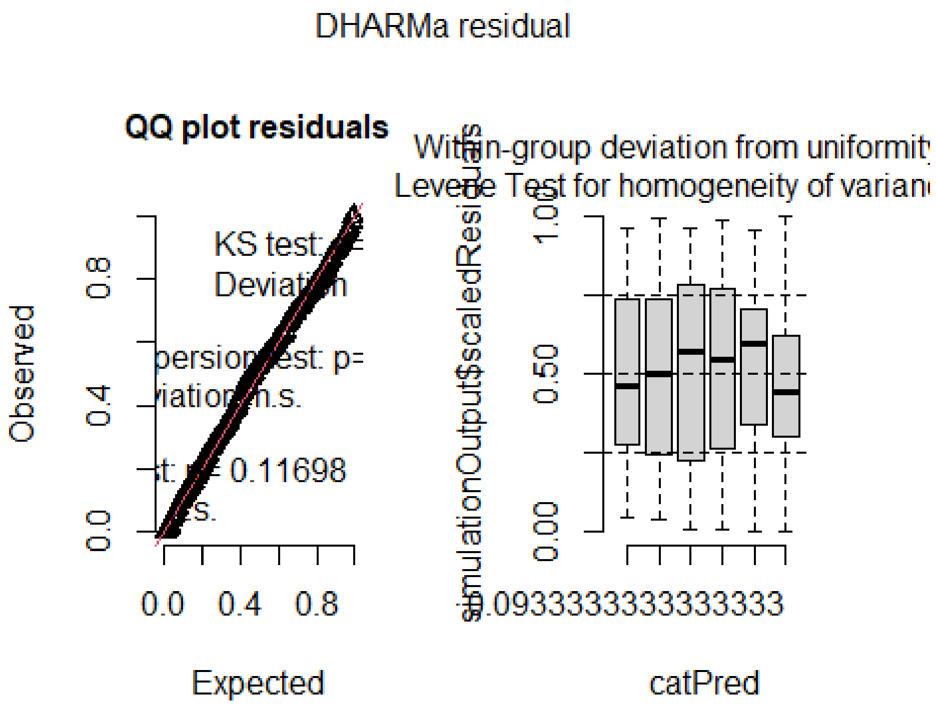
```
## `geom_smooth()` using formula = 'y ~ x'
```



Supplemental Figure 12: Effect of the dose of DEP on the food consumption per bumblebee per day. The graph shows a significant negative correlation between the two variables. The black point with whiskers represents the mean with standard error for every dose respectively.

Generalized Liner Mixed Model (GLMM) for differences in consumption after chronic exposure to DEP

```
my_glmm_con <- glmmTMB(consumption_mean ~treatment + (1|colony), data = chronic, family="Ga
mma")
summary(my_glmm_con)

##  Family: Gamma  ( inverse )
## Formula:          consumption_mean ~ treatment + (1 | colony)
## Data: chronic
##
##      AIC      BIC   logLik deviance df.resid
##   -589.1   -558.9    302.6   -605.1      314
##
## Random effects:
##
## Conditional model:
##  Groups Name        Variance Std.Dev.
##  colony (Intercept) 0.1714   0.414
## Number of obs: 322, groups:  colony, 8
##
## Dispersion estimate for Gamma family (sigma^2): 0.103
##
## Conditional model:
##                           Estimate Std. Error z value Pr(>|z|)
## (Intercept)               3.334505   0.204115  16.336  < 2e-16 ***
## treatmentsolvent control -0.764551   0.178007  -4.295 1.75e-05 ***
## treatmentDEP 0.5g/l       0.002616   0.199231   0.013     0.99
## treatmentDEP 1g/l         0.958972   0.230520   4.160 3.18e-05 ***
## treatmentDEP 2g/l         2.002963   0.269967   7.419 1.18e-13 ***
## treatmentDimethoate      -1.268922   0.171770  -7.387 1.50e-13 ***
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

sim_my_glmm_con<-simulateResiduals(my_glmm_con)

plot(sim_my_glmm_con)
```



Supplemental Figure 23*:* DHARMa plots of the residuals of the consumption data glmm after chronic exposure

```
Anova(my_glmm_con, Test="F")
```

```
## Analysis of Deviance Table (Type II Wald chisquare tests)
##
## Response: consumption_mean
##           Chisq Df Pr(>Chisq)
## treatment 273.1  5  < 2.2e-16 ***
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
```

```
mult.consumption_random <- glht(my_glmm_con, linfct = mcp(treatment = "Tukey"))
summary(mult.consumption_random,test=adjusted("holm"))
```

```
##
##   Simultaneous Tests for General Linear Hypotheses
##
## Multiple Comparisons of Means: Tukey Contrasts
##
##
## Fit: glmmTMB(formula = consumption_mean ~ treatment + (1 | colony),
##     data = chronic, family = "Gamma", ziformula = ~0, dispformula = ~1)
##
## Linear Hypotheses:
##                                      Estimate Std. Error z value Pr(>|z|)
## solvent control - control == 0    -0.764551   0.178007  -4.295 0.000105 ***
## DEP 0.5g/l - control == 0          0.002616   0.199231   0.013 0.989523


## DEP 1g/l - control == 0            0.958972   0.230520   4.160 0.000154 ***
## DEP 2g/l - control == 0            2.002963   0.269967   7.419 1.06e-12 ***
## Dimethoate - control == 0         -1.268922   0.171770  -7.387 1.20e-12 ***
## DEP 0.5g/l - solvent control == 0  0.767167   0.176600   4.344 9.79e-05 ***
## DEP 1g/l - solvent control == 0    1.723523   0.211328   8.156 5.33e-15 ***
## DEP 2g/l - solvent control == 0    2.767514   0.253840  10.903  < 2e-16 ***
## Dimethoate - solvent control == 0 -0.504371   0.144845  -3.482 0.001098 **
## DEP 1g/l - DEP 0.5g/l == 0         0.956356   0.229463   4.168 0.000154 ***
## DEP 2g/l - DEP 0.5g/l == 0         2.000347   0.269108   7.433 1.06e-12 ***
## Dimethoate - DEP 0.5g/l == 0      -1.271538   0.170134  -7.474 8.57e-13 ***
## DEP 2g/l - DEP 1g/l == 0           1.043991   0.292976   3.563 0.001098 **
## Dimethoate - DEP 1g/l == 0        -2.227894   0.205959 -10.817  < 2e-16 ***
## Dimethoate - DEP 2g/l == 0        -3.271885   0.249490 -13.114  < 2e-16 ***
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
## (Adjusted p values reported -- holm method)
```

```
chronic_corcon<- chronic_no_outliers[chronic_no_outliers$treatment%in% c("solvent control",
"DEP 0.5g/l","DEP 1g/l","DEP 2g/l"),]

chronic_corcon$treatment<-ordered(chronic_corcon$treatment, levels= c("solvent control","DE
P 0.5g/l","DEP 1g/l","DEP 2g/l"))

chronic_no_outliers$treatment<-ordered(chronic_no_outliers$treatment, levels= c("control","
solvent control","DEP 0.5g/l","DEP 1g/l","DEP 2g/l"))

levels(chronic_corcon$treatment) <- c("0", "0.5", "1","2")

levels(chronic_no_outliers$treatment) <- c("0","0", "0.5", "1","2")

chronic_corcon$treatment<-as.numeric(as.character(chronic_corcon$treatment))

chronic_no_outliers$treatment<-as.numeric(as.character(chronic_no_outliers$treatment))

str(chronic_corcon)
```

```
## 'data.frame':    165 obs. of  17 variables:
##  $ ID              : int  160 147 205 221 79 103 204 222 210 224 ...
##  $ treatment       : num  0.5 0.5 1 1 0 0 1 1 1 ...
```

```
##  $ colony          : Factor w/ 8 levels "37","38","39",..: 7 5 6 8 4 7 6 8 6 8 ...
##  $ consumption1    : num  0.778 0.925 0.897 0.861 0.873 ...
##  $ consumption2    : num  0.587 1.014 0.949 0.748 1.181 ...
##  $ consumption3    : num  -0.394 0.552 1.029 0.578 0.921 ...
##  $ consumption4    : num  0.66 0.426 0.539 0.412 1.154 ...
##  $ consumption5    : num  1.072 0.455 0.421 0.411 0.932 ...
##  $ survival        : num  10 10 10 10 10 10 10 10 10 10 ...
##  $ death           : num  0 0 0 0 0 0 0 0 0 0 ...
##  $ measurement..mm.: num  2.88 3.02 3.32 3.19 2.98 ...
##  $ fat.body..g.    : num  0.00705 0.00606 0.00527 0.00505 0.0047 0.00352 0.00496 0.0048
0.00396 0.00366 ...
##  $ fat.body..mg.   : num  7.05 6.06 5.27 5.05 4.7 3.52 4.96 4.8 3.96 3.66 ...
##  $ rel..Fat.body   : num  2.45 2.01 1.59 1.58 1.58 ...
##  $ max_dose        : num  1.1 1.1 2.2 2.2 0 0 2.2 2.2 2.2 2.2 ...
##  $ consumption_mean: num  0.27 0.337 0.384 0.301 0.506 ...
##  $ dose_ingested   : num  0.297 0.371 0.844 0.662 0 ...
```

```
str(chronic_no_outliers)
```

```
## 'data.frame':    217 obs. of  17 variables:
##  $ ID              : int  54 160 55 147 7 205 221 79 103 5 ...
##  $ treatment       : num  0 0.5 0 0.5 0 1 1 0 0 0 ...
##  $ colony          : Factor w/ 8 levels "37","38","39",..: 8 7 8 5 1 6 8 4 7 1 ...
##  $ consumption1    : num  0.958 0.778 0.67 0.925 0.807 ...
##  $ consumption2    : num  1.11 0.587 0.911 1.014 0.729 ...
##  $ consumption3    : num  0.567 -0.394 0.898 0.552 1.307 ...
##  $ consumption4    : num  0.827 0.66 0.75 0.426 0.887 ...
##  $ consumption5    : num  0.558 1.072 0.684 0.455 0.648 ...
##  $ survival        : num  10 10 10 10 10 10 10 10 10 10 ...
##  $ death           : num  0 0 0 0 0 0 0 0 0 0 ...
##  $ measurement..mm.: num  3.03 2.88 2.99 3.02 2.98 ...
##  $ fat.body..g.    : num  0.00873 0.00705 0.00715 0.00606 0.00478 0.00527 0.00505 0.0047
 0.00352 0.00466 ...
##  $ fat.body..mg.   : num  8.73 7.05 7.15 6.06 4.78 5.27 5.05 4.7 3.52 4.66 ...
##  $ rel..Fat.body   : num  2.88 2.45 2.39 2.01 1.6 ...
##  $ max_dose        : num  0 1.1 0 1.1 0 2.2 2.2 0 0 0 ...
##  $ consumption_mean: num  0.402 0.27 0.391 0.337 0.438 ...
##  $ dose_ingested   : num  0 0.297 0 0.371 0 ...
```

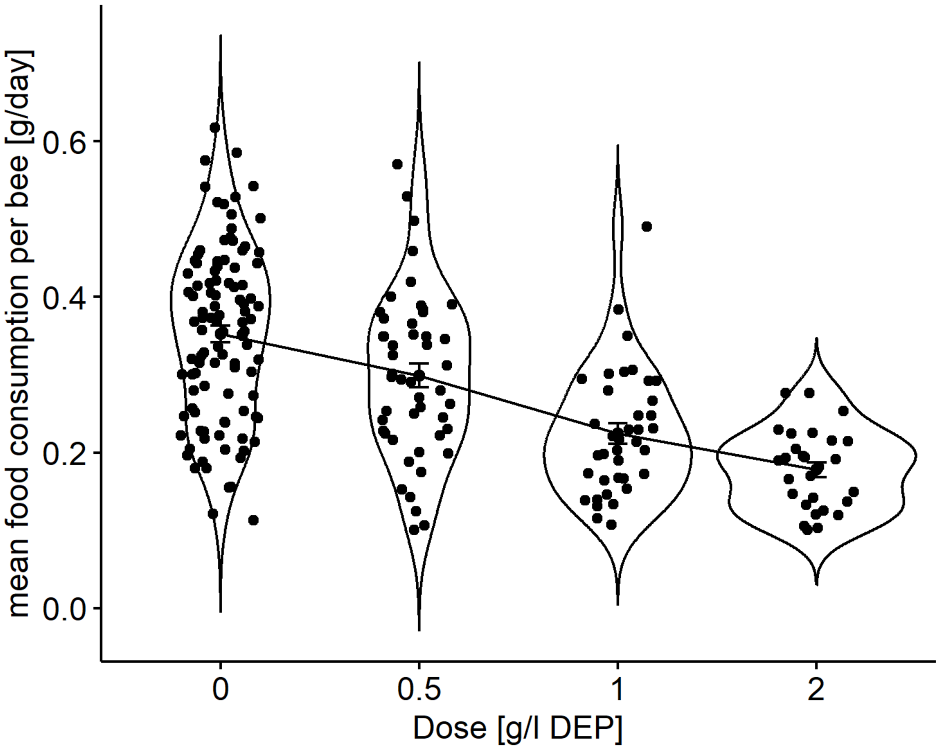
```
corcon <- cor.test(chronic_corcon$treatment, chronic_corcon$consumption_mean,
                    method = "pearson")
corcon
```

```
##
##  Pearson's product-moment correlation
##
## data:  chronic_corcon$treatment and chronic_corcon$consumption_mean
## t = -10.172, df = 163, p-value < 2.2e-16
## alternative hypothesis: true correlation is not equal to 0
## 95 percent confidence interval:
##  -0.7084793 -0.5198582
## sample estimates:
##        cor
## -0.6231478
```

```
corcon1<- cor.test(chronic_no_outliers$treatment, chronic_no_outliers$consumption_mean,
                    method = "pearson")

corcon1
```

```
##
##  Pearson's product-moment correlation
##
## data:  chronic_no_outliers$treatment and chronic_no_outliers$consumption_mean
## t = -9.3492, df = 215, p-value < 2.2e-16
## alternative hypothesis: true correlation is not equal to 0
## 95 percent confidence interval:
```

```
##  -0.6259859 -0.4356323
## sample estimates:
##        cor
## -0.5376241

ggline(chronic_no_outliers, x = "treatment", y = "consumption_mean",
       add = c("mean_se", "jitter", "violin"),
       xlab = "Dose [g/l DEP]", ylab = "mean food consumption per bee [g/day]",conf.int = T
RUE,
          cor.coef = TRUE, cor.method = "pearson")
```



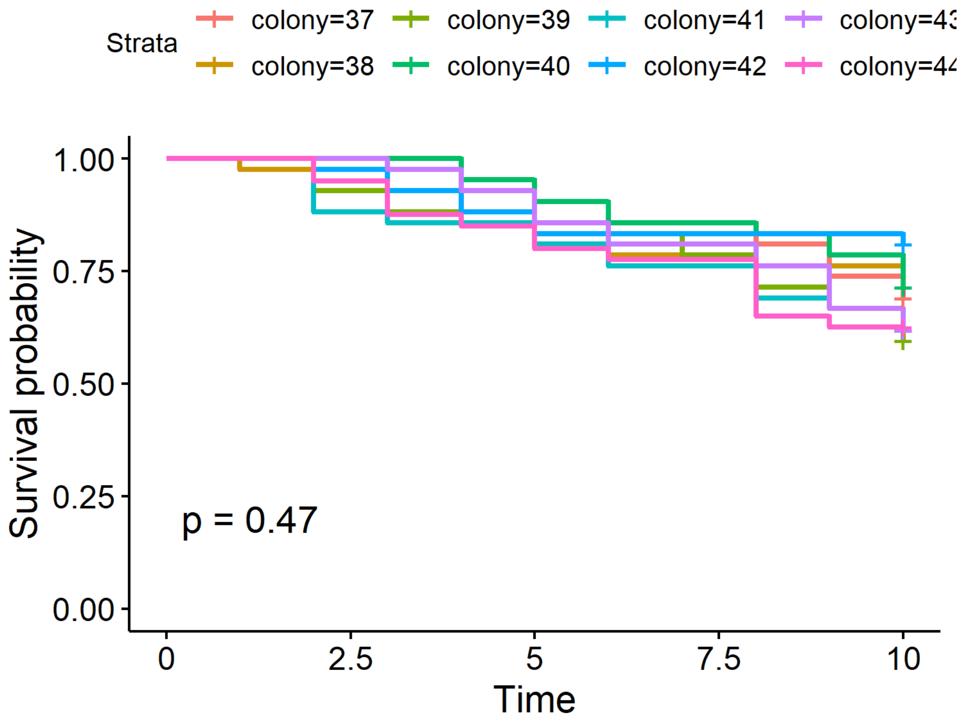
Supplemental Figure 14: Effect of the dose of DEP on the food consumption per bumblebee per day. The graph shows a significant negative correlation between the two variables. The black point with whiskers represents the mean with standard error for every dose respectively. For the dose 0 g/l we pooled the food consumption values from the control and solvent control individuals.

Good model fit according to DHARMa. Consumption rates: dimethoate > solvent control > control = soot 0.5 > soot 1 > soot 2

Colony effects

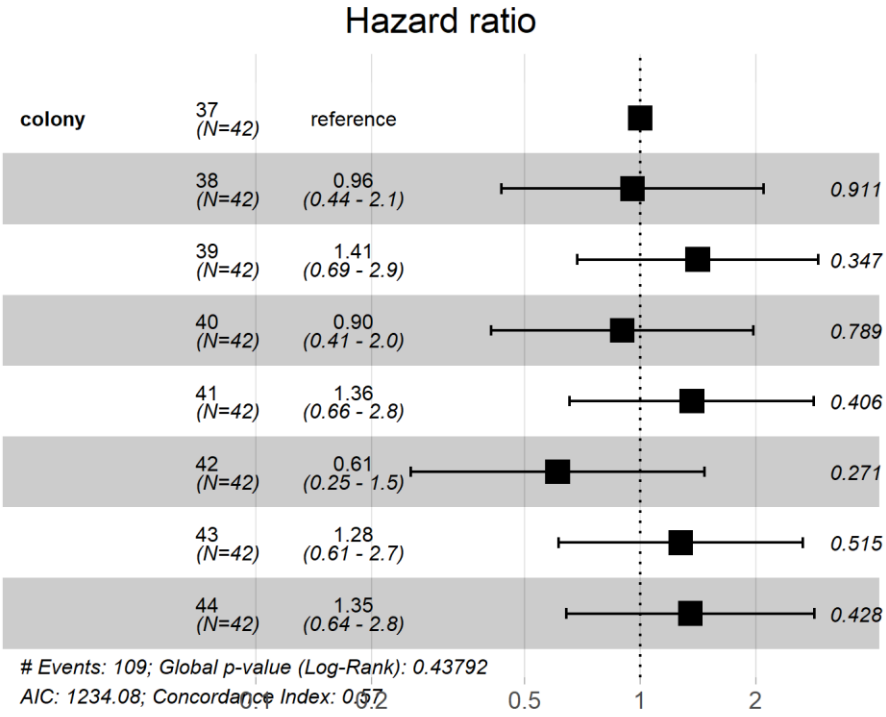
Survival

```
fit_col <- survfit(Surv(survival,death) ~ colony, data = chronic)
ggsurvplot(fit_col, data = chronic, pval = TRUE)
```



Supplemental Figure 15: Survival probability across the 10 day period for the used bumblebee colonies. The p-value indicates significant differences between the Kaplan-Meier curves of the different treatment.

```
fit_colcox <- coxph( Surv(survival,death) ~ colony, data = chronic)
ggforest(fit_colcox)

## Warning in .get_data(model, data = data): The `data` argument is not provided.
## Data will be extracted from model fit.
```

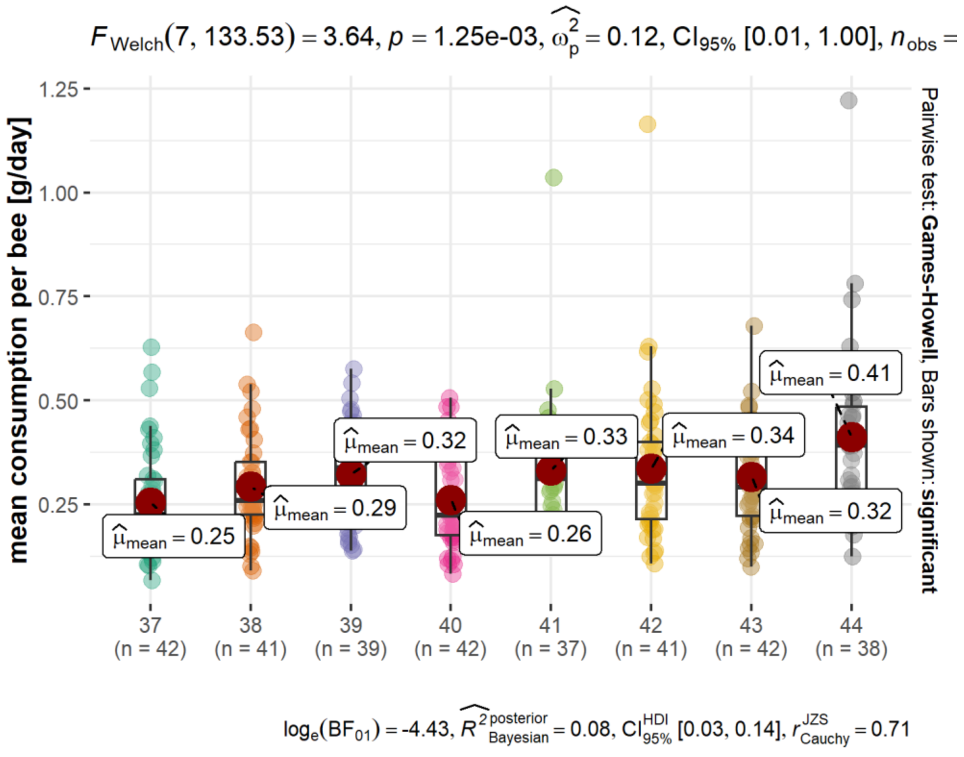


Supplemental Figure 16: Forest plot of hazard ratio from multivariable Cox proportional hazard regression model on survival of the used colonies. Bars represent 95% confidence intervals.The p-values with asterisks indicate significantly elevated mortality risk compared to control organisms.

```
anova(fit_colcox)

## Analysis of Deviance Table
##  Cox model: response is Surv(survival, death)
## Terms added sequentially (first to last)
##
##         loglik  Chisq Df Pr(>|Chi|)
## NULL   -613.50
## colony -610.04 6.9137  7     0.4379
```

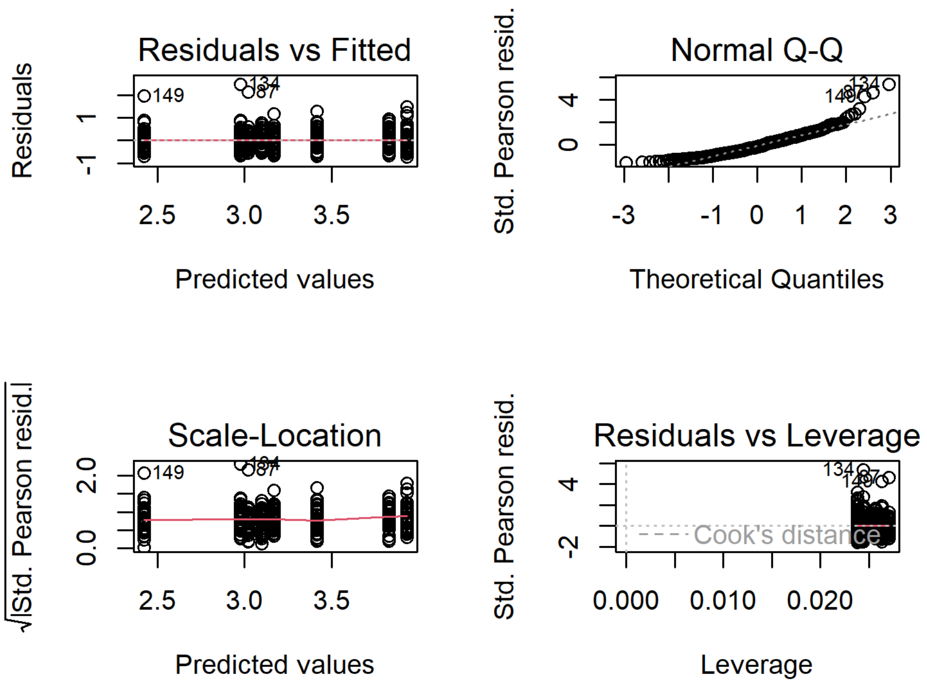
No effect of colony on survival.

Sugar water consumption



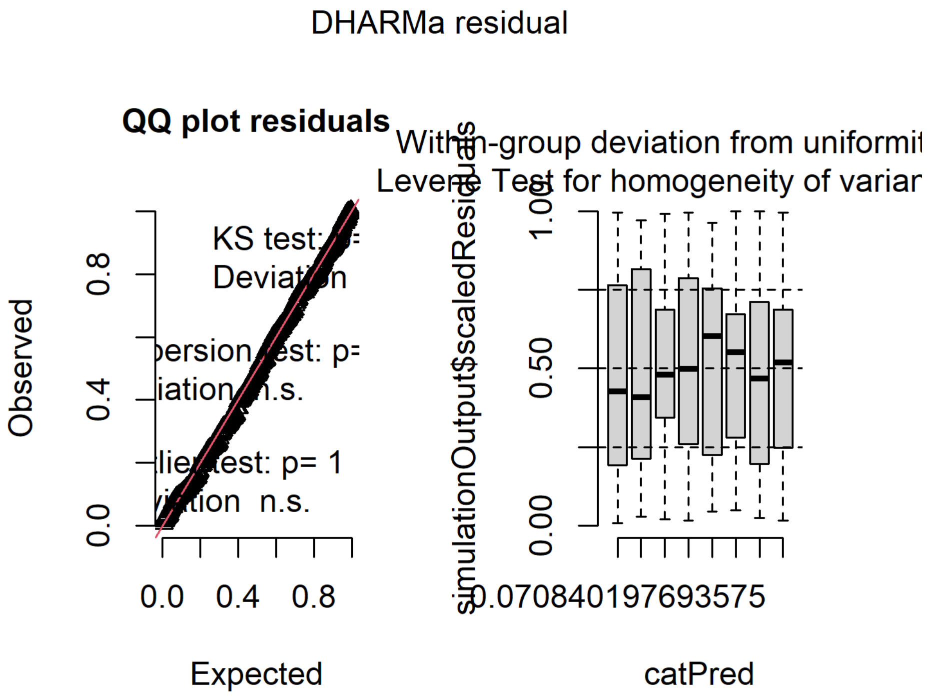
Supplemental Figure 17: Effect of colony on the daily sugar water consumption of the bumblebees after the 10- day period. Number of replicates per treatment are represented by n. The values in the boxes represent the mean relative fat body weight of the treatment. Letters indicate significance between treatments.

```
my_glm <- glm(consumption_mean ~ colony, data= chronic, family="Gamma")
par(mfrow=c(2,2))
plot(my_glm)
```



Supplemental Figure 18: plot of the residuals of the glm of the colony data from the chronic exposure experiment

```
summary(my_glm)

##
## Call:
## glm(formula = consumption_mean ~ colony, family = "Gamma", data = chronic)
##
## Deviance Residuals:
##      Min        1Q    Median        3Q       Max
## -1.08733  -0.36981  -0.08418   0.23746   1.56541
##
## Coefficients:
##             Estimate Std. Error t value Pr(>|t|)
## (Intercept)   3.9287     0.2816  13.950  < 2e-16 ***
## colony38     -0.5142     0.3751  -1.371  0.17141
## colony39     -0.8317     0.3638  -2.286  0.02293 *
## colony40     -0.1008     0.3932  -0.256  0.79780
## colony41     -0.9078     0.3641  -2.494  0.01316 *
## colony42     -0.9521     0.3549  -2.683  0.00768 **
## colony43     -0.7606     0.3618  -2.102  0.03631 *
## colony44     -1.5042     0.3357  -4.481 1.04e-05 ***
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
##
## (Dispersion parameter for Gamma family taken to be 0.2158108)
##
##     Null deviance: 70.323  on 321  degrees of freedom
## Residual deviance: 63.632  on 314  degrees of freedom
##   (14 Beobachtungen als fehlend gelöscht)
## AIC: -395.34
##
## Number of Fisher Scoring iterations: 5

sim_my_glm<-simulateResiduals(my_glm)
plot(sim_my_glm)
```



Supplemental Figure 19: DHARMa plots of the residuals of the colony data glm with Gamma after chronic exposure from the chronic data frame.

```
anova(my_glm,test="F")

## Analysis of Deviance Table
##
## Model: Gamma, link: inverse
##
## Response: consumption_mean
##
## Terms added sequentially (first to last)
##
##
##        Df Deviance Resid. Df Resid. Dev      F    Pr(>F)
## NULL                     321     70.323
## colony  7   6.6909       314     63.632 4.4291 0.0001033 ***
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

mult.consumption <- glht(my_glm, linfct = mcp(colony = "Tukey"))
summary(mult.consumption,test=adjusted("BH"))

##
##   Simultaneous Tests for General Linear Hypotheses
##
## Multiple Comparisons of Means: Tukey Contrasts
##
##
## Fit: glm(formula = consumption_mean ~ colony, family = "Gamma", data = chronic)
##
## Linear Hypotheses:
##              Estimate Std. Error z value Pr(>|z|)
## 38 - 37 == 0 -0.51415    0.37507  -1.371 0.298257
## 39 - 37 == 0 -0.83169    0.36385  -2.286 0.068274 .
## 40 - 37 == 0 -0.10082    0.39319  -0.256 0.856665
```

```
## 41 - 37 == 0 -0.90780    0.36406  -2.494 0.059015 .
## 42 - 37 == 0 -0.95214    0.35488  -2.683 0.051081 .
## 43 - 37 == 0 -0.76060    0.36177  -2.102 0.090404 .
## 44 - 37 == 0 -1.50420    0.33570  -4.481 0.000208 ***
## 39 - 38 == 0 -0.31754    0.33830  -0.939 0.487084
## 40 - 38 == 0  0.41333    0.36968   1.118 0.388356
## 41 - 38 == 0 -0.39365    0.33852  -1.163 0.380941
## 42 - 38 == 0 -0.43799    0.32864  -1.333 0.300785
## 43 - 38 == 0 -0.24645    0.33607  -0.733 0.617803
## 44 - 38 == 0 -0.99005    0.30782  -3.216 0.012119 *
## 40 - 39 == 0  0.73087    0.35828   2.040 0.091999 .
## 41 - 39 == 0 -0.07611    0.32604  -0.233 0.856665
## 42 - 39 == 0 -0.12045    0.31577  -0.381 0.820011
## 43 - 39 == 0  0.07109    0.32349   0.220 0.856665
## 44 - 39 == 0 -0.67251    0.29404  -2.287 0.068274 .
## 41 - 40 == 0 -0.80698    0.35849  -2.251 0.068274 .
## 42 - 40 == 0 -0.85132    0.34918  -2.438 0.059063 .
## 43 - 40 == 0 -0.65978    0.35618  -1.852 0.119408
## 44 - 40 == 0 -1.40338    0.32966  -4.257 0.000290 ***
## 42 - 41 == 0 -0.04434    0.31601  -0.140 0.888417
## 43 - 41 == 0  0.14720    0.32373   0.455 0.790485
## 44 - 41 == 0 -0.59639    0.29430  -2.026 0.091999 .
## 43 - 42 == 0  0.19154    0.31338   0.611 0.688633
## 44 - 42 == 0 -0.55206    0.28287  -1.952 0.101973
## 44 - 43 == 0 -0.74359    0.29147  -2.551 0.059015 .
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
## (Adjusted p values reported -- BH method)
```

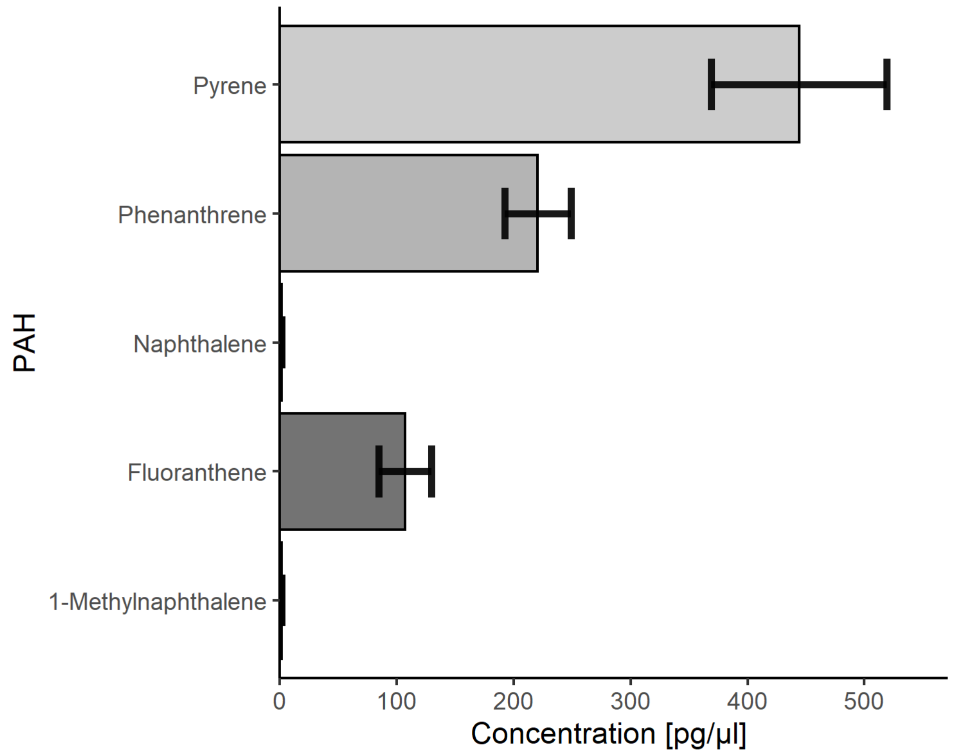
Good model fit according to DHARMa.

Colony 44 consumed approximately 40, 32 and 37 % than colonies 37, 38 and 40 respectively. The rest does not differ significantly.

```
setwd("C:/Users/Frederic/Desktop/Phd/GC-MS_Russ")

PAK<- read.csv2("PAK2.csv")
str(PAK)

## 'data.frame':    15 obs. of  2 variables:
##  $ PAH          : chr  "Naphthalene" "Naphthalene" "Naphthalene" "1-Methylnaphthalene" .
..
##  $ Concentration: chr  "1.356701127" "1.573515706" "1.613229267" "1.469663946" ...

PAK$PAH<-as.factor(PAK$PAH)
PAK$Concentration<-as.numeric(PAK$Concentration)


my_sum <- PAK %>%
  group_by(PAH) %>%
  summarise(
    n=n(),
    mean=mean(Concentration),
    sd=sd(Concentration)
  ) %>%
  mutate( se=sd/sqrt(n))  %>%
  mutate( ic=se * qt((1-0.05)/2 + .5, n-1))

p2<- ggplot(my_sum,aes(fill=PAH, y=PAH, x=mean))+
  geom_bar(position="dodge", stat="identity",color="black")+
  geom_errorbar(aes(y=PAH, xmin=mean-se, xmax=mean+se),width=0.4, colour="black", alpha=0.9
, size=1.3)+
  theme_classic()+
  xlab("Concentration [pg/µl] ")+
  scale_x_continuous(expand = expansion(mult = c(0, 0.1))) +
  scale_y_discrete(name = "PAH")+
```

```
  theme(panel.grid.major.y = element_blank(),
        legend.position = "off")

## Warning: Using `size` aesthetic for lines was deprecated in ggplot2 3.4.0.
## ℹ Please use `linewidth` instead.

p2+ scale_fill_grey()
```

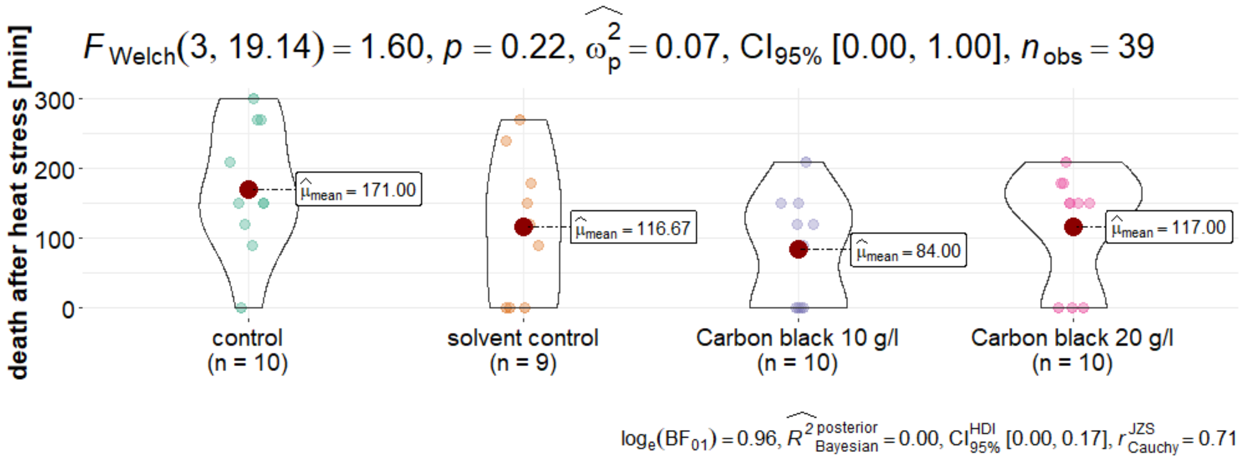
PAH analysis



Supplemental Figure 20*:* Concentration of the analysed PAHs found in the DEP sample. Error bars show the standard error. A total of six replicates were analysed via GC-MS. Naphthalene and 1-methylnaphthalene were below the limit of detection.

Particle control 72 hours with heat stress

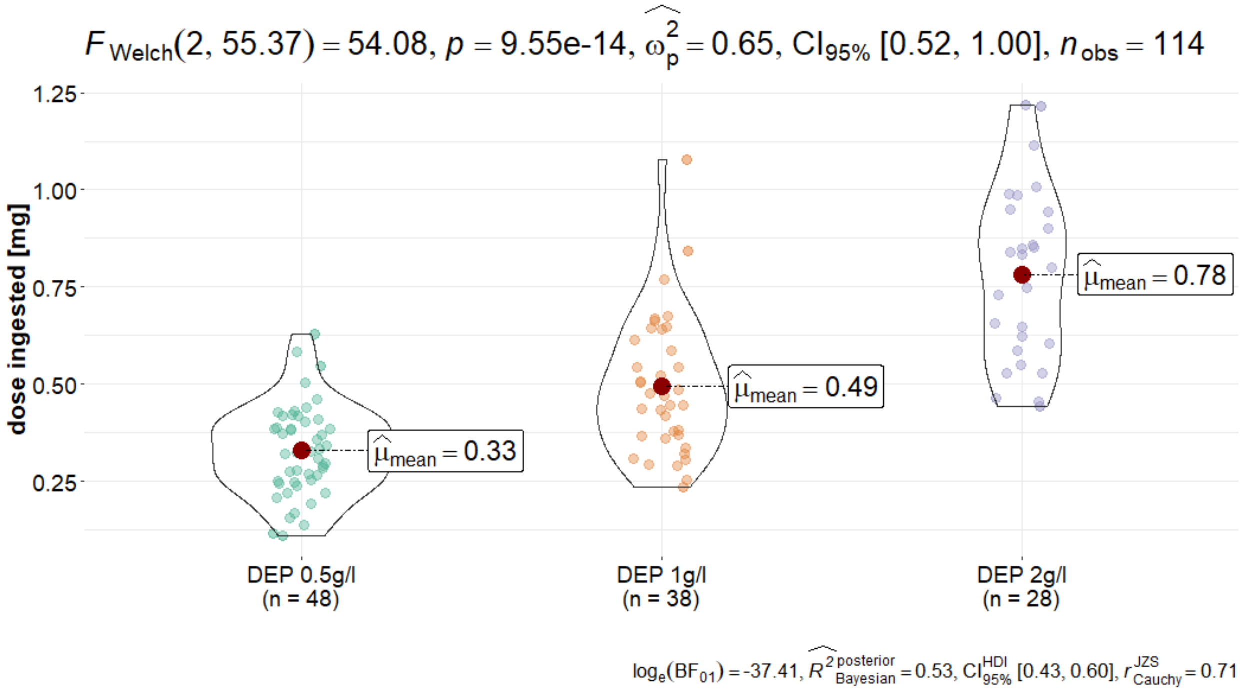
There was no significant toxic effect of 10 and 20 g/l carbon black particles after 72 hours. This is why we exposed the bumblebees to heat stress (40°C) in a heating oven, to investigate possible increased susceptibility to additional stressors. We could not find significant differences in survival times.



Supplemental Figure 21: Survivalof the bumblebees in minutes after being exposed to heat stresss and antecedent 72 hour period of oral exposure to the control, solvent control, 10 g/l carbon black and 20 g/l carbon black.

Ingested doses in chronic exposure experiment

To visualize that the dose ingested, is constantly rising despite lower consumption rate, we made a boxplot showing the ingested doses per treatment.



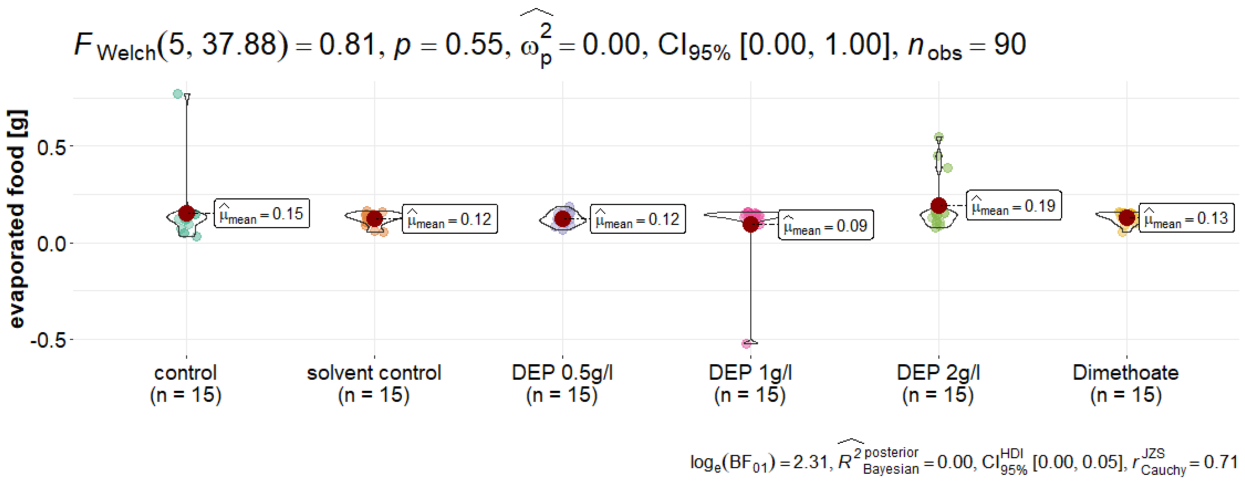
Supplemental Figure 22: Mean daily dose of DEP ingested per bumblebee over the 10 day exposure period.

Evaporation comparison of exposure solutions/suspension

```
evap<-read.csv2("evaporation.csv")


evap$Treatment<-as.factor(evap$Treatment)

evap$Treatment <- factor(evap$Treatment, levels = c("control","solvent control", "DEP 0.5g/
l","DEP 1g/l", "DEP 2g/l","Dimethoate"))
evap$ID<-as.factor(evap$ID)
evap$Day.of.measurment<-as.factor(evap$Day.of.measurment)


str(evap)

## 'data.frame':    90 obs. of  9 variables:
##  $ ID               : Factor w/ 18 levels "Dimethoate 1 ",..: 4 5 6 16 17 18 7 8 9 10 ..
.
##  $ Treatment        : Factor w/ 6 levels "control","solvent control",..: 1 1 1 2 2 2 3 3
 3 4 ...
##  $ Day.of.measurment: Factor w/ 5 levels "Day 10","Day 2",..: 2 2 2 2 2 2 2 2 2 2 ...
##  $ Evaporated       : num  0.145 0.122 0.147 0.139 0.148 0.139 0.115 0.164 0.155 0.139 .
..
##  $ X                : logi  NA NA NA NA NA NA ...
##  $ X.1              : logi  NA NA NA NA NA NA ...
##  $ X.2              : logi  NA NA NA NA NA NA ...
##  $ X.3              : logi  NA NA NA NA NA NA ...
##  $ X.4              : logi  NA NA NA NA NA NA ...
```

```
my_glm <- glm(Evaporated~Treatment, data = evap)
summary(my_glm)

##
## Call:
## glm(formula = Evaporated ~ Treatment, data = evap)
##
## Deviance Residuals:
##      Min        1Q    Median        3Q       Max
## -0.61573  -0.03737  -0.00443   0.02732   0.61813
##
## Coefficients:
##                         Estimate Std. Error t value Pr(>|t|)
## (Intercept)              0.15187    0.03056   4.969  3.5e-06 ***
## Treatmentsolvent control -0.02787    0.04322  -0.645    0.521
## TreatmentDEP 0.5g/l      -0.02773    0.04322  -0.642    0.523
## TreatmentDEP 1g/l        -0.05813    0.04322  -1.345    0.182
## TreatmentDEP 2g/l         0.04140    0.04322   0.958    0.341
## TreatmentDimethoate      -0.02233    0.04322  -0.517    0.607
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
##
## (Dispersion parameter for gaussian family taken to be 0.0140123)
##
##     Null deviance: 1.2617  on 89  degrees of freedom
## Residual deviance: 1.1770  on 84  degrees of freedom
## AIC: -120.9
##
## Number of Fisher Scoring iterations: 2

Anova(my_glm, Test="F")

## Analysis of Deviance Table (Type II tests)
##
## Response: Evaporated
##           LR Chisq Df Pr(>Chisq)
## Treatment   6.0421  5     0.3021
```

plot of evaporation control



Supplemental Figure 23: Mean evaporation over the period of two days over all treatments.

Article 2

Diesel exhaust particles alter gut microbiome and gene expression in the bumblebee *Bombus terrestris*

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RESEARCH ARTICLE

Ecology and Evolution Open Access WILEY

Diesel exhaust particles alter gut microbiome and gene expression in the bumblebee *Bombus terrestris*

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Abstract

Insect decline is a major threat to ecosystems around the world as they provide many important functions, such as pollination or pest control. Pollution is one of the main reasons for the decline, alongside changes in land use, global warming, and invasive species. While negative impacts of pesticides are well-studied, there is still a lack of knowledge about the effects of other anthropogenic pollutants, such as airborne particulate matter, on insects. To address this, we exposed workers of the bumblebee *Bombus terrestris* to sublethal doses of diesel exhaust particles (DEPs) and brake dust, orally or via air. After 7 days, we looked at the composition of the gut microbiome and tracked changes in gene expression. While there were no changes in the other treatments, oral DEP exposure significantly altered the structure of the gut microbiome. In particular, the core bacterium *Snodgrassella* had a decreased abundance in the DEP treatment. Similarly, transcriptome analysis revealed changes in gene expression after oral DEP exposure, but not in the other treatments. The changes are related to metabolism and signal transduction, which indicates a general stress response. Taken together, our results suggest potential health effects of DEP exposure on insects, here shown in bumblebees, as gut dysbiosis may increase the susceptibility of bumblebees to pathogens, while a general stress response may lower available energy resources. Those effects may exacerbate under natural conditions where insects face a multiple-stressor environment.

KEYWORDS
air pollution, brake dust, insect decline, particulate matter, pollinator, transcriptome

TAXONOMY CLASSIFICATION
Ecotoxicology, Entomology, Genomics, Microbiomics

1 | INTRODUCTION

Global biodiversity loss is one of the major challenges humanity currently faces (Díaz et al., 2006; Dirzo et al., 2014). Especially the rapid decline in insects is cause for concern, as they provide or contribute to many important ecosystem functions such as pollination, nutrient cycling, pest control, and linking trophic levels (Cardoso et al., 2020; Noriega et al., 2018). Pollution is one of the major reasons for the decline alongside intensification of land use, climate change, and invasive species (Miličić et al., 2021; Sánchez-Bayo & Wyckhuys, 2019).

Pesticides harm insects on many different levels ranging from subtle changes in the gut microbiome over behavioral changes to increased mortality (Desneux et al., 2007; Motta et al., 2018; Ndakidemi et al., 2016). Other anthropogenic pollutants might also contribute to the observed declines in insects, but their impacts are often less well-studied (Cameron & Sadd, 2020; Feldhaar & Otti, 2020; Sánchez-Bayo & Wyckhuys, 2019). Airborne particulate matter deriving from traffic or industrial processes has become ubiquitous in the environment (Gieré & Querol, 2010; Zereini & Wiseman, 2010). While the harmful effects on mammals, in particular humans, have been intensively studied, research investigating the impact on insects remains scarce (Kim et al., 2015; Valavanidis et al., 2008). Insects can encounter these pollutants in various ways, for example, by foraging in contaminated areas, consuming contaminated food, or direct deposition on the insect's cuticle (Feldhaar & Otti, 2020; Łukowski et al., 2018; Negri et al., 2015). The airborne particulate matter might enter an insect's body via oral ingestion or the tracheal system (Feldhaar & Otti, 2020; Negri et al., 2015). Social insects might be at an increased risk, as pollutants are transferred to and stored in their nests, which could lead to a higher exposure to conspecifics and the brood (Feldhaar & Otti, 2020; Hladun et al., 2016).

Vehicle brake dust and diesel exhaust particles (DEPs) are major classes of airborne particulate matter deriving from traffic released into the environment (Hamilton & Hartnett, 2013; Harrison et al., 2012; Rönkkö & Timonen, 2019). Brake dust particles contain various metals and phenolic compounds, depending on the brake lining used (Iijima et al., 2007; Thorpe & Harrison, 2008). Exposure of different invertebrate species to such particles showed mixed effects. Particulate matter contamination in soil did not affect colony founding in the ant *Lasius niger* (Seidenath et al., 2021). However, soil-feeding earthworms (*Eisenia fetida*) showed a strongly increased mortality when exposed to soil spiked with brake dust particles (Holzinger et al., 2022). DEPs have a different composition than brake dust. They are composed of an elemental carbon core with adsorbed organic compounds, such as polycyclic aromatic hydrocarbons (PAHs), and traces of metals and other elements (Greim, 2019; Wichmann, 2007). Exposure to high doses of diesel exhaust particles (1 and 2 g/L) in food over a period of 7 days reduced survival in *Bombus terrestris* workers compared to controls by nearly 50 percent (Hüftlein et al., 2023).

Many classical ecotoxicology approaches focus on the effect of a substance on mortality, growth, or reproduction. However, pollutants can also have more subtle sublethal effects on insects, which may have severe consequences in the long term (Straub et al., 2020). Direct sublethal effects include changes in physiology such as stress reactions or detoxification processes. By interacting with microorganisms inside the insect's body, oral exposure to pollutants may indirectly affect insect health.

Most eukaryotic organisms and their associated microbes form an entity, the so-called holobiont (Theis et al., 2016; Zilber-Rosenberg & Rosenberg, 2008). In insects, microorganisms can be found in the digestive tract, the exoskeleton, the hemocoel, or within cells (Douglas, 2015). The insect gut microbiome has a range of functions that include protection from pathogens, detoxification, digestion, and the production of essential nutrients (Engel & Moran, 2013). Social bumblebees (*Bombus* spp.) and honeybees (*Apis mellifera*) are model organisms to study gut microbiota as their gut microbiome is rather simple and highly conserved (Engel et al., 2016; Kwong & Moran, 2016; Zhang & Zheng, 2022). A few core bacterial taxa dominate the gut microbiome of bumblebees: *Snodgrassella, Gilliamella, Schmidhempelia*, Bifidobacteriaceae (*Bifidobacterium and Bombiscardovia*), and two clusters within Lactobacillaceae (Hammer et al., 2021; Koch & Schmid-Hempel, 2011a; Martinson et al., 2011). While many functions of the bacterial symbionts in bumblebees have been proposed, only very few have been demonstrated in experiments (Hammer et al., 2021; Zhang & Zheng, 2022). The gut microbiome of bumblebees may be important for detoxification as microbiota-free individuals had lower survival when exposed to toxic concentrations of selenate (Rothman et al., 2019). Moreover, resistance to the common trypanosomatid parasite *Crithidia bombi* is higher in bumblebees with an intact microbiome compared to microbiota-free individuals (Koch & Schmid-Hempel, 2011b). When infected with *C. bombi* the outcome varies with host microbiota composition rather than genotype (Koch & Schmid-Hempel, 2012).

Examining the effects of anthropogenic pollutants, such as airborne particulate matter, on the gut microbiome is an important tool for assessing their risk for insect health (Duperron et al., 2020). Even with a conserved gut microbiome, the relative abundance of core bacteria and the presence of other microorganisms will vary with age, diet, and changing environmental parameters (Koch et al., 2012; Kwong & Moran, 2016). Different pollutants affect the microbial composition of bee guts. In honeybee workers, pesticides or antibiotics change the relative and absolute abundance of core gut microbiota species (DeGrandi-Hoffman et al., 2017; Motta et al., 2018; Raymann et al., 2017). An array of environmental toxicants, such as cadmium, copper, selenate, and hydrogen peroxide, alter the gut microbiome of *Bombus impatiens* at field-realistic concentrations (Rothman et al., 2020). These shifts in the microbial community may affect bumblebee health. Intestinal dysbiosis, compositional and functional alteration of the microbiome, is associated with various diseases and health problems in humans and vertebrates (DeGruttola et al., 2016; Levy et al., 2017; Shreiner et al., 2015). In insects, dysbiosis negatively affects reproductive fitness, immunity, and resistance to pathogens (Ami et al., 2010; Daisley et al., 2020; Raymann et al., 2017).

Transcriptome analysis is a sensitive tool to characterize sublethal effects of potentially harmful substances on a molecular and cellular level (Prat & Degli-Esposti, 2019; Schirmer et al., 2010).

Changes in gene expression help to identify biological processes, such as stress responses and detoxification processes, at an early stage. Exposure to different pollutants have been shown to induce changes in gene expression in several insect species. Mosquitos (*Aedes aegypti*) exposed to anthropogenic pollutants (insecticides, PAHs) increased the expression of genes related to detoxification, respiration, and cuticular proteins (David et al., 2010). Fireflies (*Luciola leii*) showed a similar response when exposed to benzo(a) pyrene, a widespread PAH (Zhang et al., 2019). In different bee species, the neonicotinoids imidacloprid, thiamethoxan, and clothianidin induce an upregulation of metabolic, immune, and stress response genes (Aufauvre et al., 2014; Bebane et al., 2019; Christen et al., 2018; Colgan et al., 2019; Gao et al., 2020; Shi et al., 2017). The expression of genes related to detoxification was higher in honeybees (*A. mellifera*) exposed to heavy metals than in controls (Al Naggar et al., 2020; Gizaw et al., 2020; Zhang et al., 2018).

In contrast to pesticides, the effects of other environmental pollutants, such as particulate matter, on gene expression in bees as well as their gut microbiome are largely unclear. To address this knowledge gap, we exposed workers of the buff-tailed bumblebee *Bombus terrestris* to airborne particulate matter deriving from traffic and investigated changes in the gut microbiome and gene expression. Bumblebees were fed sugar water spiked with sublethal concentrations of brake dust or diesel exhaust particles (DEPs). Adding to this oral exposure, one group of bumblebees was exposed to DEPs via air to enable potential uptake in the tracheal system. We expect changes in the composition of the gut microbial community, as previous research showed changes due to different metals in a closely related *Bombus* species (Rothman et al., 2020). Moreover, we expect changes in the expression of detoxification and metabolic genes, indicating an increased stress level, as the toxic compounds in the particulate matter may interfere with bumblebee physiology.

2 | METHODS

2.1 | Bumblebee keeping

Four queenright colonies of *B. terrestris* were ordered from Biobest (Westerlo, Belgium) in March 2021. Colonies were kept in a climate chamber at 26°C and 70% humidity under a constant, inverted 12:12 h light: dark cycle. Colonies were provided with sugar water (50% Apiinvert, Südzucker AG, Mannheim, Germany) and pollen (Imkerpur, Osnabrück, Germany) ad libitum.

2.2 | Dose selection

The data on airborne particulate matter in terrestrial environments is sparse as it is difficult to quantify and identify the origin. Evidence for high levels of input of airborne particulate matter are often revealed only after it has settled, for example, by analyzing soil samples. Unnaturally high amounts of specific metals could be attributed to external resources such as brake dust (Alsbou & Al-Khashman, 2018; Peikertova & Filip, 2016). Isotopic analyses of urban soils in Arizona revealed up to 0.54% (w/w) as soot carbon black presumably produced by burning fossil fuels (Hamilton & Hartnett, 2013). While bees are contaminated by airborne particulate matter in the wild, we have no data or modeling on the uptake of these particles (Negri et al., 2015). In previous experiments, chronic oral DEP exposure over 7 days reduced survival of bumblebees when exposed to concentrations of 1 g/L and more (Hüftlein et al., 2023). Oral exposure to brake dust particles reduced survival after 7 days for a concentration of 8 g/L (F. Hüftlein, D. Seidenath, A. Mittereder, T. Hillenbrand, D. Brüggemann, O. Otti, H. Feldhaar, C. Laforsch, M. Schott, unpublished data). For our microbiome and transcriptome experiment we selected sublethal doses of 0.4 g/L that did not affect mortality or fat body weight in previous experiments (F. Hüftlein, D. Seidenath, A. Mittereder, T. Hillenbrand, D. Brüggemann, O. Otti, H. Feldhaar, C. Laforsch, M. Schott, unpublished data). For the flight treatment boxes were contaminated with 1.5 mg of DEP and subsequently single workers released into the boxes. DEP was dispersed by the flight movements of the workers and at this concentration we observed a substantial contamination of the bumblebees on their cuticle in this setup (see below).

2.3 | Experimental procedure

At the beginning of the experiment, adult workers from the four colonies were randomly assigned to one of six treatments. Control: fed with sugar water only (50% Apiinvert) ($n = 56$); Solvent control: fed with sugar water spiked with 0.02% (v/v) of the emulsifier Tween20 ($n = 56$); Brake dust: fed with sugar water spiked with 0.02% (v/v) of the emulsifier Tween20 and 0.4 g/L brake dust particles ($n = 56$); DEP: fed with sugar water spiked with 0.02% (v/v) of the emulsifier Tween20 and 0.4 g/L diesel exhaust particles ($n = 56$); Flight control: fed with sugar water (50% Apiinvert) and allowed to fly once per day in a plastic box (7 × 7 × 5 cm, EMSA, Emsdetten, Germany) for 3 min ($n = 24$); DEP flight: fed with sugar water (50% Apiinvert) and allowed to fly once per day for 3 min in a plastic box (7 × 7 × 5 cm, EMSA, Emsdetten, Germany) that contained 1.5 (±0.1) mg of diesel exhaust particles ($n = 24$).

The experiment was conducted in a climate chamber at 26°C and 70% humidity under a constant 12:12 h light: dark cycle. Bumblebees were kept in Nicot cages (Nicotplast SAS, Maisod, France) connected to a 12 mL syringe (B. Braun SE, Melsungen, Germany) with the tip cut off, that contained 2 mL of the respective feeding solution (ad libitum). Every day the syringes were replaced with fresh ones to prevent molding or bacterial growth in the food. The exposure lasted for 7 days. At the end of the experiment, the animals were frozen at −20°C.

Within a week after the end of the experiment, we randomly selected twelve (three workers per colony) bumblebees per treatment for transcriptome analysis ($N = 72$). Additionally, for the control, solvent control, brake dust, and DEP treatment, we randomly selected 20 bumblebees (five workers per colony) for microbiome analysis ($N = 80$), respectively.

2.4 | Generation and collection of diesel exhaust particles (DEPs)

Diesel exhaust particles were collected from a four-cylinder diesel engine (OM 651, Daimler AG, Stuttgart, Germany) during a repeating cycle of transient and stationary operating points, resembling an inner-city driving scenario with stop-and-go intervals. The engine was operated on a test bench with a water-cooled eddy-current brake as previously described in Zöllner (2019). DEP samples were collected by an electrostatic precipitator (OekoTube Inside, Mels-Plons, Switzerland). A fast response differential mobility particulate spectrometer DMS500 (Combustion, Cambridge, England) was applied to measure submicron particle size distributions of raw exhaust samples. Depending on engine load and speed during the inner-city cycle, solid particles showed a median diameter between 52.1 ± 1.8 nm and 101.9 ± 1.7 nm. DEP composition was characterized by thermogravimetric analysis (TGA, STA 449 F5 Jupiter, Netzsch-Gerätebau GmbH, Selb, Germany). A fraction of 72.2% ± 1.1% of the DEP mass was attributed to elemental carbon, 23.2% ± 0.9% w/w to organic fractions, and 4.6% ± 0.7% w/w to inorganic matter. Quantification of PAHs revealed concentrations of 444 ppm for pyrene, 220 ppm for phenanthrene, and 107 ppm for fluoranthene.

The elemental composition of the DEP samples was analyzed by inductively coupled plasma optical emission spectrometry (ICP-OES, Optima 7300 DV, PerkinElmer Inc., Waltham, United States of America) and interpreted according to Zöllner (2019). It showed fractions of calcium (1.63% w/w), zinc (0.53% w/w), and phosphorus (0.50% w/w) that can be traced back to diesel fuel and lubrication oil. Copper (1.03% w/w), aluminum (0.02% w/w), and iron (0.02% w/w) can be attributed to abrasion of piston rings, cylinder head, and engine block material, respectively. In addition, small amounts of boron (0.13% w/w), magnesium (0.10% w/w), molybdenum (0.03% w/w), natrium (0.02% w/w), and sulfur (0.17% w/w) were found.

2.5 | Generation of brake dust particles

The brake dust particles provided by the Chair of Ceramic Materials Engineering of the University of Bayreuth are derived from LowMet brake pads (provided by TMD Friction Holdings GmbH, Leverkusen, Germany) that were milled for 3 min in a vibrating cup mill with a tungsten carbide grinding set (Pulverisette 9, Fritsch GmbH, Idar-Oberstein, Germany). LowMet brake pads are common and representative of passenger cars in Europe and consist of nonferrous metals (25% (w/w)), steel wool (15% (w/w)), petrol coke (12% (w/w)), sulfides (10% (w/w)), aluminum oxide (5% (w/w)), resin (5% (w/w)), graphite (4% (w/w)), mica (4% (w/w)), silicon carbide (3% (w/w)), barite (2% (w/w)), fibers (2% (w/w)), and rubber (1% (w/w)) (Wiaterek, 2012). The particle size distribution of the milled, fine-grained powder was measured with a laser diffraction particle size analyzer (PSA 1190 LD, Anton Paar GmbH, Ostfildern-Scharnhausen, Germany). The mean particle size found was 10.19 ± 4.37 μm (D10 = 0.68 μm (10% of all particles being smaller in diameter than this size), D50 = 5.76 μm (median particle size), D90 = 25.87 μm (90% of particles being smaller in diameter than this size)).

2.6 | Bumblebee gut microbiome analysis

Prior to dissection bumblebees were defrosted and rinsed in 70% ethanol, 90% ethanol, and twice in ultrapure water. We placed each bumblebee on an autoclaved square of aluminum foil (5 × 5 cm) and opened the abdomen with sterilized tweezers and scissors. After carefully separating the midgut and hindgut from the crop and transferring it to an Eppendorf tube, we snap-froze the gut in liquid nitrogen. All samples were stored at −80°C until further processing.

2.7 | PCR amplification and sequencing of 16S rDNA fragments

Metagenomic DNA of bumblebee gut samples was purified using the NucleoMag DNA Bacteria kit (Macherey-Nagel, no. 744310, Düren, Germany) after disruption of samples with 1.4 mm (diam.) ceramic beads (no. P000912-LYSK0A, Bertin Instruments, Montigny-le-Bretonneux, France) in a FastPrep-24 bead beating device (MPbio, Irvine, USA) following the instructions of the manufacturer. The metagenomic DNA was diluted to a concentration of 5 ng/μL, and 2.5 μL DNA was used to amplify 16S rDNA fragments using primers 515F-Y (Turner et al., 1999) and 806RB (Apprill et al., 2015) as described in the 16S Metagenomic Sequencing Library Preparation protocol (Part # 15044223 Rev. B, www.illumina.com). Sample libraries were barcoded using the Nextera XT index kit (v2 set A, www.illumina.com), combined in equimolar amounts, and sequenced on Illumina's iSeq-100 platform using a 293-cycle single-end R1 mode. Demultiplexing of reads was performed by the iSeq-100 local run manager and sample-specific reads were saved in FastQ format.

2.8 | Microbiome analysis

Statistical analyses of the microbial data were performed using QIIME2 (Bolyen et al., 2019) and R 4.2.1 (R Core Team, 2022). Forward reads of 16S rDNA fragments (R1 reads) were analyzed using the QIIME2 microbiome analysis package (ver. 2021.11; Bolyen et al., 2019). Unless indicated otherwise, all analysis tools were used as plugins of the QIIME2 package. The respective parameters used along the analysis steps are readily accessible by provenance information in the QIIME2 data files (available as Appendix S1). In brief, the following analysis steps were performed: Demultiplexed reads were trimmed for 16S primer sequences (plugin cutadapt; Martin, 2011), denoised, dereplicated, and chimera-checked (plugin DADA2; Callahan et al., 2016) resulting in amplified sequence variants (ASVs). Rare ASVs were filtered using the median frequency (=6) of ASVs over all samples. Taxonomic classification of ASVs was performed (plugin feature-classifier; Bokulich et al., 2018) using the prefitted sklearn-based taxonomy classifiers based on the SILVA reference database (ver. 138.1; Quast et al., 2013; Yilmaz et al., 2014). ASVs that could not be taxonomically assigned at any taxonomic level ('unassigned') as well as samples with less than 3900 reads in total were removed prior to subsequent analysis steps. Alpha diversity metrics, such as Shannon diversity index, Faith's phylogenetic diversity, Pielou's evenness, and observed ASVs, were obtained using the QIIME2's 'core-metrics-phylogenetic' workflow (plugin diversity), rarefied to 3900 reads per sample. To assess the overall effects of treatment and colony origin on microbial composition we performed permutational multivariate analysis of variance ADONIS from the R package *vegan* (Oksanen et al., 2022) in Qiime2. To find significant differences in α-diversity we fitted generalized linear mixed models (GLMMs) with treatment as fixed factor and colony as random factor using the function *glmmTMB* from the package *glmmTMB* (Brooks et al., 2017). We checked model assumptions using model diagnostic test plots, that is, qqplot and residual vs. predicted plot from the package *DHARMa* (Hartig, 2022). We then produced statistics with the function *Anova*() from the package *car* (Fox & Weisberg, 2019) to calculate *p*-values for differences between treatments. For significant treatment effects, we ran pairwise comparisons using Tukey HSD post-hoc test with Benjamini-Hochberg correction from the package *multcomp* (Hothorn et al., 2008). Differential abundance of the rarefied data we analyzed using the package *DESeq2* with a negative binomial distribution, a significance level cutoff of $FDR < 0.01$, replacement of outliers turned off, and cooksCutoff turned off (Love et al., 2014). Compositional differential abundance analysis was performed using Aldex2 (plugin aldex2; Fernandes et al., 2013). Beta diversity of the sparse, compositional microbiome data were calculated using QIIME2's plugin DEICODE, which performs a robust Aitchison PCA (Martino et al., 2019). Significance was tested in a PERMANOVA with 999 permutations followed by pairwise PERMANOVA with Benjamini-Hochberg (BH) correction for multiple testing (Anderson, 2008). We used the packages *qiime2R* (Bisanz, 2018) and *mia* (Ernst, Shetty, et al., 2022) to import and process the microbiome data in R. Data were arranged using the package *tidyr* (Wickham & Girlich, 2022) and were plotted using the packages *ggplot2* (Wickham, 2016), *ggpubr* (Kassambara, 2020), and *miaViz* (Ernst, Borman, & Lahti, 2022).

2.9 | Transcriptome analysis of whole bumblebee abdomens

Bumblebees were defrosted and rinsed in 70% ethanol, 90% ethanol, and twice in ultrapure water prior to dissection. The abdomen was cut off with sterile scissors, placed in an Eppendorf tube, and snap-frozen in liquid nitrogen. All samples were stored at −80°C until further processing.

2.10 | RNA sequencing

Total RNA was prepared from abdomen samples using the RNeasy Lipid Tissue kit (Qiagen, no. 74804, Hilden, Germany). RNA-Seq libraries were constructed from 100ng RNA using the NEBNext Ultra II Directional Library Prep Kit for Illumina (New England Biolabs, no. E7760, Ipswich, USA) in combination with the NEBNext Poly(A) mRNA Magnetic Isolation Module (New England Biolabs, no. E7490, Ipswich, USA). The samples were combined at equimolar amounts and sent out for sequencing on an Illumina device in 150bp paired-end mode (Genewiz, Leipzig, Germany). A total of 1.470 million reads, corresponding to an average of 19.5 million reads per sample, were obtained.

2.11 | Differential expression analysis

RNA-Seq reads were further analyzed using the OmicsBox bioinformatics platform (v. 2.0.36, www.biobam.com). Unless indicated otherwise, all tools used for differential expression analyses are accessible within the OmicsBox platform. RNA-Seq reads were preprocessed by Trimmomatic (details see Appendix S1: RNAseq_1_trimmomatic_report) (Bolger et al., 2014) to remove sequencing adapters, low-quality sequences, and short reads from the dataset. The quality-trimmed reads were mapped to the *B. terrestris* genome assembly (Bter_1.0, GCA_000214255.1, downloaded from metazoa.ensembl.org) using STAR (Dobin et al., 2013). A gene-specific count table was created from the mapping files using HTseq (Anders et al., 2015) and differentially expressed genes were identified by edgeR (Robinson et al., 2010), respectively. Functional annotation of the *B. terrestris* genome was based on annotation release v. 102 (available in gff3 format from metazoa.ensembl.org). Since 4975 of the 12,008 genes did not contain any functional annotation, the functional annotation workflow of the OmicsBox platform was used to update the published annotation with additional information. In brief, the coding sequences of unannotated genes were used to extract functional annotations from refseq_protein database (www.ncbi.nlm.nih.gov) and InterProScan (www.ebi.ac.uk). These we then fed into the GO mapping and annotation tools of the pipeline and finally merged to the existing functional annotations. To assess the overall effects of treatment and colony origin on gene expression we performed permutational multivariate analysis of variance ADONIS from the R package vegan (Oksanen et al., 2022) in Qiime2. Gene Set Enrichment Analyses (GSEA; Subramanian et al., 2005) were performed using ranked list of genes ($rank = sign(logFC) * -\log_{10}(p\text{-value})$; FC: fold change) and gene sets defined by Gene Ontology's functional annotations. For the functional network analysis of enriched GO terms we used ClueGo (v. 2.5.9; Bindea et al., 2009) and CluePedia (v. 1.5.9; Bindea et al., 2013) plugins in Cytoscape (v. 3.9.1; Shannon et al., 2003). We used the packages *ggplot2* (Wickham, 2016), *ggpubr* (Kassambara, 2020), and *pheatmap* (Kolde, 2019) to plot transcriptome data in R 4.2.1 (R Core Team, 2022).

3 | RESULTS

3.1 | Effect of pollutants on the bumblebee gut microbiome

Amplicon sequencing of the bacterial 16S rDNA fragments yielded a total of 2,425,928 raw reads. After quality filtering and removal of unassigned sequences, we also removed samples with a sampling depth below 3900 reads ($n=7$), all from DEP treatment, to ensure adequate sampling depth (13 DEP replicate samples remained in the analysis). In the remaining samples we obtained 1,856,025 16S rDNA gene sequences with a mean of 25,425 reads per sample ($n=73$), corresponding to 468 amplicon sequence variants (ASVs). Sample-based rarefaction curves suggest a sufficient sequencing depth for a representative coverage of the microbiome as most of the samples reach a plateau (Figure A1). ADONIS analysis revealed a significant effect of treatment on microbiome composition ($R^2=0.423$, $p<.001$). There was no significant effect of colony origin ($R^2=0.001$, $p=.946$) on microbiome composition.

3.2 | Taxa abundance

On the genus level, the most common bacterial taxa (>1% in at least one treatment) were: *Gilliamella*, *Snodgrassella*, *Lactobacillus*, *Asaia*, *Bombiscardovia*, *Methylorubrum*, and *Bombilactobacillus*. The relative abundance of the most common genera for each sample shows a different microbial composition in the DEP treatment compared to the other treatment groups (Figure 1).

While the relative abundance of ASVs did not differ between control, solvent control, and brake dust, DEP treatment had 16 differentially abundant ASVs compared to the control, according to DESeq2 (Figure 2, Table A1). Eleven ASVs had a higher abundance in the DEP treatment than control. Five ASVs had reduced abundance in comparison to the control treatment. A more conservative approach to identify differential abundance is ALDEx2, which revealed five ASVs with significantly altered abundance in the DEP treatment compared to the control: *Snodgrassella* 1 + 2, Neisseriaceae, *Lactobacillus bombicola*, and *Bombiscardovia* (Table A2).

3.3 | α-diversity of the gut microbiome

The number of observed ASVs did not differ between treatments (GLMM with Gaussian distribution: $\chi^2=0.918$, $df=3$, $p=.821$; Figure 3a). Pielou's evenness differed between treatments (GLMM with Gaussian distribution: $\chi^2=42.697$, $df=3$, $p<.001$; Figure 3b). The DEP treatment had a significantly lower evenness than the other treatments (Tukey comparisons with Benjamini-Hochberg (BH) adjusted *p*-values: DEP vs. control $p<.001$, DEP vs. solvent control $p<.001$, DEP vs. brake dust $p<.001$; Figure 3b). Shannon diversity differed between treatments (GLMM with Gaussian distribution: $\chi^2=24.035$, $df=3$, $p<.001$; Figure 3c). The DEP treatment had a significantly lower diversity than the other treatments (Tukey comparisons with BH adjusted *p*-values: DEP vs. control $p<.001$, DEP vs. solvent control $p<.001$, DEP vs. brake dust $p<.001$; Figure 3c). Faith's PD differed between treatments (GLMM with Gaussian distribution: $\chi^2=19.062$, $df=3$, $p<.001$; Figure 3d). Faith's PD in the DEP treatment was significantly higher than in the other treatments (Tukey comparisons with BH adjusted *p*-values: DEP vs. control $p<.001$, DEP vs. solvent control $p<.001$, DEP vs. brake dust $p<.001$; Figure 3d).

3.4 | β-diversity of the gut microbiome

The community composition of the bumblebee gut microbiome differed between treatments indicated by significant differences between the robust Aitchison distances (Overall PERMANOVA

FIGURE 1 Relative abundance of the most common bacterial genera for each sample. Samples are arranged according to treatment.

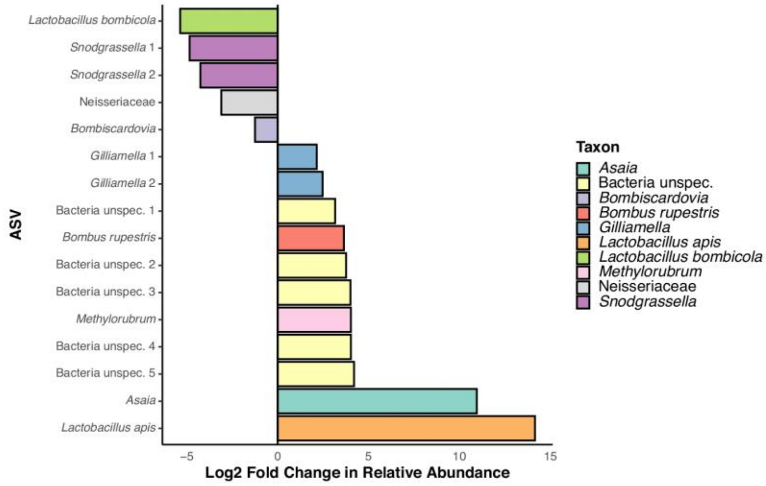

FIGURE 2 Log$_2$ fold change in relative abundance of ASVs in the DEP treatment in comparison to the control. Cutoff for inclusion of ASVs in this plot was FDR (=p_{adj}) < .01. Colors represent most specific taxonomic label.

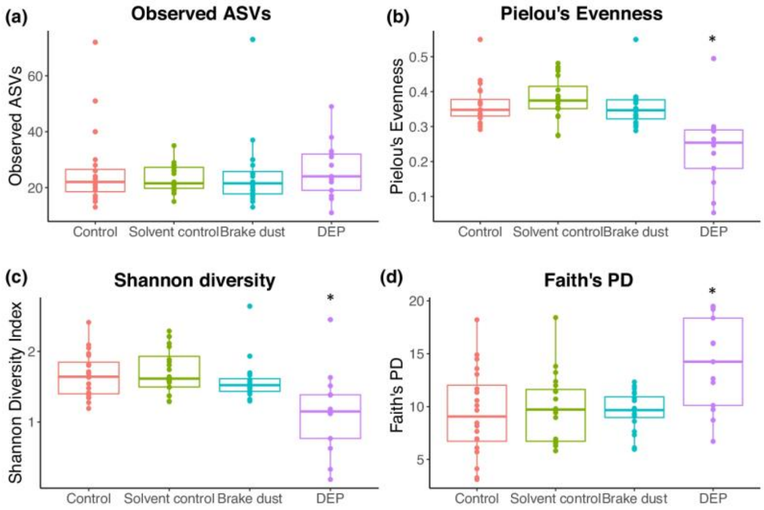

FIGURE 3 α-diversity of the bumblebee gut microbiomes for the different treatments. (a) Observed ASVs, (b) Pielou's Evenness, (c) Shannon Diversity, (d) Faith's PD. Asterisks indicate significant differences compared to the other treatments ($p < .05$). Boxplots show median, first, and third quartile. Dots represent individual data points.

pseudo-$F_{4,\,73} = 16.844$, $p = .001$). Microbial community composition of the DEP treatment differed from all other treatments (Pairwise PERMANOVA with BH adjusted p-values; DEP vs. control: pseudo-$F = 32.247$, $p = .002$; DEP vs. solvent control: pseudo-$F = 30.651$, $p = .002$; DEP vs. brake dust: pseudo-$F = 25.699$, $p = .002$). We found no differences between the other treatments (Pairwise PERMANOVA with BH adjusted p-values: $p > .05$) (Figure 4).

3.5 | Effect of pollutants on bumblebee gene expression

In the transcriptome analysis, we focused only on biologically relevant comparisons of treatments to prevent unnecessary inflation of reported results. We compared control vs. solvent control, control vs. DEP, control vs. brake dust, and flight control vs. DEP flight. The analysis for differently expressed genes (DEGs) revealed differences between our treatments. In total, 324 genes were differentially expressed in the DEP treatment compared to the control (low-count gene filter settings: CPM Filter = 1, samples reaching CPM Filter = 2). 165 genes were upregulated (LogFC > 1) and 159 genes downregulated (LogFC < −1), respectively (Table A3, Figure A2). In the brake dust treatment only one gene (lipase 3) was differentially expressed (upregulated) in comparison to the control. In the solvent control, there were no differentially expressed genes compared to the control. In the DEP flight treatment, we found no differentially expressed genes in comparison to the flight control. ADONIS analysis revealed a significant effect of treatment on gene expression ($R^2 = 0.279$, $p = .002$). There was no significant effect of colony origin ($R^2 = 0.031$, $p = .054$) on gene expression.

The variation in gene expression of bumblebee workers is clearly distinct between the control and the DEP treatment (Figure 5). The

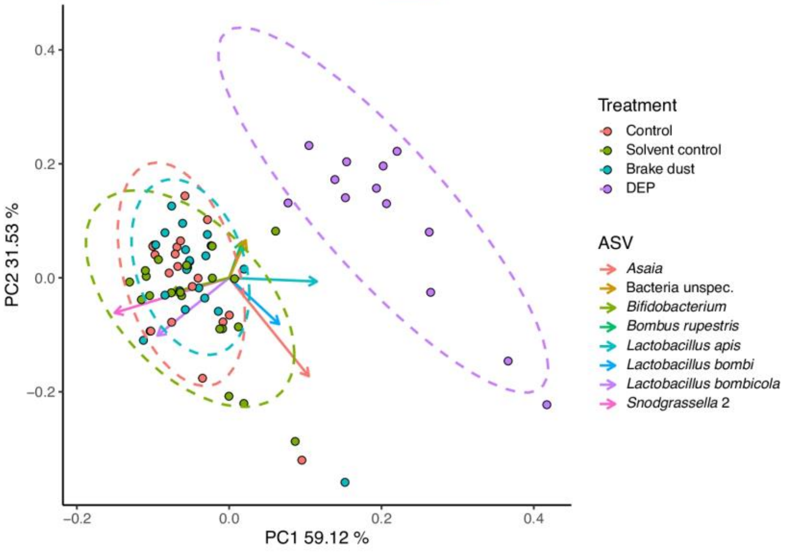

FIGURE 4 DEICODE distances based on Robust Aitchison Principal Components Analysis. Points represent single samples colored according to treatment. Arrows represent Euclidian distances from the origin and indicate ASVs with strong influence on the principal component axis. Ellipses show 95% confidence interval for multivariate t-distribution of each treatment. The ASV of the eukaryotic organism *Bombus rupestris* can be explained by a remaining nonspecificity of the used primers (as analyzed by TestPrime, www.arb-silva.de).

clear separation between the treatments across all samples indicates substantial differences in gene expression of bumblebees when exposed to DEP orally. The reliability of this difference in gene expression is confirmed by a cluster analysis, which shows a definite clustering by treatment rather than by colony (Figure 6). The other treatments are not clearly distinct in a nMDS plot and indicate no differences in gene expression (Figures A3–A5), thus we do not conduct further analyses on these comparisons.

The 324 differentially expressed genes in the DEP treatment were annotated to gene ontology (GO) terms, which describe gene properties and group each into one of three categories: Cellular component, molecular function, and biological process. We used GO enrichment analysis to find the most over- and underrepresented term. The 30 most significantly upregulated GO terms in the DEP treatment include protein-binding functions, enzyme complexes, and metabolic, especially catabolic, processes (Figure 7a). The 30 most significantly downregulated GO terms in the DEP treatment include transferase activity, mitochondrial and organelle membranes, as well as metabolic, especially biosynthetic, processes (Figure 7b).

The functional network analysis based on κ-Score ≥ 0.4 for differentially expressed GO terms with FDR ≤0.05 in the DEP treatment shows clustering to specific functional groups (Figure A6a). Upregulated functions are related to phosphorylation, regulation of metabolic process, guanyl nucleotide binding, and signal transduction (Figure A6b). Downregulated functions are related to mitochondria, lipid metabolic processes, the endoplasmic reticulum, and phospholipid biosynthetic processes (Figure A6c).

4 | DISCUSSION

In this study, we found that oral exposure to diesel exhaust particles (DEPs) changes the gut microbiome and gene expression of

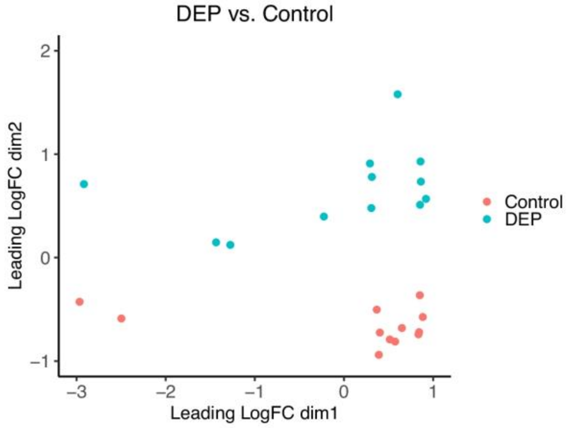

FIGURE 5 Nonmetric multidimensional scaling plot based on the $\log_2$ fold changes (FC) between control and DEP treatment. The axes of the nMDS plot represent dimensional reductions of gene expression visualizing the variability of the transcriptional changes for each treatment. Each point represents one sample, colored according to treatment.

bumblebee workers, while DEP exposure via air did not. Brake dust, the second pollutant we tested via oral exposure, did not induce changes in the gut microbiome or gene expression in the bumblebee workers.

While the composition of the microbial gut community in control, solvent control, and brake dust exposure treatment was similar, we detected major shifts in the DEP treatment. This raises several interesting questions: (1) How do DEPs affect the bacteria to induce changes in the gut microbiome composition? (2) Which components in diesel exhaust are responsible for the observed changes? Our hypothesis is that PAHs could be the component of DEP affecting bacteria directly. DEPs contain different PAHs, a

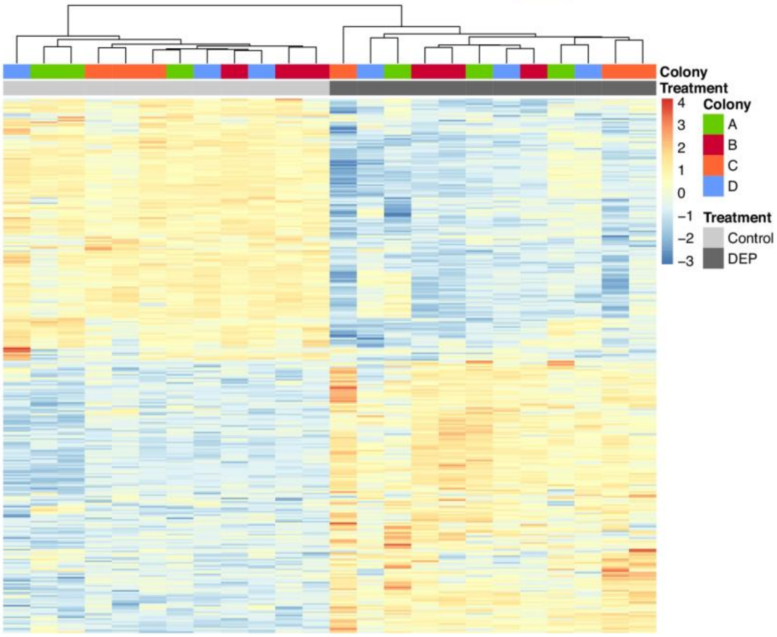

FIGURE 6 Heatmap showing hierarchical clustering of samples (*x*-axis) of differentially expressed genes for the control and DEP treatment. The heatmap was obtained using Ward's clustering with the Euclidean distance. The values represent *z*-scores of $\log_2$-transformed CPM (Counts per million reads) expression values.

class of organic compounds well-known to be toxic, mutagenic, and genotoxic to various life forms (Patel et al., 2020; Sun et al., 2021). Also, shifts in the microbial gut community due to PAH exposure have been reported in different animals, such as fish, sea cucumbers, or potworms (Enchytraeidae) (DeBofsky et al., 2020, 2021; Ding et al., 2020; Quintanilla-Mena et al., 2021; Zhao et al., 2019). Therefore, we suspect PAHs to be the leading cause of changes in the bumblebee gut microbiome in our study. However, the large amount of elemental carbon in DEPs, may itself provide another explanation. The DEPs may function like activated carbon with its large surface-area-to-volume ratio and may adsorb microbes that are then discharged by excretion (Naka et al., 2001; Rivera-Utrilla et al., 2001; Wichmann, 2007). Even though activated carbon has no direct negative impact, constant adsorption and discharge might disrupt the bacterial community resulting in the compositional and quantitative changes similar to those observed in our study.

The bacterium *Snodgrassella*, one of the dominant core bacteria in undisturbed gut microbiomes of bumblebees (Hammer et al., 2021), is nearly absent after the DEP exposure. *Snodgrassella*, together with *Gilliamella*, forms a biofilm coating the inner wall of the ileum (Hammer et al., 2021; Martinson et al., 2012). Both host and symbionts could profit from this biofilm formation as it prevents bacteria from washout and enables the formation of a syntrophic network (Kwong et al., 2014; Powell et al., 2016; Zhang & Zheng, 2022). Additionally, the biofilm could protect the host against gut parasites, such as *C. bombi*, which need to attach to the gut wall to persist (Koch et al., 2019; Näpflin & Schmid-Hempel, 2018). However, the mutualistic relationship between the microbes seems to be disrupted by DEP exposition, as *Snodgrassella* abundance is extremely diminished. In contrast, *Gilliamella* increases in relative abundance after DEP exposure. This indicates that *Gilliamella* may be able to form a biofilm independently from *Snodgrassella*. A relatively simple explanation for the higher relative abundance of *Gilliamella* might be that the reduction of *Snodgrassella* leaves *Gilliamella* as the only dominant bacterium in the gut, and therefore, *Gilliamella* might thrive better or fill the void. *Snodgrassella* seems especially prone to pollutants, as Rothman et al. (2020) already reported a decrease in its relative abundance after exposure of bees to copper, selenate, or glyphosate. Additionally, we found an unknown bacterium from the family Neisseriaceae, the same family to which also *Snodgrassella* belongs, having a lower relative abundance after DEP exposure. If this is a consistent result, it might indicate a general susceptibility of this family to DEPs.

The higher abundance of *Asaia* in the DEP treatment was driven by two samples, in which *Asaia* dominates the bacterial community with relative abundances of 99% and 67%, respectively. *Asaia* is a flower-associated acetic acid bacterium, which is commonly found in the gut of members of different insect orders, such as Hemiptera, Diptera, and Hymenoptera (Bassene et al., 2020; Crotti et al., 2009; Kautz et al., 2013). It can dominate the gut microbiome of *Anopheles* mosquitos, which is why it is considered a potential tool in malaria control (Capone et al., 2013; Favia et al., 2008). While there have been reports of *Asaia* in bumblebees, the dominance of *Asaia* in some of the DEP samples is rather uncommon (Bosmans et al., 2018). DEPs might disrupt the natural microbiome community opening the door for opportunistic bacteria such as *Asaia* (Favia et al., 2007). Even though we kept the bumblebees in this experiment indoors throughout their lives, *Asaia* bacteria may derive from pollen fed to the bumblebees before the start of the experiment.

We detected an interesting pattern in the genus *Lactobacillus*, one of the core gut bacteria of bumblebees (Hammer et al., 2021). While the species *L. bombicola*, a bumblebee-associated bacterium, has a lower abundance after DEP exposure, the abundance of the honeybee-associated *L. apis* increases. Again, the disruption of the original microbiome caused by DEPs might explain that foreign

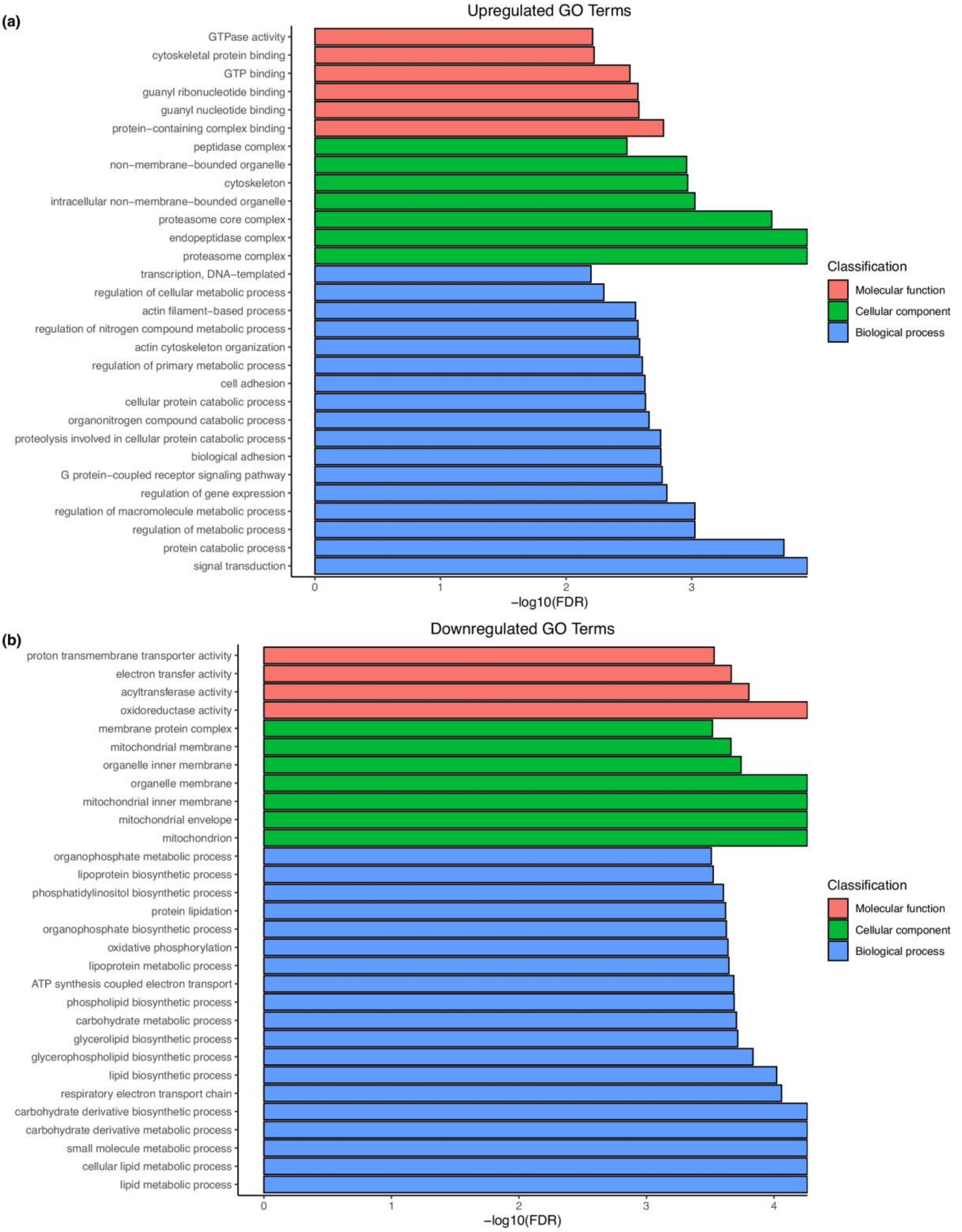

FIGURE 7 Gene ontology terms of (a) the 30 most significantly upregulated and (b) downregulated genes in the DEP treatment colored by category and sorted by $-\log_{10}$FDR.

bacteria can establish themselves in the microbiome. As the pollen fed to the bumblebees before the experiment was collected by honeybees, it could be the source of *L. apis*.

The DEP-induced changes in the gut microbiome may affect bumblebee health, as core bacteria could prevent infections by parasites. The abundance of *Gilliamella*, *Lactobacillus*, and *Snodgrassella* is negatively correlated with the parasites *Crithidia* and *Nosema*, while noncore bacteria are more abundant in infected bumblebees (Cariveau et al., 2014; Koch et al., 2012; Koch & Schmid-Hempel, 2012; Mockler et al., 2018). The biofilm formation of *Snodgrassella* and *Gilliamella* may form a physical barrier to the trypanosome *C. bombi*, which needs to attach to the ileum wall to persist (Koch et al., 2019, Näpflin & Schmid-Hempel, 2018). The disruption of this biofilm and the higher abundance of noncore bacteria, such as *Asaia*, may increase the parasite susceptibility of bumblebees exposed to DEPs.

The transcriptome analysis revealed significant changes in gene expression after oral exposure of bumblebees to a sublethal dose of DEPs. In total, 165 genes were upregulated, and 159 genes were downregulated. GO enrichment analysis and network analysis indicate that these changes could be related to a general stress response against pollutants. While upregulated GO terms involve many metabolic and catabolic processes, downregulated GO terms include metabolic and biosynthetic processes. DEP exposure might deplete stored reserves causing the observed changes as a consequence of higher energetic costs. Changes in metabolism seem to be a typical reaction to pollutants in insects, which seems reasonable as they often interfere with biochemical processes. Transcriptional changes in bumblebees and honeybees exposed to sublethal doses of neonicotinoids are mainly linked to metabolic processes (Bebane et al., 2019; Colgan et al., 2019; Gao et al., 2020; Shi et al., 2017). Exposure to heavy metals or PAHs induces similar changes in spiders, mosquitos, moths, and fireflies (Chen et al., 2021; David et al., 2010; Li et al., 2016; Zhang et al., 2019, 2021). Even though the changes differ in detail, certain processes seem commonly involved in the response to pollutants. Consistent with our findings, exposure to insecticides or PAHs affects mitochondrial functioning, an important part of the insect energy metabolism (Colgan et al., 2019; Zhang et al., 2019, 2021). This supports the idea of increased energy demand caused by pollutants (Beyers et al., 1999; Calow, 1991). We also observed an upregulation of signal transduction in our study, similar to observations in honeybees and fireflies exposed to Imidacloprid and the PAH benzo(a)pyrene, respectively (Gao et al., 2020; Zhang et al., 2019, 2021). Typically, chemical stressors, such as PAHs, insecticides, and heavy metals, affect genes associated with detoxification processes and drug metabolism (Chen et al., 2021; David et al., 2010; Gizaw et al., 2020; Zhang et al., 2019). However, in our study, we did not find any differentially expressed detoxification-related genes. Possibly the number of PAHs attached to the DEPs was not enough to trigger a reaction that would lead to a measurable increase in detoxification. Overall, the observed changes in gene expression after oral DEP exposure of bumblebees resemble a general stress response to pollutants.

As microbiome and gene expression of bumblebees significantly changed after oral DEP the question arises if and how these systems might affect each other. Metabolic changes may be caused by the DEP-induced changes in the gut microbiome, which can potentially alter the type and amount of metabolites provided to the host (Douglas, 2018). Moreover, insect immunity might be dependent on gut microbiome. In honeybees the native gut microbiome stimulates immune gene expression, inducing the production of antimicrobial peptides (Kwong et al., 2017). The function and the mechanistic underpinning of this interaction is not entirely clear, but the host might regulate the microbiota in this way. However, host health might also benefit from this interaction by priming the immune system against future infections. Pollutants altering the gut microbiome might thereby jeopardize insect health. This could explain the increased mortality in honeybees with altered gut microbiome due to antibiotic exposure (Raymann et al., 2017).

In contrast to oral exposure, we did not find any effect on gene expression after exposure of bumblebees to DEPs via the air. To cause changes, DEPs need to enter the tracheal system or attach to sensory organs, such as the antennae. The exposure of bumblebees for 3 min per day may not have been enough to affect them. Particles on the antennae may have been removed quickly by cleaning behavior and the spiracles seem to be an effective protective barrier against the uptake of particles into the tracheae (Harrison, 2009; Schönitzer, 1986). Thus, our results should be taken with care because probably only very few particles entered the tracheal system of the bumblebees.

Unlike DEPs, oral exposure to brake dust particles did not affect the gut microbial community or the gene expression of the bumblebees. However, some concerns remain about the experimental procedure. For one, we did not use brake dust from a real braking scenario, but rather artificially milled brake pads. Dust derived from them may have different physicochemical properties. Milled brake dust particles have a much higher mean particle size than DEPs (10 μm vs. 0.01 μm). As we defined treatment concentration per weight, these different physical properties lead to big differences in the particle counts of the treatment solutions, that is, solutions with brake dust contained far fewer particles than those with DEPs. Moreover, large brake dust particles tend to sink to the bottom of the feeding syringes, which might have reduced the particle uptake. While brake dust seems not to affect the bumblebees, further studies are needed to address the indicated limitations of the present study.

Another problem that needs to be addressed is how the doses used in this study relate to field-realistic concentrations encountered by bumblebees. With the still often vague knowledge of origin and quantity of airborne fine particulate matter present in terrestrial habitats, we know even less about their potential uptake by insects. Contamination of bee products is documented, but there is a need for realistic modeling of encounter rate of insects with airborne particulate matter (Conti & Botrè, 2001). The doses used in this study are presumably higher than those encountered naturally. However, our experimental setup does not include other stressors bees have to face in the wild, such as parasites, limited food availability, or abiotic factors such as drought or heat stress. Bumblebees may be able to compensate for facing one stressor but will eventually be overstrained by multiple stressors.

Taken together, the results from our microbiome and transcriptome analysis indicate potential consequences for insect health, here shown in bumblebees, after oral DEP exposure. Gut dysbiosis may increase the susceptibility of bumblebees to pathogens, while a general stress response may lower available energetic resources. This highlights the potential role of airborne particulate matter such as DEPs as a driver of insect declines.

AUTHOR CONTRIBUTIONS

Dimitri Seidenath: Conceptualization (equal); data curation (equal); formal analysis (equal); investigation (equal); methodology (equal); visualization (lead); writing – original draft (lead). **Alfons R. Weig:** Conceptualization (supporting); data curation (equal); formal analysis (equal); methodology (equal); software (equal); visualization (supporting); writing – original draft (supporting); writing – review and editing (equal). **Andreas Mittereder:** Methodology (equal); resources (equal); writing – review and editing (supporting). **Thomas Hillenbrand:** Methodology (supporting); resources (supporting); supervision (supporting); writing – review and editing (supporting). **Dieter Brüggemann:** Funding acquisition (supporting); project administration (supporting); resources (supporting); supervision (supporting); writing – review and editing (supporting). **Thorsten Opel:** Methodology (supporting); resources (supporting); writing – review and editing (supporting). **Nico Langhof:** Funding acquisition (supporting); methodology (supporting); project administration (supporting); resources (supporting); supervision (supporting); writing – review and editing (supporting). **Marcel Riedl:** Investigation (equal). **Heike Feldhaar:** Conceptualization (equal); funding acquisition (equal); project administration (equal); supervision (equal); writing – original draft (supporting); writing – review and editing (lead). **Oliver Otti:** Conceptualization (equal); formal analysis (supporting); funding acquisition (equal); project administration (equal); supervision (equal); visualization (equal); writing – original draft (supporting); writing – review and editing (lead).

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DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation. The microbiome and RNA-Seq sequencing data were deposited at NCBI's Sequence Read Archive (SRA) under Bioproject numbers PRJNA907197 (16S microbiome sequencing) and PRJNA907822 (transcriptome sequencing), respectively.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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APPENDIX A

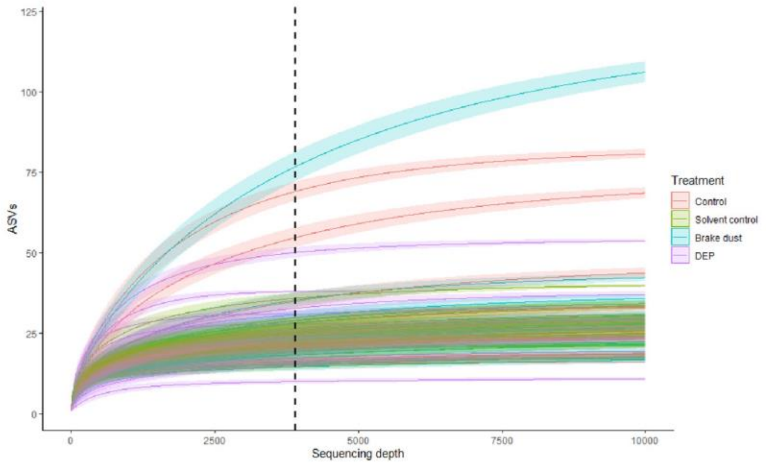


FIGURE A1 Rarefaction curve of each sample, colored according to their respective treatment. *X*-Axis is cut off at 10,000 reads. Vertical dashed line indicates sequencing depth of 3900.

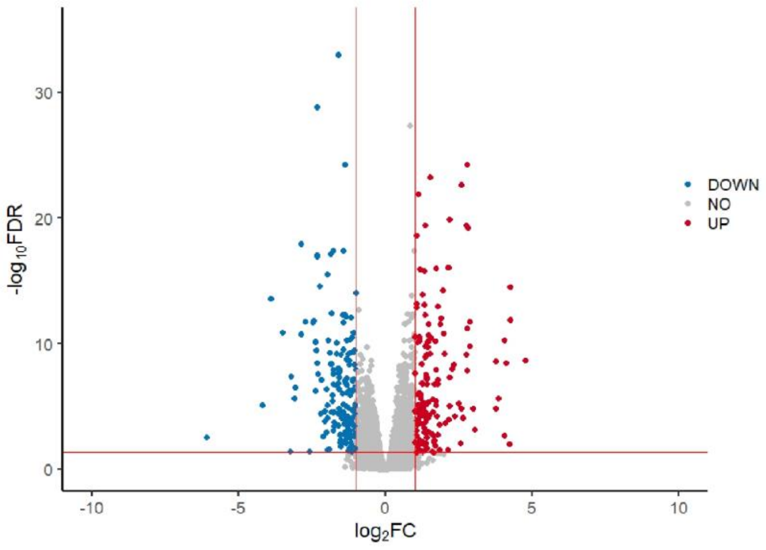


FIGURE A2 Differential expression of genes in the DEP treatment in comparison to the control. Blue dots represent significantly downregulated genes, red dots represent significantly upregulated genes. The horizontal red line marks a $-\log_{10}(FDR = 0.05)$. The two vertical red lines mark a $\log_2$FC of −1 and 1, respectively.

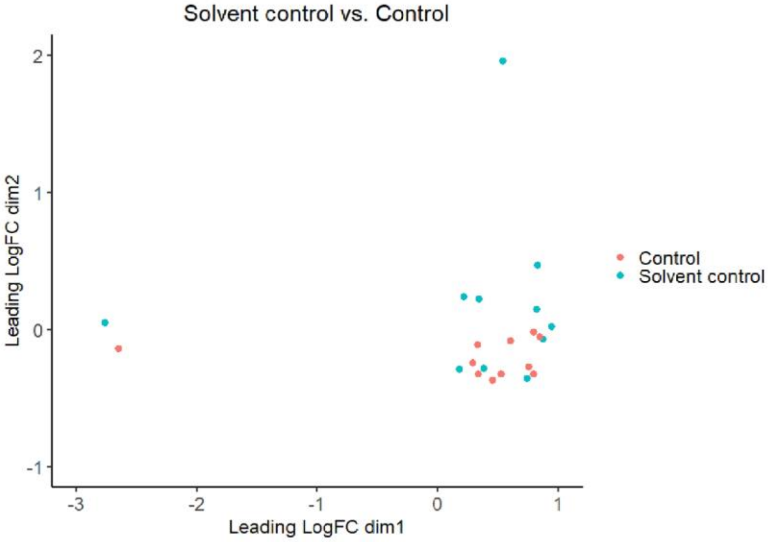


FIGURE A3 Non-metric multidimensional scaling plot based on the $\log_2$ fold changes (FC) between control and solvent control. The axes of the nMDS plot represent dimensional reductions of genes expression visualizing the variability of the transcriptional changes for each treatment. Each point represents one sample, colored according to the respective treatment.

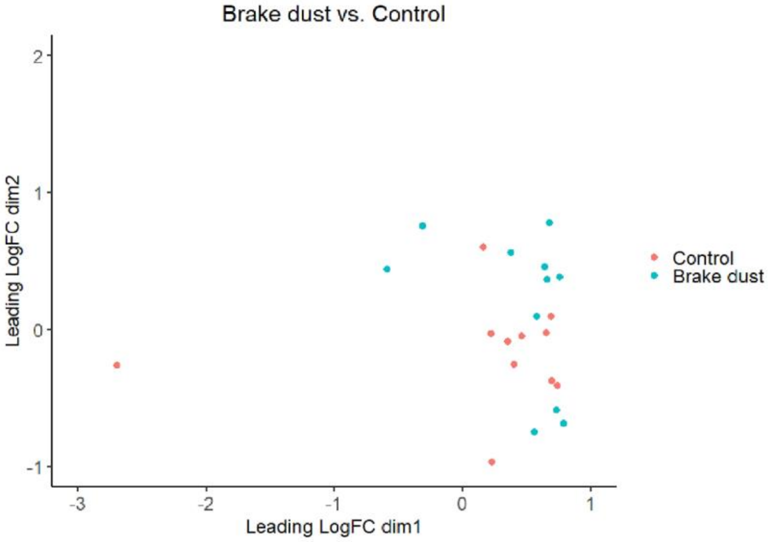
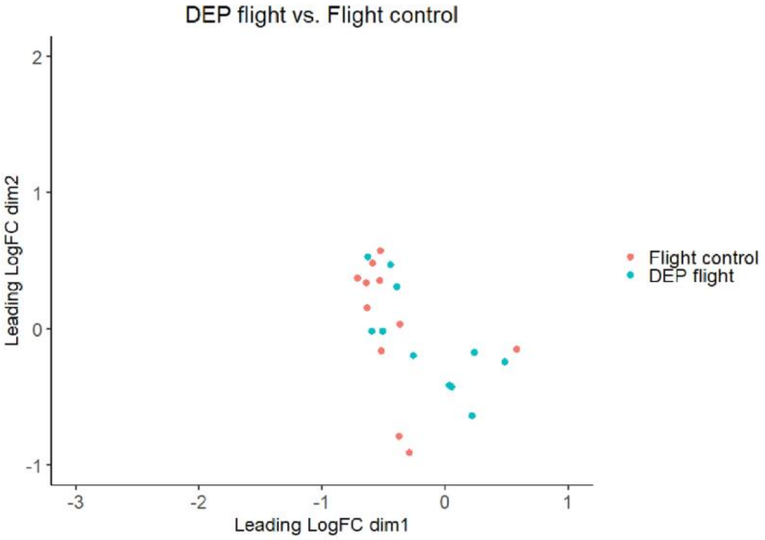


FIGURE A4 Non-metric multidimensional scaling plot based on the $\log_2$ fold changes (FC) between control and brake dust treatment. The axes of the nMDS plot represent dimensional reductions of genes expression visualizing the variability of the transcriptional changes for each treatment. Each point represents one sample, colored according to the respective treatment.

FIGURE A5 Non-metric multidimensional scaling plot based on the $\log_2$ fold changes (FC) between flight control and DEP flight treatment. The axes of the nMDS plot represent dimensional reductions of genes expression visualizing the variability of the transcriptional changes for each treatment. Each point represents one sample, colored according to the respective treatment.



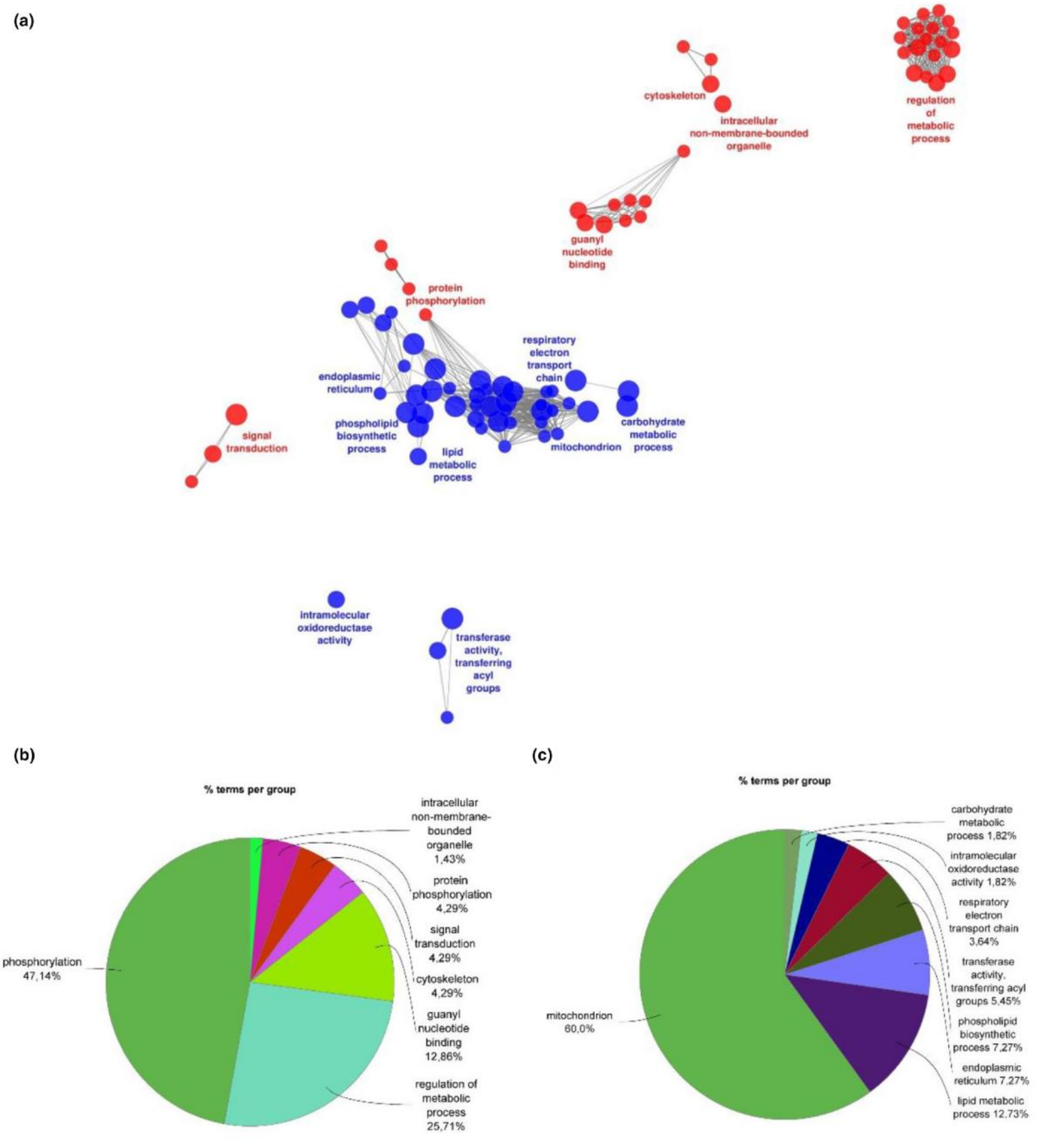


FIGURE A6 Network analysis of enriched gene terms and functional groups in the DEP treatment based on Kappa-Score ≥ 0.4 for GOs with FDR ≤ 0.05 using the ClueGo and CluePedia plugins of Cytoscape. (a) Functionally grouped network of upregulated (red) and downregulated (blue) gene ontologies. (b) pie chart with functional groups, including specific terms upregulated in the DEP treatment. (c) pie chart with functional groups, including specific terms downregulated in the DEP treatment. The area covered by each group represents the relative number of GO terms within each group. The most significant term each group is labelled.

TABLE A1 Differentially abundant ASVs comparing DEP to the control treatment, according to DESeq2 (cutoff: FDR < 0.01).

ASV	Log_2 fold change	p_{adj} (=FDR)	Feature ID
Lactobacillus bombicola	−5.372	<.001	ac3366c90455cdc1a4ad414f21215a91
Snodgrassella 1	−4.848	<.001	f9dff838e1ab76a58a54df65a2457d5a
Snodgrassella 2	−4.256	<.001	8f7166172175c35bbfc8fa4dc5ef58b8
Neisseriaceae	−3.108	<.001	f1ae3848b7e710b5da56f2a447ae0234
Bombiscardovia	−1.251	.010	bf7591505d4138d52e3a9c537c958fa1
Gilliamella 1	2.146	<.001	36aed5b1dc9b5c1a2844e58f2d34b1f5
Gilliamella 2	2.473	<.001	1e232cdf347e2b62b3b1d7347e891797
Bacteria unspec. 1	3.162	.001	6445d5095ad81f1b73aa974a171ebce6
Bombus rupestris	3.645	<.001	6d53feb4ee4fac60aba11969e1e5fc01
Bacteria unspec. 2	3.768	.004	101de948d3a66ac329a31fd5f92c00d5
Bacteria unspec. 3	4.008	<.001	7ebb40e08aa315a3ab9ae5fb0b47ae34
Methylorubrum	4.025	<.001	92f1720367db58c68a96eceb9feb416a
Bacteria unspec. 4	4.030	<.001	5c70c440562c05d292daf0c5b4694ef4
Bacteria unspec. 5	4.201	<.001	a6ddcd6498df4ed3d6c3e05663f658fb
Asaia sp.	10.960	<.001	49d46d00a93443b060707ab2db8ba82d
Lactobacillus apis	14.158	<.001	96d14363f547715b65bf7d8ad1d31d17

Note: Positive Log_2 fold changes indicate higher abundance in the DEP treatment.

TABLE A2 Differentially abundant ASVs comparing DEP to the control treatment, according to ALDEx2.

ASV	Effect	p_{adj}	Feature ID
Snodgrassella 2	−5.516	<.001	8f7166172175c35bbfc8fa4dc5ef58b8
Neisseriaceae	−2.659	<.001	f1ae3848b7e710b5da56f2a447ae0234
Lactobacillus bombicola	−2.393	<.001	ac3366c90455cdc1a4ad414f21215a91
Snodgrassella 1	−2.356	<.001	f9dff838e1ab76a58a54df65a2457d5a
Bombiscardovia	−2.092	<.001	bf7591505d4138d52e3a9c537c958fa1

Note: Negative effect indicates higher abundance in the control. p_{adj} = Expected Benjamini-Hochberg corrected *p* value of Wilcoxon test. Effect = median effect size (diff.btw/max(diff.win)).

TABLE A3 Differentially expressed genes in the DEP treatment compared to the control (low-count gene filter settings: CPM Filter = 1, samples reaching CPM Filter = 2).

Feature	Description	LogFC	LogCPM	FDR
LOC105666082	Protein IWS1 homolog	4.778	−0.174	<0.001
LOC100651567	Protein yellow-like	4.268	5.560	<0.001
LOC100644846		4.259	5.525	<0.001
LOC100644158		4.239	0.464	0.009
LOC100643093		4.101	2.086	<0.001
LOC105666427	Titin homolog	4.054	−0.636	<0.001
LOC100646940		4.045	−2.033	0.002
LOC110119163	Protein fantom-like	3.865	−1.153	<0.001
LOC110119507		3.774	−0.774	<0.001
LOC110120240		3.763	−1.264	<0.001
LOC100648995		3.034	2.508	<0.001
LOC100646947	Proline-rich protein 4	2.981	−0.210	<0.001
LOC100648170	Salivary glue protein Sgs-3-like	2.882	4.684	<0.001
LOC110120085	MATH and LRR domain-containing protein PFE0570w-like	2.865	0.639	<0.001
LOC100646909	Leucine-rich repeat protein SHOC-2-like isoform X1	2.815	2.525	<0.001
LOC100647974		2.789	9.078	<0.001
LOC100647281	Spore wall protein 2-like	2.784	0.248	<0.001
LOC100644232	MATH and LRR domain-containing protein PFE0570w-like	2.775	4.245	<0.001
LOC100647178		2.754	7.912	<0.001
LOC100645500		2.752	5.742	<0.001
LOC100647176		2.642	−1.240	<0.001
LOC100652307	Mucin-5AC-like isoform X3	2.590	3.066	<0.001
LOC100647203	Glycine-rich cell wall structural protein	2.579	−0.377	<0.001
LOC105666061	Fibrous sheath CABYR-binding protein-like	2.551	1.106	0.008
LOC100649104	Electron transfer flavoprotein beta subunit lysine methyltransferase-like	2.489	−0.274	<0.001
LOC100650993	Hybrid signal transduction histidine kinase L-like	2.367	−0.563	<0.001
LOC100642564	Proton-coupled amino acid transporter-like protein pathetic	2.340	1.911	<0.001
LOC100647041		2.281	4.208	<0.001
LOC100645710	Centrosomal protein of 290 kDa-like	2.198	4.105	<0.001
LOC100651433		2.187	8.265	<0.001
LOC100644285	Zinc finger protein 100-like	2.186	0.703	<0.001
LOC100647265		2.163	5.745	<0.001
LOC105666426	Titin homolog	2.151	−0.425	<0.001
LOC100644468	Spore coat protein SP96-like	2.129	−0.294	0.027
LOC100649167	Coiled-coil domain-containing protein 170 isoform X1	2.121	4.034	<0.001
LOC100645585	Uncharacterized protein LOC100645585 isoform X1	2.037	0.733	<0.001
LOC100643561		2.001	6.567	<0.001
LOC100645996		1.988	0.963	<0.001
LOC100652019		1.967	6.469	<0.001
LOC105666709	Uncharacterized protein LOC105666709	1.890	4.064	<0.001
LOC110119744	Uncharacterized protein LOC110119744	1.870	2.413	<0.001
LOC100647550	Cyclin-dependent kinase inhibitor 1C	1.832	−1.175	0.029

TABLE A3 (Continued)

Feature	Description	LogFC	LogCPM	FDR
LOC100650340		1.829	−0.412	<0.001
LOC100647929		1.794	−0.292	<0.001
LOC105665898	Uncharacterized protein LOC105665898	1.790	−1.126	0.002
LOC105665941	Uncharacterized protein LOC105665941	1.781	1.785	<0.001
LOC100646677		1.774	9.275	<0.001
LOC110120139	Uncharacterized protein LOC110120139	1.759	0.166	<0.001
LOC100645840		1.739	0.876	<0.001
LOC100647883		1.738	9.769	<0.001
LOC100651423	Cystinosin homolog isoform X1	1.726	7.389	<0.001
LOC110119585	Odorant receptor 49b-like	1.718	1.471	<0.001
LOC100646153		1.709	2.931	<0.001
LOC105666013	Protein Hook homolog 3-like	1.687	−0.056	0.013
LOC100645923	Uncharacterized protein LOC100645923 isoform X1	1.657	0.413	<0.001
LOC100649809	Microtubule-associated protein 10-like	1.655	−0.359	0.006
LOC100651231		1.645	3.339	0.006
LOC100648688		1.631	4.192	0.046
LOC110119618	Uncharacterized protein LOC110119618	1.630	0.823	0.002
LOC110119338		1.620	0.068	<0.001
LOC100646202		1.617	4.879	<0.001
LOC105666927	Uncharacterized protein LOC105666927	1.576	−0.631	0.041
LOC100651530		1.574	0.299	0.002
LOC100646747		1.562	5.823	<0.001
LOC100648646		1.534	6.323	<0.001
LOC100648300		1.531	7.334	<0.001
LOC110120263	Uncharacterized protein LOC110120263 isoform X2	1.496	4.036	<0.001
LOC100646922		1.473	8.312	<0.001
LOC100648283		1.472	0.806	<0.001
LOC100646009		1.471	9.026	<0.001
LOC100646896		1.449	4.249	<0.001
LOC100649615	Ataxin-7-like protein 1	1.440	5.277	<0.001
LOC105666227	LOW QUALITY PROTEIN: uncharacterized protein LOC105666227	1.439	−0.443	0.001
LOC100651732		1.439	2.514	<0.001
LOC100642884		1.436	5.710	<0.001
LOC100642438	Probable WRKY transcription factor protein 1	1.420	3.795	<0.001
VSP		1.409	9.742	<0.001
LOC105666604	Uncharacterized protein LOC105666604	1.404	0.197	0.003
LOC105665882		1.399	2.459	<0.001
LOC100645563		1.399	1.182	<0.001
LOC100646094		1.399	6.502	<0.001
LOC100645979		1.397	−0.803	0.021
LOC105665708	LOW QUALITY PROTEIN: uncharacterized protein LOC105665708	1.394	0.855	0.008
LOC100648236	Uncharacterized protein LOC100648236	1.386	3.095	<0.001
LOC100644599		1.370	4.767	<0.001
LOC100646656	Myb-like protein X	1.364	1.395	<0.001

(Continues)

TABLE A3 (Continued)

Feature	Description	LogFC	LogCPM	FDR
LOC100645702		1.361	9.861	<0.001
LOC100652183		1.357	9.345	<0.001
LOC100652258		1.351	9.815	<0.001
LOC100644734		1.344	6.064	<0.001
LOC100642770		1.339	8.595	<0.001
LOC100648102		1.336	9.620	<0.001
LOC100643215		1.335	1.766	0.012
LOC100643695	Vesicular inhibitory amino acid transporter	1.332	0.157	0.001
LOC105666799	Two pore potassium channel protein sup-9	1.331	0.806	0.033
LOC100648304		1.327	6.114	<0.001
LOC110119815		1.325	3.311	<0.001
LOC100648321	Uncharacterized protein LOC100648321	1.300	2.319	<0.001
LOC100646208	Protein PIH1D3	1.292	0.892	<0.001
LOC100642715		1.288	−0.427	0.009
LOC100647986		1.277	6.942	<0.001
LOC100646384	Pupal cuticle protein G1A-like	1.275	1.415	<0.001
LOC100645727	Prohormone-2-like	1.275	1.824	<0.001
LOC100650276		1.268	2.753	<0.001
LOC100650566		1.264	8.947	<0.001
LOC100649387		1.259	5.304	<0.001
LOC100649836		1.257	3.552	<0.001
LOC100645137		1.252	3.719	<0.001
LOC100648970		1.251	−0.611	0.006
LOC100649938		1.247	6.943	<0.001
LOC100651901		1.242	8.224	<0.001
LOC100646624		1.229	4.294	0.044
LOC100647259	Uncharacterized protein LOC100647259	1.228	5.697	<0.001
LOC100647497		1.213	−0.225	0.008
LOC100649579		1.209	9.855	<0.001
LOC100645676		1.202	7.801	<0.001
LOC100646376		1.195	−0.595	0.033
LOC100649407		1.188	9.355	<0.001
LOC100647950	Alpha-tocopherol transfer protein-like	1.184	1.030	0.046
LOC100651491		1.177	2.702	<0.001
LOC100642208	DNA ligase 1-like isoform X6	1.175	2.630	<0.001
LOC100649496	Uncharacterized protein LOC100649496	1.174	0.985	0.006
LOC100645061	Protein odd-skipped	1.171	3.722	<0.001
LOC100642957		1.162	6.871	<0.001
LOC105666369		1.162	1.689	0.004
LOC100649739		1.160	7.421	<0.001
LOC100643243		1.153	2.973	<0.001
LOC100648476		1.150	5.734	<0.001
LOC100648653		1.146	2.896	<0.001
LOC100648558		1.143	3.700	<0.001
F2	Uncharacterized abhydrolase domain-containing protein DDB_G0269086-like	1.134	−0.529	0.011

TABLE A3 (Continued)

Feature	Description	LogFC	LogCPM	FDR
LOC100648973	Protein GDAP2 homolog	1.129	9.913	<0.001
LOC105666926	Uncharacterized protein LOC105666926 isoform X2	1.120	2.081	0.0369
LOC100647147		1.115	3.715	<0.001
LOC110119847	Protein lethal(2)essential for life-like	1.114	4.426	0.039
LOC100645059	SIFamide-related peptide	1.109	−0.695	0.036
LOC100643782		1.102	0.097	0.003
LOC100644956		1.101	4.921	<0.001
LOC100651177		1.095	2.141	<0.001
LOC100647329		1.091	9.649	<0.001
LOC100646320		1.089	0.916	0.017
LOC100642883		1.081	5.822	<0.001
LOC100651656		1.075	7.739	<0.001
LOC100642484		1.074	−0.079	0.030
LOC100648879		1.072	−0.588	0.043
LOC110119508	Uncharacterized protein LOC110119508	1.072	0.436	<0.001
LOC100642826	Protein FAM151B isoform X2	1.069	9.561	<0.001
LOC100651405	Esterase B1-like	1.067	0.321	0.003
LOC110119866	Uncharacterized protein LOC110119866	1.065	0.879	0.013
LOC105666040	Uncharacterized protein LOC105666040	1.064	0.860	0.002
LOC100644862		1.052	7.371	<0.001
LOC105666834		1.045	0.993	0.010
LOC100649218	Uncharacterized protein LOC100649218 isoform X2	1.044	1.947	<0.001
LOC100645036		1.040	2.696	<0.001
LOC100649225	Basic proline-rich protein isoform X1	1.029	−0.635	0.034
LOC100648073		1.026	1.176	0.007
LOC100644397		1.023	1.507	0.015
LOC100644350	Uncharacterized protein LOC100644350	1.018	1.295	0.021
LOC100643873	Prion-like-(Q/N-rich) domain-bearing protein 25 isoform X2	1.017	8.273	<0.001
LOC100645385		1.016	2.750	<0.001
LOC100647323		1.015	6.130	<0.001
LOC100645062		1.012	−0.628	0.008
LOC100646777		1.009	3.179	0.001
LOC100645806	Growth factor receptor-bound protein 14 isoform X2	1.004	6.502	<0.001
LOC100644243	Probable salivary secreted peptide	1.002	12.695	<0.001
LOC100649384		−1.008	5.405	<0.001
LOC100650561		−1.017	7.586	<0.001
LOC100643490		−1.018	8.027	<0.001
LOC100649785		−1.019	4.479	<0.001
LOC100647616		−1.020	5.533	0.021
LOC100646229		−1.023	5.296	<0.001
LOC100649475		−1.024	10.228	<0.001
LOC105666138		−1.035	6.859	<0.001
LOC100642963	Histidine-rich glycoprotein-like	−1.045	5.154	0.002
LOC100651034		−1.045	6.727	<0.001
LOC100642358		−1.045	3.873	<0.001

(Continues)

TABLE A3 (Continued)

Feature	Description	LogFC	LogCPM	FDR
LOC100648843		−1.049	8.843	<0.001
LOC100642272		−1.054	8.741	0.021
LOC100631070	Melittin	−1.059	3.766	0.008
LOC100642297	Lysozyme-like	−1.061	10.682	0.002
LOC100649166		−1.061	4.248	<0.001
LOC100644014		−1.072	6.401	<0.001
LOC100651129	Protein G12	−1.078	10.283	0.043
LOC100645024		−1.091	8.467	<0.001
LOC100644917		−1.091	4.886	<0.001
LOC100647588	Long-chain fatty acid transport protein 4-like	−1.096	6.759	<0.001
LOC100644715	Polypeptide N-acetylgalactosaminyltransferase 2	−1.100	4.682	<0.001
LOC100651969	Uncharacterized protein LOC100651969 isoform X2	−1.100	0.890	0.002
LOC100646207		−1.101	5.682	0.002
LOC105666529	Aquaporin-11	−1.102	2.117	<0.001
LOC100644235	Uncharacterized protein LOC100644235	−1.107	1.968	<0.001
LOC100646060		−1.114	7.402	<0.001
LOC100648993		−1.115	9.557	<0.001
LOC100648212		−1.117	7.707	<0.001
LOC100646721		−1.135	5.557	<0.001
LOC100646290		−1.146	1.245	0.041
LOC100643349		−1.147	10.562	0.014
LOC100644362		−1.171	5.665	<0.001
LOC100643278	Uncharacterized protein LOC100643278	−1.185	4.474	0.007
LOC100645388		−1.195	5.780	<0.001
LOC100647598		−1.198	3.658	<0.001
LOC100643624		−1.203	8.331	<0.001
LOC100643512		−1.214	8.551	<0.001
LOC100646642		−1.217	2.851	0.003
LOC100642930		−1.218	6.323	<0.001
LOC100646691		−1.219	10.406	<0.001
LOC100649890	Alpha-tocopherol transfer protein-like	−1.230	0.789	0.023
LOC100650536		−1.236	6.399	0.002
LOC100651809		−1.236	5.354	<0.001
LOC100649409		−1.241	5.340	<0.001
LOC100645662		−1.243	9.781	<0.001
LOC100649281		−1.253	5.567	<0.001
LOC100648311		−1.261	5.069	<0.001
LOC100646687		−1.265	13.363	<0.001
LOC100643086		−1.268	6.574	<0.001
LOC100646858	Uncharacterized protein LOC100646858	−1.271	1.155	0.035
LOC100650878		−1.281	0.984	0.002
LOC100642446		−1.285	1.803	<0.001
LOC100642488	Ionotropic receptor 75a-like	−1.292	2.460	<0.001
LOC100646246		−1.311	5.411	<0.001
LOC100649270		−1.312	8.042	<0.001

TABLE A3 (Continued)

Feature	Description	LogFC	LogCPM	FDR
LOC100648174		−1.312	1.204	0.003
LOC100649872		−1.314	6.120	<0.001
LOC100648029		−1.315	6.794	<0.001
LOC100647832		−1.322	4.922	<0.001
LOC100645755		−1.332	3.458	<0.001
LOC100650947		−1.346	7.668	<0.001
LOC100652063	Trissin	−1.354	1.343	<0.001
LOC100645107		−1.356	8.581	<0.001
LOC100651500	Mid1-interacting protein 1-B	−1.357	3.443	<0.001
LOC100645894		−1.359	2.278	<0.001
LOC100645429		−1.360	6.319	<0.001
LOC100650250		−1.364	0.367	0.008
LOC100645461		−1.374	5.010	<0.001
LOC100643020		−1.381	8.281	<0.001
LOC100646701		−1.393	8.105	<0.001
LOC100647539		−1.407	8.027	0.002
LOC100649304		−1.416	6.659	<0.001
LOC100642695		−1.421	−0.396	<0.001
LOC100646080		−1.424	6.440	<0.001
LOC100647261		−1.429	6.854	<0.001
LOC100645568		−1.435	4.554	<0.001
LOC100645839		−1.441	5.714	0.007
LOC100643609		−1.444	3.461	<0.001
LOC100644742		−1.445	4.297	<0.001
LOC100652226		−1.456	6.961	<0.001
LOC100647540		−1.466	5.081	<0.001
LOC100647578		−1.482	7.434	<0.001
LOC100651196		−1.491	6.564	<0.001
LOC100644600		−1.500	6.213	0.006
LOC100652036		−1.510	0.330	0.017
LOC100648169		−1.518	5.991	<0.001
LOC100650111	Uncharacterized protein LOC100650111	−1.531	6.779	0.004
LOC100643779		−1.542	5.867	0.008
LOC100648980		−1.547	4.108	<0.001
LOC100644177		−1.561	2.197	<0.001
LOC100644225		−1.563	8.560	0.002
LOC100644459		−1.572	3.927	<0.001
LOC100644716	Proton-coupled amino acid transporter-like protein pathetic	−1.576	6.177	<0.001
LOC100647785		−1.589	9.673	<0.001
LOC100651168	Heterogeneous nuclear ribonucleoprotein A3 homolog 2-like isoform X2	−1.594	10.511	<0.001
LOC100644772		−1.598	4.675	<0.001
LOC100646078		−1.606	11.649	<0.001
LOC100652210		−1.609	7.475	<0.001
LOC100646491		−1.615	4.672	<0.001

(Continues)

TABLE A3 (Continued)

Feature	Description	LogFC	LogCPM	FDR
LOC100650628		−1.619	4.326	<0.001
LOC100644921	Proton-coupled amino acid transporter-like protein CG1139	−1.621	6.500	<0.001
LOC105667110		−1.668	4.511	<0.001
LOC100645013		−1.669	6.269	<0.001
LOC100646373		−1.674	9.611	<0.001
LOC100649608		−1.681	8.549	<0.001
LOC100645163		−1.751	9.563	<0.001
LOC100652301		−1.753	5.583	<0.001
LOC100649568		−1.763	2.324	<0.001
LOC100652268	Cysteine-rich venom protein 1-like isoform X1	−1.773	0.064	<0.001
LOC100648451		−1.778	2.360	<0.001
LOC100649144		−1.781	8.292	<0.001
LOC100646186		−1.783	4.856	<0.001
LOC105666640		−1.791	3.144	<0.001
LOC100647719		−1.794	6.790	<0.001
LOC100647796		−1.795	10.385	<0.001
LOC100646617	Uncharacterized protein LOC100646617	−1.796	8.254	<0.001
LOC100644966	Uncharacterized protein LOC100644966	−1.832	9.266	<0.001
LOC100651268		−1.833	6.843	<0.001
LOC100646752	Uncharacterized protein LOC100646752	−1.837	2.764	<0.001
LOC105666139		−1.875	6.449	<0.001
LOC100646598		−1.877	7.588	<0.001
LOC100650460		−1.900	6.629	<0.001
LOC100643115	Uncharacterized protein LOC100643115	−1.919	5.810	0.023
LOC100644713		−1.959	8.841	<0.001
LOC100644893	Neurotrimin-like isoform X1	−1.968	5.651	<0.001
LOC100648883		−1.968	8.224	<0.001
LOC100645985	—NA—	−1.985	4.826	0.029
LOC100647222		−1.996	10.755	<0.001
LOC100649178		−2.001	0.465	<0.001
LOC100645831		−2.021	0.541	0.001
LOC100650649		−2.064	7.973	<0.001
LOC100644337	Uncharacterized protein LOC100644337	−2.081	7.848	<0.001
LOC100648482		−2.130	6.922	0.002
LOC100651812		−2.184	7.630	<0.001
LOC100642508		−2.226	2.487	<0.001
LOC105666790		−2.244	3.217	<0.001
LOC100648508	Uncharacterized protein LOC100648508	−2.298	9.712	<0.001
LOC100647241		−2.314	3.493	<0.001
LOC100648563		−2.314	3.255	<0.001
LOC100645349		−2.319	5.686	<0.001
LOC100644867		−2.330	6.433	<0.001
LOC100650704		−2.350	4.803	<0.001
LOC100643391	Zwei Ig domain protein zig-8	−2.378	4.512	<0.001
LOC105667180	Uncharacterized protein LOC105667180	−2.392	3.648	<0.001

TABLE A3 (Continued)

Feature	Description	LogFC	LogCPM	FDR
LOC100647739	Cell wall protein RBR3-like	−2.444	3.621	<0.001
LOC100643622		−2.485	5.346	<0.001
LOC100646104	Endochitinase A1-like	−2.577	2.397	0.041
LOC100649907		−2.734	6.882	<0.001
LOC100643254	Uncharacterized protein LOC100643254	−2.860	3.945	<0.001
LOC100646690		−2.880	5.792	<0.001
LOC100648425		−3.060	2.570	<0.001
LOC100649744		−3.112	11.820	<0.001
LOC110119840	Lymphocyte expansion molecule-like	−3.228	−0.248	<0.001
LOC100650436		−3.240	10.056	0.039
LOC100644470		−3.506	6.307	<0.001
LOC100647759		−3.915	10.417	<0.001
LOC100644839		−4.203	3.045	<0.001
LOC100645869	Elastin-like	−6.097	3.083	0.003

Note: Positive log-fold change (logFC) indicates higher expression in the DEP treatment.

Article 3

Exposure to diesel exhaust particles impairs takeoff but not subsequent homing and foraging behavior of workers of the buff-tailed bumblebee *Bombus terrestris*

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RESEARCH ARTICLE

Exposure to diesel exhaust particles impairs takeoff but not subsequent homing and foraging behavior of workers of the buff-tailed bumblebee *Bombus terrestris*

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Abstract
The loss of insect diversity and biomass has been documented in many terrestrial ecosystems. Drivers of this insect decline include climate change, habitat degradation, and pollution. Exposure to airborne particulate matter, such as diesel exhaust particles, may be harmful, especially for insects around urban or industrial areas. Ecotoxicological experiments have shown that chronic oral uptake of diesel exhaust particles can result in higher mortality and changes in the gut microbiome in bumblebees. However, how such effects manifest under natural conditions is still largely unknown, especially effects on foraging activity. Here, we exposed workers of the bumblebee *Bombus terrestris* to diesel exhaust particles in the field at distances of 380 m and 1100 m from their colony. We measured the time until bumblebees took off, the duration of their homing flight after a one-time exposure, and subsequent foraging activity over 1.5 days, recording the number and duration of the foraging flights in comparison to untreated bumblebees. The treated bumblebees needed significantly longer to start their homing flight, caused by some workers that were even unable to take off vertically from the exposure box and performing extensive grooming behavior. Homing flight duration and the subsequent foraging activity did not differ between treated and control workers. It remains unclear why bumblebees struggled to take off after exposure to diesel exhaust particles. This observation needs further investigation to elucidate whether this behavior is induced by particulate matter in general or related to specific physico-chemical properties of the particles inducing a physiological effect.

Keywords Air pollution · Particulate matter · Insect decline · Pollinator · Homing flight

Introduction

The global decline in biodiversity and its ecological consequences have shifted into the focus of research, policy, and society. Preserving biodiversity with a wide variety of life forms and the associated individual traits and interactions contributes to maintaining the stable functioning of ecosystems (Cardinale et al. 2012, Loreau et al. 2001). Insects provide an array of ecosystem services, such as pollination, nutrient cycling, or decomposition of organic matter (Cardoso et al. 2020, Noriega et al. 2018). Hence, the ongoing loss of insect biomass and diversity observed in many regions worldwide (Cowie et al. 2022, Hallmann et al. 2017, Wagner et al. 2020) threatens the stability and resilience of ecosystems. The loss of insect biodiversity is, besides biological impacts, primarily driven by anthropogenic factors like climate change as well as habitat destruction and environmental pollution due to intensive agriculture and proceeding urbanization (Cameron and Sadd 2020, Ganivet 2020, Müller et al. 2023, Sánchez-Bayo and Wyckhuys 2019, Uhler et al. 2021, Wagner 2020). Research on the effects of environmental pollution on insects focused mainly on pesticides and fertilizers as they are extensively applied in agriculture (Sánchez-Bayo

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and Wyckhuys 2019). The recurrent use of fertilizer leads to floral homogenization and, consequently, to a simplification of associated insect biodiversity (Cameron and Sadd 2020, Sánchez-Bayo and Wyckhuys 2019). Pesticides have various negative impacts on insects depending on the dose applied. Even in non-target species such as bees and bumblebees, high doses may increase mortality, while sublethal doses can impair neurological functions responsible for memory, navigation, and motor function (Cameron and Sadd 2020, Stanley et al. 2016, Tison et al. 2017, Tosi et al. 2017), as well as the immune system (Czerwinski and Sadd 2017).

Especially in urban areas, an important contributor to environmental pollution is airborne particulate matter. It is mainly generated and emitted into the atmosphere by domestic heating, industry, and traffic (Dimitriou and Kassomenos 2014, Jandacka and Durcanska 2019). These air pollutants vary in their composition and size depending on their origin. Particles from road traffic make up around 20% of all airborne particulate matter in Western Europe and are separated into non-exhaust and exhaust particles (Hopke et al. 2020). The incomplete combustion of fuels leads to the production of a non-volatile and quantitatively large proportion of exhaust airborne particulate matter. The exhaust airborne particulate matter from diesel engines consists of elementary carbon. Due to the surface properties, it mainly binds organic components and polycyclic aromatic hydrocarbons in small amounts, as well as metals and other trace elements (Hüftlein et al. 2023, Sánchez-Piñero et al. 2022, Viteri et al. 2021, Wichmann 2007). Because of their small size of ≤ 10 or ≤ 2.5 µm (PM_{10} and $PM_{2.5}$, respectively), particles are inhaled easily and thus represent a serious health problem for humans. The harmful properties of diesel exhaust particles are associated with the large proportion of polycyclic aromatic hydrocarbons they contain and their carcinogenic, mutagenic, and immunosuppressant effects on mammals, including humans (Kim et al. 2013, Pant et al. 2017, Sánchez-Piñero et al. 2022, Viteri et al. 2021). Once taken up, reactive metabolites of the polycyclic aromatic hydrocarbons can bind to cellular proteins and DNA, which disrupts the biochemistry of the cells and consequently leads to their damage (Lee et al. 2002). There is less scientific evidence regarding the potentially debilitating effects of polycyclic aromatic hydrocarbons or diesel exhaust particles on invertebrates. However, for most of the invertebrate species that have been studied to date extended exposure to polycyclic aromatic hydrocarbons resulted in adverse effects, including insects, mussels, or annelids. The exposure typically leads to oxidative stress, resulting in a suppressed immune function, significant DNA damage, and increased mortality (Ball and Truskewycz 2013). Because insects are a very diverse group, the impact of polycyclic aromatic hydrocarbons on the metabolism can be manifold. Especially physically demanding and cognitive activities, such as flying or finding food, might be impaired under oxidative stress.

Eusocial pollinators such as honeybees and bumblebees are central place foragers and stay in the area of their colony and brood they care for. As a result, their reproductive success depends on the success in foraging and the quantity and quality of the resources in the landscape surrounding the nest (Osborne et al. 2008). Additionally, the pollution level around the nest influences pollinators (Gradish et al. 2019). Traditionally, researchers used honeybees for the assessment of ecotoxicological effects of substances on pollinators. However, honeybees and bumblebees differ in their life history, behavior, and morphology, affecting susceptibility and exposure to pollutants (Gradish et al. 2019). Solitary foraging in spring and autumn may result in high exposure to bumblebee queens. Many bumblebee species build their nest underground, exposing the colony, including the brood, to residues of pollutants in the soil (Gradish et al. 2019). The use of bumblebees for the assessment of potentially harmful substances helps to predict the impact of these substances on some of the other important wild bee species (Gradish et al. 2019). The buff-tailed bumblebee *Bombus terrestris* (Linnaeus, 1758) is common and widespread in Central and Western Europe, especially in urban areas (Goulson et al. 2008), and is commercially bred for greenhouse pollination. These aspects make it a suitable, if not necessary, test organism to study the effects of diesel exhaust particle exposure. The diesel exhaust particles can be taken up by bumblebees orally via pollen and nectar (Leita et al. 1996, Hüftlein et al. 2023, Seidenath et al. 2023, Seidenath et al. 2024) and may additionally stick to their cuticle, hairs, and wings (Balestra et al. 1992, Negri et al. 2015). Such diesel exhaust particle deposits on the body surface may impede the flight activity of bumblebees, similar to the painted lady *Vanessa cardui* showing decreased speed, flight distance, and stamina after exposure to combustion-generated airborne particulate matter (Liu et al. 2021).

Although social insects have emerged as model organisms and bioindicators to study the effects of anthropogenic pollution because of their ecological dominance and importance (Cameron & Sadd 2020, Chapman and Bourke 2001, Leita et al. 1996), only a few publications have examined the effects of diesel exhaust particles on pollinators so far. Exposure to high doses of diesel exhaust impairs associative learning abilities, memory, and tolerance to additional abiotic stress in honeybees at the individual level but also reduces colony fitness (Reitmayer et al. 2019, Reitmayer et al. 2022). We have shown increased mortality of bumblebee workers when fed with high doses of diesel exhaust particles over several days (Hüftlein et al. 2023) and a shift in the gut microbiome and transcriptome after sublethal exposure (Seidenath et al. 2023) while colony development was not affected negatively under laboratory conditions

(Seidenath et al. 2024). In addition, pollinators can be affected indirectly. Pollution with diesel exhaust leads to the rapid degradation of floral volatiles, which hinders the perception of flowers and reduces the foraging efficiency and, thus, the pollination performance of honeybees (Girling et al. 2013, Lusebrink et al. 2015, Ryalls et al. 2022).

To test if we find similar effects of airborne particulate matter in a wild bee, we exposed *Bombus terrestris* workers to diesel exhaust particles in a field experiment and tracked their flight activity. We collected workers from their colony and released them at two different distances from the nest after a one-time exposure to diesel exhaust particles. We monitored the behaviour after release and the time a worker needed to return to its colony, i.e. the homing flight. We also observed the subsequent flight activity, i.e. duration and number of foraging flights. Since diesel exhaust particles may stick to the body surface of the bumblebees, we expect that treated bumblebees initially start auto-grooming and wiping off the particles and thus need more time to start vertically from the exposure boxes compared to the untreated bumblebees of the control group. Additionally, it is conceivable that exposed bumblebees perform additional cleaning stops during the flight, have an impaired spatial orientation, a lowered motivation doing foraging flights, or are negatively affected by the harmful properties of the particles. We, therefore, expect that bumblebees treated with diesel exhaust particles need more time to fly back to their colony and assume that fewer individuals will find their way home. Finally, we expected an impaired foraging activity of the bumblebees and hypothesized that bumblebees treated with diesel exhaust particles do foraging flights less frequently and for longer than the bumblebees of the control group.

Material and methods

Bumblebee husbandry

At the beginning of August 2021, we ordered a *Bombus terrestris* colony from Biobest® (Biobest Group NV, Belgium) with an estimated 50 individuals. We kept this colony in a ventilated box (21 × 13 × 17 cm) in a climate chamber under controlled conditions (26 °C, 70% humidity) with an inverted day and night rhythm of 12:12 h of light–dark cycles. We provided the colony with ad libitum sugar water (50% Apiinivert®, Südzucker AG, Mannheim, Germany) and pollen (Imker Pur, Osnabrück, Germany). After eleven days in the climate chamber, we placed the colony in a meadow on the campus of the University of Bayreuth and left it there for 13 days to acclimatize. During this period, the workers were allowed to forage under natural conditions and get acquainted with the new environment. In addition to the meadow where we placed the colony, the Ecological Botanical Garden of the University of Bayreuth, immediately adjacent to the campus area, guaranteed the food supply of the colony.

Experimental procedure

After the acclimatization period, we conducted the field experiments on three days within one week in the early September 2021 (2^{nd} to 8^{th} September). The air temperature on these days ranged between 9.9 °C at night and 21.8 °C maximum during the day with slightly cloudy to sunny weather. Over the three days, we tagged 80 bumblebees when leaving the colony with an individual square tag for identification (see: individual identification and transport). We randomly assigned the bumblebees to either a diesel exhaust particle exposure treatment ($N = 40$) or no treatment (control group) ($N = 40$). Then, we transported half of the exposed and half of the control group to 380 m ($N = 2 \times 20$) and the other two halves 1100 m away ($N = 2 \times 20$) from the colony. There, we released the workers and automatically tracked the foraging and flight activity of the bumblebees for 1.5 days via video camera (time of release: approx. 2:30 p.m.–11:59 p.m. the following day). We defined the homing flight duration of a worker as the period between the takeoff and the first observation by the video recording device. After the first return to the colony of each bumblebee, we observed flight activity for 1.5 days. We measured the foraging duration as the time between leaving and returning to the colony. Moreover, the number of foraging flights was counted. Flights of bumblebees that either did not return to their colony or only returned the next day and thus spent the night outdoors were not included in the measurements of the foraging activity.

Individual identification and transport

To label individual bumblebees, we created AprilTags 3 (type 36h11) with 2D barcodes using the code for generating tag families from APRIL Robotics Laboratory (Edwin 2019) based on the work of Krogius et al. (2019). The 2D barcodes (4 × 4 mm) consist of a 36-bit code, which can generate a total of up to 587 unique identification numbers. The 2D barcodes were printed on white plastic film (1 mm, laserFol PETP 275 opak, creativ papier, Neuenhaus, Germany) and additionally provided with the associated identification number with a maximum of three digits in a font size of 5 pt to allow a direct readout by observers. We cut out the tags manually to a dimension of approximately 5 × 6 mm (Fig. 1).

For tagging, we transferred each bumblebee to an individual live capture jar (22 × 63 mm) and placed it on ice for 10–20 min until immobilization. Then, each bumblebee was carefully fixed between fingers and working surface to attach the individual tag to the thorax with a small drop of

Fig. 1 Image of a single frame with a top view of the arena from one of the videos recorded. Shown is a tagged bumblebee that recently entered and a stone in the center of the arena that served as a barrier for the bumblebees to prevent them from crossing the arena too fast on foot or even in flight

odorless superglue (UHU® GmbH & Co. KG, Germany) placed between the first yellow and black stripe on the mesonotum. During this process, we ensured that the lower edge of the tag was slightly above the first pair of wings and did not cover the tegulae. Like this, the tags do not impair the wing movement. Depending on the size of the bumblebees, the upper part of the tag reached over the head but not over the total length of the antennae. At the end of the procedure, we visually inspected each bumblebee to ensure normal wing and head movement. All bumblebees failing the process of tagging were excluded and replaced. After tagging, we individually transferred the bumblebees into transport cages, in which they remained until the exposure to the treatment and their release. The transport cages consisted of a Nicot®-Queen cage (Nicotplast SAS, Maisod, France) attached to a 10 ml plastic syringe, which we filled with one ml of sugar water. During this period, the bumblebees were able to recover from the previous process and ingest sucrose solution ad libitum.

Colony observation

To protect the colony and the video recording device (see below) from bad weather conditions and other external influences, we put the colony box into a gray, untransparent plastic box with a hinged lid and ventilation holes on the sides (60×40 × 33.5 cm, Auer-Packaging, Amerang, Germany; Fig. 2). The bumblebees were able to leave and enter the colony by crossing a transparent and flexible PVC tube (inner diameter 1.6 cm) that served as a passage between the colony and the outside environment (Fig. 2).

All bumblebees leaving or entering the colonies had to pass through an arena (8.54×2.5 × 8.54 cm) located inside the plastic box between the colonies and the outer flight hole (Fig. 2). The arena consisted of a white plastic base and a transparent plexiglass cover. Above the arena, we installed a tower (12.2×26.9×12.2 cm), which carried the video recording device required to track the flight activity of the bumblebees during the experiment. To identify the bumblebee individuals and their exact homing flight duration, we used a Raspberry Pi HQ Camera V1.0 2018 (12.3 megapixel, Raspberry Pi Trading Ltd. Cambridge, England) and a Raspberry Pi single-board computer (Raspberry Pi Model 3B, Raspberry Pi Trading Ltd.) to record short videos when a bumblebee passed the arena (for an overview of the camera settings see Table 1 in Appendix). We fixed the camera and single-board computer centrally above the arena at the

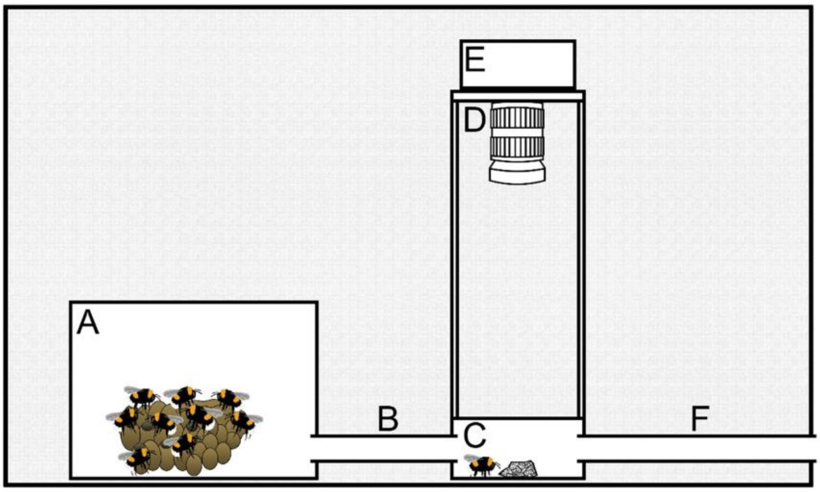

Fig. 2 Schematic of bee filming setup inside Euro container: **A** bumblebee colony, **B** tube connecting colony and **C** filming arena containing stone to hinder fast passage of arena, **D** HD-Camera, **E** Raspberry Pi computer for movement detection and recording of videos, **F** exit tube

highest point of the tower (Fig. 2). We 3D-printed the arena and the tower (for detailed information on the dimensions, see Figs. 6 and 7). We controlled the camera via a web-based interface (Melchior and Tidey 2013). As soon as the camera detected any motion in the frame, it started to record a video while the movement lasted. We stored the data as mp4 video files on an SD card for later analyses. With an LED light strip (length: 48.8 cm, 14 LEDs) attached about 4 cm above each arena at the respective outer edges of the tower, we ensured constant lighting even during twilight (Fig. 2). In addition, we placed a small stone (approx. 2 × 1.5 × 2 cm) in the center of the arena (Fig. 1) to serve as a small barrier for the bumblebees. It prevented them from crossing the arena too fast on foot or even in flight and thus improved the automatic detection of barcodes or manual reinspection of video material and identification of individual bumblebees.

Controlled production of diesel exhaust particles

We collected diesel exhaust particles from a four-cylinder diesel engine (OM 651, Daimler AG, Stuttgart, Germany) during a reaping cycle of transient and stationary operating points, resembling an inner-city driving scenario with stop-and-go intervals. We operated the engine on a test bench with a water-cooled eddy-current brake, as previously described in Zöllner (2019). We collected diesel exhaust particle samples with an electrostatic precipitator (OekoTube Inside, Mels-Plons, Switzerland). We applied a fast response differential mobility particulate spectrometer DMS500 (Combustion, Cambridge, England) to measure sub-micron particle size distributions of raw exhaust samples. Solid particles showed a median diameter between 52.1 ± 1.8 nm and 101.9 ± 1.7 nm, depending on engine load and speed during the inner-city cycle.

We characterized diesel exhaust particle composition by thermogravimetric analysis (TGA, STA 449 F5 Jupiter, Netzsch-Gerätebau GmbH, Selb, Germany). A fraction of 72.2 ± 1.1% of the diesel exhaust particle mass was attributed to elemental carbon, 23.2 ± 0.9% w/w to organic fractions, and 4.6 ± 0.7% w/w to inorganic matter. Quantification of polycyclic aromatic hydrocarbons revealed concentrations of 444 ppm for pyrene, 220 ppm for phenanthrene, and 107 ppm for fluoranthene. We analyzed the elemental composition of the diesel exhaust particle samples by Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES, Optima 7300 DV, PerkinElmer Inc., Waltham, United States of America) and interpreted it according to Zöllner (2019). It showed fractions of calcium (1.63% w/w), zinc (0.53% w/w), and phosphorus (0.50% w/w) that can be traced back to diesel fuel and lubrication oil. Copper (1.03% w/w), aluminum (0.02% w/w), and iron (0.02% w/w) can be attributed to abrasion of piston rings, cylinder head, and engine block material, respectively. In addition, we found small amounts of boron (0.13% w/w), magnesium (0.10% w/w), molybdenum (0.03% w/w), natrium (0.02% w/w) and sulphur (0.17% w/w).

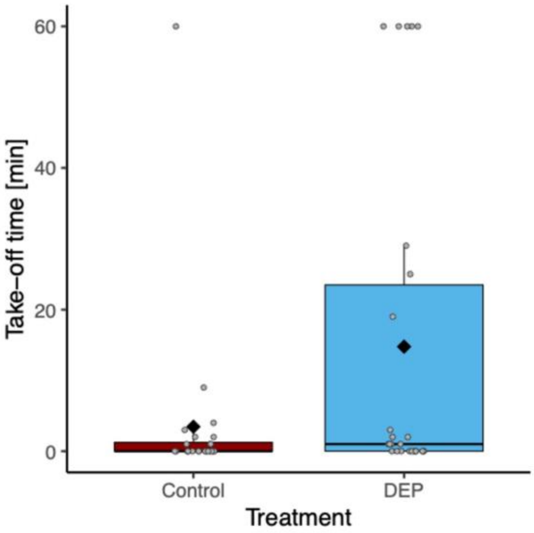

Fig. 3 Comparison of the bumblebee takeoff times (Control $n=39$, diesel exhaust particle exposed $n=37$) in minutes. The diesel exhaust particle exposed bumblebees needed significantly longer to take off vertically out of the exposure boxes than the bumblebees of the control group (Kruskal–Wallis rank-sum test: $X^2=8.85$, $df=1$, $p=0.003$). Shown are boxplots with median and the first and third quartiles (Q1-Q3). The mean values are represented by the large diamond shape in each box. Small dots represent individual data points

Diesel exhaust particle exposure and behavioral observations

The exposure to the treatment with diesel exhaust particles (DEP) and the subsequent release of the bumblebees occurred at two different locations at distances of either 380 or 1100 m southeast of the colonies. Consequently, there were four different treatment-distance combinations: Control_380m, DEP_380m, Control_1100m, and DEP_1100m. At the two exposure sites, the bumblebees were simultaneously transferred to individual square plastic boxes with a volume of 200 ml (approx. 7.5 × 5 × 7.5 cm) in a randomized order and locked in for exactly three minutes. The boxes with the diesel exhaust particle treatment contained 1.5 mg ± 0.1 mg (mean ± SD) diesel exhaust particles, while the control did not contain any particles. Inside the boxes, the diesel exhaust particles are whirled up due to the wing movement of the bumblebees, resulting in a distribution of the particles and subsequent contamination of the bumblebees' body surface. After three minutes, the boxes were opened, and the bumblebees were able to take off.

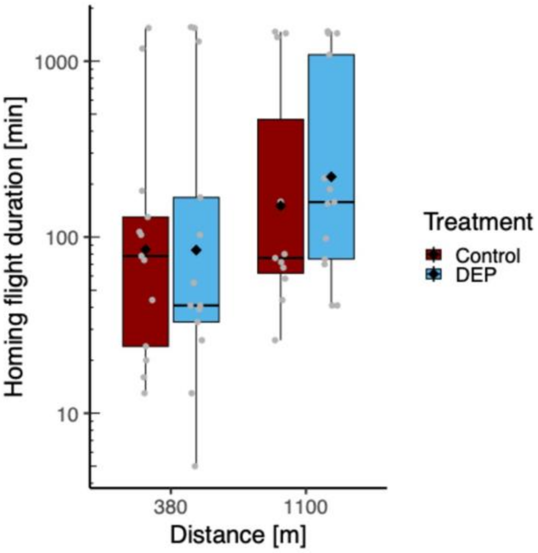

Fig. 4 Comparison of homing flight duration (Control_380m ($n=19$), Control_1100m ($n=20$), DEP_380m ($n=16$), DEP_1100m ($n=20$), $N=75$) depending on the treatment and distance combination. The line in each box represents the median. Shown are boxplots with median and the first and third quartiles (Q1-Q3). The mean values are represented by the large diamond shape in each box. Small dots represent individual data points

Exposure and release took place in the afternoon from 2:45 p.m. to 4:45 p.m. We measured the time each bumblebee needed to leave the exposure box. If a worker did not manage to leave the box within one hour, we released it exactly after 60 min on the meadow next to the box. As all those workers immediately took off and flew away, we included them in our further analysis.

Statistical analysis

We recorded a total of 56,708 individual video files during all test days. Four bumblebees (one bumblebee of the control group and three diesel exhaust particles treated bumblebees) were excluded from the statistical analysis because they lost their tags inside the transport boxes or escaped after tagging but before treatment. When examining the exact homing flight duration of the bumblebees, we had to exclude one individual treated with diesel exhaust particles and released at a 380 m distance from the analyses. Although this bumblebee returned to the colony on the day of the treatment, we could not determine the exact homing flight duration. We evaluated the videos manually using the online available 'SMPlayer' (Version 21.1.0, by Ricardo Villalba, open source). Within this process, we identified the bumblebees and the date and the time of arrival or departure from the colony.

All statistical analyses were conducted with R version 4.1.1 (R Core Team, 2021). We used Pearson's Chi-square test of independence to analyze the effect of the treatment, distance, and their interaction term on the bumblebees' ability to return to the colony. This ability we measured as the proportion of workers that found their way back to the colony. We analyzed the impact of the treatment with diesel exhaust particles on the start behavior and the homing flight duration by conducting Kruskal–Wallis rank sum tests, as the residuals were not normally distributed. The number and duration of the foraging flights were analyzed by fitting generalized linear models (GLMs) with treatment as a predictor. We checked model assumptions using model diagnostic test plots, i.e., qqplot and residual vs. predicted plot from the package *DHARMa* (Hartig 2022). For the GLMs we did F-statistics with the function *Anova*() from the package *car* (Fox & Weisberg 2019) to calculate p-values for differences between the two groups. We used the package ggplot2 (Villanueva and Chen 2019) for plotting the data.

Results

Overall, 67.11% (51 of 76 individuals) of the bumblebee workers found their way back to the colony within 1.5 days. The proportion of returnees was not affected by treatment (Pearson's Chi-squared test: $X^2=0.66$, $df=1$, $p=0.41$), distance (Pearson's Chi-squared test: $X^2=1.31$, $df=1$, $p=0.25$), or the treatment-distance combination (Pearson's Chi-squared test: $X^2=3.17$, $df=3$, $p=0.37$). However, the bumblebees from the diesel exhaust particle treatments needed significantly longer to take off from the exposure boxes (Kruskal–Wallis rank-sum test: $X^2=8.85$, $df=1$, $p<0.01$; Fig. 3).

Homing flight duration did not differ between treatments (Kruskal–Wallis rank-sum test: $X^2=0.16$, $df=1$, $p=0.69$), distance (Kruskal–Wallis rank-sum test: $X^2=3.33$, $df=1$, $p=0.07$), or treatment-distance combination (Kruskal–Wallis rank-sum test: $X^2=3.97$, $df=3$, $p=0.27$) (Fig. 4).

Foraging activity was not affected by treatment. Neither the number of foraging flights differed between treatments (GLM with Gaussian distribution: $F_{1,37}=0.72$, $p=0.40$) nor the mean duration of foraging flights (GLM with Gaussian distribution: $F_{1,30}=0.14$, $p=0.71$) (Fig. 5).

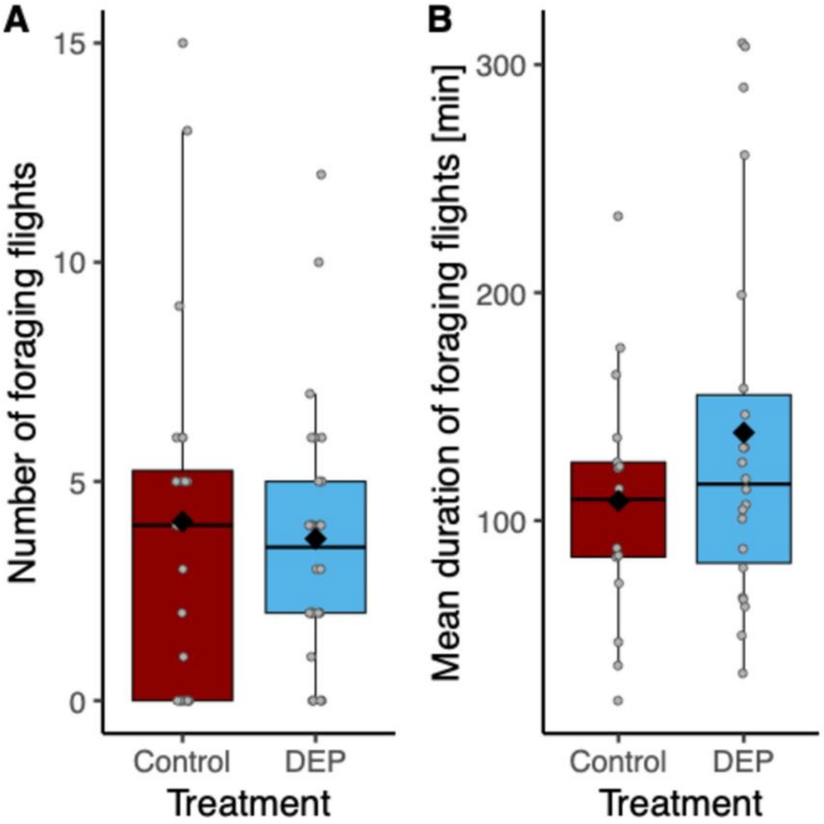

Fig. 5 Box plots showing the effect of the diesel exhaust particle exposure compared to the control group of **A** the average number of foraging flights and **B** the mean foraging flight duration for each bumblebee over 1.5 days. The line in each box represents the median. Shown are boxplots with median and the first and third quartiles (Q1–Q3). The mean values are represented by the large diamond shape in each box. Small dots represent individual data points

Discussion

In our study, we found that exposure to diesel exhaust particles increases the flight takeoff time of bumblebees after their release to perform a homing flight (Fig. 3). In contrast, the return flight to the colony was not affected by treatment (Fig. 4). Moreover, we did not see any differences in the subsequent foraging behavior (Fig. 5).

Our results show that the bumblebees exposed to diesel exhaust particles needed significantly more time to start vertically out of the exposure boxes than the untreated individuals (Fig. 3). One reason is that the treated bumblebees initially started wiping off the particles from their body surface. Hlavac (1975) described this process as auto-grooming. In this process, a set of setae (grooming structures) are arranged at a slant on legs or other movable body parts, which scrape against each other and along cuticular projections, wings, or mouth parts to transport particles from the body surface and sensory organs and eliminate them. The treated bumblebees of our experiment also distinctly showed this auto-grooming behavior, which explains the delayed takeoff start to some extent. However, we observed struggles in the diesel exhaust particle exposed bumblebees to fly vertically out of the box, causing a delayed start. We frequently observed diesel exhaust particle exposed bumblebees flying straight into the wall of the treatment box, incapable of overcoming the only 5 cm high rim to leave it. The struggle caused enormous delays in the flight takeoff time, forcing us to manually place the bumblebees outside the box after 60 min to measure the homing flight duration. The reasons for this behavior remain unclear. However, it could indicate underlying physiological malfunctions. For example, diesel exhaust particles might affect the sensory systems, especially on the antennae that carry different types of sensillae with functions in sensing chemical, thermal, mechanical, and water stimuli (Fialho et al. 2014, Rands et al. 2023). In addition, the visual perception of the bumblebees may be affected, which could impede the takeoff by failing to identify the walls of the box as a barrier and thus flying straight into them. Diesel exhaust particle deposition on mechanosensory hairs of the bumblebees may also impair the perception of electric fields, leading to motoric struggles to overcome the barrier (Sutton et al. 2016).

Contrary to our expectations, our results indicate that diesel exhaust particle exposure neither reduced the ability to return to the colony nor the homing flight duration (Fig. 4). In addition, the proportion of bumblebees that returned to the colonies did not depend on diesel exhaust particle exposure, flight distance, or their combination. We take this as evidence that a one-time exposure to diesel exhaust particles does not impair cognitive abilities and thus negatively affects spatial orientation and navigation. Besides visual landmarks (Ne'eman and Ne'eman 2017), the sun as a compass, and the polarization pattern of the sky (Wehner et al. 1996), bumblebees also rely on olfactory cues to navigate within their environment (Ne'eman and Ne'eman 2017). However, bumblebees cannot fully compensate for visual cues by other senses aiding in spatial orientation, as shown by experiments in complete darkness (Chittka et al. 1999). The observed problems in vertical takeoff by bumblebees exposed to diesel exhaust particles seem to be a short-term impairment from which the bumblebees recover rather rapidly, possibly due to the removal of the particles by grooming, as the longer homing flight is not affected.

In addition to the perception of sensory or olfactory impressions and their storage, the memory and retrieval of this information also play an important role in orientation. Previous studies show that sublethal doses or field-realistic levels of pesticides such as neonicotinoids negatively affect the learning behavior and short-term memory of honeybees (Tison et al. 2017) and bumblebees (Stanley et al 2015). Reitmayer et al. (2019) found that an acute exposure of diesel exhaust at high doses and over a long time (150–210 min; containing NO and NO_2) leads to impaired learning and memory of floral odors in honeybees. Thus, Reitmayer et al. (2019) suggested that treated bees need more repetitions to learn and accomplish the same task. As we did not see any difference in the ability to return to the colony between the treatments, our data suggest no cognitive impairment of the bumblebees in our experiment. The bumblebees seem to be able to remember the environment and landmarks they memorized during their foraging flights in the acclimatization period. In our experiment, bumblebees were exposed to diesel exhaust particles only once and for only three minutes, which might not have been enough to affect learning or memory (Dramstad et al. 2003; Goulson 2010). In addition, we exposed the bumblebees to particles filtered from diesel exhaust and not to genuine diesel exhaust, which contains other toxic volatile components such as NO and NO_2. It is also conceivable that diesel exhaust particles may have a less measurable effect on the memory processes of orientation than on the learning and memory of floral odors.

Most insects breathe through tracheae that connect the inner body to the air via spiracles. The spiracles serve as a mechanical barrier against environmental particles, which can be opened and closed actively (Chapman 1998, Hartung et al. 2004, Nikam and Khole 1989). Tan et al. (2018) found that caterpillars closed their spiracle valves for longer in poor air quality, which could decrease oxygen uptake and reduce metabolism. In our experiment, the treated bumblebees did not take longer to return to their colony than the control animals. We assume that the particle load was too low or the particle size too big to induce a closure of their spiracle valves. As we observed grooming behavior before the start in some workers, a large part of the particles may have been removed from the body surface. Therefore, we think that the oxygen intake and the metabolism were not or only slightly impaired in our setup.

For most bumblebee species flight distance depends on the size of their colonies and the food availability in their foraging area (Goulson 2010). The maximum reported foraging distances of *B. terrestris* vary from 312 m (Darvill et al. 2004) up to 10,000 m (Cresswell et al. 2000). *B. terrestris* forages around the nest in the smallest possible radius, as this seems to be the most efficient way to obtain food (Dramstad et al. 2003, Goulson 2010). However, they have been reported to navigate back to their colony from distances up to 9.8 km (Goulson and Stout 2001). Bumblebees systemically search for familiar landmarks to locate their nest. If displaced outside their familiar home and foraging range, bumblebees take longer to return and fewer find their colony than bumblebees displaced within their familiar foraging range (Goulson and Stout 2001). Our findings did not show a significant difference between the two distances, indicating that the longer distance (1100 m) lies within the foraging radius of the bumblebees. These results are surprising, as the bumblebees released further away had to cover a longer distance on their way back to the colony which should result in a prolonged homing flight duration. As we only observed a tendency to an increased homing flight duration and had varying times in both treatments, we conclude that many bumblebees do not fly straight back to their colony on the fastest route. Instead, they might go on foraging prior to their return which may mask the difference in distance from release resulting in no significant difference in homing flight duration between the two distances. Nonetheless, we attribute the trend of a slightly longer time to the fact that the bumblebees must cross a longer distance to return to their colony.

Finally, we did not find any effect of the diesel exhaust particle exposure on foraging behavior after returning home (Fig. 5). In contrast, other studies have shown that the foraging motivation is reduced by anthropogenic pollution such as pesticides (Lämsä et al. 2018, Muth and Leonard 2019). In bumblebees, a low dose of neonicotinoid insecticides leads to reduced foraging motivation and they are slower to initiate foraging and visit fewer flowers (Lämsä et al. 2018).

As we did not observe any effect, our results indicate that a single treatment with diesel exhaust particles with its harmful components might not unfold the same toxic effects, as they are known for many pesticides (Cassereau et al. 2017, Devillers 2002).

In summary, we were able to show that a single exposition with diesel exhaust particles has a negative effect on the start behavior of the bumblebee *B. terrestris* but not on the homing flight duration and success. However, as we used a semi-artificial set up in a limited time span, we must be careful in interpreting the total effect of diesel exhaust particles on the foraging behavior. In nature, bumblebees must cope with additional stressors apart from anthropogenic pollution, such as pathogens or rising temperatures (Holmstrup et al. 2010). Normally, these multiple stressors do not occur in isolation but rather have an interactive effect and may reinforce their own negative impacts synergistically (Goulson et al. 2015). Although field-realistic doses of diesel exhaust particles are not directly lethal to bumblebees in general, they might reduce the ability to manage additional stressors (Czerwinski and Sadd 2017, Reitmayer et al. 2019). The colony function of eusocial pollinators like bumblebees depends on the efficient performance of many individuals. Due to the large number of individuals, large colonies are able to buffer or compensate for some effects of stress (Bryden et al. 2013). However, in an urban environment with heavy traffic and long-term diesel exhaust particle exposure, the small and sublethal effects that are imperceptible at the individual level have the potential to add up at the colony level and can be particularly fatal for the dynamics and functioning of whole colonies (Bryden et al. 2013) or even entire ecosystems. Our findings add to the understanding of the potential role of particulate matter pollution in the global insect decline. We suggest that the observed struggle taking off needs further investigation because its cause remains unclear, and such behavior may indicate underlying physiological constraints.

Appendix

See Figs. 6 and 7; Table 1.

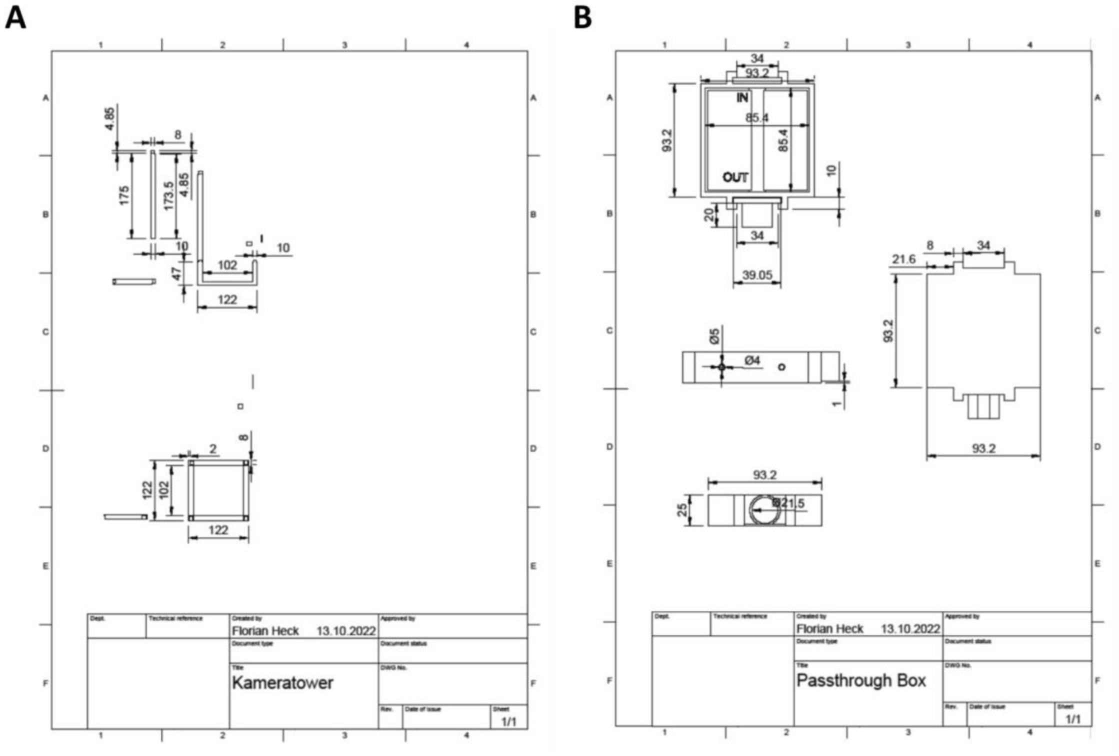

Fig. 6 Constructional drawings including the dimensions in millimeters of the individual components for the towers carrying the cameras (**A**) and the arenas (**B**) used for 3D print

Fig. 7 3D-model of the tower including the arena, which served as a template for the 3D print in general view (**A**), top view of the arena (**B**) and the view of the top of the tower where the camera was attached

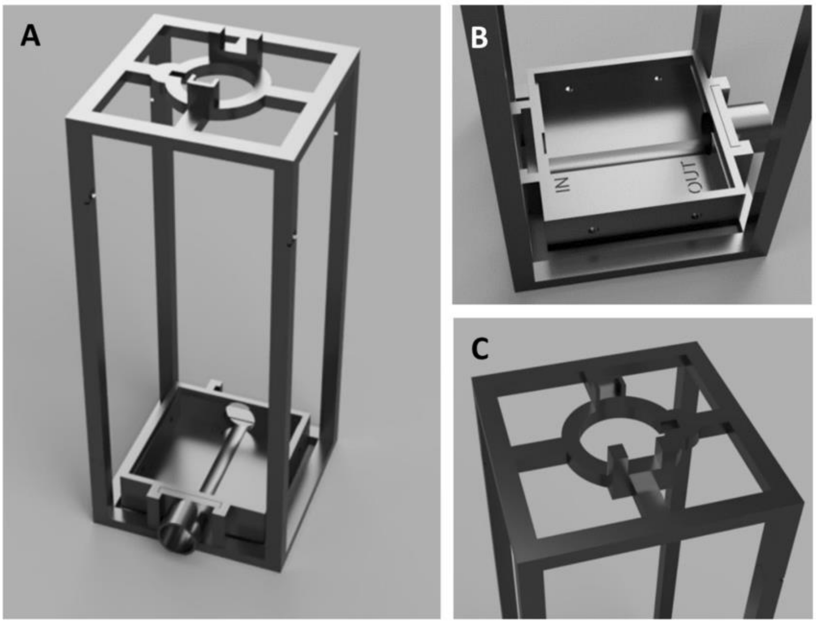

Table 1 Overview of the camera setting used in the experiment

Camera settings	First settings	Final settings	
Resolutions		Load preset Custom values: Video resolution Video fps FPS divider Image resolution	– 1296 × 972 pixel 25 recordig, 25 boxing 1 2592 × 1944 pixel
Timelapse-interval		3 s	
Video split		0 s	
Annotation		Text: OL/OR/UL/UR “daphnia1” & “daphnia2” Background	(Describes the Cam’s position) off
Annotation size		50	
Custom text colour		Disabled; y:u:v = 255:128:128	
Custom backgruond colour		Disabled; y:u:v = 0:128:128	
Buffer		0	
Sharpness		0	
Contrast	100	100	
Brightness	50	50	
Saturation		0	
ISO		0	
Metering mode	Average	Backlit	

Table 1 (continued)

Camera settings	First settings	Final settings	
Video stabilisation		Off	
Exposure compensation		0	
Exposure mode	Backlight	Auto	
White balance	Greyworld	Greyworld	
White balance gains	150	Gain_r 150; Gain_b 150	
Image effect		None	
Colour effect		Disabled; y:u:v = 0:128:128	
Image statistics		Off	
Rotation	270	Rotate_270	
Flip	Both	Both	
Sensor region		X:0; y:0 W: 65,536; h:65,536	
Shutter speed		80,000	
Image quality		10	
Preview quality		Quality: Width: Divider:	100 512 10
Raw layer		Off	
Video bitrate		17,000,000	
Minimise frag		MF:	0
Init quantisation		IQ:	25
Encoding qp		QP:	31
MP4 boxing mode		Background	
Watchdog		Interval: Errors:	3 s 3
Motion detect mode		External	
Log size lines		5000	

Author contributions DS, OO, MS, SP, CL and HF conceived the idea, designed the experiment, and wrote the manuscript. AM, TH, and DB produced and analyzed the particulate matter. DS and SP carried out the experiment. DS, SP and MS performed the data analysis. DS, SP, OO, MS, CL and HF interpreted the results. All authors read and approved of the final manuscript.

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Data availability The data that support the findings of this study are available upon request.

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Article 4

Do diesel exhaust particles in pollen affect colony founding in the bumble bee *Bombus terrestris*?

Insectes Sociaux
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Insectes Sociaux

SHORT COMMUNICATION

Do diesel exhaust particles in pollen affect colony founding in the bumble bee *Bombus terrestris*?

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Abstract
The global insect decline threatens ecosystem functioning because insects provide many essential services, such as pollination or nutrient cycling. Pollution is one of the main drivers of insect decline besides land-use change, global warming, and invasive species. Airborne particulate matter, such as diesel exhaust particles, is ubiquitous in the environment. Their effects on insects are still largely unknown. We provided queens and workers of the bumble bee *Bombus terrestris* with pollen spiked with diesel exhaust particles at the early colony founding stage and compared the colony development to control colonies. After 10 weeks, we investigated individual and colony-level life history traits. We did not see any effects of pollen spiked with diesel exhaust particles on worker count, brood count, worker size and relative fat body weight. These results indicate no harmful effects of diesel exhaust particles on colony founding in *B. terrestris* in our single-stressor setup. Our novel approach adds to the understanding of the role that airborne particulate matter plays in the global insect decline and we are looking forward to seeing similar studies with other species and additional stressors, such as heat stress or food shortages.

Keywords Air pollution · Particulate matter · Insect decline · Pollinator

Introduction

The rapid decline in insects is a major threat to many ecosystems around the globe as insects provide many vital ecosystem functions such as pollination, nutrient cycling, and linking of trophic levels (Cardoso et al. 2020; Noriega et al. 2018). Pollution is one of the main drivers of insect decline besides changes in land use, invasive species, and global warming (Miličić et al. 2021; Müller et al. 2023; Sanchez-Bayo & Wyckhuys 2019; Wagner 2020). Pesticides, specifically designed to harm certain groups of organisms, negatively affect important pollinators such as honeybees or bumble bees on different levels ranging from behavioral changes over impaired development to increased mortality (Cameron & Sadd 2020; Desneux et al. 2007; Gill & Raine 2014; Ndakidemi et al. 2016; Raine & Rundlöf 2024; Sanchez-Bayo 2021; Schuhmann et al. 2022). Effects of other anthropogenic pollutants, such as airborne pollutants, on insects are less well-studied. Airborne particulate matter is defined by size rather than chemical composition and comprises all airborne particles with diameters typically between 100 nm and 10 µm. As these particles originate from various sources such as dust and soil, wood and coal smoke, sea salt, and road traffic exhaust among others, they are highly variable in terms of chemical composition, concentration, and spatial distribution (Harrison 2020). A thorough documentation of the adverse effects of airborne particulate matter on human health exists (Kim et al. 2015; Valavanidis et al. 2008), but studies on their impacts on other organisms are scarce. Due to the small particle size, airborne particulate matter, such as diesel exhaust particles, may enter insects' bodies via tracheae or by consuming contaminated food, such as pollen, which contains airborne particulate matter at least in industrial areas (Feldhaar & Otti 2020; Papa et al. 2021). Chemically, diesel exhaust particles are composed of a carbon core with different organic and

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inorganic substances adsorbed to its surface, including polycyclic aromatic hydrocarbons (PAHs) (Hüftlein et al. 2023; Wichmann 2007), which are known to be harmful to various life forms (Patel et al. 2020; Sun et al. 2021).

One group of insects in decline and exposed to anthropogenic pollution are wild bees, especially social bees. On the one hand, social bees often have large foraging areas where they can encounter an array of pollutants, and transfer them to their nests, which could result in early exposure of their offspring (Morales et al. 2020). On the other hand, the large colony sizes may also buffer the negative effect of pollutants and contaminated patches can be avoided when larger foraging areas are available (Crall et al. 2019; Feldhaar & Otti 2020; Straub et al. 2015). The most vulnerable stage in eusocial insects is the colony founding stage because the colony's future depends solely on the survival of one or very few individuals (Helanterä 2016). As the colony grows, the risk of colony failure due to the death of single individuals decreases, leading to more effective buffering against harmful effects (Crall et al. 2019). In bumble bees, only the queens hibernate. In early spring, queens emerge from their hibernacula, start to forage for pollen and search for a suitable nesting place. Once the queen finds a good spot, she lays the first batch of eggs to start the new colony. This first generation of bumble bee workers is raised by the queen on her own (Alford 1975). During the colony founding phase, pollution might directly affect the queen or the larvae she feeds with potentially polluted pollen. In both cases, potential negative effects at such an early stage may lead to a significant reduction of the long-term colony fitness, such as a lower number of brood and workers, and a higher risk of colony failure (Baron et al. 2017). Chronic exposure to high doses of diesel exhaust particles in sugar water, as a surrogate for plant nectar, increases the mortality of workers of the bumble bee *Bombus terrestris* (Hüftlein et al. 2023), and sublethal doses cause changes in their transcriptome and gut microbiome (Seidenath et al. 2023). However, the impact such effects have on the colony level, including the brood, is still unknown. Moreover, the effects could differ when bumble bees are exposed to diesel exhaust particles via pollen rather than sugar water.

In this study, we provided bumble bees with pollen spiked with diesel exhaust particles at the early colony founding stage to test for effects of airborne particulate matter on colony development in comparison to controls fed with uncontaminated pollen. Due to the chemical composition of those particles and the observed negative effects on workers during chronic exposure earlier (Hüftlein et al. 2023), we predict negative effects of diesel exhaust particles on larval development, resulting in impaired colony development. We anticipate fewer workers to emerge and smaller broods to be produced in the colonies treated with diesel exhaust particles. Moreover, we predict the condition of workers, measured by relative fat body weight as a proxy, to be worse with diesel exhaust particle exposure than without.

Methods

Experimental procedure

Twenty queenright colonies were ordered from Biobest (Westerlo, Belgium) in January 2022. Colonies were kept in a climate chamber at 26 °C and 70% humidity under a constant, inverted 12:12 h light:dark cycle. At the start of the experiment, we removed the queen and ten haphazardly picked workers from the top of each colony with forceps. To observe colony initiation, we placed them in fresh boxes (21 × 13 × 27 cm) without any nesting material or brood and provided sugar water (50% Apiinvert, Südzucker AG, Mannheim, Germany) ad libitum. Half of the queen-worker groups were randomly assigned to the control group receiving pollen cakes made from ground pollen (Imkerpur, Osnabrück, Germany) and Apiinvert (ratio 5:1 (g:ml)) (Online Resource 1: Figure ESM1A). The other half we assigned to the treatment with diesel exhaust particles receiving pollen cakes made from ground pollen spiked with 0.2% diesel exhaust particles (w/w) and Apiinvert (ratio 5:1 (g:ml) throughout the experimental period (Online Resource 1: Figure ESM1B). At the beginning of the experiment each colony received 4 pellets (ca. 1.5 g each) of pollen cake. Afterwards, we added two fresh pollen pellets to the colonies twice per week. We replaced the sugar water once per week to prevent molding. Once per week, we removed dead workers. On day 35, 43 and 63 we removed 20% of the workers from each colony by randomly picking workers from the top, to simulate a natural mortality rate and prevent overcrowding (Otti and Schmid-Hempel 2007). After 66 days all colonies were frozen at −20 °C.

Trait measurements

After defrosting, we removed all wax and silk cover and counted the number of workers, larvae, and pupae, while eggs were excluded. For each colony, we pooled all larvae and pupae in 10 ml glass vials, respectively, and placed them at 70 °C for 96 h in a drying oven (UFE 600, Memmert GmbH + Co. KG, Schwabach, Germany) to measure larvae and pupae dry weight using a fine scale to the nearest 0.01 mg (SM1265Di, VWR, Darmstadt, Germany). We calculated brood number and dry weight by adding numbers and dry weights of larvae and pupae, respectively.

For measurements of worker size and fat body content we randomly selected ten workers per colony workers by lining all workers in a row, generating ten random numbers and then picking the bumble bees with the according position in the row. From these workers we measured the radial cell length of the right forewing under a light microscope (Leica DFC290, Leica M165, Leica, Wetzlar, Germany) to determine worker size (Medler 1962). Additionally, we measured the fat body weight of the selected workers according to Hüftlein et al. (2023). For that purpose, we separated the abdomen from head and thorax and slightly opened the sternites with tiny scissors. After placing each abdomen in a 5 ml glass vial, we dried it in a drying oven (UFE 600, Memmert GmbH + Co. KG, Schwabach, Germany) at 70 °C for 72 h. Then, we weighed the dry weight including the fat body of each abdomen on a fine scale to the nearest 0.01 mg (PLE 420-3N, Kern & Sohn GmbH, Balingen-Frommern, Germany). To remove the fat content, we added 2 ml chloroform to each vial. Every 24 h we replaced the chloroform with 2 ml fresh one. After 72 h we removed the chloroform and placed the vials in the drying oven again at 70 °C for 72 h. Then we weighed the abdomens again without the fat body. We calculated the fat body weight by subtracting the dry weight after fat body removal from the initial dry weight of the abdomen (see Online Resource 2 for regression of fat body weight in relation to radial cell size).

Generation of diesel exhaust particles

We produced samples of diesel exhaust particles on a test bench with a four-cylinder diesel engine (OM 651, Daimler AG, Stuttgart, Germany) as described in Zöllner (2019). When analyzed with a fast response differential mobility particulate spectrometer DMS500 (Combustion, Cambridge, England), raw exhaust samples showed a median diameter between 52.1 and 101.9 nm within an inner-city driving cycle. Elemental carbon accounted for 72.2 ± 1.1% of the diesel exhaust particle mass, organic matter for 23.2 ± 0.9% w/w, and inorganic matter for 4.6 ± 0.7% w/w, according to thermogravimetric analysis (TGA, STA 449 F5 Jupiter, Netzsch-Gerätebau GmbH, Selb, Germany). We analyzed the samples of diesel exhaust particles for polycyclic aromatic hydrocarbons (PAH) and detected a concentration of 444 ppm for pyrene, 220 ppm for phenanthrene, and 107 ppm for fluoranthene. The elemental composition showed fractions of calcium (1.63% w/w), copper (1.03% w/w), zinc (0.53% w/w), phosphorus (0.50% w/w), sulphur (0.17% w/w), boron (0.13% w/w) and magnesium (0.10% w/w). More details on the characterization and sampling methods of diesel exhaust particles can be found in Hüftlein et al. (2023) and Seidenath et al. (2023).

Statistical analysis

All statistical analyses were performed using R 4.2.1 (R Core Team 2022). We corrected fat body weight for body size effects by regressing fat body weight with worker size (i.e. radial cell length) and used the residuals for further analysis. Colony-level traits, i.e. worker number, larvae number, pupae number, brood number (sum of larvae and pupae), larvae dry weight, pupae dry weight and brood dry weight (total weight larvae and pupae), were analyzed fitting generalized linear models (GLMs) with treatment as a predictor. Individual traits, i.e. residual fat body weight and worker size were analyzed fitting generalized linear mixed models (GLMMS) with treatment as a predictor and colony as a random factor using the package *glmmTMB* (Brooks et al. 2017). We checked model assumptions using model diagnostic test plots, i.e. qqplot and residual vs. predicted plot from the package *DHARMa* (Hartig 2022). For the GLMs we then produced F-statistics with the function *Anova*() from the package *car* (Fox & Weisberg 2019) to calculate p values for differences between the two groups. For the GLMMs we performed chi-squared tests with the function *Anova*() from the package *car* (Fox & Weisberg 2019) to calculate p values for differences between the two groups.

Results

Overall, we did not find any differences between the control and the treatment group. All colonies survived until the end of the experiment after 66 days. The number of workers did not differ between groups (GLM with Gaussian distribution: $F_{1,18} = 0.308$, $P = 0.586$). Likewise, neither the number of larvae (GLM with Gaussian distribution: $F_{1,18} = 0.001$, $P = 0.975$) nor the number of pupae (GLM with Gaussian distribution: $F_{1,18} = 0.209$, $P = 0.653$) or the brood number (GLM with Gaussian distribution: $F_{1,18} = 0.023$, $P = 0.882$) differed between groups. We also did not find differences in dry weight for comparisons between the groups of larvae (GLM with Gaussian distribution: $F_{1,18} = 0.051$, $P = 0.823$), pupae (GLM with Gaussian distribution: $F_{1,18} = 0.229$, $P = 0.638$), or brood (GLM with Gaussian distribution: $F_{1,18} < 0.001$, $P = 0.982$). The residual fat body weight (GLMM with Gaussian distribution: $X^2 = 0.490$, $df = 1$, $P = 0.484$) and the worker size did not differ between groups (GLMM with gamma distribution: $X^2 = 0.848$, $df = 1$, $P = 0.357$) (Fig. 1).

Discussion

Our study is the first to evaluate the effects of diesel exhaust particles on whole bumble bee colonies in a laboratory setup by regularly feeding diesel exhaust particle-spiked pollen cake. We did not detect any effect of pollen contaminated with diesel exhaust particles on the colony founding of *B.*

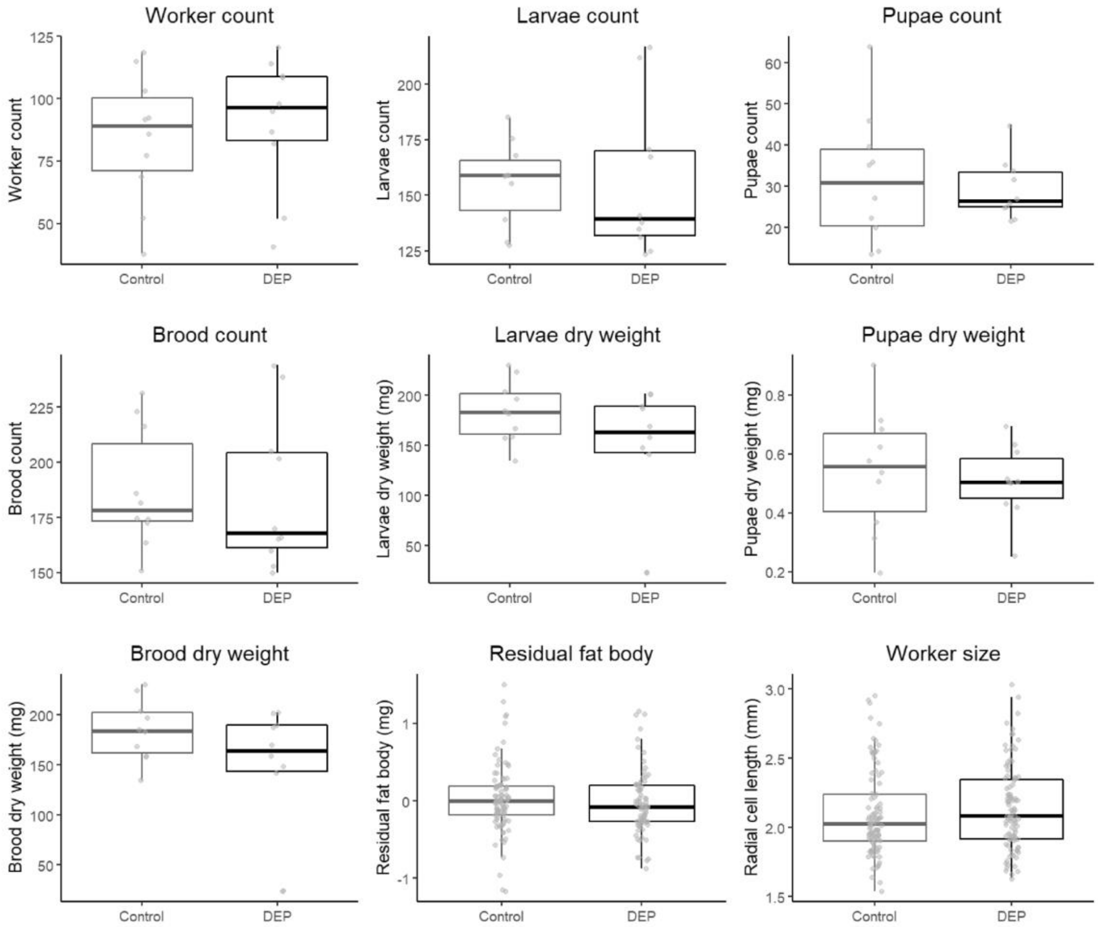

Fig. 1 Individual and colony-level traits compared between treatment and diesel exhaust particles group. Boxplots show median, first, and third quartile. Dots represent individual data points

terrestris. All the colony life history traits we measured, i.e. the number of workers and brood, as well as individual life history traits, i.e. worker size and fat body weight, were similar between the treatments.

These results indicate that diesel exhaust particle exposure via pollen alone does not impair colony and worker development at an early colony founding stage. Hence, we have no reason to expect effects at later colony stages. However, as we have not measured the production and development of sexual offspring for the next generation, we should be careful with further speculations. Hidden effects that were not investigated in this study might come to light once a complete colony cycle is investigated. Also, when fed with sugar water spiked with diesel exhaust particles, bumble bee workers had a significant shift in gut microbiome composition compared to controls (Seidenath et al. 2023). It remains speculative how such shifts affect the performance of whole colonies. As the microbiome plays an important role in detoxification and in the protection against parasites (Koch & Schmid-Hempel 2011; Rothman et al. 2019), the colony-level effects might only show when additional stressors are present. In nature, insects face a multi-stressor environment, including parasites, food limitations, heat stress and more (Cameron & Sadd 2020; Holmstrup et al. 2010; Padda & Stahlschmidt 2022; Raine & Rundlöf 2024). Adding sublethal effects due to pollution could then be the cause of a colony failure (Cameron & Sadd 2020; Raine & Rundlöf 2024). In contrast, ad libitum food provision in a controlled environment, as in this study, may enable the insects to compensate for the effects of diesel exhaust particle exposure.

Because identifying and quantifying airborne particulate matter, such as diesel exhaust particles, in terrestrial environments is very difficult, we lack reliable data on the level of exposure for insects. Isotopic analyses revealed that certain Arizona soils contain up to 0.54% (w/w) of soot black carbon, presumably produced by burning fossil fuels (Hamilton & Hartnett 2013). While reports exist of contaminated bees in the wild (Negri et al. 2015; Thimmegowda et al. 2020), we still lack realistic modelling on the uptake of these particles. Other experiments on effects of diesel exhaust particles on individual workers showed increased mortality in *B. terrestris* workers after oral diesel exhaust particle exposure via sugar water with concentrations of 1 g/l and more (Hüftlein et al. 2023). In contrast, we did not see any difference in mortality or other life-history parameters. This may be due to the different exposure route via pollen or the colony setup, which might enable the bumble bees to buffer individual negative effects (Crall et al. 2019).

B. terrestris is one of the most abundant European wild bees, especially in urban areas (Herbertsson et al. 2021; Whitehorn et al. 2022). Thus, *B. terrestris* is possibly more tolerant towards anthropogenic pollution, such as diesel exhaust particles, or has already adapted to higher levels of air pollution than other species. That a species is common might mean that it is more tolerant towards stressors than a rarer, often more specialized species (Vincent et al. 2020), and, e.g. *B. terrestris* has been shown to be less affected by pesticides in comparison to other wild bee species (Schmolke et al. 2021). We thus need to be careful when trying to extrapolate the results of our study to other rarer species with more specific dietary needs or narrower niches. Physiological differences between insect populations and species translate into variation of the susceptibility to different stressors (Chown 2001; Spurgeon et al. 2020). Therefore, in the future we should also incorporate rarer species to evaluate the impact of potentially harmful substances in a comparative approach.

In our study, we successfully exposed whole bumble bee colonies to diesel exhaust particles in the laboratory. We found no measurable effects of exposure to pollen contaminated with diesel exhaust particles on the colony founding of *B. terrestris*. Our novel approach adds to the understanding of the role that airborne particulate matter plays in the global insect decline and we are looking forward to seeing the results of future studies adding additional stressors to the setup, such as heat stress or food shortages.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s00040-024-00965-4.

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Author contributions DS, OO, and HF conceived the idea, designed the experiment, and wrote the manuscript. AM, TH, and DB produced and analyzed the particulate matter. DS carried out the experiment. DS performed the data analysis. DS, OO, and HF interpreted the results. All authors read and approved of the final manuscript.

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Data availability Data is provided in the electronic supplement.

Declarations

Conflict of interest The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Do diesel exhaust particles in pollen affect colony founding in the bumble bee *Bombus terrestris*?

Insectes Sociaux

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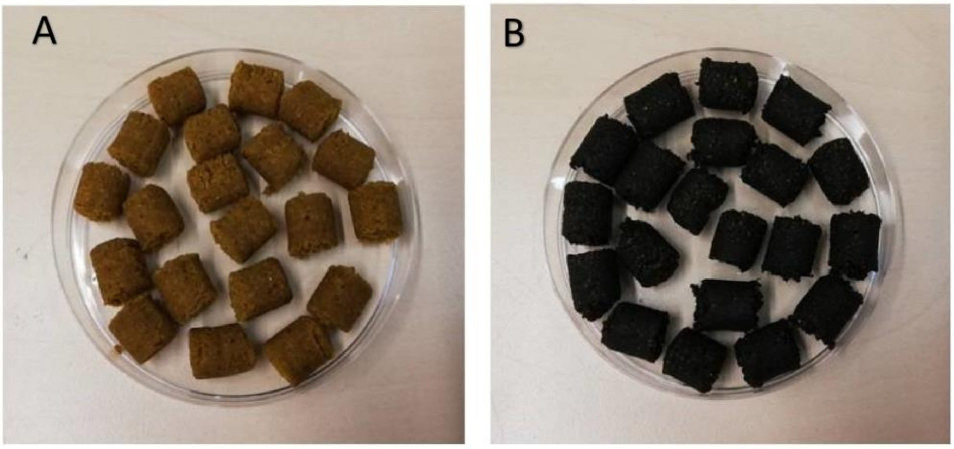

Figure ESM1: Control pollen cake (A) and pollen cake spiked with diesel exhaust particles (B).

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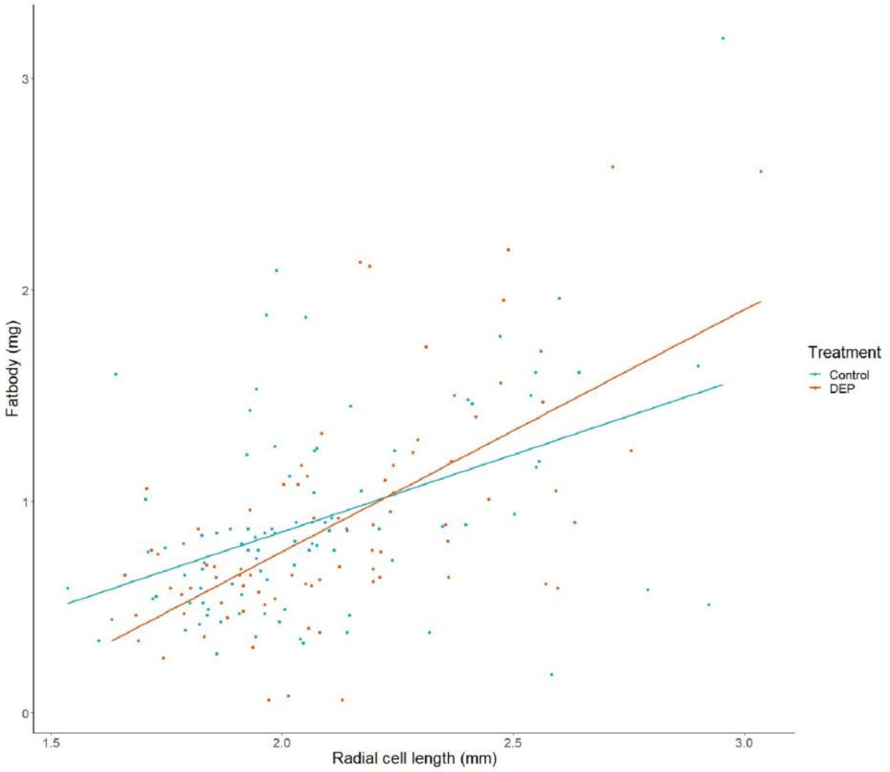

Figure ESM2: Fat body weight in relation to the size of the radial cell per bumble bee. DEP: Treatment with diesel exhaust particles.

ID_complete	colony	Treatment	worker	larvae	pupae	weight_larvae_g	weight_pupae_g	worker_estimate	brood_estim
1_45_LAB_s	45	DEP	82	212	32	24,373	0,63034	47	80
1_46_LAB_c	46	Control	92	159	27	203,367	0,57758	54	83
1_47_LAB_s	47	DEP	109	217	22	187,455	0,25282	51	70
1_48_LAB_c	48	Control	69	155	20	196,572	0,31445	42	100
1_49_LAB_s	49	DEP	87	171	34	169,129	0,50357	43	94
1_50_LAB_c	50	Control	92	128	36	167,602	0,71584	60	88
1_51_LAB_s	51	DEP	41	124	26	189,629	0,43119	42	86
1_52_LAB_c	52	Control	115	176	40	157,343	0,53626	63	91
1_53_LAB_s	53	DEP	121	125	45	200,727	0,69346	58	65
1_54_LAB_c	54	Control	77	185	46	229,733	0,68472	30	70
1_55_LAB_s	55	DEP	95	141	25	23,176	0,42066	50	69
1_56_LAB_c	56	Control	38	129	22	223,629	0,50839	30	85
1_57_LAB_s	57	DEP	114	138	27	147,677	0,50621	51	70
1_58_LAB_c	58	Control	103	159	64	181,891	0,90073	65	103
1_59_LAB_s	59	DEP	109	135	25	141,292	0,51567	45	75
1_60_LAB_c	60	Control	119	139	35	184,924	0,62404	61	100
1_61_LAB_s	61	DEP	98	167	35	201,573	0,60736	57	84
1_62_LAB_c	62	Control	86	168	14	159,066	0,19725	53	95
1_63_LAB_s	63	DEP	52	131	22	158,245	0,50079	39	80
1_64_LAB_c	64	Control	52	159	14	134,461	0,36905	42	93

ID_complete	ID	colony	Treatment	weight_before_mg	weight_after_mg	fatbody_mg	radial_cell_mm	relative_fatbody_mg/mm
1_1_54_LAB_c	1	54	Control	19,21	18,7	0,51	2,923	0,17448
1_2_64_LAB_c	2	64	Control	12,77	10,68	2,09	1,988	1,05131
1_3_52_LAB_c	3	52	Control	11,65	11,18	0,47	1,907	0,24646
1_4_48_LAB_c	4	48	Control	23,36	22,17	1,19	2,556	0,46557
1_10_54_LAB_c	10	54	Control	NA	NA	NA	2,076	NA
1_14_48_LAB_c	14	48	Control	9,61	9,15	0,46	2,146	0,21435
1_16_50_LAB_c	16	50	Control	NA	NA	NA	2,627	NA
1_19_50_LAB_c	19	50	Control	9,83	9,4	0,43	1,995	0,21554
1_23_48_LAB_c	23	48	Control	30,74	29,8	0,94	2,503	0,37555
1_25_54_LAB_c	25	54	Control	5,15	4,56	0,59	1,824	0,32346
1_26_48_LAB_c	26	48	Control	5,67	5,12	0,55	1,728	0,31829
1_31_50_LAB_c	31	50	Control	5,15	4,66	0,49	1,84	0,2663
1_32_52_LAB_c	32	52	Control	17,05	16	1,05	2,172	0,48343
1_35_54_LAB_c	35	54	Control	8,9	7,03	1,87	2,051	0,91175
1_36_48_LAB_c	36	48	Control	9,85	9,15	0,7	2,027	0,34534
1_37_52_LAB_c	37	52	Control	8,15	7,63	0,52	1,829	0,28431
1_40_54_LAB_c	40	54	Control	7,64	7,29	0,35	2,04	0,17157
1_41_48_LAB_c	41	48	Control	9,42	8,59	0,83	1,942	0,42739
1_42_52_LAB_c	42	52	Control	5,38	4,51	0,87	1,888	0,46081
1_52_54_LAB_c	52	54	Control	7,71	6,98	0,73	1,944	0,37551
1_56_52_LAB_c	56	52	Control	10,82	10,02	0,8	2,065	0,38741
1_57_52_LAB_c	57	52	Control	6,16	5,49	0,67	1,953	0,34306
1_58_50_LAB_c	58	50	Control	5,03	4,23	0,8	1,787	0,44768
1_59_52_LAB_c	59	52	Control	6,93	6,22	0,71	1,831	0,38777
1_60_48_LAB_c	60	48	Control	NA	NA	NA	2,219	NA
1_61_48_LAB_c	61	48	Control	5,25	4,47	0,78	1,747	0,44648
1_63_52_LAB_c	63	52	Control	22,65	22,47	0,18	2,583	0,06969
1_65_54_LAB_c	65	54	Control	5,95	4,94	1,01	1,704	0,59272
1_67_50_LAB_c	67	50	Control	15,45	14,6	0,85	1,962	0,43323
1_68_54_LAB_c	68	54	Control	12,53	11,76	0,77	2,112	0,36458
1_70_54_LAB_c	70	54	Control	20,05	19,18	0,87	1,926	0,45171
1_71_50_LAB_c	71	50	Control	7,96	7,4	0,56	1,912	0,29289
1_72_54_LAB_c	72	54	Control	NA	NA	NA	2,752	NA
1_75_48_LAB_c	75	48	Control	9,61	8,75	0,86	2,141	0,40168
1_80_52_LAB_c	80	52	Control	14,14	13,35	0,79	2,075	0,38072
1_82_50_LAB_c	82	50	Control	25,21	23,57	1,64	2,9	0,56552
1_90_52_LAB_c	90	52	Control	14,01	13,14	0,87	1,978	0,43984
1_91_48_LAB_c	91	48	Control	38,01	36,55	1,46	2,411	0,60556
1_92_50_LAB_c	92	50	Control	25,93	25,03	0,9	2,031	0,44313
1_97_50_LAB_c	97	50	Control	5,89	5,04	0,85	1,858	0,45748
1_99_50_LAB_c	99	50	Control	9,12	8,65	0,47	1,962	0,23955
1_101_60_LAB_c	101	60	Control	8,78	7,66	1,12	2,017	0,55528
1_103_64_LAB_c	103	64	Control	8,51	8,18	0,33	2,046	0,16129
1_105_56_LAB_c	105	56	Control	21,01	20,29	0,72	2,239	0,32157
1_107_58_LAB_c	107	58	Control	29,36	27,86	1,5	2,539	0,59078
1_108_60_LAB_c	108	60	Control	19,81	18,57	1,24	2,07	0,59903
1_109_58_LAB_c	109	58	Control	4,22	3,63	0,59	1,537	0,38386
1_112_46_LAB_c	112	46	Control	37,98	36,82	1,16	2,55	0,4549
1_113_62_LAB_c	113	62	Control	9,6	8,83	0,77	1,926	0,39979

1_116_64_LAB_c	116	64	Control	7,53	7,25	0,28	1,858	0,1507
1_122_62_LAB_c	122	62	Control	11,69	10,92	0,77	1,948	0,39528
1_123_62_LAB_c	123	62	Control	10,06	9,57	0,49	2,006	0,24427
1_124_56_LAB_c	124	56	Control	13,81	12,89	0,92	2,107	0,43664
1_125_60_LAB_c	125	60	Control	NA	NA	NA	2,378	NA
1_127_60_LAB_c	127	60	Control	6,88	6,5	0,38	2,141	0,17749
1_128_46_LAB_c	128	46	Control	8,24	6,98	1,26	1,984	0,63508
1_130_56_LAB_c	130	56	Control	7,78	6,97	0,81	2,028	0,39941
1_133_64_LAB_c	133	64	Control	13,74	12,29	1,45	2,149	0,67473
1_134_62_LAB_c	134	62	Control	15,48	14,59	0,89	2,398	0,37114
1_136_58_LAB_c	136	58	Control	13,36	12,56	0,8	1,913	0,41819
1_137_64_LAB_c	137	64	Control	30,35	28,74	1,61	2,549	0,63162
1_138_60_LAB_c	138	60	Control	4,79	4,11	0,68	1,828	0,37199
1_139_46_LAB_c	139	46	Control	102,28	99,09	3,19	2,954	1,07989
1_140_62_LAB_c	140	62	Control	11,33	10,29	1,04	2,069	0,50266
1_141_60_LAB_c	141	60	Control	NA	NA	NA	2,536	NA
1_142_56_LAB_c	142	56	Control	12,19	10,97	1,22	1,924	0,6341
1_143_62_LAB_c	143	62	Control	12,51	11,64	0,87	2,21	0,39367
1_144_60_LAB_c	144	60	Control	NA	NA	NA	2,549	NA
1_145_60_LAB_c	145	60	Control	7,3	6,45	0,85	1,984	0,42843
1_147_46_LAB_c	147	46	Control	6,94	6,33	0,61	1,892	0,32241
1_150_46_LAB_c	150	46	Control	NA	NA	NA	2,547	NA
1_152_60_LAB_c	152	60	Control	7,96	7,5	0,46	1,838	0,25027
1_154_64_LAB_c	154	64	Control	12,52	11,75	0,77	2,059	0,37397
1_155_58_LAB_c	155	58	Control	37,3	35,52	1,78	2,472	0,72006
1_156_58_LAB_c	156	58	Control	12,95	12,11	0,84	1,827	0,45977
1_157_56_LAB_c	157	56	Control	14,18	12,93	1,25	2,076	0,60212
1_158_62_LAB_c	158	62	Control	12	11,12	0,88	2,347	0,37495
1_160_56_LAB_c	160	56	Control	10,87	8,99	1,88	1,966	0,95626
1_162_64_LAB_c	162	64	Control	27,27	26,89	0,38	2,319	0,16386
1_163_62_LAB_c	163	62	Control	8,02	7,12	0,9	2,065	0,43584
1_164_58_LAB_c	164	58	Control	5,23	3,63	1,6	1,641	0,97502
1_168_64_LAB_c	168	64	Control	6,94	6,18	0,76	1,71	0,44444
1_169_58_LAB_c	169	58	Control	9,2	8,66	0,54	1,72	0,31395
1_170_46_LAB_c	170	46	Control	13,34	12,1	1,24	2,244	0,55258
1_173_56_LAB_c	173	56	Control	8,54	7,01	1,53	1,945	0,78663
1_174_46_LAB_c	174	46	Control	5,83	5,44	0,39	1,791	0,21776
1_175_58_LAB_c	175	58	Control	3,51	3,17	0,34	1,603	0,2121
1_176_56_LAB_c	176	56	Control	9,74	9,11	0,63	1,968	0,32012
1_183_56_LAB_c	183	56	Control	15,38	14,52	0,86	2,102	0,40913
1_185_64_LAB_c	185	64	Control	8,69	8,26	0,43	1,867	0,23032
1_186_46_LAB_c	186	46	Control	17,35	15,92	1,43	1,931	0,74055
1_187_62_LAB_c	187	62	Control	8,91	8,39	0,52	1,801	0,28873
1_192_64_LAB_c	192	64	Control	20,41	19,83	0,58	2,792	0,20774
1_194_60_LAB_c	194	60	Control	37,04	35,43	1,61	2,643	0,60916
1_195_58_LAB_c	195	58	Control	6,89	6,24	0,65	1,789	0,36333
1_196_56_LAB_c	196	56	Control	6,97	6,55	0,42	1,821	0,23064
1_197_46_LAB_c	197	46	Control	5,09	4,73	0,36	1,943	0,18528
1_198_58_LAB_c	198	58	Control	17,74	16,26	1,48	2,403	0,6159
1_199_46_LAB_c	199	46	Control	9,28	8,38	0,9	2,093	0,43

1_200_62_LAB_c	200	62	Control	25,26	23,3	1,96	2,6	0,75385
1_5_57_LAB_s	5	57	DEP	NA	NA	NA	2,826	NA
1_6_63_LAB_s	6	63	DEP	9,9	9,03	0,87	1,819	0,47828
1_7_57_LAB_s	7	57	DEP	7,64	6,68	0,96	1,931	0,49715
1_8_53_LAB_s	8	53	DEP	5,78	5,3	0,48	1,916	0,25052
1_9_57_LAB_s	9	57	DEP	NA	NA	NA	2,09	NA
1_11_55_LAB_s	11	55	DEP	35,66	33,47	2,19	2,49	0,87952
1_12_47_LAB_s	12	47	DEP	6,28	5,83	0,45	1,881	0,23923
1_13_63_LAB_s	13	63	DEP	4,68	4,24	0,44	1,632	0,26961
1_15_49_LAB_s	15	49	DEP	17,43	16,38	1,05	2,592	0,40509
1_17_45_LAB_s	17	45	DEP	14,02	13,43	0,59	2,597	0,22719
1_18_55_LAB_s	18	55	DEP	8,44	7,76	0,68	1,911	0,35583
1_20_57_LAB_s	20	57	DEP	NA	NA	NA	2,128	NA
1_21_63_LAB_s	21	63	DEP	NA	NA	NA	2,191	NA
1_22_49_LAB_s	22	49	DEP	28,69	27,79	0,9	2,633	0,34182
1_24_49_LAB_s	24	49	DEP	NA	NA	NA	2,452	NA
1_27_57_LAB_s	27	57	DEP	8,74	8,2	0,54	1,984	0,27218
1_28_57_LAB_s	28	57	DEP	NA	NA	NA	2,676	NA
1_29_53_LAB_s	29	53	DEP	10,64	9,72	0,92	2,069	0,44466
1_30_57_LAB_s	30	57	DEP	16,81	15,8	1,01	2,448	0,41258
1_33_57_LAB_s	33	57	DEP	NA	NA	NA	2,946	NA
1_34_55_LAB_s	34	55	DEP	40,19	37,63	2,56	3,036	0,84321
1_38_57_LAB_s	38	57	DEP	17,39	16,77	0,62	2,197	0,2822
1_39_47_LAB_s	39	47	DEP	3,75	3,49	0,26	1,743	0,14917
1_43_45_LAB_s	43	45	DEP	10,44	9,93	0,51	1,962	0,25994
1_44_47_LAB_s	44	47	DEP	11,84	11,23	0,61	2,571	0,23726
1_45_49_LAB_s	45	49	DEP	4,64	3,89	0,75	1,732	0,43303
1_46_49_LAB_s	46	49	DEP	7,42	7,36	0,06	1,971	0,03044
1_47_49_LAB_s	47	49	DEP	4,19	3,49	0,7	1,837	0,38106
1_48_55_LAB_s	48	55	DEP	NA	NA	NA	2,056	NA
1_49_63_LAB_s	49	63	DEP	11,21	10,69	0,52	1,869	0,27822
1_50_57_LAB_s	50	57	DEP	19,22	18,33	0,89	2,354	0,37808
1_51_45_LAB_s	51	45	DEP	13	12,23	0,77	2,195	0,3508
1_53_49_LAB_s	53	49	DEP	9,16	8,59	0,57	1,95	0,29231
1_54_55_LAB_s	54	55	DEP	11,4	10,32	1,08	2,034	0,53097
1_55_63_LAB_s	55	63	DEP	19,36	17,63	1,73	2,312	0,74827
1_62_45_LAB_s	62	45	DEP	9,86	9,48	0,38	2,082	0,18252
1_64_49_LAB_s	64	49	DEP	NA	NA	NA	1,916	NA
1_66_53_LAB_s	66	53	DEP	9,89	7,78	2,11	2,189	0,96391
1_69_63_LAB_s	69	63	DEP	21,08	19,52	1,56	2,473	0,63081
1_73_63_LAB_s	73	63	DEP	6,18	5,71	0,47	1,788	0,26286
1_74_45_LAB_s	74	45	DEP	12,22	11,61	0,61	2,051	0,29742
1_76_49_LAB_s	76	49	DEP	8,69	8,13	0,56	1,783	0,31408
1_77_63_LAB_s	77	63	DEP	NA	NA	NA	2,207	NA
1_78_55_LAB_s	78	55	DEP	13,95	13,08	0,87	2,139	0,40673
1_79_47_LAB_s	79	47	DEP	10,01	8,84	1,17	2,042	0,57297
1_81_55_LAB_s	81	55	DEP	NA	NA	NA	1,937	NA
1_83_53_LAB_s	83	53	DEP	5,05	4,28	0,77	1,717	0,44846
1_84_55_LAB_s	84	55	DEP	6,34	5,75	0,59	1,759	0,33542
1_85_45_LAB_s	85	45	DEP	7,27	6,58	0,69	1,854	0,37217

1_86_63_LAB_s	86	63	DEP	16,71	14,58	2,13	2,169	0,98202
1_87_45_LAB_s	87	45	DEP	5,87	5,56	0,31	1,937	0,16004
1_88_63_LAB_s	88	63	DEP	4,26	4,18	0,08	2,014	0,03972
1_89_49_LAB_s	89	49	DEP	NA	NA	NA	2,218	NA
1_93_45_LAB_s	93	45	DEP	11	10,36	0,64	2,36	0,27119
1_94_55_LAB_s	94	55	DEP	NA	NA	NA	2,343	NA
1_95_45_LAB_s	95	45	DEP	11,02	10,21	0,81	2,359	0,34337
1_96_55_LAB_s	96	55	DEP	NA	NA	NA	2,029	NA
1_98_53_LAB_s	98	53	DEP	15,13	14,18	0,95	2,235	0,42506
1_100_45_LAB_s	100	45	DEP	11,93	10,61	1,32	2,086	0,63279
1_102_51_LAB_s	102	51	DEP	15,69	13,74	1,95	2,479	0,78661
1_104_47_LAB_s	104	47	DEP	8,49	7,85	0,64	1,857	0,34464
1_106_59_LAB_s	106	59	DEP	14,69	13,52	1,17	2,241	0,52209
1_110_61_LAB_s	110	61	DEP	23,69	23,06	0,63	2,082	0,30259
1_111_61_LAB_s	111	61	DEP	NA	NA	NA	1,942	NA
1_114_61_LAB_s	114	61	DEP	4,33	3,99	0,34	1,689	0,2013
1_115_53_LAB_s	115	53	DEP	18,83	17,43	1,4	2,419	0,57875
1_117_59_LAB_s	117	59	DEP	NA	NA	NA	2,68	NA
1_118_51_LAB_s	118	51	DEP	37,31	36,07	1,24	2,755	0,45009
1_119_61_LAB_s	119	61	DEP	15,56	14,92	0,64	2,211	0,28946
1_120_51_LAB_s	120	51	DEP	18,6	17,1	1,5	2,373	0,63211
1_121_53_LAB_s	121	53	DEP	27,24	25,77	1,47	2,564	0,57332
1_126_59_LAB_s	126	59	DEP	19,09	17,8	1,29	2,293	0,56258
1_129_59_LAB_s	129	59	DEP	11,83	11,23	0,6	1,916	0,31315
1_131_61_LAB_s	131	61	DEP	NA	NA	NA	1,799	NA
1_132_59_LAB_s	132	59	DEP	7,4	6,75	0,65	1,932	0,33644
1_135_51_LAB_s	135	51	DEP	5,51	5,11	0,4	2,058	0,19436
1_146_61_LAB_s	146	61	DEP	18,02	17,26	0,76	2,214	0,34327
1_148_59_LAB_s	148	59	DEP	7,36	6,24	1,12	2,055	0,54501
1_149_61_LAB_s	149	61	DEP	14,13	14,07	0,06	2,13	0,02817
1_151_51_LAB_s	151	51	DEP	12,42	11,5	0,92	2,121	0,43376
1_153_47_LAB_s	153	47	DEP	17,42	15,71	1,71	2,561	0,66771
1_159_53_LAB_s	159	53	DEP	8,26	7,18	1,08	2,004	0,53892
1_161_47_LAB_s	161	47	DEP	6,28	5,22	1,06	1,707	0,62097
1_165_61_LAB_s	165	61	DEP	16,59	15,99	0,6	2,064	0,2907
1_166_53_LAB_s	166	53	DEP	18,46	17,27	1,19	2,367	0,50275
1_167_51_LAB_s	167	51	DEP	12,83	11,79	1,04	2,241	0,46408
1_171_61_LAB_s	171	61	DEP	13,76	13,4	0,36	1,831	0,19661
1_172_59_LAB_s	172	59	DEP	10,67	9,99	0,68	2,197	0,30951
1_177_51_LAB_s	177	51	DEP	52,8	50,22	2,58	2,716	0,94993
1_178_59_LAB_s	178	59	DEP	14,84	13,74	1,1	2,223	0,49483
1_179_51_LAB_s	179	51	DEP	8,12	7,47	0,65	1,908	0,34067
1_180_47_LAB_s	180	47	DEP	9,41	8,76	0,65	2,021	0,32162
1_181_59_LAB_s	181	59	DEP	6,32	5,86	0,46	1,684	0,27316
1_182_53_LAB_s	182	53	DEP	21,88	20,65	1,23	2,283	0,53876
1_184_51_LAB_s	184	51	DEP	13,13	12,24	0,89	2,197	0,4051
1_188_51_LAB_s	188	51	DEP	NA	NA	NA	2,363	NA
1_189_61_LAB_s	189	61	DEP	4,57	3,92	0,65	1,66	0,39157
1_190_59_LAB_s	190	59	DEP	6,78	6,09	0,69	2,124	0,32486
1_191_47_LAB_s	191	47	DEP	NA	NA	NA	1,838	NA
1_193_47_LAB_s	193	47	DEP	7,4	6,81	0,59	1,802	0,32741

Article 5

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Individual vs. Combined Short-Term Effects of Soil Pollutants on Colony Founding in a Common Ant Species

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Insects are integral to terrestrial life and provide essential ecosystem functions such as pollination and nutrient cycling. Due to massive declines in insect biomass, abundance, or species richness in recent years, the focus has turned to find their causes. Anthropogenic pollution is among the main drivers of insect declines. Research addressing the effects of pollutants concentrates on aquatic insects and pollinators, despite the apparent risk of contaminated soils. Pollutants accumulating in the soil might pose a significant threat because concentrations tend to be high and different pollutants are present simultaneously. Here, we exposed queens of the black garden ant *Lasius niger* at the colony founding stage to different concentrations and combinations of pollutants (brake dust, soot, microplastic particles and fibers, manure) to determine dose-dependent effects and interactions between stressors. As proxies for colony founding success, we measured queen survival, the development time of the different life stages, the brood weight, and the number of offspring. Over the course of the experiment queen mortality was very low and similar across treatments. Only high manure concentrations affected the colony founding success. Eggs from queens exposed to high manure concentrations took longer to hatch, which resulted in a delayed emergence of workers. Also, fewer pupae and workers were raised by those queens. Brake dust, soot and plastic particles did not visibly affect colony founding success, neither as single nor as multiple stressors. The application of manure, however, affected colony founding in *L. niger* negatively underlining the issue of excessive manure application to our environment. Even though anthropogenic soil pollutants seem to have little short-term effects on ant colony founding, studies will have to elucidate potential long-term effects as a colony grows.

Keywords: multiple stressors, soil pollution, insect decline, claustral colony founding, particulate matter, microplastic

INTRODUCTION

The loss of biodiversity worldwide poses one of the biggest threats to ecosystem functioning and consequently to human well-being in the Twenty-first century (1–3). Human-induced vertebrate declines and species extinctions are well-documented and have long captured the attention of scientists and the broader public (4). Due to an increasing number of studies showing massive declines in insect biomass, abundance, or species richness over the last decades [(3, 5–7), but see: (8, 9)], the focus has recently turned to understanding the mechanisms behind insect declines.

Insects are an integral part of terrestrial and aquatic food webs as consumers and by linking primary producers with consumers of higher trophic levels. They provide many essential ecosystem functions such as pollination, regulation of herbivores and plants, or nutrient cycling through the decomposition of leaf litter and dead wood, or removal of dung (10). Consequently, further losses in insect diversity and biomass will result in a highly uncertain development of ecological processes potentially affecting human living as we know it.

One of the main drivers of insect declines, besides habitat conversion, land-use intensification, climate change, and biological factors, is anthropogenic pollution (7, 11, 12). Pollutants can originate from traffic, industrial production and agriculture, including pesticides (13). They enter the environment via deliberate application or leakage and poor waste management (14). Because agricultural intensification is one of the most apparent reasons for the observed declines in insects, research has strongly focused on the effects of pesticides and fertilizer application on insect health and fitness (12). However, especially in or close to urban areas, industrial pollution from heavy metals, airborne particulate matter, or plastic waste may also adversely affect insect populations (15, 16). A potential sink for such pollutants is soil, as contaminants can accumulate therein over centuries (13, 17). As a result, soils may contain mixtures of pollutants originating from various anthropogenic activities over the years. For soil-dwelling insects, such as ants nesting in soil, springtails, or beetles, this could be very problematic (18, 19).

Despite the apparent risk of contaminated soils, research addressing the effects of pollutants on insects concentrates on aquatic insects and pollinators (12). The focus on pollinators is evident, not only due to their ecological and economic value but also because many of them are eusocial insects. Social insects may be especially threatened by pollution for several reasons. They often have large foraging areas and transport food to their nest as a central storage place. The storage of food also likely accumulates pollutants in the nest. Such an accumulation may lead to chronic exposure to a mixture of pollutants to adults and their offspring (16). For example, in different compartments of honeybee hives, heavy metals and pesticides have been identified and shown to have negative effects on individual bees and colony development, primarily affecting brood stages negatively (20–24).

The effect of multiple stressors on insects has recently come into focus, and experiments with two or more stressors gain momentum (25, 26). Currently, many studies focus on the interplay of two or more pesticides (27, 28) or the interplay of pesticides with another stressor, such as climate change (29, 30), or pathogens (31–33). Many insects will likely be confronted with multiple-stressor scenarios under natural conditions in human-altered landscapes. The outcomes of these studies show different interactive effects. Some stressors interact antagonistically such as temperature diminishing adverse effects of pesticides (29, 34). Other studies found evidence for synergistic effects because exposure to multiple stressors increased negative effects in a non-additive way [overview in (16, 35)]. However, we still largely lack data investigating potential interactions between stressors and the form of the interaction in soil insects. Research in aquatic environments and in bees show that non-additive effects are quite common (36, 37). Understanding these interactive effects between multiple stressors in insects is vital when trying to unravel the complexity of insect declines and to predict how a combination of particular stressors will impact insects.

Ants are a prominent insect group in most terrestrial ecosystems with regard to species diversity and biomass (38). They are important ecosystem engineers due to their functions in soil perturbation, nutrient cycling, seed dispersal and as pest controllers (39–42). Even though many reports on the decline of Hymenoptera such as wild bees exist, the evidence for ant species declines is still sparse (12, 43). Increasing land-use intensity in temperate grasslands has been shown to result in a decrease in ant species richness and abundance (44–46). The drivers of this decrease were a higher frequency of mowing or fertilization (45). In agricultural land or other strongly human-impacted habitats, such as urban parks, roadside habitats or surroundings of industrial sites, ant species richness and abundance decrease [(47), overview in (48)]. Here, habitat fragmentation, habitat loss and soil pollution may drive the decline of ant diversity and abundance (47–50). In addition, neonicotinoid insecticides that are widely used in agriculture have been shown to lower colony growth-rate of ants (51).

As long-lived organisms and central place foragers, ants can be negatively affected by pollutants in their environment. Pollutants may accumulate in their bodies (49, 52) with adverse effects on individual and thus colony-level fitness [(49), overview in (16, 51, 53)]. Some ants, such as the black garden ant *Lasius niger*, live in a variety of different habitats, including agricultural and urban areas (54). The soil in such areas may be contaminated with a mixture of anthropogenic pollutants, such as microplastic deriving from degradation of larger plastic litter, airborne particulate matter from traffic and industrial processes such as brake dust or soot, or manure as fertilizer that is commonly applied to arable land and grasslands. The number of microplastic particles in the soil varies widely between sites, with concentrations of up to 6.7% (w/w) in industrial areas (55, 56). Identifying and quantifying airborne particulate matter in soil is complicated as the elemental composition may overlap with natural soil components. However, unnaturally high amounts of metals can be attributed to external sources such as brake dust (57, 58). Isotopic analyses revealed up to 0.54% (w/w) of urban soils in Arizona as soot carbon black (59). As for manure, the European law allows application of up to 35 tons per year per hectare, resulting in large quantities on agricultural fields and grasslands (60).

In many ant species, like *Lasius niger*, queens found new colonies during a claustral phase. They build a nest in the soil and raise their first brood by metabolizing stored body reserves by histolysis of their flight muscles (38, 61). Only a minority of young queens successfully manages to found a colony. Predators catch many queens during their nuptial flight and subsequent search for a suitable nest site (38, 62). Nesting in soil, queens potentially encounter many pollutants during the claustral phase of colony founding, affecting the queen's fitness and the development of their brood. Negative effects of pollutants in the soil may therefore further diminish the ratio of queens successfully founding a colony.

To test this hypothesis, we exposed *Lasius niger* queens at the claustral colony founding stage to five pollutants in two concentrations to reveal the potential effects of different soil pollutants on ants. We simulated different soil contaminations by mixing soil with brake dust, soot, microplastic particles, microplastic fibers, and manure. We compared the effects of each pollutant alone to multiple stressor environments with combined pollutants. Finally, we measured the development time of the different life stages and queen survival. Once workers were present, we measured the brood weight and the number of offspring as a proxy for colony founding success. Except for water, the queens do not take up any food during claustral colony foundation. Therefore, we do expect marginal lethal effects of the pollutants on the queens themselves, as toxic effects would mostly be exerted via contact of the cuticle with the contaminated soil. However, since the brood and especially larvae have a thinner cuticle than the queens, these life stages may be negatively affected directly when in contact with contaminated soil. The presence of pollutants could cause stress in the queens, leading to reduced investment in the brood due to allocation costs (63), since pollutants may be taken up by the queen when cleaning the surface of larvae that have come into contact with the contaminated soil or feeding during brood care.

Moreover, pollutants may alter the microbial community in the soil, which could affect the founding process (64). Even though we expect to find negative effects of single pollutants, it might well be that significant effects only manifest when ant queens or brood are exposed to a combination of pollutants. Organisms might be able to compensate for single effects but will be overstrained when facing multiple stressors.

MATERIALS AND METHODS

Ant Queen Collection and Housing

Between 9th and 12th July 2020 we collected 600 *L. niger* wingless queens after their nuptial flight in and around Bayreuth (Bavaria, Germany). We kept them in plastic boxes containing damp paper towels until further use. On 13th July, 510 queens were randomly assigned to 16 soil treatments and one control treatment ($N = 30$ queens per treatment) using Research Randomizer (65). For this, we prepared 15 ml falcon tubes as nests for the queens with the different soils. Each tube was filled with 5 ml autoclaved water and a cotton ball pushed to the 5.5 ml mark to provide constant moisture. On top of the cotton ball, we put 5 ml of the respective soil treatment. The soils contained different types, concentrations and combinations of pollutants (For details see "Preparation of Soil Treatments"). Finally, we placed one queen into each tube, closed the screw-top loosely to ensure air circulation and then started the experiment.

During the experiment, the queens were kept in a climate chamber at 20°C and 70% humidity under a constant 12:12 h light:dark cycle. Only the regular checks for queen survival, the presence of the brood stages and burrowing depth were performed in the laboratory at room temperature. Brood stages included eggs, larvae, pupae and freshly hatched workers. We checked tubes every other day until eggs or the next brood stage appeared. Then, we checked daily for a whole week. For the checks, we used a dissecting microscope to identify brood stages clearly. Observers were blind regarding the treatment. After the first worker hatched, the tube, including the queen and the brood, was frozen at −20°C until further assessment to compare colony founding success at a defined stage. In cases where no workers emerged, queens were frozen 60 days after the start of the experiment. Finally, we sorted, counted, and weighed each queen and its brood (dry weight after 48 h at 50°C). For each queen, we calculated the development times of brood stages by deducting the days of the first emergence from each other. As we froze the brood when the first worker appeared, worker count alone is not a meaningful variable as sometimes more than one worker hatched at the same time by chance. Consequently, for brood count, we add up the number of pupae and workers to have only one variable.

Preparation of Soil Treatments

The soil used in our experiment was provided by the Ecological Botanical Garden of the University of Bayreuth and consisted of low-nutrient cultivation soil mixed with 10% organic compost. Before mixing it with pollutants the soil was dried for overnight at 70°C in a drying oven (UFE 600, Memmert, Schwabach, Germany) and sieved. We added autoclaved water (20% v/v) to the soil to establish the same moisture in each soil treatment. Then we used the following pollutants to prepare the soil treatments: brake dust particles, soot particles, polystyrene particles, polystyrene fibers and liquid manure.

Brake Dust Particles

Brake dust particles were provided by the Department of Ceramic Materials Engineering of the University of Bayreuth. Tribologically tested LowMet brake pads (provided by TMD company) were ground, after several braking cycles on a ceramic brake disc, that means after a dissipation of a total friction energy of about 15 MJ and temperatures up to 400°C. In order to reach the required fine-grained powder, 3 min in total, a vibrating cup mill with tungsten carbide grinding set up (pulverisette 9, Fritsch GmbH, Idar-Oberstein, Germany) was applied. A breakdown of the composition of such brake pads can be found in Breuer and Bill (66). The biggest fractions consist of steel wool [15% (w/w)], petrol coke [12% (w/w)], sulfides [10% (w/w)] as well as aluminum oxide and binder [both 5% (w/w)] (66). The particle sizes of the ground brake pads were measured using a laser diffraction particle size analyzer (PSA 1190 LD, Anton Paar GmbH, Ostfildern-Scharnhausen, Germany). The average particle size found was $10.19 \pm 4.37\,\mu\text{m}$ ($D10 = 0.68\,\mu\text{m}$, $D50 = 5.76\,\mu\text{m}$, $D90 = 25.87\,\mu\text{m}$).

Soot Particles

We used the carbon black PRINTEX 30 Furnace Black (Degussa AG, Frankfurt, Germany) for the soot treatments with an average primary particle size of 27 nm. Carbon black and soot are often used interchangeably even though they are distinct from each other. Carbon blacks are commercially produced elemental carbon particles with different properties (67). In contrast, soot is a by-product of relatively uncontrolled, incomplete combustions, which results in a material of varying and often unknown composition (68). In terms of particle size, there is a high degree of similarity between soot and carbon blacks (69). As we want to simulate contaminated soil and since soot is the most similar, naturally occurring pendant we henceforth refer to the carbon black as soot.

Polystyrene Particles and Fibers

Granules were ordered from Styrolution (Frankfurt am Main, Germany) and further processed to particles and fibers by the faculty of Macromolecular Chemistry I (MCI) at the University of Bayreuth. Polystyrene particles had a particle size of 125–200 µm, while the fibers had a length of 1–4 mm and a diameter of 40 µm.

Liquid Manure

Liquid manure was provided by a small dairy cattle farm in Bauerngruen near Bayreuth on 10th July 2020 (49.894071, 11.587649). The liquid manure was collected directly from the outlet of the stable for calves (where ∼20 calves are kept at a time) that do not receive any treatment with antibiotics according to the farmer. However, it is likely that the calves have received deworming treatment. As manure is typically applied in liquid form, we did not dry the manure. Also, drying the manure likely changes its properties making it less comparable to the common practice in agriculture.

We assessed individual and dose-dependent effects of each solid pollutant by using single pollutant treatments with two different concentrations [0.5 and 2% (v/v)] of each pollutant mixed into the soil. In the liquid manure treatments, we replaced the water added to the soil completely [20% (v/v)] or partly with liquid manure [5% (v/v)]. In the multiple stressor treatments, we mixed the four solid pollutants in equal proportions with three different overall concentrations [0.5, 2, and 8% (v/v)] into the soil, either with or without manure [20% (v/v)], to see combinatorial effects. We chose the concentrations to assess stressor effects and dose effects (For details see: "Statistical data analyses"; **Figure 1**). Because studies have found a wide range of pollutant concentrations in natural soils [up to 6.7% (w/w)] (56), we chose a similar range for our experiment. However, for conceptual consistency we used volume/volume concentrations, as particles differ dramatically in their bulk density.

Statistical Data Analyses

All statistical analyses were performed using R 4.0.3 (70). We excluded queens that were untraceable after some time (4.1%), mostly due to burrowing in the soil, and were not observed again until freezing from the survival analysis. The survival data were analyzed with a Cox proportional hazard regression (COXPH) with treatment as a predictor [package *survival*, (71, 72)]. Survival model assumptions were tested using Schoenfeld residuals [*survminer*, (73)].

For all subsequent analyses, we excluded queens that died during the experiment ($N = 36$), were untraceable for at least 10 days ($N = 21$) or had zero or more than five workers at the point of freezing ($N = 35$). More than five workers at the point of freezing indicated that we missed the day of first emergence because normally only few workers emerge within 1 day. Queen weight, brood weight, brood counts and development times were analyzed fitting generalized linear models GLMs with treatment as a predictor. We checked model assumptions using model diagnostic test plots, i.e., qqplot and residual vs. predicted plot from the package *DHARMa* (74). Depending on model assumptions, we then used Kruskal-Wallis tests or produced F-statistics with the function *anova()* to calculate *p*-values for differences between treatments. For significant treatment effects, we ran pairwise comparisons. In the case of a significant Kruskal-Wallis test, pairwise comparisons were done using Dunn's test for multiple comparisons with Benjamini-Hochberg correction [package "FSA," (75)]. In the case of a significant ANOVA, pairwise comparisons were made using Tukey HSD *post-hoc* test with Benjamini-Hochberg correction from the package *multcomp* (76). Even though we ran all possible pairwise comparisons, we only report the relevant comparisons because comparing different stressor types with different concentrations is not very informative. To characterize the stressor effects, we show the comparisons of each stressor type to the control, the comparisons between stressor types of the same concentration and the comparisons between the high concentration of manure and the multiple stressor treatments with manure. Finally, we compared the treatment levels within the same stressor type to identify dose effects (**Figure 1**). To measure effect sizes of the pairwise-comparisons, we calculated Hedges' G with the package *esvis* (77). Data were arranged using the package *tidyr* (78) and were plotted using the package *ggplot2* (79).

RESULTS

Overall, we found very low mortality in the queens. Thirty-six of 510 queens (7.1%) died during the experiment. Queen survival was similar across treatments (COXPH overall LR-test: $X^2 = 23.23$, $df = 16$, $P = 0.108$).

Queen weight significantly differed between the treatments [GLM with gamma distribution: $F_{(16,\ 324)} = 1.896$, $P = 0.020$]. However, only queens exposed to the high concentration of manure were significantly heavier than the queens exposed to the low concentration of manure (**Table 1**, **Supplementary Figure 1**, Tukey comparison: $P = 0.044$). Even though treatment significantly affected brood weight [GLM with gaussian distribution, $F_{(16,\ 324)} = 2.168$, $P = 0.006$], the multiple comparison analyses showed no significant differences between the treatment levels (**Table 1**, **Supplementary Figure 2**). At higher manure concentrations brood weight tended to be lower than the control. But for the other pollutants we could not identify a clear pattern.

Neither the number of eggs [GLM with gaussian distribution: $F_{(16,\ 324)} = 1.601$, $P = 0.067$], nor the number of larvae differed between treatments [GLM with gaussian distribution: $F_{(16,\ 324)} = 1.668$, $P = 0.051$; **Supplementary Figures 3**, **4**]. In contrast, the number of pupae and workers significantly differed between treatments [GLM with gaussian distribution: $F_{(16,\ 324)} = 2.852$,

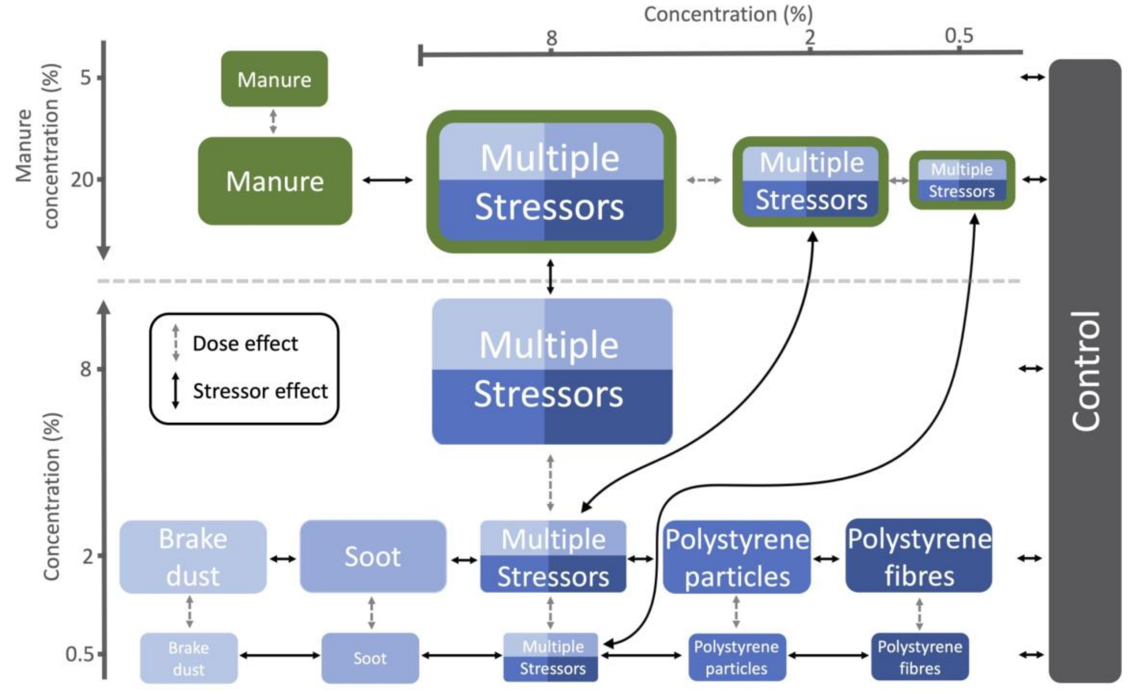

FIGURE 1 | The different treatments and their stressor concentrations (v/v). Relevant comparisons are comprised between treatments that lie on a vertical or horizontal line or are connected with arrows. Dashed arrows represent the dose effect, i.e., the comparisons within stressor type. Solid arrows represent the stressor effect, i.e., the comparisons between stressors types or stressor type and control.

$P < 0.001$]. Queens exposed to the high concentration of manure had fewer pupae and workers than control queens [Tukey comparison control vs. multiple stressors (2%) + manure: $P = 0.030$] and queens exposed to multiple stressors [Tukey comparison multiple stressors (0.5%) vs. multiple stressors (0.5%) + manure: $P = 0.004$; **Table 1**, **Supplementary Figure 5**].

The development time from egg to larvae significantly differed between treatments (Kruskal-Wallis rank sum test: $X^2 = 112.86$, $df = 16$, $P < 0.001$). The development time from egg to larvae was longer in treatments containing the high concentration of manure compared to controls (**Figure 2**, Dunn's comparisons: see **Table 1**). We also found a significant effect of treatment on the development time from larvae to pupae (Kruskal-Wallis rank sum test, $X^2 = 39.311$, $df = 16$, $P < 0.001$). However, *post-hoc* Dunn's test revealed no significant differences between the treatments (**Table 1**, **Figure 2**). The development time from pupae to worker did not differ between treatments (Kruskal-Wallis rank sum test, $X^2 = 14.417$, $df = 16$, $P = 0.568$; **Figure 2**). The overall development time from egg to worker differed among treatments (Kruskal-Wallis rank sum test, $X^2 = 88.944$, $df = 16$, $P < 0.001$). Similar to the development time from egg to larvae, the development time from egg to worker was longer in the treatments with the high concentration of manure compared to controls and multiple stressor treatments (**Figure 2**, Dunn's comparisons: see **Table 1**).

DISCUSSION

In this study, we looked at the effects of different soil pollutants on the colony founding success of *Lasius niger* ant queens in the laboratory. Ants were exposed to different concentrations and combinations of pollutants to determine dose-dependent effects and interactions between these potential stressors. While brake dust particles, soot and polystyrene microplastic (particles or fibers) did not affect any of the measured colony foundation parameters, a high concentration of manure in the soil led to delayed egg development and a smaller number of pupae and workers.

The overall ant queen mortality was very low (7.1%) and was not affected by soil treatment. This indicates that pollutants in the soil, at least those used here, do not exert toxic effects on the ant queens. During colony founding, ant queens do not consume any food as they meet their energy demands by using internal resources, such as degeneration of their flight muscles (38, 61). For a pollutant to be toxic at this stage, it would have to be lethal at a very low dose or capable of entering via the cuticle or the trachea. Consequently, most pollutants at field-realistic doses, such as insecticides and fungicides, do not increase mortality in founding ant queens [(51, 81, 82), but see (83)].

While we found no differences in queen survival, the number of offspring or brood weight, exposure to a high concentration of

TABLE 1 | *P*-values and Hedges' g of the relevant comparisons with a *p*-value below 0.1 for the different response variables.

Response	Comparison	Mean difference	*p*-value	Hedges' g
Weight (mg)				
Queen	Manure (20%) > Manure (5%)	1.524	**0.044**	1.123
Brood	Control > Manure (20%)	1.019	0.055	0.964
	Control > Multiple stressors (2%) + Manure (20%)	1.133	0.052	0.937
	Control > Multiple stressors (0.5%) + Manure (20%)	0.943	0.092	0.916
	Multiple stressors (0.5%) > Multiple stressors (0.5%) + Manure (20%)	0.908	0.092	0.938
Number of				
Eggs	–	–	–	–
Larvae	–	–	–	–
Pupae + worker	Control > Manure (20%)	4.146	0.068	0.906
	Control > Multiple stressors (0.5%) + Manure (20%)	4.313	0.068	1.005
	Control > Multiple stressors (2%) + Manure (20%)	5.174	**0.030**	0.901
	Manure (5%) > Manure (20%)	3.662	0.080	0.747
	Multiple stressors (0.5%) > Multiple stressors (0.5%) + Manure (20%)	5.950	**0.004**	1.408
	Multiple stressors (2%) > Multiple stressors (2%) + Manure (20%)	4.389	0.064	0.753
Development time (days)				
Egg to larvae	Manure (20%) > Control	1.521	**0.002**	0.893
	Manure (20%) > Manure (5%)	2.018	**<0.001**	1.298
	Multiple stressors (0.5%) + Manure (20%) > Control	2.188	**0.009**	0.944
	Multiple stressors (0.5%) + Manure (20%) > Multiple stressors (0.5%)	2.550	**<0.001**	1.207
	Multiple stressors (2%) + Manure (20%) > Control	1.632	**0.026**	0.834
	Multiple stressors (2%) + Manure (20%) > Multiple stressors (2%)	2.278	**<0.001**	1.426
	Multiple stressors (8%) + Manure (20%) > Control	0.952	0.051	0.620
	Multiple stressors (8%) + Manure (20%) > Multiple stressors (8%)	1.492	**0.002**	1.453
Larvae to pupae	Manure (20 %) > Control	0.854	0.068	0.783
	Multiple stressors (0.5 %) + Manure (20 %) > Control	1.213	**0.050**	0.613
	Multiple stressors (8 %) + Manure (20 %) > Control	1.224	0.060	0.815
Pupae to worker	–	–	–	–
Egg to worker	Manure (20%) > Control	1.958	**0.010**	0.955
	Manure (20%) > Manure (5 %)	2.478	**<0.001**	1.265
	Multiple stressors (0.5%) + Manure (20%) > Control	2.725	**0.025**	0.998
	Multiple stressors (0.5%) + Manure (20%) > Multiple stressors (0.5%)	3.650	**<0.001**	1.519
	Multiple stressors (2%) + Manure (20%) > Control	1.708	0.087	0.724
	Multiple stressors (2%) + Manure (20%) > Multiple stressors (2%)	2.167	**0.008**	0.952
	Multiple stressors (8%) + Manure (20 %) > Control	1.493	0.064	0.748
	Multiple stressors (8%) + Manure (20%) > Multiple stressors (8%)	2.163	**0.004**	1.472

According to Cohen (80) effect sizes > 0.8 indicate a large effect. Significant adjusted P-values are shown in bold.

manure (20% v/v) delayed brood development time resulting in a delayed time of first worker emergence. A slower development from egg to larvae caused this delay (**Figure 2**). While we are not aware of any study investigating the effects of manure on the development of soil-dwelling insects, the effects on soil properties, such as pH or oxygen, or on microbial soil communities are well-studied. Even though we did not measure it, manure typically changes soil pH and increases the availability of nutrients, which in turn increases microbial biomass (84, 85). This shift in pH and biomass affects the structure of the microbial soil community (86–88). Such changes in the soil may also affect the development of larger organisms. Because ant development varies with environmental conditions (89), the delayed egg development may be explained by manure-induced changes in oxygen-levels. As insect eggs depend on oxygen for their development, they have diffusion holes in the shell (90, 91). Under low oxygen levels in the immediate environment, *Tenebrio molitor* (92) and *Drosphila melanogaster* (93) show slower development. The application of manure reduces oxygen levels in the soil, sometimes locally even leading to anoxic areas (94, 95). Peak oxygen deficits in soil occur 16 h after the manure application and go back to near-normal within the following days (94). We assume this process could explain the observed delay in the development time of the brood stages in our study. Because an oxygen deficit only manifests for a short time after the manure was added, it most likely just affected

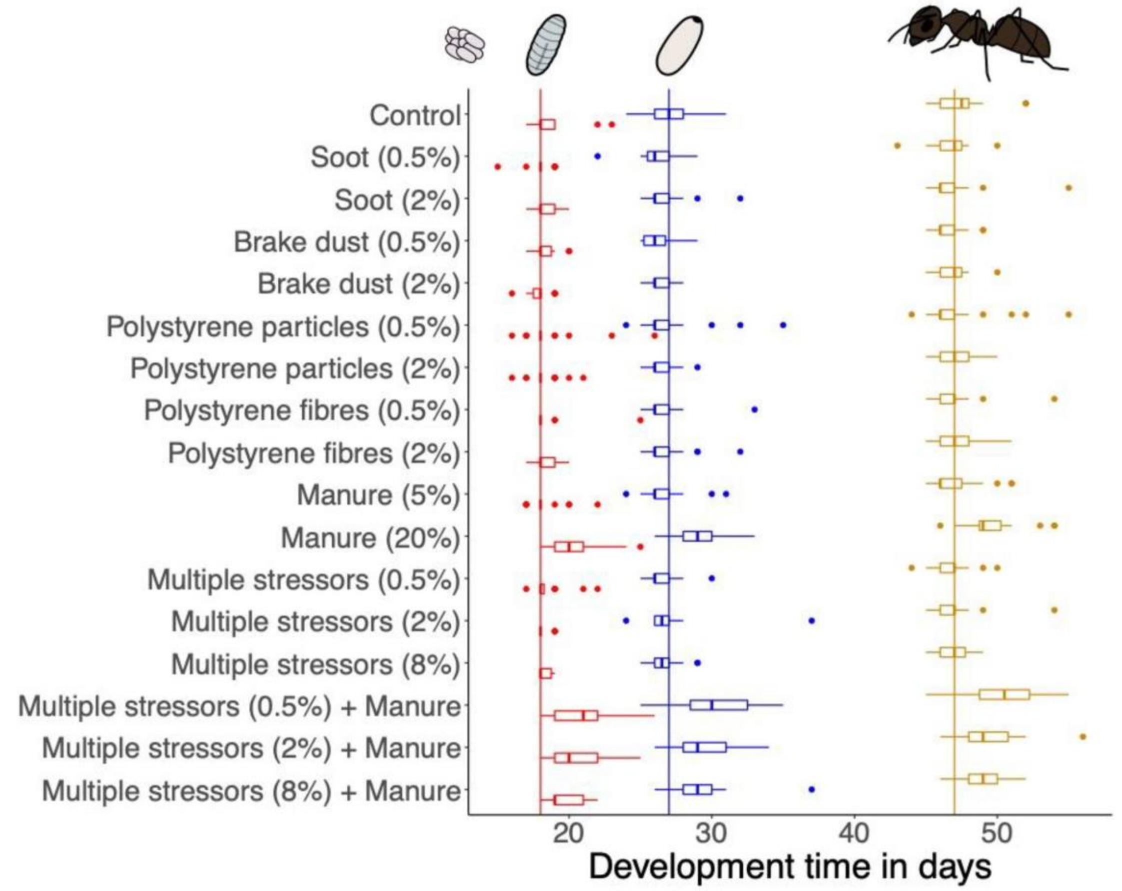

FIGURE 2 | Development time of the brood stages of each treatment (days since first appearance of eggs). Boxplots show median, first, and third quartile. Dots show outliers outside of 1.5 × Inter-quartile range. Horizontal lines indicate the median of each brood stage over all treatments (red = larvae, blue = pupae, yellow = worker).

the early brood development. As most queens (94%) laid their eggs within the first 2 days, delayed egg development could have been caused by low oxygen levels. Later brood stages were not affected since oxygen should have been back to normal after a few days. However, as we did not measure oxygen levels during our study, this explanation remains hypothetical. The smaller number of pupae and workers in the manure treatments might be explained in a similar way. Low oxygen levels at the beginning of the experiments may have not only caused delayed development but also lead to losses in the first egg clutch (91). Those early losses would result in fewer pupae and workers at the time of first worker emergence. A smaller number of workers and a delayed development could lead to less competitive ant colonies. As ants are in constant competition for resources and habitat, a smaller colony size at an early stage may reduce the survival probability of affected colonies (96). Repeated treatment of grasslands with manure can then add up to the observed slight but significant negative effects of fertilization on ant species richness and abundance (45).

The manure we used for our experiment was collected directly from the sewage of a stable that houses calves that were not treated with antibiotics. However, large-scale cattle farming typically relies on high amounts of antibiotics and dewormers (97, 98). The effect of manure that contains residues of medications could be different from the effects we observed as it is likely that that soil fauna is even more affected (99). Before application, manure is often aged in a lagoon in which bacteria degrade organic matter (100). Again, such manure could have different effects than the ones we observed, as many of the organic compounds are already decomposed (101). Because we used nearly sterile soil (dried overnight at 70°C + use of autoclaved water) in our setup, the effects of manure in our experiment could partly be attributed to microorganisms. Manure typically carries high loads of different microorganisms. Therefore, the manure treatments also represent a bacteria-rich environment, at least in comparison to our other almost sterile soil treatments. Ant queens maintain their own and their nest's hygiene by investing in external immunity, which can come at the cost of reproduction (64, 102, 103). *L. niger* queens founding a colony under high microbial pressure are actually forced to pay a substantial cost by simultaneously investing in reproduction and immunity (64). Therefore, the negative impact of manure in our experiment could also partly be caused by the similar effect of microbes on colony development described by Tragust et al. (64).

Apart from manure none of the other pollutants applied, i.e., brake dust, soot, microplastic particles and fibers, caused any changes in the investigated colony founding parameters. It is highly unlikely that initial queen weight affected this finding as we have fully randomized the assignment of the queens to the different treatments using an automated algorithm (65). Nevertheless, it would be interesting to investigate a potential effect of pollutants on the relation between initial queen weight and colony founding success. Although other studies show that fecundity and brood development of ants are sensitive to sublethal concentrations of pollutants, we did not detect any effects on those parameters in *L. niger*. In contrast, azole fungicides decrease fecundity in *L. niger* and the semi-claustral founding ant *M. rubra* (81, 83, 104). Thiamethoxam, a neonicotinoid insecticide, negatively impacted *L. niger* resulting in smaller colony size and worker weight (51). Selenium, a widespread contaminant in soils resulting from agricultural irrigation, hindered brood production in the Argentine ant *Linepithema humile* (105). A field experiment revealed negative effects of microplastic pollution on soil fauna, including ants (106). However, studies investigating the direct effects of microplastic on ants or studies that try to detect microplastic in ant bodies are still lacking. The combination of pollutants in the multiple stressor treatments revealed no additional effects other than those induced by treatments with a high concentration of manure. Therefore, we found no notable interactions among the different pollutants that would cause different effects on the ant queens.

Even though we did not find any effects of the pollutants apart from manure during our experiment, we cannot yet declare the pollutants as harmless to founding *L. niger* queens, as our experimental setup had some limitations. The commercially produced carbon black we used as soot is chemically distinct from real soot. Soot has a higher proportion of organic compounds which might have affected the ants differently (67). Similar, the brake dust in our experiment was artificially produced by grounding brake pads. Brake dust from real braking processes may differ in chemical and physical composition and thus affect ants differently. Another limitation of our study is that the period until the emergence of the first worker may not be sufficient to detect mid- or long-term effects of pollutant exposure. Several studies show that ants can compensate for stress for some time but ultimately must pay the hidden costs later in life. Some effects of a fungal pathogen in combination with physical stress on claustral colony founding ant *Crematogaster scutellaris* were only present at an early stage. In contrast, others only became evident in the long-term (107). The impact of microbe-enriched soil on *L. niger* queens just appeared when they were forced to lose their first batch of brood after hibernation (64). Another study found no effects of thiamethoxam on *L. niger* colonies before the first overwintering, but exposed colonies had fewer workers and larvae before the second winter (51).

Apart from long-term effects, other hidden costs might be present that our study design cannot uncover. The pollutants could affect worker health and immunity. Eg., heavy metal pollution suppressed the encapsulation response in wild colonies of *Formica aquilonia* (53). Ant workers may be more prone to the pollutants than the queen, as a recent study suggests a superior detoxification system in ant queens (51). In honeybees (*Apis mellifera*), queens are much more tolerant to acaricides than workers, even when adjusted for body size (108).

The black garden ant *L. niger,* a prevalent ant species across Europe, has a wide range of habitats, including urban areas and agricultural fields (54). The frequent occurrence might be explained by a higher stress tolerance of *L. niger* than other species. Higher resilience to disturbance and pollutants forms an important trait to tolerate and survive in human-altered landscapes. Genomic analysis revealed an increased potential of stress-resistance in *L. niger* compared to other ant species (109). The higher number of cytochrome P450 genes present in *L. niger* could improve its detoxification abilities of anthropogenic pollutants. Moreover, *L. niger* prefers visual information over pheromone trails for foraging, making it less vulnerable to interferences with repellent substances that could be especially present in urban environments (110, 111). These findings suggest a higher tolerance of *L. niger* against pollutants than other ants, even though we do not know of any study explicitly testing this hypothesis. Studies of *Formica* s. str. in heavy metal polluted areas showed that even closely related species can differ in their sensitivity to pollutants (49). Consequently, even though we do not find any short-term effects of pollutants on *L. niger*, we cannot conclude that there are no effects on ants in the long-term or that the pollutants studied here have more detrimental effects on other ant species. Especially rare ant species may be more vulnerable to pollution.

We could show that single and combined exposure of different soil pollutants does not affect colony founding in *L. niger* until the first workers emerge. The application of manure, however, affected colony founding by prolonging the development time from egg to larvae which ultimately led to a delayed emergence of the first workers. Moreover, fewer pupae and workers were raised by the queens in the manure treatments. These findings underline the issue of excessive manure application in our environment. Even though we did not find any effects or interactions among the other pollutants, effects on later stages of colony development cannot be ruled out. Therefore, future studies could investigate potentially hidden long-term effects of pollutants on colony development. Of similar importance might be to show if and how ant queens take up the pollutants.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

DS, AH, OO, and HF conceived the idea, designed the experiment, and wrote the manuscript. AH, NL, and TO produced the particles. DS, KK, and DL carried out the experiment. DS, KK, and OO performed the statistical analysis. DS, AH, DL, KK, OO, and HF interpreted the results. All authors read and approved of the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/finsc.2021.761881/full#supplementary-material

Supplementary Figure 1 | Queen weight of the different treatments. Boxplots show median, first and third quartile. Dots show outliers outside of 1.5 × Inter-quartile range.

Supplementary Figure 2 | Brood weight of the different treatments. Boxplots show median, first and third quartile. Dots show outliers outside of 1.5 × Inter-quartile range.

Supplementary Figure 3 | Number of eggs of the different treatments. Boxplots show median, first and third quartile. Dots show outliers outside of 1.5 × Inter-quartile range.

Supplementary Figure 4 | Number of larvae of the different treatments. Boxplots show median, first and third quartile. Dots show outliers outside of 1.5 × Inter-quartile range.

Supplementary Figure 5 | Number of pupae and workers of the different treatments. Boxplots show median, first and third quartile. Dots show outliers outside of 1.5 × Inter-quartile range.

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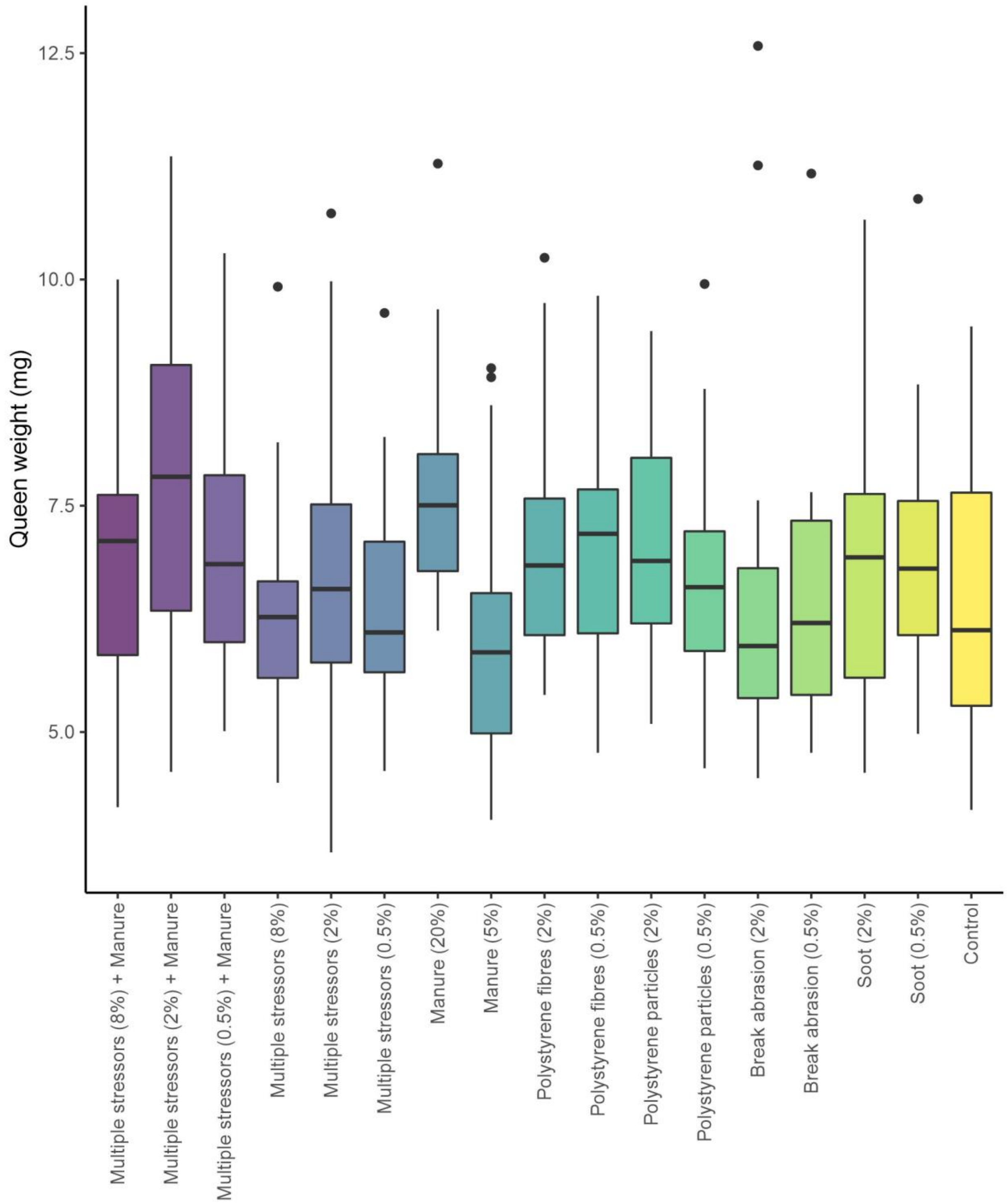
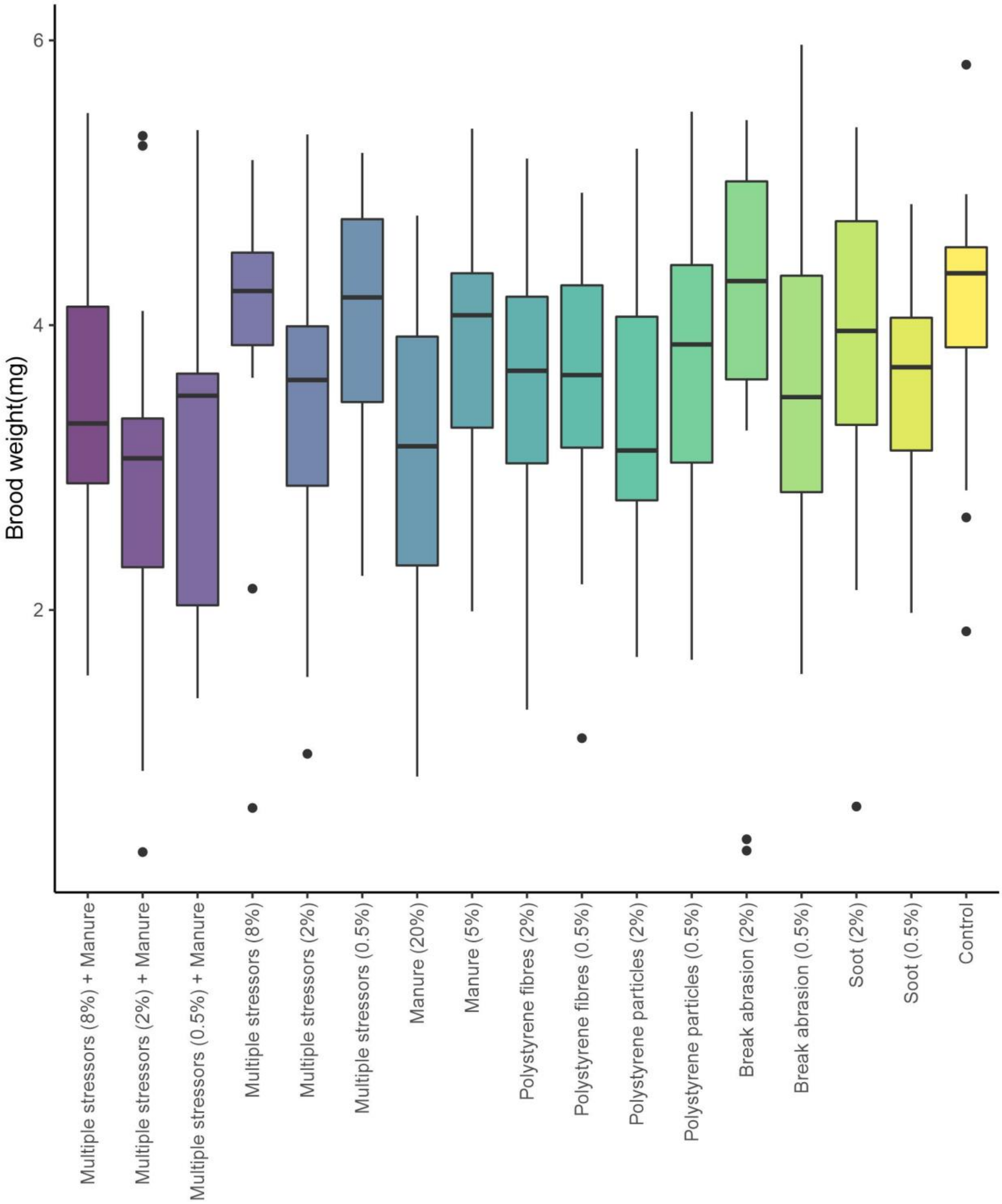
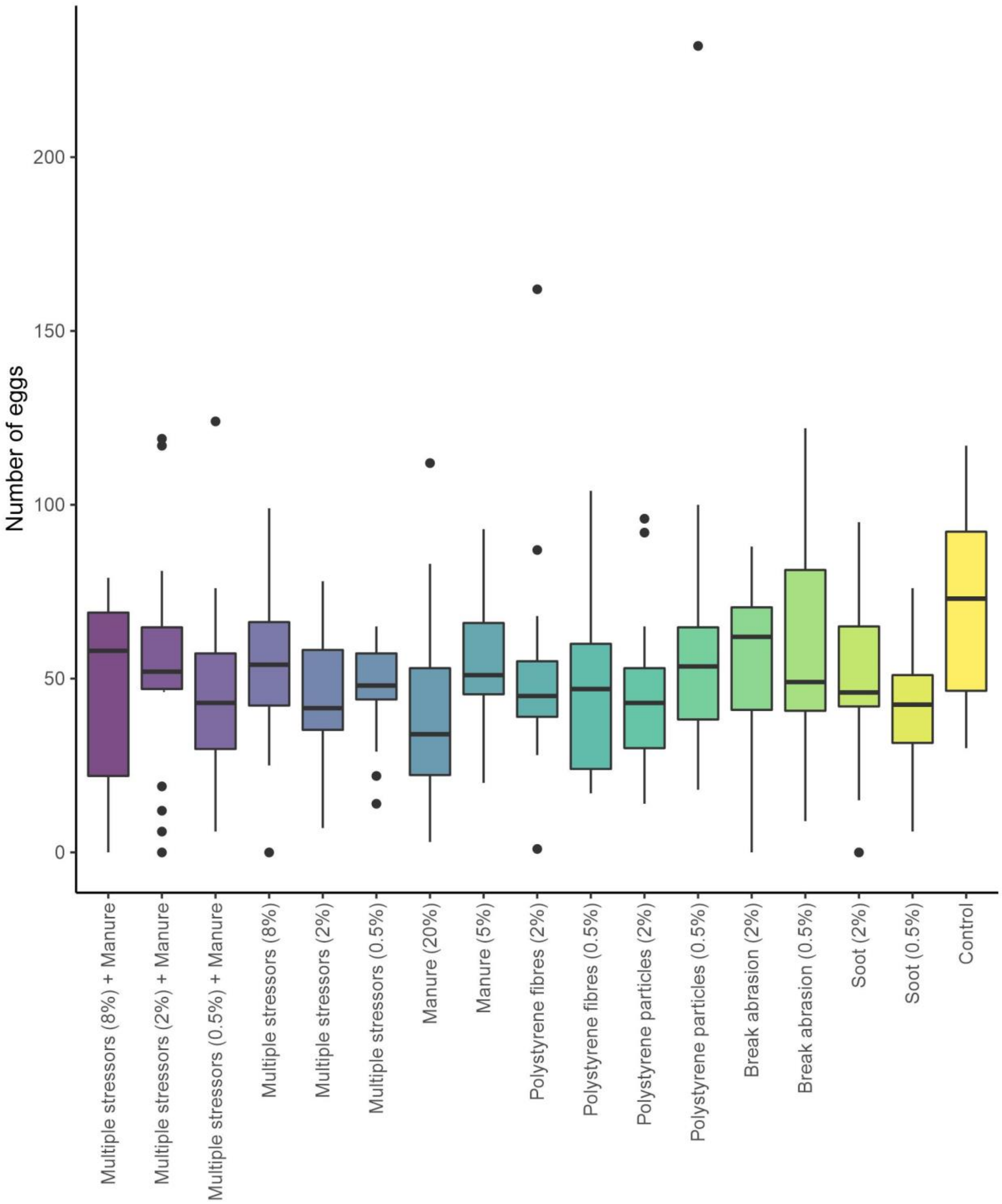
Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.



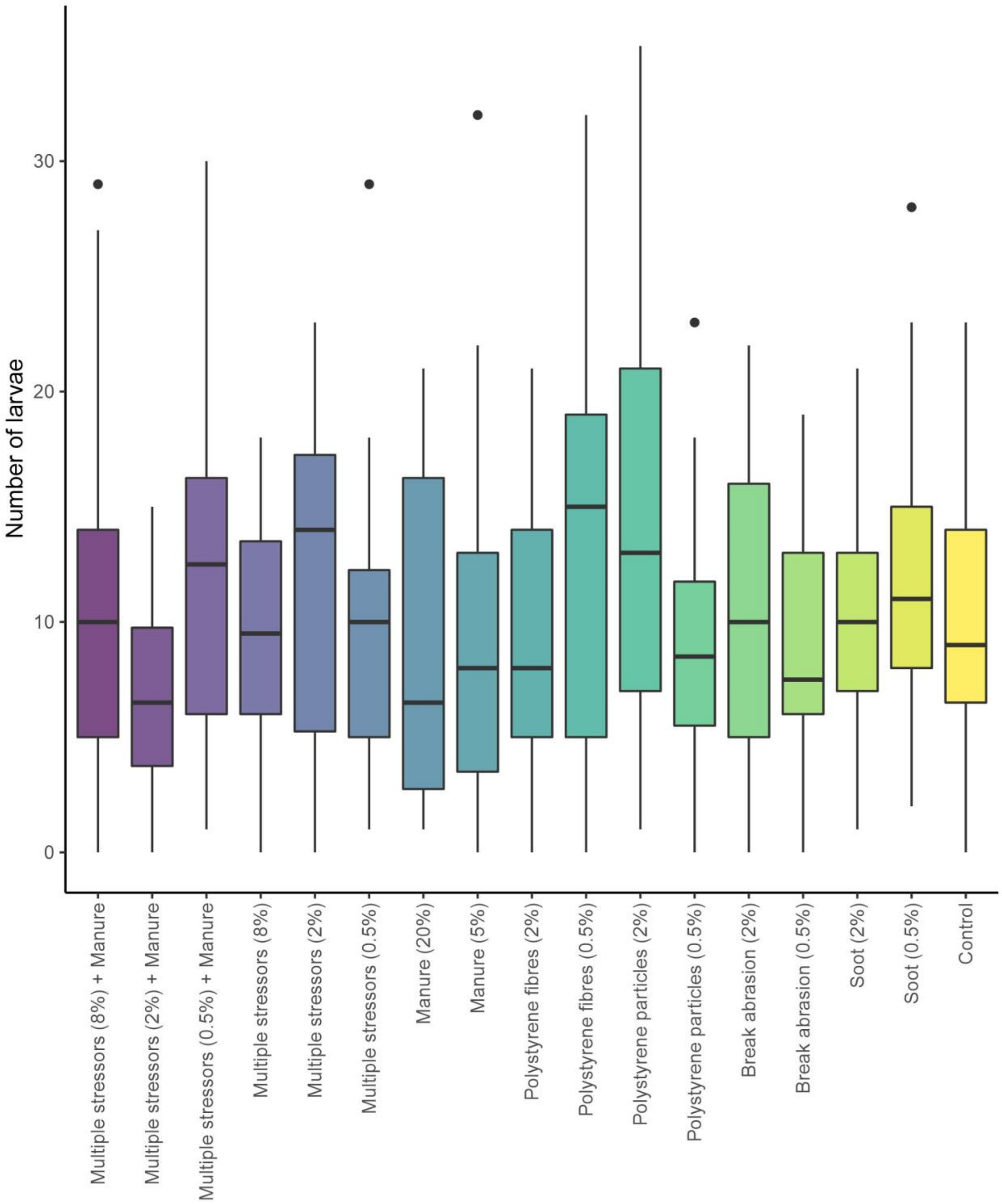
Supplementary Figure 1. Queen weight of the different treatments. Boxplots show median, first and third quartile. Dots show outliers outside of 1.5 × Inter-quartile range.



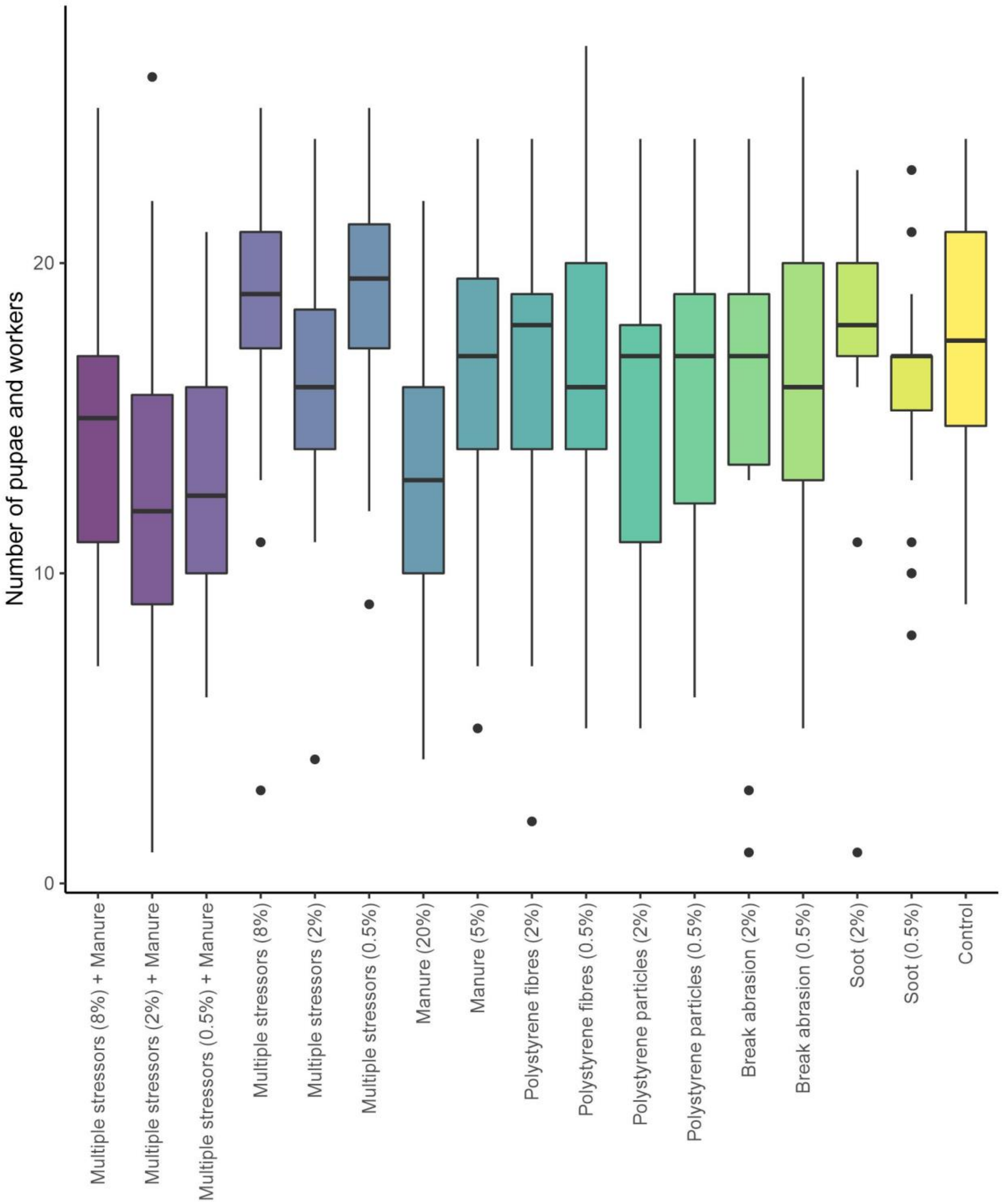
Supplementary Figure 2. Brood weight of the different treatments. Boxplots show median, first and third quartile. Dots show outliers outside of 1.5 × Inter-quartile range.



Supplementary Figure 3. Number of eggs of the different treatments. Boxplots show median, first and third quartile. Dots show outliers outside of 1.5 × Inter-quartile range.



Supplementary Figure 4. Number of larvae of the different treatments. Boxplots show median, first and third quartile. Dots show outliers outside of 1.5 × Inter-quartile range.



Supplementary Figure 5. Number of pupae and workers of the different treatments. Boxplots show median, first and third quartile. Dots show outliers outside of 1.5 × Inter-quartile range.

List of all own publications

Published in Peer-reviewed Journals:

2025:

Seidenath, D., Pölloth, S., Mittereder, A., Hillenbrand, T., Brüggemann, D., Schott, M., Laforsch, C., Otti, O., & Feldhaar, H. (2025). Exposure to diesel exhaust particles impairs takeoff but not subsequent homing and foraging behavior of workers of the buff-tailed bumblebee *Bombus terrestris*. *Insectes Sociaux*, 1-13.

2024:

Seidenath, D., Mittereder, A., Hillenbrand, T., Brüggemann, D., Otti, O., & Feldhaar, H. (2024). Do diesel exhaust particles in pollen affect colony founding in the bumble bee *Bombus terrestris*?. *Insectes Sociaux*, 71(2), 157-163.

2023:

Seidenath, D., Weig, A. R., Mittereder, A., Hillenbrand, T., Brüggemann, D., Opel, T., Langhof, N., Riedl, M., Feldhaar, H., & Otti, O. (2023). Diesel exhaust particles alter gut microbiome and gene expression in the bumblebee *Bombus terrestris*. *Ecology and Evolution*, *13*(6), e10180.

Hüftlein, F., **Seidenath, D.**, Mittereder, A., Hillenbrand, T., Brüggemann, D., Otti, O., Feldhaar, H., Laforsch, C., & Schott, M. (2023). Effects of diesel exhaust particles on the health and survival of the buff-tailed bumblebee *Bombus terrestris* after acute and chronic oral exposure. *Journal of Hazardous Materials*, *458*, 131905.

2022:

Holzinger, A., Mair, M. M., Lücker, D., **Seidenath, D.**, Opel, T., Langhof, N., Otti, O., & Feldhaar, H. (2022). Comparison of fitness effects in the earthworm Eisenia fetida after exposure to single or multiple anthropogenic pollutants. *Science of the Total Environment*, *838*, 156387.
This publication is not part of the thesis

2021:

Seidenath, D., Holzinger, A., Kemnitz, K., Langhof, N., Lücker, D., Opel, T., Otti, O., & Feldhaar, H. (2021). Individual vs. combined short-term effects of soil pollutants on colony founding in a common ant species. *Frontiers in Insect Science*, *1*, 761881.

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