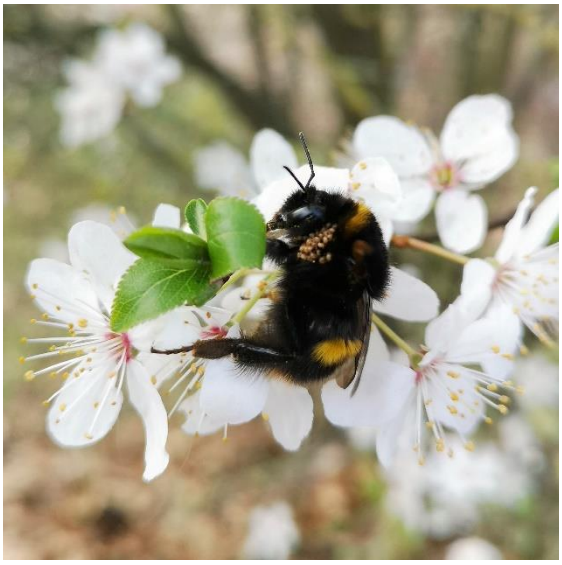
Road Verges for Bumblebee Conservation: A Green Infrastructure Opportunity or an Ecological Trap?



Thesis submitted to the School of Biological Sciences at the University of East Anglia for the degree of Doctor of Philosophy

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Abstract

My thesis discusses the potential of road verges as a tool for bumblebee conservation, focusing on *Bombus terrestris* and roadsides within the UK.

Chapter 1 is an extensive literature review on the topic of road verges as a tool for pollinator conservation. The benefits and drawbacks of roadsides are thoroughly discussed, highlighting areas requiring further investigation. The concept of, and how verges may represent, ecological traps is also covered.

Chapter 2 addresses how distance from a major road impacts the development of *B. terrestris* colonies. The reproductive success of colonies located on the verge is compared to those positioned in the surrounding landscape. The results are discussed in the context of bumblebee conservation, and the importance of large-scale ecologically realistic field studies is highlighted.

Chapter 3 investigates the impacts of two common roadside metals (copper and cadmium) on the development of *B. terrestris* micro-colonies. Micro-colonies were exposed to different levels of metal contamination via a pollen or nectar source. The main findings are presented, with emphasis on the future of the transport sector.

Chapter 4 explores the pollen collection of *B. terrestris* colonies located on verges compared to those in the surrounding landscape. Pollen loads collected from foraging workers from colonies either on the roadside or in the surrounding area were identified using microscopy. Flowers visited by bumblebees were compared to floral resources within the landscape, and the potential for roadsides as a viable forage source for bumblebees is discussed in depth.

I conclude with a summary of all three data chapters, highlighting the knowledge gaps which have been addressed. Areas of research still requiring further investigation are discussed along with conservation applications/recommendations identified by my thesis. The future of the transport industry and the likely impacts this will have on pollinator conservation along verges is also discussed.

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CHAPTER 1: A review of the literature concerning road verges and bumblebees.

This chapter details the literature surrounding the topic of road verges as a conservation tool for bees and other pollinators. I begin by discussing pollinator and bumblebee declines, followed by the use of landscapes by bumblebees. I then discuss road verges and the case for their use in pollinator conservation. I summarise the literature on the benefits of roadside habitat, primarily the potential of verges to provide viable food and nesting resources for bees. The concept of verges as ecological traps is explored, followed by an in-depth assessment into each of the main hazards and pollutants associated with roadsides and their impacts on pollinators. Literature on the management of verges for pollinators is also discussed, and I then describe the aims and structure of my thesis. I identify gaps in current knowledge which include research on pollinator nesting behaviour with respect to road verges, exposure routes of pollutants, and use of verges by bumblebees.

1.1. INTRODUCTION

Pollination is an extremely valuable service provided by the ecosystem (Bauer and Wing, 2016; Dodd et al., 1999; Genung, 2017; Goulson, 2003; Kearns et al., 1998). As angiosperms are sessile, they rely on vectors to reproduce sexually, and it has been estimated that 87% of flowering plants are pollinated by animals (Ollerton et al., 2011). Despite their ecological and economic importance however, pollinators are declining (Goulson et al., 2015; Ollerton et al., 2014; Potts et al., 2016a; Powney et al., 2019). Numerous factors are thought to contribute to these losses, including climate change and invasive species, but their current declines are most strongly associated with severe habitat loss, degradation, and fragmentation as a result of agricultural intensification and changes in land use (Dicks et al., 2021; Kennedy et al., 2013; Potts et al., 2016b).

1.1.1. Bumblebees in decline

Bumblebees, *Bombus* spp., are a group of pollinators that have shown considerable declines both internationally (Hemberger et al., 2021; Soroye et al., 2020; Williams and Osborne,

2009) and in the UK (Comont and Dickinson, 2021; Williams, 1986). Globally, over a third of known and assessed bumblebee species have declined by up to 70% (Arbetman et al., 2017). The European Red List of bees states that 45.6% of European bumblebee species are in decline; 23.6% are threatened with extinction and 4.4% are classed as near threatened (Nieto, 2014). In the UK, two bumblebee species (*Bombus cullumanus* and *Bombus subterraneus*) have been declared extinct in the past 80 years, and seven bumblebee species are currently listed on conservation priority species lists as a result of UK-wide declines (Bumblebee Conservation Trust, 2021; Department for Environment, Food and Rural Affairs, 2022; NatureScot, 2020).

1.1.2. Landscape use by bumblebees

Bumblebees are eusocial, colony-based insects, and understanding how they use and interact with landscapes is pivotal to their conservation. The foraging range of bumblebees differs between species, with individuals from five different species recorded foraging on average between 270m and 550m from the nest, with maximum distance varying between 810m and 2.8km (Redhead et al., 2016). Quantifying bumblebee use of landscapes has been very well documented in the literature, and can be achieved using various techniques such as modelling, genetic sampling, and monitoring reared colonies in the field. Colony development in response to landscape stressors has also been intensively studied, particularly concerning pesticides. I will discuss examples of all three techniques here, with emphasis on monitoring of reared colonies in response to pesticide exposure.

Timberlake et al., (2020) used a combination of genetic sampling and modelling to predict bumblebee colony density in farmland across south-western England. Tarsal samples were taken from 886 *Bombus terrestis* foragers and genotyped at 12-14 loci to estimate colony density in the landscape. A series of 18 predictive models were then performed, each one simulating a different farming scenario (for example adding one hectare of red clover cover crop to each 1km^2 of farmland). The authors found that semi-natural habitat did not influence colony density, however the proportion of gardens was a good predictor. Importantly, they revealed that nectar supply during September was a strong predictor of *Bombus terrestris* colony density the following year, explaining over half of all variation in colony density. Samuelson et al., (2018) reared colonies of *B. terrestris* from wild-caught queens and placed them in gardens in south-eastern England. Locations were classified into one of three categories (agricultural, village, or city) depending on land use within a 500m radius of the colony. They recorded various metrics of colony success and revealed that land use was a strong predictor of colony success, with colonies in the ‘urban’ category producing more reproductives and reaching greater peak size than colonies is the ‘agriculture’ category.

Whilst foraging and nesting in the landscape, bees can be exposed to pollutants that may have negative impacts on their development or overall success; a key example of this is pesticide use. Neonicotinoid pesticides and their negative effects on bees have been extensively studied, and so techniques on bumblebee monitoring in response to pesticides are well established (Rundlöf et al., 2015; Whitehorn et al., 2012; Wood and Goulson, 2017; Woodcock et al., 2017). In southern Sweden, Rundlöf et al., (2015) monitored the success of solitary bees (using three trap nests of *Osmia bicornis* at each site), bumblebees (six commercial colonies of *Bombus terrestris* at each site), and honeybees (using six *Apis mellifera* hives at each site). The authors found that bumblebee colonies in the clothianidin seed-coated treatment gained significantly less weight and produced less males and gynes than colonies in the control treatment. A study across three countries (UK, Germany and Hungary), also found negative effects of seed-coating on bumblebee colonies, with a negative correlation between gyne production and neonicotinoid residues in colony stores.

To my knowledge, there are no studies investigating landscape use by bumblebees with respect to roads. As roads are a ubiquitous feature of modern landscapes and often proposed as a tool for bumblebee conservation, it is vital to understand how bumblebees use verges compared to other habitat types. Also, it is important to understand how bumblebees respond to stressors associated with roadsides. As such, the remainder of this chapter discusses road verges and their potential as tools for bumblebee conservation.

1.1.3. Road verges

To conserve bumblebees and other pollinators, it is important to restore and improve the quality of semi-natural habitat that they rely upon for food and shelter (Senapathi et al., 2017). One such habitat that has been proposed as a key component for pollinator conservation are road verges (Gardiner et al., 2018; Phillips et al., 2020a). Road verges are areas that separate the road from adjacent land and are managed by governments, highways agencies, and local councils. The fundamental purposes of road verges are to aid driver visibility, provide pedestrian access, support road drainage, and help the road blend into the landscape (Gardiner et al., 2018). They have also been shown to provide several ecosystem services including carbon sequestration and water regulation (Phillips et al., 2020b). If managed correctly however, road verges could also present a highly valuable habitat for pollinators due to the immense area of land that they cover; 270,000km^2 (0.2%) of all land globably is classified as road verge (Phillips et al., 2020b). In the UK, road verges cover 2579km^2 (1.2%) of land, composed of short, frequently-mown grassland (24.47%), regular grassland (40.87%), woodland (18.73%), and scrub (10.66%) (Phillips et al., 2021a). For context, semi-natural grassland habitat comprises 24,900 km^2 (10%) of land in the UK (Office for National Statistics, 2022). The density of roads is highly variable between countries; in the UK road density is etimated as 172.41km of road per 100km^2 of land, compared to 36.02km in China (NationMaster, 2023).

1.1.4. The case for conservation using verges

Roadside habitat has been identified by wildlife charities both across the world (Xerces Society, 2023), and in the UK (Buglife, 2023; Butterfly Conservation, 2019; The Wildlife Trusts, 2023) as valuable habitat for pollinators. Road verges are already being managed as conservation areas by several local councils in the UK. When questioned, 78 local councils out of 148 were already maintaining verges for pollinator conservation in some respect, with a further six seeking to establish conservation areas on verges in the near future (Yeo, 2021). In 2019, Plantlife released their best practice guide for road verge mangement, which details effective strategies for promoting floral diversity along roadsides (Plantlife, 2019).

The case for road verges as a tool for pollinator conservation is multifaceted; not only do verges cover vast areas of land, but this land is structured as a connected network. If managed appropriately, verges could therefore provide a substantial quantity of nectar, pollen and nesting habitat across the landscape. These benefits are discussed in detail in the following section (**section 1.2**).

1.2. POTENTIAL BENEFITS OF ROAD VERGES

Road verges are often proposed as a key habitat for pollinators and have been shown to support a vast array of pollinator species (Phillips et al., 2020a; Villemey et al., 2018). Many rare species of pollinator have been identified along road verges. For example, four critically endangered European pollinator species (*Andrena florivaga, Halictus scabiosae, Sphecodes spinulosus* and *Nanoclavelia leucoptera)* were recorded on a 38.5km stretch of highway in Czechia (Heneberg et al., 2017). Sites along a 4-lane highway with a traffic density of 9523-38,688 vehicles/day were surveyed for communities of plants, bees, and wasps, and the selected sites were surrounded mainly by arable fields. Nine threatened herb species were identified, as well as 32 threatened species of bee and wasp. The results of this study suggest that road verges could be a valuable resource for bee and wasp communities in areas dominated by agriculture.

Road verges can provide a valuable resource in a landscape dominated by agriculture, with bumblebee numbers shown to be higher in road verges than in the field margins of arable farmland (Hanley and Wilkins, 2015). At least two studies, from Scotland and Argentina, have demonstrated that road verges provide valuable resources for pollinators in agricultural landscapes, with higher densities of foraging insects recorded than in other habitat types in the landscape. Cole et al., (2017) assessed the species richness of bees, butterflies, and hoverflies in Ayrshire, and found that road verges hosted the greatest numbers of species when compared with multiple different habitat types including farmland, rough grassland, hedgerows and woodland. Monasterolo et al., (2020) demonstrated that floral species richness and pollinator abundance in road verges increased with verge width, and so conservation priority should be given to wider roadsides.

Road verges can support pollinators in two ways; the provision of floral resources, and sites for nesting and hibernating. The verges of roads can be an important food source for pollinators in landscapes that are otherwise lacking in floral resources (Hanley and Wilkins, 2015; Osgathorpe et al., 2012). It is estimated that in the UK arable farmland only produces an average of 6.9kg of sugar/hectare/year, compared to road verges which are estimated to produce 60.63kg/hectare/year (Baude et al., 2016). In Ayrshire, Scotland, road verges have been shown to host an overall greater number of pollinators than other types of land (Cole et al., 2017), however verges will be of greater value for some species than others; for example, road verges have been shown to be a better resource for long-tongued bumblebees than short-tongued bumblebees (Osgathorpe et al., 2012).

Pollinators could also use road verges for nesting and hibernating. A study by Munguira and Thomas (1992) showed that moths and butterflies were using road verges for breeding, with different larval stages of 11 species found in close proximity to the road edge. Road verges that have multiple habitat types within them could increase pollinator diversity, for example having patches of bare soil or tussock grasses as nesting resources for wild bees. Hopwood (2008) found that bare ground on road verges resulted in greater numbers of solitary and ground-nesting bees. Further research is required to assess how pollinators nesting on road verges fare compared to those nesting away from traffic (Wojcik and Buchmann, 2012).

There are no studies, to my knowledge, that directly assess how larger-bodied insects foraging at a landscape scale, like bumblebees, use road verges. Bumblebee species are known to travel large distances to collect pollen and nectar, particularly in resource poor landscapes (Redhead et al., 2016). The ways in which bumblebees may incorporate verges into their diet relative to other habitat types remains a significant gap in the knowledge, as this will provide insight into their value as a conservation tool. While road verges have the potential to provide bumblebees and other pollinators with substantial amounts of good quality habitat, there is also the possibility of negative impacts. The negative aspects of road verges could mean that road verges act as ecological traps.

1.3. ECOLOGICAL TRAPS

An ecological trap is defined as a habitat of low quality which species prefer over a higher quality habitat (Dwernychuk and Boag, 1972). The term has been linked to source-sink theory; where two habitats are present, the use of one will increase the population growth rate (the source), whereas use of the other will result in a population decline (the sink) (Pulliam, 1988). In the context of ecological traps however, individuals fail to recognise the superior habitat as such because the environmental cues used to select suitable habitat do not accurately reflect its quality (Kristan, 2003; Schlaepfer et al., 2002). Therefore, the frequent use of traps could drive populations locally extinct (Donovan and Thompson, 2001).

Ecological traps could be a major threat to conservation, and many areas have been categorised as potential ecological traps: airports, urban habitats, and linear habitat corridors, although the extent to which these traps occur is unclear (Boal and Mannan, 1999; Henein and Merriam, 1990; Kershner and Bollinger, 1996). Over half of the research concerning ecological traps is centred on birds, with 75% of the literature originating from Europe and North America (Hale and Swearer, 2016). Research on insects has been the subject of less attention, however there are some key studies that depict insects using ecological traps.

One of the most frequently observed ecological traps in insects is the used of man-made structures as egg-laying sites (Robertson and Hutto, 2006). Polarised light is used by aquatic insects to locate suitable areas for egg deposition, and this light is often heightened on the windows of buildings, making them highly attractive to insects such as caddisflies and mayflies. However, when the larvae emerge they do not have access to an appropriate food source (Horváth et al., 2009). A study by Faldyn et al., (2018) found that in the presence of increased temperatures, the cardenolide concentrations present in tropical milkweed plants (*Asclepias curassavica*) shifted from beneficial to detrimental. This plant is one of the only sources of food for Monarch butterfly larvae (*Danaus plexippus*), who as a consequence had reduced wing length as adults. The authors conclude that the larvae continued to perceive previous cues as beneficial even though they had negative effects, and so present an ecological trap. They also state that this may be a real emergent ecological trap as climate change will mean higher temperatures for the area in the future. Timber from beech trees stored in the vicinity of the forest present an ecological trap for Rosalia longicorn (*Rosalia alpina*), who lay eggs in the bark (Adamski et al., 2018). Researchers successfully demonstrated the spatial impact that these ecological traps can have on individuals. They found that between 19.7-81.6% of beetles within the range of timber stacks were impacted by their presence, and that impacts were even more pronounced due to habitat fragmentation caused by the logging. It has also been suggested that sown wildflower strips in field margins may represent an ecological trap for insect pollinators as they are disturbed by land use, pesticides, and annual mulching, however, research on several insect groups (including bees) has shown that species richness and abundance actually increase over time when using the strips (Füglistaller et al., 2018; Schmied et al., 2022).

Road verges may represent an ecological trap for several plant and animal species. The negative effects of roads are both direct and indirect, and due to their network structure, roads can impact vast areas of habitat, leaving only small isolated fragments unaffected by their influence (Coffin, 2007). A study by Fekete et al., (2017) investigated the phenomenon of road verges as ecological traps in endangered lizard orchids (*Himantoglossum* spp.). Orchids were found in high numbers along road verges in Europe, and they occurred more closely to the road edge than would be expected by chance. However, the reproductive success of individuals diminished with increasing proximity to the road, and the authors suggest two reasons for this: (1) the presence of dust and metals from traffic in the soil, and (2) lesser numbers of insect pollinators along the road edge. The authors conclude that populations of orchids along road verges are significant and viable however, despite having reduced reproductive success compared to their non-roadside counterparts. They also state that verges are more likely a refuge for these rare orchid species, rather than an ecological trap. Ben-Aharon et al., (2022) found that roads acted as a severe ecological trap for *Oenanthe lugens*, a territorial cavity nesting bird. Due to collisions with vehicles, territories along the roadside were vacated at a significantly higher rate than territories in the surrounding landscape. These territory gaps were then also replenished at a faster rate than non-roadside territories, creating a quick turnover cycle of individuals nesting on verges. The researchers conclude that the verges were an ecological trap as individuals were unable to perceive the diminished quality habitat as a result of vehicle mortality.

To my knowledge, there are currently no studies aiming to address whether road verges act as ecological traps with regards to bumblebees and other pollinators, perhaps owing to the complex set up which would be required to answer this question. For example, one approach would be to compare bumblebee colony density and reproductive success in sites matched for floral resources and pesticide loading, but with differing road densities. Despite a lack of studies on road verges as ecological traps explicitly, there is a significant amount of research on both the positive and negative effects of roadsides assessed individually. Whilst managing road verges in a way which promotes environmental sustainability and draws wildlife to the area, this could inadvertently mean that individuals are exposed to a multitude of pollutants and risks associated with being in such close proximity to traffic. The drawbacks of these threats may outweigh the benefits of a resource rich environment, and therefore have an overall negative impact on species using the verge. Some examples of these negative effects include pollutants such as road dust, nitrogen, air and metal pollution, noise and vibrations, and light pollution.

1.4. POTENTIAL HAZARDS OF ROAD VERGES

1.4.1. Corridor or barrier

Roads and road verges could have two very different impacts on pollinator dispersal and movement, and these impacts could be perceived in different ways by different species. Roads themselves may act as a barrier, preventing or reducing the number of pollinators able to cross and utilise resources on the opposite side. Conversely, the verges may provide corridors or stepping stones of habitat that pollinators can use to navigate and disperse across the landscape.

In accordance with this duality, Hopwood et al., (2010) found that road verges had both positive and negative impacts on bumblebees. Whilst individually marked bumblebees were observed repeatedly visiting the same floral patch on a road verge, there was significantly less movement between verges on either side of the same road than would be expected if the road had no impact on movement. These findings suggest that the presence of the road is having a negative effect on bumblebees choosing to cross roads or preventing them from doing so entirely. However, a further experiment involving adhesive plates on cars revealed that no bumblebees were killed by oncoming traffic. Further research is therefore required to determine the precise cause of their restricted movement.

A study conducted in Sweden demonstrated the barrier effect of roads on bees and wasps by looking at community composition, rather than individual movement (Andersson et al., 2017). The community composition on the eastern and western sides of a busy highway were significantly different, and this difference became even more pronounced when the largest species (e.g. bumblebees) were removed from analysis. These results suggest that the road is acting as a barrier, preventing individuals from crossing and utilising resources on the opposite side. The authors also state that as larger bodied insects have greater mobility and therefore greater dispersal capabilities, the barrier effect was less pronounced. 45% of the larger bodied insects caught in this study were bumblebees, and the authors concluded that bumblebees most likely fly higher above the ground than smaller species, thus decreasing their susceptibility to traffic collisions. This study is in accordance with previous work which also illustrates the same phenomenon in butterflies. Skórka et al., (2013) found that smaller bodied butterfly species were overrepresented in roadkill samples taken along transects in Poland, again demonstrating that poorer dispersers are disproportionately affected.

Along with dispersal capabilities, other elements including the road width and traffic volume could be determining factors in whether or not pollinators choose to cross. It has been suggested that bees will cross roads in both cities and the suburbs, implying that traffic volume has little impact on road crossing, however the width of these roads were not provided (Wojcik and Buchmann, 2012). Hopwood et al., (2010) demonstrated that bumblebees would not cross a four-lane 50m wide road, but would cross a two-lane 20m wide road. A study by Valtonen et al., (2006) found that only 10% of butterflies crossed highways, whereas 40% crossed smaller roads. Although the precise reasons were not explored, butterflies have also been observed flying to the edge of a road and then turning to fly along the verge or turning back on themselves completely rather than cross (Halbritter et al., 2015). Fitch and Vaidya (2021) showed that traffic volume, road width, and bee species identity can influence road crossing, with width being the best predictor of movement.

Several studies explicitly describe pollinators using road verges as corridors. Valtonen and Saarinen (2005) found that in habitats connected by road verges, butterfly movement was doubled compared to habitats without road verges present. However, the results of this study were based on a comparison of only two sites, and so may not be generalisable to other contexts. Conversely, Öckinger and Smith (2008) found that road verges did not increase the movement of butterflies between pastures, however the authors attribute this to the large size of the verges surveyed, as butterflies tend to remain in larger patches (Marini et al., 2014). Dániel-Ferreira et al., (2022a) revealed that flower visiting insects use roadsides as a corridor. Flowers of potted plants on either side of a road were treated with either blue or red fluorescent dye, and the transfer of dye to the surrounding flowers was recorded. The authors found that movement along roadsides was more frequent than movement perpendicular from the road into adjacent habitat, supporting the notion that verges act as corridors. Interestingly, this movement was independent of floral density, suggesting that even low quality verge habitat can act effectively as corridors. There was a greater propensity for movement within the verge as opposed to between verges, again highlighting the barrier effect of the road itself. Honeybees have been shown to use linear structures, including gravel roads, in homing strategies (Menzel et al., 2019). Radar tracking of workers revealed that honeybees will use pathways in the landscape as navigation tools. Although preference is given to a pre-learned compass direction, individuals can also use these linear structures to assist with flight back to the hive.

The use of road verges by pollinators may also have effects on the pollination services they provide. Monasterolo et al., (2022) assessed pollen transfer along 16 roadsides and in their adjacent fields across two consecutive seasons. They found that floral abundance on roadsides negatively affected pollen transfer both within verges and across fields. This was likely caused by a dilution effect on individual plants and also reduced spill over into fields as a result of increased plant abundance on the verge itself. The insect visitation rate in adjacent fields however, increased with increased plant species richness in the verges, and the authors conclude that promoting floral diversity, as opposed to abundance, is vital for enhancing the pollination services of roadside habitat.

It is well recognised that pollinators use other types of habitat corridors for movement as well, and the use of other corridor systems could also provide insight into how pollinators may use road verges. Sutcliffe and Thomas (1996) showed that ringlet butterflies (*Aphantopus hyperantus*) would move through habitat patches by using woodland rides as opposed to travelling directly through the forest. Butterflies have also been shown to move between patches of woodland using 25m wide cleared corridors, compared to patches where such corridors were absent (Tewksbury et al., 2002). Two pollinators, the cinnabar moth (*Tyria jacobaeae*) and the Silvery blue butterfly (*Glaucopysche lygdamus coperi*) have expanded their ranges along road verges in Germany and Canada respectively, which also implies species dispersal along road verges (Brunzel et al., 2004; Dirig and Cryan, 1991). Bumblebees are also known to use other linear features, such as hedgerows, for dispersal (Cranmer et al., 2012).

From the literature, it is highly probable that roads and road verges are acting as both barriers and corridors simultaneously. The dispersal capabilities of species and characteristics of the road (such as traffic volume and width) are key factors in determining whether or not individuals choose to cross roads. Alongside other linear landscape features, roads have been shown to act as corridors, aiding with navigation and as a valued food resource. Much of the research in this field focuses on Lepidoptera, with a few key studies centred on bees. Whether the corridor or the barrier effect has a greater impact on pollinators remains unclear, and quantifying the balance of these very specific phenomena will require further detailed experimentation.

1.4.2. Mortality

When attempting to cross roads pollinators may be hit by oncoming vehicles, and therefore roads can increase pollinator mortality (Muñoz et al., 2015). Baxter-Gilbert et al., (2015) found that pollinators represented 71% of over 100,000 dead invertebrates found along a 2km stretch of road in Canada, with bees being killed at a rate of 26.8 individuals/km/day. Several factors can influence the mortality rates of pollinators on roads including traffic volume, floral resources, and pollinator ecology and morphology, and I will discuss each of these in turn in this section.

Generally, as the volume of traffic on a road increases, so does the rate of pollinator mortality (Muñoz et al., 2015). A study by Skórka et al., (2015) found that just 4% of total road length across three sites in Poland was responsible for 49% of all butterfly deaths resulting from traffic collisions. The results were obtained using roadkill counts, and were attributed to poor floral resources on the road verges themselves, a high abundance of surrounding grassland habitat, and high traffic volume. Some studies have found conflicting results. For example, a study by McKenna and McKenna (2001) found that butterfly deaths were most frequent where traffic volume was intermediate. Another study in Canada using sticky traps on vehicles found that diurnal flying insects were less prevalent on roads with greater traffic volume which resulted in fewer overall collisions (Martin et al., 2018). Both of these studies failed to consider the floral resources of the verges surveyed however, and this could account for their findings.

Floral provisions along road verges could be a lure for passing pollinators, and therefore the abundance of flowers may be correlated to the rate of collisions. Pollinator traffic collisions relating to floral availability are not uniform however, with different groups exhibiting different responses. Butterfly deaths tend to be highest when floral provisions are low (Skórka et al., 2013; Skórka et al., 2015). Studies have found this to be linked with constant or recent mowing, as butterflies disperse the area to find new patches (Ries et al., 2001). Bumblebees, on the other hand, show higher mortality when floral resources on verges are high. Keilsohn et al., (2018) found that there were two main ways in which resources influenced traffic collisions with pollinators; the surrounding habitat, and the vegetated median (the strip of land dividing a dual carriageway). A surrounding landscape of predominately woodland resulted in less mortality for all pollinator groups than meadows or lawn. Regardless of surrounding habitat, the presence of a vegetated median significantly increased the numbers of insects killed. However, when analysed separately, it was shown that butterfly fatalities were highest where the median was meadow, and bumblebee mortality was greatest with a lawn median, again suggesting different groups respond differently.

Dániel-Ferreira et al., (2022b) investigated the effect of both traffic volume and floral diversity on queen bumblebee mortality and behaviour. Observational transect walks were conducted along roadsides in spring and late summer to observe *Bombus* spp. queens either searching for nests or overwintering sites. Researchers found that queen mortality increased significantly with traffic volume; the probability that an observed queen was dead was four times higher when the level of traffic increased from 100 to 6000 vehicles per day. Using data from two previous studies on bumblebee density, they also estimate that between 1 and 10% of queens present in their study landscape were killed by traffic. This is the first and only study to explore the impacts of roads on queen bumblebees.

Pollinator ecology and morphology may also be a determining factor in the rate of traffic collisions. A study by Munguira and Thomas (1992) on butterflies found that traffic collisions occurred in 7% of species from open populations (mobile with a wide egg-laying distribution), whereas only 0.6-1.9% of species from closed populations were killed by traffic. As previously mentioned in **section 1.4.1**, smaller-bodied pollinators have been seen flying at low heights above roads, and this could mean they are disproportionately affected by traffic collisions (Skórka et al., 2013; Vinchesi, 2013).

Pollinator mortality on roads is influenced by a number of factors, and each of these should be considered when conducting future research and producing management plans for verges. It is important to consider pollinator ecology and morphology when assessing the risk of traffic collisions, as individual behaviours and body-size may yield different results.

Much of the research on traffic collisions and pollinators focuses on butterflies and bumblebees, and studies on a broader range of pollinators are required to draw more general conclusions. Mortality rates appear to be influenced by traffic volume, as well as the floral resources available on and around road verges, but again results are highly variable between sites and species.

1.4.3. Pollutants

Road verges have the potential to be contaminated with a variety of different pollutants associated with traffic. These pollutants include road dust, nitrogen, and metals, and can originate from exhaust fumes, tyre abrasion, brake wear, or pollutants from the road surface itself (Adamiec et al, 2016; Field et al., 2010). Here, pollutants are presented and discussed individually as represented in the literature, however in reality pollutants will occur concurrently and are likely to have synergistic effects.

1.4.3.1. Road Dust

Road dust can be defined as any organic or inorganic particulate matter found on the road surface which becomes airborne through traffic movement (Khan and Stand, 2018). Dust on the surface of roads can originate from a variety of sources including natural wind abrasion of nearby land, local crop or livestock production, and also the activity of traffic (Field et al., 2010). Despite the prevalence of road dust, there is little research on how it affects individual organisms, and currently there are only two studies to my knowledge that aim to quantify the effects of road dust on bees (Phillips et al., 2021b; Waser et al., 2017).

A study on unpaved roads in Colorado found that dust was negatively correlated with pollen deposition on stigmas (Waser et al., 2017). Airborne dust from passing traffic was deposited mainly within 30m of the road edge, and there was a negative relationship between distance from road edge and dust deposition, with samples closer to the edge experiencing greater quantities of dust deposition. As the size of dust particles overlapped with the pollen grain size of the study species, it was hypothesised that dust deposition could interfere with pollination. Plant stigmas closer to the road edge had greater quantities of dust, and in almost all cases this resulted in less pollen deposition. However, despite less pollen deposition, there was no significant effect on seed set. The authors propose four reasons for this surprising result: (1) pollen quantities exceeded the amount needed for maximum seed set, (2) undusted flowers experienced more geitonogamy (fertilisation by pollen from a flower of the same plant) as a consequence of increased pollinator visitation than dusted flowers, and geitonogamy is known to depreciate seed set, (3) resources, as opposed to pollen deposition, is the limiting factor in seed production, and (4) herbivory on undusted flowers is greater than dusted flowers.

The second study investigating the effects of road dust on pollinators is Phillips et al., (2021b). The researchers used potted plants to capture dust at varying distances from the road edge (control, 1m, and 4m) over a four-day period. Plants were then arranged in a Latin square and pollinator visitation rates and durations were noted. 176 individuals were recorded, and there were no significant effects of dust deposition treatment on pollinator visitation. Despite this lack of effect, the authors state that there may be confounding variables, such as physical disturbance and herbivore exposure, which could lead to long-term impacts not observed in this study.

There are several mechanisms through which road dust could negatively impact pollinators. The presence of road dust on flowers may disrupt bee foraging behaviour if the dust interferes with, or obscures, floral cues involving sight or scent. Bees have three types of photoreceptive cells in their eyes: green, blue, and ultraviolet (UV) (Menzel and Backhaus 1991; Peitsch et al., 1992). It is widely recognised that bees use the UV patterns on the petals of flowers as cues for landing (Chittka and Raine, 2006; Peitsch et al., 1992). Studies investigating UV and pollinating insects have shown that covering greenhouses with a material that reduces the transmission of UV wavelengths can reduce the overall activity of insects. Soler et al., (2006) found that when a UV absorbing plastic was used in the greenhouse, bumblebees spent significantly more time at the nest entrance than out foraging, and nest activity began later in the day than when the material did not interfere with UV wavelengths. Further research in this area has found that bees make twice as many foraging trips and are less likely to get lost under material which does not disrupt UV wavelength compared to bees under material which does (Morandin et al., 2001). A study by Dyer and Chittka (2004) found that whilst bumblebees do recognise if there is a change in UV radiation, their foraging efficiency is not impacted in a greenhouse pollination context, suggesting bees are learning to recognise flowers in the absence of UV.

As well as visual cues, bees respond to olfactory cues emitted by flowers (Lunau and Maier, 1995; Raguso, 2001). The chemical composition of these scents also has potential to alter bee foraging behaviour. Olfactory signals are quickly learned by bees, and evidence has shown that bees can be more attracted to flowers possessing lesser quantities of floral reward when the scents emitted by those flowers are deemed “stronger” than plants with greater floral reward (Pham-Delegue et al., 1990). This suggests that scent is likely a dominant floral characteristic used by foraging bees to detect rewards. Further research has shown that bees are highly sensitive to scent; honeybees have an estimated 170 different olfactory receptors, allowing them to distinguish between the numerous different chemical components of floral scents (Robertson and Wanner, 2006).

The chemical composition of olfactory cues are delicate, and external factors can cause changes to the scents emitted by flowers. A study by Cembrowski et al., (2012) found that bumblebees significantly altered their foraging behaviour and pollination efficiency when ant scent cues were present on flowers. It is also known that bumblebees avoid visiting flowers that have been recently visited by other bumblebees, and this can be attributed to various cues including visual, humidity electrostatic, and olfactory (Chittka and Thomson, 2001; Clarke et al., 2017; Goulson et al., 1998). As has been shown for other roadside pollutants (see **section 1.4.3.2**. on exhaust emissions), the presence of road dust on flowers may alter or reduce the strength of olfactory cues emitted by flowers, making them less attractive to foraging bees.

While it is suggested that the presence of road dust may have a negative effect on foraging behaviour, there is a lack of evidence supporting this explicitly. Currently, only two studies have addressed the question of how road dust impacts pollination, and both showed no impact on the overall reproductive success of plants or pollinator visitation rates. Plant-pollinator interactions are a key component of bee conservation, as this is where bees obtain resources for themselves and the rest of the colony. It is therefore important to know how road dust may affect a bees’ ability to locate floral resources along road verges, particularly in agricultural areas where road verges may be one of the few resources available to foragers. Further research that focusses on how the presence of road dust on flowers influences bee foraging behaviour, through obstruction of UV patterns or olfactory cues, is necessary to understand whether bees are experiencing any long-term negative impacts as a consequence of road dust.

1.4.3.2. Exhaust emissions

One of the major sources of pollution on roadsides is exhaust emissions, which include nitrogen oxides (NO_x), carbon monoxide (CO), exhaust particulate matter (PM), and volatile organic compounds (VOCs) (Liu et al., 2022). These air pollutants can disrupt olfactory cues that bees and other pollinators rely upon to locate flowers by either altering the chemical composition of floral volatiles or masking them completely (Jamieson et al., 2017; Riffell et al., 2014). They can also impact individuals directly by altering gene expression, gut microbiome, and circulatory physiology (Reitmayer et al., 2022; Seidenath et al., 2023 (*in review*); Thimmegowda et al., 2020). Several studies have attempted to quantify the impacts of both diesel and petrol exhaust emissions on pollinator physiology, behaviour, and learning.

Diesel exhaust has been shown to negatively impact plant-pollinator interactions, with pollinator abundance and frequency of flower visitation declining by 69% and 89% respectively on plants treated with diesel during visitations relative to controls (Ryalls et al., 2022). A study by Reitmayer et al., (2022) demonstrated the negative effects of repeated, short-term exposure to diesel emissions on honeybee hives. While hives did not display any differences with regards to virus prevalence, daily activity, or composition of collected pollen, colony weight was significantly impacted by exposure. During the three-week study period both control and diesel treated hives lost weight at the same rate, however in the three weeks following exposure treated hives continued to lose significantly more weight than control hives. Also, between weeks six and nine, weight of control hives increased at a significantly higher rate than treated colonies. The study also found that foragers in treated hives exhibited upregulation of Neurexin (NRX) 1 expression compared to controls. NRXs are molecules which facilitate memory formation, and prior to commencement of the experiment both control and treated bees displayed this upregulation. The authors suggest that this continued upregulation in individuals from treated hives means that there is a greater need for repeated learning of information to perform the same task as effectively as control bees. This is further supported by previous work showing that honeybees exposed to diesel emissions demonstrated impaired learning and memory of floral scents (Leonard et al., 2019; Reitmayer et al., 2019).

Diesel exhaust emissions have also been shown to impact key floral volatiles, which has negative repercussions on honeybee foraging behaviour (Lusebrink et al., 2015). A floral blend of eight common volatiles was created and exposed to either ambient air or diesel exhaust. Three volatiles in the diesel exposed blend were either reduced (myrcene), transformed (β-caryophyllene became isocaryophyllene), or disappeared completely (β-ocimene) from the mix. In the behavioural assays, a lack of β-ocimene did not impact recognition, however a reduction of myrcene lead to a 37% decrease in recognition by bees. Of all seed plants studied, myrcene occurs in 63 plant families (70%) and has been identified as a key odour in complex scent recognition by honeybees. The interference of diesel exhaust emissions is inhibiting individuals' ability to recognise floral cues which is also likely to have consequences on colony health and development, however this pathway is yet to be investigated.

A similar effect on olfactory learning in honeybees has been observed in petrol emissions (Leonard et al., 2019). Bees conditioned with floral volatiles contaminated with petrol pollutants (CO, NO and NO_2) took significantly longer to learn associations between scent and reward than control individuals. This difference became more pronounced over time, with recognition declining even further at the 24 and 48-hour post-exposure time points. The authors conclude that these sublethal effects will be more prominent in urban areas, where bees will experience reduced health and foraging efficiency.

There has been substantially less research conducted on the effects of exhaust pollution on bumblebees. Seidenath et al., (2023) (*in review*) investigated the impacts of diesel exhaust particles and brake dust on the gut microbiome and gene expression of *Bombus terrestris*. Workers were exposed to pollutants via air (allowing individuals to fly in a box containing contaminants) or ingestion (feeding individuals sugar water spiked with contaminants). Brake dust had no effect on workers but the authors note this could be a consequence of experimental design, with the large particles sinking to the bottom of the feeding syringes therefore reducing uptake by workers. Exposure to diesel exhaust particles via air had no effect either, but again the authors highlight that the three-minute exposure period may not have been enough time for an effect to be observable. Oral exposure, however, significantly altered the bacterial community composition, with *Snodgrassella*, the dominant bacterium in the microbiome, almost disappearing completely. *Snodgrassella* has a symbiotic relationship with bumblebees, as it coats the ileum with a biofilm which may help protect against infection by gut parasites such as *Crithidia* and *Nosema bombi*. The authors note that this reduction in *Snodgrassella* could lead to an increase in susceptibility and parasite prevalence in bumblebees. Gene expression was also significantly altered, with upregulation of 165 genes and downregulation of 159 genes compared to controls. These changes can be classified as a general stress response to pollutants and as an increase in energy consumption.

Evidently, the impacts of exhaust emissions on bees is complex and there are still many avenues of research that need to be addressed, for example the long-term effects of continued exposure as would be the case in a real-world scenario. As with the majority of pollinator studies, honeybees are disproportionately represented, with only one study aiming to quantify the effects of exhaust pollutants on bumblebees, and none on solitary bee species. An important consideration regarding exhaust emissions and air pollution associated with traffic is the replacement of the standard combustion engine with an electric car battery. The decarbonisation of the motor industry and the specific impacts this will have on pollinators using roadside verges are discussed in detail in **sections 2.5.4., 3.5.1.,** and **5.4.1.**

1.4.3.3. Nitrogen

Nitrogen has been identified as a key driver of primary production (Gómez Giménez et al., 2019; LeBauer and Treseder, 2008; Stevens et al., 2015). Many plants favoured by bees (such as clovers) possess nitrogen-fixing bacteria in their root nodules, and are able to exploit areas of land other plants cannot, giving them a competitive advantage in nutrient-poor soils (Ledgard and Steele, 1992). However, plants in close proximity to roads are likely to experience a surplus of nitrogen due to nitrogen emissions from vehicles, thus increasing the numbers of plants able to thrive on road verge soils. A study in Pakistan demonstrated that soils 0-3m from the road edge had significantly elevated levels of nitrogen (519-628% greater) compared to control soils located 100m from the road edge (Khalid et al., 2020). Similarly, Xu et al., (2019) showed that nitrogen pollution had a negative exponential relationship from the road edge along a roadside in southern China.

In the USA, vehicle emissions are the single greatest source of nitrogen emissions, accounting for 37% of all nitrogen deposition (USEPA, 2011). The major source of nitrogen emissions from traffic is when nitrogen gas from the air is oxidised in the high pressure and temperature conditions of car engines to form nitrogen oxide (NO) and nitrogen dioxide (NO_2) (Abdel-Rahman, 1998). The high nitrogen deposition as a result of vehicles can increase the quantities of nitrogen in areas in close proximity to roads. A comparison between areas next to roads and areas 500 metres away revealed that there is twice as much nitrogen, and three times as much ammonia and nitrogen dioxide in the air closer to roads (Kirchner et al., 2005). Davidson et al., (2010) found that nitrogen also accumulated on the verges of small residential roads, and it is estimated that between 10 and 25% of ammonia emissions from vehicles are deposited within 50m of the road edge.

Soils with excess quantities of nitrogen present can be exploited by dominating plants that are unable to fix their own nitrogen. Plants that have a competitive advantage in nitrogen-deficient soils, for example legumes such as bird’s-foot trefoil (*Lotus corniculatus*), can therefore be displaced by more aggressive competitors such as cow parsley (*Anthriscus sylvestris*) and stinging nettle (*Urtica dioica*) (Aerts, 1999; Wilson and Tilman, 1991). Management strategies can be adopted which can help discourage aggressive competitors, for example through the planting of yellow rattle (see **section 1.5**). So, while road verges have conservation potential, nitrogen pollution means that plant species favoured by bees will likely be out-competed by other species, unless verge management controls competitive species and promotes bee-friendly plants.

1.4.3.4. Metal pollution

Metals naturally occur in most soils at trace levels, however anthropogenic activities, for example driving, can result in toxic levels becoming present (Nagajyoti et al., 2010). Metals that originate from traffic can come from multiple sources including directly through exhaust emissions, tyre and road surface abrasion, motor oil, and also brake linings (Adamiec et al., 2016). Metals are classified as particulate matter (PM) pollution, and between 85-90% of PM pollution originates from non-exhaust pollutants (Timmers and Achten, 2016). Studies have found significant levels of metals such as aluminium, copper, cadmium, iron, nickel, chromium, and lead in roadside verges (Adachia and Tainoshob, 2004; Hjortenkrans et al., 2007). Typical levels measured on roadsides for cadmium are 0.2ppm, 20ppm for copper, zinc is 60ppm, and nickel 10ppm (Werkenthin et al., 2014). Detailed records of six common roadside metals are shown in **Table 3.1**.

At high levels, these metals can negatively impact organisms by inhibiting growth, impairing tissue repair processes, and limiting reproductive success (Depledge et al., 1997). Bees and other pollinators can be exposed to these pollutants through water, air, and soil, however the literature concerning pollinators and metals focusses predominantly on contamination through floral rewards. Previous studies have acknowledged that some tolerant plants are able to accumulate metals from the soil into their above-ground tissues (Van der Ent et al., 2013). Recent research however, has shown that non-hyper-accumulator plants can also translocate metals from the soil into their reproductive organs and floral rewards (Xun et al., 2017). These findings have massive implications for pollinator conservation, particularly in areas that have higher than average levels of metals present in soils.

A study by Meindl and Ashman (2013) demonstrated that metals present in nectar could alter bumblebee foraging behaviour. Bumblebees spent 75% less time foraging on flowers contaminated with 100ppm nickel compared to controls. Also, bees that visited control flowers first were more likely to visit the next closest flower than bees that foraged on nickel first. Bees foraging on flowers which were contaminated with 100ppm aluminium however, exhibited no behavioural differences relative to the control. Both metal contaminated and control flowers were equally likely to be visited, and therefore the authors concluded that bees could not detect the presence of either metal within nectar prior to visitation. As bees are unable to discriminate between contaminated and non-contaminated flowers initially, they could be ingesting trace levels of metals with every visitation which could have repercussions on their foraging behaviour and overall health. Phillips et al., (2021b) however found that some pollinators actively avoided lethal concentrations of metals in the field. Feeders were contaminated with concentrations of five common roadside metals (cadmium, copper, lead, antimony, and zinc) found at 1m and 3-8m from the road edge, with another ‘maximum’ level treatment. At maximum levels there were 41% and 75% fewer visits by honeybees and wasps respectively. They also spent less time foraging (61% less for honeybees and 56% for wasps) compared to controls.

Sivakoff and Gardiner (2017) found that bumblebees however were unable to distinguish between contaminated and non-contaminated flowers, despite contaminated flowers displaying different floral morphologies from controls. Sunflowers exposed to lead through soil contamination were 18% shorter with flower heads 38% smaller than those grown in control soil. There was no effect on visitation rate, however bumblebees spent on average 5.4 times longer foraging on sunflowers grown in uncontaminated soil compared to those grown in contaminated soil, and this effect was not mediated by floral traits (for example flower head size). These results again imply that bees can detect metals in floral rewards, but only after making a visitation.

A study by Moroń et al., (2012) investigated the survival, reproduction, and population dynamics of the solitary bee *Osmia rufa* along gradients of metal pollution in the UK and Poland. They used trap nests, and measured the concentrations of lead, cadmium and zinc in the forage of bees to determine levels of pollution. In areas with higher levels of metal contamination, females constructed less than half of the number of brood cells compared to unpolluted sites (3-4 vs 8-10 cells), and had a greater proportion of dead offspring (50-60% vs 10-30%). The population growth rate was also shown to be decreasing along the metal gradient. Negative impacts of heavy metal contamination have also been observed in bumblebees. In north-eastern Ohio, USA, Sivakoff et al., (2020) found that bumblebees positioned in urban landscapes experienced increased exposure to lead (as measured in workers and larvae) relative to a control not placed in the field. They found heavy metal contamination was positively correlated with urbanisation, and this also resulted in a decline in both the number of workers and larvae present in colonies.

Currently, only one study exists explicitly testing the impacts of metal pollution on bumblebee colony growth and reproduction (Scott et al., 2022). Colonies of *Bombus impatiens* were fed 50% sucrose solution contaminated with either arsenic, cadmium, chromium or lead, or a combination of all four metals for a 15 and a 30-day period. There were no differences found between any of the treatments with regards to the number of individuals present at each life stage (egg, larvae, pupae, adult). The proportion of dead brood present however was significantly different across all treatment at 15 days, with colonies 3 to 4 times more likely to have dead brood when fed a single metal, and this was even higher when fed all metals (9 times greater likelihood). A similar effect was observed in the 30-day treatments.

As with the majority of research on pollinators and floral rewards, research on metal contamination is heavily biased towards nectar rather than pollen. Contamination through nectar may have indirect effects on developing offspring via diminished care, however direct effects may also be possible through pollen consumption. There are currently no studies which aim to quantify the impacts of metal contamination via exposure routes other than floral rewards, for example through soil, and this remains a significant research gap. There is evidence that soil can act as an exposure route for other pollutants, for example pesticide to squash bees (*Peponapis pruinosa*) (Willis Chan et al., 2019), and so it is important that this is assessed for metal pollutants also. Although literature in this area is increasing rapidly as interest in metal contamination becomes more prominent, there are still several knowledge gaps that need to be addressed.

1.4.3.5. Noise and vibration

Noise and vibrations produced by traffic have the potential to mask or interfere with communication cues used by species. A study by Slabbekoorn and Peet (2003) found that great tits (*Parus major*) in the Netherlands sing their mating calls at higher pitches to counteract the low frequency noise of urban environments. A comparison study between two bird species with different song pitches along road verges illustrated the same phenomenon (Parris and Schneider, 2009). The Grey Shrike-thrush (*Colluricincla harmonica*) adjusted its call to a higher frequency than normal to compensate for low-frequency traffic, whereas the Grey fantail (*Rhipidura albiscapa*) continued to produce calls at its normal frequency which were already higher than traffic noise. Birds were also observed less at road verges where traffic noise was greater, and the authors conclude that noise from traffic may be a contributing factor to their absence, as it could be more difficult to establish and maintain a territory where vocal communication is impaired by traffic. Vocal interference has also been shown to disrupt spatial orientation in tree-frogs (*Hyla chrysoscelis*), with females being significantly less successful in locating calling mates when traffic noise is played (Bee and Swanson, 2007**).**

Research investigating the impacts of traffic noise has primarily focussed on vertebrate species, however there is evidence to suggest that invertebrates will also experience adverse effects. Many invertebrates detect auditory stimulus at low frequencies (around 10Hz), and are therefore susceptible to masking or interference from low-frequency traffic (Hoy and Robert, 1996). Communication through vibration is also commonplace in invertebrates, and although the detection method for recognising vibrational signals is distinct from auditory sensors, sounds can transmit and propagate in substrates which can have negative impacts on vibrational communication (Ewing, 1989; Hill, 2009).

A study by Gordon and Uetz, (2011) found that when exposed to low-frequency air-borne noise, the vibrational mating cues of a wolf spider (*Schizocosa ocreata*) were impaired, and this resulted in reduced reproductive success. Bhardwaj et al., (2019) attempted to quantify the "road-effect zone" (the area adjacent to the road where organisms are likely to experience an impact as a consequence of the road) for nocturnal flying insects. They used light traps to capture individuals at varying distances from the road, and found that Orthoptera was the only order whose biomass increased with increasing distance from the road. The authors suggest two possible reasons for this: (1) the habitat closer to the road was not optimal for grasshoppers and crickets, and (2) species of Orthoptera are reliant on auditory cues for communication, and traffic noise is inhibiting these signals.

Organisms often have to cope with natural sounds in their environment; for example, wind, rain, and calls from other animals. Some invertebrates have responded to external noise through adaptation over generations, or short-term behavioural modifications. The Australian bush cricket (*Sciarasaga quadrata*) is able to completely close its tracheal system in order to eliminate the high-frequency calls of other crickets and identify the low-frequency calls of conspecifics (Römer and Bailey, 1998). Research on the rainforest cricket, *Paroecanthus podagrosus,* shows that this species has evolved to be highly sensitive to frequencies produced by calls of conspecifics only when auditory competition is strong (Schmidt et al., 2011). These adaptions over long and short-term scales illustrate the complexities of auditory stimuli in invertebrates, but also demonstrate the flexibility of invertebrates in noisy environments.

Davis et al., (2018) revealed that while negatively affected in the short-term, larvae of the Monarch butterfly (*Danaus plexippus*) may become desensitised to noise associated with traffic. The heart rate of larvae exposed to simulated road noise increased by 17% relative to controls, indicating that traffic noise is perceived as a stressor by developing individuals. Larvae exposed over a continuous period of 7 and 12 days however showed no elevation in heart rate at the end of the study, and the authors attribute this to habituation at chronic noise levels. Playback of recorded road traffic noise had no effect on the density of pollinators or the foraging patterns of bumblebees (Phillips et al., 2021b). Two noise intensity treatments were tested; a typical road (6 vehicles passing per minute), and a busy road (24 vehicles/min). It is highlighted by the authors that there are elements of noise other than intensity which may be significant, and so they do not dismiss the possibility of effects.

How bumblebees and other pollinators respond to vibration produced by traffic remains largely unexplored. As eusocial insects, bumblebees communicate with one another, and are known to do so using pheromones and excited runs coupled with wing-fanning upon returning to the colony (Dornhaus and Chittka, 2001; Dornhauset al., 2003; Molet et al., 2008). It has been suggested however that bumblebees also communicate using vibrations, or buzzes (Röseler and Röseler, 1977). The buzzes produced by bumblebees for defence and foraging are not only distinct from each other, but are also significantly different between species (De Luca et al., 2014). Due to their complexity, buzzes produced by bees could be affected by noise and vibrations from nearby traffic, and these impacts may result in changes in foraging behaviour and communication within colonies.

The current literature on noise and vibration is primarily focussed on the impact of a single noise in isolation, and while this is logistically understandable, it is not representative of field scenarios where organisms will experience a mix of different noises at varying intervals. Study species could become habituated or tolerant to noise produced in these situations. Also, the majority of current studies concentrate on the immediate impacts of noise and vibration, not the long-term effects. There are no studies that determine whether characteristics such as age, sex or overall size, influence an organism's response or recovery time from noise exposure. Vibration pollution may be particularly problematic for insects overwintering or nesting in roadside soil (e.g. *Bombus* spp.), and this is a substantial knowledge gap yet to be explored.

1.4.3.6. Light Pollution

Another potential threat to pollinators associated with road verges is artificial light at night (Boyes et al., 2021; Macgregor et al., 2015; Macgregor et al., 2017). Artificial light at night is estimated to be increasing at a global rate of 6% annually and is strongly associated with urban development and expansion (Bruce-White and Shardlow, 2011; Hölker et al., 2010). The types of artificial light being used are ever-changing; artificial lights that emit light across a broader spectrum are increasingly popular as they aid colour detection by humans

(Bruce-White and Shardlow, 2011). LEDs are also favoured now as they are more energy efficient than types previously used (Gaston et al., 2014). Approximately 30% of all motorways and A-class roads managed by National Highways are lit at night (Highways Agency, 2012).

Artificial light at night is known to impact several species at cellular, organism, and community levels (Grubisic and Grunsven, 2021; Longcore and Rich, 2004; Navara and Nelson, 2007). The impact of artificial light at night on pollinators and pollination as an ecosystem function has been scarcely investigated, with most research focussing on nocturnal pollinators (Gaston et al., 2014; Macgregor et al., 2015). Nocturnal insect pollinators, such as moths, are known to be negatively affected by artificial light at night. Nocturnal pollination cues can be disrupted by light, for example alteration in the nocturnal colour environment or obscuring photoperiodic cues used in the release of floral volatiles, and this could lead to changes in floral visitation patterns (Macgregor and Scott-Brown, 2020). Boyes et al., (2021) revealed that the abundance of nocturnal moth caterpillars across three counties in southern England was reduced by 47% (hedgerow habitat) and 33% (grassland margins) in lit sites compared to unlit sites. They also showed that this negative effect was more pronounced under LED light compared to conventional yellow sodium lamps, suggesting that these impacts will become even more significant in the future as LEDs continue to increase in prominence.

Different colours of artificial light at night have been shown to influence fruit infestation by seed predators (Boom et al., 2020). While there was no effect of light on the proportion of fertilised fruits in *Silene latifolia* by *Hadema bicrurus*, there was a significant increase in the proportion of fruit infestation under both green and white light relative to controls (53% and 44% compared to 28%), with a lower proportion of fertilised ovules under green light also. Davies et al., (2012) found that the presence of artificial light at night can alter community composition of invertebrates. This study is the first to demonstrate that non-aerial insects can be influenced by street lighting. Ants, harvestmen, ground beetles and woodlice were found more frequently in close proximity to street lights, resulting in a community that contained more predatory insects and scavengers. The authors conclude that these effects will likely cascade to higher trophic levels, and could alter interactions within complex food webs. However, exactly what these effects will be remains unclear.

Knop et al., (2017) is the first study aiming to quantify the impacts of artificial light on both nocturnal and diurnal insect pollinators. They compared flower visitation rates by nocturnal insect pollinators to plants, and also plant fruit set between sites illuminated with street lamps and dark sites. There were 62% fewer flower visits, and 29% fewer pollinator species on lit sites compared to dark sites. In addition to this, the number of fruits produced by plants that were visited by insect pollinators was significantly less in lit sites compared to dark sites, with no significant difference in plant biomass between treatments. The study demonstrates that the pollination services of nocturnal insect pollinators are disrupted by the presence at artificial light at night produced by street lamps. The production of fewer fruits means that the reduced reproductive fitness of the plants cannot be compensated for by diurnal pollinators, which further emphasises the complementary effects of nocturnal and diurnal pollinators (Fontaine et al., 2005). Giavi et al., (2021) investigated the effects of artificial light at night on diurnal pollinators specifically and found that 19% of the plant species included in the study had changes to the number of pollinator visits during the daytime after exposure to LED street lamps. The researchers note that in only one instance was this alteration directionally positive (i.e. more pollination visits), but the mechanisms underlying this reduction in pollination service due to illumination remain unclear.

Overall, research on the impacts of artificial light at night on pollinators highlights that both nocturnal and diurnal pollinators can experience negative effects, which can also lead to a reduction in the reproductive success of plants. Additionally, insect community composition is vulnerable to artificial light at night, and this may have repercussions on organisms at higher trophic levels, for example by changing the forage availability for insectivorous birds. Increasing urbanisation and the paralleled increase in artificial light at night will likely result in more pronounced consequences for pollinator populations.

1.4.3.7. Turbulence

Turbulence generated by passing traffic has the potential to impact pollinators foraging on the road edge. Hennessey et al., (2020) demonstrated that increasing exposure to wind caused a hesitancy to take off in honeybees, which negatively affected flower visitation rates. Wind speeds of up to 16km/h have been recorded on roadsides from passing vehicles (Bani-Hani et al., 2018), and this could cause floral displacement or prevent individuals from visiting entirely.

To my knowledge, only two studies to date have investigated roadside turbulence impacts on pollinators. Dargas et al., (2016) found that increasing traffic velocity resulted in a reduction of total visits, duration of visits, and pollen deposition to flowers of *Centratherum punctatum* by bees. The road was divided into three sections corresponding to low (mean 51km/h), medium (67km/h), and high (76km/h) traffic velocity. Flower visit duration was approximately one third and two thirds lower in the medium and high velocity treatments respectively, with 84% of pollinators terminating a visit when a vehicle passed. Using an electric wind blower, Phillips et al., (2021b) showed that the total number of flowers visited and the time spent in the observation area which was affected by wind was significantly reduced relative to controls. Individuals spent 54% less time and visited 47% fewer flowers in the high turbulence treatment relative to control plots. Interestingly, of all pollutants measured in this study (metals, dust, noise, and turbulence) researchers found that turbulence was the only pollutant which did not extend its impact beyond 1m from the road edge, suggesting that only pollinators using verge habitat immediately adjacent to the road will be impacted.

While the impacts of turbulence on foraging pollinators are largely understudied, its' influence appears to be limited to within 3m of the road edge (Phillips et al., 2021b). Turbulence is likely to have disproportionate effects based on insect body size. As bumblebees are larger-bodied pollinators they will experience different impacts compared to other smaller-bodied pollinators, like hoverflies and honeybees. These variable responses across taxa present an opportunity for future research.

1.5. MANAGEMENT

While there are many potential benefits and hazards to bees and other pollinators along road verges, verge management is a key element in determining their value. Management regimes that help promote plant species richness and abundance, while also accounting for different flowering times across the season, will support more pollinators than regimes using chemicals and excessive cutting to control weeds. There are two key aspects of mowing management: the timing/frequency of cutting, and whether to leave or remove the cuttings. I will consider these here in turn.

Mowing road verges for pollinators is important as it prevents the prevalence of dominant plant species, and encourages plant species richness (Parr and Way, 1988). A study in Florida investigating roadside management strategies found that mowing frequency was negatively correlated with butterfly abundance (Halbritter et al., 2015). The interaction between mowing treatment and time had a significant effect on butterfly abundance, with the no-mowing site yielding the largest quantity of live butterflies. The results show that reducing the mowing frequency of road verges during peak butterfly activity can increase butterfly numbers. In the Netherlands, Noordijk et al., (2009) found that two annual cuttings, one at the end of June and the other in early autumn, was the best method for promoting both floral resources and the number of insects on road verges. Despite being conducted on small plots, these results have been shown in previous studies (Parr and Way, 1988).

The timing of cutting is another factor to consider when managing for pollinators. The majority of studies in this area focus on butterflies, but one study in Scotland found that the peak flower species richness correlated with the peak abundance of both bees and hoverflies (Cole et al., 2017). Humbert et al., (2012) conducted a review and meta-analysis of 24 studies relating to the timing of cutting in meadows throughout Europe and found that cutting in summer as opposed to spring had positive effects on insect abundance and diversity. A study by Valtonen et al., (2006) showed that one cut in mid-summer leads to a greater abundance of butterflies in the late season when other habitats offered fewer resources. One study in Finland found that population density of ringlet butterflies *(Aphantopus hyperantus*) was higher in late-summer mown verges compared to mid-summer mown verges (Saarinen et al., 2005). Phillips et al., (2019) found that verges cut during peak flowering time (late-May to mid-July) hosted fewer pollinators. The authors highlight the need for long-term research on cutting regimes as mortality of eggs and larvae present in roadside vegetation is likely to be elevated where mowing is increased.

Cuttings left after mowing can prevent new growth by covering gaps that could otherwise be beneficial for seed germination, and can also add nutrients to the soil promoting the growth of dominant grass species (Bonanomi et al., 2006). A study by Noordijk et al., (2009) in the Netherlands investigated how the removal of cuttings affected flower and insect abundance. The results showed that while removing cuttings after two cuts promoted a greater number of flowers, the impacts of this on insects and floral visitation rates were not significantly different. This study was conducted on a road verge that had an already high floral diversity however, and the results may be different when carried out on road verges under more typical conditions. Removal of cuttings has also been shown to promote floral species richness by producing space for seed germination through scarification during removal (Parr and Way, 1988). In a study of 19 road verges across Cornwall, UK, pollinator abundance was lower in verges where cuttings were left on the roadside (Phillips et al., 2019).

Another way to promote pollinators on road verges is using yellow rattle (*Rhinanthus* spp.). Yellow rattle is a root hemi-parasite of intermediate or low fertility grasslands, and can be planted along road verges to help control the growth of dominant plant species. Bullock and Pywell, (2005) successfully demonstrated this phenomenon, with the presence of yellow rattle reducing productivity in grassland communities by between 6 and 73%, thus increasing the proportion of forbs relative to grasses. A study by Ameloot et al., (2006) along two road verges in Belgium found that when the presence of yellow rattle was high, the growth of grasses was supressed compared to areas where yellow rattle presence was low. The presence of yellow rattle may subsequently reduce the need for frequent cutting. A study by Wrzesien and Denisow (2016) compared the floral species richness and abundance on road verges and railway embankments, and found that railway embankments hosted significantly more species favoured by bees. The authors attributed this to the presence of dominant grass species on road verges and suggest that appropriate management strategies, like introducing yellow rattle, would make road verges a valuable resource for foraging bees. Adding yellow rattle seed to sown plots has also been shown to improve the establishment and persistence of other sown species (Pywell et al., 2004; Smith et al., 2003).

The management of road verges is an exceptionally important consideration when conserving for pollinators, and the literature reflects this. Late-summer cuts along with collection of cuttings appear to be the best methods for increasing both floral and pollinator abundance and diversity in roadside habitat. There is also evidence that incorporating yellow rattle into mixes is an effective way of increasing their value for pollinators, however this remains to be tested on roadsides explicitly. Further research is required to address the long-term impacts of cutting regimes on pollinators, particularly those using verges for reproduction.

1.6. CONCLUSION AND THESIS AIMS

In light of recent bumblebee declines, road verges could prove a valuable resource for bumblebee populations. Road verges are prevalent across the globe, and if managed correctly they could provide bees with good quality forage and nesting habitat in working landscapes. However, being in such close proximity to traffic does present several concerns: bumblebees using these resources could be exposed to a multitude of pollutants, excessive noise and light, and increased mortality as a result of collisions with vehicles. Studies on road verges area have demonstrated both positive and negative effects of roads on insects, with the results variable across taxa and often dependent on management practices.

It is evident from the literature that more research investigating this topic is required. Evidence suggests that roads and road verges may be acting as both corridors and barriers, however more studies into the use of road verges as ecological corridors are needed. Landscape scale studies on the spatial movement of bumblebees as well as analysis of their forage rewards could help determine whether bees use verges for resources and navigation tools within their environment. The roads themselves could present a barrier to bees, and high mortality of insects on road verges indicates this. Studies have shown that body size is an important variable in an individual's susceptibility to collision, with bigger bodied insects representing a smaller proportion of individuals killed. As larger insects, bumblebees may be less effected by traffic mortality, and so research on how bumblebees manoeuvre across roads and traffic would add to this area of the literature.

The presence of metals in roadsides has received some attention, however studies mainly focus on foraging behaviour, with very few studies looking into bumblebee colony growth and reproductive success in response to metal contamination. No studies have investigated whether contamination via pollen impacts bumblebees in any capacity. Only two studies have surveyed how road dust effects pollination, and so this area represents a large gap in knowledge. Field and laboratory studies on this topic could be conducted to determine if dust does interfere with bees' perception of floral resources. Noise and vibration are a major source of traffic pollution, and yet no research has been conducted which assesses how this impacts pollinator foraging or nesting. Ground-nesting bees on road verges will be subjected to the constant thrum of passing traffic, and this may have an impact on colony development and reproductive success by interfering with communication signals between conspecifics. Research into how bees respond to noise and vibration at levels emitted by traffic is required to determine if road verges are suitable habitats for ground-nesting bees.

My thesis aims to address some of the key gaps in knowledge concerning the subject of bumblebees and road verges. In light of the current literature, I will address three questions relating to road verges as a tool for bumblebee conservation:

1. How does distance from the edge of a major road impact bumblebee colony growth and reproduction? (**Chapter 2**)
2. How does contamination of nectar and pollen rewards by two common roadside metals (copper and cadmium) impact colony development? (**Chapter 3**)
3. How do bumblebees use floral resources on roadsides relative to other types of habitat, and is this dependent on the location of the nest within the landscape? (**Chapter 4**).

To address question 1, I conducted research on the colony development of bumblebees on road verges using colonies of *Bombus terrestris* reared from wild-caught queens. More specifically, I recorded how bumblebees nesting on road verges compared to those nesting away from the road edge with regards to weight, number of individuals, and reproductive success. I expected to find that bumblebee colonies positioned on the road edge would experience a reduced growth rate and produce less reproductives as a consequence of close proximity to the road. Question 2 was investigated using queen-less micro-colonies of commercial *B. terrestris* exposed to levels of copper and cadmium recorded in roadsides through either a nectar or pollen source. I observed the numbers and weight of eggs, larvae, and pupae produced by micro-colonies over a three-week period to assess how these common roadside metals impacted development. At levels comparable with those currently found on roadsides, I predicted that micro-colony performance would be reduced compared to controls with regards to the number and size of offspring produced. Finally, to answer question 3, I collected pollen loads from workers returning to their nest, which were located either on a verge or in the surrounding area, and identified the species in the samples using light microscopy. Alongside this, I mapped the area surrounding each colony to classify the habitat types and carried out floral surveys of these habitats to determine what species (and their relative abundances) were present. I expected both colonies adjacent to the road edge and those away from the edge to be collecting pollen predominantly from road verge plant taxa, as verge habitat would be higher quality than other habitat types in the surrounding agricultural landscape.

CHAPTER 2: Close proximity to a major road does not impact the growth and reproductive success of *Bombus terrestris* colonies.

2.1. ABSTRACT

1. Road verges are often proposed as a key habitat for pollinator conservation; they form a vast network of connected habitat that can provide pollinators with food, nesting, and overwintering sites. However, the effects of nesting in such close proximity to traffic remain understudied.

2. We investigated how growth and reproductive success of bumblebee colonies positioned on the edge of a busy trunk road compared to those positioned at least 1km from the road edge, in the same landscape. Eight colonies of *Bombus terrestris* reared from wild-caught queens were subject to all potential positive and negative effects of verges for a total of 19 weeks in 2021.

3. There were no differences in the overall size (number of workers or weight), reproductive success (number and size of males and gynes), queen survivorship, or the onset of reproduction of bumblebee colonies, between the two treatments. Although colonies near the road were consistently heavier and produced slightly more gynes, this may be explained by local landscape composition as opposed to proximity to the road itself.

4. The overall neutral effect of verges on *B. terrestris* colonies observed in this study suggests that the benefits of nesting on roadsides may offset any negative impacts of traffic, thus creating a habitat suitable for bumblebees to nest and forage. As verge management improves for biodiversity and the automotive industry moves towards electricity as its primary fuel source, the positive aspects of verges are likely to further increase in the future.

5. Pollinator conservation strategies commonly use grassland restoration on verges as an active measure to support pollinators. We have demonstrated that such action does not negatively impact *Bombus terrestris*, to some extent allaying concerns that road verges could be an ecological trap for pollinators. More research will be needed to understand whether the neutral effect is a trade-off between benefits and harms.

2.2. INTRODUCTION

Pollinators are vital for fruit and seed production in many crops and wild flowers. It is estimated that 75% of major food crops and 87% of wild plants are dependent on animal pollinators to some extent (Klein et al., 2007; Ollerton et al., 2011; Rodger et al., 2021). Despite their ecological and economic importance, many pollinator species have shown declines in various regions throughout the world (Goulson et al., 2015; Potts et al., 2016a; Powney et al., 2019). These pollinator declines are attributed to a number of factors, including climate change and pesticide use, however, habitat loss, degradation, and fragmentation is considered the most important and well established cause of negative species trends (Dicks et al., 2021; Kennedy et al., 2013; Potts et al., 2016b). To conserve pollinators, it is therefore important to restore and improve the quality of semi-natural habitat that they rely on for food, reproduction, and overwintering (Senapathi et al., 2017).

One such habitat that has been proposed as a key component for pollinator conservation are road verges (Gardiner et al., 2018; Phillips et al., 2020a; Villemey et al., 2018). Road verges are areas of land that separate the road from the adjacent land and are managed primarily by large highway authorities, government, and local councils. The global road network extends an estimated 36 million km, and so road verges themselves consequently cover large areas of land. There are, for example, an estimated 2,500 km^2 of road verge in the UK (Phillips et al., 2021a), 50,000 km^2 in the USA (Central Intelligence Agency, 2017), and worldwide (270,000 km^2) (Phillips et al., 2020b). For context, semi-natural grassland habitat comprises 24,900 km^2 (10%) of land in the UK (Office for National Statistics, 2022).

Road verges have been shown to support pollinator populations, and in some cases have been identified as strongholds for threatened or critically endangered species (Heneberg et al., 2017; Villemey et al., 2018). Verges can support pollinators in two ways: by providing floral resources for foraging, and sites for nesting and overwintering. Roadsides can be an important food source for pollinators in landscapes which are otherwise lacking in floral resources (Hanley and Wilkins, 2015; Osgathorpe et al., 2012). It is estimated that arable farmland produces an average of 6.9kg sugar/hectare/year, compared to road verges which are estimated to produce 60.63kg sugar/hectare/year (Baude et al., 2016). Road verges have been shown to host an overall greater number of pollinators than other types of land (Cole et al., 2017; Hanley and Wilkins, 2015). They are almost certainly of greater value for some species than others; for example, roadside resources are more important for long-tongued bumblebees than short-tongued bumblebees (Osgathorpe et al., 2012). Ground nesting pollinators, such as bumblebees, may also use road verges for nesting and hibernating. Verges containing various micro-habitats can increase pollinator diversity, for example having patches of bare soil has been shown to increase numbers of solitary ground-nesting bees on roadsides (Hopwood, 2008).

There is growing concern over whether or not pollinators using road verges will experience overall positive or negative effects (Baxter-Gilbert et al., 2015; Hanley and Wilkins, 2015; Juliana et al., 2022; Muñoz et al., 2015; Phillips et al., 2019). Whilst road verges cover large areas of land and can improve landscape connectivity, pollinators using verges will also experience several negative effects from close proximity to traffic (Moroń et al., 2012; Sivakoff and Gardiner, 2017; Waser et al., 2017). These can include a barrier effect from the road itself (Andersson et al., 2017; Fitch and Vaidya, 2021), increased mortality due to vehicle collisions (Baxter-Gilbert et al., 2015; Muñoz et al., 2015), and exposure to ground and airborne pollutants associated with traffic (Phillips et al., 2021b; Ryalls et al., 2022). Though several studies have looked at these factors individually, none have explored their combined overall effect on pollinators. While it is logistically understandable to study these effects separately, in real-world scenarios these factors will not work in isolation, and it is important to understand their collective effect on pollinators, so that verges can be managed appropriately for pollinator conservation.

As a consequence of proximity to traffic, bumblebee colonies nesting on roadsides may experience an overall reduced fitness compared to colonies in the surrounding landscape. For example, foragers from roadside colonies are more likely to experience mortality due to vehicle collisions, which could have repercussions on overall colony development (Baxter-Gilbert et al., 2015). Workers may also experience direct mortality or sublethal effects from ingesting metal contaminants or road dust present along roadsides, which could impair their ability to care for larvae and pupae, and thus reduce the colony workforce even further (Phillips et al., 2021b). The onset of reproduction may also be influenced by proximity to the road edge. As queens are known to optimise their reproductive strategy (i.e. begin producing gynes as late as possible; Alaux, et al., 2005) a reduced workforce could mean this switch to reproductive production is later, as queens want to ensure they have a large enough colony to produce the maximum number of gynes possible (Santos et al., 2022).

Here, we investigate how colonies of the bumblebee species *Bombus terrestris* positioned on the edge of a major trunk road compared to colonies positioned 1 km away from the road, in their size, growth and reproductive success. While previous work on verges primarily focusses on foraging patterns in pollinators (Gardiner et al., 2018; Phillips et al., 2021b; Villemey et al., 2018), this is the first study to translate these individual-level impacts to overall colony success. Bumblebee colonies used in this experiment were exposed to all potential costs and benefits of living on roadsides, and so the collective impact of nesting on verges is explored. Given the lack of research in this area, this broad approach is an appropriate and much needed first step into the assessment of verge management as a tool for bumblebee conservation.

2.2.1. Research questions and hypotheses

We expect that roadside colonies will have an overall reduced number of workers compared to control colonies, as foraging individuals are likely to experience greater mortality due to collisions with vehicles. As a consequence of increased exposure to pollutants associated with traffic (e.g. metals, exhaust emissions, etc.) we also predict that roadside colonies will experience reduced fitness and this will result in fewer males and gynes produced compared to control colonies. Given the predicted reduction in worker number, we also anticipate that the onset of reproduction will be later in roadside colonies relative to controls, as the queen continues to build a larger workforce and must compensate for individuals lost. We specifically addressed three research questions:

1) Does distance from the edge of the A11 road impact the number of workers produced by a *Bombus terrestris* colony?
2) Does the reproductive success of a *B. terrestris* colony vary depending on distance from the road edge?
3) Does distance from the edge of the road influence the onset of gyne and male production by a *B. terrestris* colony?

2.3. MATERIALS AND METHODS

2.3.1. Queen rearing

Nest-searching *Bombus terrestris* queens were caught during March and April 2021 in a local nature reserve in Cambridge, UK (UK grid reference: 52.224357, 0.154397). On clear days between 9am and 2pm researchers used butterfly nets to catch queens, and once captured, bees were placed into labelled collection tubes and stored in a cool box for transfer to the laboratory.

In the laboratory, bees were screened for four common bumblebee pathogens (see **Appendix 1** for full screening methods) and then transferred to a controlled temperature room (26^{o}C and 50-60% relative humidity). Parasitised queens were not used in this experiment and never kept in the same rearing room as unparasitised queens to avoid cross-contamination. Queens were placed into Perspex rearing boxes (14cm x 8cm x 6cm) following a bespoke design, manufactured on demand by Allied Plastics. The base was a removable perforated polypropylene sheet (3mm thick). Rearing boxes were elevated slightly above the workbench using wooden planks to aid with ventilation. All boxes were supplied with glucose syrup *ad libitum* via a gravity feeder (Biobest, UK), and pollen in the form of a patty where the queen could begin laying eggs. Nectar and pollen were refreshed every three days to prevent wax moth infestations.

Once a queen had begun to found a nest she was provided with additional pollen in pellet form, which was, again, refreshed every three days. Queens were re-screened for parasites once a week as providing commercial nectar and pollen can lead to increased levels of parasitism. Any queen found to be parasitised at this stage was removed from the experiment.

Once ten workers had eclosed, the queen, workers, and brood were transferred to a permanent wooden nest box (30cm x 20cm x 15cm) with a clear plastic top. At opposing ends of the box were a mesh-covered ventilation hole and an entrance hole. There was also a hole in the base which allowed for access to a supply of glucose syrup via a wick connected to a tank below. Once in their new box, the colony was given seven days to adjust before being placed into the field.

2.3.2. Study site

Data collection took place between May and September of 2021 at the Elveden Estate in Norfolk (UK grid reference: 52.387325, 0.676124). The estate is a commercial farm covering 22,500 acres, producing a variety of root vegetables and cereals in rotation. The land is situated in East Anglia's Brecklands, and has nutrient deficient, sandy soils with high drainage and low water retention. The A11 trunk road runs directly through the centre of the estate and 16 sites were selected based on distance from the road and accessibility. The A11 is a 2-lane (dual carriageway), 70mph road with an average annual daily traffic volume of approximately 25,582 vehicles (Department of Transport, 2022). The verge varies in width throughout the estate from 0.5m to 14m, with an average width of 8.9m. The A11 is managed by National Highways through a cut and collect scheme, and consequently the verge supports high floral abundance and diversity compared to the surrounding agricultural landscape (see **section 4.5.** and **Table 4.1** for details).

Eight sites were located in close proximity to the A11 (within five meters of the road edge), and the remaining eight were located at least 1km away from the road edge, hereafter referred to as 'roadside' and 'control' (exact locations are shown in **Appendix 2**). 16 sites were selected in this study as a power analysis showed that eight colonies per treatment allowed for >80% statistical power with an effect size of 0.7 (SIMR; Green and MacLeod, 2016). All sites were separated by a minimum of 1km (**Figure 2.1**).

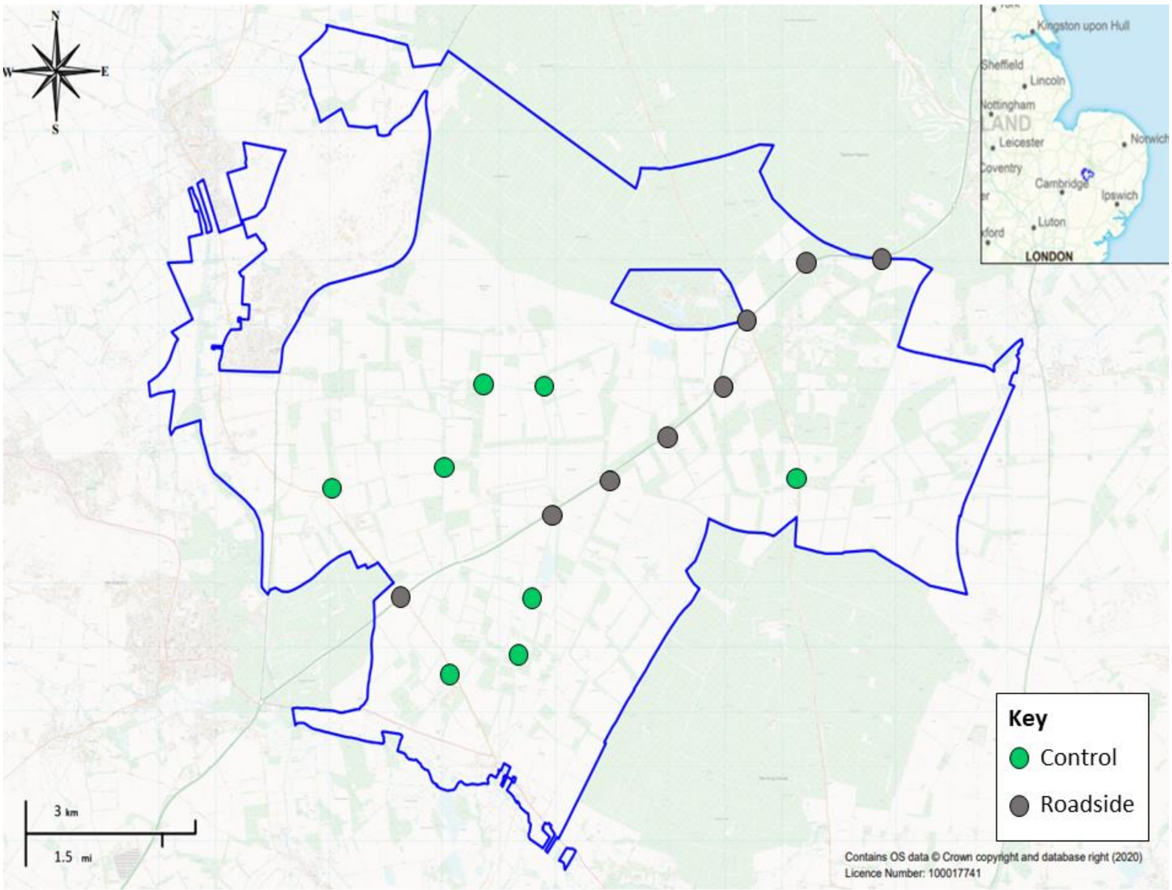


Figure 2.1: A map of the Elveden Estate. The area within the blue boundary is land owned by the Estate, and the cutout within the border is owned by Center Parcs. The 16 points on the map represent the 16 colonies used in the study (green points indicate control colonies and grey points indicate roadside colonies). Inset, top right, shows the location of the Estate in the East of England.

A bumblebee colony was placed in each of the 16 study sites. Colonies were randomly assigned a treatment, and subsequent location, using a random number generator. Prior to their placement in the field, all colonies were counted (three counts and an average taken), and the queen marked with a bee paint pen for identification. Colonies were placed into ventilated plastic boxes (40cm x 30cm x 28cm) for rain protection, and secured into the ground with metal pins which were pushed through pre-drilled holes in each corner on the base of the outer box. A secondary entrance hole was drilled in the outer box level with the wooden colony entrance hole and the two were connected with clear plastic tubing (diameter: 18mm). A ratchet strap was placed around each box to protect from badger attacks (see **Appendix 3** for a diagram of the bumblebee box set up). After a week of placement, access to the wick within the colony box was cut off so bees could no longer use the supplementary glucose syrup supply and had to forage within the landscape.

2.3.3. Data collection

Data were collected weekly during dark hours (between 9pm and 5am), to ensure all foragers were in the nest during counts. The tube connecting the colony to the outer plastic box was removed and the entrance sealed temporarily by securing a thin plastic sheet over the hole with duct tape to prevent any bees escaping. A red-light torch was used to illuminate the colony and ensure minimum disturbance.

Each week, the total number of workers was recorded (an average of three counts), along with the status of the queen (alive or dead), and the presence of any cuckoo bees. The colony was scanned for ten minutes for gynes, and then a further ten minutes for males, and any seen were counted, removed, and subsequently stored in labelled collection pots for freezing in the laboratory and subsequent size analysis. Bees were measured using digital calipers (accurate to two decimal places). The widest point of the thorax was measured three times and an average taken and this was used as a proxy for bee size (Peat and Goulson, 2005; Uthoff and Ruxton, 2022). A small sample of workers were removed each week and stored in labelled collection pots for size analysis also. The number of workers removed depended on overall colony size: one (<30 bees), three (30-50), or five (over 50). The colony weight was recorded by removing the inner wooden box and setting on digital scales (accurate to two decimal places). This was done after all other measurements as moving the colony increased activity within the nest.

Colonies were removed from the field and taken back to the laboratory for storage when there were either less than three workers and the queen remaining, or less than ten workers remaining with no queen.

2.3.4. Road impact

To take into account the potential impacts of all other roads in the area, as well as the A11, on bumblebee colony development we calculated a ‘road impact score’ adapting from methods set out by Cooke et al., (2020). The road impact score incorporates two important variables relating to road exposure: distance from the road and volume of traffic. Private roads used exclusively by the Elveden estate were not included in this analysis.

In QGIS (version 3.2) we mapped the location of the 16 colonies and created a 500-meter buffer around each colony. Shapefiles of roads located within each 500-meter buffer were created using the line feature: the A11, the B1106, the B1112, and London Road. Traffic volume data (annual average daily flow (AADF)) were obtained for each of the roads within the 500-meter buffer from the Department of Transport Traffic Counts website (Department of Transport, 2022). No traffic volume count data were available for London Road from any year, so we used the same traffic volume data as the B1106. London Road is a D-class road and would have a lower traffic volume than the B-class, however this ensures the traffic volume is not underestimated in this instance.

Kernel density estimation (KDE) was then used to determine the impact of roads on colonies. We used both the distance from the road (using markers placed every ten meters along the road and calculating the distance to the colony), and their associated traffic volumes. We also assumed a negative exponential relationship between distance from road and colony exposure, as the impacts of roads are likely to decrease with distance (for example, collisions with vehicles is a greater risk closer to the road). The estimated parameter in this instance, *k*, was selected for each road using the following methods: first, we selected a set range of values for *k* with limits of 1 and 100 as these assume that road exposure is 1% at 500m, and nears 100% at the road edge, and these are above and below what would be expected in reality.

We then ran multiple generalised additive mixed models (GAMM; Wood and Pya, 2016), narrowing the potential values of *k* through bisection searches. This was achieved by creating graphs of *k* and log-likelihood and searching for peaks. The values on either side of the highest peak were selected and the average of these two values calculated, to create three values. Further new values were generated in a repeated interval-halving method until the difference between two of the values was less than one, which was determined as the optimal value for *k* (for full KDE methods see **Appendix 4**).

Five colonies were completely out of the range of any roads and were given a road impact value of zero. Seven colonies were located within range of a single road and were therefore given a single road impact value corresponding to the appropriate road. The remaining four colonies were within range of two roads. All four belonged to the roadside treatment and so their impact score consisted of the A11 and another B-class or D-class road. These two scores were combined which provided all colonies with a single value for road impact.

2.3.5. Statistical methods

All statistical analysis was conducted in R (version 3.6.2), using the packages MASS (Venables and Ripley, 2002), pscl (Jackman, 2020), lme4 (Bates *et al*, 2015), MuMin (Barton, 2020), and survival (Therneau, 2020), with all graphs created and presented using ggplot2 (Wickham, 2016). An AICc approach was used for model selection. For each analysis the original model consisted of all variables and a step process was used to remove variables which did not explain significant variation in the model. To analyse the number of workers produced per week, a generalised linear mixed effects model with a Poisson error distribution was used. The same model was used to analyse colony weight change however with a Gaussian error distribution. Colony was nested in the model as a random factor and treatment, days since placement, and a two-way interaction between treatment and days since placement were included as fixed factors. Days since placement was also modelled as a quadratic function, to account for the expected non-linearity of colony growth over time. Worker size was analysed using one-way ANOVAs, with worker size as the dependent variable and treatment as the explanatory variable. A zero-inflated generalised linear model was used to model the number of reproductives produced as three and four colonies did not produce any males and/or gynes respectively. Again, treatment, days since placement, and a two-way interaction between them were included as fixed factors with colony included as a random factor. Queen survival and the onset of reproductive production were modelled using Cox proportional hazards survival analysis which contained treatment and days since placement as fixed factors.

2.4. RESULTS

Data collection lasted for a 19-week period; all colonies were put out between 17th May and 1st June, and collected between 16th August and 20th September. The mean colony duration was 14.63 weeks (±0.39), the shortest duration was 11 weeks, and the longest was 16 weeks. One roadside colony did not produce any new gynes, and three colonies (two control and one roadside) produced neither males nor gynes. All three colonies that failed to produce any reproductives lost their queen between weeks three and five.

Roadside colonies each produced a total of 313 (±31.1) new workers (an average of 19 new workers per colony per week), and control colonies produced 325 (±24.03) (21 per colony). The total number of males and gynes produced by roadside colonies was 99 (±8.53) and 41 (±4.76) respectively, while control colonies produced 102 (±9.38) males and 37 (±4.7) gynes. Only two cuckoo bumblebees were observed; both *Bombus vestalis* individuals were from control colonies and were found, and subsequently removed, within the first two weeks of placement.

2.4.1. Bee size

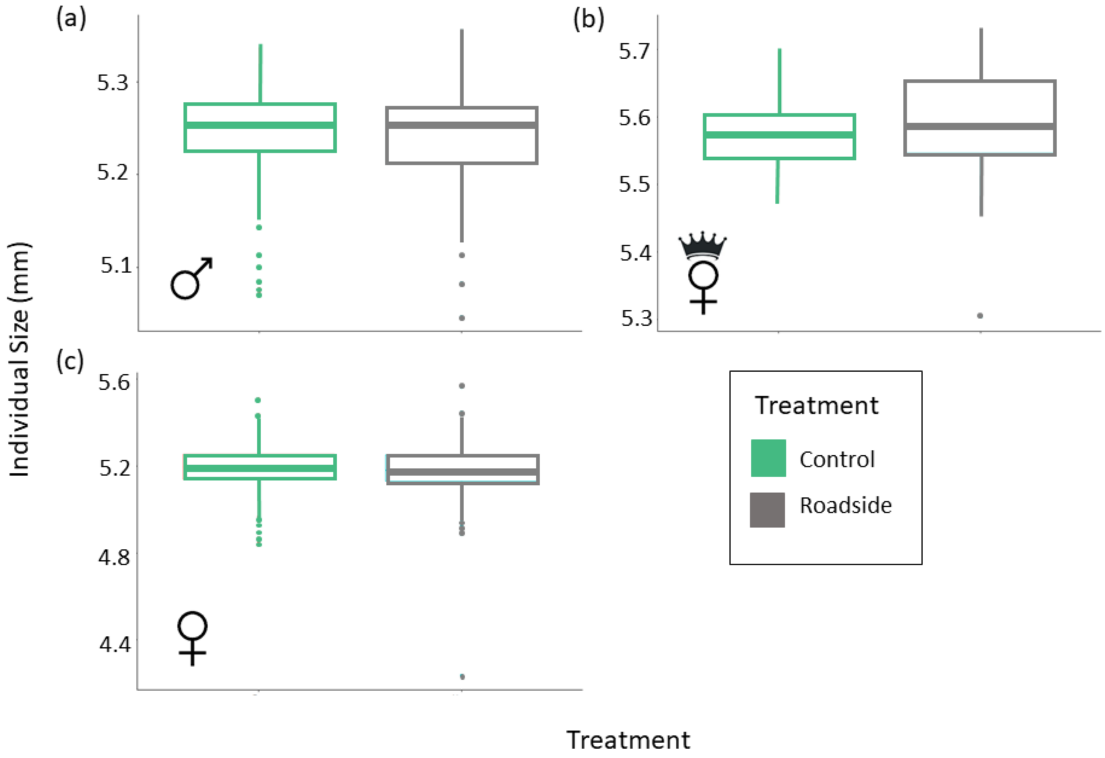


Figure 2.2: The average size (in mm) of **(a)** males, **(b)** gynes, and **(c)** workers produced by the *B. terrestris* colonies across the 19-week study period. Grey represents roadside colonies, and green shows control colonies.

The size of workers produced by roadside and control colonies did not significantly differ (**Figure 2.2**) (aov, F(1,14)=2.17, *p*=0.14). There was also no statistical difference in the size of males (aov, F(1,14)=0.01, *p*=0.98) or gynes (aov, F(1,14)=0.67, *p*=0.42) produced by colonies either. The average size of workers produced by roadside colonies was 5.17mm (±0.007) and for control colonies it was 5.19mm (±0.005). Males in roadside colonies were on average

5.23mm (±0.008), and 5.24mm (±0.006) in control colonies. Gynes were 5.64mm (±0.03) on average in roadside colonies, and in control colonies they were 5.61mm (±0.02).

2.4.2. Worker number

The average number of workers in a colony did not significantly differ between treatments (glmer, parameter estimate=-0.04, s.e.=0.13, z=-0.28, *p*=0.78). Across all 16 colonies the number of workers showed the same general trend, increasing steadily to a peak before declining. Roadside colonies produced an average of 19 (±3.93) new workers per colony per week and control colonies produced an average of 21 (±3.9). The most productive colony with regards to worker number was a control colony, producing 352 (±14.2) individuals over a period of 14 weeks. The smallest roadside colony contained 69 (±7.88) workers at its peak, and this was 80 (±9.38) for control colonies. In both treatments, the smallest colony lost the queen in week four (roadside) and week six (control).

2.4.3. Colony weight

Despite colonies in the roadside treatment remaining consistently heavier than control colonies, there was no significant difference in colony weight between treatments (glmer, parameter estimate=-0.04, s.e.=0.23, z=-0.55, *p*=0.21). Across both treatments, colony weight remained stable initially, then increased steadily to a peak before declining slightly towards the end of the colony lifecycle (**Figure 2.3**). Roadside colonies gained an average of 113.1 (±8.52) grams per colony and control colonies an average of 105.49 (±7.96) grams per colony. The colony to gain the most weight was from the roadside treatment and gained 168.32 (±4.14) grams, whereas the colony that gained the least weight (66.96 (±3.54) grams) was from the control treatment.

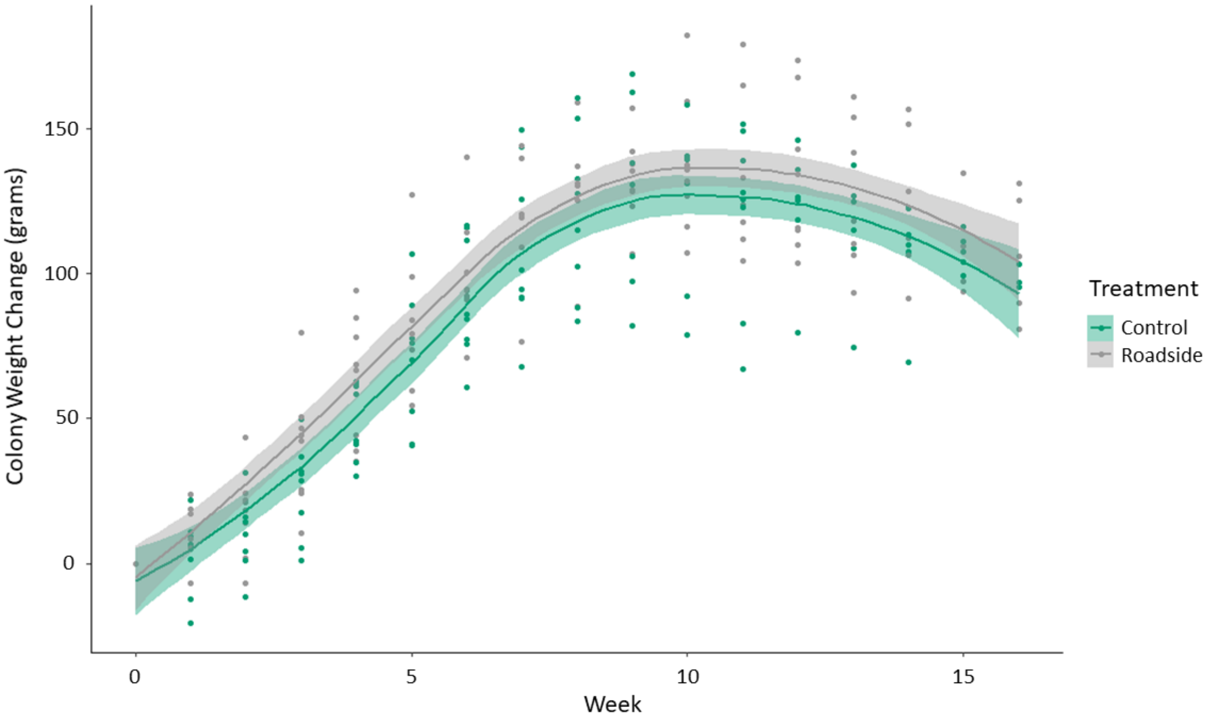


Figure 2.3: The weight change (in grams) of the *B. terrestris* colonies across the study period. Individual points represent the raw data, and the fitted lines show the mean values for each treatment from the model fits. The shaded areas correspond to the 95% confidence intervals. The grey line and points depict the colonies from the roadside treatment and green, the control colonies.

2.4.5. Reproductive success

The reproductive success of a colony was calculated using RS=M+3G, where M is the number of males and G is the number of gynes. The coefficient of differential investment was set to 3 to represent the per capita investment into the production of gynes compared to males (Pelletier and McNeil, 2003).

Despite roadside colonies producing slightly more gynes than control colonies there was no statistical difference in reproductive success between treatments (zeroinfl, parameter estimate=-0.79, s.e.=1.36, z=-0.59, *p*=0.56). The average number of males produced by roadside colonies was 12.38 (±2.91), and 12.75 (±2.85) was the average for control colonies. The maximum number of males produced by a colony in the roadside treatment was 22 compared with 19 from the control treatment. For gynes, the average number produced by a roadside colony was 5.13 (±1.47) and 4.93 (±1.32) for the control. The greatest number of gynes produced in each treatment was 8 (roadside) and 10 (control) (**Figure 2.4**).

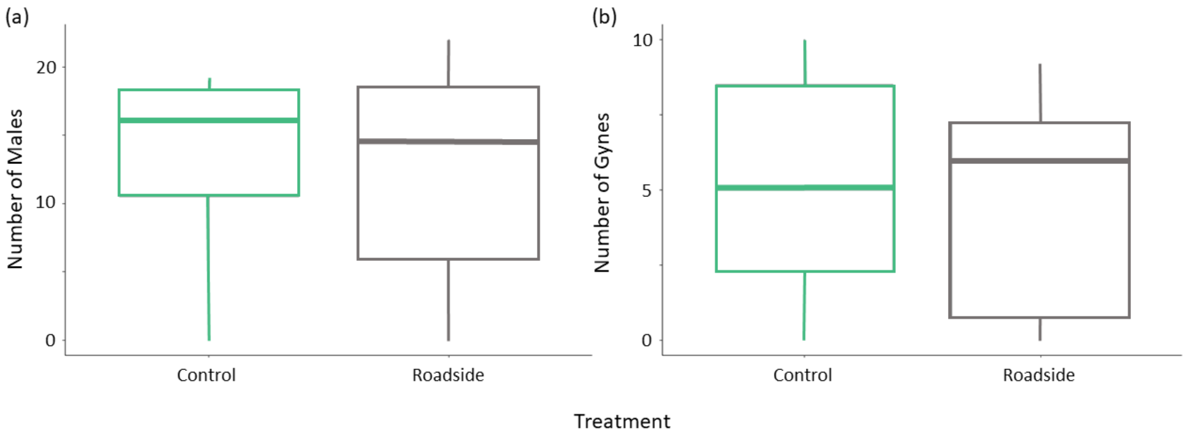


Figure 2.4: The average number of **(a)** males and **(b)** gynes produced by the *B. terrestris* colonies over the 19-week period of data collection. Roadside colonies are represented by grey, and control by green.

2.4.6. Queen survivorship

There was no statistical difference in queen status between treatments (coxph, hazard ratio=0.82, s.e.=0.12, z=-1.63, *p*=0.103). Queens from roadside colonies were 19% less likely to die at any given point compared to control colonies. The concordance value of the model was 0.86 and this was statistically significant on all tests (p(likelihood ratio test)=<0.05, p(wald test)=<0.05, p(score test)=<0.05). Roadside colonies lost their first queen in week three, whereas control colonies lost their first queen in week five, with all queens from control colonies dying first. The only surviving queen at the end of the experiment was from a roadside colony.

2.4.7. Week of reproductive onset

The onset of the production of males and gynes was not statistically different between roadside and control colonies (coxph, hazard ratio=0.88, s.e.=0.12, z=-0.32, *p*=0.75). The model had a significant concordance value of 0.72 (*p*(likelihood ratio test)=<0.05, *p*(wald test)=<0.05, *p*(score test)=<0.05). Control colonies were 4% more likely to have started producing reproductives at any given point than roadside colonies. Colonies from both treatments began producing males on week seven, and the first gyne was observed in a control colony on week eight. Roadside colonies followed a week later with three colonies producing gynes on week nine.

2.5. DISCUSSION

This is the first study to look at how bumblebee colonies respond to being positioned on the edge of a busy road in comparison to colonies positioned in the surrounding landscape. Despite roadside and control colonies showing slight differences in their overall colony size and reproductive success, this was not significant. Interestingly, roadside colonies remained consistently heavier and produced more gynes than the control, which was contrary to our expectations. Although non-significant, these slight differences may be a product of the study site itself as opposed to treatment.

The estate where the study took place is a commercial farm, and the landscape surrounding the area of road verge in this study was predominately large fields of crops (including wheat, barley, and onion) and grazing pasture for livestock, with small patches of coniferous forest sparsely spaced throughout. As there were minimal floral resources on the estate itself, it is highly likely that bees from both roadside and control colonies were using the food resources on the roadsides. The verges bordering the stretch of A11 running through the estate are grassland managed for biodiversity. We noted on site that the verges were rich in floral resources, including *Trifolium* spp., *Echium* sp., and *Rhinanthus* sp., which are known to be prominent in bumblebees' diet. Bumblebees are known to travel large distances for good quality pollen and nectar, and foraging distance correlates negatively with floral resources available in the landscape (Redhead et al., 2016). Although colonies were placed as far away from the verge as possible, this was never further than 2.5km, which *B. terrestris* has been shown to travel (Hagan et al., 2011; Redhead et al., 2016). Therefore, despite there being generally more bees in the roadside colonies, the expense of travelling a greater distance for food could explain the reduced weight and lesser number of gynes produced by the control colonies. However, there is no evidence collected in this study which would support this theory, and further research would be required to determine if this was the case.

A crucial element of this research was the use of bumblebee colonies reared from wild-caught queens. While commonplace in many research studies, the use of commercially reared bumblebee colonies does not allow for a comprehensive assessment of how colonies may behave in real-world scenarios. It is important to capture the entirety of the colony lifecycle to draw relevant conclusions and make thorough recommendations relating to bumblebee conservation, and catching queens from the wild is the most accurate way to represent this in research. Queens used in this study were local to the area, which accounts for any possible local adaptations to several biotic and abiotic factors which have previously been observed in bees (such as parasites and climate; Jackson et al., 2020; Kovačić et al., 2020), and would not be observed using commercial colonies. Sivakoff et al., (2020) also highlights the importance of using wild caught queens to generate ecologically realistic data. Our use of reared colonies from wild-caught queens ensures that any changes to the colony throughout the season were in response to their treatment as opposed to their conditions prior to placement in the field, and therefore our results are more widely applicable.

While the use of wild-caught queens improves the realism of our results, our findings are still constrained by species. *Bombus terrestris* is becoming the model bumblebee species, and while it may be possible to generalise our results to other bumblebees and potentially even ground-nesting solitary bee species, this is not the case for all pollinators. Further research is still required to confirm whether our results are consistent across other pollinator taxa. The use of *B. terrestris* as a model species is discussed in detail in **section 5.4.1.**

2.5.1. Queen survivorship

Queen survivorship was not significantly influenced by treatment. Queen bumblebees are more vulnerable to external stressors during their overwintering or nest-founding period than they are once they have founded a colony (Baron et al., 2014; Vesterlund et al., 2014). As colonies already contained a minimum of ten workers upon placement, queens had already undergone a significant portion of their lifecycle before the study even began. Therefore, the difference observed between colonies, although not significant, was likely to be complete chance. It is also worth noting that these queens were wild-caught, and their subsequent colonies reared in laboratory conditions. The success rate of rearing *B. terrestris* colonies in the laboratory is approximately 10-25% (Sharma et al., 2022) (in our study 22 colonies were successfully reared out of 164 queens caught), and so it can also be argued that the queens used in this study were already very resilient to external stressors.

2.5.2. Onset of reproduction

The onset of gyne and male production by colonies was also not influenced by treatment, with the first emergence of males recorded in the same week for both treatments. Gynes were first recorded from control colonies, with the first instances of gynes in roadside colonies the following week. While these values are representative of colonies, they may be underestimated for two reasons: 1) as there was a week between each instance of data collection it is possible that males and/or gynes left the nest and were therefore not recorded, and 2) we did not record the presence of pupae or larvae, which are often included in research of this kind (e.g. Samuelson et al., 2018; Siviter et al., 2018), due to time constraints with only one field recorder. It may be that colonies were producing reproductives earlier than noted but they were not successful and removed from their cells before eclosure. While it is possible to examine the colony brood to determine which cells contained gynes, it is much more difficult to ascertain the cells which contained males. This method of measuring reproductive success was therefore not implemented in this case to maintain consistency. Future research should aim to have more regular colony visits and record the presence of pupae and larvae to mitigate this problem. Despite this, however, there is no reason to suspect that this underestimation would be different in roadside or control colonies.

2.5.3. Cuckoo bumblebees

Rates of invasion by cuckoo bumblebees are usually between 30 and 50% (Erler and Lattorff, 2010), however here we only recorded two instances of cuckoo bumblebees within nests. This low rate of invasion could be due to our study landscape. Previous research on *Bombus terrestris* colonies has revealed that the availability of floral resources in the landscape is a strong predictor of cuckoo invasion (Carvell et al., 2008). Colonies positioned in landscapes with abundant floral resources were more likely to be parasitised than colonies where resources were lower. As previously mentioned, the landscape in our study was lacking in floral resources, and this could be a contributing factor to the distinct absence of cuckoos. It is also possible that natural bumblebee density in our landscape was already low, and therefore cuckoo density is lower than in other studies (Koch et al., 2021; Sramcova and Ayasse, 2009).

2.5.4. The future

While our results suggest an overall neutral effect of roads on bumblebee colonies, it is important to consider the future of the automotive industry when inferring any conservation measures. It is widely accepted that our current use of petrol and diesel powered vehicles is unsustainable, and alternative fuel solutions are being explored. The frontrunner is electricity, with the electric vehicle rapidly increasing in popularity amongst drivers. Electric vehicle sales doubled in 2021 to 6.6 million, and electric cars accounted for an estimated 10% of all global car sales (IEA, 2022). This switch to electricity is an effective way of significantly decarbonising the transport sector, and as car manufacturers and governments continue to promote and subsidise electric vehicle use, this influx of greener transport will continue to accelerate. This will more than likely have a positive impact on pollinators using verges; pollution from exhaust emissions will no longer be present and reduced noise may also have beneficial effects. It is therefore highly likely that, alongside roadside management strategies promoting biodiversity, the move towards increased electric vehicle use will make verges even more beneficial as a tool for pollinator conservation.

2.6. IMPLICATIONS FOR MANAGEMENT

Our study has revealed that *B. terrestris* colonies nesting on roadsides do not differ from colonies positioned away from the road edge with regards to colony weight, number of workers/males/gynes produced, queen survivorship, or the onset of reproduction. Previous work on roadsides and pollinators suggests that, overall, the positive impacts of well-managed verges outweigh costs (Phillips et al., 2020a), and while we do not show a positive effect of verge on colony development here, the neutral effect shown in our study does support the notion of verges as a conservation tool as no negative impacts were observed. We can recommend, therefore, that where appropriate, roadsides be managed for bumblebee conservation, as any negative effects of traffic on individuals does not appear to translate to diminished colony fitness. We concur with previous literature on roadside management and suggest that priority be given along roads with lesser traffic volumes and areas at least 2m from the road edge where negative effects on individuals will be reduced (Phillips et al., 2021b).

CHAPTER 3: Exposure to roadside levels of copper and cadmium does not impact the development of *Bombus terrestris* micro-colonies.

3.1. ABSTRACT

Road verges are often proposed as a key habitat for pollinator conservation, however there can be negative effects on individuals associated with traffic, including elevated exposure to metal pollutants. Whether these individual level impacts of metals extend to colony success remains unclear. We investigated the success of *Bombus terrestris* micro-colonies when fed on various diets consisting of two common roadside metals (copper and cadmium). Micro-colonies were reared in a controlled environment facility and subject to contamination via either a pollen or a nectar source for a three-week period. The results suggest that exposure to copper and cadmium at levels currently detected on roadsides does not have any significant impact on the number or weight of eggs, larvae and pupae produced by micro-colonies. There was a difference in the number of dead offspring for cadmium at levels slightly higher than those currently found in roadsides, which aligns with previous research. Our data fills an important research gap on the impacts of metal roadside pollutants on bumblebee colony development.

Implications for insect conservation. This study supports current research in favour of road verges as a tool for pollinator conservation, as the presence of copper and cadmium in floral rewards at levels detected on roadsides had little to no impact on queen-less *Bombus terrestris* micro-colonies. Levels in the field should be continually monitored however, as a slight increase in cadmium contamination has negative effects on the number of dead offspring.

3.2. INTRODUCTION

Pollination is an extremely valuable service provided by the ecosystem (Klein et al., 2007). Animals account for 87% of all pollinators, and their annual economic contribution to crop production is estimated to be between USD$235 and $577 billion worldwide (Ollerton et al., 2011; Potts et al., 2016). Ecologically, 350,000 animal pollinators are responsible for the pollination of 300,000 angiosperm species (Ollerton, 2017). Despite their monetary and environmental importance, various regions across the globe have reported declines in multiple pollinator species (Goulson et al., 2015; Potts et al., 2016a; Powney et al., 2019). Many different factors have been identified as contributors to their decline, such as pesticide use and climate change, however, the loss, degradation, and fragmentation of habitat is regarded as the most important and well established reason for their reductions in populations (Dicks et al., 2021; Kennedy et al., 2013; Potts et al., 2016b). To aid in their conservation, it is essential to increase both the quantity and quality of semi-natural habitat that pollinators depend upon for food, reproduction, and hibernating (Senapathi et al., 2017).

Road verges are often proposed as a key habitat for pollinator conservation (Gardiner et al., 2018; Phillips et al., 2020a Villemey et al., 2018). Verges are defined as areas of land that separate a road from the adjacent land and are managed by government organisations and local councils. Roads are estimated to span 36 million km worldwide, with verges subsequently covering approximately 270,000km^2 of land globally (Forman et al., 2003; Phillips et al., 2020b). There is growing concern over the use of verges as a tool for pollinator conservation however, with several studies highlighting the potentially negative consequences of living and foraging in such close proximity to traffic. Individuals can experience increased levels of mortality (Skórka et al., 2015), a ‘barrier’ effect (Andersson et al., 2017), and exposure to a multitude of different pollutants (Bhardwaj et al., 2019; Waser et al., 2017).

One such group of pollutants are metals. Metals occur naturally in most soils at trace levels, however anthropogenic activities, such as driving, can result in toxic levels being reached (Nagajyoti et al., 2010). Metals originating from traffic can come from multiple sources including directly through exhaust emissions, tire and road surface abrasion, motor oil, and brake linings (Adamiec et al., 2016). Studies have found significant levels of metals such as cadmium, chromium, copper, nickel, lead, and zinc in road verges (Adachi and Tainosho, 2004; Hjortenkrans et al., 2007; Krailertrattanachai et al., 2019). The accumulation of metals on roadsides is dependent on several factors including road exposure, drainage, and distance from road edge, and as such levels are highly variable between sites. **Table 3.1** shows the median values of six common roadside metals from studies across Europe at varying distances from the road edge (Werkenthin et al., 2014).

Table 3.1: The median levels of six common roadside metals detected across 27 European based metal contamination studies (Werkenthin et al., 2014). All values are presented in parts per million (ppm).

Metal	**Distance from the road edge**		
	0-5m	**5-10m**	**10-25m**
Cadmium	0.73	0.39	0.20
Chromium	28.00	9.98	13.82
Copper	47.90	15.05	15.05
Nickel	24.50	9.90-	6.93
Lead	106.00	40.60	24.00
Zinc	179.50	179.50	57.70

At high levels, these metals can negatively impact organisms by limiting growth or reproductive success (Depledge et al., 1997). Bees and other pollinators can be exposed to metals through various routes including air, soil, and floral rewards such as nectar and pollen, with the majority of research focusing exclusively on oral ingestion of contaminated floral rewards. There are two ways in which floral rewards can become contaminated with metal pollutants on roadsides: (1) through airborne road dust, and (2) uptake from the soil by plants. With regards to the former, dust deposition can be influenced by floral morphology, as flowers with an open inflorescence (e.g. *Leucanthemum vulgare*) are more susceptible to deposition than tubular species (e.g. *Echium vulgare*) (Szczęsna, 2007). Concerning the latter, soil type and plant species are known to impact plant uptake rates. Xun et al., (2017) recently demonstrated that non-hyperaccumulator plants were able to translocate metals from the soil into their reproductive organs and floral rewards, which has major implications for conservation efforts on roadsides. Notably, dandelions, a very important early-flowering resource for newly emerging bees, have been shown to accumulate cadmium (Tomczyk et al., 2020). *Bombus terrestris*, like many other bumblebee and solitary bee species, nests underground and is therefore also subject to contamination through direct contact with soil, however this exposure route has not yet been explored in the literature.

Much of the research pertaining to metal pollution and pollinators is focused on honeybees and contamination of honey products (Cunningham et al., 2022; Taha and Kahtani, 2020). A few key studies have explored the impacts of different metals on foraging behaviour in bumblebees and other pollinator species (Meindl and Ashman, 2013; Shepard et al., 2020; Sivakoff and Gardiner, 2017), but there is limited knowledge on how these individual level impacts translate to overall colony success (Sivakoff et al., 2020). Biological scale is an important consideration in colony-based species, like *Bombus* spp.; how metal contamination translates across levels of organisation (i.e. from individual to colony to population) will determine the severity of the stressor, and therefore its importance when planning conservation strategies (Vanbergen et al., 2013).

We only know of three studies that address the question of metal contamination at this higher colony level. Honeybee hives have been shown to respond negatively to copper and cadmium contamination at field-realistic levels (25ppm and 0.24ppm respectively) with regards to the number of dead pupae, but, conversely, the impacts on worker and queen weight were not different from controls despite accumulation of these two metals in workers (Hladun et al., 2015). Moroń et al., (2012) found that populations of wild solitary bees were negatively affected by metal contamination, with the number of individual bees emerging from trap nests, as well as bee species richness, declining significantly across the gradient for three common metals; cadmium: 0.8 - 9.3ppm, lead: 42.0 - 356.2ppm, zinc: 56.0 - 492.4ppm. A recent study on *B. impatiens* colonies found no significant contamination effect of four metal contaminants (arsenic: 0.894ppm, cadmium: 0.276ppm, chromium: 0.245ppm, and lead: 0.265ppm) on the number of individuals and colony weight, however there was an effect on the proportion of dead brood (Scott et al., 2022). These few studies addressing metal contamination on colony development suggest that impacts are varied across metals and taxa, and more thorough research is required.

When considering conservation strategies for colony-based species (like bumblebees), it is vital to know if these behavioral changes are limited to the individual, or if they have repercussions at the colony level. This will determine whether the stressor (in this case metals) will have impacts on population dynamics (Vanbergen et al., 2013). Here, we aim to quantify the impacts of two common roadside metals (copper and cadmium) on micro-colony development in *Bombus terrestris*. Copper and cadmium are two of the most common metals along roadsides, and their prevalence in the literature reflects this (Hladun et al., 2015; Phillips et al., 2021b; Scott et al., 2022). Despite this however, there are no studies that explore their impacts on bumblebee colony development via a pollen resource.

3.2.1. Research questions and hypotheses

We expect that contamination of provisions by metals at roadside levels (Cu: 20ppm, and Cd: 0.2ppm) will incur a fitness cost to micro-colonies. This will be evident through a reduction in the number and size of offspring produced by micro-colonies exposed to environmentally relevant levels of metal contamination relative to controls. We also predict there will be more pronounced negative impacts on colony performance with increasing contamination levels, particularly for cadmium which has a higher toxicity in bees (LD_{50} Cu: 19-96ppm, Cd: 1-6ppm; Roman, 2010). We specifically address the following research questions:

1. Does copper and cadmium at typical roadside levels (Cu: 20ppm, and Cd: 0.2ppm) impact the number and size of eggs, larvae and pupae produced by micro-colonies?
2. Is there a difference in micro-colony performance based on exposure route (i.e. through nectar or pollen)?
3. Does micro-colony composition (proportion of eggs, larvae, and pupae) differ between treatments?
4. Does the consumption of nectar and pollen by micro-colonies differ depending on level of contamination?

3.3. MATERIALS AND METHODS

Data collection took place over two periods: between January and April 2021, and between October and December of 2021. Seven commercial colonies of *Bombus terrestris audax* (Biobest, UK) were used per study period in this experiment, with each colony representing a 'repeat'. Seven was selected as a power analysis showed that seven colonies would allow for >80% statistical power with an effect size of 0.75 (SIMR; Green and MacLeod, 2016). Colonies were screened for parasites prior to the start of the experiment, and excluded from the study if any parasites were recorded (see **Appendix 1** for further details).

3.3.1. Micro-colony setup

All micro-colonies were reared in a controlled temperature (CT) room which was set at 26°C and 50-60% relative humidity. The CT room was kept in complete darkness except for when handling/observing the bees, in which case the room was illuminated using red light. Work benches were covered with tissue roll, with lengths of wood wrapped in cling film, upon which the micro-colony boxes were placed to keep them off of the bench surface and aid with ventilation (**Figure 3.1**). Custom built Perspex micro-colony boxes measured 13.8cm x 7.7cm x 5.7cm with the top on, with a base of perforated sheet plastic measuring 7cm x 16cm x 0.3cm.

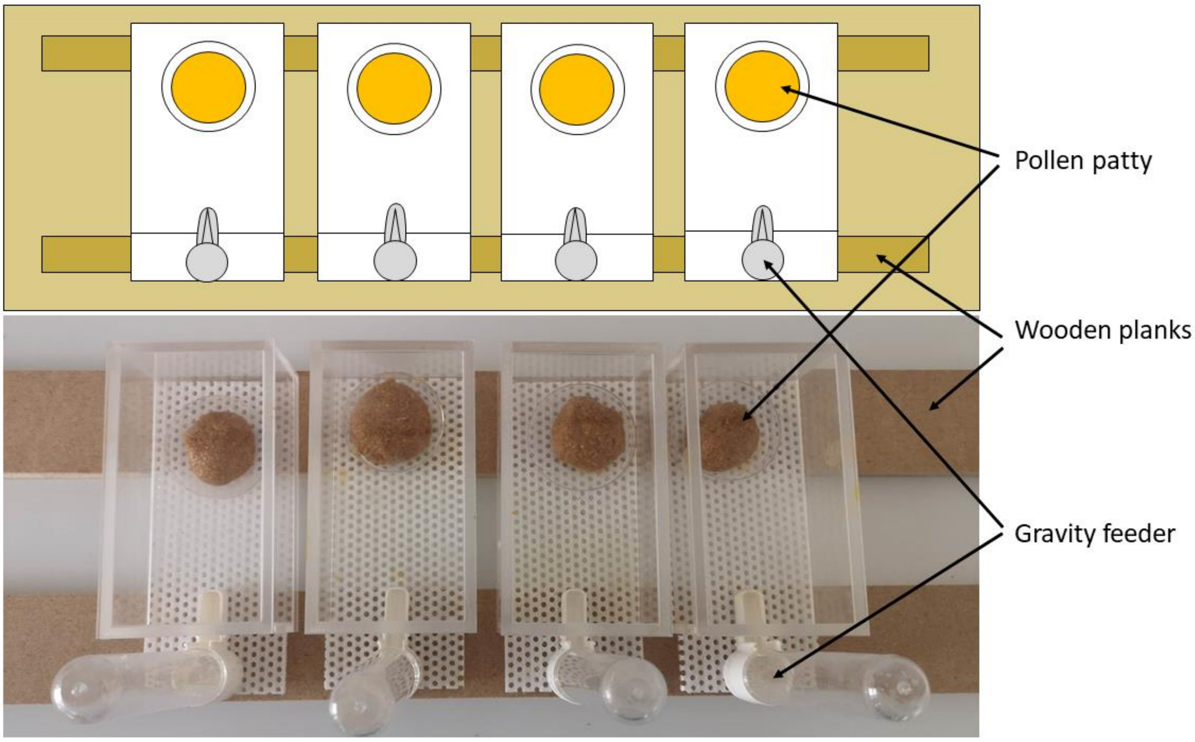


Figure 3.1: An aerial view of the experimental set up. Perspex rearing boxes were placed upon two lengths of wood on the workbench. Colonies had access to a pollen patty and nectar solution via a gravity feeder. Only four micro-colonies are shown in this diagram for illustrative purposes.

Four two-day old workers from a single source colony were randomly selected to form each micro-colony. All micro-colonies were fed with 50% sucrose solution and pollen *ad libitum* for a three-week period. Sucrose solution was provided via a gravity feeder, and pollen in the form of pollen patties placed on petri dishes. Once bees began brood formation on the pollen patty, a secondary pollen source was provided on a petri dish. Gravity feeders contained a pipette tip in the base to prevent bees becoming trapped in the feeder. Pollen and nectar were refreshed every two - three days to avoid contamination. Any pollen and nectar which remained during refreshing were weighed, and fresh pollen and nectar were weighted prior to placement in the micro-colony. This difference in weight between refreshing was recorded as a measure of micro-colony food consumption. Any micro-colonies who did not produce eggs after one week were removed from the experiment.

3.3.2. Contamination

Colonies were subjected to 11 different contamination treatments: a control treatment where bees were exposed to no copper or cadmium, and contamination through either a nectar or pollen source at five different levels for each metal (copper: 1ppm, 20ppm, 50ppm, 100ppm, and 500ppm, and cadmium: 0.01ppm, 0.2ppm, 0.5ppm, 1ppm, and 5ppm). All metal contaminants were prepared using metal chlorides ($CuCl_2$ and $CdCl_2$) (see **Appendix 5** for further details). Roadside concentrations (Cu: 20ppm, and Cd: 0.2ppm) were selected on the basis of typical levels of copper and cadmium recorded in roadsides (Phillips et al., 2021b). We also tested concentrations at intervals both above and below the typical values recorded in verges to account for variation between different roads e.g. traffic volume. These levels, although not typical, are not beyond the range of values recorded on roadsides (Cu: 7-413ppm, and Cd: 0.1-10.5ppm; Werkenthin et al., 2014).

3.3.3. Dissection

Once the three-week study period was complete, all micro-colonies were frozen in a deep freezer at -80°C for 15 minutes to euthanize the workers. Micro-colonies were then transferred to a -18°C freezer for storage. The four workers were removed from each box and their size was measured using digital calipers (three counts of thorax width with an average taken).

Micro-colonies were then dissected to record variables indicative of colony success. The number of eggs, larvae, pupae (**Figure 3.2**), were counted as well as their status (alive or dead). Offspring status was determined through appearance, with dead offspring showing distinctly different morphologies and darker colouration (see **Appendix 6**). After defrosting for 30 minutes, larvae and pupae were also weighed on scales and measured using digital calipers (larval length, and pupal thorax width). All eggs were approximately the same length and due to their small size and the sensitivity of the scales (three decimal places), weight differences between each egg was negligible. Therefore, only the number of individual eggs is considered in this study.

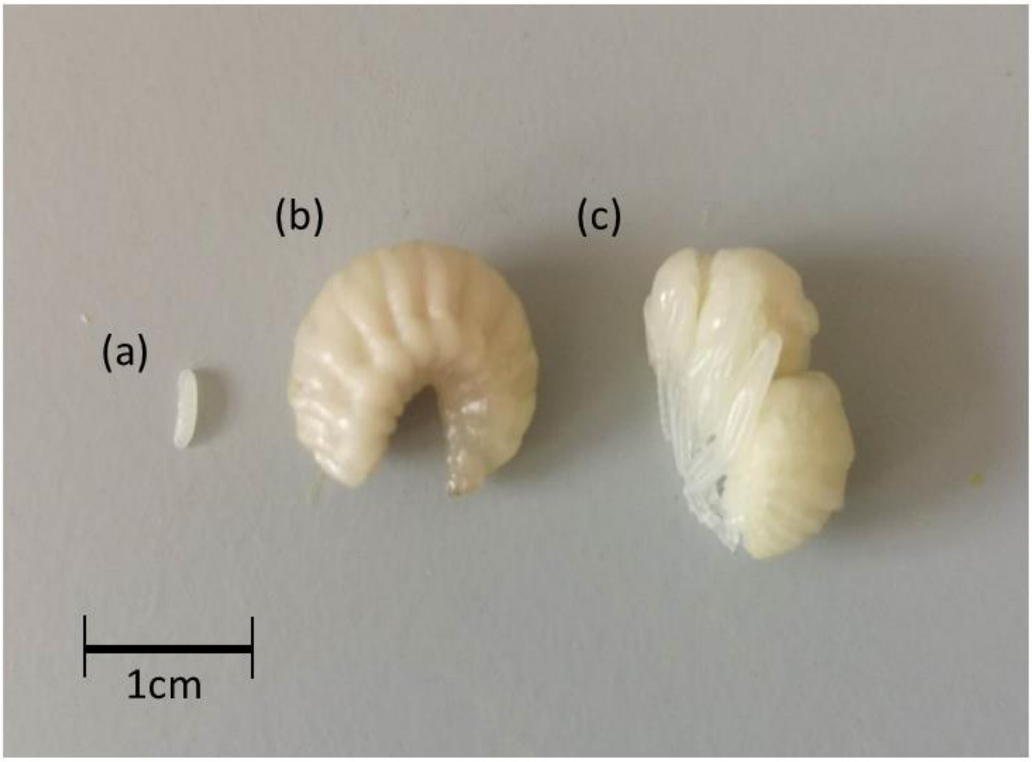


Figure 3.2: An **(a)** egg, **(b)** larvae, and **(c)** pupae of *Bombus terrestris* produced by micro-colonies. A scale bar is provided for reference.

3.3.4. Statistical methods

All statistical analysis was conducted in R (version 3.6.2), using the packages MASS (Venables and Ripley, 2002), pscl (Jackman, 2020), lme4 (Bates et al., 2015), MuMin (Barton, 2020), and vegan (Oksanen et al., 2013), with all graphs created and presented using ggplot2 (Wickham, 2016). An AICc approach was used for model selection.

For each analysis the original model consisted of all variables and a step process was used to remove variables which did not explain significant variation in the model. Generalised linear mixed effects models (GLMMs) with a Poisson error distribution were used to analyse the effect of three fixed categorical variables of metal (two levels: copper and cadmium), treatment (six levels: Cu: control, 1ppm, 20ppm, 50ppm, 100ppm, and 500ppm, and Cd: control, 0.01ppm, 0.2ppm, 0.5ppm, 1ppm, and 5ppm), and exposure route (two levels:

nectar and pollen) on the number of offspring produced and nectar/pollen consumption by micro-colonies. The same model was run for larval and pupal weight, but with Gaussian error distribution in place of Poisson. Colony of origin was nested in the model as a random factor. Prior to analysis all data were checked for normality and over dispersion, and data transformed when assumptions were violated.

The number of dead offspring was analysed using zero-inflated generalised linear models as many micro-colonies did not produce any dead offspring. Again, treatment, metal, and exposure route were included as fixed factors and colony as a random factor. Brood composition (i.e. the proportion of eggs, larvae, and pupae) were compared using permutational multivariate analysis of variance (perMANOVA), with distinct perMANOVAs performed using metal, treatment, exposure route, and their interactions as variables to determine any differences in micro-colony development.

3.4. RESULTS

A total of 154 micro-colonies were used in this experiment; 22 per commercial colony. All micro-colonies produced eggs and larvae, however at least one micro-colony from each treatment did not produce any pupae. There was no difference between exposure routes (via either a nectar or pollen source) and so all graphs contain both nectar and pollen data. All values are presented as the average ± the standard error unless stated otherwise.

3.4.1. Exposure route

Exposure route had no significant effect on any of the variables recorded. Brood composition (perMANOVA: $F_{2,10}=4.09$, $p=0.18$), offspring number (GLMM: $z=0.35$, $SE=0.56$, $p=0.11$), offspring weight (GLMM: $z=0.72$, $SE=0.39$, $p=0.25$), resource consumption (GLMM: $z=1.16$, $SE=0.27$, $p=0.38$), and number of dead offspring (zeroinfl, parameter estimate=-0.22, s.e.=2.06, $z=-0.33$, $p=0.29$) showed no differences across any of the treatment levels regardless of whether metal contamination was provided via nectar or pollen.

3.4.2. Brood composition

There was no statistical difference in the composition of brood between treatments (perMANOVA: $F_{1,11}=5.98$, $p=0.39$; **Figure 3.3**). Control colonies consisted of 65.2% (±5.41) eggs, 29.1% (±3.55) larvae, and 5.68% (±0.49) pupae, compared to those in the roadside cadmium (eggs: 62.19% (±7=4.45), larvae: 33.14% (±2.99), pupae: 4.27% (±0.23)) and copper (eggs: 62.12% (±6.22), larvae: 29.75% (±3.54), pupae 4.87% (±0.49)) treatments. Micro-colonies in the highest treatment had a smaller proportion of pupae for both metals, although these differences were not significant.

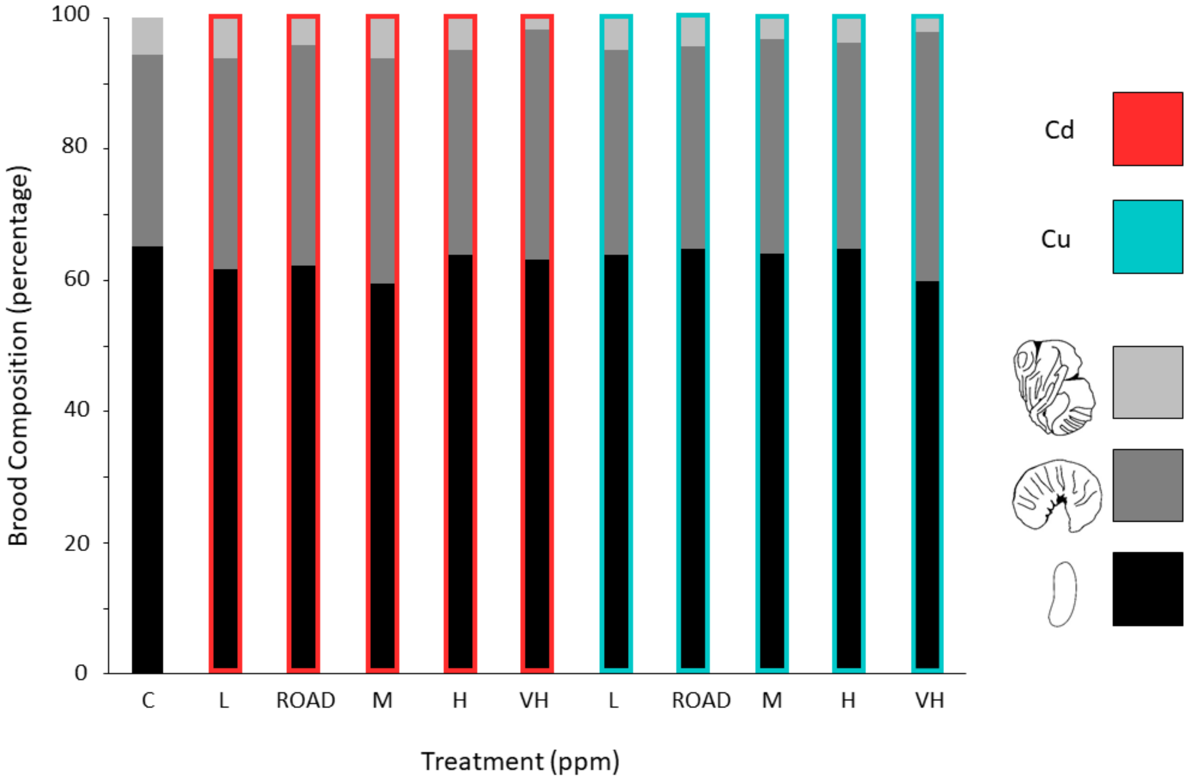


Figure 3.3: Percentage of offspring (eggs/larvae/pupae) in each treatment for both copper (Cu) and cadmium (Cd). Eggs are shown in black, larvae in dark grey, and pupae in light grey. Copper treatments are outlined in blue, cadmium in red, and control has no outline. As exposure route had no effect, data for both exposure routes (via nectar or pollen) are shown together. Treatment values for Cu and Cd are as follows: L (1ppm, 0.01ppm), Road (20ppm, 0.2ppm), M (50ppm, 0.5ppm), H (100ppm, 1ppm), VH (500ppm, 5ppm).

3.4.3. Offspring number

The number of larvae produced by micro-colonies contaminated with copper did not significantly differ between treatments (GLMM: z=0.11, SE=0.46, *p*=0.28). There was an overall decline in the number of eggs, larvae, and pupae with increasing concentration (**Figure 3.4**), but this was only significant for eggs (GLMM: z=0.42, SE=1.08, *p*=<0.01) and pupae (GLM: z=0.53, SE=0.95, *p*=<0.01) at the highest level. The only treatment to have a significant negative effect on all offspring numbers was 5ppm cadmium (GLM: z=0.66, SE=0.25, *p*=<0.01). Micro-colonies fed the highest level of cadmium contaminated nectar and pollen produced only 16.28 ±1.06 (11 ±0.95 eggs, 5.14 ±0.74 larvae, and 0.14 ±0.14 pupae) and 16.29 ±0.97 (9.57 ±1.38 eggs, 6.29 ±0.52 larvae, and 0.43 ±0.3 pupae) respectively, compared to 39 ±1.56 (25.43 ±1.11 eggs, 11.36 ±0.82 larvae, and 2.21 ±0.37 pupae) produced by the control.

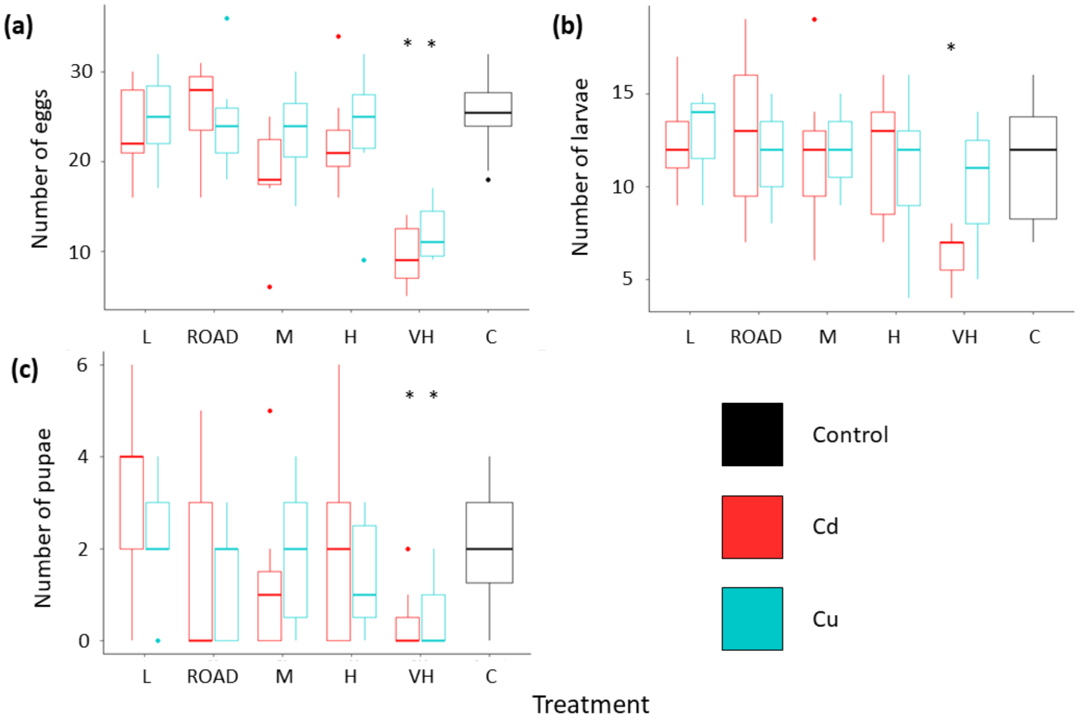


Figure 3.4: Number of **(a)** eggs, **(b)** larvae, and **(c)** pupae produced by micro-colonies in each treatment over the three-week study period. Blue and red bars represent copper and cadmium contamination respectively with black showing the control. Treatment values for Cu and Cd are as follows: L (1ppm, 0.01ppm), Road (20ppm, 0.2ppm), M (50ppm, 0.5ppm), H (100ppm, 1ppm), VH (500ppm, 5ppm). Statistical significance from the control is indicated by an asterisk.

3.4.4. Offspring weight

There was no statistical difference in the weight of larvae and pupae (GLMM: z=0.54, SE=0.28, *p*=0.39) produced by roadside treatments of Cd and Cu compared to the controls. Control larvae weighed 0.159 ±0.008g, compared to 0.142 ±0.006g and 0.161 ±0.01g in the 0.2ppm cadmium nectar and pollen treatments, and 0.143 ±0.007g and 0.143 ±0.009g in the nectar and pollen 20ppm copper treatments. Pupal results were similar, with control pupae weighing 0.263 ±0.015g, cadmium contaminated pupae weighing 0.243 ±0.011g (nectar) and 0.253 ±0.017g (pollen), and copper contaminated pupae weighing 0.246 ±0.009g (nectar) and 0.278 ±0.015g (pollen).

3.4.5. Resource consumption

Consumption of both nectar and pollen was not significantly different across treatments (GLMM: z=1.75, SE=0.47, *p*=0.26; **Figure 3.5**). Micro-colonies in the control treatment consumed 53.46 ±0.88g of pollen over the 3-week period compared to 51.89 ±2.21g in the roadside level cadmium treatment, and 52.95 ±2.51g in the roadside level copper treatment. The same trend was observed in nectar consumption, with control micro-colonies consuming 29.37 ±0.45g, compared to 29.41 ±0.88g for 0.2ppm cadmium, and 29.01 ±0.83g in the 20ppm copper treatment.

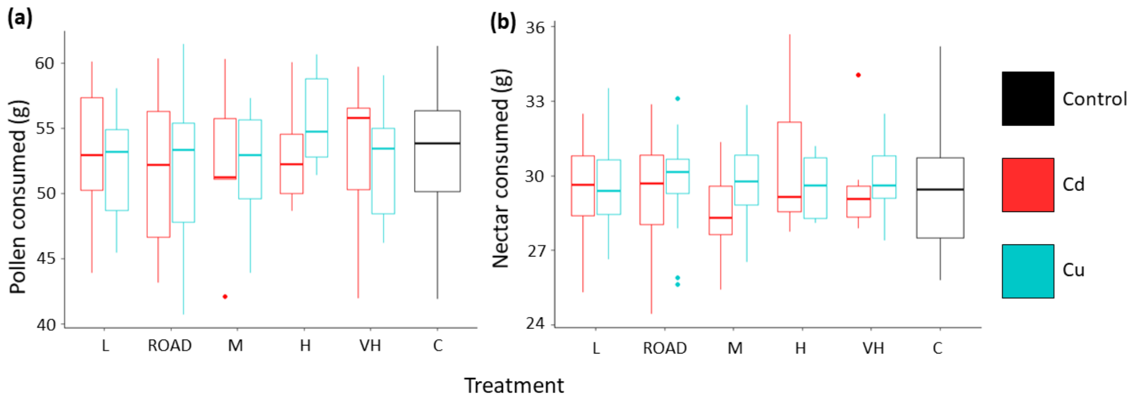


Figure 3.5: Pollen (a) and nectar (b) consumed by micro-colonies in each treatment over the 3-week study period. Blue and red bars represent copper and cadmium contamination respectively, with the control shown in black. Treatment values for Cu and Cd are as follows: L (1ppm, 0.01ppm), Road (20ppm, 0.2ppm), M (50ppm, 0.5ppm), H (100ppm, 1ppm), VH (500ppm, 5ppm).

3.4.6. Dead offspring

The number of dead offspring did not vary significantly between the control treatment and copper/cadmium treatments at roadside levels (zeroinfl, parameter estimate=-0.64, s.e.=1.42, z=-0.21, *p*=0.17; **Figure 3.6**). At slightly higher cadmium treatments (0.5ppm), micro-colonies contained 17 ±2.9 dead individuals compared to controls which contained 5 ±0.75 dead offspring. Cadmium treatments consistently had more dead offspring than copper treatments, and the only copper treatment to differ from the control was the highest treatment (500ppm) which contained 13 ±1.6 dead individuals.

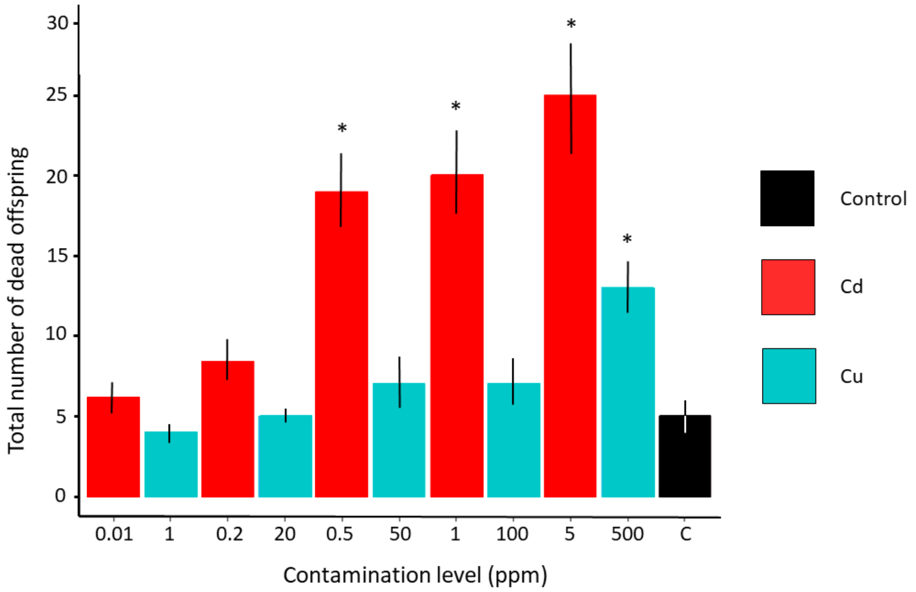


Figure 3.6: The number of dead offspring produced by micro-colonies in each treatment over the three-week period. Cadmium, copper, and control treatments are shown through the red, blue, and black bars respectively. Treatments which are significantly different from the control are indicated with an asterisk.

3.5. DISCUSSION

Our results indicate that exposure to levels of copper and cadmium currently found in roadsides, in the absence of other stressors, does not have direct effects on the ability of *Bombus terrestris* to reproduce, as measured by worker male production in queen-less micro-colonies. There were no significant differences between roadside level treatments and the control, with regards to number and weight of offspring which aligns with previous research in the field (Hladun *et al*, 2015; Scott *et al*, 2022). At very high levels (Cd: 5ppm and Cu: 500ppm) negative impacts were observed on weight and number of offspring, however this is not representative of metal contamination levels in roadside plants. The number of dead offspring in the roadside treatments also did not differ significantly from controls, although at slightly elevated levels this was significant for Cd (0.5ppm).

Based on consumption of resources and supplied dose, in our study *B. terrestris* workers consumed on average 0.16ppm Cd and 1.5ppm Cu at roadside level treatments during the 3-week study period. Both values are well below the published LD_{50} concentrations for these metals observed in honeybees (1-6ppm for Cd and 19-96ppm for Cu; Roman, 2010), which could explain the lack of effect at these field-realistic doses. At the highest concentrations, bees consumed metals at doses within the ranges of the reported LD_{50} (Cd: 2.74ppm and Cu: 40.5ppm). This could provide a reason for the significant difference in the total number of offspring produced at the highest level.

These results should be taken in their experimental context and applied cautiously. Here, we worked with queen-less micro-colonies, in a laboratory setting with other stressors removed (e.g. pathogens, food resource limitation), so we cannot infer much about the responses of whole colonies living in the wider environment.

Micro-colonies were exposed to contaminants for a period of three weeks and accessed metals only through means of nectar and pollen sources provided to them. In a natural setting, bees would be exposed throughout the entire colony lifecycle and potentially through other exposure routes (e.g. through direct contact with the soil in ground-nesting bee species). Regardless of treatment, micro-colonies used in this study did not differ in the quantities of nectar and pollen consumed across the three-week study period. The extent to which bumblebees detect or avoid metal contaminated nectar and pollen remains understudied, however some evidence suggests that bumblebees will spend less time foraging on flowers contaminated with nickel whilst subsequently avoiding nearby contaminated flowers (Meindl and Ashman, 2013). In contrast, honeybee workers have been recorded feeding from flowers contaminated with field-realistic concentrations of arsenic, lead, and zinc at the same rate as those without metals (Monchanin et al., 2022). Bees in our study were not given the choice between a contaminated and uncontaminated food source and this lack of autonomy does not reflect an ecologically realistic scenario. It also may be a reason for the absence of variation in pollen and nectar consumption between metals and treatment levels.

The long-term impacts of Cu and Cd pollution on bumblebee colony health remain understudied. The three-week timeframe of our experiment means that offspring production can be compared between treatments, but as no individuals eclosed during the experimental period, we were unable to record variables that have been shown to be affected by metal contamination in other pollinator species, such as wing formation (Szentgyörgyi et al., 2017; Mielczarek et al., 2021). It has been suggested that some bee species could be used as bio-indicators for metal contamination in the environment (Skorbiłowicz et al., 2018). Solitary bees (de Matos Barbosa, 2021), honeybees (Goretti et al., 2020), and bumblebees (Szentgyörgyi et al., 2011) have all been shown to accumulate metals in their bodies, which can have sublethal impacts on their memory and behaviour (Burden et al., 2016). Micro-colony performance was affected by metal contamination at higher levels, but in the field this would only be seen in the latter stages of colony cycle as bees accumulated metals throughout the season. It has been shown previously in honeybees that queens, workers, developing offspring, and honey accumulate copper and cadmium when exposed over prolonged periods of time (Hladun et al., 2015), and so future research investigating this phenomenon in bumblebee colonies should be conducted.

While the use of micro-colonies in this instance allows for a neat comparison between different concentrations, it is also imperative to note that they are incapable of producing workers or gynes, and therefore all energy expenditure was directed towards the production of males. It is estimated that rearing a new queen takes up to three times more colony resources than rearing a male (Pelletier and McNeil, 2003), and our study design provides no information on how this variation in resource allocation to offspring may impact colony development. Another important factor to consider is that in a real world scenario, bees would be exposed to an amalgamation of different metal pollutants at varying concentrations. While our study addresses an important question about exposure to copper and cadmium specifically, it does not consider the impacts of metal pollutants in an ecological context, where they are likely to have synergistic effects (Yoo et al., 2021). Scott et al., (2022) demonstrated this relationship in *Bombus impatiens*, where bees fed a diet of mixed metals (Ar, Cd, Cr, and Pb) had significantly more dead brood than those fed a single metal. It is worth noting however, that these are all non-essential heavy metals, and many metals found on road verges are essential metals (such as Zn). Whether this extends to other bee species and with other common roadside metals remains a highly significant research gap, which should be thoroughly explored before inferring any major conservation strategies or policy changes concerning roadside habitat.

An aspect of bumblebee ecology that has not been thoroughly investigated is the possible interactions between metal pollutants and the prevalence of pathogens or parasites. Szentgyörgyi et al., (2011) remains, to our knowledge, the only study addressing this issue. The authors found no significant correlation between heavy metal contamination (Pb, Zn, and Cd) and the frequency of *Nosema bombi* infections in bumblebees sampled. The authors expected that infection rate would be higher in individuals exposed to metal pollutants, as contamination has been shown to compromise the immune system in other invertebrate species (Sorvari et al., 2007). They identify two potential reasons for this unexpected result however; time of sampling, and the recent introduction of a new subspecies of the target pathogen. They conclude that much more detailed research is required to address this highly complex relationship. Our bees were screened for pathogens prior to the commencement of the study (**Appendix 1**), but this was not investigated further. It is possible that some individuals were infected after the start of the experiment because commercial pollen fed to bumblebees is collected from honeybee hives and is known to contain pathogens that can introduce themselves into the bumblebee microbiome (Pereira et al., 2019). Even with sterilised pollen, this is extremely difficult to control for, and remains a prominent issue in laboratory based bumblebee studies.

3.5.1. Electric vehicles

It is vital to consider the future of the transport sector when discussing conservation measures involving any form of pollution associated with roads. The sales and use of electric vehicles (EVs) have increased significantly over the past 20 years, and by 2035 it is projected that more than half of all new vehicle sales will be electric (BNEF, 2022). Alongside this, the cost of production has decreased, with the price of lithium-ion cells falling by 97% since 1991 (Ziegler and Trancik, 2021). The rapid uptake of EVs in an effort to decarbonise the automotive industry will have overwhelmingly positive impacts on pollution associated with exhaust emissions, however the outcome on metal pollution in verges remains uncertain. Non-exhaust emissions (i.e. road surface abrasion, brake wear etc.) account for between 85-90% of particulate matter (PM) pollution from traffic. Metals (including copper and cadmium) are classified as PM, and their presence on roadsides is highly correlated to non-exhaust emissions. Both cadmium and copper are present in the tread of tires, and it is estimated that a new car tire will lose 10-20% of its rubber mass within 3 years due to road surface abrasion (Adamiec et al., 2016). Cadmium in particular is used in the brake coatings of vehicles, and the friction generated during the braking process causes Cd particles to be released into the environment (Talebzadeh et al., 2021). Due to the battery, EVs are approximately 24% heavier than their combustion engine counterparts, and this increase in weight will result in an increase in non-exhaust emissions as greater pressure is exerted between tires and road surface, as well as on brakes and steering mechanisms (Timmers and Achten, 2016). It is also projected that as they become more commonplace, the weight of EVs will continue to increase as they accommodate larger batteries and frames to power longer journeys (Shaffer et al., 2021). A combination of more, and heavier, vehicles on the road means that metal contamination on roadsides is likely to become more pronounced. There are several suggestions on how best to address the issue of EV weight (including a weight tax and use of lighter materials), but due to costs these have yet to be implemented. It is highly likely that the typical levels of copper and cadmium currently found in roadsides will increase with the transition to EVs. Therefore, in light of our results that Cd at levels slightly higher than those currently typically found in road verges had significant negative impacts on the number of dead offspring, it is essential to continue monitoring the effects that this rapidly evolving industry has on metal pollution in roadsides.

3.6. CONCLUSION

Overall, our results show that the presence of cadmium or copper at roadside levels in foraging rewards has little to no impact on the ability of *Bombus terrestris* queen-less micro-colonies to produce male offspring. Our research supports previous work showing that at current levels, bees are unlikely to experience any short-term negative effects of metal pollution. There remain significant research gaps, such as the interacting effects of metal exposure when combined with parasites and alternate exposure routes. Given the potential we have shown for slightly higher levels of cadmium than currently measured at roadsides to have a small negative impact on *B. terrestris* reproduction, we recommend that, where not already in place, authorities at both local and national levels have programs to periodically monitor roadside soils and vegetation for metal pollutants. The transition to EVs is likely to lead to elevated levels of metal contamination in road verges, and so this process is vital to assess the continued suitability of roadsides as viable habitat for bumblebees.

CHAPTER 4: Late-season pollen collection by *Bombus terrestris* colonies is not affected by proximity to a major road.

4.1. ABSTRACT

1. Roadside verges are a ubiquitous feature of anthropogenic landscapes, and their potential as a valuable resource is a current topic of interest in bee conservation. Verges have been proposed as an important resource for bumblebees, particularly in intensively farmed landscapes where flowers may be lacking.

2. Here, we investigated the floral preferences of *Bombus terrestris* colonies positioned on a roadside compared to those located at least 1 km away from the verge with respect to pollen collection in an agricultural landscape. Landscape composition surrounding each colony was classified and floral units in each habitat recorded over an 8-week period. Pollen loads were collected from foragers reared in colonies from wild-caught *B. terrestris* queens to determine the foraging preferences of bees.

3. Road verges were the most florally diverse habitat type surveyed in our landscape, and this remained consistent over the duration of the study (H= 2.12-2.4). We found no significant effect of distance from road on the pollen diet of bumblebee colonies used in this study. Foragers from both treatments exhibited similar preferences, with *Phacelia tanacetifolia* constituting a major component of colony diet (>29%).

4. Several plant taxa (including *Phacelia*) were visited more or less frequently than predicted by our null model, however the majority of flowers present in corbicular loads were recorded as expected by their relative abundance in the field.

5. Whilst not comprising the major taxa of the diet, we found that plant communities associated with verge habitat diversified the pollen content of samples (18-47%), and are therefore still a valuable resource in an otherwise florally poor landscape.

6. We have demonstrated that when managed appropriately, roadside habitat is one of the most florally diverse habitats in agricultural landscapes. In line with previous research, we show the significance of mass-flowering cover crop in bumblebee foraging preferences. We have also highlighted the importance of road verges as a pollen resource in agricultural

landscapes, as a wider breadth of pollen provides a greater variety of nutrients for developing colonies. Our findings support a role for roadside habitat in bumblebee conservation.

4.2. INTRODUCTION

Pollinating insects are globally important both ecologically and economically, however evidence shows they are exhibiting declines (Goulson et al., 2015; Potts et al., 2010; Potts et al., 2016a). Declining pollinator populations are of global concern, and evidence has shown that increasing agricultural intensification leading to a loss of semi-natural habitat is the most important and well-established reason for declines (Dicks et al., 2021; Kennedy et al., 2013; Potts et al., 2016b). Several widely adopted changes in farming practices, including increased weed control, the loss of leguminous cover crops, and the shift to large monocultures, have resulted in landscapes with lesser quantities of floral resources present (Brown and Schulte, 2011; Hicks et al., 2016; Rasmont and Iserbyt, 2013). As a consequence, multiple pollinator species have experienced negative effects, including bumblebee populations (Bommarco et al., 2012; Scheper et al., 2014; Timberlake et al., 2019; Timberlake et al., 2020).

Road verges have been proposed as a key habitat for bumblebees and other pollinators, particularly in agricultural landscapes (Cole et al., 2017; Gardiner et al., 2018; Phillips et al., 2020a). Road verges are areas of land typically managed by councils or highway authorities that separate the road from neighbouring land. Their main purpose is to aid driver visibility; however, they could be a valuable resource for pollinators if managed in a way that promotes floral diversity. In the UK, it is estimated that verge habitat produces 60kg nectar/hectare/year, compared to arable farmland which only produces 6kg/hectare/year, and so it is likely that verges constitute an important part of pollinator diet in arable landscapes (Baude et al., 2016). Several studies have investigated how bumblebees and other pollinators use resources on road verges, recording foraging individuals present on the roadside (Hanley and Wilkins, 2015; Heneberg et al., 2017).

Roadsides cover approximately 270,000km^2 (0.2%) of land worldwide, 2500km^2 of which is in the UK, and a crucial feature of verge habitat is connectivity, which is an important factor to consider in conservation strategies (Phillips et al., 2020b, Phillips et al., 2021a). Roadsides form a network of connected habitat across a landscape, which has been shown to facilitate movement of individuals across the landscape (Dániel-Ferreira et al., 2022; Valtonen and Saarinen, 2005). Despite this, there are several negative impacts associated with road verges such as collisions with vehicles (Baxter-Gilbert et al., 2015), increased exposure to pollutants (Phillips et al., 2021b), and a barrier effect (Anderson et al., 2017) that could limit their potential as suitable habitat for bees and other pollinators. Understanding the preferences and behaviour of bumblebees in agricultural landscapes interspersed with verge habitat is important for determining the value of road verges as a conservation tool for bees.

Bumblebees, *Bombus* spp., are central place foragers, gathering floral resources from the landscape and returning them to the colony. Different bumblebee species are able to find suitable forage at different distances from the nest, with the mean forage distance for five UK *Bombus* spp. ranging from 273m – 551m (Redhead et al., 2015). Foraging range varies according to floral resource availability (Hardman et al., 2016), and *Bombus terrestris* have been shown to fly over 1km for good quality resources (Osborne et al., 2008), although their typical foraging range is also within 500m of the nest (Wolf and Moritz, 2008). With pollen, bees accumulate grains as they visit flowers and then gather them in corbicular loads on their hind legs for transport back to the colony. Pollen is a colonies' protein source, and it is vital for larval development as it also contains several important vitamins, minerals, and lipids (Roulston and Cane, 2000). The nutritional quality of pollen is highly variable, ranging from 2-61% protein content dependent on species (Roulston et al., 2000). Bumblebees are generalists, collecting pollen and nectar from a wide variety of plant species, and a more diverse collection of pollen has been shown to support overall greater colony fitness (Hass et al., 2018; Schweiger et al., 2022), most likely by increasing the probability of a nutrient balanced diet (Moerman et al., 2017). Higher quality pollen (with a higher percentage protein content) results in a greater growth rate of colonies compared to low quality pollen diets (Moerman et al., 2016), and as bees are able to determine pollen quality within the landscape, this is likely to impact their foraging preferences and behaviour (Ruedenauer et al., 2016).

There are several studies investigating how bumblebees use pollen floral resources within the landscape (Hass et al., 2018; Kämper et al., 2016; Leonhardt and Blüthgen, 2012; Piko et al., 2021; Potter et al., 2019; Schweiger et al., 2022). Leonhardt and Blüthgen (2012) found that bumblebees (*B. terrestris* and *B. pascourum*) not only collected pollen from twice as many plant taxa as honeybees, but also selected pollen from the landscape with a higher protein content. A study in Germany investigated species present in the pollen stores of 30 *B. terrestris* colonies and found that the pollen community between colonies was largely similar despite differences in the landscape composition within a 500m radius of individual colonies (Kämper et al., 2016). They also found that colonies collected 80% of pollen from woody plants as opposed to herbaceous plants. The authors emphasise that this result is likely due to timing (data was collected from early May-June) and may not translate across the entire season. Identification of bee pollen loads sampled from foraging individuals along sown wildflower strips revealed that *Phacelia*, *Chrysanthemum*, and *Papaver* were important pollen resources for *B. terrestris* in July (Potter et al., 2019). As the majority of corbicular samples contained plant species not present in the flower strips, the results highlight the ability of pollen identification to detect foraging interactions on a larger spatial scale than observation alone. Piko et al., (2021) showed similar results, with less than half of all pollen samples collected from bumblebees foraging on strips containing sown wildflower plant species. A study in California found that the presence of more natural habitat at the landscape scale did not influence the pollen richness of corbicular loads of *Bombus vosnesenskii* foragers (Jha et al., 2013). Pollen loads of individual foragers generally contain between one and two species, and this is also reflected at the colony level, with foraging efforts focused on one or two major species, however bumblebee diet is then also supplemented from several minor taxa (Kleijn and Raemakers, 2008).

Landscape scale studies on *Bombus* spp. predominantly use commercially reared colonies (Benner et al., 2023; Klatt et al., 2020; Rundlöf et al., 2015; Whitehorn et al., 2012). Colonies of *Bombus terrestris* and *Bombus impatiens*, the most common UK and US species respectively, are available to purchase from suppliers primarily for supplementary pollination services for growers, however they are also prevalent in bee research (Scott et al., 2022; Sivikoff et al., 2020). The use of commercial colonies, although commonplace, does not allow for a thorough assessment of bumblebee colony performance and behaviour in a real-world setting for three primary reasons: 1) the history of the colony is unknown prior to its obtainment, 2) local adaptations to factors such as climate and parasites are not accounted for (Jackson et al., 2020; Kovačić et al., 2020), and 3) wild and commercial bumblebees may experience different selection pressures leading to differences in morphology and/or behaviour of individuals (Kardum Hjort et al., 2022). To avoid these issues, colonies can be reared from wild-caught queen bumblebees. Nest-searching queens can be caught in early spring and set up in a controlled environment facility for rearing in the laboratory. This approach is becoming increasingly popular in bumblebee research as it produces results which are more ecologically realistic and applicable than those generated from commercial colonies (Kaushal et al., 2022; Samuelson et al., 2018; Siviter et al., 2018).

The floral preferences of bumblebees within a landscape can be studied observationally by recording foraging individuals on plants in the field, however observations may be biased towards plant species where bees are more easily observed, for example it is easier to record active foragers on grassland flowers than those in tree-canopies (Allen and Davies, 2023). The supply and demand of nectar resources in the landscape has been widely studied, with seasonal gaps in nectar identified in late summer (Baude et al., 2016; Timberlake et al., 2019; Timberlake et al., 2021a). Pollen preferences can be studied through taxonomic identification of pollen grains collected by bees. Pollen can be identified primarily in two ways; (1) using light microscopy, and (2) through DNA sequencing (metabarcoding and whole genome sequencing). Both methods have several advantages and disadvantages, for instance, microscope identification often has lower taxonomic resolution, but greater certainty about the relative abundance of pollen types that can be distinguished (Carneiro de Melo Moura et al., 2022; Bell et al., 2017; Hawkins et al., 2015; Umber et al., 2022).

4.2.1. Research questions and hypothesis

Here, we use a combination of floral transects and morphological identification of pollen samples to determine what species are present in the landscape, and to what extent *Bombus terrestris* foragers use them as a pollen resource, during a critical foraging period from mid to late summer. As our site is predominately agricultural, we predict that road verge habitat will be one of, if not the most, florally diverse habitat type. We also expect certain plant species to be more strongly associated with particular habitat classifications, for example woodland plants will be recorded predominately in woodland habitat. We anticipate that bumblebee colonies positioned on verges and those located in the surrounding area will be using plants associated with verge habitat at a higher rate than taxa from other habitat types. Our study specifically addresses the following research questions:

1. Is the road verge plant community more or less florally diverse/abundant than other habitats surveyed in our study landscape?
2. Are particular plant species more or less strongly associated with verge habitat than other habitat types?
3. Does the pollen community collected by *Bombus terrestris* colonies differ between colonies positioned on a large trunk road and those located away from the verge?

4.3. METHODS

4.3.1. Study sites

Data collection was carried out on the Elveden Estate in Norfolk (UK grid reference: 52.387325, 0.676124). Elveden is a commercial farm producing both cereal and vegetable crops in rotation. The farm covers approximately 22,500 acres of Breckland habitat, with sandy, nutrient deficient soils. The road of focus for this study was the A11; a 4-lane, 70mph road with an annual daily traffic volume of 25,582 vehicles (Department of Transport, 2022). 16 sites were selected on the basis of distance from the edge of the A11 and accessibility/safety (**Figure 4.1**). Eight were positioned as close to the edge of the road as possible (within 5m), and the other eight were positioned away from the road edge (>1km); these colonies are hereafter referred to as ‘road’ and ‘control’ respectively (for exact locations see **Appendix 2**). All colonies were also at least 1km apart.

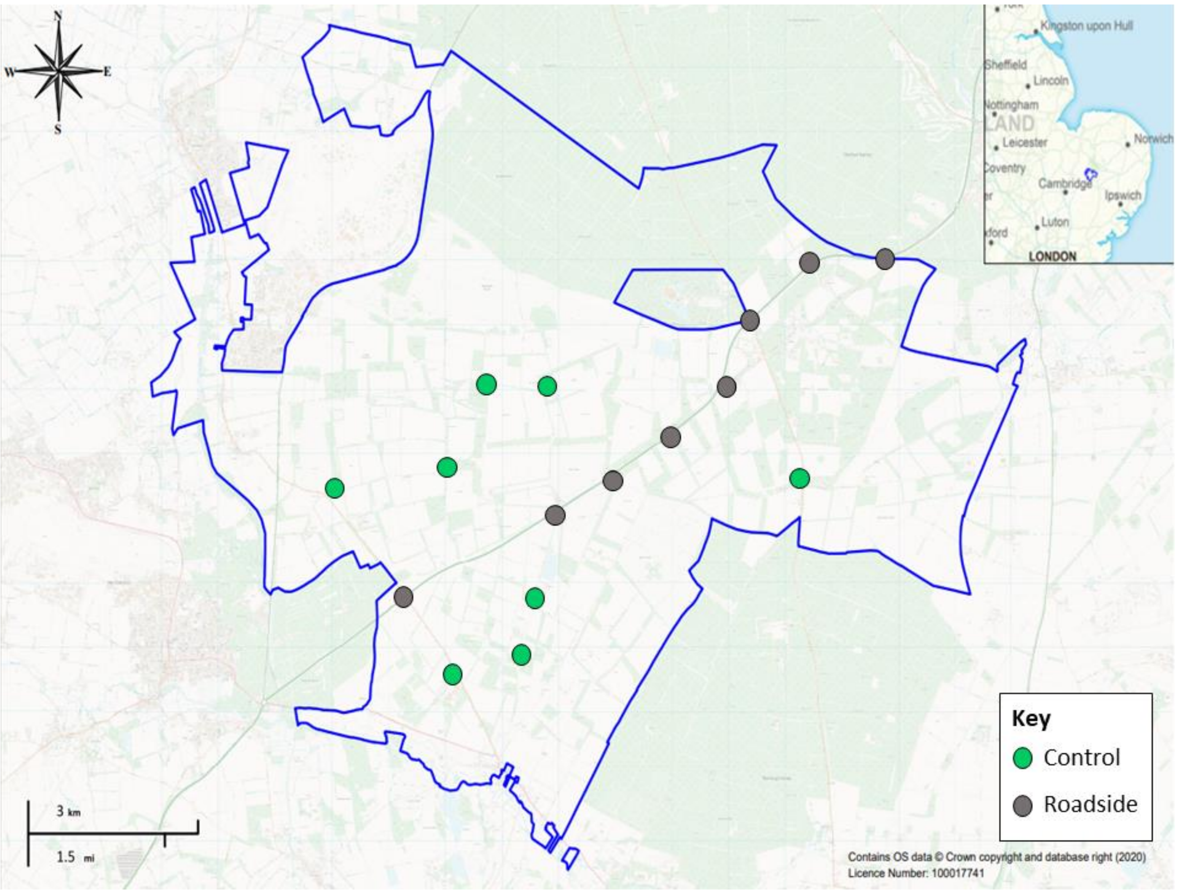


Figure 4.1: A map of the Elveden estate. The area within the blue boundary is land owned by the estate, and the cutout within the border is owned by Center Parcs. The 16 points show the location of the 16 colonies used in the study (green points are control colonies and grey points roadside colonies). The top right inset shows the location of the Estate in the East of England.

Floral transects and pollen sampling took place bi-weekly over an 8-week period from July to September 2021. There were a total of four sampling windows: 12th July –25th July, 26th July – 8th August, 9th August – 22nd August, 23rd August – 5th September. As pollen sampling was weather dependent, floral surveys could be carried out either before or after pollen collection, but both were always completed within a 4-day period.

4.3.2. Queen rearing and colony set-up

All 16 colonies used in this study were reared from wild caught *Bombus terrestris* queens following methods by Siviter et al., (2018). Queens were captured while nest-searching during March and April 2021 on mild, sunny days between 9am and 2pm at a local nature reserve in Cambridge, UK. Individual bees were caught using butterfly nets and transferred into labelled collection tubes. They were then stored on ice in a cool box before being transported back to the lab.

All queens were first screened for parasites (see **Appendix 1**) and then set up in a controlled temperature facility set to 26^0C and 50-60% relative humidity. Bees with parasites were reared in a different room to prevent cross-contamination, although enough colonies were reared from non-parasitised individuals for all colonies used in the study to be parasite free upon placement. Queens were reared in clear perspex boxes (14cm x 8cm x 6cm) with a removable perforated plastic base and supplied with glucose syrup *ad libitum* (British Bee Feeds, UK) through a gravity feeder. Pollen (Agralan, UK) was supplied via pollen patties where the queen could begin laying eggs and subsequently incubate her brood. Rearing boxes were raised slightly from the workbench with wooden planks to allow ventilation. Nectar was refreshed every three days and pollen every seven days to prevent outbreaks of wax moths. Queens were supplied with supplementary pollen pellets on a petri dish once they had begun founding nests, and this was refreshed every three days.

After ten workers had eclosed, the colony was moved into a wooden nest box (30cm x 20cm x 15cm) with a transparent plastic lid. One end of the box contained a mesh covered ventilation hole, and the other the nest entrance hole. Another hole was present in the base of the box which enabled bees to access nectar solution via a tank below the colony. Bees were allowed to settle into their new box for seven days before placement in the field. Once in the landscape, wooden nest boxes were placed into ventilated plastic boxes to protect them from rain. The outer plastic boxes were held in place with ground pins and a ratchet strap was secured around the box for protection from badgers. Access to the supplementary nectar supply was closed off after seven days in the field.

4.3.3. Floral sampling

Habitat types located within a 500m radius of each of the 16 colonies were mapped using QGIS v3.2 (QGIS, 2022; **Figure 4.2**). These habitat types were classified using a combination of Google satellite imagery, maps provided by the estate, and ground-truth surveys.

Figure 4.2: An aerial map of the Elveden estate farm. That habitat types within a 500m radius of each colony are classified by colour as indicated by the key on the right-hand side. Control colonies are shown with a green outline, and road colonies are indicated by a grey outline. The marked road is the A11 and the scale bar is provided for reference.

There were 21 different habitat types, including: road verges, field margins, arable crops, and pasture (see **Appendix 7** for full list of habitats surveyed). Here, road verges are classified into two distinct groups; those located along the edge of the A11 (‘VergeA11’), and all other verges in the landscape (‘VergeOther’). 18 of these habitat types were surveyed (developed areas, roads, and water bodies were not sampled) relative to their area within the landscape surrounding each colony i.e. if 50% of habitat cover within the 500m radius of a colony was woodland, then 250m of the transect would be conducted in woodland habitat (full percentage cover for each colony can be found in **Appendix 8**). This was carried out at colony level, with transects tailored to each colony to account for differences in habitat composition between colonies. All hedgerows surveyed were hawthorn (*Crataegus monogyna)* or blackthorn (*Prunus spinosa*), and therefore not flowering at the time of data collection, although visits were still made at each sampling period and flowers within their edges recorded.

Transect starting locations and direction were randomly assigned in QGIS. For safety reasons, road verge transects were always run parallel to the road. To avoid disturbing crops, transects in crop habitats were run along field borders, with quadrats always placed on the inside of the field. 1m wide transects equating to a length of 500m were run in each of the buffers surrounding the colonies, with a 1x1m^2 quadrat being placed every 5m for a total of 100 quadrats per colony. The flowering plant species were identified to species level using floral identification keys (Rose, 2006; Stace, 2010) and floral units in each quadrat counted following Baude et al., (2016). These were averaged to obtain a unit value per m^2, which was then multiplied by habitat area for a landscape estimate.

4.3.4. Pollen collection

Pollen sampling was carried out for each of the 16 colonies between 9am and 5pm when there was no rain and temperatures were above 14^{0}C. The entrance to the colony was closed for up to one hour, and returning foragers were intercepted. Workers were captured in collection tubes before being transferred to a honeybee queen marking tube, where both corbicula loads were removed using a mounted needle and transferred into a pre-labelled Eppendorf tube. Both needle and marking tube were cleaned of pollen and sterilised with alcohol wipes between individuals. The entrance to the colony was opened again once 12 workers had been sampled or an hour had passed. Using this approach ensured no bees were sampled more than once during a single sampling period and also minimised colony disturbance. Samples were stored on ice in a cooler on site and then transferred to a -20^{0}C freezer within 10 hours where they were stored until identification.

4.3.5. Pollen preparation and identification

Following methods by Smart et al., (2017), 75µl of glycerine and 10µl of Calberla's stain were added to the corbicular sample in the Eppendorf tube. The sample was pipetted up and down to suspend the pollen and then 20µl was placed onto a labelled microscope slide and topped with a coverslip. The sample was left for at least 20 minutes before visualisation to allow the pollen to absorb the stain. Samples were then viewed at 100x and 400x magnification under a compound light microscope, and 100 pollen grains were counted and identified from their morphology, to the highest taxonomic resolution possible, with the aid of reference samples, guidebooks and online photographs (PalDat, 2023; PollenWiki, 2023; Sawyer and Pickard, 1981). This process was repeated three times for a total of 300 grains counted per pollen load. In some cases, it was only possible to identify pollen grains to genus or family level, rather than to species (Holt and Bennett, 2014).

4.3.6. Statistical analysis

All statistical analyses were carried out in R (version 3.6.2; R Core Team, 2022) using the packages mglm (Kim et al., 2018), mvabund (Wang et al., 2012), vegan (Oksanen et al., 2013), muMIN (Barton, 2020), and econullnetr (Vaughan et al., 2018) with all data visualised using the ggplot2 package (Wickham, 2016). Prior to analysis, all data were checked for normality and over dispersion, with transformations performed when model assumptions were violated.

To check floral sampling completeness, we first visualised species accumulation curves for each habitat type. Then, to assess the differences in floral diversity between habitat types we calculated the Shannon-wiener diversity index for each habitat type across the study period and then compared the diversity using a one-way ANOVA and post-hoc Tukey’s tests. To determine the associations between plant taxa and habitat types in the landscape, we ran chi-squared tests of independence for each survey period. The expected values of plant species were calculated by assuming an even distribution across habitat types, according to the overall abundance of each species recorded.

Multivariate generalised linear models (MvGLMs) were used to investigate the effect of sampling round and treatment on pollen abundance and diversity. To account for any large variation in the abundance of each taxa within samples, the proportion of species within pollen loads were calculated using total number of pollen grains present as opposed to just presence or absence. Model-based community analyses (like MvGLMs) are being more frequently used over distance-based methods (e.g. PCoA) due to their increased statistical power (Jones et al., 2022; Jupke and Schäfer, 2020; Warton et al., 2012). Using the ‘manyglm’ function, we tested the effect of sampling round and treatment on pollen composition with a block design to also include colony as a variable in the model.

A null modelling approach was used to determine whether foragers from both treatments collected pollen from plant taxa more or less than would be expected by chance relative to their availability in the local surrounding landscape. Null models are a popular method to determine species preferences as they account for both sample incompleteness and differences in resource abundance (Blüthgen et al., 2008; Cecala and Wilson Rankin, 2021; Davies et al., 2022; Ollivier et al., 2020). Bipartite interactions were modelled to predict whether they were significantly different from the null model where foragers collect pollen in proportion to plant species' abundance in the landscape. Individual sampling periods were tested independently to account for seasonal changes. To assess whether there were differences in pollen collection between road and control colonies we used two sampled t-tests with composition of pollen as the response variable and, again, separate analyses were run for each time period to account for changes across sampling rounds. We also calculated Bray-Curtis dissimilarity matrices for each colony using the proportion of plant taxa present in corbicular loads and plotted the beta diversity using non-metric multidimensional scaling (NMDS).

4.4. RESULTS

Across the 8-week study period, 64 x 500m transects were run, and a total of 61 flowering plant species were recorded. Across the 18 surveyed habitat types, the number of floral species present ranged from 0 to 23 with the most prevalent species' occurring across 6 of the habitat types. Floral sampling completeness ranged from 85 – 100% across the 18 habitat types. The total number of floral units recorded within the landscape buffer surrounding each colony ranged from 328 to 11,892.

4.4.1. Landscape variation

There was a significant difference in floral diversity between the habitat types surveyed (ANOVA $F_{(17,72)} = 1.021$, p=0.02). The most diverse habitat type surveyed was 'VergeA11', followed by 'VergeOther' (**Table 4.1**). The least diverse habitat types were all crop ('Onion', 'Potato', 'Rye', 'Spring Barley'), with no flowering plant species recorded at all.

Table 4.1: The 18 habitat types surveyed across all 16 colonies with their corresponding floral richness, floral evenness, and diversity index. The values were calculated from data collected across the entire 8-week sampling period. The number of quadrats surveyed in each habitat type across all 16 colonies is also shown. The two road verge habitat types ('VergeA11' and 'VergeOther') are highlighted in bold.

Habitat Type	No. of Quadrats	Floral Richness	Floral Evenness	Shannon Diversity Index (*H'*)
Carrot	60	2	0.496	0.344
Cover Crop	38	3	0.395	0.434
Field Margin	20	7	0.816	1.590
Grassland	122	12	0.798	1.980
Hedge Plantation	18	2	0.796	0.552
Hedgerow	6	2	0.204	0.141
Onion	122	0	0.000	0.000
Parsnip	38	1	-	-
Pasture	110	4	0.429	0.595
Potato	184	0	0.000	0.000
Rye	89	0	0.000	0.000
Scrubland	34	7	0.554	1.080
Spring Barley	140	0	0.000	0.000
Verge A11	**41**	**23**	**0.766**	**2.400**
Verge Other	**27**	**17**	**0.803**	**2.120**
Winter Barley	124	1	-	-
Winter Wheat	31	3	0.210	0.231
Woodland	396	6	0.713	1.280

4.4.2. Species associations

We found that plant species were not evenly distributed among habitat types (X^2 (18, N= 172)= 6.9, *p*= 0.03; **Figure 4.3**). Certain species were only found in particular habitat types, for example *Phacelia tanacetifolia* was only surveyed within the 'CoverCrop' habitat.

Similarly, *Echium vulgare* (viper's bugloss) was most strongly associated with 'VergeA11' habitat (occurring in 70.45% of quadrats), and *Erica* spp. (heathers) with 'Scrubland' (90.91%). While recorded in three habitat types, *Rhinanthus minor* (yellow rattle) was most commonly surveyed in 'VergeA11' also (46%). There were, however, several other species which were found across multiple habitat types; for example, *Taraxacum officinale* (dandelion) was surveyed in a third of all quantified habitats. Several plant taxa, including *Trifolium repens* (white clover) and *Papaver rhoeas* (field poppy) were fairly evenly distributed between grassland habitats, but predominantly found in verges.

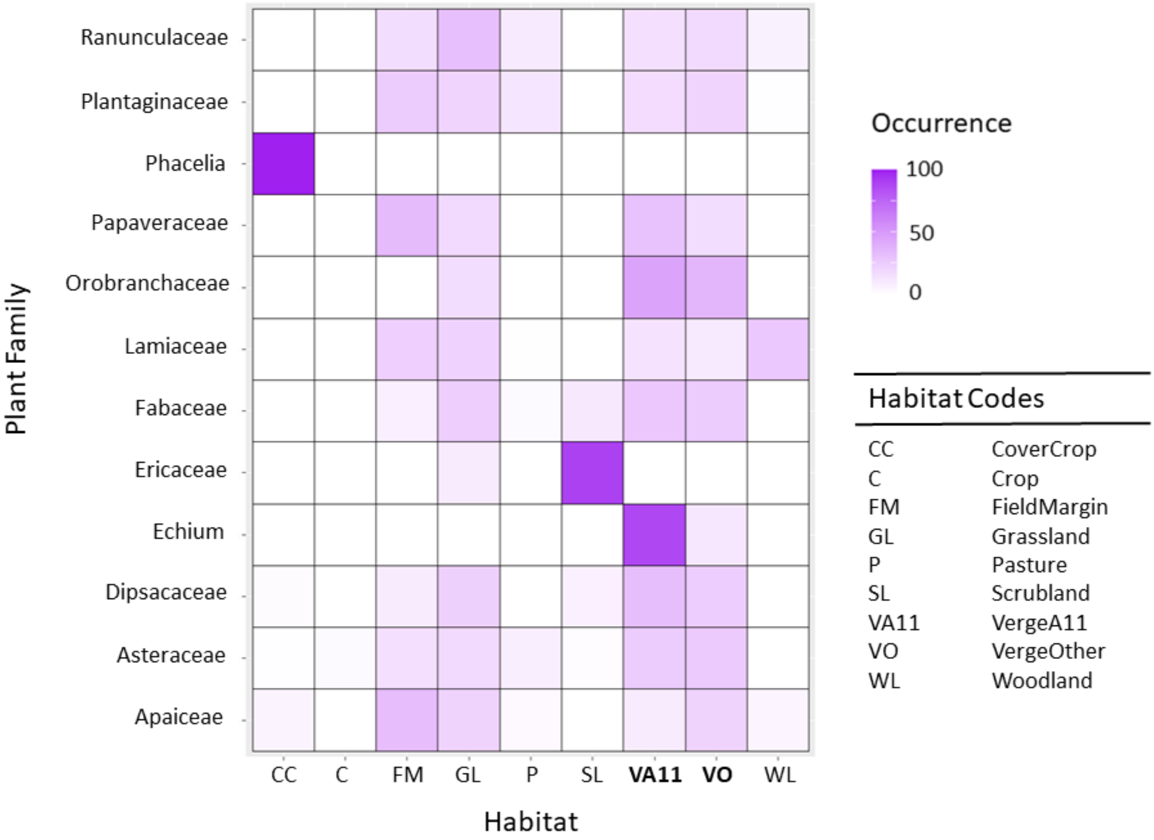


Figure 4.3: The interactions between habitat type and major plant families. The colour scale represents the number of quadrats a plant taxa was recorded in in the corresponding habitat type. Darker shades indicate more frequent occurrence of a plant species, and lighter shades less frequent occurrence. *Phacelia* and *Echium* are shown separately due to their specific associations in the landscape. All crop habitats are combined into one variable, 'Crop', and plant species found in <5% of surveys are not included.

4.4.3. Pollen foraging

575 pollen samples were obtained from returning foragers; 281 taken from road colonies and 294 from control colonies. As is to be expected, the rate of collection decreased towards the end of the season, with an average of 4.125 (± 0.11) pollen samples per colony obtained in the final week, compared to 10.19 (± 0.14) in the first week. Pollen samples collected by bees were taxonomically classified via microscopy into 26 different species, 17 genera, and 9 families. The average number of taxa per sample was 1.29 (± 0.67). Examples of common species surveyed in corbicular loads collected by bumblebees in this study are shown in **Appendix 9**.

Neither sampling round ($LR_{6,521}$=213 *p*= 0.17) nor treatment ($LR_{3,562}$=189 *p*= 0.31) were significant predictors of corbicular sample composition. While there was a significant difference in pollen foraging between individual colonies (PerMANOVA: $F_{2,15}$ = 1.98, *p*= 0.01; **Figure 4.5**), this did not extend to treatment, with workers from road and control colonies showing no significant differences from each other in terms of pollen collected. This was true of all four sampling occasions, with foragers from both treatments collecting similar pollen taxa at each time period (t-test: sampling round 1: t(14)=2.17, *p*= 0.33; sampling round 2: t(13)=1.76, *p*=0.21; sampling round 3: t(14)=1.44, *p*=0.18; sampling round 4: t(11)=2.05, *p*=0.14; **Figure 4.4**).

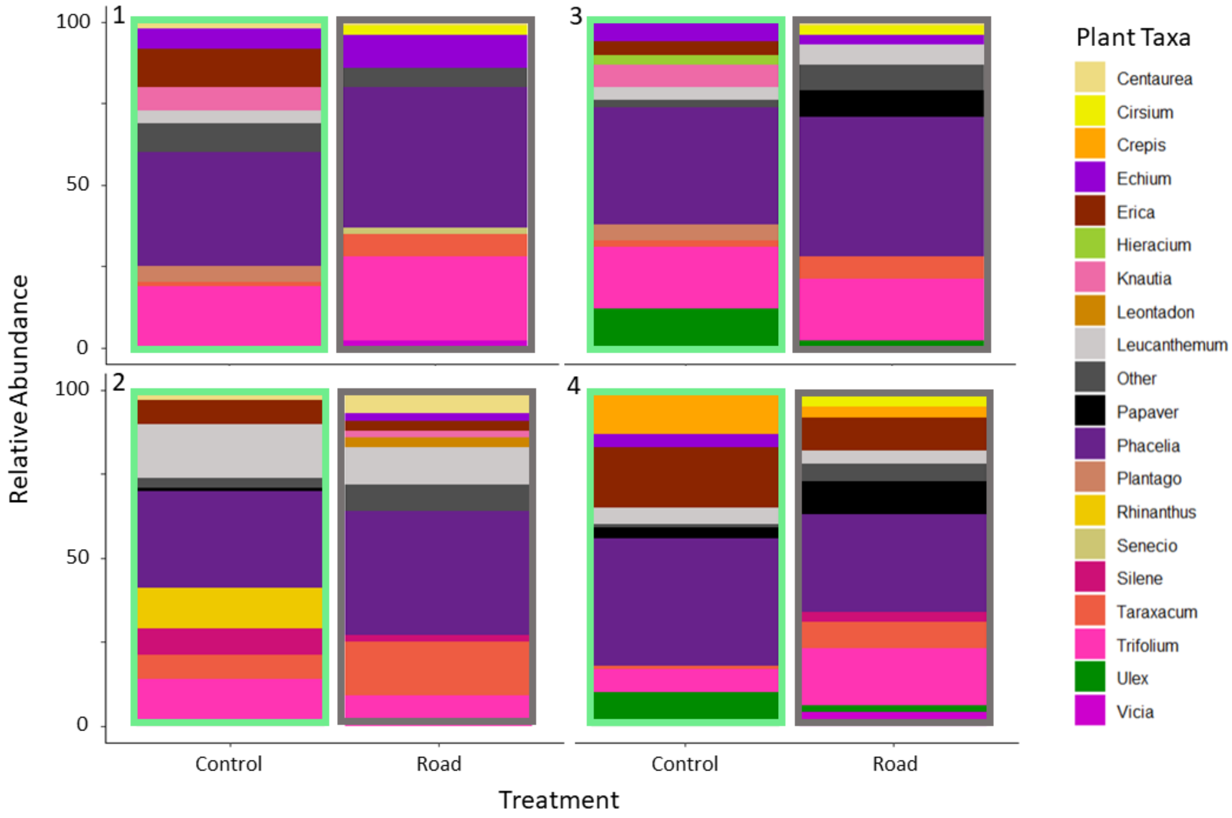


Figure 4.4: The relative proportion of plant genera present in pollen samples collected by bees. The four plots signify the four biweekly sampling occasions: 1 from mid to late-July, 2 from late-July to early-August, 3 from early to late-August, and 4 from late-August to early-September. Bars represent the treatment; those outlined in green are pollen samples from the control colonies, and grey are the roadside colonies. ‘Other’ incorporates taxa that were present at <5% abundance at colony level.

The average species richness of pollen in colonies per sampling round was 7.92 (± 0.91). Regardless of time sampled, *Phacelia* was the dominant flower species present in 195 corbicular loads across all colonies. This was followed by the Fabaceae family, which comprised of six identifiable taxonomic groups (*Trifolium repens*, *Trifolium pratense*, *Trifolium* spp. *Ulex* spp, *Vicia* spp., and *Lotus* spp.), found in 114 pollen loads (19.8%). There were no distinct groupings by treatment in the NMDS ordination of pollen dissimilarities (**Figure 4.5**).

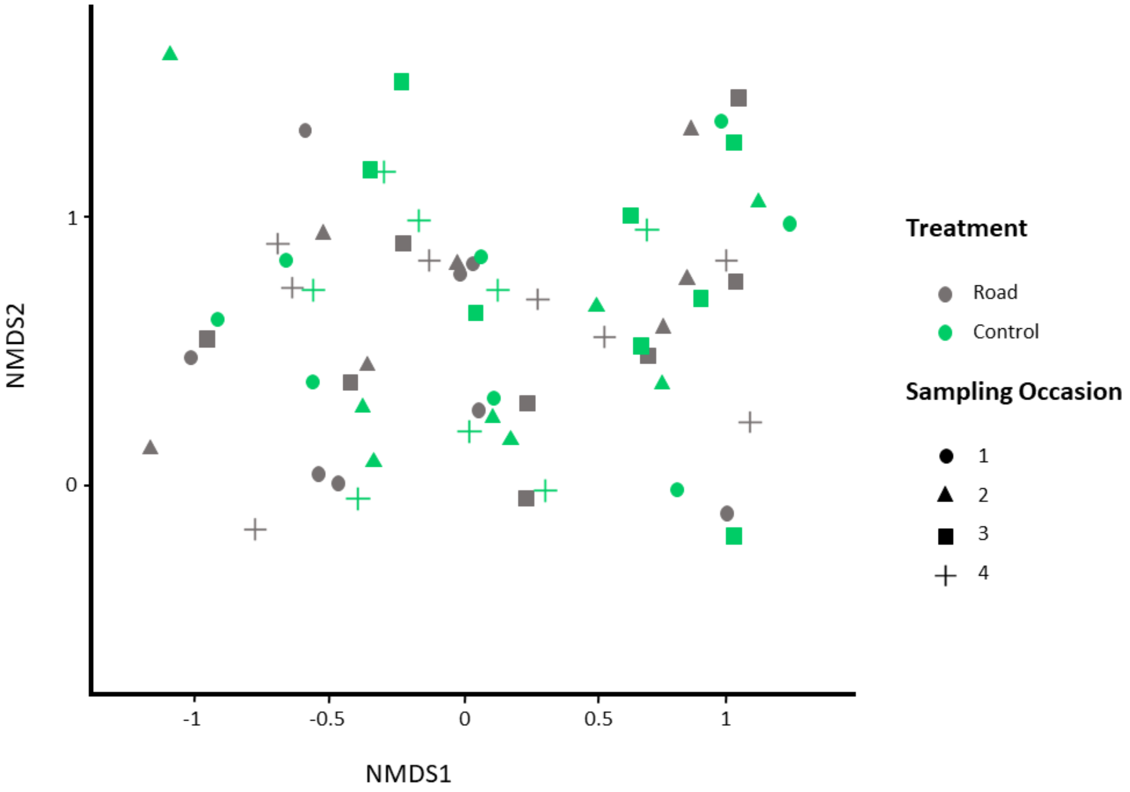


Figure 4.5: NMDS plot illustrating the diversity of corbicular loads collected by bees over the 8-week sampling period. Bumblebee colonies are grouped by sampling occasion and treatment (road or control). Stress = 0.138.

When compared to the abundance of floral resources available in the landscape, bumblebees collected proportions of the majority of plant taxa (46 taxa) that would be expected by chance. For a few plant taxa (n = 5), bees collected either more or less than predicted by the null model (**Figure 4.6**). Across all sampling occasions, bees consistently collected from *Phacelia* at a slightly higher rate than expected. Foragers also collected from *Erica* spp. and *Ulex* spp. at a greater frequency than predicted by the null model. Relative to their abundance in the landscape, *Taraxacum* spp. and *Vicia* spp. were visited less frequently than would be expected by chance. The remaining 14 groups were present in pollen samples in proportion to their abundance in the landscape as predicted by the null model.

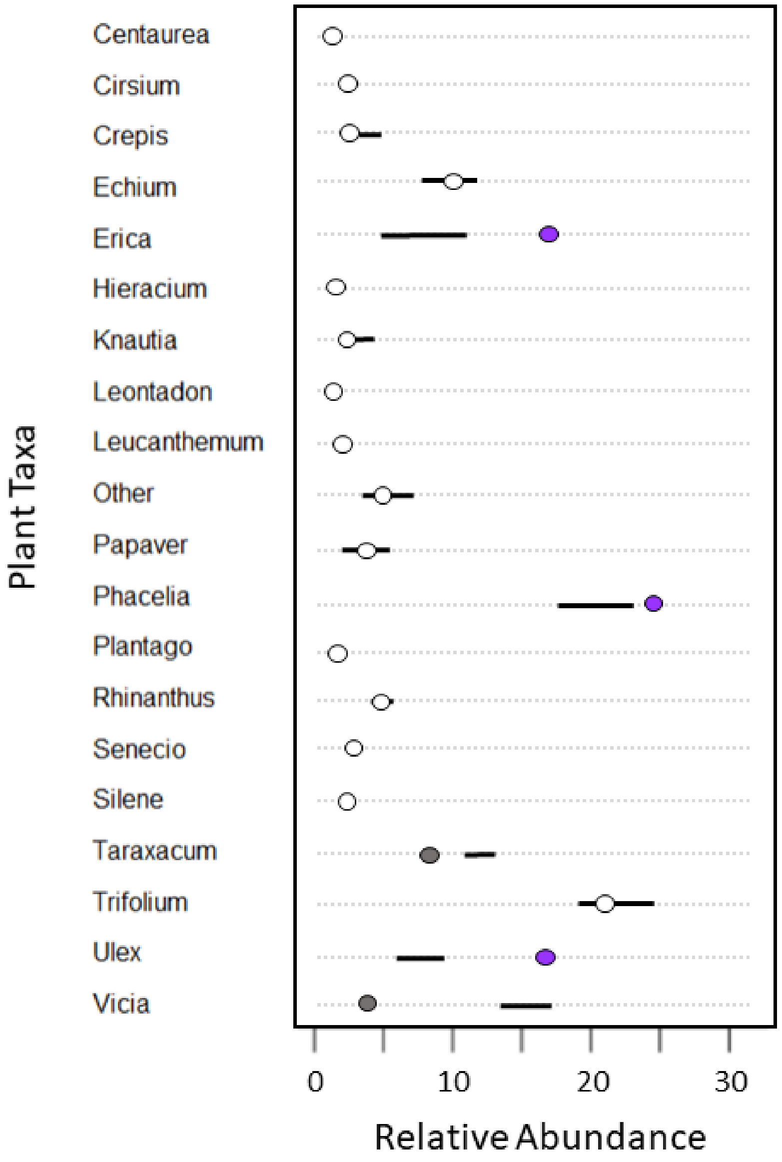


Figure 4.6: A comparison between observed and expected relative abundances of plant taxa in pollen samples, calculated using a null modelling approach. 19 of the most commonly observed taxa are shown, and the category 'Other' represents plant taxa which were present in samples at <5%. The horizontal lines represent the 95% confidence limits for the expected relative abundance of pollen grains of a particular plant taxon, according to the relative abundance of flowers in the landscape. Circles show the observed proportion of the same plant taxon in bumblebee-collected corbicular samples; grey circles indicate lower than expected frequency, white is as expected, and purple higher than would be expected.

4.5. DISCUSSION

Our study revealed that road verge habitat along the A11 (‘VergeA11’) was significantly more diverse than all other habitat types surveyed in this study. This was, perhaps unsurprisingly, followed by ‘VergeOther’, and both classifications had a diversity index over 2. The A11 verge is managed by National Highways, a government owned corporation responsible for all motorways and major (A-class) roads in England (National Highways, 2023). Grass cutting along the A11 is provided by Chevron Green Services, who, between 2019 and 2022 were trailing a ‘cut and collect’ approach along the A11 verge. Verges were cut in mid-September each year and the cuttings were bailed and removed by an automated robot. It is well-documented in the literature surrounding roadside management that collection of cuttings promotes biodiversity, as it both removes nutrients from the verge enabling wildflowers to thrive and allows space for seeds to germinate (Bonanomi et al., 2006; Parr and Way, 1988). It has also been shown that cutting later in the season has positive impacts on floral abundance and diversity as plants are able to set seed before cutting (Humbert et al., 2012). Alongside this, yellow rattle (*R. minor*), was predominately recorded in A11 verge habitat (46%). The presence of yellow rattle is known to promote floral diversity, as it is a root hemi-parasite of dominant grasses and thus inhibits their growth, creating space for wildflowers to grow (Bullock and Pywell, 2005). While we had no control over management in this study, a combination of cutting removal, a later cut, and the presence of yellow rattle could provide an explanation as to why this stretch of verge is so diverse. Further work would be necessary to determine if this was the case.

We found that particular plant species were more strongly associated with particular habitat types, for example *Phacelia tanacetifolia*, purple tansy, was only ever recorded in ‘CoverCrop’. Similarly, we can conclude that pollen from *Erica* spp. and *Ulex* spp. most likely came from scrubland, and *Echium* spp. pollen from the A11 verge. While we cannot rule out the possibility of bees collecting this pollen from different habitats in the landscape completely, it is highly likely that foragers were visiting these areas to collect this pollen. Future studies could confirm this through bee tagging and observation of pollen foraging individuals within different habitat types.

We found that generally, bumblebees collected pollen from plant taxa at rates which would be expected based on their relative abundance in the landscape. There were several exceptions to this however, with both *Erica* spp. and *Ulex* spp. (which were correlated to scrubland habitat) occurring in pollen samples at a greater rate than predicted. In contrast, both *Taraxacum* spp. and *Vicia* spp. were found less frequently than expected, but this could be a consequence of their very wide distribution within habitat types. Floral units of both *Erica* spp. and *Ulex* spp. are found in dense clusters, and so once located, bees can collect from flowers en masse which may explain their overrepresentation in pollen samples.

Regardless of treatment, the predominant plant species present in pollen samples collected by all bumblebees in this study was *P. tanacetifolia*. We also found that bees visited *Phacelia* at a higher rate than would be expected by chance, which implies that foragers are actively seeking out this plant for pollen collection. *Phacelia* is a non-native, hardy annual plant sown by land managers that produces clusters of purple flowers in the late summer and early autumn, which correlates directly with our sampling period. It is a popular cover crop, providing multiple ecosystem services including weed control and nutrient assimilation (Cottney et al., 2022; Schappert et al., 2019).

It is widely recognised that bumblebees will modify their foraging behaviour in response to resource availability and the presence of mass-flowering crops (Hemberger and Gratton, 2018). Westphal et al., (2006) found that *B. terrestris* foragers from colonies positioned adjacent to mass-flowering *Phacelia* had shorter foraging trips than those in landscapes with fewer floral resources. While we did not measure trip duration here, based on pollen sample composition, we can conclude that bees elected to visit *Phacelia* even when this plant was not present in the typical foraging range of workers. It has also been shown that colonies of *Bombus impatiens* positioned adjacent to *Phacelia* plantings gained more mass and produced more gynes than those without *Phacelia* present in the landscape (Hemberger et al., 2022). Corbicular loads of foragers returning to the nest contained between 25-40% *Phacelia*, and the authors emphasise the importance of mass-flowering cover crops in an otherwise low quality landscape. Here, we demonstrate the same phenomenon from a different perspective, where despite the presence of a more diverse habitat (road verge) within the foraging range of the colony, bees still choose to visit *Phacelia* at a higher rate than predicted by the landscape.

Whilst the high prevalence of *Phacelia* in corbicular loads is likely a result of a resource poor landscape, it is also possible that bees were selecting *Phacelia* over other plant taxa due to its nutritional value. In late-summer, queen bumblebees switch to producing males and gynes as opposed to more new workers, and it is estimated that it requires three times the investment cost to successfully rear a queen relative to males or workers (Pelletier and McNeil, 2003). *Phacelia* pollen has a particularly high protein content (30.1%) when compared to other cover crop species flowering at the same time, for example sunflower (14.86%) (Pernal and Currie, 2000). *Phacelia* was also higher in protein content compared to several other plant taxa present in our floral surveys (*Trifolium repens*: 22%, *Taraxacum officinale*: 16%, and *Bellis perennis*: 13%; Cinbirtoğlu and Güney, 2021). The high protein content of this species could be a contributing reason to its significant prevalence in pollen loads collected. Conversely, the diversity of samples collected by colonies indicates that all nutritional requirements of bumblebees are not met by this species alone, and foragers continue to visit a wide range of plant taxa.

Regardless of sampling period, individual pollen loads taken from foragers contained between one and two floral species, and these species were unevenly represented, with one constituting a greater proportion of the corbicular sample. While the minor proportion was made up of a wide variety of plant species, the major component was comprised predominantly of *Phacelia* (>29%), and this remained consistent across the 8 weeks. *Trifolium* spp. also made up a significant portion of pollen samples, however this was far more variable between samples (between 7-26%). These results suggest that bees in our study relied greatly on select species but collected from other taxa within the landscape at lower levels, perhaps to supplement their diet with additional nutrients (Moerman et al., 2017). This diverse pollen diet has been shown previously in bumblebees, with foragers collecting pollen from twice as many plant taxa as honeybees (Leonhardt and Blüthgen, 2012).

Although the major species present in bumblebee diet was not found on either road verge habitat surveyed in our study, many of the minor components were strongly associated with roadside habitat. This suggests that these plant taxa do still contribute significantly to the diversity and breadth of colony diet, and without their presence in the landscape, the composition and nutritional value of pollen collected by foragers would be lower. This reduced diversity of pollen collected by bees could negatively impact the development of the colony, and therefore we can conclude that verges play an important role for all bumblebee colonies in our landscape regardless of treatment. These ‘roadside’ plant taxa occurred in pollen samples as was predicted by the null model, and so future studies could compare the pollen diet of bumblebees in landscapes with verges of varying floral abundance and diversity to empirically test if this alters their frequency in corbicular loads.

There was no significant difference in the abundance of *Phacelia* present in corbicular samples between sampling periods in our study, which suggests that this plant remained a consistently valuable floral resource throughout the latter half of the season. Floral surveys support this, with *Phacelia* in constant bloom throughout the 8-week period. Treatment was also not a significant predictor of pollen diversity, which shows regardless of location (roadside or not), bumblebees in this study use available pollen resources in similar ways. While there were differences between individual colonies with respect to habitat structure within the 500m radius, the overall habitat uniformity between treatments could be an underlying reason for this lack of variance.

There were several limitations in this study; the first being scale. For logistic and safety purposes, the A11 road was only accessible from certain areas of the estate, and this limited where colonies could be placed in the landscape. Due to this, it was necessary to place some colonies only 1km apart from each other, and therefore they were likely to overlap to some extent in their foraging ranges. Although the typical flight range of *B. terrestris* is around this (average 551m; Redhead et al.,2015), foragers are known to travel greater distances when resources are scarce (Osborne et al., 2008). Here, we showed that all colonies did forage on *Phacelia,* even when cover crop (which *Phacelia* was strongly associated with) was not present within the 500m radius. This suggests that bees are visiting flowers outside of our mapped radius, and so future studies should aim to increase both the distance between colonies and the mapped radius surrounding each colony to verify our results.

Another important point to note is that our null model approach used floral units as an estimate of available resource, however this may not be the most ecologically realistic measure. Using mean pollen volume as opposed to floral units could better explain variation in the foraging preferences of individuals, however pollen data for the main plant species collected by bees (*Phacelia*) was unavailable, and we were unaware of its importance during data collection, so this approach was not adopted (Timberlake et al., 2021b).

4.6. CONCLUSION

This study is, to our knowledge, the first to investigate how bumblebees located on roadsides use pollen resources within the landscape. It is also the first to both measure the pollen collected by bumblebee foragers returning to the nest and the flowers available in the landscape. We have revealed that, despite verges being the most florally diverse habitat type surveyed, the pollen diet of *B. terrestris* in our study consisted largely of a mass-flowering cover crop species (*Phacelia*). Even when cover crop habitat was not present within a 500m radius of the colony, pollen samples still contained a high proportion of *Phacelia*, suggesting individuals are actively seeking out this plant species. Whilst *Phacelia* was evidently a valuable resource for foragers in our study, bees did also collect pollen from plants strongly associated with verge habitat, however these taxa were present in corbicular samples in proportion to their relative abundance in the landscape (i.e. foragers visited verge plants as would be expected by chance). Our findings suggest that *Phacelia* is a key pollen resource for bumblebees during a period known to have low floral resources in many UK landscapes.

CHAPTER 5: Conclusions and applications

Many bumblebee species are declining across the UK due to loss of habitat. Roadside habitats represent large areas in the UK (2579km^2 (1.2%) of land) and are often managed to provide large numbers of flowering resources (Phillips et al., 2021a). There is therefore a huge opportunity for verges to provide important habitats for bumblebee conservation. However, the usefulness of verge habitats for bumblebee foraging is limited by an understanding of bumblebee colony foraging and success in these habitats. This thesis addressed three key questions concerning road verges and their potential as a tool for bumblebee conservation. Firstly, I wanted to assess how distance from the edge of a major road impacted colony growth and development. Secondly, I wanted to quantify the effects of two common roadside metals on bumblebee colony development, using micro-colonies as a proxy. Finally, I set out to determine how bumblebee colonies positioned on the edge of a major road used pollen resources in the landscape relative to those located away from the road edge.

This thesis provides valuable insights to how bumblebees are utilising road verge habitats by contributing empirical work that demonstrates:

- Bumblebee colony development and foraging preferences in road verge habitats
- The impact of common road-sourced heavy metal contaminants on bumblebee colony health

In this final chapter, I discuss both how my results have addressed these specific research questions and how my thesis contributes to the wider body of literature on road verges and bumblebee conservation. I also discuss the limitations of my research, considerations for the future of road verge research, and management recommendations.

5.1. CHAPTER 2

Chapter 2 addressed the first aim of my thesis; investigating how distance from the edge of a major road impacts bumblebee colony development and reproductive success. This was achieved by placing *Bombus terrestris* colonies reared from wild-caught queen bumblebees at varying distances from the edge of a major road (the A11), and recording variables which were indicators of overall colony development and reproductive success on a weekly basis. Data collection took place over a 19-week period in 2021, and the entire colony cycle was captured. The main result from this chapter was, that despite roadside colonies remaining consistently heavier and producing slightly more gynes than control colonies, this was not significant.

This is the first study to show that proximity to the road edge has no impact on the growth or reproductive success of *B. terrestris* colonies. It is also, to my knowledge, the first to test the collective impacts of multiple stressors associated with roadsides on bumblebee colonies, which was a significant gap in current literature. Previous work has assessed the response of pollinators to several pollutants individually (Phillips et al., 2021b), but none have looked at the combined effects, which has been highlighted as an important topic requiring study (Phillips et al., 2020a). This, coupled with the use of colonies reared from wild-caught queens, mean that my results are widely applicable in a real-world context and a highly valuable addition to the research concerning road verges and pollinators. The lack of a negative effect supports the current general consensus that road verges can provide bumblebees with good-quality habitat.

Both road properties (e.g. traffic volume, width, speed, etc.) and verge management can influence capacity of bumblebees to utilise roadside habitats. It is possible that different types of roads will have different impacts on colonies and future work in this area should test the effects of varying traffic volumes, speeds, and road width on colony development and reproductive outputs. Another important consideration for conservation is whether or not bumblebees choose to nest in roadside habitat at all. I placed colonies onto the roadside artificially, and it may be the case that queen bumblebees actively avoid roadsides for nesting due to proximity to traffic. Dániel-Ferreira et al., (2022b) surveyed queen bumblebees along roadsides with varying traffic volumes and found that 24% of queens observed were performing nest-searching behaviour, with only one individual looking for an overwintering site. In roadsides with high-flowering plant diversity, traffic volume had no influence on the likelihood of an observed queen being dead, however in verges with low-flowering diversity, the chance of an observed queen being dead dramatically increased with increasing traffic volume. These results highlight the importance of verge management practices, particularly along roadsides where traffic volume is greater. Future research should quantify bumblebee nest density in roadside habitats compared to other types of habitat to determine the value of verges as suitable nesting habitat for queens.

5.2. CHAPTER 3

Road verges are known to be sources of heavy metal contamination (Werkenthin et al., 2014) and may make roadside poor quality habitats for bumblebees. In **chapter 3**, the impacts of two common roadside metals on colony development were assessed. Copper and cadmium were fed to micro-colonies composed of four commercial *B. terrestris* workers through either a nectar or pollen source at six different levels (Cu: control, 1ppm, 20ppm, 50ppm, 100ppm, and 500ppm, Cd: control, 0.01ppm, 0.2ppm, 0.5ppm, 1ppm, and 5ppm). These concentrations reflect current background levels typically found in roadsides, as well as those high and low extremes to ensure an effect was observable. Micro-colonies were allowed to develop for a period of 3 weeks, after which they were frozen and dissected to assess various metrics of overall success.

In alignment with previous literature, we found no significant effect of field-realistic levels of metal contamination on micro-colony development (Scott et al., 2022). With the exception of the highest contamination level, *B. terrestris* workers from all treatments performed the same with respect to brood composition, the amount of pollen and nectar consumed, and the number and weight of offspring produced. At slightly elevated levels of cadmium contamination (0.5ppm compared to 0.2ppm), the number of dead offspring in micro-colonies was significantly higher. While current environmental cadmium levels are below this threshold, the increased prominence of electric cars may increase the significance of this metal as an environmental contaminant in the future (**section 5.4.1**).

There are several topics for future consideration concerning the impacts of metals on bumblebees. From a much broader perspective, it is important to continue monitoring the levels of roadside metals in soils, plant tissues and floral rewards, as levels are likely to increase with more electric vehicles on the road. A next step for investigating bumblebee colony development specifically would be to subject whole colonies to metal contaminants and monitor their development across an entire field season. Also, ground nesting bee species, including bumblebees, spend a large proportion of their life cycle in contact with the ground, and soil-based pathways may be equally important for exposure to contaminants. However, no studies have investigated the influence of soil contamination on bumblebee health, and this remains a significant knowledge gap.

5.3. CHAPTER 4

The foraging patterns of individuals on roadsides has been extensively studied (Hass et al., 2018; Kämper et al., 2016; Schweiger et al., 2022). This thesis builds on this body of literature by providing the first assessment of the relative use of different landscapes by bumblebees nesting on roadsides. In **chapter 4**, I assess the foraging preferences of colonies reared from wild-caught queens of *B. terrestris* positioned on the edge of a major road and those located away from the road edge. Plant species within corbicular loads were identified with light microscopy and compared with the available floral resources in the landscape.

There was no influence of treatment on the plant taxa collected by bumblebee colonies. Instead, *Phacelia tanacetifolia*, a mass-flowering cover crop, comprised a significant portion of bumblebee pollen diet across the study period. *Phacelia* has previously been shown to be an important late-flowering resource for bumblebees (Hemberger et al., 2022; Westphal et al., 2006), and my results also demonstrate this, with foragers visiting this species more than would be expected by chance. Bees also visited this species regardless of whether it was present within the 500m colony buffer, suggesting that foragers travel outside of their typical range (*B. terrestris*: 550m; Redhead et al., 2016) to access this resource. *Phacelia* pollen is exceptionally rich in protein (~30%; Pernal and Currie, 2000), which was considerably higher than other pollen types recorded in corbicular samples. It can be concluded, therefore, that foragers were actively seeking out this plant for pollen in my study, perhaps due to its nutritional value.

While this chapter shows that *Phacelia* is an important flowering resource late in the season (from July until September), this resource is not flowering at the beginning of this colony cycle, and future studies should aim to quantify floral resources and pollen collection by bumblebees early in the season to capture the full colony cycle, as verges could still play a significant role in bumblebee diet during their growth phase.

While there are a handful of studies that have assessed pollen collection with reference to availability of surrounding habitats (Kämper et al., 2016), or assessed bumblebee density in relation to landscape floral density (Timberlake et al., 2020), no studies have attempted to directly compare the plant taxa collected by bumblebees to the available floral resources in the environment using colonies at this scale. The novel approach to landscape quantification in my chapter allows for a more detailed and thorough assessment on landscape use by bumblebees with reference to pollen collection.

It could be expected that bumblebees travelling further to obtain resources would develop at a reduced rate and produce less reproductives than those visiting resources close to the colony. While there were no significant differences in colonies from roadside and control treatments, the results of **chapter 4** suggest that *Phacelia*, rather than plants associated with roadside habitat, was a key component of bumblebee diet during the latter stages of the colony cycle when males and gynes are produced. *Phacelia* was not planted in roadsides, but is a key component of cover crops used in agricultural areas (Westphal et al., 2006). Therefore, there may be differences in development and reproductive success of colonies depending on distance to *Phacelia*, which was not investigated in my thesis. An important next step for this research would be to map all *Phacelia* cover on the estate in 2021 and measure the shortest, as well as the mean, distance to *Phacelia* for each colony. This information could then be used to determine forager range and then could be used as a variable to predict colony success, as it may be a significant contributing factor to the development of the colonies.

5.4. FUTURE DIRECTIONS

5.4.1. Electric vehicles

As briefly discussed in **chapters 2** and **3**, the transport sector is moving away from the traditional combustion engine towards the use of electric car batteries (BNEF, 2022). This shift in technologies is happening at a rapid rate and will have significant repercussions on the suitability of roadsides as viable pollinator habitat.

To my knowledge, no studies which investigate the impacts of roadsides on bumblebees and other pollinators discuss the implications of vehicle decarbonisation on the future applicability of their results. All research concerning roadsides should consider if their findings will remain relevant as the levels and forms of pollution associated with traffic will change as a result of transport electrification. Furthermore, study design should reflect this, with priority given to pollution which is likely to increase (metal contamination), as opposed to decrease or cease altogether (exhaust emissions). This lack of acknowledgement is a crucial gap in literature, which two of my data chapters' attempt to address.

In particular, **chapter 3** of my thesis specifically addresses how metal pollution impacts micro-colony development. As previously discussed in **section 3.5.1.** emission of metals is a form of pollution associated with traffic that is very likely to increase as electric vehicles become more commonplace. The majority of particulate matter pollution (80-90%) originates from non-exhaust emissions, such as wear to the brakes, clutch, tires, and steering mechanisms of cars (Timmers and Achten, 2016). Metals are classed as particulate matter pollution, and as vehicles become heavier due to the reinforced structural components required to house an electric car battery, metal pollution on roadsides will increase (Shaffer et al., 2021). My results show that at 0.5ppm, cadmium had a significant negative impact on the number of dead offspring in micro-colonies. While not the current level recorded on the majority of roadsides (0.2ppm is typical), 0.5ppm could become the new standard as electricity becomes the primary fuel source for vehicles. This specific result highlights the need to consider the future of the transport sector, as its importance, and potential future relevance, would not have been emphasised in the chapter otherwise.

5.4.1. *Bombus terrestris* as a model species

Across all three data chapters of my thesis, I have used *Bombus terrestris* as a study species. In **chapters 2** and **4**, bumblebee colonies were reared from wild-caught queens, and **chapter 3** used commercially purchased bumblebee colonies from Biobest, UK. Ideally, colonies from wild-caught queens would be used for all data collection as data collected from these colonies are more ecologically realistic than data from commercial colonies (details in **section 4.2**), however timing of the research meant that commercial colonies were used for **chapter 3**.

B. terrestris is a eusocial insect native to Eurasia and Northern Africa. It is the most common bumblebee species in the UK, and trends indicate that populations of *B. terrestris* continue to increase, despite declines in other species (Comont and Dickinson, 2021). *Bombus* spp. can be divided into two groups relating to their brood set up: pocket-makers and pollen-storers (Cameron et al., 2007). Pocket-makers feed their larvae collectively; in early development pollen is pushed into pockets underneath the brood mass, forming a cushion upon which the larvae feed. In the latter stages of development, the queen creates holes in the wax cap covering the brood and regurgitates a mixture of nectar and pollen onto the larvae. In pollen-storers, the larvae form individual cells from wax and silk and larvae are fed on a mixture of regurgitated nectar and pollen for the majority of their development. *B. terrestris* is a pollen-storer, and thus the response of pocket-maker bumblebee species to my research questions may be entirely different.

B. terrestris is fast becoming the model species for bumblebee research in the UK due to its robust nature and ease of acquirement (Samuelson et al., 2018; Whitehorn et al., 2012). While this species does provide insight into bumblebee ecology and unique opportunities for study, *B. terrestris* should not be considered a well-rounded representation of all bumblebee species. It is precisely because of its desirable rearing qualities (generalist feeder, hardy climate resilience) that it may fail to demonstrate the very specific and delicate requirements of other bumblebee species. Qualities including early emergence and large foraging range have resulted in *B. terrestris* becoming an invasive species in many parts of the world including Japan, Mexico, China, and Chile (Dafni et al., 2010; Fontúrbel, et al., 2021), successfully illustrating how adaptive this species is.

Therefore, while applicable, the results of my research should be considered in their experimental context. Generalisations can be made from my findings to an extent, however, research focused on other species is still required to provide a comprehensive review of road verges as a tool for bumblebee conservation, as one species is not an accurate representation of the entire genera. This predicament is not unique to my thesis however, and research on bumblebee ecology is highly biased towards a few model species (predominantly *B. terrestris* and *B. impatiens*) (Cnaani et al., 2002; Samuelson et al., 2018; Scott et al., 2022; Whitehorn et al., 2012). To obtain more applicable results, it is imperative to test how other bumblebee species, respond to placement on roadside habitat. Pocket-makers are particularly lacking in current research, but are particularly difficult to study, since no one has, to my knowledge, successfully reared a pocket-maker bumblebee species in the laboratory.

5.4.3. Management of road verges for bumblebee conservation

Previous work on road verges and pollinators have demonstrated that management practices are an important factor when considering the value of roadside habitat for conservation (Jakobsson et al., 2018). As discussed in **section 1.5**, the degree to which verges can support bumblebee populations will be greatly influenced by management, specifically the frequency and timing of cuts as well as the collection of cuttings (Phillips et al., 2020a).

The results of **chapter 4** show that verge habitat can be one of the most florally diverse habitat types in agricultural landscapes. Although bumblebees in my study visited plants that were associated with other habitat types, foragers still collected pollen from plant taxa that were predominantly found on verges. Verges may play a larger role in bumblebee diet during the spring and early summer months, or in agricultural landscapes where cover crops (in my study *Phacelia*) are not sown.

In light of my results that bumblebee colonies positioned on roadsides do not differ from those away from the verge with respect to development and reproductive success (**chapter 2**) or pollen collection in the latter stages of the colony cycles (**chapter 4**), nesting on verges does not appear to have major negative consequences for bumblebees at the colony level. This finding has huge implications for the future of road verge management, as authorities and councils can promote floral species richness on roadsides with the intention of providing resources for bumblebees.

The results from **chapter 3** however, indicate that metal pollution may become an issue for bumblebee colonies on roadsides in the future. While more thorough research is still required, one approach to counter this issue would be to manage verges for wildlife conditionally. A 1-2m wide strip of land directly adjacent to the road edge can be mown more frequently, whilst leaving the remainder of the verge away from the edge for bees and other pollinators. Different forms of pollution associated with traffic have been shown to negatively correlate with proximity to the road (Phillips et al., 2021b), and so pollutant exposure on verges would be less with increasing distance from the road. This conditional conservation management would also satisfy the safety requirements of highways authorities, as driver visibility and roadside access would not be impaired.

5.7. CONCLUSION

My PhD project provides significant and timely insights into bumblebee colony development and foraging on roadsides, by addressing several important gaps in the current literature.

The results from all three data chapters support previous research that road verges can be used a tool for bumblebee conservation, when managed appropriately. Colony development and reproductive output were not affected by proximity to the road edge, suggesting that while individual bees may experience negative effects of traffic, this does not translate to overall colony success. The pollen collection of foragers originating from colonies on roadsides also did not differ from those in the surrounding landscape, demonstrating that, in the latter stages of the colony cycle and in the presence of other high-quality pollen resources, roadsides have a neutral effect on pollen foraging patterns in *Bombus terrestris*. The presence of metals in floral resources at levels currently associated with roadsides had no effect on micro-colony development, however at slightly elevated levels there was an effect on the number of dead offspring for cadmium. This result highlights the importance of future-orientated studies in road verge research, and shows that researchers must consider the applicability of their results in light of a rapidly changing transport industry.

Appendices

Appendix 1: Parasite screening

Bumblebees are known to host several parasite species which may impact their development (Schmid-Hempel, 1998). To account for this, bumblebees were screened for four of the major parasites (the neogregarine *Apicystis bombi*, the trypanosome *Crithidia bombi*, the microsporidian *Nosema bombi*, and the nematode *Sphaerularia bombi*; **Figure A1**) using the following methods.

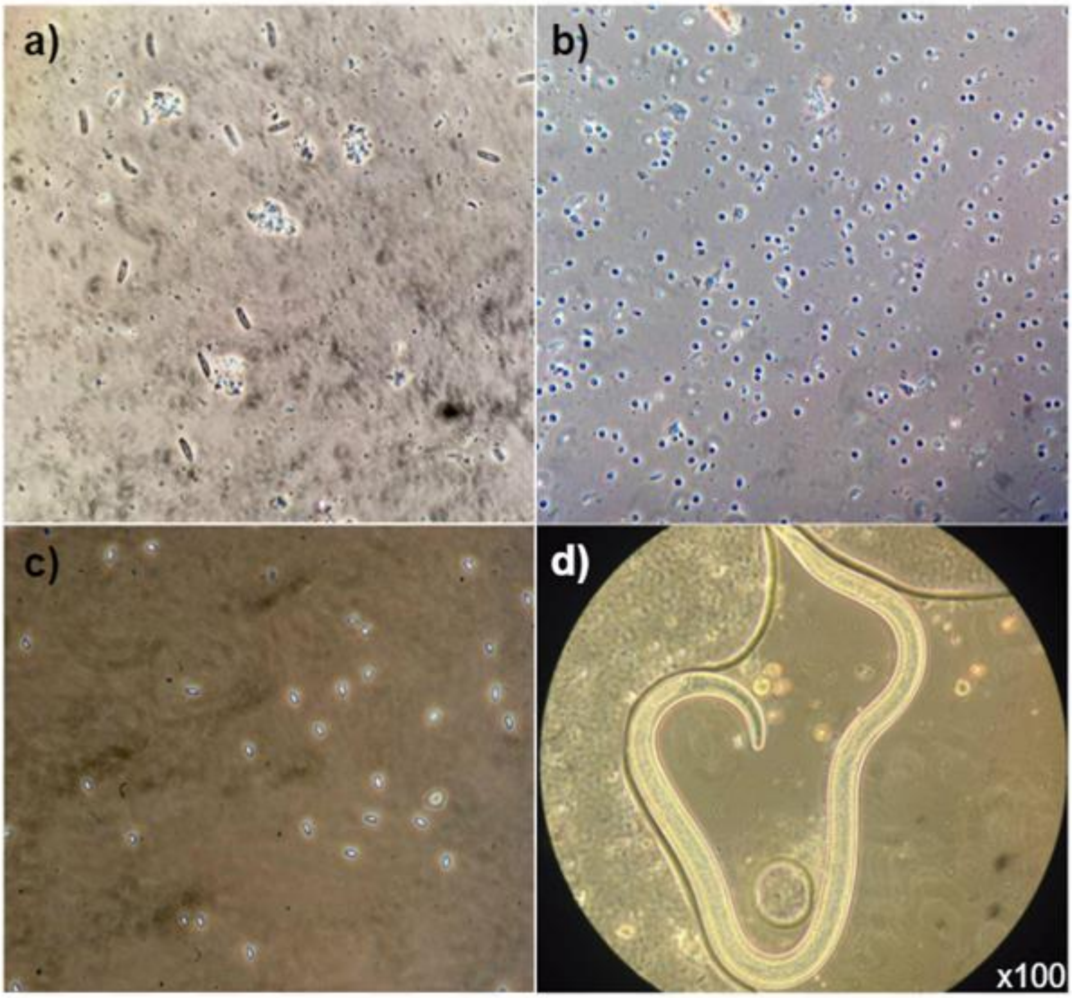


Figure A1: Photographs of the four major bumblebee parasites: (a) *Apicystis bombi*, (b) *Crithidia bombi*, (c) *Nosema bombi*, and (d) *Sphaerularia bombi* under a phase contrast microscope at 400x magnification (unless stated otherwise). Photo credit to Arran Folly.

Wild colonies

Queens were screened on the day of collection where possible. Between capture and arrival at the laboratory, bees have usually defecated in their collection tube. Where they had not, queens were encouraged to defecate by either blowing directly into the tube or banging the tube against the work bench. This was tried again after a period of ten minutes where queens did not defecate, and if this still did not trigger defecation queens were set up in the quarantine room to try again the next day.

After defecation, a micro-capillary tube was inserted through one of the ventilation holes in the tube lid to uptake the sample. This was then blown out of the micro-capillary tube onto a numbered microscope slide corresponding to the queen number and a coverslip was placed on top.

The slide was placed under a phase contrast light microscope (400x magnification, Phase 2) and samples were examined using a zigzag motion from one side of the sample to the other. Where the pollen count was too high (thus inhibiting observation), bees were set up in the quarantine room with just sugar syrup and no pollen and screened again the next day. The bumblebee microbiome is complex, and it is possible for bees to be infected with more than one parasite at a time (Rutrecht and Brown, 2008), so even where one parasite was immediately found, thorough screening continued.

C. bombi infection increases significantly after feeding with commercial sugar syrup and pollen (Logan et al., 2005), and therefore it is important to rescreen queens after feeding. This took place three weeks following the initial screening using the same methods, and if queens were clean on the second screening they were considered free from infection. Queens were placed back into collection tubes to collect the sample and this was done using flame-sterilized tongs.

Prior to moving into the wooden colony boxes from the plastic rearing boxes, five randomly selected workers were screened for parasites to ensure the colony was parasite free before use in any experiments. Again, removal of workers from the colony and into collection tubes was conducted using flame-sterilized tongs.

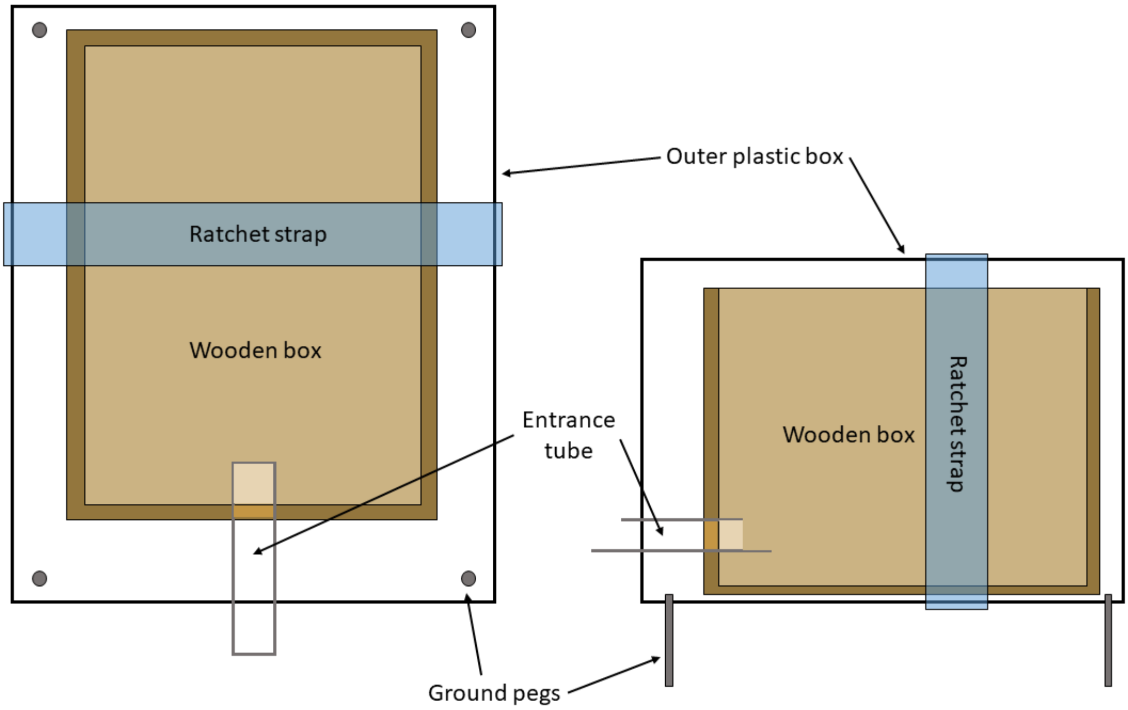
Commercial colonies

On the day of delivery, five randomly selected workers were removed from the colony using flame-sterilized tongs and transferred to collection pots. The method of sample collection and microscope screening were the same for commercial and wild reared colonies. If any of the workers were infected, then the colony was deemed infected.

Appendix 2: The location of the 16 colonies used in **chapter 2** and **chapter 4**. The treatment randomly assigned to each colony (road or control) along with the X and Y co-ordinates of the colonies are provided.

Colony Number	Treatment	Location (X,Y)
1	Road	52.372273, 0.63991
2	Road	52.388452, 0.664123
3	Control	52.350246, 0.605086
4	Road	52.363181, 0.612474
5	Control	52.36742, 0.557526
6	Control	52.369199, 0.585331
7	Road	52.396527, 0.700464
8	Road	52.377708, 0.653329
9	Control	52.379692, 0.609859
10	Road	52.351881, 0.571336
11	Control	52.340979, 0.584412
12	Control	52.366848, 0.676267
13	Control	52.38148, 0.594826
14	Road	52.396312, 0.680246
15	Control	52.341561, 0.600901
16	Road	52.367788, 0.627027

Appendix 3: A diagram of the bumblebee box set up for **chapter 2** and **chapter 4**.



Appendix 4: Kernel density estimation methods used in **chapter 2**.

This KDE method was adapted from Cooke et al., (2020). In QGIS, we mapped the location of the 16 colonies and created a 500m buffer around each colony. We then mapped all roads (excluding private roads used only by the Elveden estate) within the 500m buffer, and placed points at intervals of 10m along each road (**Figure A2**). We then attributed the corresponding traffic level along the road to each point.

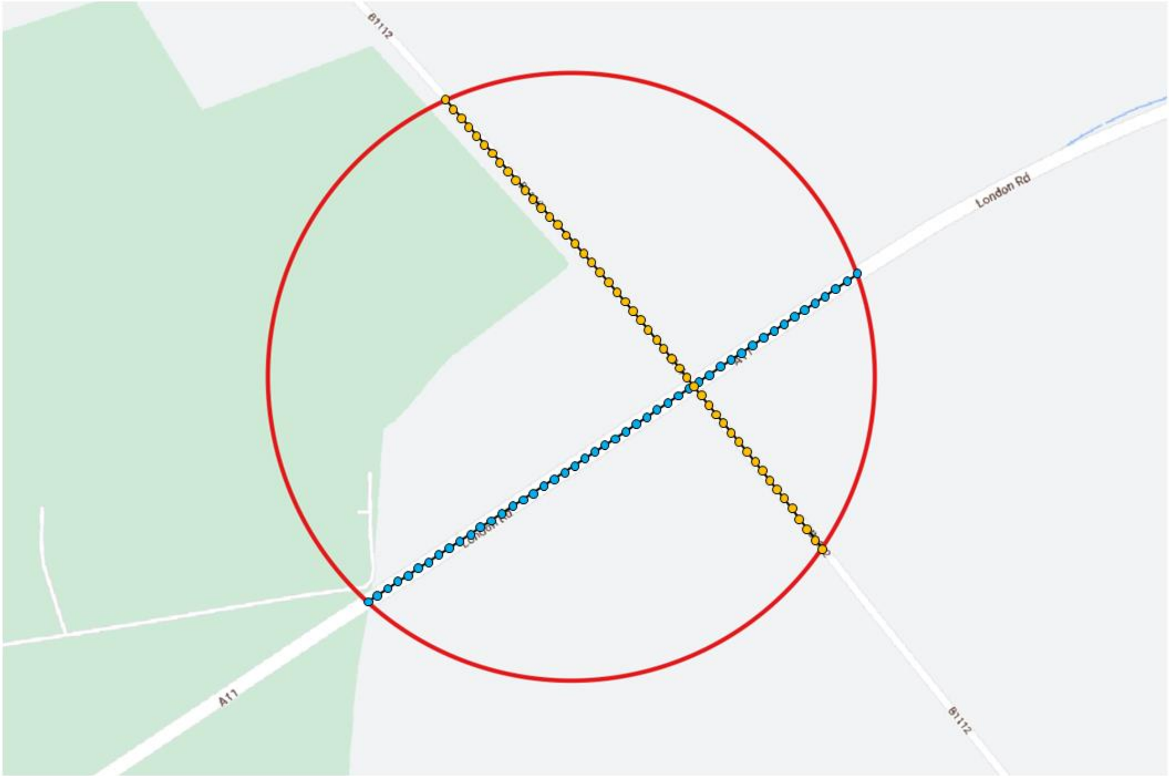


Figure A2: An example of a colony with points placed every 10 m along roads within the 500m colony buffer.

To determine road exposure for each colony we assumed a negative exponential relationship between potential road impacts and distance from the road edge. We used the following formula to calculate road exposure for each colony:

Road exposure (RE) = $\sum(t_i * \exp(-d_i * k))$

Where:

di = distance from the colony to kernel point (i)
ti = traffic level at kernel point (i), measured as annual average daily flow (AADF)
k = parameter determining the scale of the relationship between road exposure and distance from a road

The values of *k* were optimised using bisection searches in GAMMs. For each colony we first tested values by selecting a range of values for *k* (1-20, in intervals of 1; 25-45, in intervals of 5; and 50-100, in intervals of 10). Limits of 1 and 100 were selected as these assume that road exposure (when not weighted by traffic) is 1% at 500m, and nears 100% at the road edge, and we thought these were above and below the distance expected in reality. A GAMM was then run with all covariates.

Next, a graph of *k* and log-likelihood of the GAMM was created and inspected for peaks. Peaks were defined as points in the graph where points on either side were both lower. If there was more than one peak identified, the highest was selected. Values which fell on either side of the peak were selected and set as *k*1 and *k*3. *K*2, a middle *k* value, was calculated as the average of *k*1 and *k*3.

To calculate new values for *k*1 and *k*3, the difference between *k*3 and *k*2 was divided by two and this new value was added to *k*2 to produce a new value for *k*3. This new *k*3 value was subtracted from k2 to create a new value for *k*1. This process was repeated until the difference between *k*2 and *k*3 was below one, and this was set as the optimum value of *k* for the colony.

Appendix 5: Metal contamination preparation for **chapter 3**.

Contamination of nectar solution and pollen by copper (Cu) and cadmium (Cd) were carried out using the following methods.

Nectar

50% w/v sucrose solution was first prepared by weighing out 50g of sugar using digital scales. The sugar was then added to 100ml of deionized water and shaken until dissolved. This was repeated as needed.

A stock nectar solution was created for both metals equivalent to 1 gram of elemental material per liter (or 1000ppm). To achieve this for copper, 2.4483 grams of copper (II) chloride was added to 1 liter of 50% sucrose solution. For cadmium, 1.8274 grams of cadmium (II) chloride was added. A further 10ppm stock solution for Cd was created first by adding 900ml of sucrose solution to 100ml stock (creating 100ppm stock), and then adding another 900ml of sucrose solution to 100ml of the new 100ppm stock solution.

Both metal chlorides were weighed on digital scales accurate to four decimal places. The powder was removed from the jar using a spatula and placed into a weighing boat on the scale before being added to the sucrose solution and inverted 20 times. The stock solution was then diluted down using 50% sucrose solution to the desired concentration for each metal (**Table A1**).

Table A1: The dilution ratios for each level of metal contamination used in **chapter 3**. Unless stated otherwise stock solution used is 1000ppm.

Treatment	**Ratio of Stock: Sucrose solution**
Cu 500ppm	50:50
Cu 100ppm	10:90
Cu 50ppm	50:50 (from new 100ppm stock)
Cu 20ppm	20:80 (from 100ppm)
Cu 1ppm	1:99 (from 100ppm)
Cd 5ppm	50:50
Cd 1ppm	10:90
Cd 0.5ppm	50:50 (from new 1ppm stock)
Cd 0.2ppm	20:80 (for 1ppm)
Cd 0.1ppm	1:99 (from 1ppm)

Pollen

Commercial pollen pellets (Agralan, UK) were first ground into a fine powder using an electric coffee grinder until 1kg of powder was obtained. Again for copper, 2.4483g were added to this powder and mixed thoroughly in a beaker using a long spatula for 10 minutes to achieve an even distribution of material. 1.8274g of cadmium (II) chloride were added and the same mixture method applied.

To create the pollen patties for bees to begin laying eggs in, the equivalent 50% sucrose solution (Cu or Cd) was added in increments and stirred until a dough-like texture was achieved. The pollen was then rolled into a 'ball' shape with a small depression to prevent rolling when in the box.

Appendix 6: A comparison between a live larvae (left), and a dead larvae (right).

Appendix 7: All 20 habitat types mapped in **chapter 4** with definitions of each classification.

Habitat	Description
Carrot	Arable field growing carrots
Cover Crop	Areas sown with *Phacelia tanacetifolia* to provide ecosystem services
Developed Area	All built-up areas, including al buildings and car parks
Field Margin	Land immediately surrounding the edge of a field
Grassland	All open areas of land not used for production purposes
Hedge Plantation	Hedge grown from commercial purposes
Hedgerow	Hedge grown to create a barrier between two areas
Onion	Arable field growing onions
Parsnip	Arable field growing parsnip
Pasture	Fields with grazing livestock (sheep and cows)
Potato	Arable field growing potato
Road	All paved linear surfaces used for vehicle movement
Rye	Arable field growing rye
Scrubland	Areas consisting of scrub vegetation
Spring Barley	Arable field growing spring barley
Verge A11	The strip of land between the road edge and adjacent farmland along the A11 road
Verge Other	The strip of land between the road edge and adjacent farmland along all roads excluding the A11
Water	Natural and man-made water bodies including ponds and rivers
Winter Barley	Arable field growing winter barley
Winter Wheat	Arable field growing winter wheat
Woodland	Land covered with trees (predominantly coniferous)

Appendix 8: A list of the habitat types within a 500m radius of each colony in **chapter 4** and their percentage cover.

Table A2: Habitat types within 500m radius of colony 1 (treatment: road).

Habitat Type	Percentage Coverage
Carrot	24
Field Margin	2
Grassland	1
Potato	17
Rye	4
Spring Barley	8
Verge A11	5
Woodland	27

Table A3: Habitat types within 500m radius of colony 2 (treatment: road).

Habitat Type	Percentage Coverage
Field Margin	1
Grassland	17
Pasture	3
Scrubland	1
Spring Barley	25
Verge A11	12
Verge Other	1
Winter Barley	7
Woodland	33

Table A4: Habitat types within 500m radius of colony 3 (treatment: control).

Habitat Type	Percentage Coverage
Field Margin	1
Grassland	19
Onion	9
Potato	23
Rye	3
Scrubland	2
Spring Barley	4
Winter Barley	22
Woodland	17

Table A5: Habitat types within 500m radius of colony 4 (treatment: road).

Habitat Type	Percentage Coverage
Carrot	8
Cover Crop	5
Field Margin	2
Grassland	4
Onion	8
Pasture	48
Scrubland	1
Verge A11	7
Winter Barley	6
Winter Wheat	5
Woodland	6

Table A6: Habitat types within 500m radius of colony 5 (treatment: control).

Habitat Type	Percentage Coverage
Field Margin	1
Pasture	28
Potato	45
Rye	11
Verge Other	2
Woodland	13

Table A7: Habitat types within 500m radius of colony 6 (treatment: control).

Habitat Type	Percentage Coverage
Cover Crop	3
Field Margin	2
Grassland	4
Hedgerow	1
Parsnip	14
Potato	11
Spring Barley	25
Winter Barley	7
Winter Wheat	11
Woodland	22

Table A8: Habitat types within 500m radius of colony 7 (treatment: road).

Habitat Type	Percentage Coverage
Cover Crop	3
Field Margin	1
Grassland	11
Onion	16
Scrubland	1
Spring Barley	8
Verge A11	7
Woodland	53

Table A9: Habitat types within 500m radius of colony 8 (treatment: road).

Habitat Type	Percentage Coverage
Carrot	18
Field Margin	1
Grassland	3
Hedge Plantation	3
Verge A11	8
Winter Barley	34
Woodland	33

Table A10: Habitat types within 500m radius of colony 9 (treatment: control).

Habitat Type	Percentage Coverage
Carrot	10
Field Margin	3
Grassland	1
Parsnip	24
Potato	14
Rye	23
Scrubland	1
Spring Barley	17
Woodland	7

Table A11: Habitat types within 500m radius of colony 10 (treatment: road).

Habitat Type	Percentage Coverage
Cover Crop	4
Field Margin	1
Grassland	1
Hedge Plantation	12
Onion	23
Rye	10
Scrubland	1
Spring Barley	9
Verge A11	5
Verge Other	2
Winter Barley	15
Woodland	17

Table A12: Habitat types within 500m radius of colony 11 (treatment: control).

Habitat Type	Percentage Coverage
Cover Crop	3
Field Margin	2
Grassland	6
Hedgerow	1
Onion	22
Pasture	11
Potato	13
Scrubland	1
Spring Barley	8
Verge Other	1
Winter Barley	12
Woodland	20

Table A13: Habitat types within 500m radius of colony 12 (treatment: control).

Habitat Type	Percentage Coverage
Field Margin	1
Grassland	2
Potato	26
Verge Other	1
Winter Barley	19
Winter Wheat	15
Woodland	36

Table A14: Habitat types within 500m radius of colony 13 (treatment: control).

Habitat Type	Percentage Coverage
Cover Crop	4
Field Margin	1
Grassland	7
Onion	18
Pasture	1
Rye	28
Scrubland	3
Spring Barley	23
Woodland	15

Table A15: Habitat types within 500m radius of colony 14 (treatment: road).

Habitat Type	Percentage Coverage
Cover Crop	4
Field Margin	1
Grassland	21
Hedge Plantation	2
Onion	20
Rye	9
Scrubland	1
Verge A11	9
Woodland	33

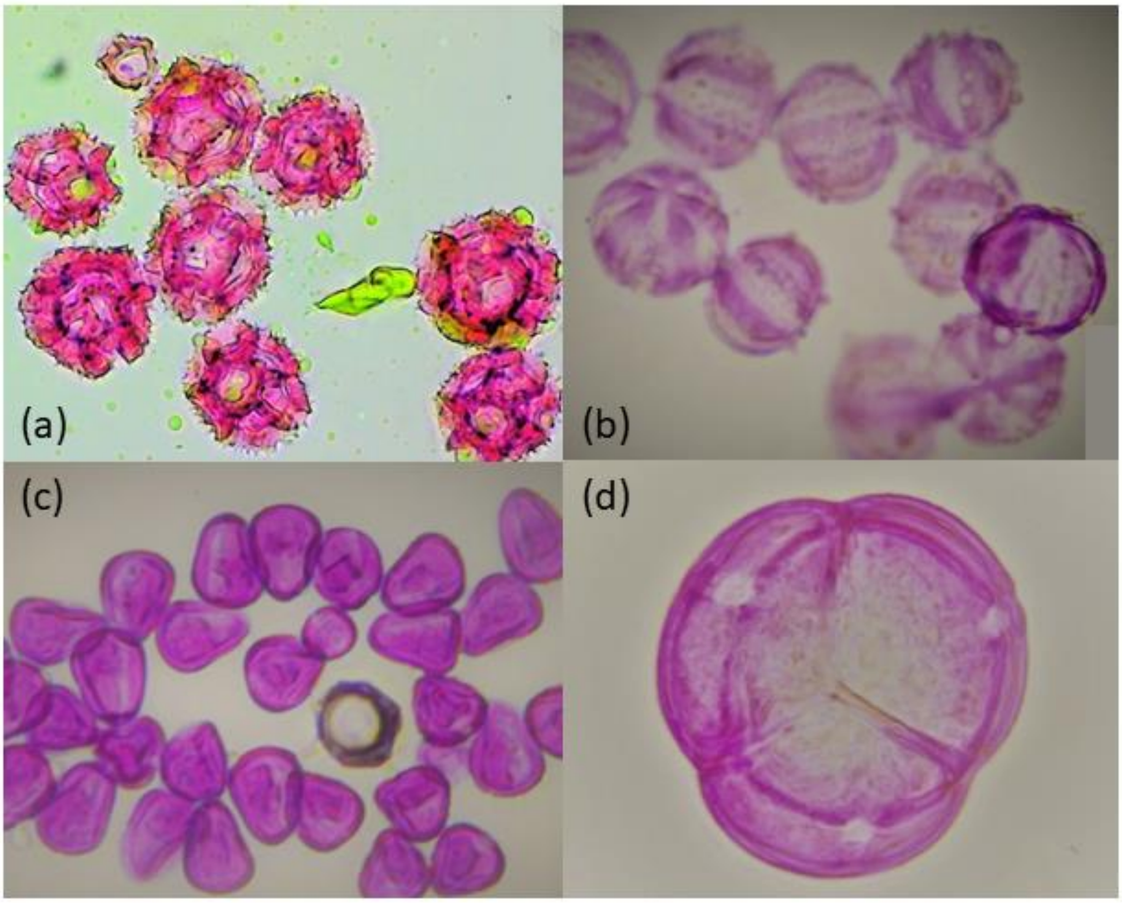
Table A16: Habitat types within 500m radius of colony 15 (treatment: control).

Habitat Type	Percentage Coverage
Grassland	20
Pasture	20
Potato	21
Scrubland	2
Woodland	37

Table A17: Habitat types within 500m radius of colony 16 (treatment: road).

Habitat Type	Percentage Coverage
Grassland	1
Pasture	47
Potato	14
Spring Barley	11
Verge A11	5
Woodland	22

Appendix 9: Photographs of four common pollen species recorded in **chapter 4**. (a) *Taraxacum* spp. (dandelion), (b) *Phacelia tanacetifolia*, (c) *Echium vulgare* (viper’s bugloss), and (d) *Erica* spp. (heather). Photo credit to Ian Jobson.



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