

The Effects of Landscape and Experience on the Navigation and  
Foraging Behaviour of Bumblebees, *Bombus terrestris*

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## Abstract

Bumblebees live in an environment where the spatial distribution of foraging resources is always changing. In order to keep track of such changes, bumblebees employ a variety of different navigation and foraging strategies. Although a substantial amount of research has investigated the different navigation and foraging behaviours of bumblebees, much less is known of the effects that landscape features have on bumblebee behaviour. In this thesis, a series of experiments were conducted in order to investigate the role that landscape features have on the navigation and foraging behaviour of *Bombus terrestris* and whether individuals' experience influences such behaviour. A hedgerow situated next to the colony was not found to significantly shape the flight paths or foraging choices of naïve bumblebees. Homing success was investigated and used as a proxy for foraging range in different environment types. Both the release distance and the type of environment were found to have a significant effect on the homing success of *Bombus terrestris* workers. Previous experience of the landscape was also found to significantly affect the time it took bumblebees to return to the colony (homing duration) as well as the likelihood of staying out overnight before returning to the colony. When focusing on the first five flights of a naïve bumblebee worker, experience was not found to significantly affect flight duration. Experience, however, significantly affected the weight of pollen foraged. The observed behaviour of bumblebee gynes provisioning their maternal colony with pollen was also investigated. The influx of pollen into the colony was found to affect this behaviour, suggesting that gynes will provision the maternal colony in response to its nutritional needs. The overall results are also discussed within the context of informing landscape management practices. The results presented in this thesis point to the critical role that factors such as the physical landscape and individual experience play in influencing bumblebee behaviour.

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## **Author's Declaration**

This thesis was funded by a Natural Environment Research Council (NERC) CASE studentship, secured by Prof Natalie Hempel de Ibarra and Mr Roger English, representing the CASE partner.

I am the main contributor of the research presented in this thesis. The work was supervised by Prof Juliet Osborne and Prof Natalie Hempel de Ibarra who contributed to the design, arrangement of fieldwork, analysis and interpretation. Mr Roger English contributed to the design and arrangement of fieldwork. Prof Juliet Osborne, Prof Natalie Hempel de Ibarra and Prof Darren Croft all provided feedback on my written chapters. Dr Rosalind Shaw supervised the statistical analysis. Dr Pizza Ka Yee Chow, Mr Harold Edmonds, Mr Thomas Garnham, Ms Katie Hall, Prof Natalie Hempel de Ibarra, Ms Lucy Nevard, Ms Aparajitha Ramesh, Dr Theo Robert, Mr Sajesh Vijaya and Mr Owen Wright all contributed to the collection of field data.



# Chapter One

## General Introduction

Bumblebees are among the most important insect pollinators, providing pollination services to both crops and wild flowers (Corbet, 1987; Plowright & Lavery 1987; Corbet, Williams & Osborne, 1991; Willmer, Bataw & Hughes, 1994; Holzschuh *et al.*, 2011; Kovács-Hostyánszki *et al.*, 2013). Due to agricultural intensification over the past few decades, however, many natural environments have experienced both habitat loss and fragmentation (Saville *et al.*, 1997; Vanbergen, 2013). Habitat fragmentation is defined as occurring when a large expanse of habitat is transformed into smaller patches, with each patch isolated from the other by a matrix of habitats which are different from the original (Wilcove, McLellan & Dobson, 1986). It is important to note that habitat fragmentation per se can occur independently of habitat loss (Fahrig, 2003). Habitat loss coupled with habitat fragmentation can pose navigational and foraging challenges to wild bumblebees and it is believed that such changes to landscape composition and configuration are some of the leading causes of wild bumblebee population declines (Williams, 1982; Rasmont, 1988; Corbet, Williams & Osborne, 1991; Osborne & Corbet, 1994; Kosior, 1995; Rasmont, 1995; Buchmann and Nabhan, 1996; Westrich, 1996; Williams & Osborne, 2009). To tackle this issue and maintain and promote wild bumblebee populations, it is necessary to have an in-depth understanding of the interaction between bumblebees and their physical environment.

As such, the underlying question that this thesis aims to tackle is: what effects does the physical environment have on bumblebee navigation and foraging behaviour and what role does a bumblebee's individual experience play in shaping this behaviour?

This introductory chapter begins by exploring the challenges that wild bumblebees face as a result of modern agricultural practices and the initiatives which are being proposed to tackle these challenges. The navigation and foraging strategies of bumblebees in their natural environment and the environmental cues that they use are then summarised. An in-depth review of these strategies is also provided in the introductions to Chapters Two to Five. The hypothesised mechanisms that underlie the navigation abilities of bumblebees, and eusocial

insects more generally, are also explored as well as the effects of individual experience. The thesis objectives are then introduced. This chapter concludes with an overview of each of the subsequent data chapters.

### **1.1 Agricultural Intensification, Habitat Loss and Habitat Fragmentation**

Since the middle of the 20<sup>th</sup> century, changes to modern farming practices have resulted in the overall intensification of agriculture. In the United Kingdom, it is estimated that thousands of miles of hedgerows were removed in an effort to increase agricultural field sizes following the Second World War (Newby, 1958). Many farmers started to plough up to their field boundaries, while cutting hedgerows very low to the ground (Marshall & Smith, 1987). Among the effects of these practices were the loss of lowland and grassland which was not treated with mineral fertilisers or lime (Fuller, 1987; Howard *et al.*, 2003). This has resulted in a loss of 97% of wildflower rich grassland since the 1930s in England and Wales and a fragmentation of the landscape more widely (Natural England, 2011). For bumblebee species, habitat loss results in a direct removal of key nesting and foraging habitats. Coupled with the fragmentation of the landscape, this has resulted in a decline in species abundance and a reduction in distribution ranges (Williams, 1982; Rasmont, 1988; Buchmann & Nabhan, 1996; Cameron *et al.*, 2011). Such changes in the abundance and distribution ranges of different bumblebee species directly impact the quantity and quality of pollination services in a particular landscape (Kwak, Velterop & van Andel, 1993) and can lead to the isolation and mutual destruction of both plant and insect populations (Rathke & Jules, 1993; Peterson, Bartish & Peterson, 2008; Bailey *et al.*, 2010; Geert, Rossum & Triest, 2010; Shuey, 2013).

With their need for undisturbed nesting, mating and hibernation sites and an uninterrupted supply of pollen and nectar sources throughout a colony's life cycle, wild bumblebees are particularly susceptible to the effects of habitat loss and habitat fragmentation as a result of intensive farming (Carvell *et al.*, 2007). Bumblebees, like all bee species, are central place foragers (Plowright & Lavery, 1984). Bumblebees return to their colony between foraging trips. Even if suitable nesting sites exist within a landscape, suitable food sources must be within a bumblebee's maximum foraging range in order for these food sources to be discovered and exploited. For bumblebees, the foraging ranges of different

species ranges between 750 m and 3 km (Osborne *et al.*, 1999; Walther-Hellwig & Frankl, 2000; Chapman, Wang & Bourke, 2003; Westphal & Tschardt, 2003; Kreyer *et al.*, 2004; Knight *et al.*, 2005; Osborne *et al.*, 2008; Hagen *et al.*, 2011; Osborne *et al.*, 2013). As such, habitat loss and fragmentation, if left unchecked, can pose both critical navigational and nutritional challenges for wild bumblebee populations.

In response to such habitat loss and fragmentation and the risks it poses, landscape management initiatives in the United Kingdom are currently focusing on both enhancing the abundance and diversity of flowering plant species within arable farming systems (Carvell *et al.*, 2007) as well as linking together habitat patches in fragmented landscapes through the creation of new habitats, or habitat 'stepping-stones' (Lawton *et al.*, 2010). To increase their chances of success, however, such initiatives must be based on scientific evidence and be subject to scientific evaluation (Kleijn & Sutherland, 2003; Knop *et al.*, 2006; Gill *et al.*, 2016). As a basic example, the placement of a habitat 'stepping-stone' within a fragmented landscape must take into account the flight ranges of the pollinators within that landscape. For bumblebees specifically, that 'stepping-stone' must be placed within reach of their nest. The floral composition of 'stepping-stones' is also important and should be considered. 'Stepping-stones' must contain food sources which are suitable for the pollinators that are found within the landscape. As such, an in-depth understanding of the interaction between bumblebees and their physical environment is needed. Specifically, an understanding of bumblebee movement within different landscapes can directly feed into such landscape management initiatives, promoting the conservation of bumblebees and insect pollinators more widely.

## **1.2 Bumblebee Navigation and Foraging Strategies**

As colonial insects living in environments where their rewarding food sources vary both spatially and temporally, bumblebees keep track of these fluctuations in order to maximise the survival and reproductive success of the colony. In order to do so, bumblebees employ a variety of different navigation and foraging strategies.

### 1.2.1 Learning Flights

When a bumblebee first leaves its nest or a rewarding food source, it performs a distinct flying behaviour, flying in a series of arcs and loops which increase in area as it flies off. Observed in ants, honeybees and wasps, this behaviour is collectively known as orientation or learning walks/flights (bumblebees: Collett & Zeil, 1996; Baddeley *et al.*, 2009; Hempel de Ibarra *et al.*, 2009; Phillipides *et al.*, 2013; Robert *et al.*, 2017; 2018; solitary wasps: Zeil, 1993; Stürzl *et al.*, 2016; wasps: Collett & Lehrer, 1993; wood ants: Nicholson, *et al.* 1999; desert ants: Müller & Wehner, 2010; honeybees: Capaldi & Dyer, 1999). It is the primary mechanism by which these insects learn the location of important goals and gain knowledge of their surroundings.

At certain points during this behaviour, these insects will turn their body orientation to face the nest, the food source, or even prominent nearby landmarks (Lehrer, 1993). It is thought that at these specific turning points, the insects encode visual information as well as the spatial relationship that exists between nearby landmarks and their goal (Collett & Zeil, 1996; Collett *et al.*, 2006). Upon returning to a goal location, eusocial insects approach it with a zigzag flight path which matches the positions that they took during their orientation/learning flights. As experience of the environment is gained, orientation/learning flights become rarer and turning back to face a goal is no longer observed (Free, 1955a; Lehrer, 1993; Zeil, 1993; Robert *et al.*, 2018; R.Herascu, personal observation). For bumblebees specifically, foraging flight trajectories straighten out with experience (Osborne *et al.*, 2013).

### 1.2.2 Traplining

Bumblebees use a variety of navigation strategies which are thought to maximise their foraging efficiency. During a foraging trip, bumblebees are observed making repeated circuits through a particular set of food patches, a behaviour termed traplining (Manning, 1956; Heinrich, 1976). Although first observed in wild bumblebees, laboratory experiments further revealed the complexities of this behaviour (Thomson, 1996; Thomson, Slakin & Thomson, 1997). When presented with a uniform array of rewarding flowers, naïve bumblebees sampled a large number of different flowers, taking a variety of different flight routes between them. As bumblebees gained experience of the floral array, however,

their foraging route became more repeatable and efficient. When compared to naïve bumblebees, experienced foragers travelled faster between patches and achieved higher rates of nectar intake (Ohashi, Leslie & Thomson, 2008). As such, experienced foragers decreased the total distance that they travelled within the floral array by up to 80% (Lihoreau *et al.*, 2012). Traplining is considered to be a sophisticated foraging behaviour (Ohashi, Thomson & D'Souza, 2007) and bumblebees are thought to learn and follow specific navigational routes between different foraging patches (Chameron *et al.*, 1998). When moving within a foraging patch, traplining is thought to potentially be based on a bumblebee's memory of the locations of specific flowers coupled with their memory of a specific motor pattern (Collett, Fry & Wehner, 1993).

### 1.2.3 Flower and Site Constancy

Traplining is closely linked to flower and site constancy, a behaviour in which individual bumblebees restrict their foraging visits to single flower types (Free, 1970; Heinrich, Mudge & Deringis, 1977; Waser, 1986; Gegear & Laverty, 2005) or foraging sites (Bowers, 1985; Waser, 1986; Dramstad, 1996; Saville, *et al.*, 1997). Heinrich (1976) observed that when bumblebees first left their nests, they sampled a variety of rewarding and non-rewarding flowers before specialising on a particular flower type. The particular flower type differed among individual bumblebees. Heinrich (1976) also observed that individuals seemed to have primary and secondary foraging strategies. This led him to term primary specialisations as a bumblebee's 'majors' and secondary specialisations as a bumblebee's 'minors'. A bumblebee's 'major' was defined as the flower type that a bumblebee was predominantly observed foraging from, as measured by the number of flowers/inflorescences visited. Heinrich (1976) hypothesised that having foraging expertise at more than one flower type would help bumblebees to keep track of changing floral resources through time and be able to quickly switch between specialties if need be. Although the 'majoring' and 'minoring' hypothesis of individual foraging specialisations is an attractive one, Heinrich's (1976) observations suffer from several flaws. Although Heinrich (1976) marked the individual bumblebees that he observed, he did not keep track of their foraging choices throughout the entire duration of their foraging trips. The foraging preferences that he reported are only based on a portion of a foraging trip. It is entirely possible that had the entire foraging trip been observed, the

foraging preferences of individual bumblebees would be entirely different. A better method of keeping track of an individual bumblebee's foraging choices throughout an entire foraging trip would have been to consider the pollen and nectar that a bumblebee brought back to their nest. By identifying the pollen and nectar that a bumblebee brought back to their nest and matching this to the floral sources in the landscape, it would have been possible to gain an idea of the floral sources that a bumblebee predominantly foraged from. Heinrich's (1976) observations do not consider a bumblebee's subsequent foraging trips, once again missing out on key foraging information. The time period of specialisation is also not defined. For example, do 'majoring' and 'minoring' only occur at the level of an individual foraging trip or are they maintained throughout a bumblebee's foraging career? Furthermore, aspects of the observations lack standardisation as different bumblebees are observed on different dates and for different amounts of time. This lack of standardisation makes it very difficult for concrete conclusions to be drawn from his observations.

Although the concept of 'majoring' and 'minoring' is not well established from Heinrich's (1976) observations, flower constancy in bumblebees has been repeatedly observed (Free, 1970; Heinrich *et al.*, 1977; Waser, 1986; Gegear & Lavery, 2005; Chapter Three). Constancy to a particular flower type or forage site is only maintained as long as it continues to be rewarding (Chittka, Thomson & Waser, 1999; Osborne & Williams, 2001). As a result, bumblebee workers are often observed bypassing equally rewarding flowers on route to their preferred site (Osborne *et al.*, 1999; Osborne *et al.*, 2013). Experienced foragers, tracked with harmonic radar, have also been seen to be constant to the compass bearing they take over successive trips (Osborne *et al.*, 1999), suggesting that flight paths taken remain largely constant once they are established.

To date, the most popular explanations for constancy point to a cognitive limitation which restricts the number of different flower types and foraging sites which a bumblebee can process at any one time (Waser, 1986; Lewis, 1993; Dukas, 1998; Goulson, 2000). This has also led to the suggestion that bumblebee workers use search images when foraging, selectively attending to particular visual characteristics in a preferred set of flower types (Goulson, 1999; Goulson, 2000). Overall, given cognitive processing constraints, flower constancy could minimise search times and increase foraging efficiency. As with the particular set

of sites that a bumblebee traplines between, it is not clear how such preferred sites are established in the first place, especially given that these sites vary between individual workers.

### **1.3 Environmental Cues**

In addition to the navigation and foraging strategies that bumblebees employ, a multitude of environment cues are thought to play a role in initially attracting bumblebees to specific types of flowers. These include visual, olfactory and social cues which can also influence the movements of bumblebees throughout their surrounding environment.

Visual attractors can manifest on various scales. When presented with a choice of different flowering crop plant species, bumblebees prefer to forage on oilseed rape (*Brassica napus*), however the specific attractants of this plant species are still unknown (J.L. Osborne, unpublished). Within a foraging patch, bumblebees preferentially visit individual plants with higher secretion rates (Cartar, 2004), relying on their previous experience to restrict their foraging to rewarding flowers (Burns & Thomson, 2006). The density of a foraging patch can also act as an attractant (Comba, 1999 but c.f. Kwak, 1987 and Sowig, 1989 who suggest this attraction may actually be species dependent). This effect does not, however, extend to individual flower sizes as well (Blarer *et al.*, 2002; Hudon & Plowright, 2011). Floral shape can also act as an attractant, and bumblebees seem to prefer flowers with symmetrical shapes (Rodriguez *et al.*, 2004). In terms of attraction to specific colours, the photoreceptors of bees more generally have spectral sensitivities which peak around 350, 450 and 550nm. These peaks correspond to the UV, blue and green regions of the colour spectrum respectively (Peitsch *et al.*, 1992). As such, these physiological characteristics equip bees with excellent colour vision (Hempel de Ibarra, Vorobyev, & Menzel, 2014) and laboratory experiments have revealed that honeybees are capable of colour learning which in turn determined their specific foraging choices (Menzel, 1967, 1968, 1969; Giurfa *et al.*, 1995, Gumbert, 2000). Taken together, bumblebees are thought to use a variety of visual floral cues which convey specific information regarding the colour, brightness, size, shape and symmetry of a particular flower. Such information can guide their local movements and influence their foraging choices.

Odour is also thought to be an important attractant of bumblebees to different flower species (Raguso, 2008; Lawson *et al.*, 2018) as are social cues (Kawagushi, Ohashi & Toquenaga, 2006; Leadbeater & Chittka, 2009; Dawson & Chittka, 2012; R.Herascu, unpublished). Although a variety of different cues spanning several modalities are used by bumblebees when exploring their natural environment, insufficient information is available at present to determine the exact nature of the recruitment patterns of bumblebees to specific foraging patches. It is still to be determined whether such patterns are a result of passive encounter rates or active choices on the part of the bumblebees (Free & Butler, 1959; Goulson, 2010).

The quantity and nutritional quality of pollen and nectar in a particular flower also act as an attractant for bumblebees. In flowers where the anthers are clearly visible, it has been suggested that bumblebees are able to assess the pollen content of a flower visually (Zimmerman, 1982; Cresswell & Robertson, 1994). Bumblebees may also be potentially able to estimate a flower's nectar secretion rate based on a flower's age/stage or condition (Cartar, 2004). Bumblebees also prefer to visit plants which produce the highest quality pollen (Hanley *et al.*, 2008) and will select pollen of specific nutritional properties. Throughout a variety of different pollen foraging experiments, bumblebees seem to prefer pollen which has a high protein content (Kitoaka & Nieh, 2009; Leonhardt & Blüthgen, 2012) and a high phytosterol content (Somme *et al.*, 2015). Vaudo *et al.* (2016) have also demonstrated that, when given a choice, bumblebees will prefer to collect pollen which has a high protein to lipid ratio. It is highly likely that the nutritional requirements of the colony will ultimately influence the pollen foraging choices of bumblebees on a given foraging trip (Hendriksma, Toth & Shfir, 2019).

With regards to nectar rewards, bumblebees may be able to visually detect the nectar content of flowers with an open structure (Thorp *et al.*, 1975; 1976; Kevan, 1976). For most flowers, however, the nectar is not visible from outside the flower (Goulson, 2010). Some authors have proposed that nectar volumes may be assessed from its scent or from the scent of fermenting products in the nectar (Heinrich, 1979b; Williams, Hollands & Tucknott, 1981 but c.f. Raihan & Kawakubo, 2013 whose results suggest this may be species specific). For example, there is a positive association between the quantity of nectar and pollen in *Brassica rapa* flowers and the floral scent compound phenylacetaldehyde



which they emit (Knauer & Schiestl, 2015). In behavioural experiments, bumble bees developed a preference for this compound over other scent compounds after foraging on *Brassica rapa* flowers (Knauer & Schiestl, 2015). Whether bumblebees are able to detect all nectar and pollen scents and use them to guide their foraging choices is currently unknown. What has been observed is that bumblebees are selective in their foraging choices based on the quantity and quality of nectar rewards. Konzmann & Lunau (2014) have found that bumblebees preferentially forage from flowers with large quantities of nectar and from which the nectar has a high sucrose content. Both pollen and nectar cues may also be coupled with floral visual cues as in many plant species, floral size is positively correlated with the quantity and quality of pollen and nectar rewards (Harder *et al.*, 1985; Cresswell & Galen, 1991; Armbruster, Antonsen & Pélabon, 2005; Fenster *et al.*, 2006; Gómez *et al.*, 2008).

#### **1.4 Navigational Mechanisms**

Bumblebees are thought to rely on a set of guidance systems which underlie the navigation capabilities previously discussed. These guidance systems are also thought to be shared by other eusocial insects. At present, it is hypothesised that eusocial insects possess two types of guidance systems: memory-based guidance systems and path integration systems. The underlying characteristic of memory-based guidance systems is that they are based on the fact that eusocial insects compare what they are currently experiencing, their current sensory input, with a sensory input that they had previously experienced, a memory or an encoding of a sensory input (Collett, Chittka & Collett, 2013). When moving towards a goal in their environment, this comparison allows eusocial insects to make sure that they are heading in the correct direction. In practical terms, this comparison allows insects to navigate in their environment successfully without getting lost or veering off course.

When a eusocial insect is at a particular goal, such as their nest, for the very first time, they are thought to extract certain characteristics of the visual scene that they are experiencing and commit these to memory (Collett, Chittka & Collett, 2013). This extraction of information is thought to occur during specific phases of their orientation/learning flights. When the insect returns to the goal, they can use their memory of the goal and the objects around it as a direct guide back to the

goal, comparing what they are currently experiencing visually with that of their encoded memory. Such image matching is not limited to objects around a particular goal and could also be used to apply to the panorama of a particular landscape (Collett, Chittka & Collett, 2013). By comparing the current panoramic view with that of a memory of that panorama, a eusocial insect would be able to navigate over large distances, traversing novel terrain, as long as the overall panorama of the landscape remained the same. This kind of panorama matching could also be used by eusocial insects to maintain their position along a habitual route, relying on the overall panorama of the landscape to navigate rather than a variety of individual landmarks at intervals along a route. Overall, such image matching has been studied in a wide variety of social insects from which the above hypotheses have been derived (honeybees: Cartwright & Collett, 1983; Cartwright & Collett, 1987; ants: Wehner & Räber, 1979; Åkesson & Wehner, 2002; Durier, Graham & Collett, 2004; bumblebees: Hempel de Ibarra *et al.* 2009, Baddeley *et al.* 2009; Philippides *et al.*, 2013, wasps: Zeil, 1993; Stürzl *et al.*, 2016).

A second type of guidance system that is thought to run alongside and complement image matching is a form of dead reckoning known as path integration. In this form of navigation, eusocial insects can estimate their whereabouts in their environment by keeping a running tally of the total distance and direction that they have travelled from a starting point, such as the nest (Mittelstaedt & Mittelstaedt, 1982; Collett & Collett, 2000; Wehner & Srinivasan, 2003). This form of navigation would allow eusocial insects to return to their nest in the absence of distinct panorama cues or landmarks, such as when they are exploring a relatively featureless environment. Insects are able to gauge the total distance and direction that they have travelled by using a variety of different methods. Directional information is calculated using the sun's polarisation and azimuth angle (Wehner & Müller, 2006) while the distance travelled is calculated by monitoring the optic flow, the amount that an image appears to move across the retina as the position of the observer moves (Esch & Burns, 1996; Srinivasan *et al.*, 1996; Esch *et al.*, 2001). The distance travelled can also be extracted by monitoring inputs from their own movements, such as the number of steps taken (Wittlinger, Wehner & Wolf, 2006).

The underlying mechanisms that insects use to navigate within their environment are central to the debate of how the spatial memories of insects are actually organised within their brain. This debate centres upon two competing theories. The spatial memories of insects could be linked together, forming an internal representation of their external environment. In this internal representation, locations in the external environment would be related to one another in a common reference frame (Collett & Collett, 2002; Wiener *et al.*, 2011; Wystrach & Graham, 2012; Collett, Chittka & Collett, 2013). Such organisation within the brain is commonly referred to as a cognitive map (Tolman, 1948). Alternatively, insects could be relying on a set of mechanisms, such as the guidance systems previously discussed. In this case, spatial memory would develop in a more passive manner as insects explore their environment (Thomson & Chittka, 2001). It is also thought that landmarks within an environment play different roles depending on the organisation of spatial memories. Within a cognitive map framework, landmarks would inform an insect of their exact position within an environment. Within a guidance system framework, however, landmarks would act primarily as signposts, prompting particular directional actions (Collett & Collett, 2002). In this sense, landmarks could actually serve to segment a route, linking spatial memories, improving navigational accuracy and buffering against mistakes (Collett & Collett, 2002). Different navigational theories have varied suggestions on which objects would be most useful for navigation (reviewed in Chan *et al.*, 2012). Currently, it is not known which physical features or objects within a landscape are used by bumblebees to navigate.

Historically, the benchmark test for whether an animal possessed a cognitive map was whether it was able to compute a novel shortcut back to a known location after being displaced. When honeybees displaced from their nest were seen flying along a novel route towards their familiar feeding station, Gould (1986) concluded that honeybees may indeed possess a cognitive map. However, this conclusion is largely criticised for not acknowledging that visual based guidance systems can also account for novel route taking (Dyer, 1991; Cartwright & Collett, 1983; Wehner, Michel & Antonsen, 1996; Collett & Collett, 2002). This is especially true if the panorama near a particular goal shares sufficient similarities with the panorama of the current environment. In a similar experiment, displaced honeybees were able to return to the nest using a novel shortcut only when they

had been displaced within an open field environment. In contrast, when displaced in within quarry, where the panorama was substantially different from that of the nest, honeybees were no longer able to return (Dyer, 1991). Furthermore, honeybee homing is thought to be enhanced when prominent horizon landmarks are present (Southwick & Buchman, 1995) and that such landmarks can act as beacons for navigation (Pahl *et al.*, 2011). As such, without direct neurophysiological evidence, it is very difficult to distinguish what specific mechanisms underlie eusocial insect navigation when the ability to compute novel shortcuts is considered (Collett & Collett, 2002; Cruse & Wehner, 2011; Cheung *et al.*, 2014).

In addition to the guidance systems discussed, eusocial insects also use celestial and terrestrial cues when navigating (Srinivasan, 2015). Collectively known as compass cues, these cues can provide directional information on an earth wide scale (Able, 2001; Wiltschko & Wiltschko, 2009; Dovey, Kemfort & Towne, 2013). Honeybees, for example, possess at least three different compass mechanisms. Their primary compass mechanism, the sun compass, uses direct light from the sun as well as polarised skylight (von Frish, 1967; Labhart, 1980; Rossel & Wehner, 1986; Wehner, 1997; Kraft *et al.*, 2011). Honeybees are able to detect polarised skylight using a group of specialised ommatidia situated in the dorsal rim area of their compound eyes (Menzel & Snyder, 1974; Labhart & Meyer, 1999). It is theorised that information about the sun's azimuth is combined with polarisation orientation information by special neurons to generate a neural celestial compass (Wehner & Mueller, 2006; Cheung *et al.*, 2014; el Jundi *et al.*, 2014). Behavioural experiments have also shown that honeybees are able to use the information that the polarised skylight provides as a compass cue to navigate (Kraft *et al.*, 2011).

In addition to their compound eyes, bees also possess a number of single lensed eyes known as their ocelli. In honeybees, it is not currently known whether ocelli, like the dorsal rim area of the compound eyes, are also able to detect polarised light (Zeil, Ribi & Narendra, 1994). Behavioural evidence suggests that the ocelli of honeybees are not sensitive to polarised light (Rossel & Wehner, 1984; Wehner & Strasser, 1985). More recent anatomical investigations, however, suggest that honeybee ocelli should be able to detect polarised light (Ribi, Warrant & Zeil, 2011; Ogawa *et al.*, 2017). Anatomical investigations have also

revealed that the ocelli of orchid bees should be able to detect polarised light (Gavin *et al.*, 2016). In bumblebees, Warrington (1974) has shown that the western bumblebee, *Bombus terrestris*, can use its ocelli alone or in conjunction with the tops of its compound eyes to detect and navigate using polarised light. Furthermore, laboratory experiments have shown that bumblebees are able to detect and learn polarisation patterns as indicators of food reward (Foster *et al.*, 2014). As such, polarised skylight seems to be a compass cue used by honeybees, bumblebees and potentially orchid bees during navigation.

Although not studied or demonstrated in bumblebees, honeybees also use a back-up compass system on cloudy days. This system is based on a honeybee's memory of the sun's movements over time in relation to the landscape (Dyer & Gould, 1981; Towne & Moscrip, 2008; Dovey, Kemfort & Towne, 2013). Honeybees also possess a magnetic compass, with research currently focusing on its specific mechanism (Lindauer & Martin, 1968; 1972; Walker & Bitterman, 1985; 1989 a,b; DeJong, 1982; Collett & Baron, 1994; Schmitt & Esch, 1993; Frier *et al.*, 1996; Válková & Vácha, 2012; Lambinet *et al.*, 2017). In bumblebees, a magnetic compass has also been suggested, as bumblebees are able to maintain correct directionality when walking in complete darkness without odour marks or odour cues (Chittka *et al.*, 1999). It should also be noted that although widely studied independently, the interactions of the compass systems and their integration with the guidance systems previously discussed are presently unknown.

## **1.5 The Role of Experience**

In addition to the variety of environmental cues which influence bumblebee behaviour throughout an environment, bumblebee foraging and navigation behaviour changes as workers gain a more in-depth experience of their environment. By the sixth foraging flight outside the nest, individual flight paths straighten, the average groundspeed increases, the maximum displacement distance from the nest increases and constancy to both foraging site and compass bearings upon take-off is observed (Osborne *et al.*, 2013). Traplines between constant foraging sites are established by the 26<sup>th</sup> flight (Lihoreau *et al.*, 2012) while it takes on average 30 flights outside the nest for a worker's foraging

efficiency to reach a plateau (Peat & Goulson, 2005). Similarly, when presenting bumblebees with natural flowers at a set distance in a greenhouse experiment, Raine and Chittka (2007) found that the rate of pollen collection increased throughout the course of a day. Furthermore, preferences for particular flower colours are flexible and change depending on the current foraging environment. In addition, the number of different flowers visited during a foraging trip increases with flight experience while the number of revisits to depleted flowers decreases (Lihoreau *et al.*, 2012). It is not known, however, how forage availability and landscape structure interact with experience with regard to foraging rate.

Although naïve bumblebee workers seem to improve their foraging and navigation skills with experience, memory retention in bumblebees is not always perfect. For example, when Keasar *et al.*, 1996 trained naïve *Bombus terrestris* workers to forage from artificial flowers with unequal profitabilities, the bumblebees learned to approach and probe profitable flowers faster as they gained experience foraging throughout the course of a day. The foraging performance of bumblebees decreased, however, following an overnight break in testing. Heinrich (1977) observed similar results using *Bombus ternarius* and *Bombus terricola*: bumblebees increased their percent of rewarding flower choices over consecutive foraging trips during the day but decreased their performance overnight. In their investigations on the rate of pollen collection, Raine & Chittka (2007) also observed a decrease in foraging performance following an overnight break. In contrast, Dukas & Real (1991) found no reduction in the proportion of visits to rewarding flower species from the end of the first day of testing to the beginning of the second day of testing. Whether or not a bumblebee's memory is significantly affected following an overnight break may depend on the frequency that it performed a particular task throughout the day or the nature of that task. For example, when trained bumblebees were given at least 400 trials of an associative task, no decline in overnight performance was observed (Chittka & Thomson, 1997). Similarly, Chittka (1998) only observed a decline in memory recall of a sensorimotor task when bumblebees were tested after a delay of several weeks. Overnight memory retention tests also show that individual bumblebees that are faster at initially learning an association are also better at retaining this information and can also reverse learnt associations more readily (Raine & Chittka, 2012). The type of information may also affect memory

retention. Naïve bumblebees seem to retain social information significantly better than non-social information for up to a period of eight hours (Abts, 2016). Lastly, pesticide exposure can also significantly affect the learning and memory capabilities of bumblebees. Following chronic pesticide exposure, bumblebees that were exposed to field realistic levels of thiamethoxam were slower to learn odour associations and had their short-term memory significantly impaired (Stanley, Smith & Raine, 2015).

Bumblebee workers are often seen returning to the nest with forage on their very first flight (Hempel de Ibarra, *et al.*, 2009; Osborne *et al.*, 2013), suggesting that, unlike honeybees, they do not have completely separate flights for exploration and foraging. There is not enough evidence currently, however, to establish whether such dual-purpose flights outside the nest are typical throughout a worker's lifetime or whether they are concentrated within a knowledge acquisition phase at the beginning of a worker's foraging career.

A substantial amount of research has investigated the different navigation and foraging behaviours, as well as the mechanisms which underlie them, that bumblebees employ when exploring their surrounding environment. Much less is known, however, of the effects that the physical environment has on bumblebee behaviour.

Evidence suggests that particular landscape features, both natural and man-made, seem to affect bumblebee flight (Cranmer, McCollin & Ollerton, 2012; Bhattacharya, Primark & Gerwein, 2003). Further support comes from analysing the flight paths of honeybees which were tracked with harmonic radar. Honeybees were observed to preferentially follow contrasting ground features, such as paths, roads and field margins when placed both in novel and familiar environments (Riley *et al.*, 2003; Degen *et al.*, 2015; Menzel *et al.*, 2019). Honeybees also use the skyline to navigate (Towne *et al.*, 2017) as well as prominent landmarks to visually pinpoint the location of a goal (Cartwright & Collett, 1982; Dyer, 1996). Given the existing evidence from honeybee studies, landscape features, in particular linear landscape features such as hedgerows and roads, could strongly determine the overall movement of bumblebee workers throughout a landscape. As a result, such features could have a strong influence

on the formation of the habitual foraging routes of bees more generally (Collett & Graham, 2015).

As such, the underlying question that this thesis aims to tackle is: what effects does the physical environment have on bumblebee navigation and foraging behaviour and what role does a bumblebee's individual experience play in shaping this behaviour?

## **1.6 Thesis Objectives**

The main objectives of this thesis are:

- To find the effect that landscape features have on the flight paths and foraging choices of bumblebee workers
- To find how experience affects the development of pollen foraging behaviour in bumblebees, specifically in terms of their flight durations and amount of pollen foraged
- To find whether homing success is landscape and/or experience dependent (using homing as a proxy for foraging range)

## **1.7 Overview of Data Chapters**

In order to address the objectives listed in the previous section, a series of experiments were conducted. These experiments and their results are presented in Chapters Two to Six.

In the experiment presented in Chapter Two, the effects that hedgerows, a common-place landscape feature, have on shaping the flight paths and foraging choices of bumblebees was investigated. Previous work has found that wild bumblebees are more likely to fly parallel to hedgerows the closer they are to them (Cranmer, McCollin & Ollerton, 2012). It is not known, however, what effects hedgerows may have on naïve bumblebees who have never previously foraged. Commercial bumblebee colonies were placed on either side of a hedgerow, either on the same side, or on the opposite side, as a mass flowering crop. Bumblebee workers were individually marked using numbered tags. The vanishing bearings of bumblebees exiting the colony (the compass bearing at the moment when the bumblebee vanished from human sight, as described in Dyer (1991)), were recorded in order to investigate the effects of hedgerows on the initial flight paths of bumblebees. The pollen that individual bumblebees returned with in their



corbiculae (pollen baskets) was also analysed and the plant species from which it originated was identified. By matching this to the known location of different foraging sources within the landscape, it was possible to investigate the role that hedgerows may play in shaping the foraging choices of individual bumblebees.

In Chapter Three, the focus shifted to the role that experience has on bumblebee flight and foraging behaviour. Specifically, changes in the trip duration and the weight of pollen foraged throughout an individual bumblebee's first five flights outside the colony were investigated. By tracking changes at the level of an individual bumblebee, the experiment presented in this chapter also shed light on the variability that exists between the workers within a bumblebee colony.

In Chapter Four, the effect that different landscape types may have on bumblebee navigation and foraging was investigated in urban and rural environments. Previous experimental work investigating this has focused on the homing abilities of *Bombus terrestris*, albeit in a single landscape (Goulson & Stout, 2001). In order to build on Goulson & Stout's work, Radio Frequency Identification (RFID) tagged bumblebees were displaced at 300 m, 1000 m and 2500 m from their colony after a period of habituation within the surrounding environment of their nests. Successful homing from a particular distance can be seen as a proxy for familiarity with that area and by extension, habitual foraging range (Greenleaf *et al.*, 2007; Chapter Four: Section 4.2: Introduction). As such, this experiment was conducted in order to shed light on the effects that different landscapes may have on the homing abilities and, by extension, the foraging ranges of bumblebees.

The experiment presented in Chapter Five followed up on the results presented in Chapter Three and Chapter Four by focusing on the role that experience plays in the homing ability of bumblebees. Conducted in a single landscape and using a slightly modified experimental set-up, marked bumblebees were released from 300 m and 1000 m from their colonies. In this experiment, the amount of experience that individual bumblebees had prior to release was manipulated and bumblebees were given one, two or five flights outside their colony. In this way, it was possible to investigate how familiarity with the surrounding landscape develops within the first five flights.

The first four data chapters all focused on the navigation and foraging behaviour of bumblebee workers. In Chapter Six, following field observations, the foraging

behaviour of bumblebee gynes was investigated. It is not clear from previous research whether gynes habitually provision their colony before leaving it to mate and hibernate or whether this behaviour only occurs during periods of nutritional shortages (Chapter Six: Section 6.2: Introduction). As such, in order to investigate the potential underlying causes of this behaviour, the amount of pollen that was entering a bumblebee colony was manipulated and the subsequent behaviour of a colony's gynes was observed. The evolutionary implications of such behaviour are also discussed.

## Chapter Two

### The Role of Hedgerows in *Bombus terrestris* Navigation and Foraging Behaviour

#### 2.1 Abstract

Living in an environment where the spatial distribution of resources is always changing, bumblebees rely on a variety of different environmental cues to help guide their navigation and foraging. Among these environmental cues are physical features of the landscape. Although not widely studied, they have the potential to affect bumblebee behaviour. These include prominent land masses, forests and even man-made structures. In lowland agricultural environments, hedgerows form a predominant and widespread linear landscape feature. Although bumblebees are more likely to fly parallel to hedgerows the closer they are to them, the effect of hedgerows on the flight paths and subsequent foraging behaviour of naïve bumblebees is unknown. To investigate the potential effects of hedgerows on naïve bumblebees, *Bombus terrestris* colonies were placed on either side of a hedgerow, either on the same side, or on the opposite side, as a mass flowering crop in three agricultural sites throughout South West England. Naïve workers from each colony were individually tagged and allowed to forage freely. The vanishing bearing of each bumblebee when it first exited the colony was recorded and any foraged pollen that it returned with was identified. Bumblebees were not more likely to fly alongside the hedgerow adjacent to their colony nor were they more likely to fly towards the mass flowering crop. The hedgerow did not have a significant effect on the pollen that bumblebees foraged for across the two colonies, either on their first flight or on subsequent flights. The results suggest that hedgerows near the colony do not significantly shape the flight paths or foraging choices of naïve bumblebees. When flowering, however, hedgerows are an important food source for bumblebee workers that will be utilised despite flowering crops present within their vicinity.

## 2.2 Introduction

As central place foragers, bumblebees gather pollen and nectar from flowers within their surrounding environment and bring it back to a central nest (Plowright & Lavery, 1984; Goulson, 2010). Bumblebees live in an environment where their rewarding food sources vary spatially as well as temporally throughout the day and the flowering season. As such, a multitude of different environment cues are thought to play a role in initially attracting bumblebees to specific foraging sources. At present, a substantial amount of research has investigated the different navigation and foraging behaviours, as well as the mechanisms that underlie them, that bumblebees employ when exploring their surrounding environment. This research will be subsequently discussed throughout this introduction. However, much less attention has been given to the effects that specific aspects of the physical environment have on shaping bumblebee flight and foraging behaviour.

Although little research thus far has focused on the effects of the physical environment on bumblebee flight and foraging behaviour, bumblebees are known to use a variety of different navigation and foraging strategies. For instance, when a bumblebee first leaves its nest or a rewarding food source, it performs a distinct behaviour, flying in a series of arcs and loops which increase in area as it flies off. Observed in ants, honeybees and wasps, this behaviour is collectively known as orientation or learning walks/flights (Collett & Zeil, 1996; Baddeley *et al.*, 2009; Hempel de Ibarra *et al.*, 2009; Phillipides *et al.*, 2013; Robert *et al.*, 2017; solitary wasps: Collett & Lehrer, 1993; wood ants: Nicholson *et al.*, 1999; desert ants: Müller & Wehner, 2010; honeybees: Cartwright & Collett, 1983). It is the primary mechanism by which these insects learn the location of important goals and gain knowledge of their surroundings.

Once outside the nest, a variety of visual, olfactory, electromagnetic and social cues are thought to influence the foraging choices of bumblebees. Visual attractants can manifest at various scales. At the landscape scale, the density of flowers within a foraging patch can act as an attractant (Comba, 1999) but this attraction may actually be species dependent (Kwak, 1987; Sowig, 1989). Although some studies have observed a positive relationship between plant visitation rate and plant density in a patch (Klinkhamer & de Jong, 1990; Kunin,

1997; Ohashi & Yahara, 2002; Grindeland, Sletvold & Ims, 2005), different bumblebee species seem to be affected by plant abundance in different ways (Stout, Allen & Goulson, 1998) making it difficult to generalise across the entire bumblebee spectrum. What is known is how flower patches in a landscape are detected by a bumblebee's visual system. Bumblebee vision, and insect vision more generally, is constrained by the poor optical resolution of the insect compound eye (Hempel de Ibarra, Langridge & Vorobyev, 2015). Compound eyes, unlike single lens eyes, are not able to focus on objects at different distances. This means that compound eyes have the same angular resolution across different viewing distances. Whether or not an insect can detect a distant target, and resolve it using its compound eyes, depends on the visual angle that the target subtends on the insect's eye (Giurfa & Lehrer, 2001). This angle depends on both the size of the target and the distance of the target from the insect. Depending on the distance of the target from the insect, and thus the visual angle of the target, different signals are used by the insect to visually detect the target. In bees, the visual angle of a target governs whether the bee is able to use chromatic or achromatic cues in order to detect the target (Giurfa & Lehrer, 2001). Honeybees can only use chromatic cues to detect a target if that target subtends a visual angle that is less than 13-15° (Giurfa *et al.*, 1997; Hempel de Ibarra, Giurfa & Vorobyev, 2002). This means that a honeybee would need to be at a viewing distance of 11 centimetres in order for it to detect a one-centimetre diameter flower using chromatic cues alone (Hempel de Ibarra, Langridge & Vorobyev, 2015). In bumblebees, chromatic cues, and thus colour vision, functions only when a target subtends a visual angle that is less than 2.7° (Dyer, Spaethe & Prack, 2008). This suggests that floral colour is a relatively short distance visual cue in both honeybees and bumblebees. When a target has a visual angle that is greater than the chromatic threshold, bees seem to use achromatic cues instead (Giurfa *et al.*, 1996; 1997; Hempel de Ibarra, Giurfa, Vorobyev, 2001; 2002; Chittka & Raine, 2006). This suggests that characteristics of a floral patch such as size, brightness and contrast with the surrounding environment are the long-distance cues that bees use when detecting floral targets.

When foraging for nectar within a flower patch, Cartar (2004) found that plants with higher nectar production rates attracted more bumblebees and had more of their flowers visited. He suggests two non-mutually exclusive ways in which bumblebees can determine which flowers have the highest secretion rates: the 'local experience hypothesis' and the 'memory hypothesis'. In the 'local experience hypothesis', based on the ideas of a foraging model proposed by Best & Bierzychudek (1982), bumblebees can sample flowers from different plants and visit more flowers at plants that offer higher rewards. By visiting more flowers at plants that offer higher rewards, bumblebees can respond to current environmental conditions, exploiting richer patches and ultimately increase their foraging gains. In the 'memory hypothesis', bumblebees can remember the individual plants that were the most rewarding on previous foraging trips and return preferentially to these plants on future foraging trips. In other words, bumblebees can use their past experiences to preferentially exploit richer patches and once again, increase their foraging gains. Cartar's (2004) results support both of his proposed hypotheses. Furthermore, Cartar (2004) suggests that his results may point to the different foraging strategies employed by naïve and experienced bumblebee foragers. Naïve foragers would be more likely to use a plant's size and flower age/stage when choosing which plants to visit. They should also use a plant's nectar standing crop in order to decide how many flowers to sample before moving on to the next plant. In contrast, experienced foragers have the additional knowledge gained from past foraging trips. When plants are cryptic, experienced foragers should discount cues such as plant size and flower age/stage and instead assess plants based on previous experience in order to maximise their foraging gains.

Furthermore, experienced foragers may also use the scent-marks left on flowers by previous foragers when assessing whether or not to probe a particular flower (Cameron, 1981; Schmitt, Lubke & Francke, 1991; Goulson, Hawson & Stout, 1998; Goulson *et al.*, 2000). Bumblebees secrete a substance from their tarsus which consists of a mixture of scented hydrocarbons (Schmitt, 1990; Goulson *et al.*, 2000; Jarau *et al.*, 2012). These secretions are thought to help bumblebees adhere to surfaces and reduce desiccation (Lockey, 1988). As bumblebees seem to deposit these secretions whenever they land on a surface (Schmitt, Lubke & Franke, 1991; Saleh *et al.*, 2007), scent-marks can act as cues for other foragers.

When presented with a choice of flowers that either had scent-marks deposited on them or not, naïve bumblebees do not exhibit an innate preference or avoidance behaviour for either type of flower (Leadbeater & Chittka, 2011). This suggests that rather than acting as signals, scent-marks are used as associative cues by experienced foragers (Leadbeater & Chittka, 2011). As a depleted flower will contain the scent-marks of its recent forager(s), bumblebees can use scent-marks on flowers as indicators of floral reward (Goulson, Hawson & Stout, 1998; Stout, Goulson & Allen, 1998; Stout & Goulson, 2001; Goulson, Chapman & Hughes, 2001; Stout & Goulson, 2002). In addition to detecting their own scent-marks and those of nest-mates and conspecifics, bumblebees are able to use the scent-marks of heterospecifics to guide their foraging choices (Goulson, Hawson & Stout, 1998; Grawle, Zimmermann & Eltz, 2005; Reader *et al.*, 2005; Ballantyne & Wilson, 2012; Pearce, Giuggioli & Rands, 2017). Furthermore, Stout & Goulson (2002) found that the duration of repellence of scent-marks is inversely correlated to the rate of nectar secretion in different floral species. The use of scent-marks in bumblebee foraging has thus has the potential to significantly improve foraging efficiency (Williams, 1998).

Once within the range of colour detection, floral colour is also thought to play a significant role in attraction (Dyer & Chittka, 2004). The photoreceptors of bees more generally have spectral sensitivities which peak around 350, 450 and 550 nm and these peaks correspond to the UV, blue and green regions of the colour spectrum respectively (Peitsch *et al.*, 1992). Bumblebees also seem to prefer flowers with symmetrical shapes (Rodriguez *et al.*, 2004). In addition to floral colour, floral odour is also an important attractant of bumblebees to different flower species which can function on many spatial scales (Raguso, 2008). Floral odour can be broadly defined as the bouquets of volatiles that flowers emit (Kunze & Gumbert, 2001). These volatiles constitute hundreds of different compounds which, in turn, give most floral scents a unique composition (Dobson, 1994). Furthermore, the different parts of a flower in a wide range of species show differences in the quantity and diversity of the floral scents that they produce (Pichersky *et al.*, 1994; Bergström, Dobson & Goth, 1995; Flamini, Cioni & Morelli, 2002; Dötterl & Jürgens, 2005). For example, Dobson, Danielson & Van Wesep (1999) found that bumblebees which forage on *Rosa rugosa* primarily use the scent of the plant's pollen coupled with visual stimuli from the stamens, and

secondarily use the scent and colours of a flower's petals. Bumblebees are also able to learn and distinguish between the different spatial arrangements of the same scent (Lawson *et al.*, 2018). Studies have also shown that floral scent can facilitate bumblebee learning. For example, *Bombus impatiens* foragers have been shown to learn coloured artificial flowers more quickly and accurately when they are scented (Kulahci, Dornhaus & Papaj, 2008). More generally, bumblebees seem to be better at object discrimination when they are presented with multimodal cues, for example a visual cue paired with an olfactory cue (Kunze & Gumbert, 2001; Gegear & Lavery, 2005). Floral scent also seems to play a role within the nest. Although bumblebees do not appear to communicate the location of rewarding food sources to their nest mates as honeybees do, the scent of predominant pollens in a bumblebee nest may encourage pollen gatherers to seek them (Free, 1955). Laboratory experiments have also found that bumblebees prefer the odours of flowers that are brought back into the nest by returning foragers (Dornhaus & Chittka, 1999). Floral scents, especially when part of multisensory cues, seem to play an important role in guiding the foraging behaviour of bumblebees.

A relatively recent discovery has been that bumblebees also use electromagnetic cues when foraging (Clarke *et al.*, 2013; Sutton *et al.*, 2016). Flying insects, including pollinators such as bees, were theorised to carry a positive electrostatic charge (Erickson, 1975; Yes'kov & Sapozhnikov, 1976; Vaknin *et al.*, 2000). This is due to the fact that as bees fly through the air, they collide with charged particles in the atmosphere. Such collisions strip electrons from the bee's surface, resulting in its body having a positive electric potential. In contrast, flowers were theorised to exhibit a negative electric potential (Corbet, Beament & Eisikowitch, 1982; Bowker & Crenshaw, 2007). This potential difference between a bee's body and a flower would result in the generation of an electric field. In practical terms, this electric field could promote pollen transfer and adhesion between a flower's reproductive organs and a bee's body (Corbet, Beament & Eisikowitch, 1982; Erikson & Buchmann, 1983; Gan-Mor *et al.*, 1995; Vaknin *et al.*, 2000). Bumblebees have been shown to be able to detect electromagnetic fields (Clarke *et al.*, 2013) using mechanosensory hairs on their bodies (Sutton *et al.*, 2016). Specifically, Clarke *et al.* (2013) have shown that bumblebees are able to discriminate between the electromagnetic fields of flowers with different



geometries. In their study, bumblebees were able to assess floral rewards and discriminate between different flowers using electromagnetic fields. Furthermore, the combination of two floral cues, in this case the electromagnetic field of a flower and its hue, enhanced a bumblebee's ability to discriminate between different flowers. The results of these studies suggest that electric fields form part of the multimodal floral cues used by foraging bumblebees (Clarke, Morley & Robert, 2017).

Studies also suggest that social cues, in the form of other bees, also influence the foraging choices of individual bumblebees. Naïve bumblebees that had never foraged before were more likely to land on flowers that were occupied by other bumblebees (Kawaguchi, Ohashi & Toquenaga, 2006; Leadbeater & Chittka, 2009) or even honeybees (Dawson & Chittka, 2012). Specifically, foraging-naïve bumblebees seem to be innately attracted to the particular combination of colour, shape and odour of a conspecific (R.Herascu, unpublished). Such behaviour suggests that workers are attuned to social cues when they have no previous knowledge of a particular flower type, taking advantage of the knowledge of the more experienced bees around them. In contrast, experienced bumblebees have been found to avoid landing on flowers that were occupied by other bumblebees (Kawaguchi, Ohashi & Toquenaga, 2007). As such, social information use in bumblebees is flexible and very much context dependent (Plowright *et al.*, 2013).

In addition to specific floral cues in the environment, the spatial arrangement of foraging patches within the landscape and their distance from a bumblebee's nest also affects foraging behaviour. If foraging patches are distributed relatively homogeneously within the landscape, then optimum foraging theory predicts that bumblebees should forage close to the colony, in order to minimise their travel costs (Heinrich, 1979a). Various studies, however, have observed bumblebees not to forage within very close proximity of their nest despite rewarding forage being available (Hobbs *et al.*, 1961; Dramstad, 1996; Saville *et al.*, 1997; Osborne *et al.*, 1999; Walther-Hellwig & Frankl, 2000; Osborne *et al.*, 2013). Furthermore, the proximity varies between different species of bumblebees. For example, using a mark-recapture study, Dramstad (1996) found that the majority of *Bombus terrestris* workers were foraging at least 50 metres away from their nest. Similarly, Walther-Hellwig & Frankl (2000) found that the majority of *Bombus terrestris* workers were foraging at least 500 metres away from their nest. Using

harmonic radar to track *Bombus terrestris* workers, Osborne *et al.* (1999) found that the flight tracks of workers ranged between 70 and 631 metres. For *Bombus lucorum* workers, Saville *et al.* (1997) did not observe any workers foraging within 250 metres from their nest. For *Bombus lapidarius* workers, Walther-Hellwig & Frankle found the majority to forage within 500 metres of their nest while for *Bombus muscorum*, the authors found that the majority of workers foraged within 100 metres from their nest. Foraging patches are rarely distributed homogeneously within the landscape and foraging further away from the nest could prove adaptive by decreasing the risk of predation, parasitisation and competition (Dramstad, 1996). Furthermore, a food source's quality, in terms of its nutritional rewards, will also come into play. A particularly rewarding food source can offset the costs of long-distance travel or the risks of short-distance foraging. Further evidence also suggests that the foraging range of bumblebees is species dependent (Kreyer *et al.*, 2004; Westphal *et al.*, 2006; Greenleaf *et al.*, 2007). As such, the distance that bumblebees will forage from their nest is likely an interplay between a multitude of different factors which include colony need, the spatial arrangement, quality and quantity of the foraging sources within the landscape, and the bumblebee species itself.

Large physical landscape features, such as forests, have been found to influence bumblebee flight. Interestingly, their effect seems to be species dependent. When profitable food sources were laid out within the forest canopy as well as in the open fields adjacent to it, Kreyer *et al.* (2003) found that bumblebee workers exploited the different foraging patches in a species-specific manner. In this case, *Bombus terrestris* workers were only observed foraging in open fields on either side of the forest, suggesting that workers of this species preferentially fly over or around the forest canopy. By contrast, *Bombus pascuorum* workers were observed foraging both within the forest canopy as well as in the adjacent open fields. Furthermore, man-made structures, such as roads and railways, were also found to have an effect on bumblebee flight. Although workers were able to cross these structures in search of rewarding food sources, when given a choice, they preferred to forage alongside these structures rather than to cross them (Bhattacharya, Primark & Gerwein, 2003).

Smaller physical landscape features are also thought to have an effect. At its simplest, any individual object which protrudes above the herb layer has the

potential to divert the flight path of a foraging bumblebee. Bumblebees do not tend to fly over and above individual objects, but rather approach and deviate around them (Plowright & Galen, 1985). In lowland agricultural landscapes, the predominant visual cue from an insect's perspective is hedgerows (Burel, 1996, Barr & Gillespie, 2000). Hedgerows are known to affect the wind speed and air flow in their vicinity (Rider, 1952) and they have been found to influence the flight paths of various pollinators including butterflies (Fry & Robson, 1994; Dover & Fry, 2001), moths (Merckx *et al.*, 2010; Coulthard, McCollin & Littlemore, 2016) and bumblebees (Cranmer, McCollin & Ollerton, 2012). Hedgerows do not seem to pose a barrier to bumblebee movement (Krewenka *et al.*, 2011) and bumblebees have been found to fly parallel to hedgerows the closer they are to them (Cranmer, McCollin & Ollerton, 2012). It is hypothesised that insects are largely observed flying in the vicinity of hedgerows due to underlying physical causes (Lewis & Dibley, 1970), and that this behaviour may be driven by the fact that insects have greater flight control in areas with reduced windspeed (Pasek, 1988).

Hedgerows are also known to affect the pollen flow within a particular landscape. A greater number of pollen grains are deposited on the stigma of flowers which are highly connected by hedgerows and the effect is seen in hedgerows which are both natural (Klaus *et al.*, 2015) and manmade (Cranmer, McCollin & Ollerton, 2012). This suggests that hedgerows may somehow direct pollinators to preferentially forage in highly connected flower patches. As the effect of hedgerows varies across pollinator species, however, their effects may not always be positive and highly dense and tall hedgerow networks have been found to negatively impede pollen flow in some plant species by creating a physical barrier for pollinators (Campagne *et al.*, 2009).

Taken together, the results of previous studies suggest that particular linear landscape features such as hedgerows could influence the pollen distribution in a landscape by affecting the overall flight paths of pollinators. As bumblebees are known to develop constancy to particular foraging patches (Heinrich, 1979a; Goulson, 2010), hedgerows may play a significant role in the formation of their habitual foraging routes (Collett & Graham, 2015). A further suggestion of this potential effect comes from analysing the flight paths of honeybees which were tracked using harmonic radar. Honeybees were observed to preferentially follow

ground features, such as paths, roads and field margins when placed in both novel and familiar environments (Riley *et al.*, 2003; Degen *et al.*, 2015; Menzel *et al.*, 2019).

Furthermore, models of pollinator foraging behaviour are currently being used to estimate the spatial patterns of pollination (wild bees: Lonsdorf *et al.*, 2009; Olsson *et al.*, 2015; honeybees: Becher *et al.*, 2014; bumblebees: Becher *et al.*, 2018). The foraging rules which govern the assumptions used in such models are based on the current knowledge of bee behaviour, specifically in terms of flight paths taken, foraging ranges, and their trade-offs with energy requirements. At present, however, the models do not take into account the physical features of the landscape and their potential effects on flight and foraging behaviour. As such, this experiment was conducted in order to investigate the effects that a hedgerow may have on the flight paths of naïve bumblebees and subsequent foraging behaviour on a mass flowering crop. Specifically:

1. Does the presence of a hedgerow affect the direction in which bumblebees fly for their first flight?
2. Does the presence of a hedgerow affect whether bumblebees fly towards the nearest mass flowering crop providing nectar and pollen?
3. Does the presence of a hedgerow affect the proportion of bumblebees returning to their colony with mass flowering crop pollen on their first and subsequent flights?
4. Does increasing the distance of a colony from the mass flowering crop reduce the proportion of bumblebees returning with mass flowering crop pollen?

In order to explore these questions, bumblebee colonies were placed on the ground, adjacent to hedgerows in three agricultural sites in the South West of England. These sites all had fields bordered by hedgerows where the predominant foraging resource was a mass flowering crop. Colonies were placed on either side of a hedgerow, either on the same or the opposite side as the mass flowering crop. Furthermore, the hedgerows used were located at various distances from the mass flowering crop. Although the flight paths of bumblebees have been tracked directly using technologies such as harmonic radar (Riley *et al.*, 1996; Osborne *et al.*, 1999; Woodgate *et al.*, 2016), one of the radar's

requirements is that it is operated in a landscape without physical obstructions due to signal interference (Goulson & Osborne, 2006). As the presence of hedgerows in the landscapes chosen here invalidates this requirement, different methods to investigate the flight paths and foraging choices of bumblebees were employed instead. Firstly, in order to investigate the initial effects of the hedgerow on the flight paths of bumblebees, the vanishing bearings, or the compass bearing at the moment when a bumblebee vanishes from human sight, were taken. This is a method that is commonly used in studies of insect orientation (Gould, 1986; Dyer, 1991, 1993; Dyer *et al.*, 1993). Secondly, the experimental sites chosen for this experiment had a relatively simple foraging landscape. At all the study sites used, there was only one mass flowering crop, the species of which was known to attract bumblebees, within a 900 m radius of the hedgerow under investigation. Depending on the study site, a number of different flower species were also flowering, either at the base of the hedgerows, in the green lanes around the colony, or within the hedgerows themselves. As such, the effects of the hedgerow on the foraging choices of bumblebees could be investigated by analysing and identifying the pollen loads of bumblebees returning to their colonies. Specifically, whether bumblebees foraged on the mass flowering crop, and whether the hedgerow had an effect on this.

If bumblebee workers first engage in exploration flights before beginning to forage on a particular floral patch and if they do so while flying above the hedgerows in the landscape, then the side of the hedgerow that the colony is located on should not have a significant effect on whether or not they forage on the mass flowering crop. Radar tracking studies have found tagged *Bombus terrestris* workers to fly between one to three metres above the ground (Osborne *et al.*, 1999) and *Bombus terrestris* workers are suspected of being able to fly above the forest canopy (Kreyer *et al.*, 2003). Whether bumblebee workers habitually fly above hedgerows, however, remains unknown.

If, on the other hand, bumblebee workers do not normally fly above the hedgerows in the landscape; and if the immediate sensory cues upon leaving their colony are largely shaping their flight paths and subsequent foraging choices; then the side of the hedgerow that the colony is placed on should have a significant effect on both their flight paths and their foraging choices. In this scenario, the presence of the hedgerow would present a different landscape

panorama for workers on either side of it. In practical terms, for colonies that are not on the same side as the mass flowering crop, the hedgerow would block their direct line of sight to the crop as they first exit their colony. This, in turn, could reduce the number of bumblebees that will forage on this crop. Furthermore, as hedgerows affect the air flow in their vicinity (Rider, 1952), they have the potential to change the odour composition of their immediate surroundings. As floral odour forms part of the multisensory cues that bumblebee workers will use to make foraging choices (Leonard, Dornhaus & Papaj, 2011); and given that floral odours are a relative long-distance signal (Dukas & Real, 1993; Sprayberry, 2018); differences in the odours in a colony's vicinity may influence the subsequent foraging choices of bumblebee workers in that colony.

Our present knowledge of bumblebee behaviour, however, does not allow us to tease these two different hypotheses apart.

In order to try to isolate the effects that the hedgerow may have on the foraging patterns of bumblebees, the effect of the distance of the colonies from the mass flowering crops was also investigated. Additional colonies were placed within the mass flowering crop itself. As bumblebee workers have been previously found to forage on a mass flowering crop the closer their colony is situated to it (Osborne *et al.*, 2008), it was hypothesized that workers from colonies within the mass flowering crop field would be more likely to forage on the mass flowering crop than those which were placed further away.

## 2.3 Materials and Methods

### 2.3.1 Experimental Sites

The experiment took place on one arable and two mixed farm sites in the South West of England.

#### *Site One*

The experimental Site One was made up of eight adjacent fields, six of which had flowering oilseed rape (*Brassica napus*) in them (Figure 2.1). The six flowering crop fields measured approximately 150 m in an east-west direction and 11000 m north-south. The total area of the mass flowering crop was approximately 19 ha, providing a huge area of available nectar and pollen. East of the most southern field which made up the crop area was a hedgerow which measured approximately 250 m in an east-west direction. The height of the hedgerow was approximately 2.50 m.

A *Bombus terrestris audax* colony (Koppert Biological Systems, UK) was placed on each side of this hedgerow (Figure 2.1: Colonies A and B). The distance from the colonies to the nearest corner of the mass flowering crop field was 65 m. The colonies were tested between 17<sup>th</sup>-20<sup>th</sup> May 2016.<sup>1</sup> The colonies were not tested on 18<sup>th</sup> May 2016 due to inclement weather. The six oilseed rape fields were the only mass flowering crop within a 900 m radius of the colonies (determined by matching current farm records with satellite imagery). In addition, during the testing period, the hedgerows in the landscape contained hawthorn (*Crataegus monogyna*), which was in full bloom.

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<sup>1</sup> A second period of testing also took place between 23<sup>rd</sup>-27<sup>th</sup> May 2016. However, the data gathered during this period of testing was not comparable and as such, could not be used. More information regarding this can be found in Appendix A.



**Figure 2.1** | Experimental Site One with *Bombus terrestris* colony placement (A-B). The black bordered grey areas represent the fields of oilseed rape (four shown here). The white border represents the hedgerow. 'A' and 'B' represent the individual colonies used. Copyright information: Google Earth V 7.3.2. (June 22, 2018). Eye altitude: 769 m. <https://www.earth.google.com> [December 15, 2018].



## Site Two

The experimental Site Two was made up of two adjacent fields, one of which had flowering red clover (*Trifolium pratense*) (Figure 2.2). The flowering red clover field measured approximately 150 m in an east-west direction and 120 m north-south. The total area of the mass flowering crop was approximately two hectares. The southern edge of the field was bordered by a hedgerow whose height was approximately 2.50 m.

Four *Bombus terrestris audax* colonies (Koppert Biological Systems, UK) were used. At this study site, two colonies were placed on each side of the hedgerow described above (Figure 2.2). As the hedgerow under investigation bordered the mass flowering crop, the distance from the colonies to the nearest edge of the mass flowering crop field was 0.50 m. All colonies were tested simultaneously between 16<sup>th</sup> -19<sup>th</sup> August 2016. The red clover field was the only mass flowering crop field within a 900 m radius of the colonies (determined by matching current farm records with satellite imagery). In addition, during the testing period, a variety of wildflowers were blooming in the hedge banks and green lanes in the landscape around the colonies.



**Figure 2.2** | Experimental Site Two with *Bombus terrestris* colony placement (C-F). The black bordered grey area represents the red clover field. The white border represents the hedgerow. 'C', 'D', 'E' and 'F' represent the individual colonies used. Copyright information: Google Earth V 7.3.2. (June 22, 2018). Eye altitude: 1060 m. <https://www.earth.google.com> [December 15, 2018].

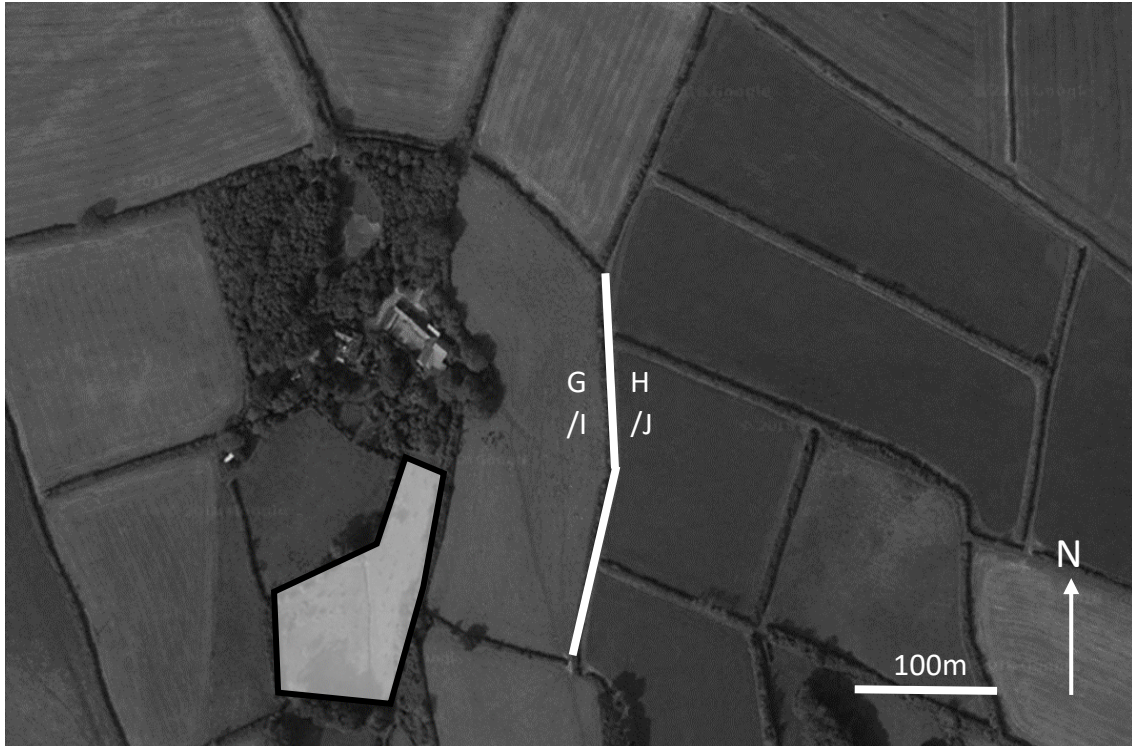
### Site Three

The experimental Site Three was made up of three adjacent fields, one of which was an orchard (Figure 2.3). In the orchard, apple, cherry and plum trees were flowering (*Rosaceae* spp.). Along the orchard, Norway Maple trees were also flowering (*Acer platanoides*). The orchard measured approximately 62 m in an east-west direction and 155 m north-south with an approximate total area of 0.80 ha. Approximately 100 m east of the orchard was a hedgerow with a length of approximately 210 m running in a north-south direction. The hedgerow had a height of approximately two metres.

Four *Bombus terrestris audax* colonies (Koppert Biological Systems, UK) were used in total. At this experimental site, a colony was placed on either side of the hedgerow described above (Figure 2.3) and only two colonies were tested at any one time. The first set of colonies was tested between 24<sup>th</sup>-27<sup>th</sup> April 2017 while the second set of colonies was tested on the 28<sup>th</sup> and 29<sup>th</sup> April 2017 and the 2<sup>nd</sup> and 3<sup>rd</sup> May 2017.<sup>2</sup> The flowering trees in the orchard were the only mass flowering resource within a 900 m radius of the colonies (determined by matching current farm records with satellite imagery). In addition, during the testing period, gorse (*Ulex* spp.) and blackthorn (*Prunus spinosa*) plants were flowering in the hedgerows and on the coastal slopes in the landscape around the colonies.

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<sup>2</sup> Colony I was not tested on 3<sup>rd</sup> May 2017.



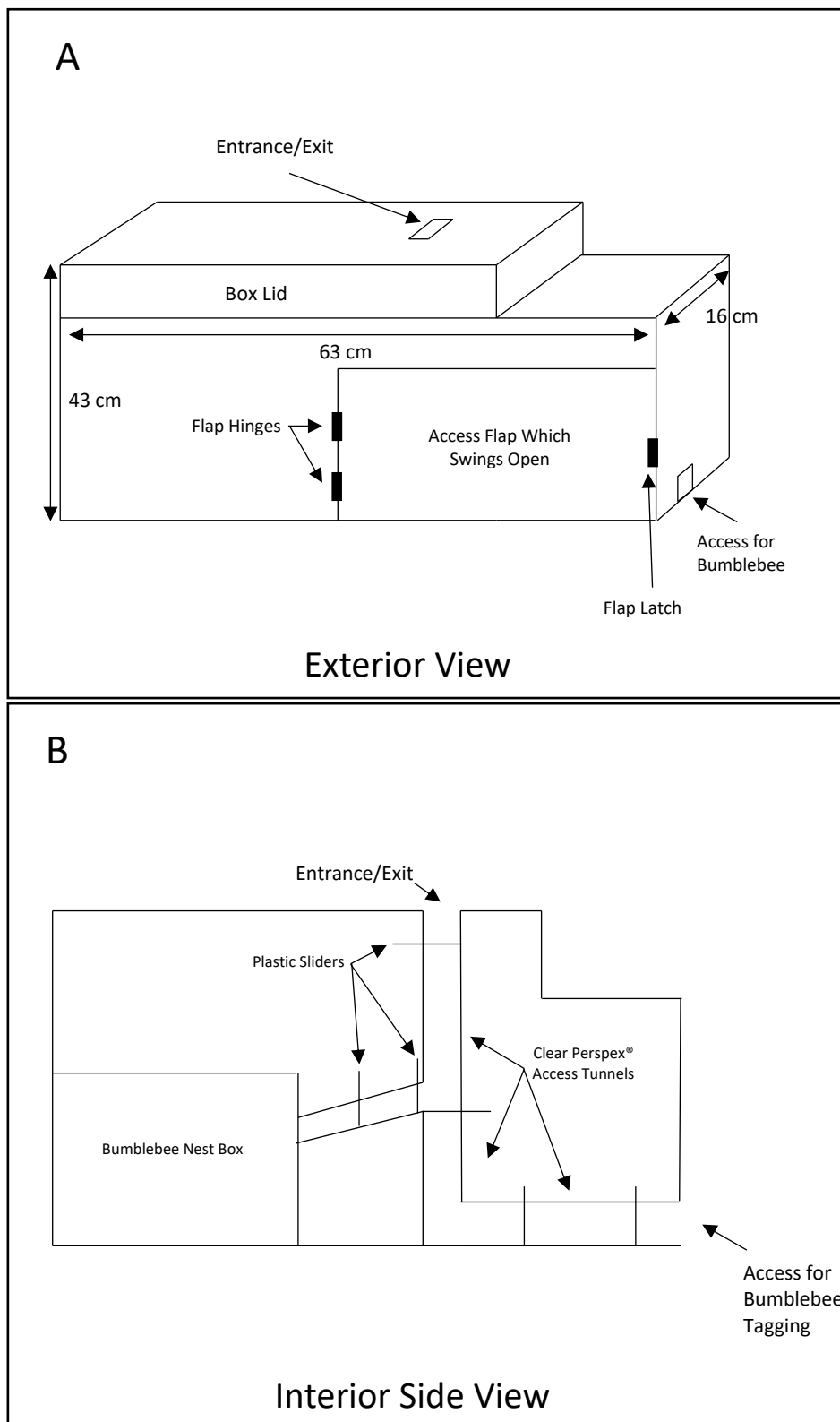
**Figure 2.3** | Experimental Site Three with *Bombus terrestris* colony placement (G-J). The black bordered grey area represents the orchard. The white border represents the hedgerow. 'G', 'H', 'I' and 'J' represent the individual colonies used. Copyright Information: Google Earth V 7.3.2. (June 22, 2018). Eye altitude: 778 m. <https://www.earth.google.com> [December 15, 2018].

### 2.3.2 Temperature Conditions and Sampling Regime

Experiments at all three sites were conducted on dry days, with air temperatures varying from 11.0 to 26.0° C. Wind direction and speed were recorded using an automated weather station (ProData Weather Systems, UK) at all three sites and was variable throughout. Colonies were tested continuously from mid-morning to dusk at all three sites on testing days. Colonies were closed outside of testing times.

### 2.3.3 Individual Tagging & Recording

At each site, the bumblebee colonies were placed in a wooden nest box (Figure 2.4). The bumblebee colony was connected to the outside of the nest box using a clear, Perspex® tube. The movement of individual bumblebees both inside and outside the colony was controlled using plastic spacers, located at regular intervals throughout the tube. Purchased colonies contained a queen and at least 50 naïve workers of mixed age that had never flown outside the plastic box in which they had arrived. Workers were released from the nest throughout the testing day and tagged using honeybee queen marking tags upon their return (EH Thorne (Beehives) Ltd, UK). Tagging was conducted in order to investigate the effects of the hedgerow on both a bumblebee's first and subsequent flights outside their colony. All nest traffic was recorded throughout the testing days.



**Figure 2.4** | Wooden bumblebee box. **A.** Exterior view of the box, showing the entrance/exit and access points as well as the box's general construction. **B.** Interior side view of the box, showing the network of access tunnels that the bumblebees would use to enter and exit the box. The plastic sliders could be moved up and down in order to allow or block movement throughout the tunnels.

#### 2.3.4 Vanishing Bearings

To determine whether the hedgerow had an effect on the flight paths of bumblebees leaving the colony, the vanishing bearing of each bumblebee was recorded. In an effort to avoid observer bias, at least two observers would watch the learning flight of each bumblebee to determine its vanishing bearings. Upon first exiting their colonies, or even when departing from a newly discovered food source, bumblebees are known to perform a distinct flying behaviour, collectively known as orientation/learning flights (Collett & Zeil, 1996; Baddeley *et al.*, 2009; Hempel de Ibarra *et al.*, 2009; Phillipides *et al.*, 2013; Robert *et al.*, 2017). This behaviour involves flying in a series of arcs and loops around an object of interest before taking off in a distinct direction away from it. In this experiment, the compass bearing at the moment when the bumblebee vanished from human sight following a series of loops around their colony was measured with a compass, as described in Dyer (1991).

In total, the vanishing bearings of 321 bumblebees on their first flight outside their colony were recorded (N=40 at Site One; N=119 at Site Two; N=162 at Site Three).

#### 2.3.5 Pollen Foraging

To determine whether the hedgerow had an effect on the foraging choices of the bumblebees returning to their colony, the pollen loads of returning bumblebees were analysed. In order to encourage the colonies to forage predominantly for pollen, the colonies had access to the sugar solution that was provided by the manufacturer inside the colony box *ad libitum*. When bumblebees returned to the wooden nest box, they were first stripped of their pollen loads before being allowed to return to their colony. This was done by removing all the pollen present in each of the bumblebee's corbiculae. Pollen was stripped from each bumblebee on each of their returning flights. Non-pollen foraging trips were also recorded. The stripped pollen was frozen at the end of each testing day.

At Site One, a total of 23 pollen loads were collected throughout the testing days. A mean of 4.6 pollen loads were collected from each hive per day (range: 1-16). At Site Two, a total of 492 pollen loads were collected throughout the testing days. A mean of 30.75 pollen loads were collected from each colony per day (range: 5-76). At Site Three, a total of 506 pollen loads were collected throughout the

testing days. A mean of 24.09 pollen loads were collected from each colony per day (range: 1-53).

### 2.3.6 Distance Effects

As bumblebee workers have been previously found to forage on a mass flowering crop the closer their colony is situated to it (Osborne *et al.*, 2008), any potential effects of the hedgerow may be obscured by how far away from the mass flowering crop the bumblebee colonies were situated. To try and isolate the effects of the hedgerow on the flight paths and foraging patterns of bumblebees, the effect of bumblebee colony distance from a mass flowering crop was also investigated. In order to test whether distance of the colony from the mass flowering crop affected foraging patterns, additional *Bombus terrestris audax* colonies (Koppert Biological Systems, UK) were also placed in the mass flowering crop fields at two of the experimental sites: Site One and Site Three. At Site One, two additional colonies were placed in the southernmost field that contained oilseed rape (Figure 2.5; Colonies K and L). The colonies were tested from the 24<sup>th</sup>-27<sup>th</sup> May 2016.<sup>3</sup> As such, four colonies in total at two different distances from the mass flowering crop were used to investigate the effects of distance at Site One: two colonies were located zero metres from the mass flowering crop and two colonies were located 66 m from the mass flowering crop (Figure 2.5).

At Site Three, two additional *Bombus terrestris audax* colonies (Koppert Biological Systems, UK) were placed in the orchard (Figure 2.6; Colonies M and N). The colonies were tested 28<sup>th</sup>-29<sup>th</sup> April 2017 and the 2<sup>nd</sup> - 3<sup>rd</sup> May 2017.<sup>4</sup> As such, six colonies in total at two different distances from the orchard were used to investigate the effects of distance at Site Three: two colonies were located zero metres from the orchard and four colonies, tested two at a time, were located 146 m from the orchard (Figure 2.6). The testing procedure used in this investigation was identical to the one outlined in the previous section.

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<sup>3</sup> Colony L was not tested on the 24<sup>th</sup> May 2016.

<sup>4</sup> Colony N was only tested on 2<sup>nd</sup> -3<sup>rd</sup> May 2017.





**Figure 2.5** | Experimental Site One with *Bombus terrestris* colony placement for distance effect investigation. 'A', 'B', 'K' and 'L' denote the location of the four colonies in this experiment. Colonies K and L were placed in the oilseed rape field. The black border grey areas represent the fields of oilseed rape (four shown here). The white border represents the hedgerow. Copyright Information: Google Earth V 7.3.2. (June 22, 2018). Eye altitude: 769 m. <https://www.earth.google.com> [December 15, 2018].



**Figure 2.6** | Experimental Site Three with *Bombus terrestris* colony placement for distance effect investigation. G', 'H', 'I', 'J', 'M' and 'N' represent the individual colonies used in this experiment. Colonies M and N were placed in the orchard. The black bordered grey area represents the orchard. The white border represents the hedgerow. Copyright Information: Google Earth V 7.3.2. (June 22, 2018). Eye altitude: 778 m. <https://www.earth.google.com> [December 15, 2018].

### 2.3.7 Pollen Analysis

Returning bumblebees were stripped of their pollen loads and these samples were frozen at -20.0 °C at the end of each testing day. Frozen samples were then defrosted and classified by their colour under natural light. Samples were then stained and mounted using the techniques described in Sawyer (1981). As such, a lateral cross section of each pollen load was taken and placed in a petri dish. A lateral cross section was taken in order to ensure that even if the pollen sample contained strata of pollen from different plant species, this would be accounted for in the sub-sample placed into the petri dish. A few drops of distilled water were added to this to form a thin slurry. Using a glass pipette, a 2.0 ml sample of this slurry was smeared onto a microscope slide. The microscope slide was then warmed on a hot-plate in order for the water to evaporate. A pre-made mountant of glycerine jelly and basic fuchsin (Brunel Microscopes Ltd, UK) was also warmed to 40 °C. The basic fuchsin in the mountant stained the pollen grains pink, allowing morphological features to be distinguished. A drop of warmed mountant was then added to each warmed slide and then a cover slip was positioned on top. The slide was then left on the hot-plate at 30 °C for 10 minutes. After 24 hours, the mountant had set and the cover slip was sealed around the edges with clear nail varnish (The Boots Company Plc, UK). This procedure was repeated for every sample that had been collected in the field, with one slide prepared for each sample collected. All slides were then analysed with the use of a compound light microscope (Leica Microsystems, Germany). In order to minimise sampling bias, all slides were firstly marked before analysis using a non-descriptive numbering system. The order in which the slides were analysed using the compound light microscope was then randomised.

Each sample was identified to the species level where possible using a variety of different techniques. The sample's original colour was used to identify the pollen by matching it to the colour samples provided in Hodges (1952). The morphology of each sample, when viewed under the microscope, was then compared to reference samples taken from plants found within a 900 m radius from the colonies at each experimental site. The reference samples were prepared using the same mounting and staining technique described above. The pollen for the reference samples was obtained by gathering flowers whose anthers contained pollen grains. Pollen samples were further identified using Sawyer's (1981)

identification key. Lastly, the pollen samples were compared with microscopic photographs of pollen from three online repositories: Pollen-Wiki([https://pollen.tstebler.ch/MediaWiki/index.php?title=PollenWiki:%C3%9Cber\\_Pol-len-Wiki](https://pollen.tstebler.ch/MediaWiki/index.php?title=PollenWiki:%C3%9Cber_Pol-len-Wiki)), SAPS Pollen Image Library (<https://www-saps.plantsci.cam.ac.uk/pollen/>) and The Pollen Grains Reference Library (<https://blogs.cornell.edu/pollengrains/>).

At Site Two, the pollen grains of mass flowering *Trifolium pratense* were distinguished from other *Trifolium* species in the landscape, such as *Trifolium repens*, based on their size. This was done with the aid of the pollen reference samples obtained at Site Two. The mass flowering *Trifolium pratense* had larger pollen grains than the other *Trofolium* species found in the landscape.

Based on the pollen identification, individual bumblebees were then assigned as preferring a particular pollen source on each of their foraging trips. In total, 1,243 samples were collected and analysed. In 90.19% of all samples collected (1,121/1,243), the pollen loads only contained a single plant species. In 9.81% of all samples collected (122/1,243), however, the pollen loads contained more than one plant species. For these samples, the preferred pollen source was assigned as the plant species that made up the highest percentage of the pollen load. This was determined by taking a cross section count of the pollen grains. It should be noted that due to varying pollen grain shapes and volumes in a mixture, these numerical counts should only be taken as an estimate of relative pollen abundance (Buchmann & O'Rourke, 1991; Cane & Sipes, 2006). In 5.73% of these mixed samples (7/122), there was not one plant species that made up more of the pollen load than another. As such, these samples were not included in the data analysis. In the mixed samples that were included in the analysis, the dominant plant species comprised on average 76.43% of the pollen load (range: 37%-98%). In five samples, the pollen, or the location of its source at the experimental site, could not be identified and as such, these samples were not included in the final analysis. It should also be noted that the pollen samples collected at Site One from 23<sup>th</sup>-27<sup>th</sup> May 2016 were not included in the final analysis.

## 2.4 Statistical Analysis

All the statistical analysis was carried out in R 3.5.2 (R Core Team, 2015).

### 2.4.1 Effects of a Hedgerow on Vanishing Bearings

In order to investigate the effect of the hedgerow on the initial flight path of bumblebees, the vanishing bearings of bumblebees exiting their colonies on either side of the hedgerow were analysed. This analysis was only carried out for the first flights of bumblebees outside their colony. To explore whether bumblebees were more likely to fly alongside the hedgerow upon exiting their colonies, the vanishing bearings of bumblebees were assigned as either flying alongside the hedgerow or not. In order for a bumblebee to be assigned as flying alongside the hedgerow, its vanishing bearings had to be within  $\pm 20^\circ$  of the coordinate directions of the hedgerow. The proportion of bumblebees that flew alongside the hedgerow was then compared to the expected proportion of bumblebees that would fly alongside the hedgerow by chance, assuming that bumblebees were flying off equally in all directions. The expected proportion of bumblebees that would fly alongside the hedgerow by chance was calculated by dividing  $360^\circ$  into  $40^\circ$  blocks, resulting in nine categories of direction. As such, if bumblebees were flying in two of these categories of direction, it was expected that 22.2% of bumblebees would fly alongside the hedgerow by chance. This was done using a one-proportion z-test. This was calculated for each site, with the bumblebees from each colony being pooled together.

To explore whether bumblebees whose colonies were situated on the same side as the mass flowering crop were more likely to fly off towards the crop than those situated on the other side of the hedgerow, the vanishing bearings of bumblebees were assigned as either flying towards the mass flowering crop or not using a two-proportions z-test. In order for a bumblebee to be assigned as flying towards the mass flowering crop, its vanishing bearings had to be within  $\pm 20^\circ$  of the coordinate directions of the edges of the mass flowering crop field (or the coordinate directions of the adjacent hedgerow, whichever was greatest).

### 2.4.2 Effects of a Hedgerow on Pollen foraging

Data were analysed using generalised linear models (GLM) and residuals were checked for fit and homoscedasticity of variance using a simulation approach

('DHARMA' package, Hartig, 2019). Overdispersion was tested for and, if present, accounted for using a quasibinomial approach. Data collected at Site One was excluded from this analysis due to the very low number of bumblebees that returned to their colony (Appendix A: Tables A1-A2).

### *First Flight*

To determine whether the hedgerow had an effect on whether bumblebees foraged on mass flowering crop pollen on their first flight, the relationship between the proportion of bumblebees that foraged predominantly on mass flowering crop pollen on their first flight outside the colony and the side of the hedgerow that the colonies was placed on was modelled using a GLM. A quasibinomial error structure and logit link were used due to overdispersed data. The explanatory variables used in the following order were the site that the experiment took place and the side of the hedgerow on which the colony was placed. These were entered into the model with an interaction term.

### *Overall Flights*

To determine whether the hedgerow had an effect on whether bumblebees foraged on mass flowering crop pollen overall, the relationship between the proportion of bumblebees that foraged predominantly on the mass flowering crop pollen and the side of the hedgerow on which the colony was placed was modelled using a GLM. A binomial error structure and logit link were used. The pollen that each bumblebee predominantly foraged on was calculated as the type of pollen that they returned with on the majority of their foraging trips. In 7.73% of bumblebees, no single type of pollen was predominantly foraged for throughout all of their foraging trips and, as such, these bumblebees were not included in the analysis. The explanatory variables used in the following order were the site that the experiment took place and the side of the hedgerow that the colonies were placed on and their interaction.

### 2.4.3 Distance Effects from the Mass Flowering Crop

Ideally, the effects of distance from the mass flowering crop would be included in the pollen foraging analysis described above. Due to the fact that no additional colonies were placed at Site Two to investigate the effects of distance and that only three bumblebees on one side of the hedgerow returned to their colony at

Site One (Appendix A: Table A.3); the decision was made to analyse the effects of distance separately. As such, this analysis only included the data collected at Site Three. As the width of the hedgerow was less than one metre at all sites, colonies placed on either side of the hedgerow were assumed to be situated at the same distance from the mass flowering crop.

#### *First Flight*

To determine if the distance from the mass flowering crop had an effect on whether bumblebees foraged on the mass flowering crop on their first flight, the proportion of bumblebees that returned with mass flowering crop pollen from each colony on their first flight at each distance was compared using an independent t-test.

#### *Overall Flights*

To determine if the distance from the mass flowering crop had an effect on whether bumblebees foraged on mass flowering crop pollen, the proportion of bumblebees that foraged predominantly on mass flowering crop pollen from each colony throughout all their flights at each distance was compared using an independent t-test.

## **2.5 Results**

The mean number of released bumblebees from each colony was 51.7 (range: 23-73; Appendix A: Tables A.1-A.2). The mean number of returned bumblebees throughout the experimental testing days from each colony was 38.8 (range: 3-69; Appendix A: Table A.2). The mean proportion of bumblebees that returned to each colony was 0.71 (range: 0.13-0.98; Appendix A: Table A.2).

Of the total number of returned bumblebees, the mean proportion that returned with pollen on their first flight from each colony was 0.82 (range: 0.33-1.00; Appendix A: Table A.2). Of the total number of returned bumblebees, the mean proportion that performed more than one flight during the experimental testing days from each colony was 0.47 (range 0-0.69; Appendix A: Table A.2).

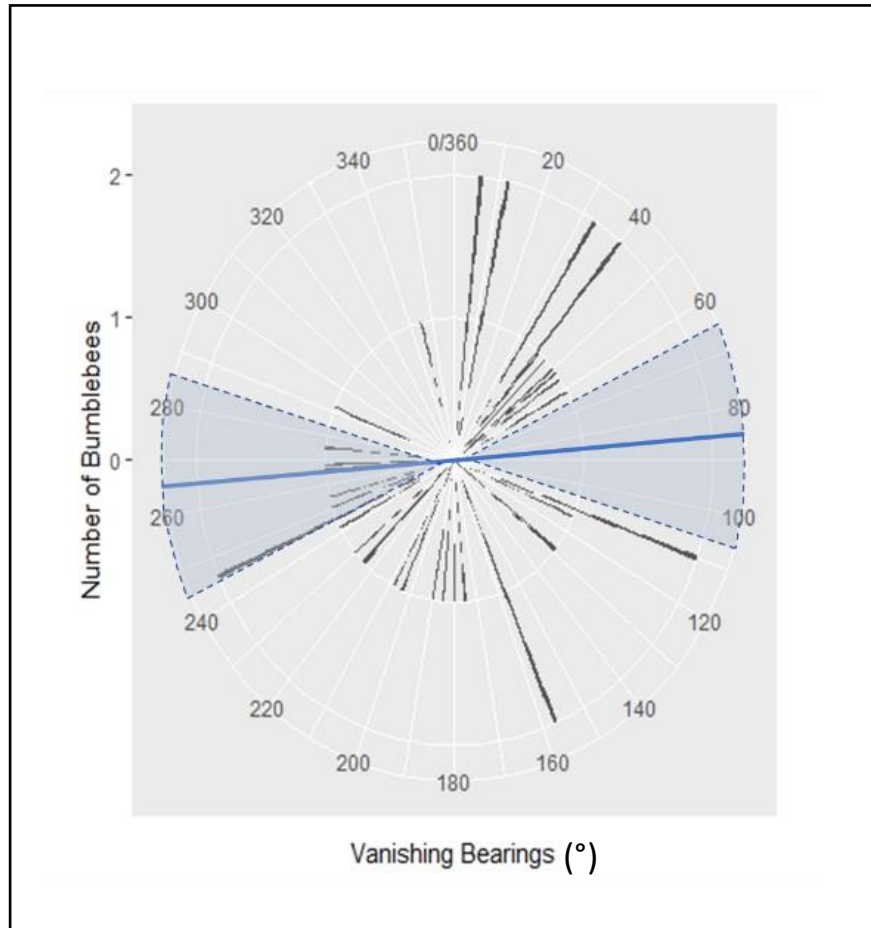
### 2.5.1 Effects of a Hedgerow on Vanishing Bearings

#### *First Flights*

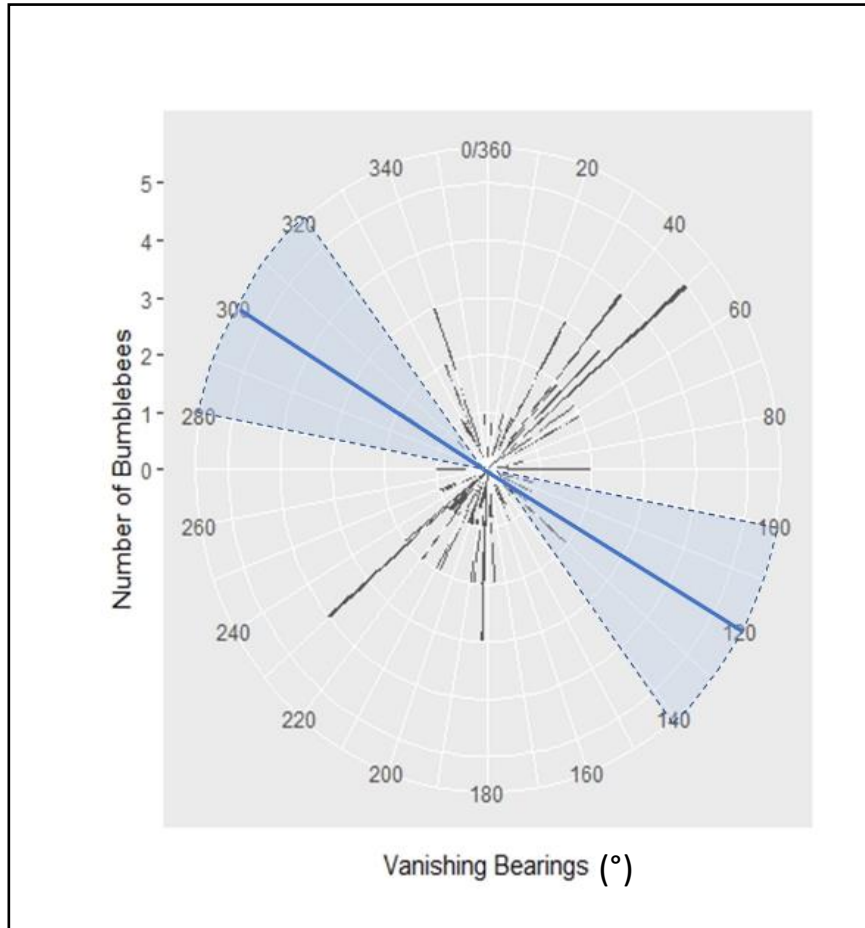
To assess whether naïve bees showed a bias for departing from the colony in line with the hedgerow, the proportion of bumblebees that vanished alongside the hedgerow on their first flight outside the colony was compared to the proportion of bumblebees that were expected to vanish alongside the hedgerow by chance (assuming bumblebees dispersed equally around their colony). A one proportion z-test found that bumblebees were not more likely to fly alongside the hedgerow when leaving their colonies for the very first time (Site One:  $N=40$ ,  $X^2 = 0.51$ ,  $df=1$ ,  $p=0.76$ ; Site Two:  $N= 119$ ,  $X^2=14.76$ ,  $df=1$ ,  $p=0.99$ ; Site Three:  $N= 162$ ,  $X^2 =1.77$ ,  $df=1$ ,  $p=0.092$ ; Figures 2.7.i-iii).

To assess whether naïve bumblebees showed a bias for departing from the colony towards the mass flowering crop, the proportion of bumblebees that vanished towards the mass flowering crop on their first flight outside the colony from colonies placed on either side of the hedgerow were also compared. A two-proportions z-test found that bumblebees from colonies placed on the same side of the hedgerow as the mass flowering crop were not more likely to fly in the direction of the mass flowering crop on their first flights outside the colony than bumblebees from colonies placed on the opposite side of the hedgerow (Site One:  $N=40$ ,  $X^2 = 1.44$ ,  $df=1$ ,  $p=0.12$  ; Site Two:  $N= 119$ ,  $X^2 = 15.48$ ,  $df=1$ ,  $p>0.99$ ; Site Three:  $N= 162$ ,  $X^2 = 1.88$ ,  $df=1$ ,  $p=0.91$ ; Figures 2.8.i-iii).

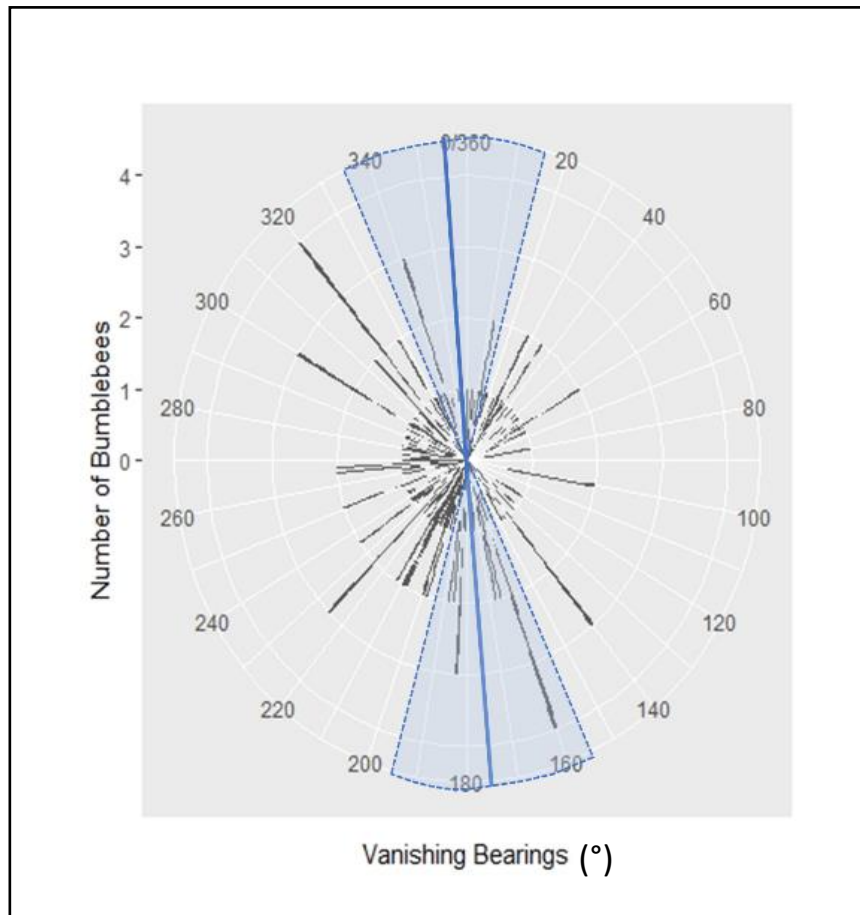




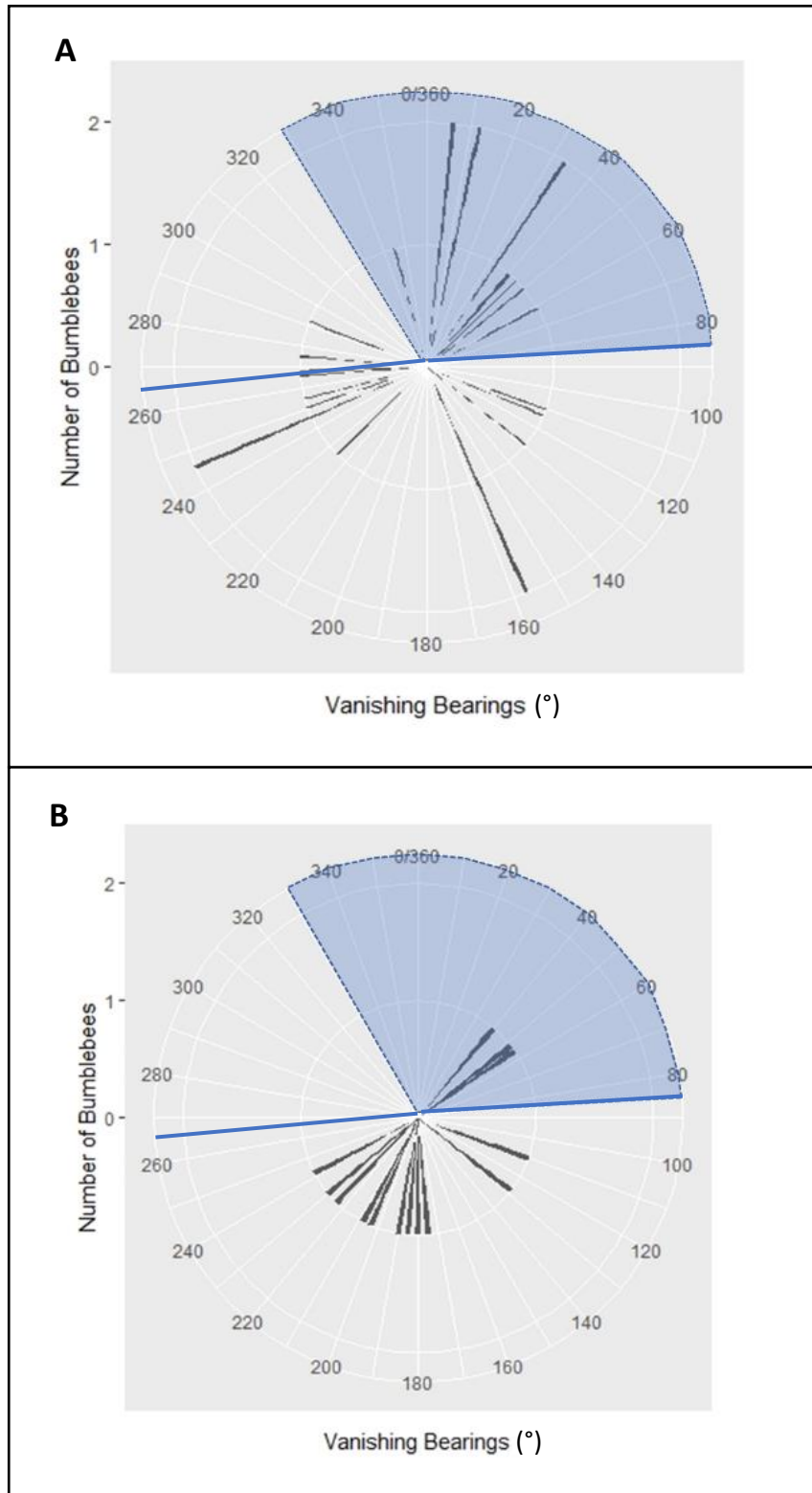
**Figure 2.7.i** | Vanishing bearings of bumblebees (in degrees) on their first flight outside the colony at Site One.  $N=40$ . The scale of black lines is illustrated on the left of each figure and corresponds to the number of bumblebees that had a particular vanishing bearing. The blue line represents the position of the hedgerow. If a bumblebee had a vanishing bearing that fell within the shaded blue area, then it would be classified as flying alongside the hedgerow.



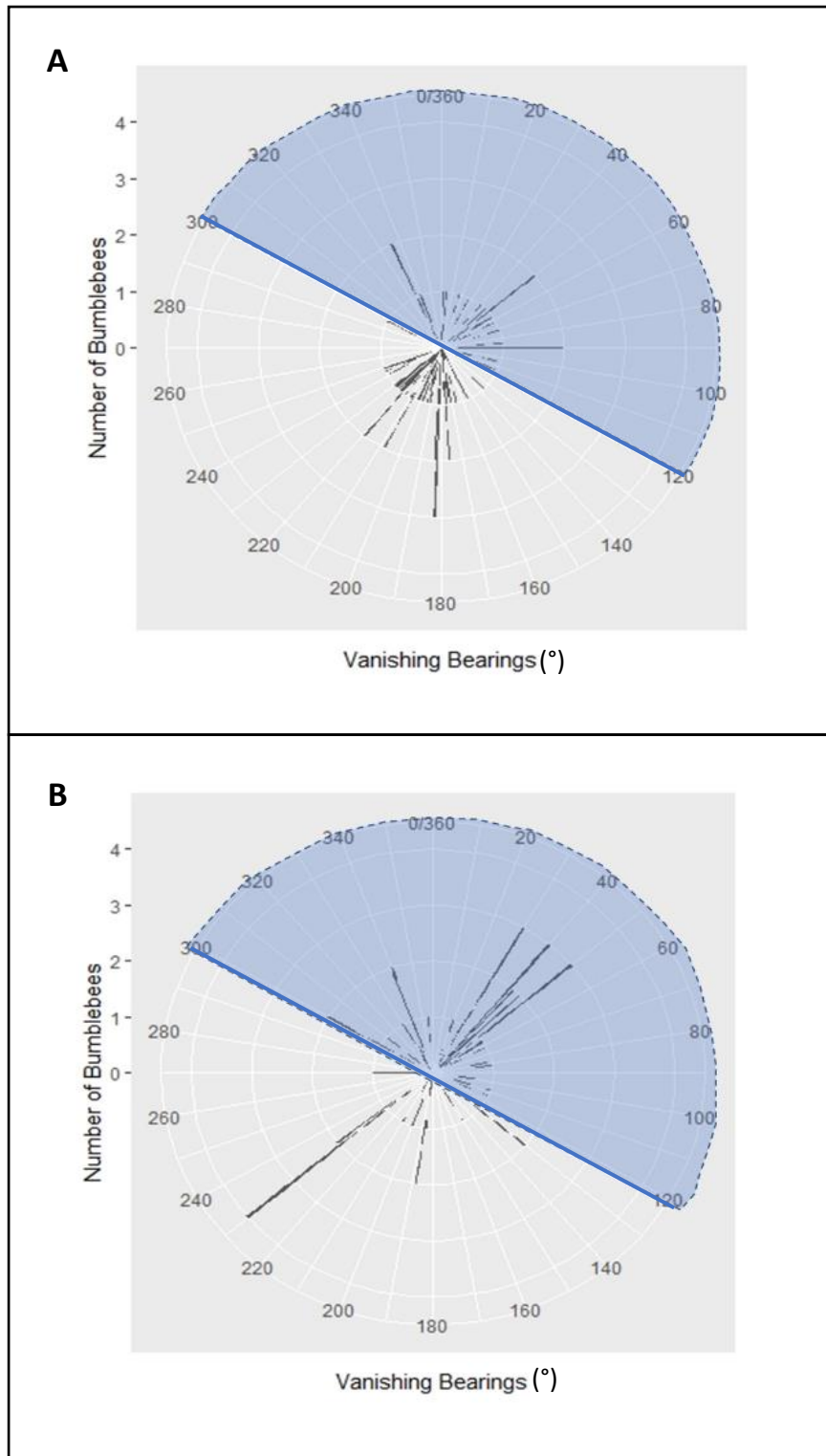
**Figure 2.7.ii** | Vanishing bearings of bumblebees (in degrees) on their first flight outside the colony at Site Two.  $N=119$ . The scale of black lines is illustrated on the left of each figure and corresponds to the number of bumblebees that had a particular vanishing bearing. The blue line represents the position of the hedgerow. If a bumblebee had a vanishing bearing that fell within the shaded blue area, then it would be classified as flying alongside the hedgerow.



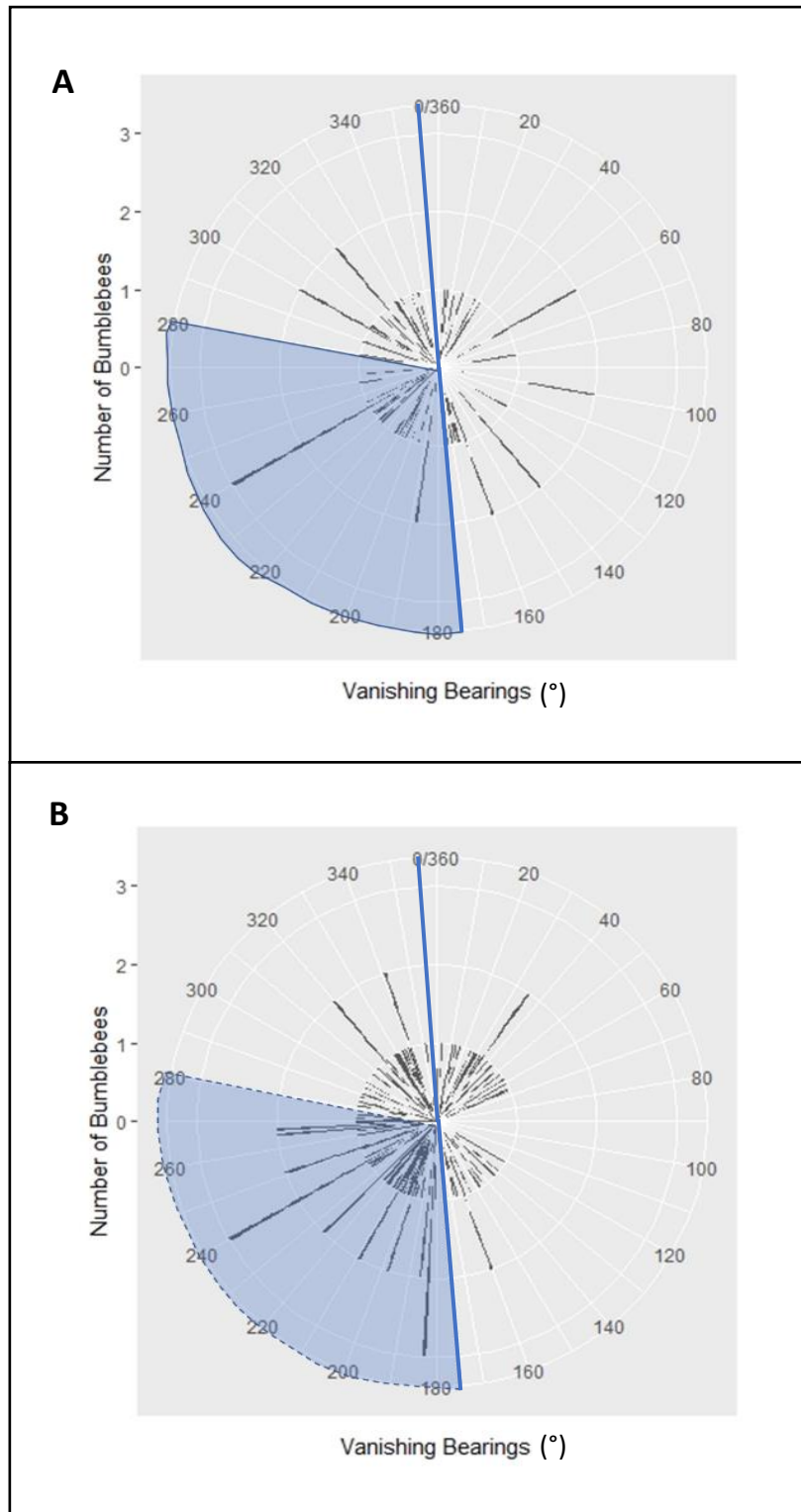
**Figure 2.7.iii** | Vanishing bearings of bumblebees (in degrees) on their first flight outside the colony at Site Three.  $N=162$ . The scale of black lines is illustrated on the left of each figure and corresponds to the number of bumblebees that had a particular vanishing bearing. The blue line represents the position of the hedgerow. If a bumblebee had a vanishing bearing that fell within the shaded blue area, then it would be classified as flying alongside the hedgerow.



**Figure 2.8.i** | Vanishing bearings of bumblebees (in degrees) on their first flight outside the colony at Site One. **A.** The vanishing bearings of bumblebees from colonies placed on the same side of the hedgerow as the mass flowering crop.  $N=26$ . **B.** The vanishing bearings of bumblebees from colonies placed on the opposite side of the hedgerow as the mass flowering crop.  $N=14$ . The scale of black lines is illustrated on left of each figure. The blue line represents the position of the hedgerow. If a bumblebee had a vanishing bearing that fell within the shaded blue area, then it would be classified as flying towards the mass flowering crop.



**Figure 2.8.ii** | Vanishing bearings of bumblebees (in degrees) on their first flight outside the colony at Site Two. **A.** The vanishing bearings of bumblebees from colonies placed on the same side of the hedgerow as the mass flowering.  $N=62$ . **B.** The vanishing bearings of bumblebees from colonies placed on the opposite side of the hedgerow as the mass flowering crop.  $N=57$ . The scale of black lines is illustrated on left of each figure. The blue line represents the position of the hedgerow. If a bumblebee had a vanishing bearing that fell within the shaded blue area, then it would be classified as flying towards the mass flowering crop.



**Figure 2.8.iii** | Vanishing bearings of bumblebees (in degrees) on their first flight outside the colony at Site Three. **A.** The vanishing bearings of bumblebees from colonies placed on the same side of the hedgerow as the mass flowering crop.  $N=57$ . **B.** The vanishing bearings of bumblebees from colonies placed on the opposite side of the hedgerow as the mass flowering crop.  $N=105$ . The scale of black lines is illustrated on left of each figure. The blue line represents the position of the hedgerow. If a bumblebee had a vanishing bearing that fell within the shaded blue area, then it would be classified as flying towards the mass flowering crop.

## 2.5.2 Effects of a Hedgerow on Pollen Foraging

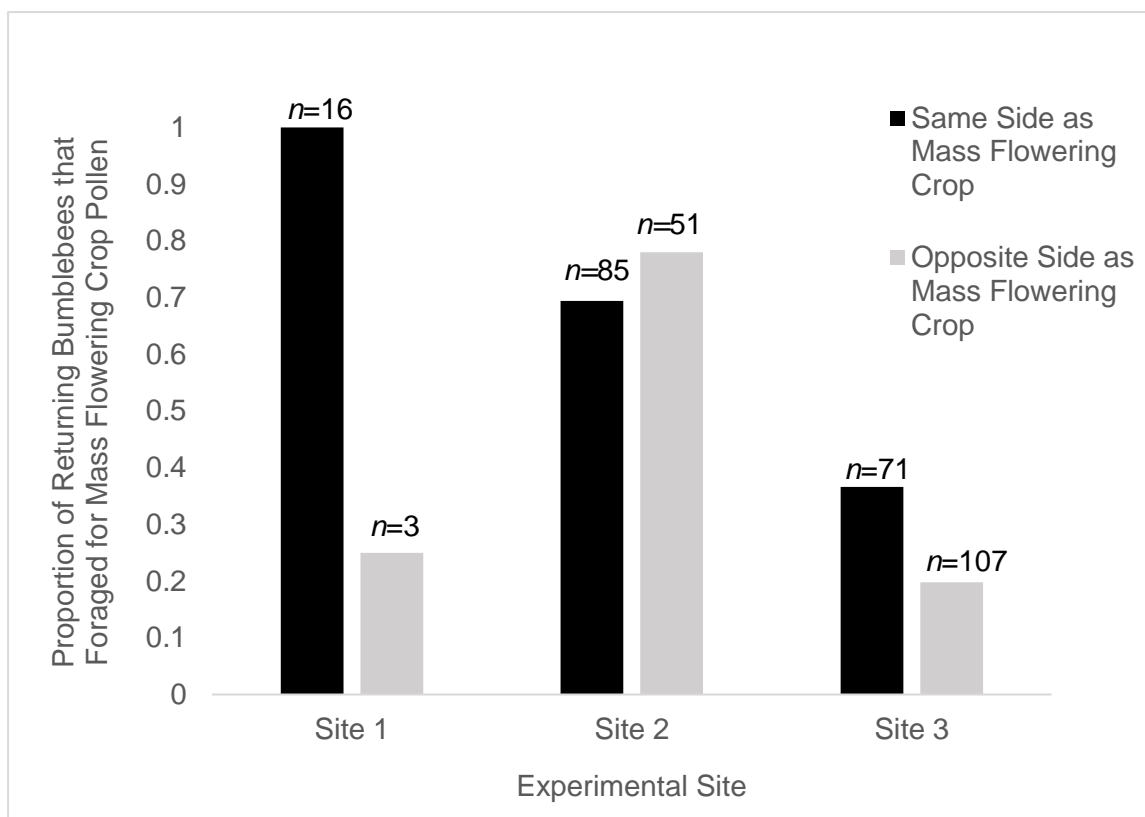
### *First Flight Pollen*

#### *Site Two and Site Three (with Site One excluded due to low numbers)*

When comparing the proportion of bumblebees that returned predominantly with mass flowering crop pollen on their first flight when placed on either side of the hedgerow, there was no significant interaction between the site that the experiment took place and the side of the hedgerow that the colonies were placed (Table 2.1; Figure 2.9). There was a significant difference in the proportion of bumblebees that returned with mass flowering crop pollen between the two experimental sites. A greater proportion of bumblebees at Site One returned predominantly with the mass flowering crop pollen on their first flight compared to those at Site Three (Table 2.1; Figure 2.9). There was also no significant effect of the side of the hedgerow that the colonies were placed (Table 2.1; Figure 2.9).

**Table 2.1. Model results from a linear model testing the effect of the experimental site and the side of the hedgerow that the colony was placed on the proportion of bumblebees that foraged on mass flowering crop pollen on their first flights. Variables, effect sizes  $\pm$  standard error,  $t$ -values and  $p$ -values from the fitted model. Site Three was used as the reference level.**

Variable(s)	Effect Size $\pm$ Standard Error	$t$ Value	$p$ Value
Intercept	-1.41 $\pm$ 0.30	-4.68	0.009
Site	2.70 $\pm$ 0.52	5.21	0.006
Hedgerow Side	0.86 $\pm$ 0.43	2.01	0.12
Site * Hedgerow Side	-1.22 $\pm$ 0.67	-1.82	0.14



**Figure 2.9** | Proportion of returning bumblebees that brought back mass flowering crop pollen on their first flight when placed on either side of the hedgerow at each site.  $N=333$ . The numbers in the graph represent the total number of returned bumblebees for each side of the hedgerow and at each experimental site. Although data from Site One was not included in the statistical analysis due to small sample size, it is still shown here.



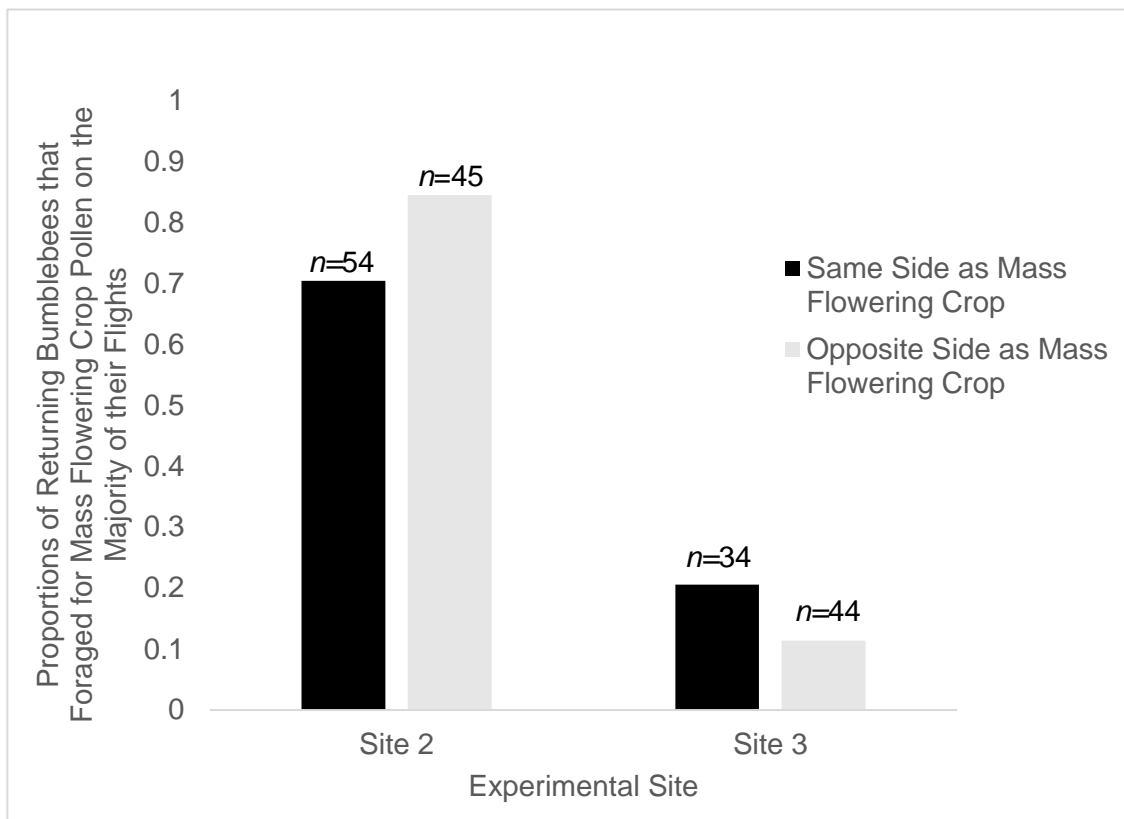
## *Overall Flight Pollen*

### *Site Two and Site Three (with Site One excluded due to low numbers)*

For all flights monitored, when comparing the proportion of bumblebees that returned predominantly with mass flowering crop pollen throughout their flights when placed on either side of the hedgerow, there was no significant interaction between the site that the experiment took place and the side of the hedgerow that the colonies were placed on (Table 2.2; Figure 2.10). There was a significant difference in the proportion of bumblebees that foraged predominantly for mass flowering crop pollen throughout their flights between the two experimental sites. A greater proportion of bumblebees at Site Two foraged predominantly for mass flowering crop pollen than those at Site Three (Table 2.2; Figure 2.10). There was also no significant difference in the proportion of bumblebees that foraged predominantly for mass flowering crop pollen throughout their flights depending on the side of the hedgerow that the colonies were placed on (Table 2.2; Figure 2.10).

**Table 2.2. Model results from a linear model testing the effect of the experimental site and the side of the hedgerow that the colony was placed on the proportion of bumblebees that foraged on mass flowering crop pollen on their flights overall. Variables, effect sizes  $\pm$  standard error, z-values and p-values from the fitted model. Site Three was used as the reference level.**

<b>Variable(s)</b>	<b>Effect Size <math>\pm</math> Standard Error</b>	<b>z Value</b>	<b>p Value</b>
Intercept	-2.05 $\pm$ 0.48	-4.32	<0.0001
Site	3.75 $\pm$ 0.63	5.96	<0.0001
Hedgerow Side	0.70 $\pm$ 0.64	1.11	0.27
Site*Hedgerow Side	-1.53 $\pm$ 0.81	-1.88	0.06



**Figure 2.10** | Proportion of returning bumblebees that brought back mass flowering crop pollen on the majority of their flights when placed on either side of the hedgerow.  $N=177$ . The numbers in the graph represent the total number of returned bumblebees that had more than one flight for each side of the hedgerow and at each experimental site. Only bumblebees on either side of the hedgerow at Site Two and Site Three completed more than one flight outside their colony.

### 2.5.3 Distance Effects from the Mass Flowering Crop

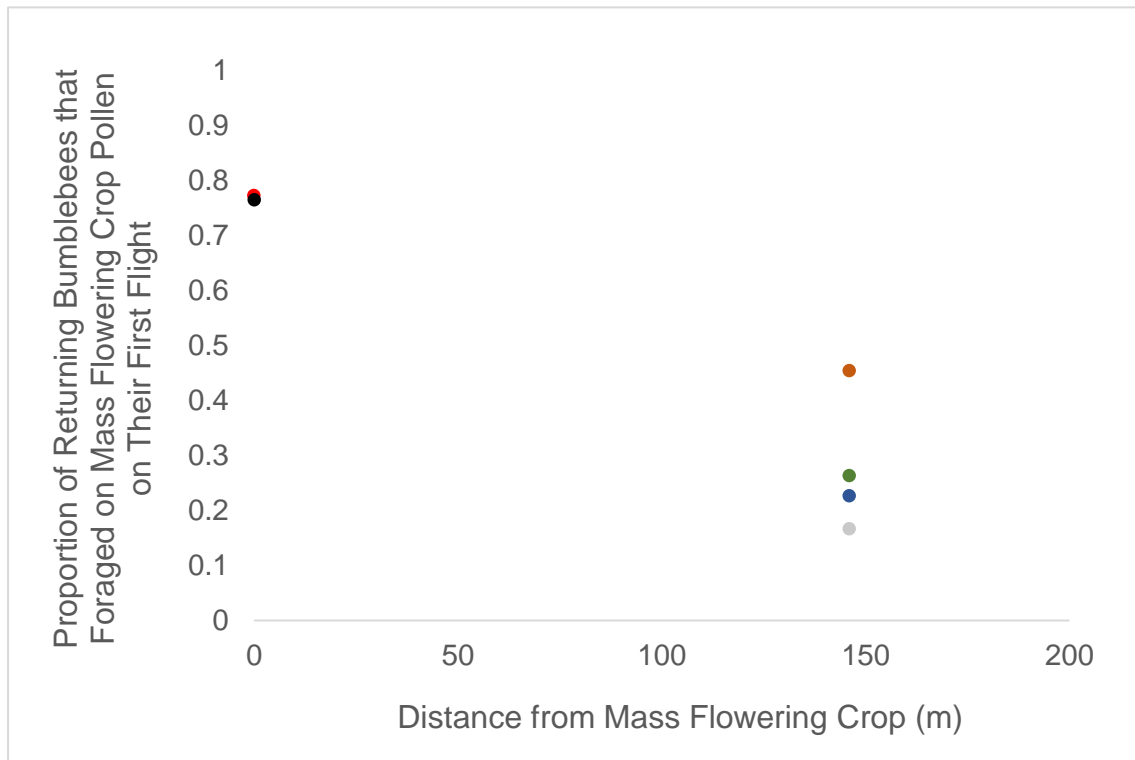
#### *Site Three (with Site One excluded due to low numbers)*

For the colonies used in the distance effects analysis, the mean number of released bumblebees for each colony was 50 (range: 19-69; Appendix A: Tables A.1-A.4). The mean number of returned bumblebees throughout the experimental testing days from each colony was 38 (range: 18-55; Appendix A: Tables A.2 and A.4). The mean proportion of bumblebees that returned to each colony was 0.81 (range: 0.46-0.98; Appendix A: Tables A.2 and A.4).

Of the total number of returned bumblebees, the mean proportion that returned with pollen on their first flight from each colony was 0.99 (range: 0.98-1.00; Appendix A: Tables A.2 and A.4). Of the total number of returned bumblebees, the mean proportion that performed more than one flight during the experimental testing days from each colony was 0.39 (range 0.11-0.68; Appendix A: Table A.2 and A.4).

#### *First Flight Pollen*

At Site Three, a greater proportion of bumblebees from colonies situated closer to the mass flowering crop returned with mass flowering crop pollen on their first flight. Specifically, 76.9% of bumblebees from colonies situated in the mass flowering crop field returned with mass flowering crop pollen on their first flight. In contrast, 23.6% of bumblebees from colonies situated at 146 m from the mass flowering crop returned with mass flowering crop pollen on their first flight. An independent t-test found a significant difference between the proportion of bumblebees that returned with mass flowering crop pollen on their first flight from each colony at the two distances ( $t_4=5.34$ ,  $p=0.006$ ; Figure 2.11).

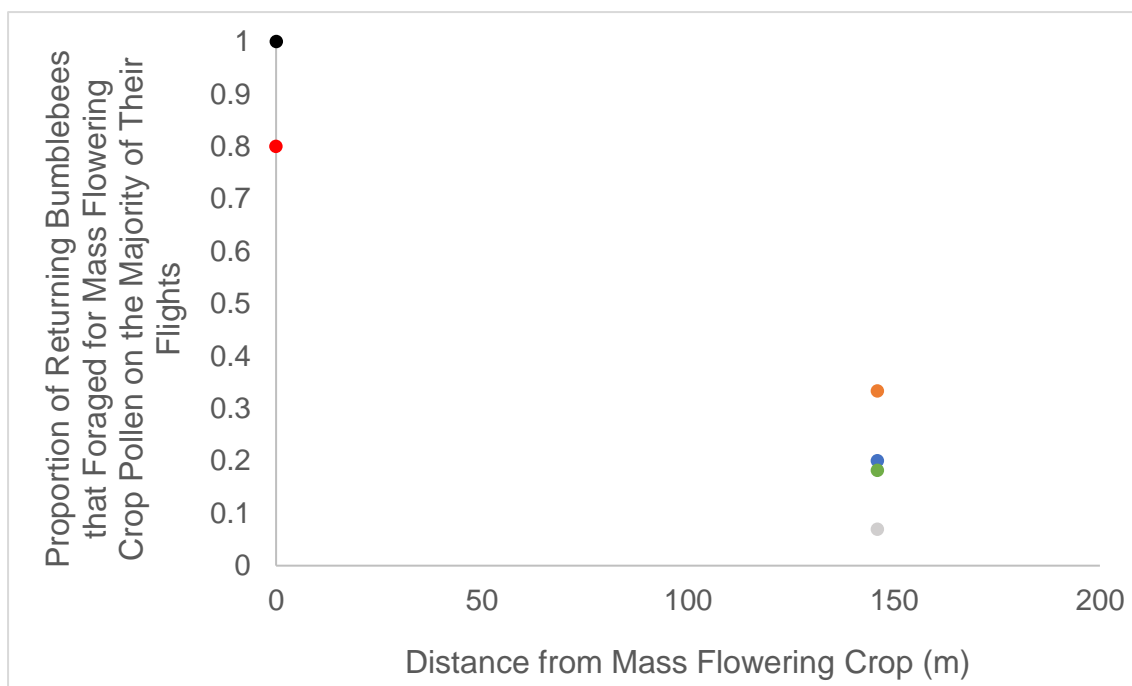


flowering crop pollen on their first flight when placed at various distances from the mass flowering crop field.  $N=217$  bumblebees from six colonies. Each data point represents a separate colony.

## Overall Flight Pollen

### Site Three (with Site One excluded due to low numbers)

For all flights monitored at Site Three, a greater proportion of bumblebees from colonies situated closer to the mass flowering crop foraged predominantly on mass flowering crop pollen. Specifically, 85.7% of bumblebees from colonies situated in the mass flowering crop field foraged predominantly on mass flowering crop pollen throughout their flights. In contrast, 15.4% of bumblebees from colonies situated at 146 m from the mass flowering crop foraged predominantly on mass flowering crop pollen throughout their flights. An independent t-test found a significant difference between the proportion of bumblebees that returned with mass flowering crop pollen from each colony at the two distances ( $t_4=6.97$ ,  $p=0.002$ ; Figure 2.12).



**Figure 2.12** | Proportion of returning bumblebees that preferentially foraged on mass flowering crop pollen on the majority of their foraging flights when placed at various distances from the mass flowering crop field.  $N=85$  bumblebees from six colonies. Each data point represents a separate colony.

## 2.6 Discussion

In this experiment, the effect of a hedgerow on the flight paths and foraging behaviour of *Bombus terrestris* workers in landscapes dominated by mass flowering crops was investigated. On their first flight outside their colony, naïve bumblebees from colonies placed on the ground, adjacent to a hedgerow were not more likely to fly alongside the hedgerow than in any other direction (Figures 2.7i-iii). Furthermore, for each of the three sites, the naïve bumblebees from the colony placed at the base of a hedgerow on the same side as a mass flowering crop were not more likely to fly off in the direction of the mass flowering crop than bumblebees from the colony placed on the opposite side (Figures 2.8i-iii). It should be noted that the z-tests only compare the vanishing bearings between colonies, and any differences found are not necessarily attributable to them being on different sides of the hedgerow but could be due to other factors. But in this case there were no significant differences in vanishing bearings between the pairs of colonies at any of the three sites leading us to a tentative interpretation that landscape features such as hedgerows are not guiding or restricting the flight paths of bumblebees exiting their colony at these sites. If the lack of difference in vanishing bearings observed is due to the fact that the hedgerow was not guiding or restricting the flights paths of bumblebees exiting their colony, this result differs from the results observed by Cranmer, McCollin and Ollerton (2012). In their experiments, wild bumblebees have been found to fly parallel to hedgerows the closer they are to them. The differences between the results obtained in this experiment and those obtained by Cranmer, McCollin and Ollerton (2012), however, may be due to the fact that their experiment was observing a different stage in a bumblebee's flight history. In the present experiment, the first flights of bumblebees were focused upon. On their first flights outside the colony, the initial flight segments of bumblebees are marked by a series of arcs and loops around the colony. Termed orientation or learning flights, this behaviour is thought to allow bumblebees to learn and memorise the position of their colony. In order to achieve this, it is possible that bumblebees need to memorise both objects within the vicinity of the colony as well as the overall landscape panorama. As such, bumblebees may need to fly above landscape features such as hedgerows when first exiting the colony in order to gain its positional information within the landscape.

During their very first flights, bumblebees are often returning with forage for the colony (Hempel de Ibarra *et al.*, 2009; Osborne *et al.*, 2013). This was also observed in the present experiment, suggesting that unlike honeybees, exploration and foraging do not occur on separate flights. When tracked with harmonic radar, the flight segments of bumblebees have been broadly categorised into ‘exploration’ and ‘exploitation’ flights (Woodgate *et al.*, 2016). ‘Exploration’ flights were characterised by long, circuitous flights around the landscape while ‘exploitation’ flights were characterised by straight tracked flights to and from a single foraging location. Furthermore, as experience is gained, orientation/learning flights when exiting the colony become rarer and flight paths become straighter (Osborne *et al.*, 2013). As such, naïve bumblebees exiting their colony as well as those undergoing ‘exploration’ flights or flight segments may choose to fly over landscape features such as hedgerows in order to survey the landscape and the horizon. On the other hand, experienced foragers as well as those undergoing ‘exploitation’ flights or flight segments, in which a particular destination is the end goal of their flight, may instead fly parallel to hedgerows in the landscape. Unlike the present experiment which investigated the vanishing bearings of naïve bumblebees, Cranmer, McCollin and Ollerton (2012) were observing the behaviour of wild bumblebees in the vicinity of a hedgerow. As such, they would be more likely to observe bumblebees undergoing ‘exploitation’ flights or flight segments. Bumblebees with a particular foraging destination as the end goal of their flight may fly parallel to hedgerows in order to take advantage of the latter’s unique characteristics. Hedgerows are known to affect the wind speed and air flow in their vicinity (Rider, 1952) and as a result, bumblebees may be observed flying parallel to them as insects in general have a greater flight control in areas with reduced windspeed (Pasek, 1988). When faced with turbulent wind conditions, bumblebees are observed employing a variety of different physiological responses which are all linked to an increase in the energetic costs of flying (Crall *et al.*, 2017). During ‘exploitation’ flights or flight segments, it is entirely possible that bumblebees will seek out areas of reduced windspeed within the landscape in order to minimise their energetic output while maximising their flight control. In lowland agricultural landscapes, hedgerows will be the landscape features which will provide such areas of reduced windspeed,



leading to the observation that bumblebees are more likely to fly parallel to hedgerows the closer they are to them.

It should also be noted that any conclusions regarding the vanishing bearings of bumblebees need to be made with caution. Vanishing bearings, or the compass bearing at the moment when a bumblebee vanishes from human sight, can be very subjective. This is due to the fact that human observers can differ in the amount of time that they can hold a bumblebee within their line of sight. This is especially true in landscapes that are characterised by abundant dark foliage in which the sight of a bumblebee can be easily lost (R.Herascu, personal observation). Furthermore, as naïve bumblebees are performing their orientation/learning flights when exiting the nest, the bumblebees may still be performing this arcing behaviour when they vanish from human sight. In that case, the vanishing bearings observed will not be a true reflection of the compass bearing that the bumblebee chose to take following their orientation/learning flights.

In this experiment, the hedgerow did not have a significant effect on the pollen that bumblebees foraged for on their first flight as well as throughout the time that the experiment took place. Bumblebees from colonies placed on the same side of the hedgerow as the mass flowering crop were not more likely to forage on that crop than those placed on the other side (Figures 9 & 10). Although hedgerows have been found to have a barrier effect in terms of the widespread movement of pollinators such as butterflies (Fry & Robson, 1994; Dover & Fry, 2001) and carabid beetles (Mauremooto *et al.*, 1995), the results of this experiment suggest that hedgerows do not pose a barrier to foraging *Bombus terrestris* workers. This supports the results of Krewenka *et al.*, (2011) who found that in a lowland agricultural landscape in Germany, hedgerows did not pose a barrier to either foraging solitary bees or foraging bumblebees. The difference in the effect that hedgerows will have on different pollinator species may be due to the physiological differences that exist between them. These include factors such as size and flight capacity. For example, for a relatively small and light pollinator, a 2.50 m hedgerow and the air flow that surrounds it may prove an insurmountable flight barrier. This effect may be compounded for non-flying pollinators. For both solitary bees and bumblebees, however, even larger landscape features such as forests have not been found to be a barrier to flight although species-specific

differences have been observed (Kreyer *et al.*, 2004; Zurbuchen *et al.*, 2010). For example, Kreyer *et al.* (2004) found that when faced with a 600 metre wide forest, *Bombus terrestris* workers were more likely to bypass the forest by flying above the canopy in search of food sources while *Bombus pascuorum* workers were more likely to fly and forage within the forest. The maximum foraging range of these two bumblebee species differs with *Bombus terrestris* habitually foraging at distances over 600 m from its colony. In contrast, *Bombus pascuorum* is known to habitually forage well within 600 m from its colony (Knight *et al.*, 2005). As such, the differences observed in the effects that the forest had on both bumblebee species could be explained by the differences that exist between their maximum foraging ranges. Maximum foraging range in bumblebees is thought to be physiologically bound (Greenleaf *et al.*, 2007). This suggests that the effects that hedgerows may have on different bumblebees and different pollinator species more generally will be species specific and largely driven by fundamental physiological differences.

Although the hedgerows investigated in this experiment did not seem to have a barrier effect on the foraging choices of bumblebees, the plant species found within the hedgerows were an important foraging resource in themselves. At all three experimental sites, bumblebees were foraging for pollen from both the mass flowering crop and the flower species found in the hedgerows and green lanes around the colonies (Figures 2.9 & 2.10). Furthermore, at Site Three, the majority of bumblebees from colonies placed on either side of the hedgerow were foraging for pollen from plant species found in the hedgerows and not from the mass flowering orchard (Figures 2.9 & 2.10). This is in contrast to Site Two, where the majority of bumblebees foraged for pollen from the mass flowering crop (Figures 2.9 & 2.10). The differences between the two experimental sites may stem from a variety of factors. For instance, the differences observed could be due to the differences in the overall availability of forage at each site. At Site Two, although a small number of plant species at the base of the hedgerows and in the green lanes around the colonies were flowering, the hedgerows themselves were not. At Site Three, however, bumblebees could choose to forage from both plants in the mass flowering orchard as well as plants located within the hedgerows themselves as the latter were also in full bloom during the experiment.

The differences observed could also be due to the nutritional quality of pollen that bumblebees forage for. For example, Vaudo *et al.* (2018) found that bumblebees placed in three different habitats foraged for pollen which had similar nutritional quality. In all three habitats, bumblebees preferred to forage on pollen which had a 4:1 protein to lipid ratio. It is possible that in the present experiment, the choice of pollen by bumblebees at each site depended on nutritional content. At Site Two, the pollen originated largely from the mass flowering crop while at Site Three, the pollen originated largely from the plant species found in the hedgerows. The accessibility of pollen from flowers in the surrounding landscape (Lunau, 2000; Koch, Lunau & Wester, 2017) as well as the morphology of pollen and its ease of packing into the bumblebees' corbiculae (Lunau *et al.*, 2015; Konzmann, Koethe & Lunau, 2019) may have also influenced the foraging choices of bumblebees at each experimental site. Another factor which may have accounted for the differences observed between experimental sites is if pesticides were differentially used at each site, as bumblebees have been shown to distinguish between foods with and without neonicotinoid pesticides (Kessler *et al.*, 2015; Arce *et al.*, 2018). We cannot extrapolate this theory directly to our results as we do not know the pesticide management of the crops, but this should be accounted for in future studies.

When investigating the role that distance from a mass flowering crop has on the pollen foraging choices of bumblebees, bumblebees at Site Three were significantly more likely to forage for pollen from the mass flowering orchard the closer they were situated to it. This result was observed both for bumblebees on their first flight outside their colony as well as for all the flights that they conducted throughout the experimental period (Figures 2.11 & 2.12). This result should be interpreted with caution, however, as only two different locations were compared. As such, the effects observed could be due to other differences between the colonies. If the results observed are indeed due to the effects of distance, this supports previous findings in which bumblebees were found to forage on a mass flowering crop the closer they were situated to it (Osborne *et al.*, 2008). From a forager's perspective, the energy costs associated with travelling to a particular food resource must be offset by the rewards encountered there. Given equally rewarding food sources located at different distances from the colony, optimal foraging theory predicts that bumblebee workers should choose to forage on the

closest available resource to minimise their energetic costs (Heinrich, 1979a). If bumblebees are actively surveying the landscape during 'exploratory' flight segments, then the result at Site Three suggests that the plant species found in the orchard and in the hedgerows were of similar quantity and quality. Alternatively, a bumblebee's choice of forage could also be the result of a passive encounter in the environment (Goulson, 2010). For example, bumblebee workers could begin to forage on the first plant species that they encounter following their orientation/learning flights. At Site Three, bumblebees would be more likely to passively encounter the mass flowering resource that was closest to their colony. As the mechanisms governing specific foraging choices are not presently known, it is not possible to tease these two alternative explanations apart.

#### 2.6.1 Behaviour of Commercial Colonies in this Experiment

Throughout this experiment, a low proportion of released bumblebees returned to the colony throughout the experimental testing days. Although the overall mean proportion of released bumblebees that returned to the colony in the hedgerow investigation was 0.71, this ranged widely from 0.13 to 0.98 depending on the colony. In the distance investigation at Site Three, the overall mean proportion of released bumblebees that returned to the colony was 0.81. This too, however, ranged widely from 0.464 to 0.981 depending on the colony. As the colonies used were not at their reproductive stage and therefore not producing males or gynes, this points to the fact that in many of the experimental colonies used, bumblebee foragers left their colonies but never returned. This observation is in line with that reported by Evans, Smith and Raine (2017), who found that 42% of their released bumblebees never returned to their colonies. Such losses of foragers and wide variations between colonies placed in the same environment suggest that the underlying reasons for these losses lie within the colonies themselves. In both this experiment and that of Evans, Smith and Raine (2017), the bumblebees used were laboratory reared colonies purchased from well-established suppliers. Furthermore, agricultural growers who use commercial bumblebee colonies for pollination services are also observing large forager losses (J.Osborne, personal communication). The mass rearing of bumblebees and their global distribution can have many negative effects, as commercial colonies have been found to carry diseases which are easily transmitted to both wild bumblebees and honeybees (Graystock *et al.*, 2013; Owen, 2016). The mass rearing of

bumblebees could also result in a large degree of inbreeding. In this scenario, it could be possible that detrimental genetic mutations associated with navigation, foraging skills and even motivational levels would not be selected against and allowed to continue throughout subsequent generations. For example, certain genetic mutations within a bumblebee's mushroom bodies (*corpora pedunculata*), the regions of densely packed neuropils which serve as high-level sensory integration centres involved in learning and memory (Heisenberg, 1998), could have extremely detrimental effects. This is especially relevant as a correlation between the density of the synaptic complexes in a region of the mushroom bodies and visual discrimination in bumblebees has been found (Li *et al.*, 2017).

Observations pointing to the possibility of high levels of inbreeding present in mass reared colonies come from the amount of colony drift observed in commercial colonies as well as a knowledge of commercial bumblebee rearing practices. Colony drift, in which bumblebee workers from different colonies leave their maternal colony for another, is reported as being as high as 24% in commercial colonies (Birmingham and Winston, 2004). Colony drift was observed in the present experiments as well, although this was not formally quantified. Bumblebees use odour cues to differentiate between kin and non-kin (Whitehorn, Tinsley & Goulson, 2009) and non-kin bumblebees are normally attacked when they first enter a colony (Free, 1958). As such, in wild colonies, only around 3% of workers have been identified as drifters (Zanette *et al.*, 2014). Such high colony drift in commercial colonies suggests that individuals within each colony, especially those originating from the same supplier, are highly related and that overall, a low genetic diversity within the colony stocks exists. The little information that is available on the exact rearing practices of the commercial suppliers suggests that although wild caught bumblebees were first used to replenish the supplier's stocks, commercial suppliers are now able to continually produce reproductives without further replenishment (Velthuis & van Doorn, 2006). Taken together, these observations point to the fact that a high degree of overall loss might be expected when using commercially reared bumblebee colonies, both in field experiments and for pollination services. Future research could test the hypothesis of genetic inbreeding in commercial bumblebee colonies and the potential adverse impacts on navigation and foraging. At

present, caution should be taken if results obtained using commercially reared bumblebees are generalised to the behaviour of wild bumblebees. To counteract the present situation, rearing from locally wild caught queens could prove to be a viable alternative and may need to be adopted more widely.

#### 2.6.2 Experimental Limitations and Improvements

This experiment and the validity of its results could be improved by increasing the number of experimental sites used. Although hedgerows were not found to significantly affect the flight paths of naïve *Bombus terrestris* workers or their pollen foraging choices at the three sites used here, this may not be the case in different landscapes. This is especially true if landscapes containing hedgerows of different heights are used, in which higher hedgerows pose a flight barrier to bumblebees. If it had been possible, experimental sites should have allowed colonies to be placed at similar distances to the mass flowering crop and to ensure that additional colonies were placed within the mass flowering crop fields at each site. The latter would have allowed for an integrated analysis investigating both the effects of the hedgerow and the distance from the mass flowering crop on the pollen foraging behaviour of bumblebee workers. Furthermore, this would have also considered whether an interaction between these two factors was also present. Where resources permit, replication of this experiment, and experimental bumblebee research more generally, should consider using workers from colonies established using locally wild caught queens. This would greatly improve experimental sample sizes. Alternatively, a greater number of commercially reared colonies should be used. This would also allow for any differences between colonies to be considered. As evidence suggests that the foraging range of bumblebees is species specific; and that *Bombus terrestris* has one of the largest foraging ranges (Kreyer *et al.*, 2004; Westphal *et al.*, 2006; Greenleaf *et al.*, 2007); it is entirely possible that hedgerows may differentially affect bumblebee species with smaller foraging ranges as these species may employ different strategies when exploring the landscape. The use of different bumblebee species in related experiments can shed light on whether the effect of landscape features such as hedgerows is also species specific.

## 2.7 Conclusion

In the experiments presented in this chapter, naïve bumblebees exiting their colony were not more likely to fly alongside the hedgerow adjacent to their colony or towards a mass flowering crop. This does not rule out the possibility that bumblebees may choose to fly alongside hedgerows in order to minimise energetic costs related to air turbulence when they are experienced or have a specific location as the end goal of their flight. Furthermore, hedgerows were not found to have a barrier effect to bumblebee flight, and bumblebees were more likely to forage on mass flowering resources the closer their colony was situated to them. Overall, in lowland agricultural landscapes, flowering hedgerows represent a crucially important foraging resource and bumblebees will utilise this resource even when crops are flowering within their vicinity.

The experiments presented in this chapter focused on the effects of hedgerows on the behaviour of naïve bumblebees. In Chapter Three, using the same experimental set-up, the focus shifted to exploring the effect of experience on the foraging behaviour of *Bombus terrestris*. Specifically, the flight duration and weight of pollen foraged during a bumblebee's first five flights was investigated.

## Chapter Three

### Examining the Effect of Experience on *Bombus terrestris* Foraging Behaviour

#### 3.1 Abstract

Maximising the rate of resource acquisition is critical if bumblebees are to successfully exploit the floral resources around their colony. Naïve bumblebees will quickly improve their flower handling skills and a worker's first few flights outside the colony likely represent a significant developmental period. To shed light on this knowledge acquisition phase, the pollen foraging behaviour of individual bumblebees during their first five flights outside the colony was investigated. Naïve *Bombus terrestris* workers were individually tagged and allowed to forage freely in three agricultural sites in South West England. A worker's first five flights outside the colony were characterised by recording flight duration and the weight of pollen foraged for each flight. The number of previous flights did not have a significant effect on flight duration and large individual differences between subsequent flights were observed during the experiment. The weight of pollen foraged significantly increased as the number of flights outside the colony increased. In a complementary experiment in which *Bombus terrestris* workers were marked with RFID tags, the number of previous flights was also found to not have a significant effect on flight duration. Taken together, this is the largest investigation to date which has focused on the development of pollen foraging behaviour, starting with a bumblebee's first flight. The results suggest that the first five flights outside the colony represent a period in which different foraging parameters, such as flower handling skills and displacement distance from the colony, are developing at different rates. Furthermore, large variations exist both within and between individual bumblebees in terms of foraging flight development.



### 3.2 Introduction

Bumblebees use a variety of different strategies in order to maximise their foraging efficiency. By minimising their travel times both within and between flower patches and maximising their floral rewards, bumblebees can maximise their rate of resource acquisition (Charnov, 1976). These strategies are not innate and foraging efficiency is very much experience dependent. In field observations, inexperienced bumblebees on their first flight outside the colony were inefficient foragers and did not restrict their foraging to specific routes or tracks (Heinrich, 1979a). In lifelong harmonic radar tracking of bumblebee workers, the flight segments of bumblebees on their first flights were also characterised by long, circuitous exploration of the landscape (Woodgate *et al.*, 2016). As bumblebees gain more experience of their environment, their foraging and navigation behaviour changes. By the sixth flight outside the nest, individual flight paths straighten, average groundspeed increases, the maximum displacement distance from the colony increases and constancy to both foraging site and compass bearing upon take off is observed (Osborne *et al.*, 1999; Osborne *et al.*, 2013). In contrast to naïve bumblebees, experienced foragers also have flight segments which are dominated by 'exploitative' behaviour, characterised by straight tracked flights to and from a single foraging location (Woodgate *et al.*, 2016).

As experience is gained, two distinct behaviours emerge. In laboratory studies which presented bumblebees with a uniform array of rewarding flowers, naïve bumblebees who had never foraged beforehand sampled a large number of different flowers, taking a variety of different flight routes between them. Experienced foragers, on the other hand, established a particular flight route and travelled faster between flower patches (Ohashi, Leslie & Thomson, 2008; Klein *et al.*, 2017a). As naïve bumblebees gained experience, they decreased the total distance that they travelled within the floral array by up to 80% (Lihoreau *et al.*, 2012). "Semi-natural" studies of bumblebees tracked with harmonic radar also show similar results. When presented with an artificial floral array which was set in a natural environment, the bumblebees' total flight distance within the array as well as their total duration of flights reduced with experience. Flight paths also became straighter and 'exploratory' behaviour, flight segments outside the floral array boundary, decreased. Furthermore, this reduction in 'exploratory' behaviour

accounted for around half the observed reduction in total flight duration (Woodgate *et al.*, 2017). This behaviour, of making repeated, non-random circuits between food patches, is termed trapline foraging (Manning, 1956; Heinrich, 1976) and has the overall effect of increasing foraging performance (Ohashi, Leslie & Thomson, 2008). Trapline foraging is also closely linked to flower and site constancy, a behaviour in which individual bumblebees restrict their foraging visits to single flower types (Waser 1986) or foraging sites (Bowers, 1985; Waser, 1986; Dramstad, 1996; Saville *et al.*, 1997). As bumblebees gain experience of their environment, traplines between constant foraging sites are established by the twenty-sixth flight outside the nest (Lihoreau *et al.*, 2012). Both trapline foraging and flower constancy are thought to be flexible behaviours, with bumblebees adding or discarding floral patches to their flight routes in line with environmental conditions and colony need (Thomson & Chittka, 2001). The extent of trapline foraging also varies between individual bumblebees and not all bumblebees exhibit this behaviour (Woodgate *et al.*, 2017).

Experience also has an effect on a bumblebee's flower handling skills. Bumblebees take a number of visits to a plant species to learn to efficiently extract both pollen and nectar from specific flowers (Lavery, 1980; Lavery and Plowright, 1988) and the number of different flowers visited during a foraging trip increases with flight experience (Lihoreau *et al.*, 2012). The number of daily foraging trips also increases with age and older bumblebees have also been observed collecting larger pollen loads (Cartar, 1992). Nectar collection also seems experience dependent as more experienced bumblebees exhibit a higher nectar collection rate (Evans, Smith & Raine, 2017). Lastly, it takes on average 30 trips outside the nest for a bumblebee worker's foraging efficiency to reach a plateau (Peat & Goulson, 2005).

In bumblebees, behavioural differences between the different castes are largely recognised and documented (Alford, 1975; Heinrich, 1979a; Goulson, 2010). There is evidence now emerging that social insects also display a level of behavioural variability within each caste (Pinter-Wollman, 2012; Walton & Toth, 2016). For example, harmonic radar tracking studies of bumblebees have found significant inter-individual differences in their behaviour, both in experiments using free-flying bees (Woodgate *et al.*, 2016) as well as those using artificial floral arrays (Woodgate *et al.*, 2017). Laboratory and field studies have also

shown that the daily number of foraging trips taken by individual bumblebees varies widely between individual workers (Woodgate *et al.*, 2016; Evans, Smith & Raine, 2017). Furthermore, bumblebee workers also exhibit variation in their learning performance (Smith & Raine, 2016; Walton & Toth, 2016) as well as their decision speed in flower discrimination tasks (Chittka *et al.*, 2003; Burns & Dyer, 2008). As each bumblebee worker will have a unique experience of its surrounding environment, it seems likely that each individual worker's behaviour is unique (Thomson & Chittka, 2001). Such individual variations in foraging and navigation behaviour of bumblebee workers within a colony could improve overall foraging efficiency and adaptability by buffering the colony against factors brought on by environmental change (Ohashi & Thomson, 2005; Klein *et al.*, 2017a; Klein *et al.*, 2017b). Specifically, workers who are more prone to developing trapline foraging behaviour may perform better in stable environments which are characterised by highly predictable rewards while workers who are more prone to exploration may perform better in highly variable reward environments (Klein *et al.*, 2017a). As such, a colony with workers that exhibit a diversity of different foraging behaviours and strategies can exploit a variety of different environments.

Although the effects of experience on bumblebee navigation and foraging have been widely documented in both laboratory and field studies, the knowledge acquisition phase in individual bumblebees throughout their first flights outside the colony remains largely unknown. As central place foragers (Plowright & Lavery, 1984), bumblebees must be able to return to their colony on their first flight outside the nest. Bumblebee workers are also often seen returning to the colony with forage on their very first flight (Hempel de Ibarra *et al.*, 2009; Osborne *et al.*, 2013). It seems likely that the first few flights of a bumblebee outside its colony represent a period where significant navigation and forage handling skills are gained. In order to investigate the behaviour of bumblebee workers during their first few flights outside the colony and the role that experience may play throughout these flights, the pollen foraging behaviour of individual bumblebees during their first five flights outside the colony was investigated. Specifically:

1. Does flight duration change during a bumblebee worker's first five flights as workers gain more experience of their environment?

2. Does the weight of pollen foraged change during a bumblebee worker's first five flights as workers gain increased flower handling skills?
3. How variable are the changes within individual bumblebee workers?

In order to answer these questions, bumblebee colonies were placed in two different agricultural sites in the South West of England. Individual naïve bumblebees were marked and monitored throughout their first five flights outside their colony. The duration of their flights (including orientation/learning flights and foraging trips) and the weight of the pollen that they foraged for on a particular trip were recorded. A number of different scenarios were hypothesised. Although it has been previously found that bumblebee workers will increase their maximum displacement distance from their colony throughout successive flights (Osborne, *et al.* 2013), field studies have also observed that the majority of a colony's foraging flights are made towards a single foraging destination (Osborne *et al.*, 1999; Woodgate *et al.*, 2016). In this experiment, all of the experimental sites chosen comprised a single mass flowering crop which was the only mass flowering crop within a 900 m radius of the colonies. Additional forage was scattered throughout the hedgerows and green lanes in the landscape. Specifically, the bumblebee colonies were placed within 300 m of this mass flowering crop as bumblebees are known to exploit mass flowering crops the closer they are situated to them (Westphal, Steffan-Dewenter & Tscharnkte, 2006; Osborne *et al.*, 2008). If the majority of bumblebee workers chose to forage predominantly on the mass flowering crop and site constancy develops within their first five flights outside the colony, then the flight duration of workers should decrease as they gain more experience of their environment. This would be due to a variety of factors: an increase in the average groundspeed of bumblebee workers, which has been found to increase throughout successive flights (Osborne *et al.*, 2013), a decrease in the number of exploratory flight segments (Woodgate *et al.*, 2017), and an increase in their flower handling skills (Raine & Chittka, 2007). This scenario, however, assumes that the time spent foraging within a foraging patch remains constant. Bumblebee workers could increase the number of flowers sampled within a foraging patch as they gain more experience with successive flights (Lihoreau, *et al.* 2012). In this scenario, flight duration should increase throughout the first five flights as a greater number of flowers are sampled.

Such an increase in the number of flowers handled and its associated time costs, however, could be off-set by a gradual improvement in flower handling skills. There is evidence that a trade-off in bumblebee foragers exists between finding the shortest possible route and prioritising visits to more rewarding flower sources (Lihoreau, Chittka, and Raine, 2011) and between the accuracy of route following and travel speed (Ohashi, Leslie and Thomson, 2008; Ohashi & Thomson, 2013). As such, flight duration may remain relatively unchanged throughout the first five flights as various factors off-set each other. As flight duration and its development are likely to be influenced by a variety of different factors, our current knowledge does not allow us to disentangle the various possible hypotheses. The exact mechanisms at play are also likely to vary among individual bumblebees. If individual bumblebees are consistent to a particular foraging strategy, then foragers, and their flight durations, may naturally fall into distinct categories. If, however, individual bumblebees change their foraging strategies with successive flights, then there should be large variations in individual bumblebees' flight durations.

If constancy to a particular foraging source develops early on, then the weight of pollen foraged should increase throughout the first five flights as bumblebees improve their flower handling skills. As inter-individual variability has been observed in nectar foraging bumblebees, with larger workers collecting greater amounts of pollen (Goulson *et al.*, 2002), it is hypothesised that variation in the amount of pollen foraged will be observed. As the availability of pollen will be dependent on both daily fluctuations (Raine & Chittka, 2007) and depletion rates, it is also hypothesised that the amount of pollen foraged for between a bumblebee's successive flights will also vary.

Investigating the pollen foraging behaviour of individual bumblebees necessitated that the pollen collected by each forager is removed from their corbiculae on each of their foraging trips. In order to try and control for the effects of handling the individual bumblebees, the data gathered were compared to that obtained from a separate investigation where less handling was required. In this second investigation, additional colonies were placed in two of the experimental sites. Bumblebee workers in these colonies were marked with RFID tags (Microsensys GmbH, Germany). RFID tags are passive tags which function without a power source and contain a unique identification number. When present within the

vicinity of a RFID reader, an electronic record is made of the tag's identification number as well as the date and time of the reading. By fitting RFID readers at the colony entrances, the flight activity of tagged bumblebees could be electronically obtained. As such, RFID bumblebee workers in these colonies were allowed to exit and enter their colonies freely without any additional handling beyond their initial tagging. RFID tagging has been widely used in previous bumblebee behaviour studies and has not been found to affect a bumblebee's natural behaviour (Streit *et al.*, 2003; Molet *et al.*, 2008; Gill, Ramos-Rodriguez & Raine, 2012; Evans, Smith & Raine, 2017). Although the amount of pollen foraged could not be investigated, the duration of a worker's first five flights outside the colony was automatically recorded. This could then be used as a comparison data set. This additional investigation is described in Part B.

### **3.3 Part A: Effect of Experience on Flight Duration and Pollen Foraging**

#### **3.3.1 Materials and Methods**

##### 3.3.1.1 Experimental Sites

The experimental sites used in this experiment were identical to those used and described in Chapter Two: Site One, Site Two and Site Three. Data for this experiment were collected from the same colonies of bumblebees during the same periods as in Chapter Two.

##### 3.3.1.2 Temperature Conditions and Sampling Regime

Experiments at both sites were conducted on dry days, with temperatures varying from 11.0 to 26.0°C. Wind direction and speed were recorded at all three sites using an automated weather station (ProData Weather Systems, UK) and was variable throughout. Colonies were tested continuously from mid-morning to dusk at both sites on testing days. Colonies were closed outside of testing times.

##### 3.3.1.3 Individual Tagging and Flight Duration Recording

The experimental procedure used in this experiment was identical to that described in Chapter Two. At each site, the bumblebee colonies were placed in a wooden nest box (Chapter Two: Figure 2.4). The bumblebee colony was connected to the outside of the nest box using a clear, Perspex® tube. The movement of individual bumblebees both inside and outside the colony was

controlled using plastic spacers, located at regular intervals throughout the tube. Purchased colonies contained a queen and at least 50 naïve workers of mixed age that had never flown outside the plastic box in which they had arrived.

Naïve, untagged workers were released from the nest throughout the testing day. A number of bumblebee workers from Site Three were tagged *prior* to being released on their first flight. As such, there is an accurate record of the duration of the first flight of these bumblebees. All other bumblebees, however, were only tagged upon their return to the colony after their first flight. This was done as a result of observations made in a pilot study. In a pilot study, the exit flap on the nest box of purchased colonies was opened in the laboratory. A number of bumblebees exited the colony and these bumblebees were tagged before being placed back into the colony. The nest boxes were then transported to the experimental site. Unfortunately, once at the experimental site, the majority of bumblebees that were tagged in the laboratory never emerged from the nest box. Because of this, the decision was made to only tag bumblebees upon their return to the colony after their first flight. This would ensure that the tagged bumblebees were those that were coming out of their nest box in order to forage at the experimental site. As such, there is no accurate record for the majority of workers of their first flight duration. There is, however, an accurate record of the weight of the pollen loads of all returning bumblebees on their first flight. All tagging was done using honeybee queen marking tags (EH Thorne (Beehives) Ltd, UK).

Newly tagged workers were placed back into the wooden nest box and allowed to return to their colony. As soon as a worker, tagged or otherwise, exited their colony into the plastic tunnel system, an experimenter would manipulate the plastic spacers to allow the worker to exit the wooden nest box. All nest traffic, including the number of flights outside the colony that each tagged worker had made and the duration of each of those flights from exit to return, was manually recorded by experimenters throughout the testing days. It should be noted that the body size of foraging bumblebees was not measured or recorded.

#### 3.3.1.4 Pollen Recording

In order to stimulate workers to predominantly forage for pollen, the colonies had access to the sugar solution that was provided by the manufacturer inside the colony box *ad libitum*. Upon return to the wooden nest box, all bumblebees were

captured and stripped of their pollen loads before being allowed to return to their colony. Pollen was stripped from each bumblebee on each of their returning flights. This was done by removing all of the pollen from the corbiculae of each bumblebee using a wooden toothpick while the bumblebee was immobilised in a honeybee queen marking cage (EH Thorne (Beehives) Ltd, UK). Non-pollen foraging trips were also recorded. Pollen loads were frozen at -20.0 °C at the end of each testing day. Frozen pollen loads were then defrosted and freshly weighed.

### **3.3.2 Statistical Analysis**

The first five flights of bumblebees were used in the following statistical analysis. Throughout the experiment, the total number of flights differed between individual bumblebees. As such, only bumblebees that took at least five consecutive flights during the sampling period, and which had a record of the duration of each of these flights, or a record of their pollen foraging on these flights, were used (Appendix B: Table B.1). In order to investigate whether the decision to restrict the analysis to the first five consecutive flights biased the results presented, an additional analysis was also performed. In this additional analysis, the flight duration and weight of pollen foraged on all the flights collected throughout the experiment were used. Details of this analysis can be found in Appendix B.

Data collected at Site One were entirely excluded from the analysis as only one bumblebee completed five consecutive flights outside its colony (Appendix B: Table B.1). For the flight duration analysis, bumblebees which had at least one overnight flight were excluded (19.5% of bumblebees which completed five consecutive flights and had a complete record of these flights; Appendix B: Tables B.1). This is due to the fact that the record of the duration of overnight flights is not entirely accurate. As testing did not begin at sunrise, bumblebees which had stayed out overnight may have attempted to return to their colony before an experimenter was present to let them into the colony. When bumblebees cannot enter their colony, they fly off and attempt to enter it at a later time (R.Herascu, personal observation). Furthermore, when these inaccurate data points are included in the statistical analysis, the models do not converge. For reference, however, a graph which includes these overnight flights, but which does not include any statistical analysis, is shown in Appendix B (Appendix B: Figure: B.2).



All statistical analysis was carried out in R 3.5.2 (R Core Team, 2015). Data were analysed using linear mixed models (LMMs) ('lme4' package; Bates *et al.*, 2015). Visual inspection was used to check residual plots for fit and homoscedasticity. P-values were obtained using the Satterthwaite's degrees of freedom method ('lmerTest' package; Kuznetsova, Brockhoff & Christensen, 2017). Response variables were transformed where required in order to meet model assumptions.

#### 3.3.2.1 Flight Duration

##### *Second to Fifth Flights*

Due to the way in which the experiment was conducted, the majority of bumblebees did not have an accurate record of the duration of their first flight. As such, only the duration of the second to fifth flights was analysed.

To determine whether the flight number outside the colony had an effect on the flight duration, the relationship between the duration of each flight outside the colony and the flight number was modelled using an LMM. As fixed effects, the experimental site and the flight number (entered as a numeric variable) were entered into the model with an interaction term. As a random effect, a random intercept model for the individual bumblebees was used (as a random slope model resulted in the model failing to converge). The flight duration was logarithmically transformed in order to meet model assumptions (Ives, 2015).

##### *First to Fifth Flights*

Five bumblebees at Site Three also had an accurate record of the duration of their first flight outside the colony. As such, the duration of the first to fifth flights of *these* bumblebees was graphed. Due to the low sample size, however, the relationship between flight number outside the colony and flight duration was not formally tested.

#### 3.3.2.2 Weight of Pollen Foraged

To determine whether the flight number outside the colony had an effect on pollen foraging, the relationship between the weight of pollen foraged on each flight outside the colony and the flight number taken was modelled using an LMM, with a gaussian distribution. As fixed effects, the experimental site and the flight number were entered into the model with an interaction term. A random intercept

model was used for individual bumblebees (as fitting a random slope model for the effect of flight number on each bumblebee did not give a significantly better fit to the data;  $\chi^2(2) = 0.37$ ,  $p = 0.83$ ). Post-hoc tests for estimated slopes for each site as well as differences between factors were also carried out ('emmeans' package; Lenth, 2019).

### **3.3.3 Results**

Of the total number of released bumblebees, the mean proportion of returning bumblebees from each colony was 0.71 (range: 0.13-0.98, please also refer to Chapter Two). Of the total number of returned bumblebees, the mean proportion of bumblebees that completed five flights throughout the experimental testing days from each colony was 0.05 (range: 0-0.20) (Appendix B: Table B.1).

Of the total number of bumblebees that returned with pollen on all five flights, 52.9% (27/51) remained completely constant and foraged from a single plant species throughout. 41.2% (21/51) had one flight where they foraged from a different plant species than that of their first flight. 3.98% (2/51) had two flights where they foraged from a different plant species than that of their first flight while 1.96% (1/51) had three flights where they foraged from a different plant species than that of their first flight. Furthermore, 33.3% (17/51) of bumblebees returned having foraged from more than one flower species during a single flight outside the colony.

#### **3.3.3.1 Flight Duration**

##### *Second to Fifth Flights*

When looking at each experimental site, bumblebees at Site Two had flight durations of between 10 and 157 minutes on their second to fifth flights outside the colony ( $n = 14$ ; Figure 3.1) while bumblebees at Site Three had flight durations of between 9 and 126 minutes on their second to fifth flights outside the colony ( $n = 19$ ; Figure 3.1). Furthermore, 90% of flight durations at Site Two ranged between 21 and 108 minutes while 90% of flight durations ranged between 17 and 84 minutes at Site Three.

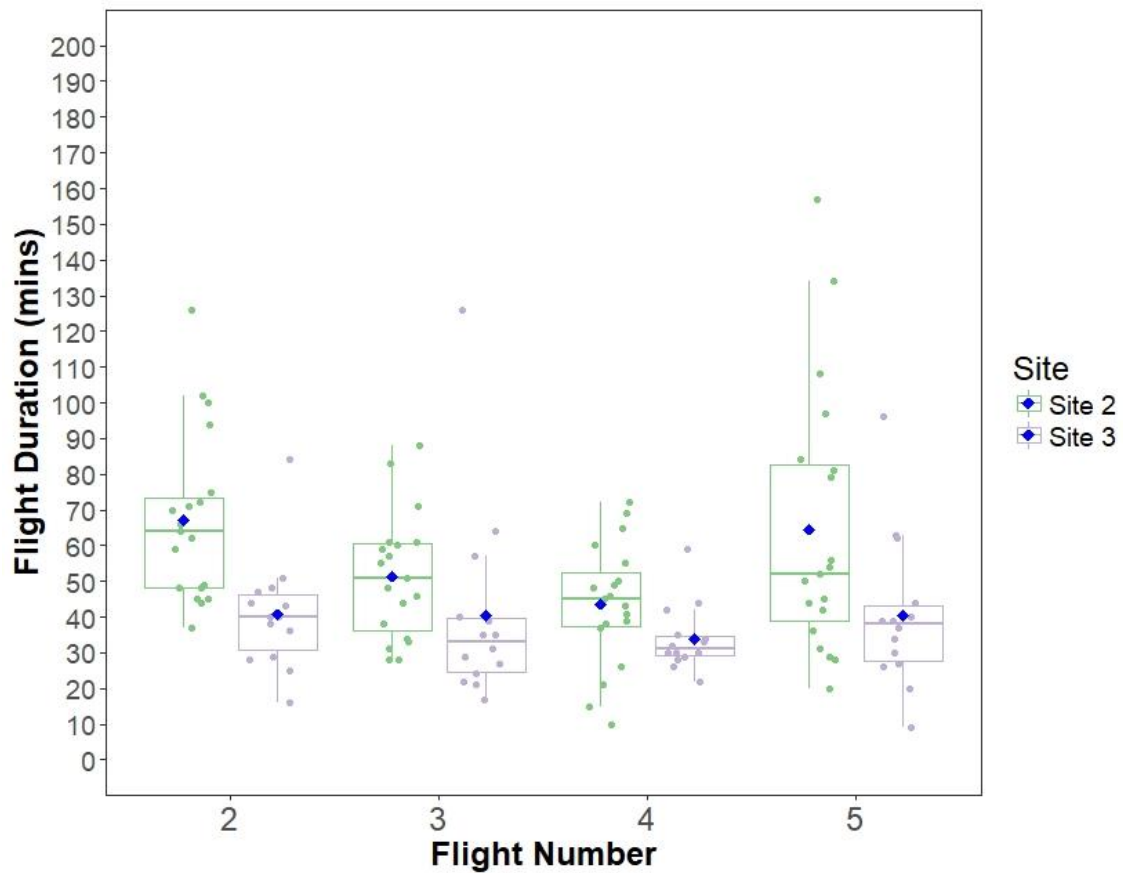
When comparing the flight duration of bumblebees throughout their second to fifth flights outside the colony, there was no significant effect of the flight number on the duration of flights outside the colony (Table 3.1; Figure 3.1) and there was

no significant effect of the experimental site on the flight duration (Table 3.1; Figure 3.1). There was also no significant interaction between the site and the flight number (Table 3.1; Figure 3.1).

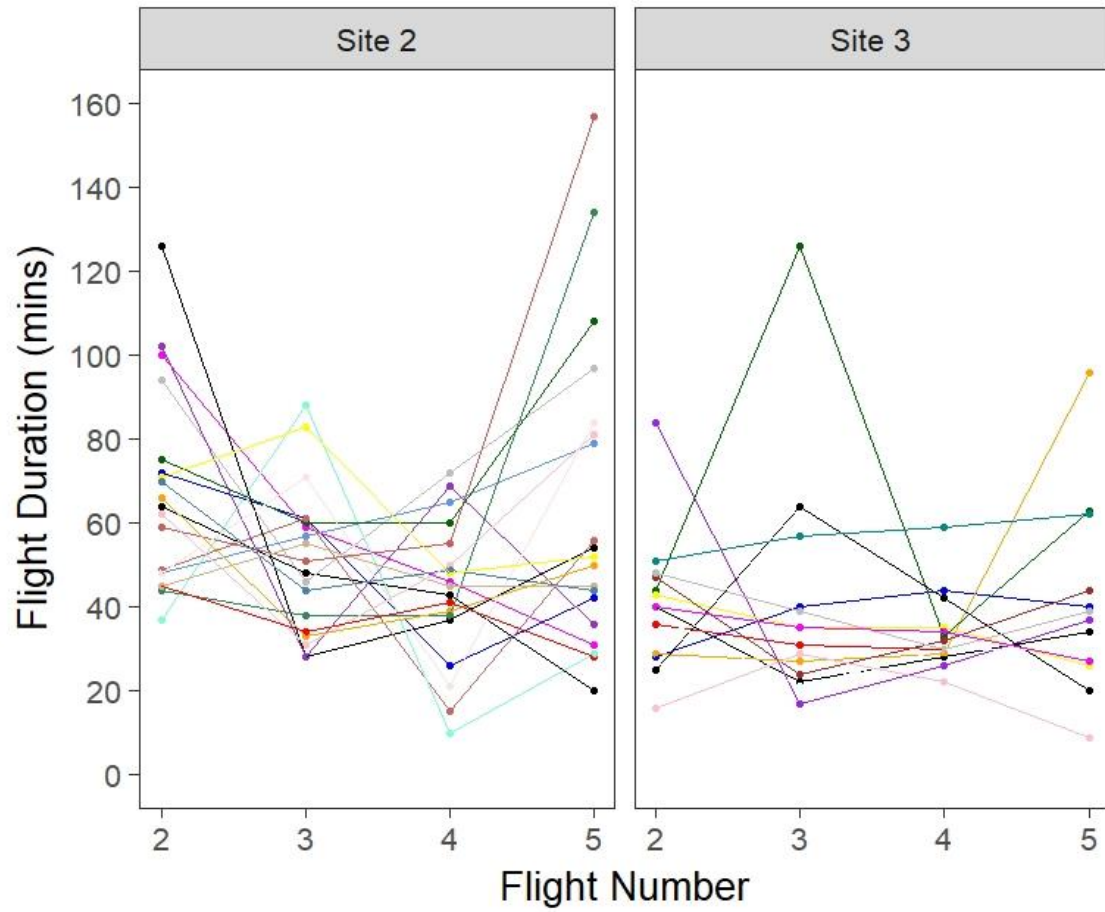
There was also large variation among the successive flights of individual bumblebees (Figure 3.2).

**Table 3.1. Model results from a linear mixed effects model testing the effect of the flight number and experimental site on the flight duration. A random intercept model with individual bumblebee identity was used. The flight duration was logarithmically transformed to meet model assumptions. Variables, effect sizes  $\pm$  standard error, degrees of freedom, *t*-values and *p*-values from the fitted model. Site Three was used as the reference level.**

<b>Variable(s)</b>	<b>Effect Size <math>\pm</math> Standard Error</b>	<b>Degrees of Freedom</b>	<b><i>t</i> Value</b>	<b><i>p</i> Value</b>
Intercept	3.66 $\pm$ 0.20	118.38	18.51	<0.0001
Site	0.48 $\pm$ 0.26	118.38	-0.49	0.06
Flight Number	-0.02 $\pm$ 0.05	97.00	0.51	0.61
Flight Number * Site	-0.03 $\pm$ 0.07	97.00	-0.49	0.62



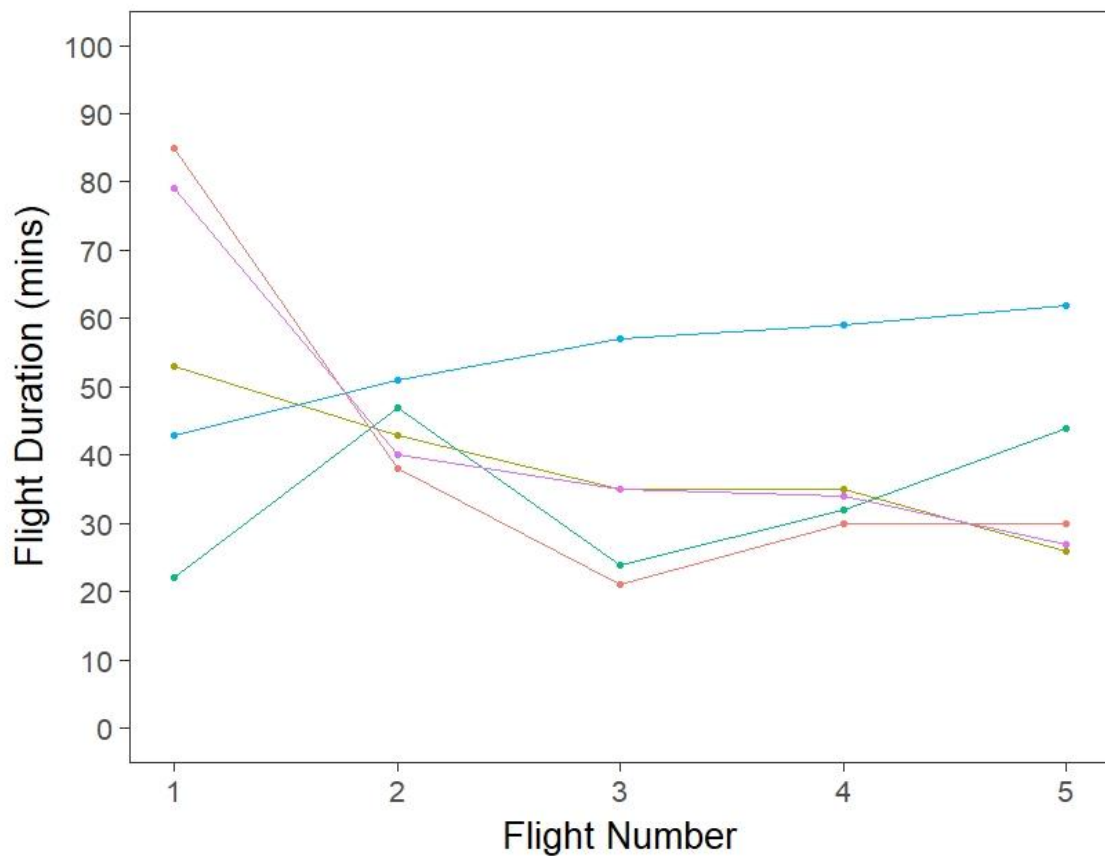
**Figure 3.1** | Box and whiskers plot of the flight duration (minutes) of bumblebees from their second to fifth flight outside the colony. Individual data points are superimposed on the plots.  $N=33$  with  $n=19$  at Site Two and  $n=14$  at Site Three. Blue diamonds denote the mean. A small value of random noise was added to each data point for plotting purposes to aid visual representation.



**Figure 3.2** | Flight duration (minutes) of individual bumblebees from their second to fifth flight outside the colony.  $N=33$  with  $n=19$  at Site Two and  $n=14$  at Site Three.

### *First to Fifth Flights*

For five bumblebees at Site Three, there was a complete record of the duration of their first five flights outside the colony. The flight duration of these bumblebees ranged from 21 and 85 minutes on their first five flights outside the colony and the first flight showed the greatest variation (Figure 3.3).



**Figure 3.3** | Flight duration (minutes) of individual bumblebees from their first to fifth flight outside the colony at Site Three.  $N=5$ .

### 3.3.3.2 Weight of Pollen Foraged

When looking at each experimental site, bumblebees at Site Two foraged for pollen weighing between 0 and 93.00 mg ( $n = 36$ ) on their first five flights outside the colony. Bumblebees at Site Three foraged for pollen weighing between 4.90 and 82.10 mg ( $n = 15$ ) on their first five flights outside the colony.

90% of the pollen loads which individual bumblebees foraged for at Site Two weighed between 0 mg and 65.60 mg on their first five flights outside the colony while 90% of pollen loads which individual bumblebees foraged for at Site Three weighed between 12.20 mg and 74.90 mg on their first five flights outside the colony.

At Site Two, the main pollen source was red clover (*Trifolium pratense*), white clover (*Trifolium repens*) and bird's-foot trefoil (*Lotus corniculatus*). At Site Three, the main pollen sources were gorse (*Ulex spp.*), Norway maple (*Acer platanoides*) and flowering fruit trees (*Rosaceae spp.*). When comparing the weight of pollen foraged throughout the first five flights outside the colony, there was a significant interaction between the site where the experiment took place and the flight number (Table 3.2; Figure 3.4). There was a significant effect of flight number on the weight of pollen foraged (Table 3.2; Figure 3.4). There was no significant effect of site on the weight of pollen foraged (Table 3.2; Figure 3.4).

The weight of pollen foraged increased significantly at both sites with flight number (Table 3.3; Figure 3.4). There was a greater increase in the weight of pollen collected during flights at Site Three compared to flights at Site Two (contrast estimate =  $3.75 \pm 1.49$ ,  $df = 202.00$ ,  $t\text{-ratio} = 2.52$ ,  $p = 0.013$ ).

At both sites, individual bumblebees varied greatly in the weight of pollen foraged throughout their first five flights with no clear patterns between bumblebees or within successive flights (Figure 3.5).

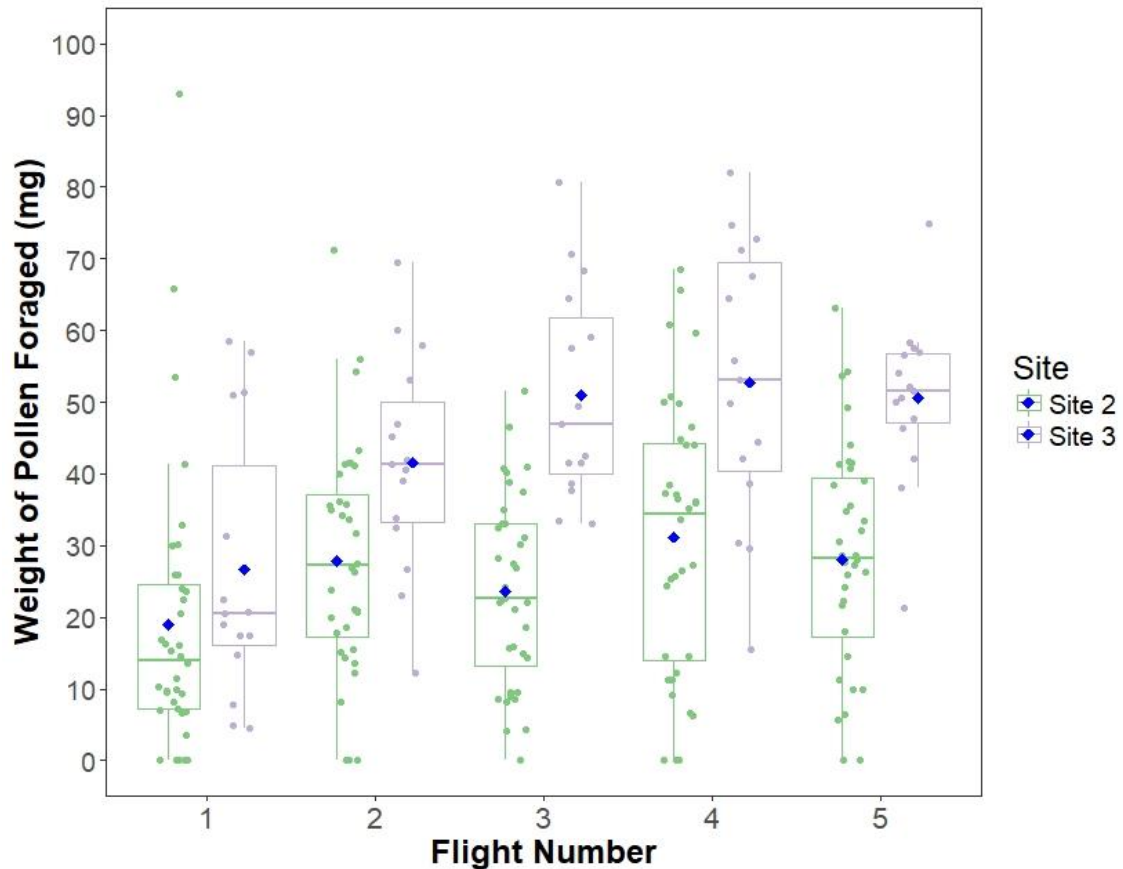


**Table 3.2. Model results from a linear mixed effects model testing the effect of the flight number and experimental site on the weight of pollen foraged. A random intercept model with individual bumblebee identity was used. Variables, effect sizes  $\pm$  standard error, degrees of freedom, *t*-values and *p*-values from the fitted model. Site Three was used as the reference level.**

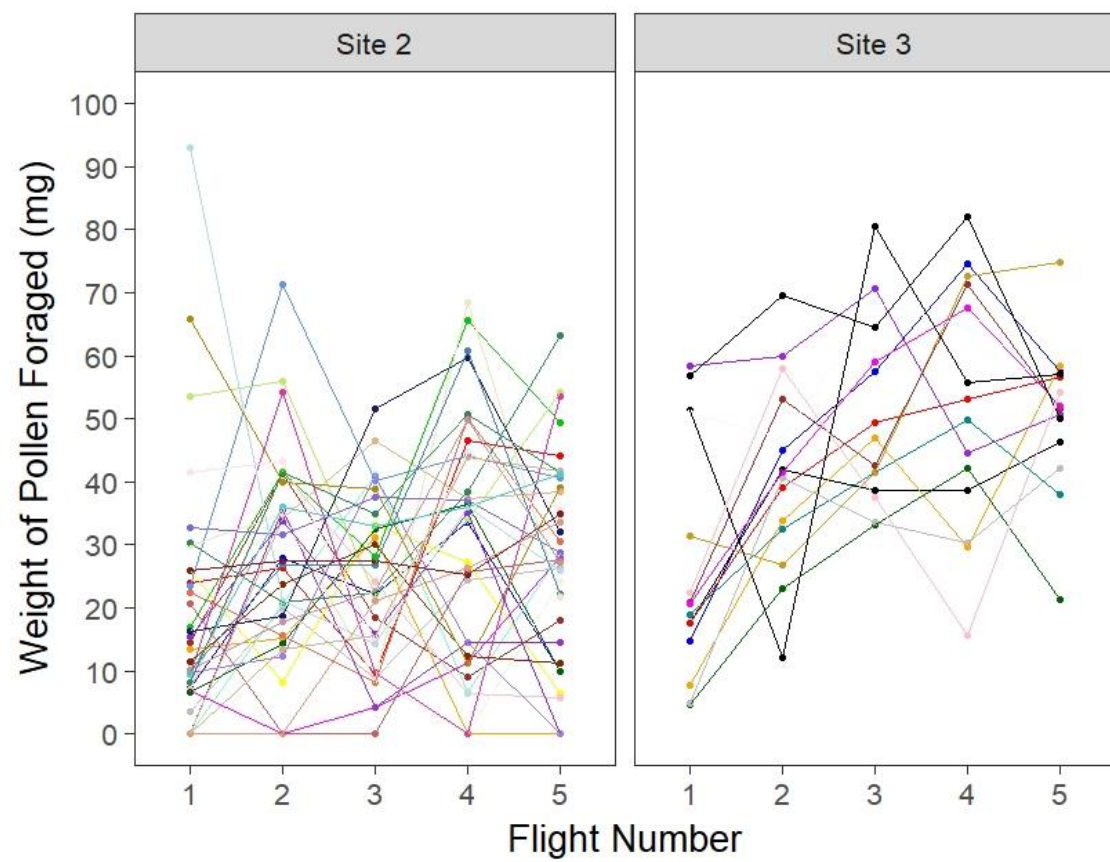
<b>Variable(s)</b>	<b>Effect Size <math>\pm</math> Standard Error</b>	<b>Degrees of Freedom</b>	<b><i>t</i> Value</b>	<b><i>p</i> Value</b>
Intercept	26.76 $\pm$ 4.82	48.98	5.56	<0.0001
Site	-7.32 $\pm$ 5.42	228.80	-1.35	0.18
Flight Number	5.92 $\pm$ 1.25	202.00	4.72	< 0.001
Flight Number*Site	-3.75 $\pm$ 1.49	202.00	-2.52	0.013

**Table 3.3 Estimated slopes of the relationship between weight of pollen foraged and flight number for each site, degrees of freedom  $\pm$  standard error and upper and lower confidence intervals.**

<b>Site</b>	<b>Estimated Slope <math>\pm</math> Standard Error</b>	<b>Degrees of Freedom</b>	<b>Lower Confidence Interval</b>	<b>Upper Confidence Interval</b>
Site Two	2.16 $\pm$ 0.81	202.00	0.57	3.76
Site Three	5.92 $\pm$ 1.25	202.00	3.45	8.39



**Figure 3.4** | Box and whiskers plot of the weight of pollen foraged (mg) by bumblebees from their first to fifth flight outside the colony.  $N=51$  with  $n=36$  at Site Two and  $n=15$  at Site Three. Individual data points are superimposed on the plots. Blue diamonds denote the mean. A small value of random noise was added to each data point for plotting purposes to aid visual representation.



**Figure 3.5 |** Weight of pollen foraged (mg) by individual bumblebees on their first five flights outside the colony.  $N=51$  with  $n=26$  at Site Two and  $n=15$  at Site Three.

### **3.4 Part B: RFID Experiment: Testing the Effect of Experience on Flight Duration**

In order to try to control for the effects of handling bumblebees when stripping the pollen that they had collected in the experiment outlined in Part A, a similar but separate investigation with less handling was conducted.

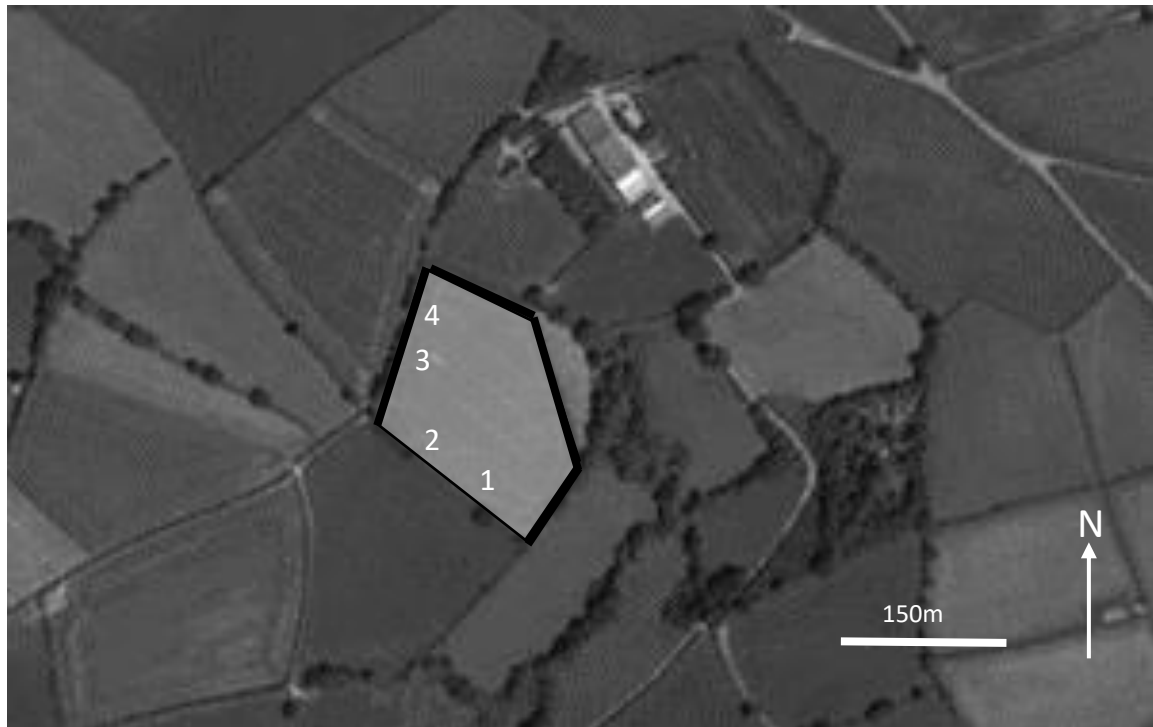
#### **3.4.1 Materials and Methods**

##### **3.4.1.1 Experimental Sites**

The experiment took place at Site Two and Site Three.

##### *Site Two*

The experiment at Site Two took place in the same agricultural fields that were used for the experiment in Part A. Four *Bombus terrestris audax* colonies were purchased (Koppert Biological Systems, UK). One colony was placed at the base of the southern hedgerow that bordered the flowering red clover field while the other three were placed at the base of the western hedgerow which bordered the flowering red clover field (Figure 3.6). All colonies were placed at least 7.50 m away from each other. All colonies were tested simultaneously from 27<sup>th</sup> July to 3<sup>rd</sup> August 2016. The red clover field was the only mass flowering crop field within a 900 m radius of the colonies (determined by matching current farm records with satellite imagery). In addition, during the testing period, a variety of wild flowers were blooming in the hedge banks and green lanes in the landscape around the colonies.



**Figure 3.6** | Experimental Site Two with *Bombus terrestris* colony placement (1-4). The black bordered grey area represents the red clover field. '1', '2', '3' and '4' represent the individual colonies used. Copyright Information: Google Earth V 7.3.2. (June 22, 2018). Eye altitude: 1060 m. <https://www.earth.google.com> [December 15, 2018].

### Site Three

The experiment at Site Three took place in the same agricultural fields that were used for the experiment in Part A. Three *Bombus terrestris audax* colonies (Koppert Biological Systems, UK) were used in total. The colonies were placed at the base of a hedgerow (Figure 3.7). All colonies were placed at least 7.50 m away from each other. All colonies were tested simultaneously from 14<sup>th</sup> - 17<sup>th</sup> June 2017. During the testing period, there were no mass flowering crops blooming within a 900 m radius of the colonies (determined by matching current farm records with satellite imagery). A variety of wild flowers, however, were blooming in the hedge banks and green lanes in the landscape around the colonies.



**Figure 3.7** | Experimental Site Three with *Bombus terrestris* colony placement (5-7). Copyright Information: Google Earth V 7.3.2. (June 22, 2018). Eye altitude: 778 m. <https://www.earth.google.com> [December 15, 2018].

#### 3.4.1.2 Temperature Conditions and Sampling Regime

The experiment was conducted regardless of the weather with temperatures varying from 10.0°C to 20.0°C. Colonies were tested continuously from before sunrise to after sunset in an effort to capture the full range of daily colony activity. Colonies were closed outside of testing times.

#### 3.4.1.3 RFID Flight Duration Recordings

At each site, the bumblebee colonies were placed in a wooden nest box (Chapter Two: Figure 2.4). The bumblebee colony was connected to the outside of the nest box using a clear Perspex® tube. RFID recording equipment was placed on the top of the wooden nest box (Microsensys GmbH, Germany). The RFID equipment consisted of two readers and a logger, each connected to each other and battery powered supplied. The readers had the shape of rectangular blocks, with a 2.0 cm diameter hole running through them. When an RFID tag (mic3-Tag, 64 bit read-only transponder, carrier frequency: 13.56 MHz, measuring 2.0 x 1.6 x 0.5 mm, mass: 4.00 mg) would be present in the reader's hole, the reader would detect this tag, and the electronic information from this tag would be registered on the logger. This electronic information consisted of the unique identification number of the tag, the time at which that tag was in the vicinity of the reader, and the unique identification number of the reader itself. As the readers were specifically designed for use with bees, the reader would only detect an RFID tag when it passed through the hole running through each reader. Two readers were placed on top of each other, with their holes aligned to match the entrance/exit hole of the wooden nest box. As such, when a tagged bumblebee would exit the wooden nest box, it would have to pass through the two readers on its way out. When a tagged bumblebee would enter the wooden nest box, it would again have to pass through the two readers. Each time a bumblebee passed through the readers, two electronic records would be generated, one from each reader. As the readers had unique identification numbers, the direction of travel of the bumblebee (in or out of the nest box) could be inferred by comparing the two electronic records and matching them with the order in which the readers were physically placed on top of the wooden nest box.

#### 3.4.1.4 Individual Tagging

Purchased colonies contained a queen and at least 50 naïve workers of mixed age that had never flown outside the plastic box in which they had arrived. In order to encourage workers to forage in the landscape, the sugar solution that was provided by the manufacturer was removed. Naïve bumblebees were released from the nest box throughout the testing days. Upon return, they were captured and marked with RFID tags (Mircosensys GmbH, Germany) using Araldite® two-part epoxy resin (Huntsman Advanced Materials GmbH, Switzerland). Once again, bumblebees were only tagged upon their return as a result of observations made in a pilot study (please see Part A: Section 3.3.1.3: Individual Tagging and Flight Duration Recording). Marking only occurred on the first two experimental days. As such, there is no accurate record of the first flight duration of workers. Newly tagged workers were placed back into the wooden nest box and allowed to return to their colony. From this point onwards, the RFID recording equipment was turned on and the plastic spacers within the tunnels of the wooden nest box were completely opened. As such, tagged bumblebees could exit and enter their nest boxes and colonies freely, their movements unhindered by the experimenter. The RFID recording equipment logged the activity of the individually tagged bumblebees throughout the testing days.

#### 3.4.1.5 Flight Duration

##### *Second to Fifth Flights*

In order to determine the flight duration of individual bumblebees, the time that a tagged bumblebee left the colony and the time that it returned was manually extracted from the electronic data files. From this, the duration of each individual flight outside the colony was calculated. Unfortunately, in 30.1% of bumblebees that returned to their colony, the electronic record was incomplete. Both readers had not accurately scanned the bumblebee each time it had gone through them. In these cases, the direction of travel of the bumblebee and/or the duration of its flight outside the colony could not be ascertained. As such, only bumblebees which had a complete, unambiguous electronic record of their second to fifth flights were used in this analysis (20.9% of returning bumblebees; Appendix C: Table C.2). Data collected at Site Three were entirely excluded from the analysis as only one bumblebee had a complete electronic record of their second to fifth



flights outside the colony (Appendix C: Table C.2). Furthermore, although an accurate record of overnight flights exists, bumblebees which had at least one overnight flight were also excluded from the analysis (n=3) in order for the data to be comparable to that in Part A (Appendix C: Table C.2).

### **3.4.2 Statistical Analysis**

All statistical analysis was carried out in R 3.5.2 (R Core Team, 2015). Data were analysed using GLMMs ('lme4' package; Bates *et al.*, 2015). Visual inspection was used to check residuals for fit and homoscedasticity. P-values were obtained using the Satterthwaite's degrees of freedom method ('lmerTest' package; Kuznetsova, Brockhoff & Christensen, 2017). Response variables were transformed where required in order to meet model assumptions.

To determine whether the flight number outside the colony had an effect on flight duration, the relationship between the duration of each flight outside the colony and the flight number was modelled using an LMM, with a gaussian distribution. As the fixed effect, the flight number was entered into the model (entered as a numeric variable). As a random effect, a random slope model for the individual bumblebees was used. The flight duration was logarithmically transformed in order to meet model assumptions (Ives, 2015).

### **3.4.3 Results**

The mean number of released bumblebees from each colony in total across both sites was 75 (range: 44-126; Appendix C: Table C.2). The mean number of returned bumblebees throughout the experimental testing days from each colony was 32.57 (range 16-59; Appendix C: Table C.2). The mean proportion of returning bumblebees from each colony was 0.397 (range: 0.32-0.60). This is in comparison to 0.71 (range: 0.13-0.98) in the experiment described in Part A.

At Site Two, of the total number of returned bumblebees, the mean proportion that completed at least five flights through the experimental testing days from each colony was 0.18 (range: 0.06-0.25; Appendix C: Table C.2). This is in comparison to 0.05 (range: 0-0.20; Appendix C: Table C.2) in the experiment described in Part A.

### *Second to Fifth Flights*

At Site Two, individual bumblebees had flight durations of between 6 and 557 minutes ( $n = 29$ ; Figure 3.8). This is in comparison to 10-57 minutes at Site Two, Part A ( $n=14$ ; Figure 3.1) and 9-126 minutes at Site Three, Part A ( $n=19$ , Figure 3.1).

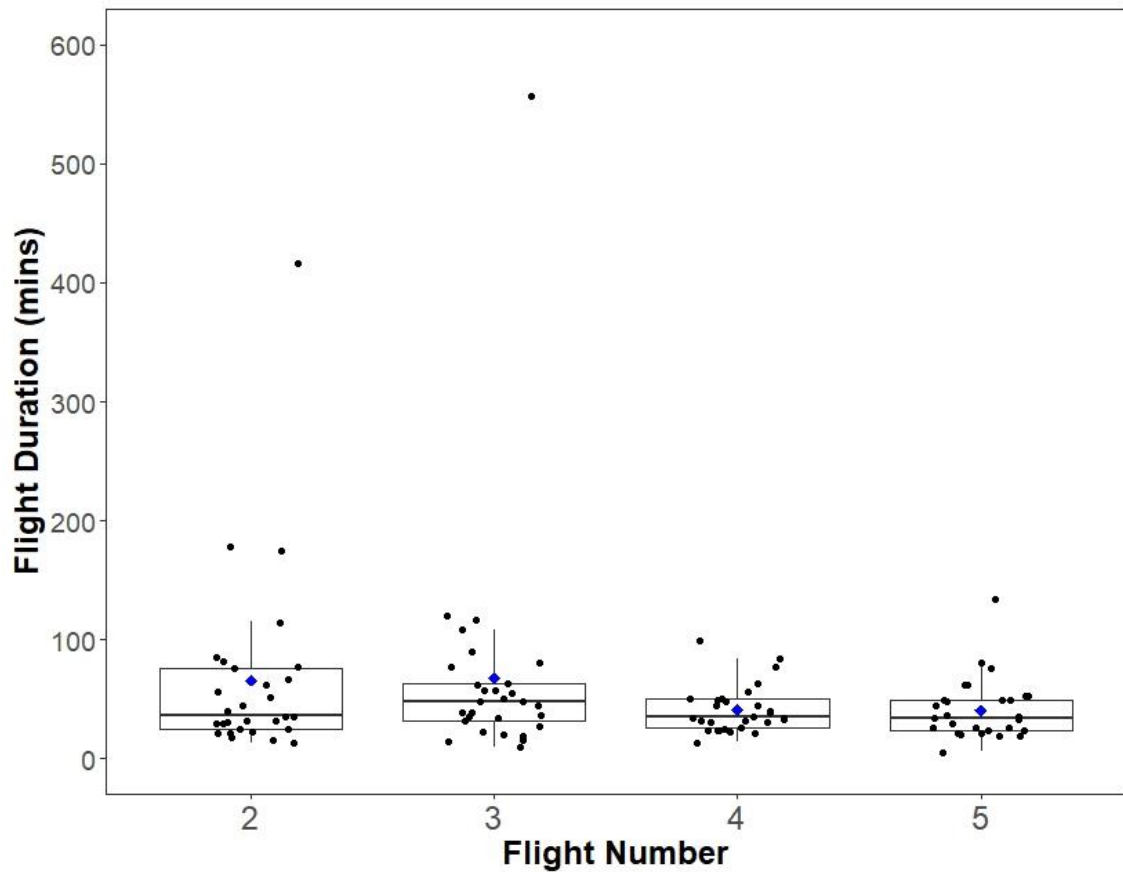
90% of flights ranged between 16 and 120 minutes. This is in comparison to 21 and 108 minutes at Site Two, Part A and 17 and 84 minutes at Site Three, Part A.

When comparing the flight duration of bumblebees throughout their second to fifth flights, there was no significant effect of the flight number outside the colony on the duration of flights (Table 3.4; Figure 3.8).

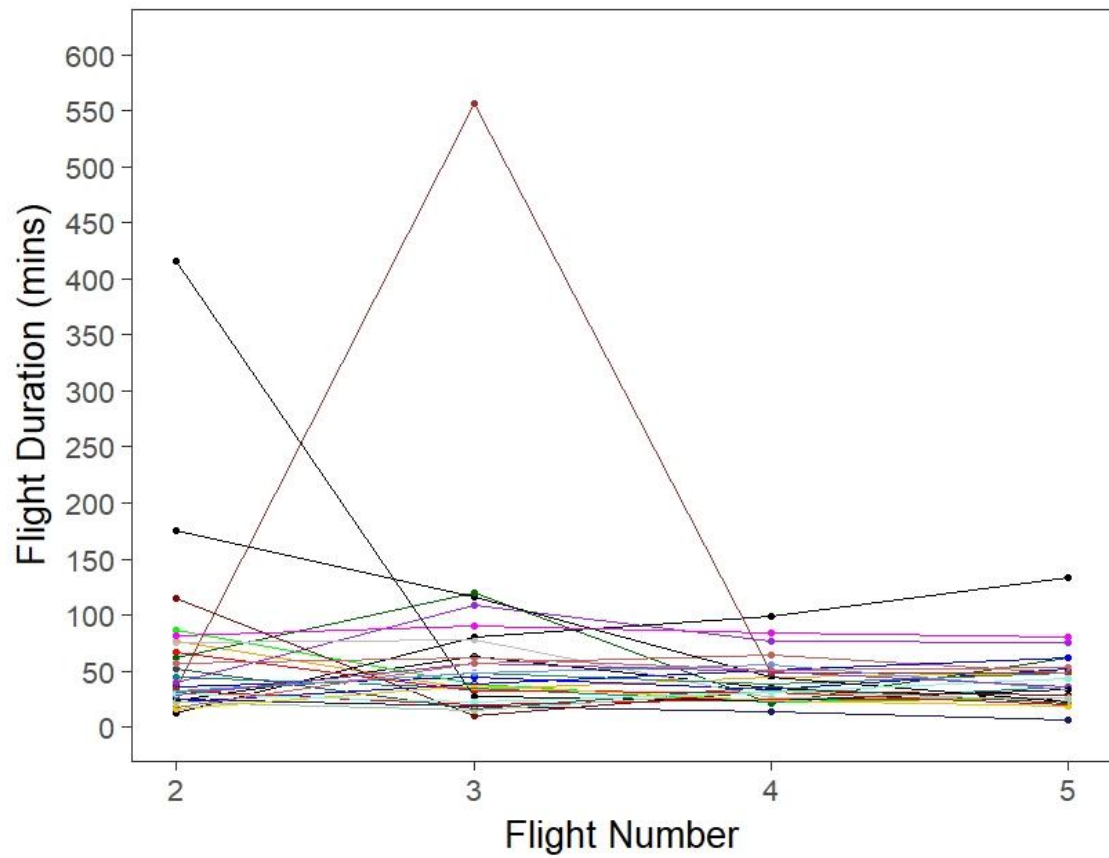
Individual bumblebees also varied greatly among themselves in the duration of their second to fifth flights. Furthermore, there was also evidence of a large variation in the duration of an individual bumblebee's successive flights (Figures 3.9.i and 3.9.ii). This was comparable to what was observed in the experiment described in Part A.

**Table 3.4. Model results from a linear mixed model testing the effect of the flight number on the flight duration. A random slope model with individual bumblebee identity was used. The flight duration was logarithmically transformed in order to meet model assumptions. Variables, effect sizes  $\pm$  standard error, degrees of freedom, *t*-values and *p*-values from the fitted model.**

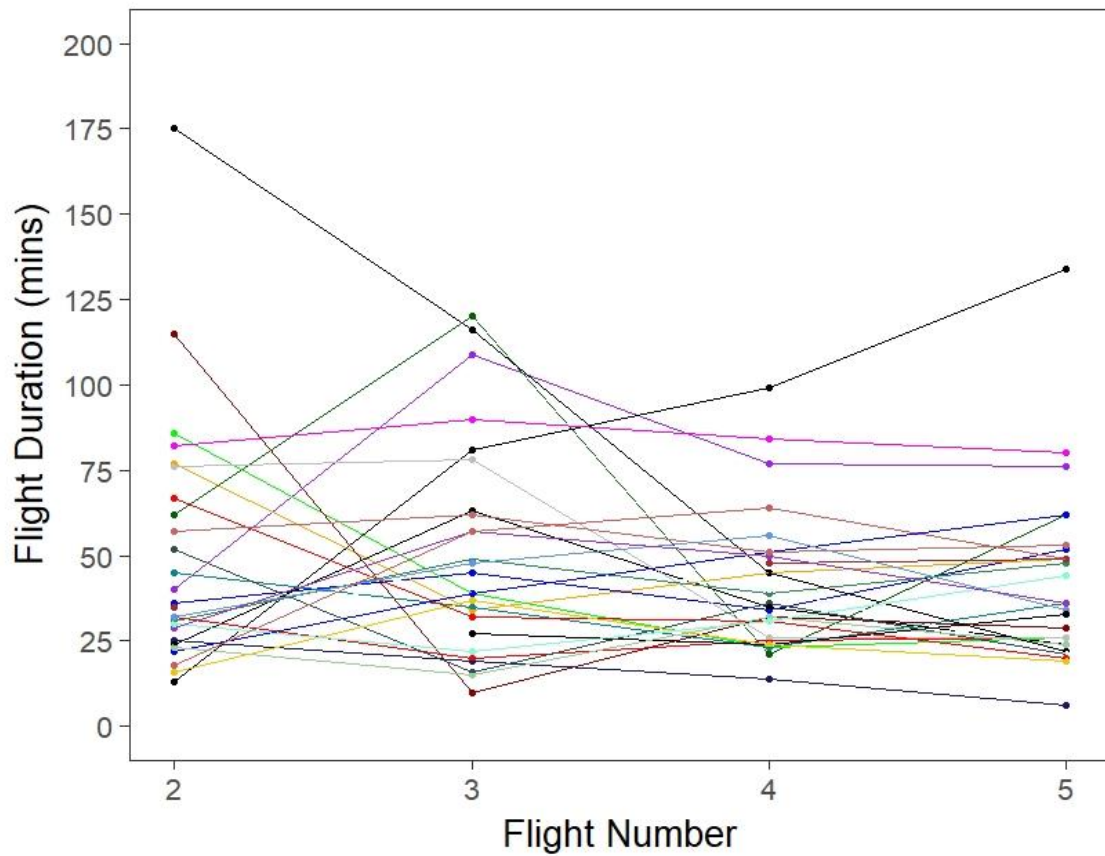
<b>Variable(s)</b>	<b>Effect Size <math>\pm</math> Standard Error</b>	<b>Degrees of Freedom</b>	<b><i>t</i> Value</b>	<b><i>p</i> Value</b>
Intercept	4.07 $\pm$ 0.22	28.00	18.12	<0.0001
Flight Number	-0.10 $\pm$ 0.06	28.00	-1.72	0.097



**Figure 3.8** | Box and whiskers plot of the flight duration (minutes) of bumblebees from their second to fifth flight outside the colony.  $N=29$ . Individual data points are superimposed on the plots. Blue diamonds denote the mean. A small value of random noise was added to each data point for plotting purposes to aid visual representation.



**Figure 3.9.i** | Flight duration (minutes) of bumblebees from their second to fifth flight outside the colony.  $N=29$ .



**Figure 3.9.ii** | Flight duration (minutes) of bumblebees from their second to fifth flight outside the colony (omitting outliers).  $N=29$ . Flight durations greater than 200 minutes were removed for greater visual clarity.

### 3.5 Discussion

In the experiment outlined in Part A, the pollen foraging behaviour of *Bombus terrestris* workers on their first five flights outside the colony was investigated. Specifically, when investigating the duration of their second to fifth flights outside the colony, the number of flights that individual bumblebees had made did not have a significant effect on their flight durations. This was the case at both Site Two and Site Three and the results did not differ between the data set chosen conservatively to ensure balance, with a complete record of individual bumblebee flight histories and the full dataset in Appendix B. If bumblebees were simply increasing their maximum displacement distance from the colony, as bumblebees have been observed doing as they gain more experience of their environment (Osborne *et al.*, 2013), then an overall increase in flight durations may have been observed. An increase in flight durations may have also been observed if bumblebees simply increased the number of flowers visited as they gained more experience of their environment (Lihoreau *et al.*, 2012). If, on the other hand, the average groundspeed of individual bumblebees was simply increasing as they gained more experience of their environment (Osborne *et al.*, 2013), or if the flower handling skills of individual bumblebees improved with each successive flight (Raine & Chittka, 2007), then an overall decrease in flight durations may have been observed. Similarly, the successive flight durations of individual bumblebees may have also decreased if bumblebees were simply decreasing the number of exploratory flight segments throughout (Woodgate *et al.*, 2016). All the scenarios outlined above, however, only take into account a change in one specific foraging component. Bumblebee foraging is a multifaceted behaviour made up of a variety of different components which likely trade-off against each other (Lihoreau, Chittka & Raine, 2011; Ohashi, Leslie & Thomson, 2008; Ohashi & Thomson, 2013). As such, a lack of significant net change in overall trip duration throughout the second to fifth flights does not rule out that changes in the individual components that make up foraging behaviour are indeed taking place. For example, an increase in average ground speed coupled with an increase in the number of flowers sampled may not result in any significant observable differences to overall trip durations, even though changes in two components are taking place. From the colony's perspective, both decreases and increases in overall trip durations can result in ultimate benefits to the colony in

terms of energy intake. A decrease in overall trip durations may result in more foraging trips being made per day which can increase the energy input into the colony. Similarly, an increase in overall trip durations may result in an increase in the number of flowers visited per trip and thus the amount of forage collected which can also increase the energy input into the colony. Investigating and monitoring all of the individual components of foraging behaviour was beyond the scope of this experiment and, at present, presents an unfeasible task for any experimenter.

Although flight durations did not change significantly throughout the second to fifth flights, flight durations varied widely both between and among individual bumblebees. This is also the case for the five bumblebees at Site Three for which a record of their first flight duration exists. This suggests that changes in the different foraging components were indeed taking place, albeit at the individual level. This also points to the degree that individual bumblebees are responding uniquely to their environment and suggests that the amount of experience gained throughout each flight outside the colony, as well as how this experience then influences future behaviour, may also be unique to each bumblebee. Individual bumblebees have been found to vary largely among themselves both in terms of flight duration (Free, 1955; Osborne *et al.*, 2013; Woodgate *et al.*, 2016; Evans, Smith & Raine, 2017; Woodgate *et al.*, 2017) and in the proportion of their flights that are made up of exploratory and exploitative flight segments (Woodgate *et al.*, 2016). Statistical models have also found that individual bumblebees seem to learn foraging routes through cycles of exploitation and exploration flights (Kembro *et al.*, 2019). As such, in this experiment, some workers may have continued to have flights which predominantly explored the landscape well beyond their fifth flight outside the colony while others may have had flights in which they predominantly foraged within the first five flights. Individual bumblebees may switch between particular foraging behaviours, resulting in large variations between successive flights.

In this experiment, the range of flight durations observed were very similar at each site with flights ranging between 10 and 157 minutes at Site Two and nine and 126 minutes at Site Three. Interestingly, the range of flight durations observed in this experiment are very similar to those found in other bumblebee studies. The four radar tracked *Bombus terrestris* workers investigated in



Woodgate *et al.*'s (2016) study had flight durations which ranged between 10.1 and 255 minutes throughout their second to fifth flights outside the colony. Similarly, throughout 20 non-consecutive testing days, Evans, Smith and Raine (2017) found that the 58 *Bombus terrestris* workers that they investigated had flight durations which ranged between 21 and 106 minutes. A variety of different reasons could account for the similarities observed in flight durations between the two experimental sites in this experiment as well as the similarities found between these results and those reported by various different studies. The similarities could suggest that, from a bumblebee's perspective, the landscapes that each experiment took place in were largely comparable in terms of both forage layout and availability. This may have resulted in comparable travel times both within and between foraging patches as well as similarities in flower handling times. The similarities across different experimental sites observed may also suggest that the range of total flight durations of individual bumblebees is also bound by innate, species specific factors. It has been previously observed that mean flight durations differ significantly between different bumblebee species (Free, 1955). As such, maximum flight durations observed could be linked to a bumblebee's maximum foraging range. In each of the experiments discussed, the same bumblebee species was used, and maximum foraging range is known to be species specific (Greenleaf *et al.*, 2007).

A general trend was also observed in the number of bumblebees that stayed out overnight before returning to their colony. For the bumblebees that had a complete record of the duration of their second to fifth flights, the highest number of overnight flights was observed on the second flight outside the colony (Appendix B: Figure B.2). When the full dataset in Appendix B is considered, the number of overnight flights decreased as flight number increased (Appendix B: Figure B.3). Overnighting behaviour in bumblebees is not uncommon and has been previously observed by several authors (Free, 1955b; Hobbs, Numni & Virostek, 1962; Roulston, 2015). Its causes, however, remain unknown. The trend observed in this experiment suggests that there may be a potential link between experience and staying out overnight before returning to the colony with this behaviour decreasing as more experience of the environment is gained. The potential effects of experience on overnighting behaviour is subsequently explored in the experiment outlined in Chapter Five.

In this experiment, the weight of pollen collected significantly increased throughout the first five flights for bumblebees at both sites. Specifically, the effect was stronger at Site Three than at Site Two. This suggests that the experience accrued throughout the first five flights in terms of flower handling skills is cumulative, but that the specific flower species present in an environment may also influence this effect. In this experiment, flower constancy was observed throughout the first five flights and 52.9% of bumblebees across both experimental sites were constant to a single flower species. As such, the majority of bumblebees were visiting the same kinds of flowers throughout successive flights and thus would have had the opportunity to improve their flower handling skills. As experience resulted in an increase in the pollen foraged for at both sites, but that this effect was stronger at Site Three, this suggests that the flower species at each site may have varied in complexity. Long term accrued experience could improve the handling of all flowers but result in larger observable differences in the handling of flowers which were initially difficult for bumblebees to forage on ('simple' versus 'complex' flowers; Lavery, 1994). One way in which the flower handling skills of the bumblebees in this experiment could have been tested would have been by investigating their skills in a controlled environment, such as the laboratory. A laboratory experiment could use the same flower types that were present in the landscapes at the two experimental sites. Different floral arrays could be set up in the laboratory and the flower handling times of bumblebees could be monitored and compared.

During the first five flights, the weight of pollen foraged increased significantly at each experimental site. When the full dataset is analysed, however, a site-specific difference emerges. During the entire length of the experiment, the weight of pollen foraged increased significantly at Site Three but not at Site Two (Appendix B: Tables B.1 & B.2; Figure B.4). This suggests that the amount of pollen gathered overall by individual bumblebees may have also been subject to the variations in the pollen standing crop at each site as well as the level of external competition present. When comparing the two experimental sites, bumblebees at Site Three foraged for a greater amount of pollen throughout when compared to those at Site Two. As such, the pollen standing crop at Site Two could have been more readily depleted throughout the course of the experiment compared to that at Site Three. This could have been due to natural variations in the pollen

standing crop as well as an increase in the number of pollinators foraging on that specific pollen source. Increases in competition for a particular pollen source could in turn be due to variations in the flowering times of different plant species in the landscape. The pollen standing crop at each site could have been roughly estimated by taking a random sample of the flowers in each landscape at set times throughout the day. A mean pollen amount from the random sample could have been calculated. This could have then been used to estimate the pollen standing crop for a certain measurable area with further extrapolation for the entire landscape. The estimated pollen standing crop throughout the experiment at each site could have then been compared. This could have shed light on the differences observed between the two sites.

As bumblebees are able to monitor the pollen stores of the colony and adjust their foraging effort accordingly (Cartar, 1992; Kitoaka & Nieh, 2009; Hendriksma, Toth & Shafir, 2019), it is also entirely possible that the site-specific differences observed overall were due to differences in the energy demands of each colony. One way in which the energy demands of each colony could have been assessed would have been by investigating the number of larvae present in the colony throughout the experimental period. Foraging bumblebees collect pollen mainly to feed the developing larvae (Pereboom, Velthuis & Duchateau, 2003) and bumblebees prefer to collect pollen which has a high protein to lipid ratio (Vaudo *et al.*, 2016). Kraus *et al.* (2019) have also found that protein regulation in bumblebee colonies seems to be influenced by the presence of brood. Monitoring the larvae numbers in each colony could have served as an indirect measure of energy demands. If monitoring the larvae numbers during the experiment would have proved too disruptive, the larvae in each colony could have also been counted after the experiment was complete. Furthermore, the colony's pollen stores could have been monitored directly throughout the experimental period as another measure of energy demand.

The site-specific differences observed overall may also be due to the differential use of pesticides at each site. At Site Two, the weight of pollen significantly increased throughout the first five flights. Overall, however, this effect was not observed (Appendix B: Figure B.4). Neonicotinoid exposed bumblebees have been found to return to their colonies with significantly smaller pollen loads per foraging bout when compared to non-exposed bumblebees (Gill, Ramos-

Rodriguez & Raine, 2012). Unlike non-exposed bumblebees who improved their foraging performance as they gained more experience, Gill and Raine (2014) observed that exposed foragers became worse with experience. Neonicotinoid exposure also seems to negatively affect the motivation of bumblebees, specifically with regards to the initiation of subsequent foraging bouts (Muth & Leonard, 2019). It is possible that the mass flowering crop at Site Two (commercially sown red clover) may have contained neonicotinoid pesticides and that this may have accounted for the change in foraging behaviour observed. Furthermore, as has been discussed in Chapter Two, bumblebees have been observed to prefer foods which contain neonicotinoid pesticides (Kessler *et al.*, 2015). This preference, however, is coupled with less overall food consumption (Kessler *et al.*, 2015). In other words, bumblebees may prefer to forage on pesticide laced food, but may consume less of this food overall. From the results of Chapter Two, bumblebees preferred to forage from the mass flowering crop at Site Two. However, when given more than five foraging flights, their overall consumption of the mass flowering crop at Site Two decreased. This result may suggest that neonicotinoid pesticides were used on the mass flowering crop at Site Two (commercially sown red clover), but not on the orchard trees at Site Three.

Similar to the variability observed in terms of flight durations, the weight of pollen foraged by individual bumblebees varied both between and among bumblebees at each experimental site. Notwithstanding the differences in the physical environment that are present on each foraging trip, variation among bumblebees may largely be due to underlying differences in both morphology and neurophysiological processes. Although the body size of bumblebees was not recorded in the present experiment, larger bumblebees are known to be faster fliers (Spaethe, Tautz & Chittka, 2000) and have better visual acuity (Spaethe & Weidenmüller, 2002). Larger bumblebees are also able to carry heavier pollen loads in their corbiculae (Fisher, 1987). Differences in neurophysiological processes, such as learning ability and flower detection, will also result in variability in the potential amount of pollen that each bumblebee is capable of foraging for. Lastly, the motivation levels of each bumblebee will also vary. Individual bumblebees are known to respond differently to colony need (Cartar, 1992) and have the potential to vary on factors such as sampling effort and giving up threshold (Thomson & Chittka, 2001), which will result in differences in the total amount of pollen foraged

for. As the majority of individual bumblebees in this experiment were constant to a particular flower species, switching between flower species and its consequences on the amount of pollen foraged for can be ruled out as accounting for the variability observed within individual bumblebees. Rather, the variability observed most likely points to the variability in the amount of pollen available on each successive trip. The variability seen within individual bumblebees throughout their successive trips also suggests that the signal to return to the colony whilst foraging does not seem to be dependent on the weight of pollen foraged. In other words, bumblebees are not foraging until a maximum or set weight is reached before returning to the colony on each foraging trip.

At both Sites Two and Three, flight durations did not change significantly throughout the second to fifth flights. The weight of pollen collected, however, did significantly increase throughout the first five flights at Site Three. As such, the rate of pollen foraged throughout the first five flights differed between sites. Although Raine and Chittka (2007) had previously found that the rate of pollen collected by bumblebees increased throughout the course of a day, several key differences exist between their experiment and the experiment outlined here. Raine and Chittka (2007) used bumblebees that had never foraged for pollen before, but that had flown and fed from gravity feeders dispensing sucrose solution. The bumblebees used would have been somewhat familiar with their surrounding environment (a greenhouse). Having previous experience of their environment and finding themselves in a relatively small space, their flights would have probably had less exploratory flight segments than the bumblebees used in the present experiment, who were completely naïve. Raine and Chittka (2007) also used a single type of flower at a set distance from the bumblebee nest. As such, the increase in the pollen collection rate observed in their experiment would have been largely due to an improvement in a bumblebee's flower handling skills. In the present experiment, the fact that the rate of pollen collection increased over successive flights at Site Three, but not at Site Two, may have been due to a variety of different factors, as previously discussed.

In the experiment outlined in Part B, flight number was also not found to significantly affect the flight duration of individual bumblebees. In contrast to the methodology used in Part A, the use of RFID tagging eliminated the need for handling the bumblebees beyond their initial tagging. In this experiment, 90% of

flights ranged between 16 and 120 minutes. This is very similar to the results obtained in the experiment outlined in Part A where 90% of flights ranged between 21 and 108 minutes at Site Two and 17 and 84 minutes at Site Three. The similarity between the two sets of results suggest that the handling of bumblebees and the removal of their foraged pollen did not have a significant effect on their flight durations. Furthermore, large variations in the flight durations both between and within the RFID tagged bumblebees were also observed, pointing to the fact that such variation is not an artefact of the experimental methodology used in Part A. It should also be noted that the colonies used in the RFID experiment were not specifically encouraged to forage solely for pollen and did not have access to a sugar solution in their colony. As such, the range of durations observed in this experiment were made up of both pollen and nectar foraging trips. Such comparable results, regardless of pollen or nectar foraging, could indicate that trip duration is largely independent of the resource that is being collected.

In both the experiments outlined in Part A and Part B, only a minority of bumblebees completed five flights outside their colonies throughout the experimental testing period. In the experiment outlined in Part A, the mean proportion of returning bumblebees which completed at least five flights in each colony was 0.05 (range: 0-0.20). In Part B, the mean proportion of returning bumblebees that completed at least five flights outside their colonies throughout the experimental testing period was 0.178 (range: 0.06-0.25). This could have been due to the fact that the experiment was only conducted for four days and as such, did not give individual bumblebees enough time to complete five flights outside their colony. Taking into account that bumblebee reserves are known to amount to only six and 0.3 days of net input for honey and pollen respectively (Heinrich, 1979a), a bumblebee worker taking longer than four days to complete five flights outside the colony seems to be extremely maladaptive behaviour. In previous studies involving tagged bumblebees, Woodgate *et al.* (2017) observed that the bumblebees in their experiment performed at least six flights a day while Spaethe & Weidenmüller (2002) observed that the mean daily number of flights ranged from 3.3 to 5.8. Similarly, Woodgate *et al.*, (2016) found that the mean daily number of flights outside the colony ranged from 1.73 to 13, depending on the individual bumblebees. Given these results, four experimental days should

have been sufficient for a bumblebee to complete at least five flights outside the colony. Taken together, the fact that so few bumblebees completed five flights throughout four days of observation does suggest that the number of daily flights varies largely between individual workers in a colony.

Although stripping the bumblebees of the pollen that they had foraged for in the experiment outlined in Part A did not seem to negatively impact their flight durations, it could have impacted their motivation levels. When bumblebee foragers return to the colony with nectar and pollen, they are known to deposit this directly into the colony's stores (Alford, 1975). Returning foraging bumblebees that have discovered a profitable nectar source are observed performing a behaviour termed 'excited runs' in which they move rapidly within the nest, touching and bumping into other bumblebees (Dornhaus & Chittka, 2001). This behaviour is coupled with the distribution of a pheromone signal (Dornhaus, Brockmann & Chittka, 2003) and previously inactive bumblebees begin to search for food. If a similar case exists for pollen entering into the colony then bumblebees stripped of their pollen loads may have behaved differently when entering the colony. It is unknown, however, whether individual bumblebees were aware of the fact that their pollen loads were taken off them before they deposited them in the colony. As pollen was not supplemented throughout the experiment, the colony would have been rendered in a state of pollen deprivation for a period of four days. A viable alternative would have been to weigh each bumblebee automatically when they exited and entered the colony, a procedure which has been successfully used in other studies (Peat & Goulson, 2005; Evans, Smith & Raine, 2017). Furthermore, both the experiments outlined in Part A and Part B used only a single bumblebee species, *Bombus terrestris*. As almost all of the studies to date investigating bumblebee behaviour use this species, it is not possible to know whether the results obtained represent the foraging flight durations and pollen foraging behaviour of bumblebees more generally or if they represent species-specific behaviour.

### 3.5.1 Experimental Limitations and Improvements

The experiment outlined in Part A could be improved by standardising the pollen sources that the bumblebees foraged from and by increasing the number of experimental sites. By choosing experimental sites with the same mass flowering

crop and performing the experiment at the same time of year, this would help to reduce the amount of inherent variation that exists between the pollen yield of different crops. The number of days that the experiment was conducted for could also be increased, allowing for a greater number of bumblebees to perform at least five flights outside the colony. The experiment could also be repeated at sites with similar mass flower crops, allowing further investigations into the effects that a specific mass flowering crop has on bumblebee foraging behaviour. As discussed in Chapter Two: Section 2.6.2, the use of bumblebee colonies reared from wild caught queens should be considered as it has the potential to greatly increase the number of bumblebees that return to the colony in field experiments. In the experiment outlined in Part B, the RFID readers failed to scan the majority of tagged bumblebees which exited and entered the wooden nest box. In many cases, experienced bumblebees were passing through the readers at such a speed and at an angle that did not facilitate scanning (R.Herascu, personal observation). For future experiments utilising this technology and the wooden nest boxes, the side exit of the wooden nest box could be used instead as the main entrance to the colony (Chapter Two: Figure 2.4). RFID readers could then be positioned in the clear Perspex® access tunnel adjacent to this side entrance. Coupled with a landing platform, this would allow bumblebees to first land on the platform and force them to crawl, rather than fly, into the access tunnel for scanning. This would maximise the likelihood that tagged bumblebees are successfully scanned.

### **3.6 Conclusion**

In the experiments presented in this chapter, experience was not found to have a significant impact on the overall flight durations of bumblebees throughout their first five flights outside the colony. Experience was found to have a significant effect on the weight of pollen foraged throughout the first five flights. Individual bumblebees were also found to vary widely in their behaviour, both between and among themselves. As individual bumblebees seem to experience, and respond, to their environment in a unique manner, it is entirely possible that experience does play a significant role in both flight duration as well as pollen foraging, but that this role is only observable at some point beyond the five-flight mark. Overall, the large individual variability observed suggests that bumblebee foragers are not



automata reacting to the needs of the colony and that the investigation of individual differences may prove a fruitful avenue for future research.

The experiments presented in this chapter focused on different behavioural parameters and the role that experience plays. In Chapter Four, the focus shifted to homing which can be seen as a behaviour that integrates previous experience of the landscape, both in terms of exploration and foraging, with navigation ability. Furthermore, the effects of the physical landscape more generally were explored as homing success is investigated within the context of two contrasting environment types.

## Chapter Four

### Finding Home: *Bombus terrestris* Homing in Urban and Rural Environments

#### 4.1 Abstract

In order for a bumblebee colony to survive, its workers must successfully navigate to and from their colony to forage. *Bombus terrestris* workers have been found to return to their colonies when displaced from up to 9.8 km; while estimates of their foraging range vary from several hundred metres to 2.2 km. Little is known, however, about the effects that a particular environment may have on how far bumblebees travel from their colony to forage and their consequent knowledge of the landscape. Using a homing experiment as a proxy for how far bumblebees are likely to explore in a landscape, the homing ability of *Bombus terrestris* workers was investigated in two rural and two urban sites in South West England. Bumblebee colonies were first introduced into the chosen environments and marked with Radio Frequency Identification (RFID) tags. Following a period of habituation, tagged bumblebees were then released at distances of 300 m, 1000 m and 2500 m from their colony in all four cardinal directions. At all sites, the proportion of returning bumblebees significantly decreased as the release distance increased. A significantly lower proportion of bumblebees were also observed returning to their colony in the two urban environments compared to the two rural environments. Bumblebees were also significantly faster to return to their colonies the closer they were released to them. Furthermore, a significantly higher proportion of bumblebees stayed out overnight in the urban environments. It is hypothesised that the differences observed both between the different release distances as well as the two environment types may relate to the resource availability around the colonies and the distances that each bumblebee had flown prior to being experimentally displaced. It may also be the case that the structure of the urban environment may be more challenging to navigate.

## 4.2 Introduction

In order for animals to successfully survive and reproduce, rewarding food sources must be available within their foraging range. As central place foragers, this is especially true for bumblebee workers, who return to their colony between foraging trips. As such, the foraging ranges of bumblebee workers directly determine the extent of the pollination services that a single colony can provide in a particular environment (Kremen, 2005).

The foraging ranges of bumblebee workers, however, are not entirely fixed. They are thought to be flexible and depend on a variety of factors with both the bumblebee species themselves as well as the distribution of forage around the colony having a substantial effect. The foraging ranges of different bumblebee species can be said to lay on a spectrum with *Bombus terrestris* workers having been found to have the largest foraging range of between 750 m and 3 km (Osborne *et al.*, 1999; Walther-Hellwig & Frankl, 2000; Chapman, Wang & Bourke, 2003; Westphal & Tscharntke, 2003; Kreyer *et al.*, 2004; Knight *et al.*, 2005; Osborne *et al.*, 2008; Hagen *et al.*, 2011; Osborne *et al.*, 2013). At the other end of the spectrum, *Bombus pratorum* workers have been found to only forage within 250 m from their nest (Westphal, Steffan-Dewenter & Tscharntke, 2006). As both bumblebee body size and average colony size vary significantly between species (Benton, 2006), these factors have been suggested as being strongly associated with the differences in foraging range observed (Greenleaf *et al.*, 2007; Rundloef, Nilsson & Smith, 2008; Goulson & Osborne, 2009). This suggests that within individual bumblebee species, maximum foraging range is likely bound by physiological constraints.

Although individual bumblebee species seem to exhibit a maximum foraging range, the area around the colony that they will habitually explore is much more variable and depends on a variety of different factors. These include both the distribution of food resources around the colony as well as experience of the environment surrounding the colony. For example, in an experiment spanning several months, the foraging ranges of *Bombus terrestris* workers from the same colony changed throughout and were dependent on the major plant species that were in bloom (Osborne *et al.*, 1999). Furthermore, the maximum displacement

distance of *Bombus terrestris* workers from their colony during a foraging trip increased significantly with the number of foraging flights that a worker had previously taken (Osborne *et al.*, 2013). Taken together, the results of various foraging range experiments suggest that factors such as the unique physiological characteristics of a bumblebee species, the distribution of rewarding forage around the colony and the individual foraging experience of workers all influence foraging range.

Although a variety of factors are thought to influence the foraging ranges of bumblebees in a particular environment, little is known of the role that abiotic features of the environment may play. These features include aspects such as topography, prominent landmarks, hedgerows (see Chapter Two) or man-made structures. The most straightforward method of investigating this would be to directly track the flight paths of foraging bumblebees in different landscapes. At present, however, due to the small size of bumblebees, flight paths cannot be tracked in their entirety and consequently, a variety of different experimental techniques have been used to indirectly measure foraging range. These include harmonic radar (Osborne *et al.*, 1999; Osborne *et al.*, 2013; Woodgate *et al.*, 2016), radio tracking (Hagen, Wikelski & Kissling, 2011), mark recapture studies (Walther-Hellwig & Frankl, 2000; Kreyer *et al.*, 2004; Osborne *et al.*, 2008; Wolf & Moritz, 2008) and the use of genetic markers (Chapman, Wang & Bourke, 2003; Darvill, Knight & Goulson, 2004; Knight *et al.*, 2005; Rao & Strange, 2012). Although harmonic radar technology would appear to be the ideal choice in investigating bumblebee movement within a particular environment, the radar's signal range does not extend beyond 1000 m (Goulson & Osborne, 2009), potentially falling short of the habitual foraging range of different bumblebee species. Furthermore, the radar's signal is unreliable when a bumblebee flies over an obstacle such as a hedgerow or lands on the ground (Osborne *et al.*, 1999; Osborne *et al.*, 2013). Due to these factors, its use in investigations of foraging range in different environments is limited. Mark recapture studies, in which marked bumblebees are searched for in foraging patches surrounding the colony, may appear to be a better choice. Using this technique, larger areas of the surrounding landscape can be investigated. This methodology, however, requires significant manpower as the potential area in which bumblebees are

foraging increases with the square root of the distance from the colony (Osborne *et al.*, 2008). As an alternative, a homing experimental methodology, based on the current theories of bumblebee navigation, can be used as an approximate measure of foraging range.

Bumblebees, and eusocial insects more generally, are thought to rely on a set of guidance systems which underlie their navigation capabilities (Chapter One: Section 1.4: Navigational Mechanisms). Using their image matching systems, eusocial insects are thought to compare what they are currently experiencing, their current sensory input, with a sensory input that they had previously experienced, a memory or an encoding of a sensory input (Collett, Chittka & Collett, 2013). When moving towards a goal in their environment, this comparison allows eusocial insects to make sure that they are heading in the correct direction towards their goal. In practical terms, this comparison allows insects to navigate in their environment successfully without getting lost or veering off course.

In a homing experimental paradigm, bumblebees are artificially displaced at various distances from their colony. As central place foragers, it is assumed that bumblebees would be motivated to return to their colony. When displaced, it is hypothesised that a bumblebee will compare its current surroundings with its memories of those which it had previously experienced in order to try and generate a direction heading towards the colony. As a result, bumblebees would be more likely to return to their colonies from areas which they have previously experienced and explored. If artificially displaced to an unfamiliar area in which no previous memory exists, then bumblebees would be effectively lost. As honeybees are observed exhibiting searching behaviour when artificially displaced (Reynolds *et al.*, 2007; Degen *et al.*, 2018), it is very likely that bumblebees will also search the landscape until they reach a familiar area from which a direction heading towards the colony can be generated. As a result, bumblebees which are artificially displaced to an unfamiliar area would be less likely to return to their colony or take a significantly longer time to do so. As a bumblebee's foraging range in a particular environment will directly affect the area that it will explore, bumblebees would be more likely to return to the colony from displacement points which are within their habitual foraging range and less likely to return to the colony from displacement points which are outside it. In this

way, a homing experimental paradigm can be used as an indirect measure of a bumblebee's habitual foraging range.

As such, in order to investigate the foraging ranges of bumblebees in environments which differ in their abiotic characteristics, the homing success of bumblebees was investigated in two different environment types: urban and rural. Specifically:

1. What effect does the release distance following artificial displacement have on the proportion of returning bumblebees within each environment type?
2. What effect does the release distance following artificial displacement have on the duration of return flights within each environment type?
3. How does the homing success of bumblebees compare between environment types?

In order to answer these questions, *Bombus terrestris* colonies were placed in two agricultural and two city sites in the South West of England. Individual naïve bumblebees were given five days of experience in their respective environment and marked with RFID tags. Tagged bumblebees were displaced and released from 300 m, 1000 m and 2500 m from their colony. The proportion of returning bumblebees as well as the duration of their return flights was recorded. In the only comparable bumblebee homing experiment, Goulson & Stout (2001) found that the proportion of returning bumblebees significantly decreased as the release distance from the colony increased. Given that *Bombus terrestris* has been found to have a foraging range of between 750 m and 3000 m (Osborne *et al.*, 1999; Walther-Hellwig & Frankl, 2000; Chapman, Wang & Bourke, 2003; Westphal & Tschardt, 2003; Kreyer *et al.*, 2004; Knight *et al.*, 2005; Osborne *et al.*, 2008; Hagen *et al.*, 2011; Osborne *et al.*, 2013), it was hypothesised that in both environment types, the majority of bumblebees would return to the colony when released from 300 m and 1000 m. In contrast, 2500 m would represent a distance that was outside the habitual foraging range for most bumblebees and as such, fewer bumblebees would successfully return from this release distance.

It was also hypothesised that the duration of return flights for bumblebees within each environment would increase as the release distance increased.

Flight ranges between the two environment types may vary for a variety of different reasons. Firstly, flight ranges may be directly linked to the availability of forage around the bumblebee colonies. Although not formally measured, it was clear from the landcover surveyed that the urban and rural environments differed in the distribution of floral resources with a greater abundance in the urban environments. As foraging range has been observed to be linked to the distribution of forage (Osborne *et al.*, 1999; 2008a), it was hypothesised that a greater proportion of bumblebees in the rural environments would be exploring the landscape further away from their colony than those in the urban environments. Secondly, flight ranges may be directly linked to the abundance of physical structures around the colonies and their potential to affect bumblebee navigation. Specifically, the two environment types differed in their abundance of man-made structures such as buildings and roads. Bumblebees are known to prefer to forage on foraging patches alongside roads rather than across them (Bhattacharya, Primack & Gerwein, 2003) and the species composition of bees and wasps has been found to vary widely on either side of large roads (Andersson *et al.*, 2017). Furthermore, although structures such as hedgerows and forests do not seem to pose a flight barrier to *Bombus terrestris* (Krewenka, *et al.* 2011; Kreyer *et al.*, 2003; Chapter Two), the effects of large groups of buildings on bumblebee flight is presently unknown. It is likely that the presence of road and building networks would encourage bumblebees in the urban environments to forage closer to their colonies than those in the rural environments. It is hypothesised that this would be reflected in the homing success of bumblebees between the two environment types. A greater proportion of bumblebees released from 2500 m in the rural environments was predicted to return to their colony than those released from 2500 m in the urban environments. Both the differences in the forage availability and abundance of physical structures around the colonies between the two environment types have the potential to influence bumblebee homing success. It should be noted, however, that this experiment cannot tease apart the effects of each factor.

The hypotheses regarding the duration of return flights between the two environment types depend upon the height at which bumblebees fly within a landscape. In the rural agricultural landscapes used in this experiment, the predominant structures above the herb layer are hedgerows and as the results of Chapter Two suggest, bumblebees seem to routinely fly over such structures if necessary. It is entirely possible that after artificial displacement in the rural environments, bumblebees will fly over the hedgerows, both when flying towards, and searching for, their colony. In the urban environments, it is currently unknown whether bumblebees fly above the building networks in a city when homing, whether they will fly within the building networks or whether they do both. Even if displaced to a familiar location, the way in which bumblebees fly in the urban environments will affect the duration of their return flights. If they predominantly weave in and out of building networks, going around large structures rather than over them, then it is hypothesised that the return flights of bumblebees in the urban environments will be significantly slower than those in the rural environments. If, however, bumblebees fly above the building networks, then the duration of return flights between the two environment types should be comparable.

### **4.3 Materials and Methods**

#### **4.3.1 Experimental Sites**

Four locations in the South West of England were chosen for this experiment, two rural sites and two urban sites. The rural sites comprised two mixed farm sites: Site RA and Site RB. The two urban sites comprised two small cities: Site UA and Site UB. A minimum of three experimental sites would have ideally been chosen for each environment type. This was not possible, however, due to the resources and time available in this instance.

*Site RA* (Site Two in Chapters Two and Three)

Four *Bombus terrestris audax* colonies (Koppert Biological Systems, UK) were placed along the western margin of a field, equidistant from the bordering hedge and a flowering crop (Colonies A, B, C and D). Colonies were placed at least 7.50 m from each other. The experiment took place from the 25<sup>th</sup> August - 1<sup>st</sup> September 2016 with maximum temperatures ranging between 18.0°C to 25.0°C.



The flowering crop in the field in which the colonies were placed was red clover (*Trifolium pratense*). Apart from this flowering crop, there were few floral resources in the landscape surrounding the colony. The clover field was cut three days after the start of the experiment. Weather conditions were scattered cloud throughout.

#### *Site RB* (Site Three in Chapters Two and Three)

Four *Bombus terrestris audax* colonies (Koppert Biological Systems, UK) were placed along the eastern margin of a field, approximately 0.50 m from the bordering hedge (Colonies E, F, G and H). Colonies were placed at least 7.50 m from each other. The field in which the colonies were placed was managed pasture with very few floral resources. The experiment took place from the 14<sup>th</sup>-20<sup>th</sup> June 2017 with maximum temperatures ranging between 25.0°C to 30.0 °C. Weather conditions were sunny and clear throughout.

#### *Site UA*

Five *Bombus terrestris audax* colonies (Koppert Biological Systems, UK) were placed in an urban garden (Colonies I, J, K, L and M). Colonies were placed at least three metres from each other. The experiment took place from the 16<sup>th</sup> – 24<sup>th</sup> May 2017 with maximum temperatures ranging between 18.0°C to 25.0°C. Weather conditions were variable and ranged from sunny to overcast throughout. Floral resources were located in both the garden containing the colonies as well as in the gardens and public parks in the surrounding area.

#### *Site UB*

Five *Bombus terrestris audax* colonies (Koppert Biological Systems, UK) were placed along the eastern edge of an urban cemetery (Colonies: N, O, P, Q and R). Colonies were placed at least three metres from each other. The experiment took place from the 1<sup>st</sup> – 9<sup>th</sup> August 2017 with maximum temperatures ranging between 18.0°C to 23.0°C. Weather conditions ranged from predominantly overcast to scattered cloud throughout. Floral resources were located throughout the cemetery as well as in the gardens and public parks in the surrounding area.

#### 4.3.2 Individual Tagging and Recording

At each site, the bumblebee colonies were placed in a wooden nest box (Chapter Two: Figure 2.4). The bumblebee colony was connected to the outside of the nest box using a clear, Perspex® tube. Purchased colonies contained a queen and at least 50 naïve workers of mixed age that had never flown outside the plastic box in which they had arrived. The commercial sugar solution that accompanied the colonies from the supplier was removed in order to motivate the bumblebees to forage outside the colony for nectar and pollen. Prior to testing, the colonies were opened, and individual bumblebees were allowed to forage freely for a period of five days in order for them to gain experience of their surrounding environment. On the third day, individual bumblebees were marked with RFID tags (Microsensys, GmbH, Germany) using Araldite® two-part epoxy resin (Huntsman Advanced Materials GmbH, Switzerland) upon their return to the colony. A total of 875 bumblebees were tagged across all four sites. A detailed description of the RFID equipment can be found in Chapter Three: Section 3.4.1.3: RFID Flight Duration Recordings. Two different RFID readers and a corresponding logger were placed at the entrance/exit hole of the wooden nest box. As such, bumblebees could also habituate themselves with the RFID equipment present at their nest boxes.

Following the five days of habitation, individual bumblebees that had an RFID tag were captured at the nest box as they were preparing to exit. Following the capture of bumblebees, only the entrance to the colonies was left open. In this way, bumblebees could enter their colonies when returning but no bumblebees could exit. The RFID equipment was also switched on at this point in order to record any returning bumblebees. The number of bumblebees captured at each nest box varied depending on the activity levels of each colony.

#### 4.3.3 Releases

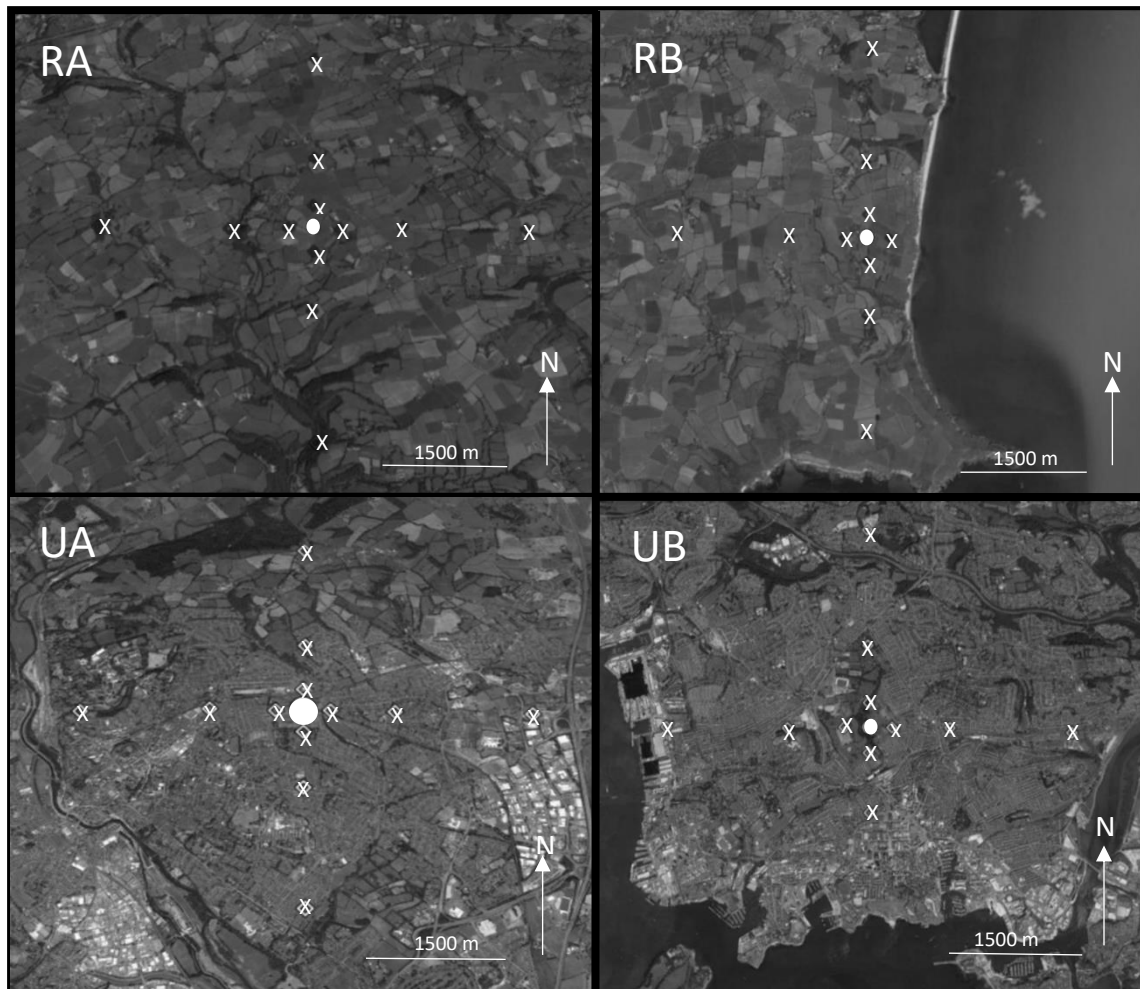
In preparation for testing to take place, captured bumblebees were placed in an opaque, polystyrene box and transported to their release location. Transport was accomplished by foot or by car depending on the release location. The total amount of time from capture to release did not exceed one hour at all sites and for all release locations. Releases were conducted at 300 m, 1000 m and 2500 m from each colony in all four cardinal points as long as the release point was

not in water (Figure 4.1 & Appendix D: Tables D1 & D2). As there is evidence to suggest that prominent topographical landmarks, such as mountains, can affect the homing abilities of honeybees (Southwick & Buchmann, 1995; Pahl *et al.*, 2011), bumblebees were released in all four cardinal points to try and control for any differences in landscape topography and prevailing wind direction which may have biased the areas previously explored by the bumblebees.

In order to account for differences in the motivation levels of individual bumblebees and to encourage them to return to their colonies without stopping to forage, bumblebees were fed commercial sugar solution (67% Brix) *ad libitum* prior to release. Individual bumblebees were released facing north at arm's length at a height of approximately 130 cm. Their release time was recorded as well as the compass bearing at which they vanished from human sight (their 'vanishing bearing'; Gould, 1986; Dyer, 1991; Dyer, 1993; Dyer *et al.*, 1993). As each nest box was equipped with two RFID readers and a corresponding logger, the RFID equipment electronically recoded the return time and identity of each bumblebee. Release locations and release times were randomly scattered throughout the day with the latest release occurring at 19:40 GMT+1 at Site RB.<sup>5</sup> All released bumblebees were only tested once. At each site, the colonies were kept open for returns until sunset each day. The colonies were completely closed at night. As normal foraging activity was halted during testing days, colonies were supplemented with 10.00 ml of commercial sugar solution (67% Brix) every day and 2.00 g of honeybee pollen every other day. Colonies were left open for returns during the daytime for four days following the last release.

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<sup>5</sup> Release times were not found to significantly affect homing success.



**Figure 4.1** | Aerial views of the colonies and release points at each site. Release points are at 300 m, 1000 m and 2500 m from the colonies. The white circle denotes the location of the colonies while the white 'X's denote the individual release locations. Copyright information: RA. Google Earth V 7.3.2. (June 22, 2018). Eye altitude: 8160 m. <https://www.earth.google.com> [December 15, 2018]. RB. Google Earth V 7.3.2. (June 22, 2018). Eye altitude: 8720 m. <https://www.earth.google.com> [December 15, 2018]. UA. Google Earth V 7.3.2. (June 22, 2018). Eye altitude: 5500 m. <https://www.earth.google.com> [December 15, 2018]. UB. Google Earth V 7.3.2. (June 22, 2018). Eye altitude: 6800 m. <https://www.earth.google.com> [December 15, 2018].

## 4.4 Statistical Analysis

Only bumblebees that displayed normal flying behaviour at each release site and which, upon release, were not observed crashing into neighbouring objects were used in the following statistical analyses (Appendix D: Table D.1). For the flight duration analysis, bumblebees which did not return within the same day as being released were excluded (30.29% of the total number of returning bumblebees; Appendix D: Table D.1). This is due to the fact that the record of the duration of overnight flights is not entirely accurate. As testing did not begin at sunrise at the urban experimental sites, bumblebees which had stayed out overnight may have attempted to return to their colony before colonies were opened and the RFID equipment was turned on to record their returns. When bumblebees cannot enter their colony, they fly off and attempt to enter it at a later time (R.Herascu, personal observation). In order to account for bumblebees that stayed out overnight before returning to their colony, the proportion of returning bumblebees that stayed out overnight was also investigated (*“Proportion of Overnight Stays”*).

All the statistical analysis was carried out in R 3.5.2 (R Core Team, 2015).

### 4.4.1 Proportion of Returns

#### *Cardinal Points*

To determine whether the differences in the landscape between each cardinal point had an effect on the homing success of bumblebees, the proportion of returns from each cardinal point at each release distance was compared for each experimental site. This comparison was carried out using a z-test.

#### *Overall Returns*

To determine whether the release distance and the environment type had an effect on the homing success of bumblebees, the relationship between the proportion of released bumblebees that returned, and their release distance and environment type was modelled with an GLMM ('lme4' package, Bates *et al.*, 2015). Due to low sample sizes, individual colonies could not be included in the model and bumblebees were pooled across colonies. A binomial error structure and logit link were used. As fixed effects, the release distance (entered as a numeric variable) and the environment type were entered into the model with an interaction term. As random effects, there was a random intercept model for the

experimental site and for the cardinal point. Residuals were checked for fit and homoscedasticity of variance using a simulation approach ('DHARMA' package, Hartig, 2019). Overdispersion was also tested for.

#### 4.4.2 Duration of Returns

To determine whether the release distance and experimental site had an effect on the duration of return flights for bumblebees that returned to the colony on the same day as being released, an LMM was initially used ('lme4' package, Bates *et al.*, 2015) with a gaussian distribution. Specifically, the relationship between the duration of same day return flights and the release distance and experimental site was modelled. Due to low sample sizes, individual colonies could not be included in the model and bumblebees were pooled across colonies. As fixed effects, the release distance (entered as a numeric variable) and the environment type were entered into the model. As random effects, there was a random intercept model for the experimental site and for the cardinal point. There was not enough data to support this random effect model, as the model resulted in a singular fit. The random model was simplified to only include one random effect: an intercept for the experimental site. The model still resulted in a singular fit. As such, both random effects were dropped from the model. An LM was used with the explanatory variables of release distance (entered as a numeric variable) and the environment type. These were entered into the model with an interaction term. The response variable was logarithmically transformed in order to meet the model assumptions (Ives, 2015). Visual inspection was used to check residual plots for fit and homoscedasticity.

#### 4.4.3 Proportion of Overnight Stays

To determine whether the release distance and the environment type had an effect on bumblebees staying out overnight before returning to their colonies, the relationship between the proportion of released bumblebees that stayed out overnight before returning and their release distance and environment type was modelled with an GLMM ('lme4' package, Bates *et al.*, 2015). Due to low sample sizes, individual colonies could not be included in the model and bumblebees were pooled across colonies. A quasibinomial error structure and logit link were used due to overdispersed data. As fixed effects, the release distance (entered as a numeric variable) and the environment type were entered into the model with

an interaction term. As random effects, there was a random intercept model for the experimental site and for the cardinal point. There was not enough data to support this, so the model resulted in a singular fit. The model was simplified to only include one random effect: a random intercept model for the experimental site. This model still resulted in a singular fit. As such, both random effects were dropped from the model. A GLM was used with the explanatory variables as the release distance (entered as a numeric variable) and the environment type. These were entered into the model with an interaction term. A quasibinomial error structure and logit link were used due to overdispersed data. Visual inspection was used to check residual plots for fit and homoscedasticity.

## 4.5 Results

### 4.5.1 Proportion of Returns

#### *Cardinal Points*

To assess whether differences within each landscape had an effect on the homing success of bumblebees, the proportion of returning bumblebees from each cardinal point was compared for each release distance and experimental site. At Site RA, z-tests found no significant difference in the proportion of returns from each cardinal point at every release distance (300 m: 100% returns throughout; 1000 m:  $N=40$   $X^2= 6.06$ ;  $df=3$ ;  $p=0.11$ ; 2500 m:  $N=34$   $X^2= 1.22$ ;  $df=3$ ;  $p=0.74$ ). At Site RB, z-tests found no significant difference in the proportion of returns from each cardinal point at every release distance (300 m:  $N=55$   $X^2= 0.75$ ;  $df=3$ ;  $p=0.86$ ; 1000 m:  $N=44$   $X^2= 3.06$ ;  $df=2$ ;  $p=0.22$ ; 2500 m:  $N=44$   $X^2= 1.76$ ;  $df=2$ ;  $p=0.41$ ). At Site UB, z-tests found no significant difference in the proportion of returns from each cardinal point at every release distance (300 m:  $N=45$   $X^2= 4.63$ ;  $df=3$ ;  $p=0.20$ ; 1000 m:  $N=40$   $X^2= 5.17$ ;  $df=3$ ;  $p=0.16$ ; 2500 m:  $N=39$   $X^2= 1.92$ ;  $df=2$ ;  $p=0.38$ ). In contrast, at Site UA, a z-test found a significant difference in the proportion of returns from each cardinal point at 2500 m ( $N=47$   $X^2= 11.25$ ;  $df=3$ ;  $p=0.01$ ). Bumblebees released from the north and east were more likely to return than those released from the south and from the west (Appendix E: Table E2). At 300 m and 1000 m at Site UA, however, z-tests found no significant difference in the proportion of returns from each cardinal point (300 m:  $N=44$   $X^2= 1.63$ ;  $df=3$ ;  $p=0.65$ ; 1000 m:  $N=47$   $X^2= 2.56$ ;  $df=3$ ;  $p=0.67$ ).

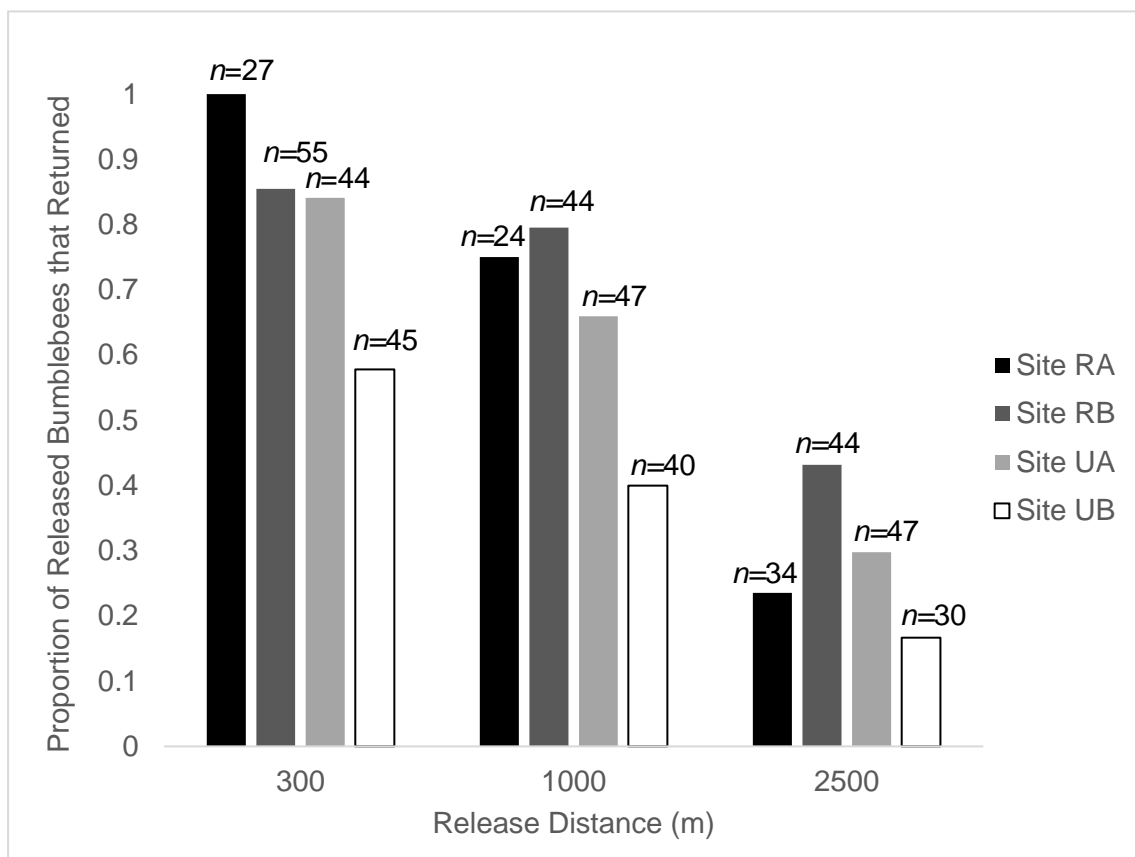
### *Overall Returns*

When comparing the proportion of released bumblebees that returned to their colony, there was no significant interaction between the release distance and the environment type (Table 4.1; Figure 4.2). The type of environment significantly affected the proportion of bumblebees that returned to their colonies after being released. Bumblebees were more likely to return to their colonies after being released in the rural environments than in the urban environments (Table 4.1; Figure 4.2). There was also a significant effect of the release distance on the proportion of returning bumblebees. Bumblebees were more likely to return to their colonies the closer they were released to them (Table 4.1; Figure 4.2).

**Table 4.1. Model results from a generalised linear mixed effects model testing the effect of environment type and release distance on the proportion of returning bumblebees. A random intercept model with experimental site and cardinal point was used. Variables, effect sizes  $\pm$  standard error, z-values and p-values from the fitted model. The rural environment type was used as the reference level.**

Variable(s)	Effect Size $\pm$ Standard Error	z Value	p Value
Intercept	4.01 $\pm$ 0.61	6.61	<0.0001
Type	-1.90 $\pm$ 0.61	-2.51	0.012
Distance	-1.52 $\pm$ 0.23	-6.73	<0.0001
Type * Distance	0.45 $\pm$ 0.29	1.53	0.13





**Figure 4.2 |** Proportion of bumblebees that returned when released from various distances from the colony.  $N=481$ . Rural sites: RA and RB. Urban sites: UA and UB. The numbers shown on the graph represent the total number of bumblebees that were released at each distance and experimental site.

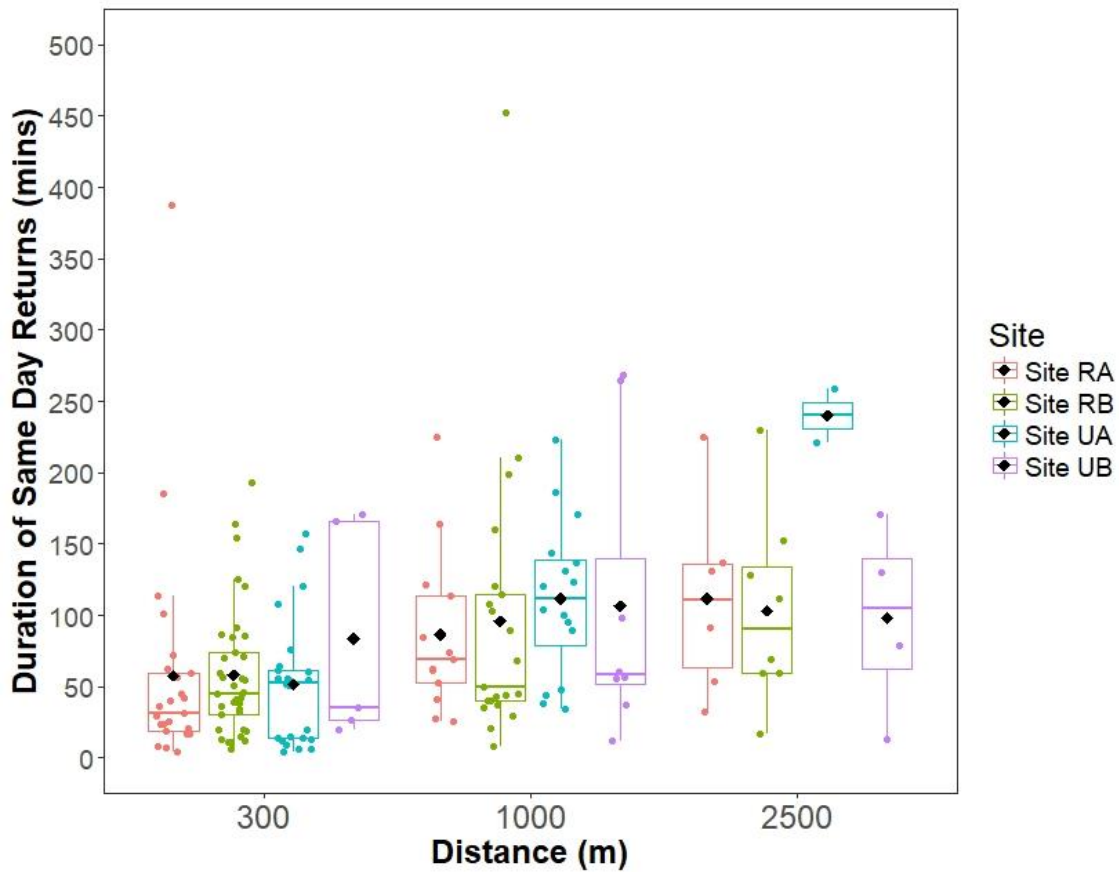
#### 4.5.2 Duration of Returns

##### *Same Day Returns*

When comparing the duration of return flights to the colony, there was no significant interaction between the release distance and the duration of return flights (Table 4.2; Figure 4.3). There was no significant effect of environment type (Table 4.2; Figure 4.3). There was a significant effect of release distance on the duration of return flights to the colony. Bumblebees were significantly faster to return to their colonies the closer they were released to them (Table 4.2; Figure 4.3).

**Table 4.2. Model results from a linear model testing the effect of environment type and release distance on the duration of return flights. Variables, effect sizes  $\pm$  standard error,  $t$ -values and  $p$ -values from the fitted model. Return flight durations were logarithmically transformed to meet model assumptions. The rural environment type was used as the reference level.**

<b>Variable(s)</b>	<b>Effect Size <math>\pm</math> Standard Error</b>	<b><math>t</math> Value</b>	<b><math>p</math> Value</b>
Intercept	3.62 $\pm$ 0.13	28.04	<0.0001
Type	-0.06 $\pm$ 0.23	-0.25	0.80
Distance	0.0004 $\pm$ 0.0001	3.29	0.001
Type * Distance	0.0001 $\pm$ 0.0002	0.91	0.36



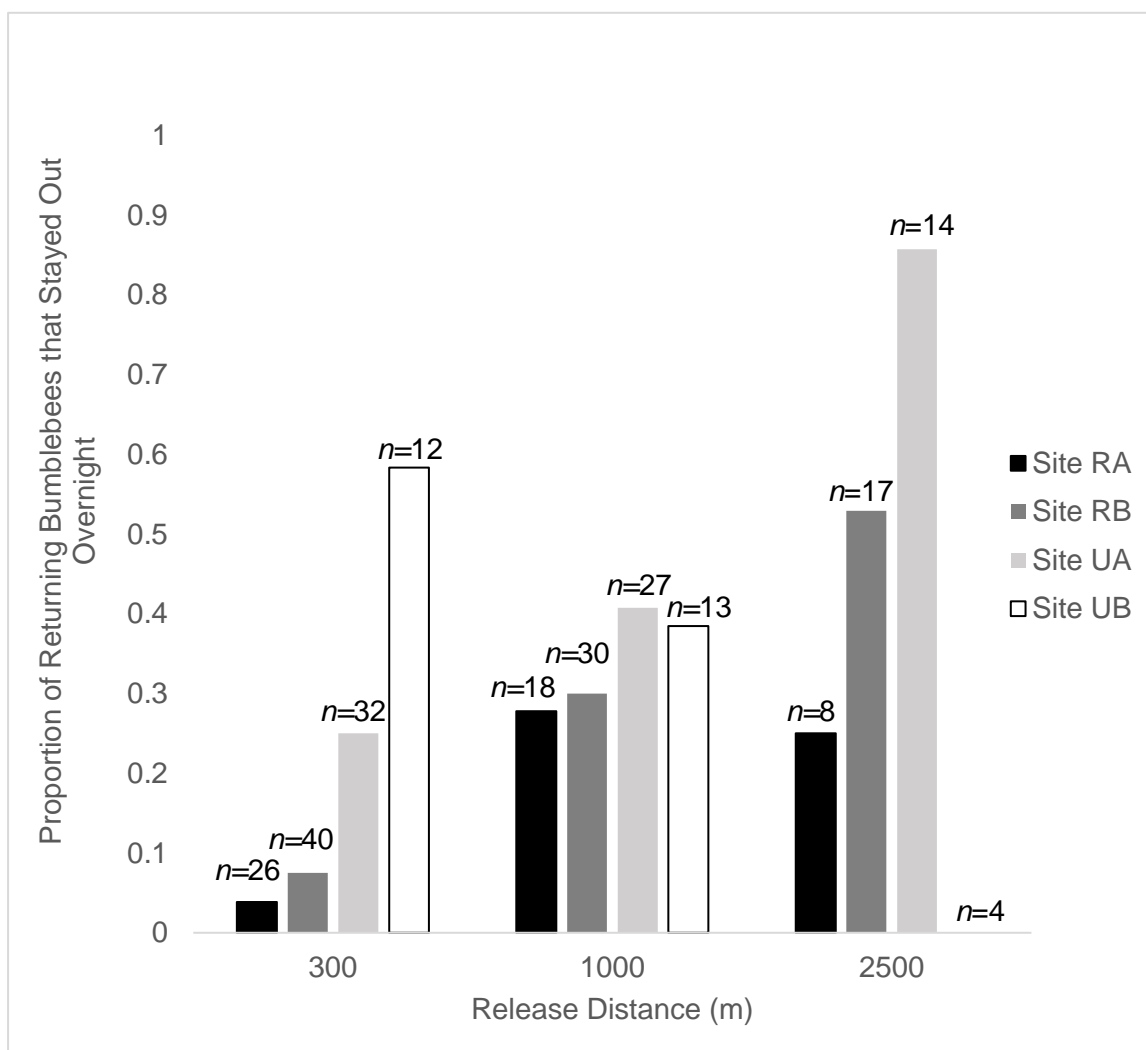
**Figure 4.3** | Boxplot of the duration of the return trip of bumblebees that returned before the first nightfall after being released (in minutes).  $N=169$  (300 m: Site RA:  $n=25$ ; Site RB:  $n=36$ ; Site UA:  $n=24$ ; Site UB:  $n=5$ . 1000 m: Site RA:  $n=13$ ; Site RB:  $n=21$ ; Site UA:  $n=16$ ; Site UB:  $n=8$ ; 2500 m: Site RA:  $n=6$ ; Site RB:  $n=8$ ; Site UA:  $n=2$ ; Site UB:  $n=4$ . Black diamonds denote the mean. A small value of random noise was added to each data point for plotting purposes to aid visual representation.

### 4.5.3 Proportion of Overnight Stays

When investigating the proportion of returning bumblebees that stayed out overnight before returning to their colony, there was no significant interaction between the release distance and the environment type (Table 4.3; Figure 4.4). There was a significant effect of the environment type on the proportion of bumblebees that stayed out overnight. Bumblebees were more likely to stay out overnight before returning to their colonies in the urban environments compared to the rural environments (Table 4.3; Figure 4.4). There was also a significant effect of the release distance on the proportion of returning bumblebees. Bumblebees were less likely to stay out overnight before returning to their colonies the closer they were released to them (Table 4.3; Figure 4.4).

**Table 4.3. Model results from a linear model testing the effect of environment type and release distance on the proportion of returning bumblebees that stayed out overnight before returning. Variables, effect sizes  $\pm$  standard error, *t*-values and *p*-values from the fitted model. The rural environment type was used as the reference level. The duration of return flights was logarithmically transformed to meet model assumptions.**

<b>Variable(s)</b>	<b>Effect Size <math>\pm</math> Standard Error</b>	<b><i>t</i> Value</b>	<b><i>p</i> Value</b>
Intercept	2.38 $\pm$ 0.46	5.22	<0.0001
Type	-1.47 $\pm$ 0.61	-2.43	0.02
Distance	-0.0010 $\pm$ 0.0002	-3.19	0.002
Type * Distance	0.0003 $\pm$ 0.0004	0.78	0.44



**Figure 4.4 |** Proportion of returning bumblebees that stayed out at least once overnight before returning.  $N=241$ . Rural sites: Site RA and Site UB. Urban sites: Site UA and Site UB. The numbers shown on the graph represent the total number of bumblebees that returned from each release distance and at each experimental site.

## 4.6 Discussion

In this experiment, the homing success of *Bombus terrestris* in rural and urban environments was investigated. Across all experimental sites, the proportion of released bumblebees that returned to their colony significantly decreased as the release distance increased (Figure 4.2). Furthermore, there was a significant difference in the proportion of bumblebees that returned to their colony between the two environment types with a greater proportion of bumblebees returning to their colonies in the rural environments compared to the urban. If bumblebees are more likely to return from areas that they have previously experienced, then workers would be more likely to return to their colony when released from distances within, or close to, their habitual foraging range. In this way, homing distance can be taken as a proxy of habitual foraging range (Greenleaf *et al.*, 2007).

Across all experimental sites, the majority of bumblebee workers returned to their colony when released from 300 m and 1000 m, suggesting that the majority of bumblebee workers were habitually exploring and foraging within 1000 m of their colony. There was a steep decline in the proportion of bumblebees that returned when released from 2500 m, suggesting that this distance was beyond their habitual foraging range (after five days of experience). These results are in line with studies which found that depending on their environment, *Bombus terrestris* workers will forage between 750 m and 3000 m from their colonies (Osborne *et al.*, 1999; Walther-Hellwig & Frankl, 2000; Chapman, Wang & Bourke, 2003; Westphal & Tschardt, 2003; Kreyer *et al.*, 2004; Knight *et al.*, 2005; Osborne *et al.*, 2008; Hagen *et al.*, 2011; Osborne *et al.*, 2013). As such, although foraging range is somewhat environmentally dependent, it is hypothesised to be ultimately bound by physiological constraints (Greenleaf *et al.*, 2007).

Even though the effects of release distance were broadly similar between environment types, a greater proportion of bumblebees were observed returning to their colonies in the rural environments compared to the urban. Although replication is low with only two sites per environment type, one hypothesis is that these observed differences may have been directly related to the differences in the availability of foraging resources around the colonies. In the urban environments, there was an abundance of floral resources surrounding the

colonies in the form of public and private gardens, flowering road verges and large municipal parks. In this environment type, the majority of bumblebee workers may have found and exploited floral resources largely within 100 m of their colonies. In contrast, in the rural environments there were very few foraging resources available when testing took place. The one exception to this was a red clover field at Site RA. This was cut after the first three days of the experiment, however. Even if bumblebee workers had been utilising this mass flowering crop, they would have been forced to forage elsewhere after it was cut. These results closely mirror those of Redhead *et al.* (2016), who found that even within the same species of bumblebee, worker foraging range is influenced by the spatial distribution of foraging resources around the colony. Bumblebee colonies in areas with low floral coverage and high fragmentation of semi-natural vegetation had, on average, workers foraging further away from their colony than colonies that were situated in areas with high floral coverage and low fragmentation. If the distribution of foraging resources around the colony affected the foraging range of bumblebee workers in this experiment, then a greater proportion of bumblebee workers in the rural environments may have experienced, and been familiar with, a larger area of the landscape surrounding their colonies compared to bumblebee workers in the urban environments.

In the only comparable homing experiment to date, Goulson and Stout (2001) found that the relationship between the proportion of returning bumblebees and the release distance was characterised by the function  $y=0.56 - 0.048x$ . Given this relationship, this would have resulted in 54.5%, 51.2% and 44.0% of bumblebees returning to their colony when released from 300 m, 1000 m and 2500 m respectively. At 300 m and 1000 m, these percentages are much lower than those observed in the current experiment (Figure 3; the only exception being at Site UB at 1000 m, where Goulson and Stout's (2001) percentage is higher). At 2500 m, the trend is reversed, and a greater percentage of bumblebees returned in Goulson and Stout's (2001) experiment compared to the current one. Goulson and Stout (2001) placed their colonies in private gardens in a largely suburban area in southern England. Their results are much closer to those observed in the urban environments in this experiment. This suggests that the suburban environment in Goulson and Stout's (2001) experiment and urban

environments in this experiment share particular characteristic(s) which are affecting the homing abilities of bumblebees in a certain way.

In this experiment, the cardinal point of release did not have a significant effect on the proportion of returning bumblebees in all but one experimental site and release distance. This suggests that any heterogeneity of the landscape around the colonies, with regards to the different features of the landscape, was not a barrier for returning bumblebees workers. This specific result also suggests that although individual bumblebee workers are known to show constancy to both flower type (Heinrich, 1976, Waser, 1986) and forage site (Heinrich, 1976; Bowers, 1985; Waser, 1986; Dramstad 1996; Saville *et al.*, 1997), the flowers and sites themselves can vary between different bumblebees. As such, the colony as a whole could still have explored large areas of the landscape even if individual bumblebees were constant to flower type and forage site. In studies with radar tracked bumblebees, different workers were found to explore the landscape in various directions around their nest (Osborne *et al.*, 2013) even though workers were individually constant to both compass bearing and destination over successive trips (Osborne *et al.*, 1999). Taken together, the results of the present experiment suggest that at the colony level, exploration of the landscape is performed in all directions around the colony. At the release distance of 2500 m at Site UA, bumblebees were more likely to return to the colony when released from the north and from the east. This may have been due to the fact that these release locations had less physical man-made structures between them and the colonies than those located at 2500 m south and west (Figure 4.1: UA). This points to the fact that a greater abundance of physical structures may be negatively affecting the homing success of bumblebees.

When comparing the return flights of bumblebees to their colonies, there was no significant difference in flight duration between the two environment types for bumblebees that returned on the same day as being released (Figure 4.3). When artificially displaced, honeybees are observed exhibiting searching behaviour (Reynolds *et al.*, 2007; Degen *et al.*, 2018). It is very likely that bumblebees employ the same behaviour, searching the landscape until a familiar area or familiar landmarks are reached. It is hypothesised that once such a familiar area or landmark is reached, bumblebees will then be able to find their way back to



the colony from there. Both in this experiment and in that of Goulson & Stout (2001), bumblebees were observed flying in a looping pattern upon release, suggesting that they immediately begin this searching behaviour. As such, bumblebees which are lost and searching for the colony are assumed to take longer to return than those which are released in familiar surroundings. Across both environment types, bumblebees were faster to return to their colonies the closer they were released to them (Figure 4.3).

Given that experienced bumblebees have an average groundspeed of 7.1m/s when tagged with harmonic radar (Osborne *et al.*, 1999; Riley *et al.*, 1999), released bumblebees in this experiment would have been capable of returning to their colony within six minutes from 2500 m if they were to fly straight back. As the fastest returning bumblebee from 2500 m had a flight duration of 13 minutes (Figure 4.3), this suggests that at least half of its returning flight time was spent on other activities. These activities include searching, foraging and resting. Bumblebees could also be flying at lower speeds or take be taking a non-optimal route back to the colony. Even for bumblebees released from the same distance, there is large variation in the return times of individual bumblebees (Figure 4.3). Part of this variation may reflect the degree to which each a bumblebee is familiar with its release location. It is also entirely possible that part of this variation is due to differing levels of motivation to return to the colony. Even though each bumblebee was fed prior to release, some bumblebees may have chosen to forage, particularly for pollen, before returning to the colony.

Although environment type did not have a significant effect on the returning flight duration for bumblebees which returned within the same day as being released, significant differences were observed in the proportion of bumblebees that stayed out overnight before returning (Figure 4.4). Bumblebees were also significantly more likely to stay out overnight as the release distance increased. This once again suggests that the majority of bumblebee workers were habitually exploring and foraging within 1000 m of their colony. Bumblebees were significantly more likely to stay out in the urban environments than in the rural environments. More time away from the colony will increase a bumblebee's risk of predation and exhaustion. Bumblebees that took longer than a day to return to the colony were

not only likely to be lost upon release, but also continued to be lost as the light intensity dropped with nightfall. As such, bumblebees that did not return to the colony within the same day as being released but that returned subsequently likely represent those bumblebees which found their way back to the colony after systematic searching. The fact that overnight stays were significantly more prevalent in the urban environment could be due to several different reasons. Bumblebees could simply be more likely to be lost in the urban environments compared to the rural as, given more abundant floral resources, they do not need to forage and explore as far away from their nest. Certain aspects of the urban environments, such as the abundance of man-made structures, could also increase search times and/or flight times in general. As bumblebees are known to fly around single objects rather than over them (Plowright & Galen, 1985), and bumblebees may be forced to weave in and out of objects such as automobiles and buildings.

The presence of urban street lighting may have also impacted the duration of returns. Reber *et al.* (2015) have found that as light levels fall, so too does the flight speed of bumblebees. Furthermore, Reber *et al.* (2015) tested the flight behaviour of bumblebees under light intensity which fell from 600 to 3.4 lux. At light levels of 6 and 3.4 lux, the flight paths of bumblebees became more uneven and longer overall. This suggests that at these light intensities, it becomes increasingly difficult for bumblebees to control their flight position. Data for an urban council in the United Kingdom report their typical street lighting levels to be 10 lux (Wigan Council, 2019). If similar street lighting levels were found at the urban sites in this experiment, this suggests that bumblebees may have found it increasingly difficult to fly under the urban street lighting. The urban street lighting could have caused bumblebees to become 'trapped' within the radius of a dimly lit area, as bumblebees cease flight when light intensity drops below a certain level (Reber *et al.*, 2015). This may have drained a bumblebee's energy reserves during the night. Certain species of moths, for example, are attracted to streetlights and will perch beneath them or circle around them until they become exhausted or are predated upon (Frank, 1988). Urban street lighting is a form of artificial light at night (ALAN) which is thought to negatively impact the movement, foraging, development and reproduction of a wide range of insects (reviewed in

Owens *et al.*, 2019). The effects of street lighting on bumblebee movement and flight behaviour remain currently unknown and future work could investigate these effects.

At first glance, the proportion of bumblebees that stayed out overnight before returning to the colony at Site UB showed a different pattern to the other experimental sites. Here, the proportion of bumblebees that stayed out overnight decreased as the release distance increased (Figure 4.4). The reason behind this observation may lie with the very low number of bumblebees that returned overall at Site UB. At the release distance of 2500 m for example, only four bumblebees returned during the experimental period and all four bumblebees returned within the same day as being released. As Site UB had the lowest proportion of returning bumblebees from all release locations, it is entirely possible that a greater proportion of bumblebees were still searching for the colony at the end of the experimental period at this experimental site. As such, a different trend may have been observed had the bumblebees had more than four days to return to their colony.

Even though bumblebees were more likely to be lost in the urban environments, particular characteristics of the urban environments may have also prolonged their search times. In long range homing, honeybees are thought to utilise the overall landscape panorama (Pahl *et al.*, 2011; Towne *et al.* 2017) and large horizon landmarks such as mountains have also been observed to aid orientation (Southwick & Buchmann, 1995). Similarly, ants are also thought to use landscape panoramas as navigational cues (Graham & Cheng, 2009) and even small changes in the overall nest panorama can lead to major navigational disruptions, with ants walking slower and using less direct paths (Narendra & Ramirez-Esquivel, 2017). Bumblebees have been shown to use both panorama and local cues in an experimental study (Jin *et al.*, 2014). If they also employ similar navigational mechanisms when homing as those employed by honeybees and ants, then differences in the visibility of the landscape panorama and large horizon landmarks between the two environment types may have resulted in differences in overall searching times. Specifically, the rural environments, characterised by relatively open spaces with few man-made structures, may have

proved easier to navigate using a relatively unchanging landscape panorama. The high density of man-made structures in the urban environments, in contrast, may have proven to be an incredibly complex visual environment. In such a landscape, bumblebees may have needed to constantly fly above the urban canopy in order to access the landscape panorama, a feat which may be both energetically costly and time consuming.

Evidence that urban and rural environments have different effects on navigation and on foraging behaviour also comes from differences observed in honeybee homing. When trying to validate a method to determine the effects of sublethal doses of pesticides on the homing abilities of honeybees, Pascal, Volles & Jeker (2016) observed that mean homing flight durations were higher in the urban environment compared to the rural. This result was observed even when the mean return rate for both environments from a release distance of 1000 m was 94%. Similar to the experiment presented in this chapter, Pascal, Volles & Jeker's (2016) results cannot distinguish whether the differences observed were due to the distribution of foraging resources around the colonies or the structure of the environments.

To try and isolate the effects that the structure and complexity of a landscape may have on bumblebee homing success, future work can focus on varying this structure while controlling for the effects of the distribution of forage. This could help in teasing apart the effects of the physical structures in a landscape and the effects of forage availability. The effect of physical structures in a landscape could be isolated by testing homing success at a time when very little forage is available. For example, homing success could be tested in landscapes with varying proportions of man-made structures towards autumn in the northern hemisphere. A control condition could also be introduced by testing homing success in a landscape that is free of both physical structures and forage. For example, this could be done in a largely agricultural setting in the autumn. The effects of the distribution of forage around the colony could also be isolated by testing homing success in a landscape with few physical structures but where the distribution of forage around the bumblebee colony is experimentally manipulated. Different factors could also be manipulated such as forage type and

forage density. Such experimental manipulation would shed light on the effects that forage distribution has on bumblebee homing success. Future work should consider such investigations.

#### 4.6.1 Experimental Limitations and Improvements

One of the main limitations of this experiment is the total number of experimental sites used. Ideally, at least three experimental sites would have been used for each environment type in order to increase the validity of the results. Similarly, overall sample size could have also been increased by using a greater number of bumblebee colonies at each experimental site. This would have allowed the individual colonies to be included as a factor in the statistical analysis and would have increased the chances of being able to conduct a statistical analysis on the number of bumblebees that stayed out overnight before returning to their colonies at each experimental site. In order to investigate the importance of the landscape panorama and its role in bumblebee homing and navigation more broadly, future research may consider using a natural environment that is as flat, uniform and featureless as possible. Similarly, future research may also consider investigating bumblebee homing success over water. In this experiment, resources did not permit releases over water at Site RB or Site UB, even though release locations over water were within the distances investigated. Investigations into the navigation and homing abilities of honeybees over water have been conducted (Heran & Lindauer, 1963; Tautz *et al.*, 2004; Pahl *et al.*, 2011) and bumblebees have been observed flying over open water (Heinrich, 1979a; Mikkola, 1984). Whether such behaviour is habitual and whether bumblebee navigation is in any way compromised or facilitated over water, however, remains unknown.

#### **4.7 Conclusion**

In the experiment presented in this chapter, the homing ability of bumblebees was found to be affected by both the displacement distance as well as the environment type. Bumblebees were significantly more likely to return to their colonies, and be faster to do so, the closer they were released to them. Bumblebees were also significantly more likely to return to their colonies and do so within the same day as being released in the rural environments compared to the urban. The differences observed between the two environment types may

have been due to differences in resource availability around the colony as well as differences in the inherent structure of each environment type. Overall, the ability to successfully home across all four experimental sites, however, suggests that *Bombus terrestris* workers are able to forage and navigate within a wide range of different landscapes.

The results of this experiment also revealed that some bumblebees will stay out overnight before returning to their colony and that this may be linked to the unfamiliarity of their surroundings. In Chapter Five, a follow-up homing experiment is presented which was conducted in order to try to investigate overnighting behaviour and the role that experience plays in the homing success of bumblebees more widely.

## Chapter Five

### The Role of Experience in *Bombus terrestris* Homing Success

#### 5.1 Abstract

As bumblebees gain experience of their environment, behavioural changes are observed. Flight paths straighten and maximum displacement from the colony, average groundspeed and foraging rate all increase. The development of spatial knowledge at the larger landscape scale, however, remains largely unknown. In this experiment, a homing paradigm was used as a proxy for the information gathering and exploration that occurs within the first few flights outside the colony. Naïve *Bombus terrestris* workers were tagged and given one, two or five flights of experience outside their colony before their homing abilities were tested. This is the first experiment that has used a homing paradigm to investigate bumblebees of differing and known experience. Following their flights, workers were released at either 300 m or 1000 m from their colonies with each worker only being released once. The number of previous flights experienced did not have a significant effect on the proportion of bumblebees that returned from each release distance. The release distance did, however, have a significant effect on the proportion of bumblebees that returned to their colony. Bumblebees released from 300 m were more likely to return to their colony than those released from 1000 m. When released from 300 m, bumblebees that returned on the same day that they were released were significantly faster to return to their colony than those released from 1000 m. Homing flight duration significantly decreased as levels of previous experience increased. Bumblebees were also significantly less likely to stay out overnight before returning to their colony when released from 300 m compared to 1000 m. The proportion of bumblebees that stayed out overnight before returning to the colony was also observed to significantly decrease with increasing levels of previous experience. Furthermore, bumblebees were significantly more likely to forage for pollen before returning to their colony when released from 300 m compared to 1000 m. Bumblebees were also significantly more likely to forage for pollen before returning to the colony the more experienced they were. The results suggest that flight ability, navigation skills and flower handling skills are all substantially developed within the first five flights.

## 5.2 Introduction

Bumblebees use a variety of different means to learn about their surrounding environment. When leaving their colony for the first time, bumblebees are observed performing orientation or learning flights (Collett & Zeil, 1996; Baddeley *et al.*, 2009; Hempel de Ibarra *et al.*, 2009; Philippides *et al.*, 2013; Robert *et al.*, 2017). This distinct behaviour, which consists of flying in a series of arcs and loops around the colony, is also observed when bumblebees leave a food source and is also more widely observed in ants, bees and wasps (solitary wasps: Collett & Lehrer, 1993; wood ants: Nicholson *et al.*, 1999; desert ants: Müller & Wehner, 2010; honeybees: Cartwright & Collett, 1983). At certain points during this flight, these insects will turn their body orientation to face the nest, the food source, or even prominent nearby landmarks (Lehrer, 1993; Boeddeker *et al.* 2015). It is thought that at these specific turning points, the insects are encoding visual (and perhaps also olfactory) information as well as the spatial relationship that exists between nearby landmarks and their goal (Baddeley *et al.*, 2009; Philippides *et al.*, 2013; Collett & Zeil, 1996). Upon returning to a goal location, eusocial insects will approach it with a zigzag flight path which matches the positions that they took during their orientation/learning flights. Harmonic radar tracking of bumblebees also confirms this behaviour (Osborne *et al.*, 2013; Woodgate *et al.*, 2016). As bumblebees gain experience of their surroundings, this behaviour is no longer observed (Free, 1955a, R.Herascu, personal observation). When tracked with harmonic radar, the flight paths of bumblebees, both to and from the colony, straighten by the sixth flight outside the nest (Osborne *et al.*, 2013). Further evidence suggesting that this distinct behaviour represents a learning mechanism comes from the fact that orientation/learning flights can be triggered in experienced bumblebees if their nest is artificially displaced (Free, 1955a).

Once their orientation/learning flights are complete, individual bumblebees will explore the landscape in search of rewarding food sources. The flight paths of bumblebees tagged with harmonic radar show that during their first flight, bumblebees will explore at least 100 m away from their colony. By the second and third flights, this increases to at least 300 m away (Osborne *et al.*, 2013; Woodgate *et al.*, 2016). The flight paths also show that although individual bumblebees will remain constant to a particular compass bearing when leaving the colony, the compass bearings themselves will vary widely among individual



bumblebees. Although an individual bumblebee may not explore the entire landscape surrounding their colony, the workforce as a whole has a greater chance of doing so. As experience is gained outside the colony, the maximum displacement distance and groundspeed of individual bumblebees also increase (Osborne *et al.*, 1999; Osborne *et al.*, 2013). The distance to which an experienced bumblebee worker will routinely travel in search of foraging resources, their foraging range, is dependent on the spatial distribution of forage within the landscape, in terms of its location, quality and quantity, as well as species-specific physiological constraints (Greenleaf *et al.*, 2007).

Although specific aspects of a bumblebee's spatial learning have been investigated, the information gathering that occurs at the larger landscape scale remains largely unknown. This is particularly true with regards to how a bumblebee's spatial knowledge develops with its experience of the landscape. Unlike honeybees, who seem to have separate flights for exploring, foraging and even re-orientating within the landscape (Capaldi *et al.*, 2000; Degen *et al.*, 2018), harmonic radar tracking shows that bumblebee flights are largely multi-purpose (Woodgate *et al.*, 2016). Coupled with the fact that individual bumblebees are regularly observed returning to the colony with forage even on their very first flight (Hempel de Ibarra *et al.*, 2009; Osborne *et al.*, 2013, Chapters Two & Three), this suggests that the first few flights outside the colony represent a critical knowledge acquisition phase. In order to investigate how knowledge of the environment develops during the first few flights outside the colony, the homing ability of bumblebee workers with differing levels of experience was investigated. Specifically:

1. What effect does a bumblebee's previous experience of their environment have on their ability to return to their colony after artificial displacement?
2. What effect does a bumblebee's previous experience of their environment have on the duration of their return flight to their colony after artificial displacement?
3. What effect does a bumblebee's previous experience of their environment have on their likelihood of pollen foraging on their return flight to their colony after artificial displacement?

In order to answer these questions, *Bombus terrestris* colonies were placed in an agricultural site in the South West of England. Naïve bumblebee workers were tagged and triaged into groups which took one, two or five flights outside the colony. Tagged bumblebees were then displaced and released from 300 m and 1000 m from their colony. The proportion of returning bumblebees, the duration of their return flights and whether or not they had foraged before returning to their colony, as evidence by the presence of pollen in their corbiculae, was recorded. In this investigation, a homing paradigm was used as a proxy measurement of previous landscape exploration (Goulson & Osborne, 2009; Chapter Four: Introduction). Using this paradigm, it was assumed that bumblebees would be more likely to return to their colony from areas that they had previously experienced and explored and do so more quickly.

The hypotheses regarding the homing abilities of bumblebee workers based on their previous experience of the landscape depended upon the assumptions of how naïve bumblebees first explore the landscape. If bumblebee workers increase their displacement distance from the colony gradually over successive flights, as suggested by the previous studies using harmonic radar (Osborne *et al.*, 2013; Woodgate *et al.*, 2016), then their homing ability ought to improve with more experience. In this scenario, bumblebees with a greater number of flights outside the colony would be more likely to return to the colony following artificial displacement. Bumblebees with more flights outside the colony would also be more likely to return to the colony from a greater displacement distance than those with less experience. Even if displacement distance from the colony is dependent upon experience, it is possible that it would take longer than five flights for bumblebees to explore beyond 1000 m especially if rewarding forage is available closer to the colony. In that case, there may not be any observable effect of experience on the homing success of bumblebees released from 1000 m. Alternatively, depending on the landscape and the distribution of rewarding forage around the colony, bumblebees may explore at large distances from their colony even on their first flight. In this scenario, a high proportion of returns may be observed at the release distance of 1000 m regardless of experience.

A variety of different scenarios are also possible with regards to the duration of return flights to the colony. As the results of Chapter Four suggest, a large variation exists between the duration of return flights of homing bumblebees. Even if bumblebees are familiar with the area in which they are released, the motivation levels of bumblebees will differ, and some may choose not to fly straight back to the colony. If all released bumblebees that were familiar with the release locations flew straight back to the colony, then bumblebees released closer to the colony should be quicker to fly back than those released further away. As the groundspeed of bumblebees is known to increase with experience (Osborne *et al.*, 2013), more experienced bumblebees should also be quicker to return to their colony than those with less experience. Furthermore, as bumblebees which are unfamiliar with their release locations are thought to engage in searching behaviour until a known area is found (Goulson & Stout, 2001; Chapter Four: Section 4.6: Discussion), it is probably that bumblebees with less experience are more likely to engage in searching behaviour upon release as they are more likely to be unfamiliar with their release sites. Bumblebees who engage in lengthy searching behaviour are also more likely to stay out overnight before returning to their colony, as they may not find their colony before nightfall. As such, it was hypothesised that bumblebees with less experience will be more likely to stay out overnight before returning at both release distances and that overall, a greater proportion of bumblebees will stay out overnight before returning when released from 1000 m compared to when released from 300 m.

Although it is assumed that, as central place foragers and eusocial insects, bumblebees should be motivated to return to their colony as soon as possible when artificially displaced, this may not be the case. It is entirely possible that more experienced bumblebees in familiar surroundings may instead choose to forage for the colony before returning. As such, bumblebees with more experience could be more likely to return to their colony with pollen in their corbiculae than those with less experience. If the majority of released bumblebees are more familiar with the areas up to 300 m from their colony compared to areas up to 1000 m; and if bumblebees are more likely to forage in familiar surroundings before returning to their colony; then released bumblebees

should be more likely to forage for pollen before returning to their colony when released from 300 m compared to when released from 1000 m.

### **5.3 Materials and Methods**

#### **5.3.1 Experimental Site**

The experiment was conducted at a mixed farm site in the South West of England: Site Four. Four *Bombus terrestris audax* colonies (Koppert Biological Systems, UK) were placed along the northern and eastern margins of a field, equidistant from the bordering hedge (Figure 5.1: Colonies A, B, C and D). Colonies were placed at least 7.50 m from each other. The experiment took place from the 23<sup>rd</sup>-29<sup>th</sup> August 2017 with maximum temperatures ranging between 20.0°C to 27.0°C. There were no flowering crops within a 900 m radius of the colonies (determined by matching current farm records with satellite imagery). Bramble flowers and fruit (*Rubus* spp.) were scattered throughout the hedgerows in the landscape. Weather conditions were varied but dry with periods of sun and scattered cloud. The wind was negligible with a mean speed of 0.14 km/h.

#### **5.3.2 Tagging and Pre-test Experience**

Bumblebee colonies were placed in a wooden nest box (Chapter Two: Figure 2.4). The bumblebee colony was connected to the outside of the nest box using a clear, Perspex® tube. Purchased colonies contained a queen and at least 50 naïve workers of mixed age that had never flown outside the plastic box in which they had arrived. The commercial sugar solution that accompanied the colonies from the supplier was removed in order to motivate the bumblebees to forage outside the colony for nectar and pollen. Prior to testing, individual bumblebees from each colony were tagged using numbered honeybee queen marking tags (EH Thorne (Beehives) Ltd, UK). RFID technology was not used in this experiment following the unreliability of the technology as experienced in the experiment presented in Chapter Three: Part B. In the experiments presented in Chapter Three: Part B, the RFID readers failed to scan the majority of tagged bumblebees which exited and entered the wooden nest box. As human experimenters were available to aid with this experiment, the decision was made to use direct observation instead of the RFID technology. A total of 314 bumblebees were tagged across all four colonies. Tagged bumblebees were

triaged sequentially and allowed to conduct one flight, two flights or five flights outside their colony. This was done in a stratified way, aiming to have equal numbers from each colony allocated to each level of experience and distance from the colony. In some cases, however, a bumblebee that was allocated to having a certain number of flights did not continue to exit their colony and undergo foraging flights. When this occurred, the bumblebee was substituted with another bumblebee from the same colony. When this was not possible, for example if no new foragers were emerging from the colony in question, then a bumblebee would be chosen from a more active colony. Once the allocated number of flights was reached, the tagged bumblebee workers were captured at the nest box entrance, as they were preparing to exit. The number of bumblebees captured at each nest box varied depending on the activity levels of each colony.

### 5.3.3 Releases

Once captured at the nest box, the tagged bumblebees were placed in an opaque, polystyrene box and transported to their release location. For all releases, the amount of time from capture to release did not exceed one hour. Releases were conducted at 300 m and 1000 m east and west from each colony (Figure 5.2). As there is evidence to suggest that prominent topographical landmarks can affect the homing success of honeybees (Southwick & Buchmann, 1995; Pahl *et al.*, 2011), bumblebees were released in more than one cardinal point in order to try and control for any topographical differences present as well as accounting for any prevailing wind direction.<sup>6</sup> In order to account for differences in the motivation levels of individual bumblebees and to encourage them to return to their colonies without stopping to forage, bumblebees were fed commercial sugar solution (67% Brix) *ad libitum* prior to release. Individual bumblebees were released facing north at arm's length at a height of approximately 130 cm. Their release time was recorded as well as the compass bearing at which they vanished from human sight (their 'vanishing bearing'; Gould, 1986; Dyer, 1991; Dyer, 1993; Dyer *et al.*, 1993).

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<sup>6</sup> The cardinal points of north and south were not tested due to a lack of resources.

A total of 164 bumblebees were released. Not all bumblebees that were tagged were released. This is because after tagging, not all bumblebees emerged once again from their nest box during the experimental period.

Experimenters observing the wooden nest boxes recorded the return time of individual bumblebees in order to calculate the duration of their return flights. Experimenters also recorded whether pollen was present in the corbiculae of returning bumblebees. Release times were randomly scattered throughout the day with the last release always occurring before 18:21 GMT +1.<sup>7</sup> All released bumblebees were only tested once. At each site, colonies were left open for returns during the daytime for four days following the last release and monitored continuously. The colonies were completely closed at night. As normal foraging activity was halted during testing days, colonies were supplemented with 10.00 ml of commercial sugar solution (67% Brix) every day and 2.00 g of honeybee pollen every other day.

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<sup>7</sup> Release times were not found to significantly affect homing success.



**Figure 5.1** | Layout of the *Bombus terrestris* colony placement at Site Four (A-D). Copyright information: Google Earth V 7.3.2. (June 22, 2018). Eye altitude: 610 m. <https://www.earth.google.com> [December 15, 2018].



**Figure 5.2** | Aerial views of the colonies and release points at Site Four. Release points were at 300 m and 1000 m from the colonies, east and west. The white circle denotes the location of the colonies while the white 'X's denote the individual release locations. Copyright information: Google Earth V 7.3.2. (June 22, 2018). Eye altitude: 8160 m. <https://www.earth.google.com> [December 15, 2018].



## 5.4 Statistical Analysis

Bumblebees which did not return within the same day as being released were excluded from the flight duration analysis (14.65% of returning bumblebees; Appendix E: Table E.1). This is due to the fact that the record of the duration of overnight flights is not entirely accurate. As testing did not begin at sunrise each day, bumblebees which had stayed out overnight may have attempted to return to their colony before colonies were opened. When bumblebees cannot enter their colony, they fly off and attempt to enter it at a later time (R.Herascu, personal observation). In order to account for bumblebees that stayed out overnight before returning to their colony, the proportion of returning bumblebees that stayed out overnight was also investigated (*“Proportion of Overnight Stays”*).

All the statistical analysis was carried out in R 3.5.2 (R Core Team, 2015).

### 5.4.1 Proportion of Returns

To determine whether the release distance and a bumblebee's previous experience of its environment had an effect on the homing success of bumblebees, the relationship between the proportion of released bumblebees that returned, and their release distance and number of previous flights taken was modelled with a generalised linear model (GLM). Due to low sample sizes, individual colonies could not be included in the model and bumblebees were pooled across colonies. A quasibinomial error structure and logit link were used due to overdispersed data. The explanatory variables used in the following order were the cardinal point of release, the release distance (entered as a numeric variable) and the number of flights that a bumblebee had taken prior to release (entered as numeric variable). An interaction between the release distance and the number of flights that a bumblebee had taken prior to release did not significantly improve the model fit ( $X^2(1) = 2.88$ ,  $p=0.23$ ). Residuals were checked for fit and homoscedasticity of variance using a simulation approach ('DHARMA' package, Hartig, 2019).

#### 5.4.2 Duration of Returns

To determine whether the release distance and a bumblebee's previous experience of its environment had an effect on the duration of return flights for bumblebees that returned to the colony on the same day as being released, the relationship between the duration of same day return flights and the release distance and number of previous flights taken was modelled with a linear model. Due to low sample sizes, individual colonies could not be included in the model and bumblebees were pooled across colonies. The explanatory variables used in the following order were the cardinal point of release, the release distance (entered as a numeric variable) and the number of flights that a bumblebee had taken prior to release (entered as a numeric variable). An interaction between the release distance and the number of flights that a bumblebee had taken prior to release did not significantly improve the model fit ( $X^2(1) = 0.0007$ ,  $p=0.98$ ). Visual inspection was used to check residual plots for fit and homoscedasticity. The response variable was logarithmically transformed in order to meet the model assumptions (Ives, 2015).

#### 5.4.3 Proportion of Overnight Stays

To determine whether the release distance and a bumblebee's previous experience of its environment had an effect on the proportion of bumblebees that stayed out overnight before returning to their colonies, the relationship between the proportion of bumblebees that stayed out overnight before returning and the release distance and number of previous flights taken was modelled with a GLM. Due to low sample sizes, individual colonies could not be included in the model and bumblebees were pooled across colonies. The explanatory variables used in the following order were the cardinal point of release, the release distance (entered as a numeric variable) and the number of flights that a bumblebee had taken prior to release (entered as a numeric variable). The model failed to converge with an interaction between the release distance and the number of flights that a bumblebee had taken prior to release. A binomial error structure and logit link were used. Residuals were checked for fit and homoscedasticity of variance using a simulation approach ('DHARMA' package, Hartig, 2019). Overdispersion was also tested for.

#### 5.4.4 Proportion of Pollen Foraged Before Returning

To determine whether the release distance and a bumblebee's previous experience of its environment had an effect on the proportion of returned bumblebees that foraged for pollen before returning, the relationship between the proportion of bumblebees that returned to their colony with pollen after being released and the release distance and number of previous flights taken was modelled with a GLM. Due to low sample sizes, individual colonies could not be included in the model and bumblebees were pooled across colonies. The explanatory variables used entered in the following order were the cardinal point of release, the release distance (entered as a numeric variable) and the number of flights that a bumblebee had taken prior to release (entered as a numeric variable). An interaction between the release distance and the number of flights that a bumblebee had taken prior to release did not significantly improve the model fit ( $X^2(1) = 1.47$ ,  $p=0.22$ ). A binomial error structure and logit link were used. Residuals were checked for fit and homoscedasticity of variance using a simulation approach ('DHARMA' package, Hartig, 2019). Overdispersion was also tested for.

### **5.5 Results**

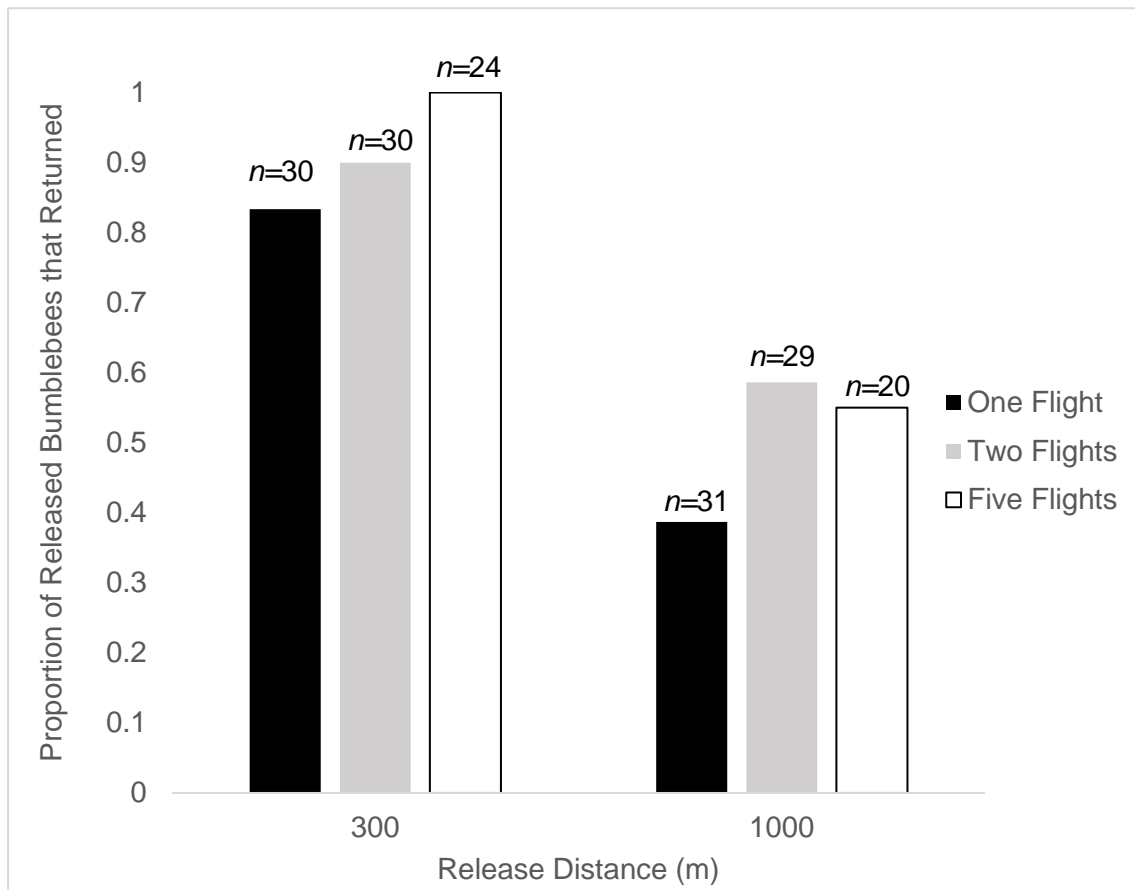
#### 5.5.1 Proportion of Returns

When released from 300 m, 83.3%-100% of released bumblebees with different experience levels returned to their colony (Figure 5.3). When released from 1000 m, 38.7%-58.6% of released bumblebees with different experience levels returned to their colony (Figure 5.3).

When comparing the proportion of released bumblebees that returned to their colony, there was no significant effect of the cardinal point that the bumblebees were released from (Table 5.1; Figure 5.3). There was a significant effect of the release distance on the proportion of released bumblebees that returned to their colony (Table 5.1; Figure 5.3). Bumblebees were more likely to return to their colony when released from 300 m compared to 1000 m (Figure 5.3). There was also no significant effect of the number of flights taken prior to release on the proportion of bumblebees that returned to their colony (Figure 5.1; Figure 5.3).

**Table 5.1. Model results from a linear model testing the effect of the cardinal point, the release distance and the number of flights taken prior to release (experience) on the proportion of bumblebees that returned to their colony. Variables, effect sizes  $\pm$  standard error, *t*-values and *p*-values from the fitted model. Cardinal point 'East' was used as the reference level.**

<b>Variable(s)</b>	<b>Effect Size <math>\pm</math> Standard Error</b>	<b><i>t</i> Value</b>	<b><i>p</i> Value</b>
Intercept	2.18 $\pm$ 0.91	2.39	0.04
Cardinal Point	1.32 $\pm$ 0.62	2.12	0.067
Release Distance	-0.004 $\pm$ 0.001	-3.57	0.007
Experience	0.28 $\pm$ 0.20	1.40	0.201



**Figure 5.3** | Proportion of released bumblebees that returned to their colony. This is based on the release distance from their colony as well as the number of flights that they experienced prior to being released.  $N=164$ . The numbers shown on the graph represent the total number of bumblebees that were released at each distance and for each level of experience.

### 5.5.2 Duration of Returns

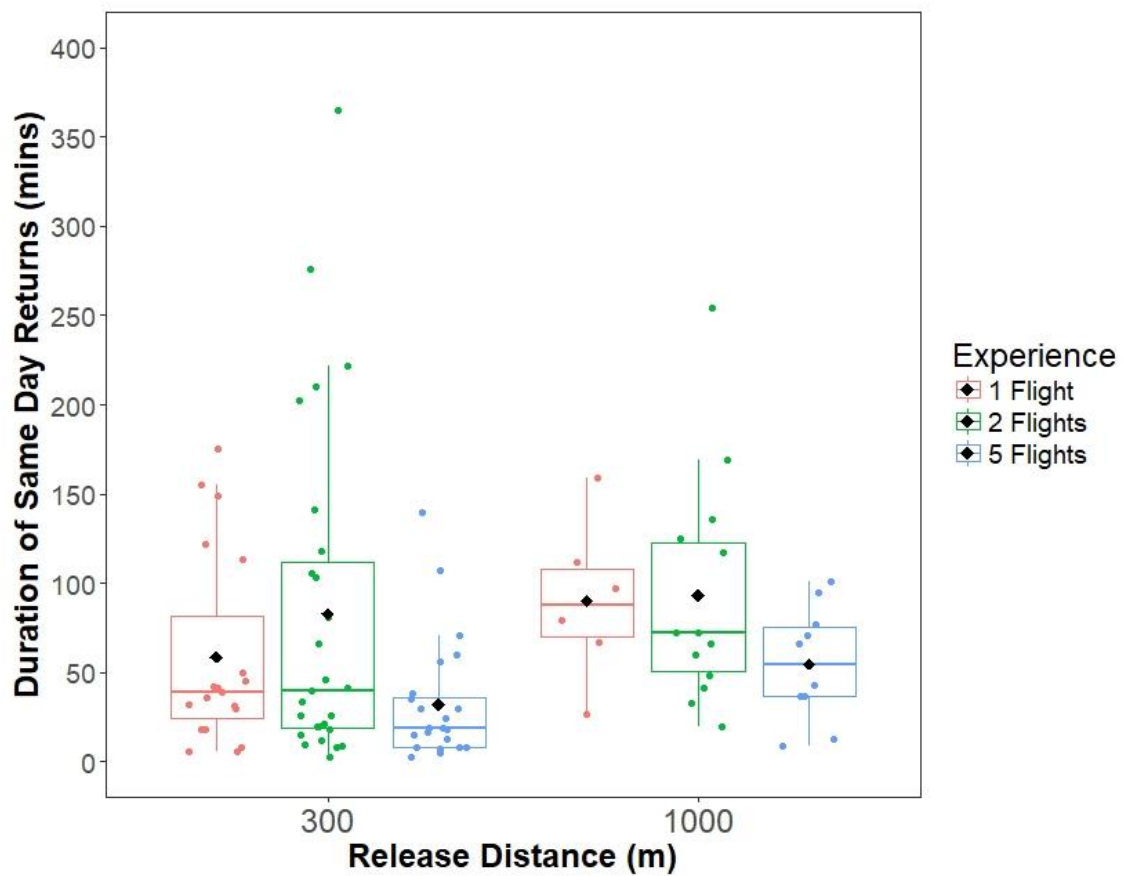
#### *Same Day Returns*

When released from 300 m, bumblebees that returned to their colony within the same day as being released had flight durations of between 3 and 365 minutes ( $n=70$ ; Figure 5.4). When released from 1000 m, bumblebees that returned to their colony within the same day as being released had flight durations of between 9 and 254 minutes ( $n=30$ ; Figure 5.4).

When comparing the duration of return flights to the colony, there was no significant difference in the duration of return flights between the two cardinal points (Table 5.2; Figure 5.4). There was a significant difference in the duration of return flights between the two release distances (Table 5.2; Figure 5.4). Bumblebees were faster to return to their colonies when released from 300 m compared to when they were released from 1000 m (Figure 5.4). There was also a significant effect of the number of previous flights that a bumblebee had taken before being released on the duration of their return flight to their colony (Table 5.2; Figure 5.4). Bumblebees which quicker to return to their colony the more experienced they were (Figure 5.4).

**Table 5.2. Model results from a linear model testing the effect of the cardinal point, the release distance and the number of flights taken prior to release (experience) on the duration of return flights. The return flight durations were logarithmically transformed in order to meet model assumptions. Variables, effect sizes  $\pm$  standard error,  $t$ -values and  $p$ -values from the fitted model. Cardinal point 'East' was used as the reference level.**

<b>Variable(s)</b>	<b>Effect Size <math>\pm</math> Standard Error</b>	<b><math>t</math> Value</b>	<b><math>p</math> Value</b>
Intercept	3.51 $\pm$ 0.26	13.43	<0.0001
Cardinal Point	0.36 $\pm$ 0.21	1.75	0.083
Release Distance	0.0009 $\pm$ 0.0003	2.68	0.009
Experience	-0.18 $\pm$ 0.06	-2.93	0.004



**Figure 5.4** | Boxplot of the duration of the return trip of bumblebees that returned before the first nightfall after being released (in minutes) depending on the number of flights they had before being released.  $N=100$ . Both return flights at east and west are shown. A small value of random noise was added to each data point for plotting purposes to aid visual representation.



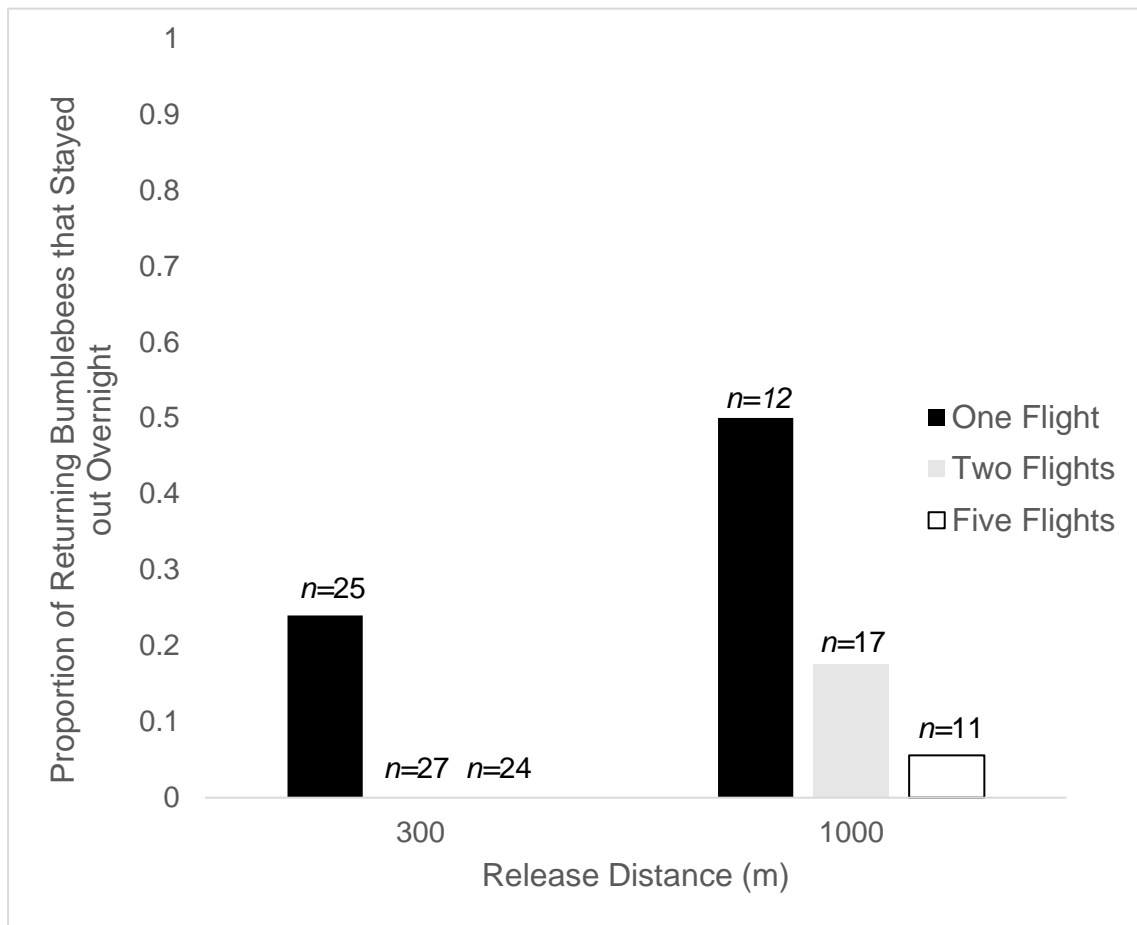
### 5.5.3 Proportion of Overnight Stays

When released from 300 m, 0% to 28.0% of returning bumblebees stayed out overnight before returning to their colony (Figure 5.5). When released from 1000 m, 5.55% to 50.0% of returning bumblebees stayed out overnight before returning to their colony (Figure 5.5).

When comparing the proportion of returning bumblebees that stayed out overnight before returning to their colony, there was no significant effect of the cardinal point that the bumblebees were released from (Table 5.3; Figure 5.5). There was a significant effect of the release distance on the proportion of returning bumblebees that stayed out overnight before returning to their colony (Table 5.3; Figure 5.5). Bumblebees were more likely to stay out overnight before returning to their colony when released from 1000 m compared to 300 m (Figure 5.5). There was also a significant effect of the number of flights taken prior to release on the proportion of returning bumblebees that stayed out overnight before returning to their colony (Table 5.3; Figure 5.5). The greater the number of flights taken prior to release; the less likely bumblebees were to stay out overnight before returning to their colony (Figure 5.5).

**Table 5.3. Model results from a linear model testing the effect of the cardinal point, the release distance and the number of flights taken prior to release (experience) on the proportion of bumblebees that stayed out overnight before returning to their colony. Variables, effect sizes  $\pm$  standard error, z-values and *p*-values from the fitted model. Cardinal point 'East' was used as the reference level.**

<b>Variable(s)</b>	<b>Effect Size <math>\pm</math> Standard Error</b>	<b>z Value</b>	<b><i>p</i> Value</b>
Intercept	1.10 $\pm$ 0.86	1.28	0.20
Cardinal Point	0.51 $\pm$ 0.61	0.85	0.40
Release Distance	-0.0023 $\pm$ 0.0008	-2.60	0.009
Experience	0.98 $\pm$ 0.41	2.38	0.017



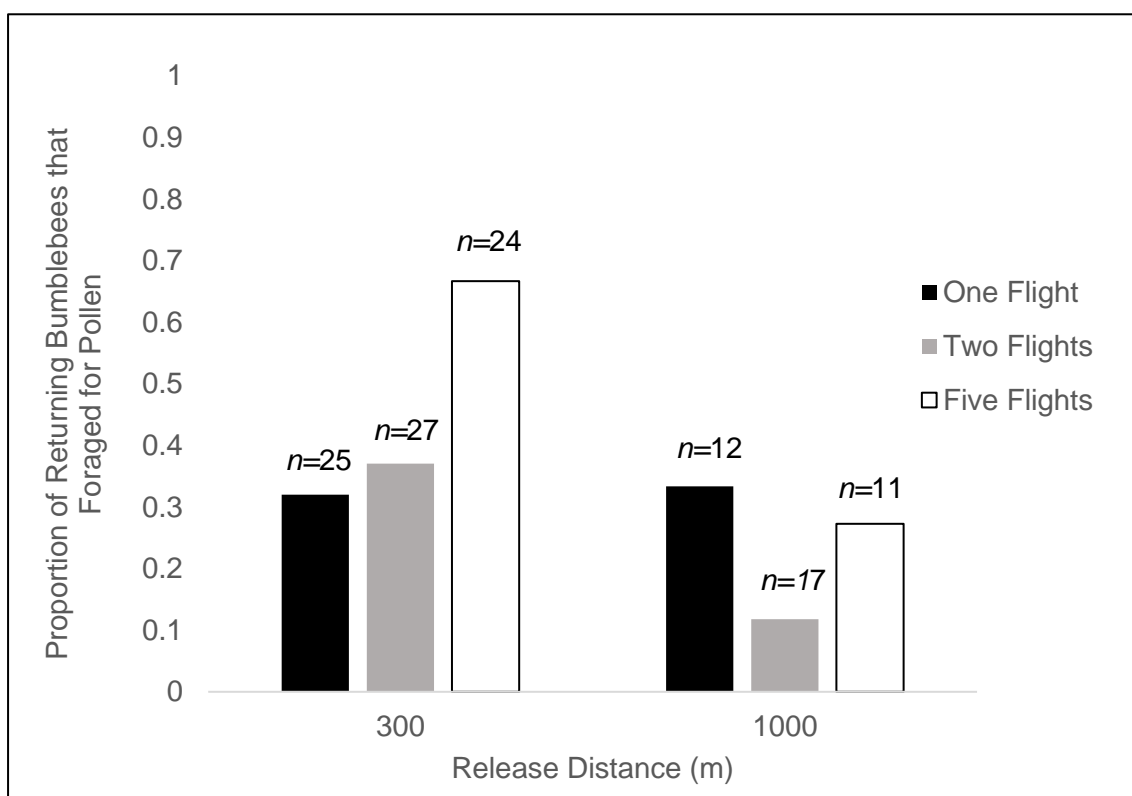
**Figure 5.5** | Proportion of returning bumblebees that stayed out overnight before returning. This is based on the release distance from their colony as well as the number of flights that they experienced prior to being released.  $N=116$ . Both returning bumblebees released from east and west are shown. The numbers shown on the graph represent the total number of bumblebees that returned at each distance and for each level of experience.

#### 5.5.4 Proportion of Pollen Foraged Before Returning

When comparing the proportion of returning bumblebees that foraged for pollen before returning to their colony, there was no significant effect of the cardinal point that the bumblebees were released from (Table 5.4; Figure 5.6). There was a significant effect of the release distance on the proportion of returned bumblebees that foraged for pollen before returning to their colony (Table 5.4; Figure 5.6). Bumblebees were more likely to forage for pollen before returning to their colony when released from 300 m compared to 1000 m (Figure 5.6). There was also a significant effect of the number of flights taken prior to release on the proportion of returned bumblebees that foraged for pollen before returning to their colony (Table 5.4; Figure 5.6). The greater the number of flights taken prior to release; the more likely bumblebees were to forage for pollen before returning to their colony (Figure 5.6).

**Table 5.4. Model results from a linear model testing the effect of the cardinal point, the release distance and the number of flights taken prior to release (experience) on the proportion of bumblebees that foraged for pollen before returning to their colony. Variables, effect sizes  $\pm$  standard error, z-values and *p*-values from the fitted model. Cardinal point 'East' was used as the reference level.**

Variable(s)	Effect Size $\pm$ Standard Error	z Value	<i>p</i> Value
Intercept	-0.33 $\pm$ 0.51	-0.65	0.51
Cardinal Point	-0.41 $\pm$ 0.41	-0.99	0.32
Release Distance	-0.0013 $\pm$ 0.0006	-2.10	0.04
Experience	0.28 $\pm$ 0.12	2.30	0.02



**Figure 5.6** | Proportion of returning bumblebees that foraged for pollen before returning. This is based on the release distance from their colony as well as the number of flights that they experienced prior to being released.  $N=116$ . Both returning bumblebees released from east and west are shown. The numbers shown on the graph represent the total number of bumblebees that returned at each distance and for each level of experience.

## 5.6 Discussion

In this experiment, the effects of experience and distance on the homing success of *Bombus terrestris* workers was investigated. Experience had no significant effect on the proportion of bumblebees that returned to their colonies. Release distance did prove, however, to have a significant effect on the proportion of bumblebees that returned to their colonies. A greater proportion of bumblebees returned to their colonies when released from 300 m compared to 1000 m (Figure 5.3); and this aligns with the results for experienced bumblebees in Chapter Four. When released from 300 m, 83.3%-100% of released bumblebees returned to their colony (Figure 5.3). In contrast, when released from 1000 m, only 38.7%-58.6% of released bumblebees returned to their colony (Figure 5.3). If bumblebees are more likely to return from areas that they have previously experienced, then this result suggests that throughout the first five flights outside the colony, only half of bumblebee workers (50.77%), on average across all experience levels, explored up to 1000 m from the colony. The majority explored and foraged within an area of at least 300 m, but less than 1000 m, around the colony. This suggests that the majority of bumblebee workers explored and foraged somewhere between 300 m and 1000 m from their colonies. When tracked with harmonic radar, bumblebees on their first flight were observed flying only within 200 m from their colony. On their second and third flights, this increased to 400 m. Only if bumblebees had more than six flights outside the colony did they explore an area of up to 500 m (Osborne *et al.*, 2013). As such, Osborne *et al.*'s (2013) results and the results of the present experiment suggest that landscape exploration seems to be a gradual process throughout a worker's career.

The suggestion that a bumblebee may concentrate its acquisition of landscape knowledge within its first flights also comes from foraging observations. Individual bumblebees are regularly observed returning to the colony with forage on their first flight (Hempel de Ibarra *et al.*, 2009; Osborne *et al.*, 2013, Chapters Two & Three) and bumblebees are known to be constant to both flower type (Heinrich, 1976; Waser, 1986) and forage site (Heinrich, 1976; Bowers, 1985; Waser, 1986; Dramstad 1996; Saville *et al.*, 1997). Furthermore, in the experiment outlined in Chapter Three, flower constancy developed well within the first five flights, as evidenced by the constancy of pollen collection. As such, new bumblebee

workers may prioritise finding a viable food source during their first flights and only switch food sources, and thus extend their exploration, only when their initial food source is no longer profitable. Rather than extensive exploration and sampling, such a strategy, if taken up by the entire colony's workforce, may ensure a constant flow of pollen and nectar into the colony. This may prove critical as bumblebees only store a few days' worth of pollen and nectar in their colonies (Heinrich, 1979a).

When examining the flights of bumblebees that returned within the same day as being released, bumblebees were significantly faster to return from 300 m than from 1000 m (Figure 5.4). Bumblebees were also significantly faster to return with increasing levels of experience (Figure 5.4). As experience seems to decrease overall flight duration, the differences observed may be due to an increase in flight speed. Bumblebees tracked with harmonic radar have been found to increase their average groundspeed as they gain more experience flying (Osborne *et al.*, 2013). It also cannot be ruled out that a decrease in return flight durations could be due to a decrease in overall search times, rather than changes in flight speed. Even if a bumblebee is not familiar with the specific release location, its overall search times will decrease the more familiar it is with areas adjacent to the release location. Another possibility is that inexperienced bumblebees, in novel situations, are more likely to be stressed and that this stress can have a negative effect on their cognitive abilities.

Returning bumblebees were also significantly more likely to stay out overnight when released from 1000 m and/or when they were less experienced (Figure 5.5). Bumblebees who do not return within the same day as being released are presumably at a greater risk of predation and exhaustion. Bumblebee workers are known to sometimes stay out overnight on foraging trips (Free, 1955b) and it is hypothesised that bumblebees will remain in the field overnight if the light intensity suddenly drops below that required for flight (Hobbs, Nummi & Virostek, 1962). Under normal foraging circumstances, Roulston (2015) has found that staying out of the colony overnight increased steadily with experience, however the reasons behind this phenomenon remain unclear. In the context of homing, it is likely that returning bumblebees who stayed out overnight represent those bumblebees who were unfamiliar with their surroundings upon release and continued searching for their colony within the landscape, even as night fell.

Rather than an active behaviour that bumblebees seek to engage in, staying out overnight in this experiment seems to be a consequence of prolonged searching for the colony.

The differences in the duration of return flights between the two release distances does not mirror the difference in distance. In other words, bumblebees returning from 1000 m did not take 3.33 times longer than those returning from 300 m. As it seems reasonable that the majority of bumblebees should have explored up to 300 m during their first five flights, this result suggests that bumblebees released from 300 m were actually more likely to engage in other activities before returning to their colony. This is evidenced by the fact that bumblebees released from 300 m were significantly more likely to forage for pollen before returning to the colony than those released from 1000 m (Figure 5.6). Bumblebees with more experience were also significantly more likely to forage for pollen before returning to the colony (Figure 5.6). This suggests that when in a familiar location, bumblebees may not be primarily motivated to return to their colony as quickly as possible when displaced.

It may also be tempting to conclude that the bumblebees who foraged for pollen before returning to their colony when released from 1000 m would have been the ones that were familiar with their release location. It is entirely possible, however, that lost bumblebees, upon extensive searching and finding themselves in a familiar area, may choose to resume foraging for the colony before returning. What does seem likely is that a motivational switch can occur when bumblebees find themselves in familiar surroundings. As colonial insects, when lost upon release, it seems feasible that bumblebees will prioritise returning to the colony over other activities such as colony provision. This is especially true given that each bumblebee was fed sugar solution *ad libitum* immediately prior to release. Finding itself in a familiar location, a released bumblebee may no longer prioritise returning to the colony but may instead resume foraging. After all, this presumably was its primary motivation when it was captured leaving its colony. The fact that bumblebees were observed returning to the colony with pollen, rather than just nectar, further suggests that this foraging was undertaken for colony provisioning. It would be interesting to investigate whether the bumblebee maintains the flower and site constancy from its previous trip(s) after displacement and subsequent reorientation. This would shed light on the circumstances which can influence



foraging decisions. This could be investigated by monitoring the pollen foraging preferences of bumblebees before and after their displacement. Similar to the technique employed in Chapter Two, the pollen loads of bumblebees could be sampled when bumblebees return to the colony. A survey of the plant species in the surrounding landscape could be done and the pollen samples could then be matched to their source. The pollen preferences of bumblebees before and after displacement could then be compared.

#### 5.6.1 Experimental Limitations and Improvements

This experiment and the validity of its results could be improved by releasing bumblebees from release locations in all four cardinal points, as was the case in the experiment presented in Chapter Four. Although this was not possible given the resources available in this instance, releasing at all four cardinal points would have helped to account more fully for differences in topography weather conditions. In this experiment, bumblebees were given one, two or five flights of experience prior to their release. From the results obtained, only half of bumblebees (50.77%), on average across all experience levels, seemed to explore up to 1000 m from the colony within their first five flights. Future experiments could increase the number of flights prior to release in order to ascertain when this displacement distance is reached.

As this experiment took place at a single experimental site, it is not possible to conclude whether the results obtained are indicative of *Bombus terrestris* behaviour more generally. As such, future experiments could increase the number of experimental sites, including sites with differing resource distribution and topography. By manipulating the distribution of foraging resources, the relationship that exists between the spatial arrangement of forage, bumblebee exploration and experience of the landscape can be investigated. For example, it is entirely possible that a bumblebee's exploration during its first five flights is predominantly influenced by the distribution of forage around their colony. In landscapes where the only available foraging resources are located around 1000 m from the colony, it may be the case that the majority of workers would be familiar with this area within their first five flights or sooner.

## 5.7 Conclusion

In the experiment presented in this chapter, the homing success of bumblebees did not seem to be significantly affected by a bumblebee's previous level of experience. This, however, was not the case for homing duration. Homing duration was found to be significantly affected by both displacement distance and previous experience. Bumblebees were faster to return to their colonies when released from 300 m compared to 1000 m if they returned the same day as being released. Homing duration decreased with increasing levels of previous experience. Staying out overnight before returning was also significantly more likely in less experienced bumblebees. Furthermore, when released from 300 m, bumblebees were also significantly more likely to forage for pollen before returning to the colony the more experienced they were. Taken together, the results point to the importance of the first five flights as a critical information gathering period in a bumblebee's lifetime.

The results of this experiment shed light on the role that experience plays on the homing success of bumblebees as well as their landscape exploration. Up to this point, the experiments presented all involved only one caste in a bumblebee colony: the workers. In Chapter Six, the foraging behaviour of future queens, the gynes, was investigated. Specifically, this was in response to field observations made during the experiments presented in Chapter Two and Chapter Three in which gynes were observed returning to their maternal colony with pollen loads in their corbiculae.

## Chapter Six

### Feeding the Family: The Foraging Behaviour of *Bombus terrestris* Gynes

#### 6.1 Abstract

A division of labour exists within bumblebee colonies, with castes and subcastes carrying out specific roles. Unlike workers, males do not forage for the colony and are seen permanently leaving the nest shortly after emerging from pupae. There is little consensus, however, on the behaviour of female bumblebee reproductives (gynes). Observations have been made of both gynes behaving similarly to males as well as foraging and provisioning their maternal colony before mating and entering hibernation. To explore whether pre-mated gynes habitually provision the maternal colony, the effect of pollen influx on *Bombus terrestris* gyne foraging behaviour was investigated in the first experiment of its kind. Bumblebee colonies at their reproductive stage were subjected to one of three experimental conditions in which the amount of pollen entering the colony was manipulated. In all conditions, a proportion of gynes returned to the maternal colony after initially leaving and did so with pollen in their corbiculae. In the first experimental condition, foraging bumblebee workers and gynes were stripped of their pollen loads when they returned to their colonies after a foraging trip. In the second experimental condition, only gynes were stripped of their pollen loads when they returned to their colonies after a foraging trip. In the third experimental condition, the control, no bumblebees were stripped of their pollen loads when they returned to their colonies after a foraging trip. A significant difference was found in the proportion of gynes that foraged between Condition One and the control and between Condition Two and the control. No significant difference was found in the proportion of gynes that foraged between Condition One and Condition Two. No significant correlation was found between the number of larvae in the colony and the proportion of foraging gynes. The results suggest that gynes can respond to pollen shortages in the colony and provision their colonies with pollen. As a proportion of gynes were observed provisioning their colonies with pollen in all experimental conditions, the results also suggest that individual physiological factors of the gynes may affect gyne foraging behaviour.

## 6.2 Introduction

Like many social insects, a bumblebee colony is made up of different castes. In a bumblebee colony, these are the workers and the reproductives. A colony's annual life cycle begins with a queen laying the first brood. Workers emerge and, uniquely among the social bees, exhibit a large size variation. Smaller workers tend to engage in colony tasks while larger workers provision the colony with pollen and nectar (Goulson *et al.*, 2002; Peat & Goulson, 2005). At a certain point in the colony's life cycle, reproductives, in the form of males and female gynes, are produced. Gynes, defined as unmated queens without a colony, are usually only reared when a high worker-to-larva ratio is reached (Richards, 1946; Duchateau & Velthuis, 1988; Röseler, 1991). In *Bombus terrestris* colonies, male production may precede gyne production (protandry) or succeed it (protogyny), depending on the colony (Amsalem *et al.*, 2015). Reproductives leave the colony to mate and the end of a colony's life cycle is marked by the death of the founding queen and the rapid deterioration of the remaining workers.

Two major social phases mark the colony's life cycle: the pre-competition and the competition phase (Duchateau & Velthuis, 1988; Cnaani, Schmid-Hempel & Schmidt, 2002; Amsalem *et al.*, 2009). During the pre-competition phase, reproduction is exclusive to the founding queen and diploid eggs are laid. At a certain 'switch point', the founding queen will begin laying haploid, male-destined eggs (Duchateau & Velthuis, 1988; Alaux, Jaisson & Hefetz, 2006). Although the majority of eggs laid will be male-destined, some diploid eggs may still be laid following the 'switch point' (van der Blom, 1986; van Doorn & Heringa, 1986; Duchateau & Velthuis, 1988). After a certain period of time, which varies depending on the colony, the competition phase will follow (Duchateau, Velthuis & Boomsma, 2004). In this phase, workers will compete with the founding queen and lay their own eggs. This phase is marked by physical conflict and aggression in which egg laying workers and the founding queen will attempt to destroy each other's eggs, in some cases even leading to the workers killing the founding queen (van Honk & Hogeweg, 1981; van Honk *et al.*, 1981; van der Blom, 1986; van Doorn & Heringa, 1986; Bourke, 1994).

The physical aggression that marks the competition phase is due to the asymmetry in relatedness that exists between members of the colony (Amsalem,

*et al.* 2009). The founding queen and the workers are in competition for the production of male, but not female, reproductives. This is best explained by coupling the haploid/diploid sex-determination system in Hymenoptera with inclusive fitness theory (Hamilton, 1964; 1972; Trivers & Hare, 1976). In a bumblebee colony, haploid males are produced from unfertilised eggs and can thus be laid by the founding queen as well as by the workers. In contrast, diploid females can only be laid by the founding queen who has mated. As a result, workers are more closely related to gynes, their sisters (coefficient of relatedness ( $r$ ) = 0.75), than they are to males, their brothers ( $r$  = 0.25). Furthermore, workers are more closely related to their own sons ( $r$  = 0.50) than they are to their brothers ( $r$  = 0.25). The founding queen is equally related to males ( $r$  = 0.50) and gynes ( $r$  = 0.50), her sons and daughters. She is more closely related to her sons ( $r$  = 0.50), however, than she is to the sons of the workers ( $r$  = 0.25), her grandsons. In such a system, the founding queen's inclusive fitness will benefit more from producing her own sons and suppressing the reproductive capabilities of her daughters. In contrast, the worker's inclusive fitness will benefit more from producing their own sons rather than helping to rear their brothers. Workers will thus be selected to compete with the founding queen over male production as well as among each other for access to reproduction (Bourke & Franks, 1995; Crozier & Pamilo, 1996; Bourne & Ratnieks, 2001).

Although the founding queen and the workers will compete over male production, the presence of reproductive workers does not result in decreased gyne production (Lopez-Vaamonde *et al.*, 2003). This is again due to the asymmetry in relatedness between the castes, as workers are more related to gynes ( $r$  = 0.75), their sisters, than they are to their sons ( $r$  = 0.50). As such, it is in their kin selected interest to reproduce only after gyne production is secure. The precise mechanism which governs the onset of the competition phase remains unknown (Amsalem *et al.*, 2009), but experimental evidence suggests that workers will postpone the competition phase until they can be certain that diploid larvae are committed to develop into gynes (Alaux, Jaisson & Hefetz, 2004; Alaux *et al.*, 2004; Duchateau, Velthuis & Boomsma, 2004; Alaux, Jaisson & Hefetz, 2005; Alaux, Jaisson & Hefetz, 2006). Caste determination in diploid larvae is under the founding queen's control and is mediated by a non-volatile pheromone which she produces (Bourke & Ratnieks, 2001). Larvae enter different developmental

pathways once caste is determined (Goulson, 2010) and gyne larvae have a longer instar development than worker larvae (Cnaani & Hefetz, 2001). This results in gynes being observably larger than workers (Alford, 1975; Cnaani *et al.*, 1997). Unlike workers, gynes will undergo hibernation and need considerable fat stores to sustain them throughout this period of diapause (Fliszkiewicz & Wilkaniec, 2007).

Castes differ in both physiological and behavioural characteristics. Unlike workers, males are not known to engage in foraging tasks and are observed leaving the colony a few days after emergence (Goulson, 2010). Although the males of some bumblebee species have been observed returning to their colony (Frison, 1917, 1928; Wild, 1924), it is generally accepted that males do not normally return to the colony after leaving and concentrate instead on feeding and mating (Goulson, 2010). Like males, gynes also leave their maternal colony and compared to workers, are much longer lived (Alford, 1975). There is mixed opinion, however, on the extent to which gynes participate in colony tasks following emergence but before leaving the maternal colony to mate and hibernate. While some authors claim that gynes do not take part in any colony tasks (Röseler & Röseler, 1988), others have observed gynes taking part in activities such as brood incubation and colony defence (Frison, 1928; Plath, 1934; Free & Butler, 1959). Furthermore, gynes have been observed provisioning their maternal colony with nectar and pollen (Free & Butler, 1959; Milliron, 1971; Heinrich, 1979a). Such provisioning behaviour, however, has only been observed when a colony is largely depleted of its workers and/or food stores. It is argued that such behaviour is not generally observed under any other circumstances (Milliron, 1971; Alford, 1975; Allen, 1978; Heinrich, 1979a; Goulson, 2010).

Unlike workers, gynes will ultimately leave the maternal colony to mate, hibernate and reproduce through the establishment of their own colony. As such, it would not seem beneficial for gynes to join the workers against the founding queen and reproduce during the competition phase. Gynes could, however, increase their inclusive fitness by aiding their sisters in bringing up both fellow gynes as well as their sister's sons. Due to the asymmetric relatedness that exists, gynes are more closely related to their nephews ( $r=0.375$ ), their sisters' sons, than they are to their brothers ( $r=0.25$ ). As it increases their inclusive fitness, gynes may provision the colony when adequate food stores are not available rather than solely

foraging and consuming nectar and pollen at the food source. Whether this can outweigh the risks associated with foraging before hibernation is not known.

As the topic of gyne behaviour prior to mating and hibernation has been largely overlooked in recent decades, this experiment was conducted in order to investigate this phenomenon. In order to investigate whether bumblebee gynes do indeed provision their maternal colonies before hibernation as well as the circumstances in which they do so, the foraging behaviour of bumblebee gynes was observed. Specifically:

1. Do bumblebee gynes forage for pollen and provision their maternal colony before leaving the colony to mate and hibernate?
2. Are bumblebee gynes more likely to forage for pollen and provision their maternal colony when there is a deficit in the amount of pollen entering the colony?
3. Does a relationship exist between bumblebee gyne foraging and the total number of workers or larvae present in the maternal colony?

In order to answer these questions, *Bombus terrestris* colonies which were undergoing gyne production were placed at two different sites in the South West of England. Colonies were assigned to two different experimental conditions and one control, designed to modify the perceived pollen status of the colony. In Condition One, all incoming bumblebees were stripped of their pollen loads upon returning to their colony. This greatly reduced the amount of pollen available to the colony. In Condition Two, only foraging gynes were stripped of their pollen loads upon returning to their colony. As such, there was only a small reduction in the amount of pollen available to the colony. It is not known, however, whether the act of depositing pollen from the corbiculae directly into the colony's stores forms an essential part of the mechanism which signals the completion of a foraging trip for a bumblebee. Stripping gynes of their pollen loads before they were deposited in the colony may have influenced gynes to continue foraging, regardless of the overall amount of pollen that was coming into the colony. This possibility was accounted for in a control condition, where no incoming bumblebees were stripped of their pollen loads upon returning to their colony. The duration of flights and whether pollen was collected was also recorded.

A number of different scenarios were hypothesised. If gynes only provision the maternal colony when there is a lack of pollen resources, then a greater proportion of bumblebee gynes should forage in the first treatment, compared to the second treatment and the control. Foraging is an energetically costly and risky behaviour in which a bumblebee is exposed to an increased risk of mortality. As a female reproductive, engaging in the hazardous task of foraging before mating and entering hibernation does not seem to be beneficial if adequate food stores are available in the colony.

Gynes may also be observed foraging in the control condition when presumably adequate pollen stores are available if there are benefits to exploring and foraging in the landscape before hibernation. Benefits may be obtained in the form of increased knowledge of the surrounding environment which may prove useful when searching for a location to hibernate or to establish a colony. Newly emerged queens have been observed dispersing at least 600 m from their maternal colony (Makinson *et al.*, 2019) and have been estimated to disperse between three to five kilometres from it (Lepais *et al.*, 2010). As such, experience of the environment and flying in general may aid future nest searching behaviour. Returning to the maternal colony while engaging in this behaviour may provide adequate shelter and warmth, decreasing thermoregulatory expenditure. It is unknown, however, whether a detailed memory of the environment surrounding the maternal colony would persist following hibernation and whether such experience would be beneficial.

## **6.3 Materials and Methods**

### **6.3.1 Experimental Sites**

The experiment took place at two sites in the South West of England: Site A and Site B. As only two experimental sites were used, the results obtained should be viewed with some caution. Different experimental treatments were tested over different experimental time periods, which may have led to a variety of factors confounding the results (please see 'Discussion: Experimental Limitations and Improvements').



### Site A

A total of six *Bombus terrestris audax* colonies were used (Koppert Biological Systems, UK) at a community orchard across three different testing periods. During each testing period, one to three colonies were placed in a clearing in the orchard (Figure 6.1). Colonies were placed at least 7.50 m from each other. Two testing periods comprised four consecutive days while one testing period comprised three consecutive days (Appendix F: Table F.1). Testing took place throughout the month of June in 2016. Air temperatures ranged from 16.0 to 28.1 °C. The weather on testing days ranged from dry and clear to overcast while the wind speed ranged from 0 to 12km/h. The starting time of the experiment ranged from 11:40 GMT+1 to 12:24 GMT+1 while the end time was always 19:00 GMT +1. All daily testing was conducted for a minimum of six hours. Outside of these experimental times, the colonies were closed.

### Site B

A total of 12 *Bombus terrestris audax* colonies were used (Koppert Biological Systems, UK) at Site B across six different testing periods (Appendix F: Table F.1). During each testing period, one to two colonies were placed in an open lawn on the university's campus (Figure 6.2). Colonies were placed at least 7.50 m from each other. All testing periods comprised four days and were consecutive in all but one case due to inclement weather. Testing took place throughout the months of June, July and September in 2016 and throughout the months of June and July in 2017. The order in which the treatments were run was randomly assigned. Once a treatment was assigned, multiple colonies were tested under that treatment and colonies were always tested simultaneously. It should be noted that different treatments were unfortunately not run simultaneously, which in retrospect would have been an improved experimental design. Air temperatures ranged from 12.7 to 32.5 °C. The weather on testing days ranged from dry and clear to overcast while the wind speed ranged from 0 to 12.7km/h. The starting time of the experiment ranged from 09:30 to 15:30 GMT+1 while the end time ranged from 15:30 GMT + 1 to 21:00 GMT+1. All daily testing was conducted for a minimum of four hours. Outside of these experimental times, the colonies were closed.



**Figure 6.1** | Experimental site A with *Bombus terrestris* colony placement. The white circles denote the placement of the individual colonies. Copyright information: Google Earth V 7.3.2. (June 22, 2018). Eye altitude: 650 m. <https://www.earth.google.com> [December 15, 2018].



**Figure 6.2** | Experimental site B with *Bombus terrestris* colony placement. The white circles denote the placement of the individual colonies. Copyright information: Google Earth V 7.3.2. (June 22, 2018). Eye altitude: 730 m. <https://www.earth.google.com> [December 15, 2018].

### 6.3.2 Experimental Procedure

Purchased colonies were left to reach the reproductive stage in their life cycle with the production of gynes. The presence of gynes was determined visually (please see Section 6.3.2.1: Establishing Caste Differences). Once this stage was reached, the bumblebee colonies were transported to each site. At each site, the colonies were placed in a wooden nest box (Chapter Two: Figure 2.4). The bumblebee colony was connected to the outside of the nest box using a clear, Perspex® tube. In order to encourage the bumblebees in each colony to forage for pollen and nectar, the commercial sugar solution that accompanied the colonies from the supplier was removed. The colonies were supplemented on the first and third day of testing with 3.78 g of dried honeybee pollen (EH Thorne (Beehives) Ltd, UK) in order to insure against the scenario of extreme resource depletion and colony death.

Workers, gynes and males were released from the nest throughout the testing day. Upon their return to the colony, gynes were tagged using honeybee queen marking tags (EH Thorne (Beehives) Ltd, UK). Each colony was assigned to one of two testing conditions or the control. In Condition One, the pollen loads of all returning bumblebees were removed before the bumblebees could return to their colony. This was done by removing any pollen that was present in the corbiculae of each bumblebee. In Condition Two, only the pollen loads of returning gynes were removed. In the control, no pollen loads from any returning bumblebees were removed.

The activity patterns of each colony were recorded with human experimenters marking each individual bumblebee's exit and entry into their colony. Differentiation between workers and gynes was assigned visually upon release based on size differences (please see Section 6.3.2.1: Establishing Caste Differences). Whether or not a returning bumblebee carried a pollen load was also recorded. In the context of this experiment, gynes were considered as having foraged only if they returned with a pollen load. In this experiment, the pollen foraging behaviour of bumblebees was focused on rather than their nectar foraging behaviour. This was due to the fact that previous pilot experiments showed that forcing bumblebees to expel the contents of their crop, a requirement if nectar foraging was to be monitored, caused them such physiological stress

that they were unable to resume normal foraging behaviour for prolonged periods of time (R.Herascu, personal observation).

#### 6.3.2.1 Establishing Caste Differences

The only observable defining feature that differentiates workers and gynes seems to be a size bimodality which is particularly apparent in pollen storing species such as *Bombus terrestris* (Alford, 1975). *Bombus terrestris* gynes have been observed to be one to three times as large as the average size of a worker (Cumber, 1949) and this seems to be the result of longer instar durations in gyne larvae (Cnaani & Hefetz, 2001). As such, castes were visually assigned to bumblebees in the field based on their body size. There can be substantial differences in worker and gyne sizes between colonies; therefore, gynes were usually observed as the larger individuals within that colony, relative to the workers.

Unlike workers, gynes undergo hibernation and need considerable fat bodies to sustain them through this period of diapause (Fliszkiewicz & Wilkaniec, 2007). As fat body growth will begin as early as the first few days of life (Dylewska, 1996), gynes should be relatively heavier than workers for their size. As a result, several authors suggest that the size bimodality between *Bombus terrestris* workers and gynes is best demonstrated by considering their weights (Richards, 1946; Cumber, 1949; Alford, 1975). Michener (1962) also recommends that due to allometry, differences in the proportions of body parts will exist among differently size individuals and, as such, multiple traits should be used when distinguishing between castes. Other features such as ovary development or the size of the corpora allata do not prove useful in caste differentiation. The ovaries of gynes do not develop until after undergoing an obligatory diapause while, under conditions of abundant food stores, the ovaries of workers may become functional (Free, 1957). The size of the corpora allata, on the other hand, scales with body size (Röseler, 1967). As such, in terms of physical features, size differences remain the best current indicator of caste differences in *Bombus terrestris*.

In order to establish whether caste differences had been correctly assigned during the experiment, each colony was frozen at the end of the experimental period. It should be noted that the number of workers left in each colony may have been variable due to lost individuals during the experimental period.

Bumblebee workers and gynes inside the colony were once again visually divided and the following physical characteristics were measured: weight, total body length (including the head), thorax width, distance between the wing buds, wing length and total wingspan (lengths of both wings + distance between the wing buds).<sup>8</sup> A total of 221 gynes and 815 workers were measured. Allometric log-log plots were created for each physical trait plotted against body weight (Figures 6.5i-v). Body weight was also plotted for each visually assigned caste (Figure 6.6). It would have also been useful at this point to measure the remaining pollen stores present in each colony.

#### 6.3.2.2 Establishing Colony Condition

In previous studies, gynes have only been observed provisioning their maternal colony when the colony's food stores or worker numbers had been largely depleted (Allen *et al.*, 1978). In order to establish the overall state of each colony in this experiment, different colony characteristics were measured after the colonies were frozen. This included the weight of the colony, the total number of workers and gynes and the state of the brood.<sup>9</sup> These characteristics were plotted against the proportion of released gynes that had returned with a pollen load in each colony.

### **6.4 Statistical Analysis**

All the statistical analysis was carried out in R 3.5.2. (R Core Team, 2015).

#### 6.4.1 Effect of Pollen Status on the Proportion of Foraging Gynes

To determine whether the experimental treatment had an effect on gynes provisioning their maternal colonies with pollen, a Fisher's exact test was used to compare the proportion of gynes that returned with pollen across the different experimental treatments. Post-hoc Fisher's exact tests between the different experimental treatments were conducted using Bonferroni adjusted alpha levels of 0.0167 (0.05/3) (McDonald, 2014).

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<sup>8</sup> This was possible for 16 of the 18 bumblebee colonies used.

<sup>9</sup> It was only possible to obtain the weight of the colony and the total number of workers in 11 of the 18 colonies used. Furthermore, it was only possible to obtain the state of the brood in 7 of the 18 colonies used.

#### 6.4.2 Confirming Caste Differentiation

To confirm that gynes were correctly identified, analysis was done to check whether there was a significant size difference in the visually assigned castes. An ANCOVA was used to investigate the relationship between each trait measured (body length, thorax width, wing bud distance, wing length and wingspan) and body weight for the visually assigned castes. The explanatory variables used were body weight (entered as a numeric variable) and caste, and the interaction term was included. The difference in intercept for the slope for each caste was used as confirmation that castes could be visually distinguished based on size. The interaction term in the model showed whether the slopes were significantly different between castes and hence whether there was a difference in the *allometric relationship* between body weight and each trait for the differently assigned castes. Visual inspection was used to check residual plots for fit and homoscedasticity.

On visual inspection of the plotted data, it was clear that there was a single outlier ( $\log(\text{body weight}) < 1$ ; Figures 6.4.i-6.4.v). Its influence was checked by comparing the results with the same analysis when the outlier was excluded. The results of this second analysis can be found in Appendix F (Tables F.4 & F.5; Figures F.1.i-v). Both analyses, with and without the outlier, yielded similar results and conclusions so the full analysis including the outlier are described in the main results.

To determine whether there was a significant difference between the weights of the visually assigned castes, a Welch's t-test was performed.

#### 6.4.3 Effect of Colony Condition on Proportion of Foraging Gynes

To determine whether the state of each colony had a significant effect on gyne foraging, a Pearson's correlation analysis was performed between the total number of workers in each colony and the proportion of returning gynes that foraged for pollen. A Pearson's correlation analysis was also performed on the total number of larvae in each colony and the proportion of returning gynes that foraged for pollen as well as on the larva/worker ratio in each colony and the proportion of returning gynes that foraged for pollen.

## 6.5 Results

### 6.5.1 Effect of Pollen Status on the Proportion of Foraging Gynes

For Condition One, where all incoming bumblebees were stripped of their pollen, the mean number of released gynes for each colony was 11 (range: 5-22; Appendix F: Table F.2). The mean number of returned gynes throughout the experimental testing days from each colony was 8 (range: 3-13; Appendix F: Table F.2). The mean proportion of gynes that returned from each colony was 0.72 (range: 0.53-1; Appendix F: Table F.2). Of the total number of returned gynes, the mean proportion that returned with pollen was 0.77 (range: 0.25-1; Appendix F: Table F.2). In all of the colonies that were measured following the experiment, there were gynes present that had not left the colony during the experimental period (Appendix F: Table F.2).

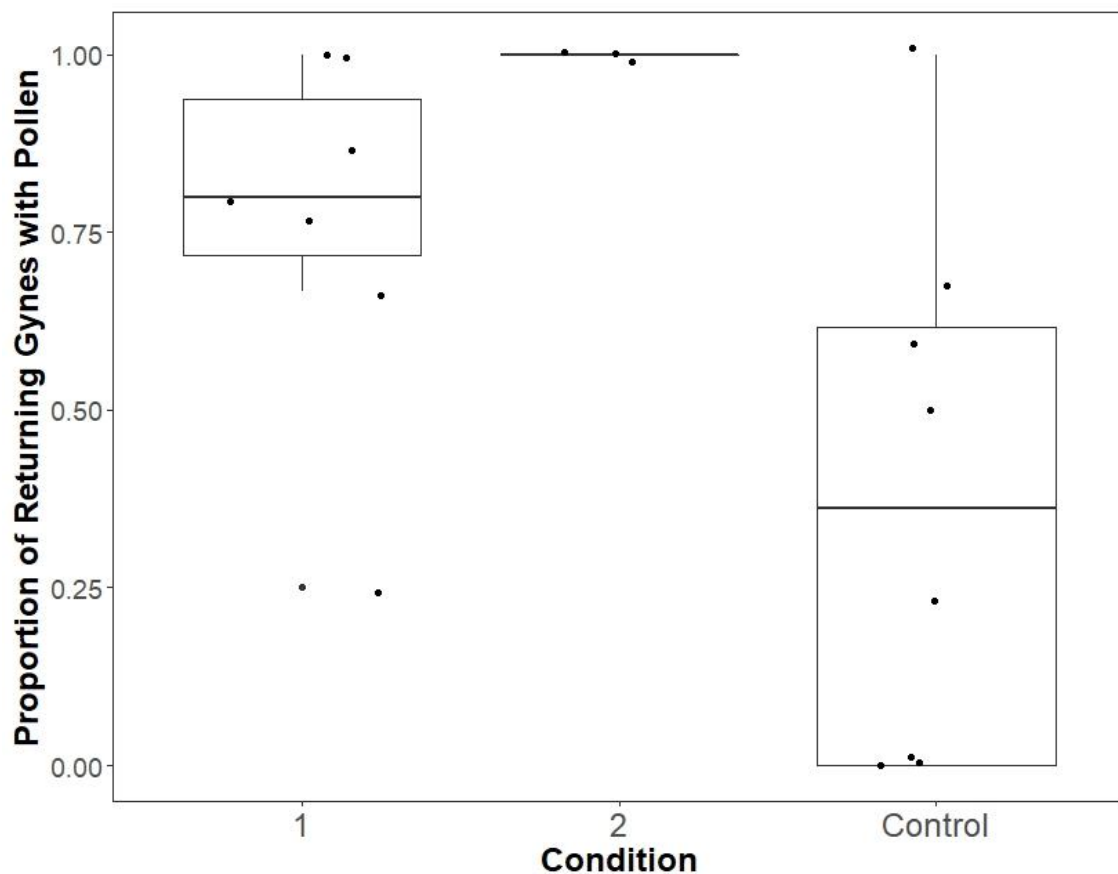
For Condition Two, where only incoming gynes were stripped of their pollen, the mean number of released gynes for each colony was 6 (range: 3-10; Appendix F: Table F.2). The mean number of returned gynes throughout the experimental testing days from each colony was 3.33 (range: 2-4; Appendix F: Table F.2). The mean proportion of gynes that returned from each colony was 0.62 (range: 0.40-0.80; Appendix F: Table F.2). All returning gynes returned with pollen (Appendix F: Table F.2). In the one colony that was measured following the experiment, there were gynes present that had not left the colony during the experimental period (Appendix F: Table F.2).

In the control, where no incoming bumblebees were stripped of their pollen, the mean number of released gynes for each colony was 5.35 (range: 0-12; Appendix F: Table F.2). The mean number of returned gynes throughout the experimental testing days from each colony was 4 (range: 0-12; Appendix F: Table F.2). The mean proportion of gynes that returned from each colony was 0.62 (range: 0-1; Appendix F: Table F.2). Of the total number of returned gynes, the mean proportion that returned with pollen was 0.37 (range: 0-0.67; Appendix F: Table F.2). In 50% of the colonies measured following the experiment (2/4), there were gynes present that had not left the colony during the experimental period (Appendix F: Table F.2).

A Fischer's exact test found an overall significant difference in the proportion of gynes that foraged for pollen before returning to their colonies across the different



experimental conditions ( $p=0.0004$ ; Figure 6.3). A post-hoc Fisher's exact test found a significant difference in the proportion of gynes that foraged for pollen before returning to their colonies between Condition One and the control ( $p=0.0024$ ; Figure 6.3). A post-hoc Fisher's exact test found a significant difference in the proportion of gynes that foraged for pollen before returning to their colonies between Condition Two and the control ( $p=0.0022$ ; Figure 6.3). A post-hoc Fisher's exact test did not find a significant difference in the proportion of gynes that foraged for pollen before returning to their colonies between Condition One and Condition Two ( $p=0.1862$ ; Figure 6.3).



**Figure 6.3** | Boxplot of the proportion of gynes that foraged for pollen in each colony and in each experimental condition: 1) pollen removed from returning workers and gynes, 2) pollen removed from returning gynes only, and 3) no pollen removed.  $N=95$  returning gynes from 18 separate colonies in total. A small value of random noise was added to each data point for plotting purposes to aid visual representation.

### 6.5.2 Confirming Caste Differentiation

The mean, standard deviation and range of the morphological traits measured for visually assigned gynes and workers are presented in Table 6.1. The traits of 221 gynes and 815 workers were measured across 16 colonies.

**Table 6.1. Mean, standard deviation (S.D.) and range of morphological traits measured for gynes and workers.**

Caste	Variables	Weight (mg)	Body length (mm)	Thorax width (mm)	Wing bud distance (mm)	Wing length (mm)	Wingspan (mm)
<b>Gynes</b>	Mean	531.32	22.61	6.87	6.43	15.23	30.57
	S.D.	199.37	2.44	0.64	0.83	1.38	2.76
	Range	87.60- 1060.50	16.46- 29.64	4.85- 8.49	3.42- 8.76	10.87- 18.54	21.74- 37.04
<b>Workers</b>	Mean	175.10	14.86	4.53	3.98	10.39	20.77
	S.D.	81.76	2.34	0.64	0.75	1.45	2.88
	Range	6.90- 650.00	3.98- 22.36	2.38- 8.80	1.88- 12.09	3.72- 17.86	7.44- 35.72

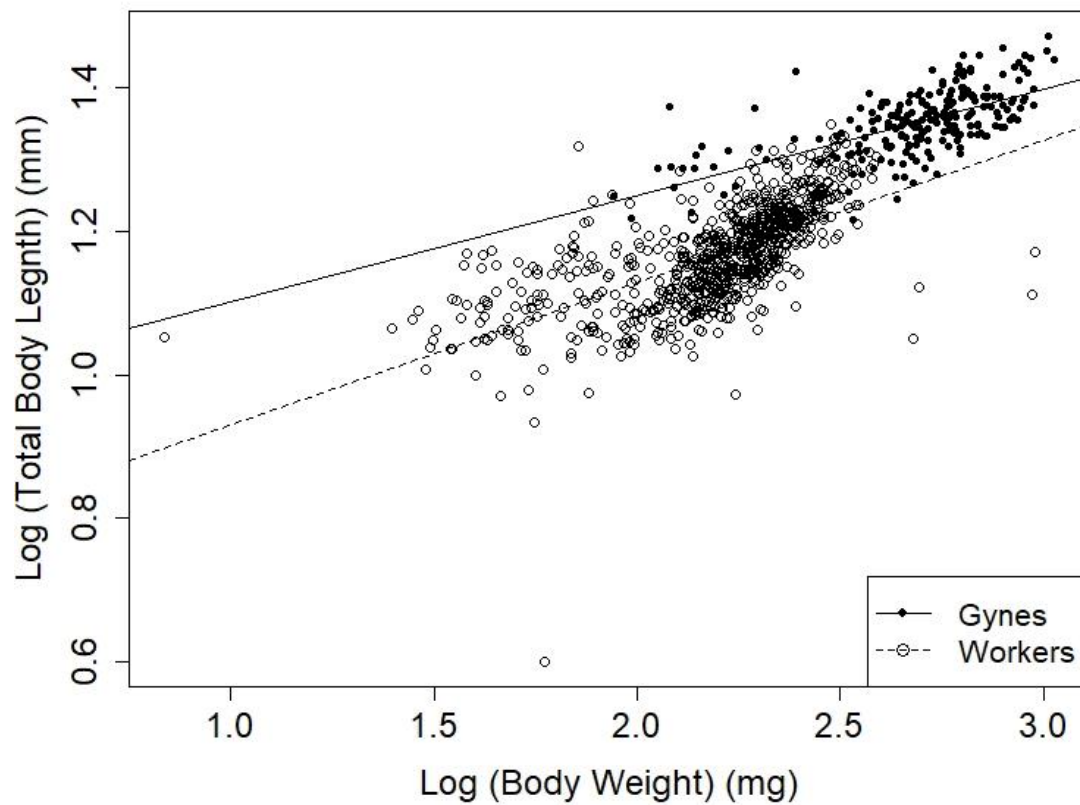
In order to determine whether a size difference did indeed exist between the visually assigned castes of workers and gynes, a variety of different morphological traits were compared using allometric scaling relationships.

For all the measured traits, the intercept for the slope for each caste was significantly different ("Caste" effect in Table 6.2). For two traits (body length and wing bud distance), there was also a significant interaction between caste and body weight, suggesting the allometric scaling relationship of these traits also varied between the assigned castes. The results together indicate that the gynes were significantly larger than the workers (Table 6.2; Figures 6.4.i-v). There was also a significant difference between the body weights of the two visually assigned castes ( $t(240.38)=25.98$ ,  $p<0.0001$ ; Figure 6.5). These results together confirm that the visual assignment of the castes of worker and gyne was justified.

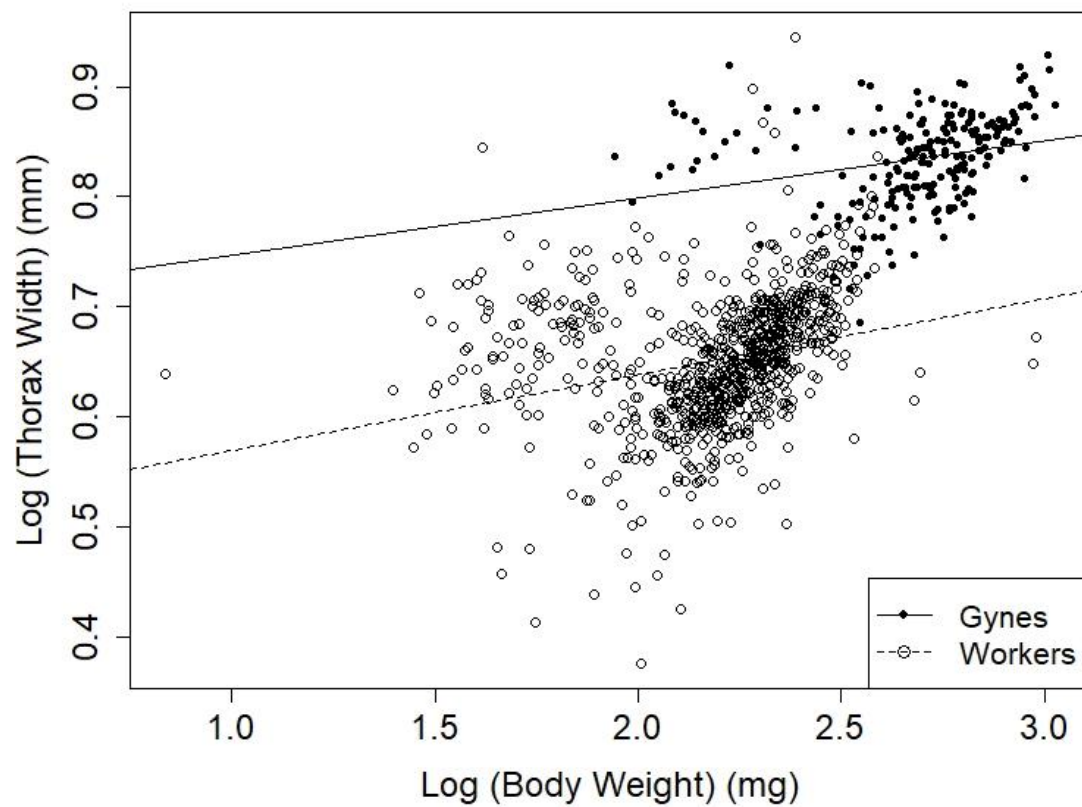
**Table 6.2. Model results from a linear model, ANCOVA, testing the relationship between the log a) body length, b) thorax width, c) wing bud distance, d) wing length and e) total wingspan and the log body weight for each caste. Variables, effect sizes  $\pm$  standard error, *t*-values and *p*-values from the fitted model. The ‘gyne’ caste was used as the reference level [“Intercept” values denote if the gyne intercept is significantly different from zero, and “Caste” values denote if the worker caste intercept differed significantly from the gyne caste intercept].**

<b>Variable(s)</b>	<b>Effect Size <math>\pm</math> Standard Error</b>	<b><i>t</i> Value</b>	<b><i>p</i> Value</b>
a) Body Length			
Intercept	0.95 $\pm$ 0.05	21.34	<0.0001
Body Weight	0.15 $\pm$ 0.01	8.98	< 0.0001
Caste	-0.22 $\pm$ 0.05	-4.61	<0.0001
Body Weight * Caste	0.05 $\pm$ 0.02	-2.70	0.007
b) Thorax Width			
Intercept	0.70 $\pm$ 0.05	14.34	<0.0001
Body Weight	0.05 $\pm$ 0.01	2.89	0.004
Caste	-0.20 $\pm$ 0.05	-3.76	0.0002
Body Weight * Caste	0.02 $\pm$ 0.02	0.88	0.38
c) Wing Bud Distance			
Intercept	0.70 $\pm$ 0.06	11.19	<0.0001
Body Weight	0.04 $\pm$ 0.02	1.60	0.11
Caste	-0.31 $\pm$ 0.07	-4.58	<0.0001
Body Weight * Caste	0.05 $\pm$ 0.03	2.05	0.04
d) Wing Length			
Intercept	0.96 $\pm$ 0.05	19.81	<0.0001
Body Weight	0.08 $\pm$ 0.02	4.56	<0.0001

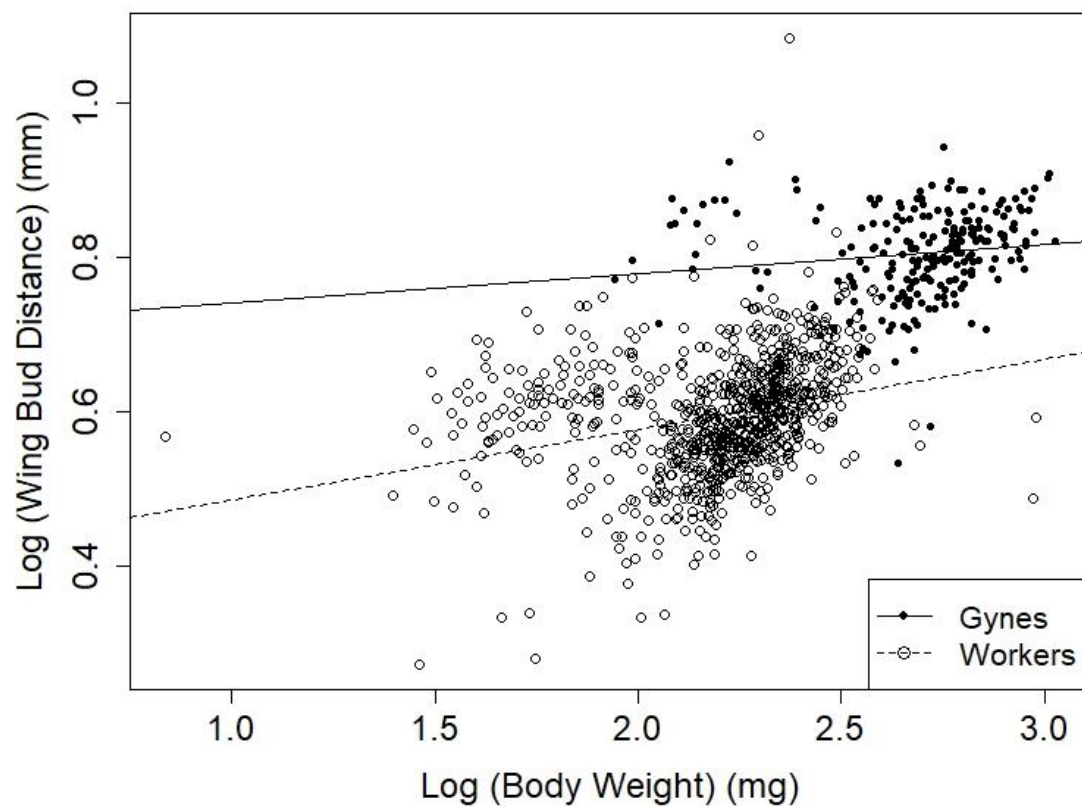
<b>Variable(s)</b>	<b>Effect Size <math>\pm</math> Standard Error</b>	<b>t Value</b>	<b>p Value</b>
Caste	-0.17 $\pm$ 0.05	-3.28	0.001
Body Weight *	0.02 $\pm$ 0.02	0.96	0.34
Caste			
e) Total			
Wingspan			
Intercept	1.26 $\pm$ 0.05	26.02	<0.0001
Body Weight	0.08 $\pm$ 0.02	4.56	<0.0001
Caste	-0.17 $\pm$ 0.05	-3.28	0.001
Body Weight *	0.02 $\pm$ 0.02	0.96	0.34
Caste			



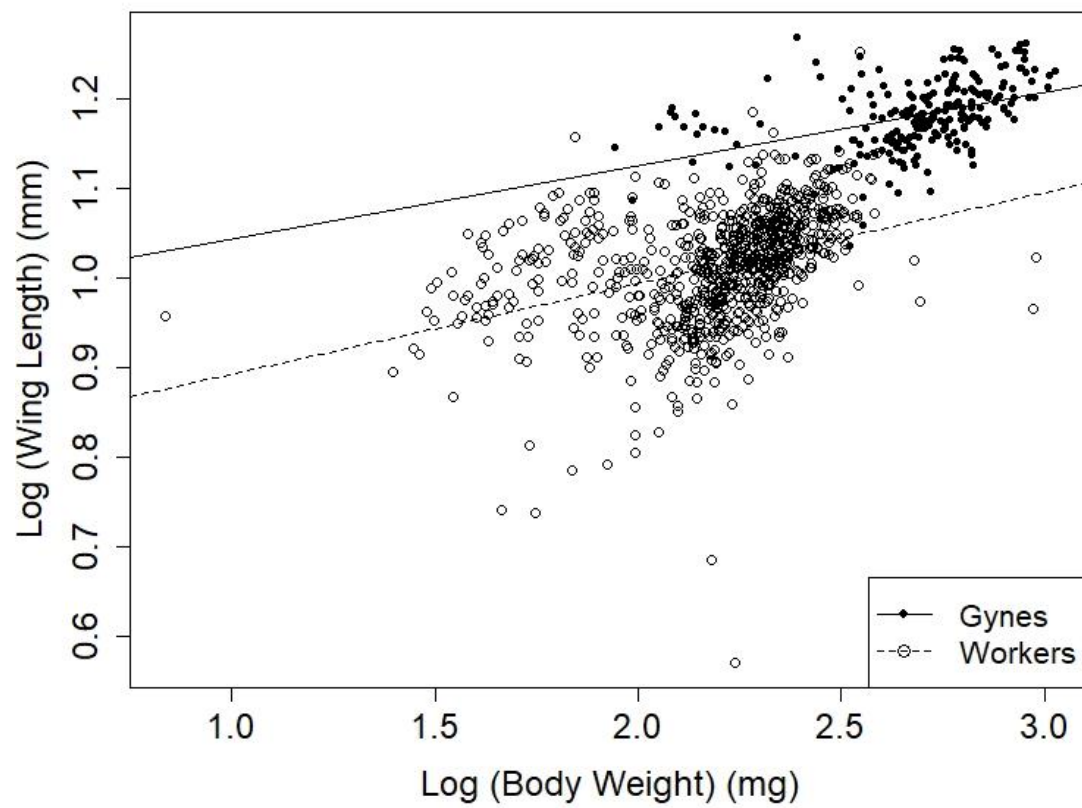
**Figure 6.4.i** | Allometric (log-log) plot of total body length (mm) plotted against body weight (mg).  $n=221$  gynes and  $n=815$  workers taken from 16 colonies.



**Figure 6.4.ii** | Allometric (log-log) plot of thorax width (mm) plotted against body weight (mg).  $n=221$  gynes and  $n=815$  workers taken from 16 colonies.

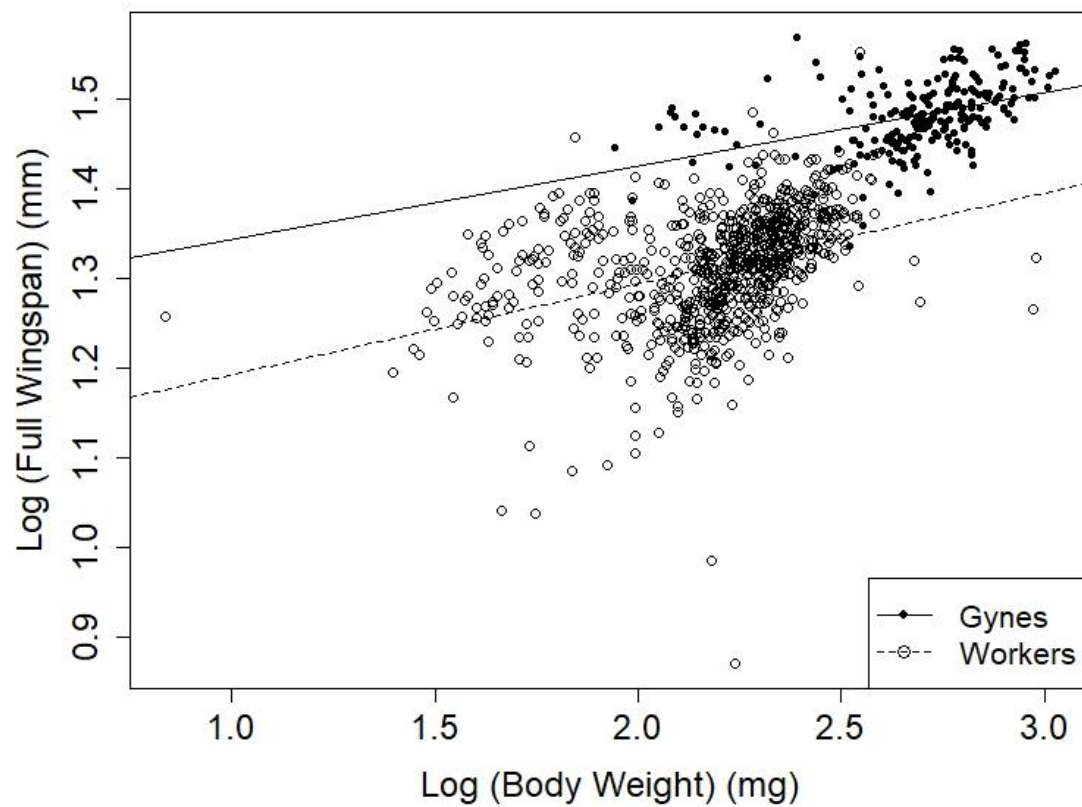


**Figure 6.4.iii** | Allometric (log-log) plot of wing bud distance (mm) plotted against body weight (mg).  $n=221$  gynes and  $n=815$  workers taken from 16 colonies.

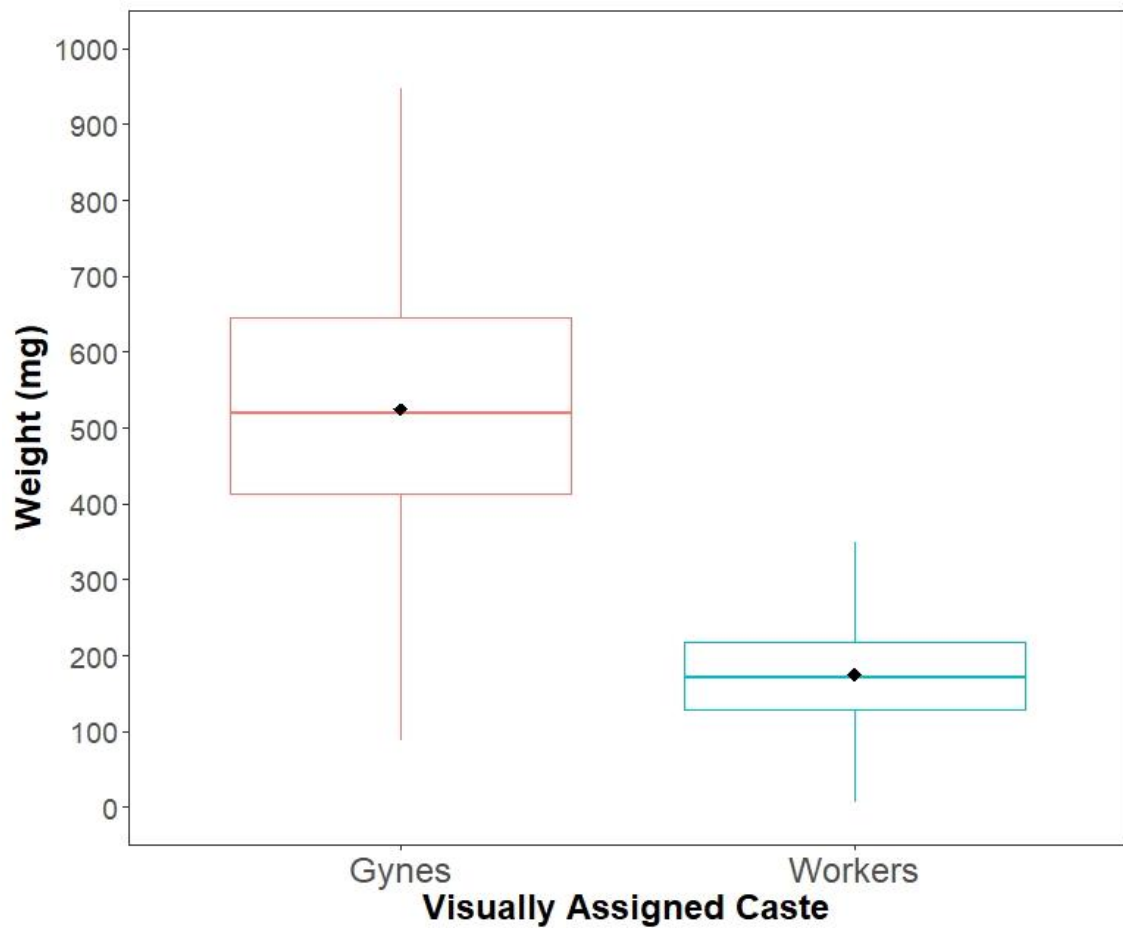


**Figure 6.4.iv** | Allometric (log-log) plot of wing length (mm) plotted against body weight (mg).  $n=221$  gynes and  $n=815$  workers taken from 16 colonies.





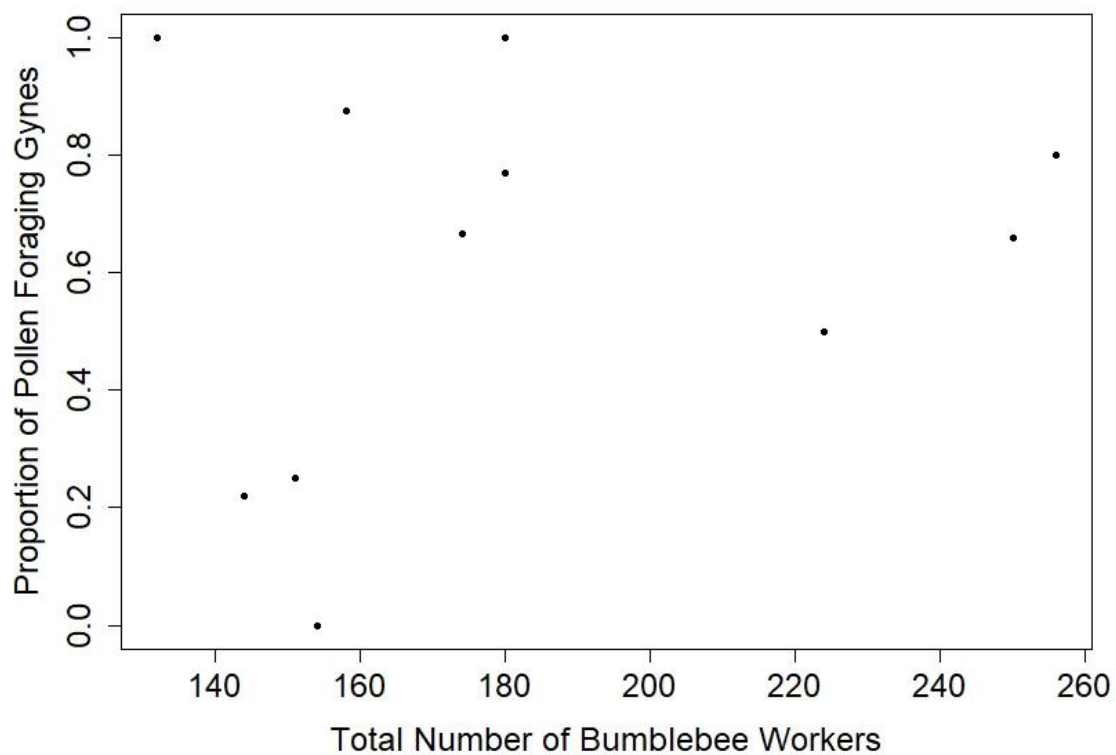
**Figure 6.4.v** | Allometric (log-log) plot of full wingspan (mm) plotted against body weight (mg).  $n=221$  gynes and  $n=815$  workers taken from 16 colonies.



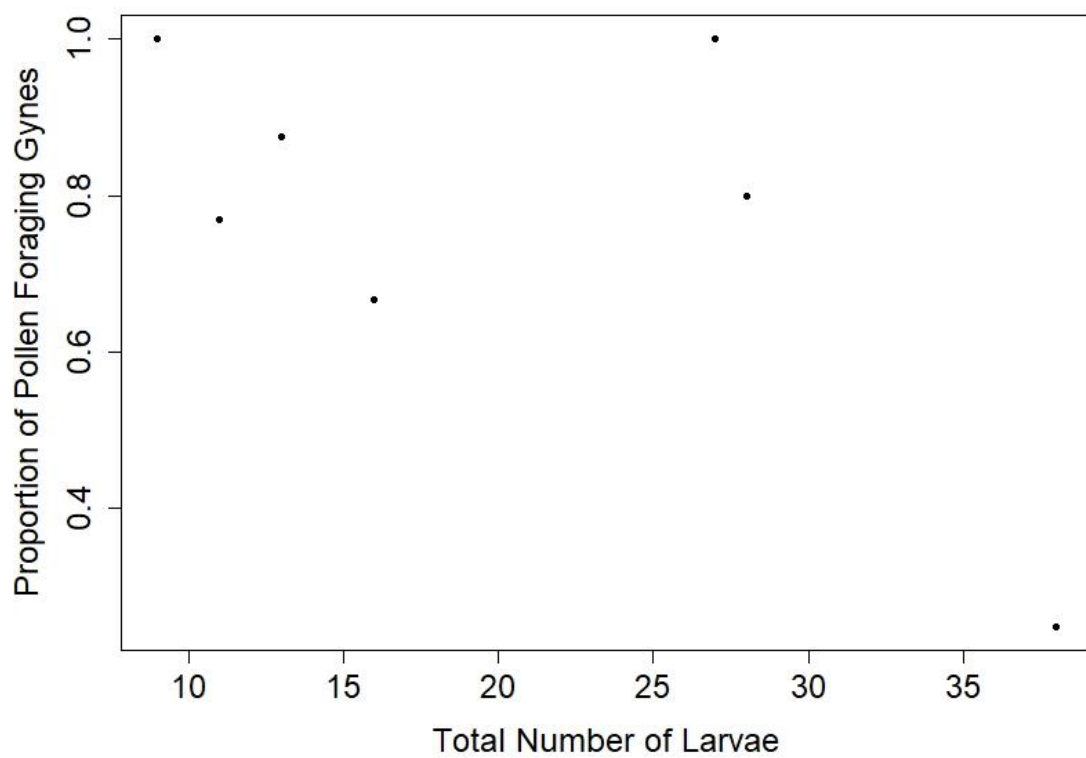
**Figure 6.5** | Box and whiskers plot of the total body weight (mg) of each potential caste.  $n=221$  gynes and  $n=815$  workers taken from 16 colonies.

### 6.5.3 Effect of Colony Condition on Proportion of Foraging Gynes

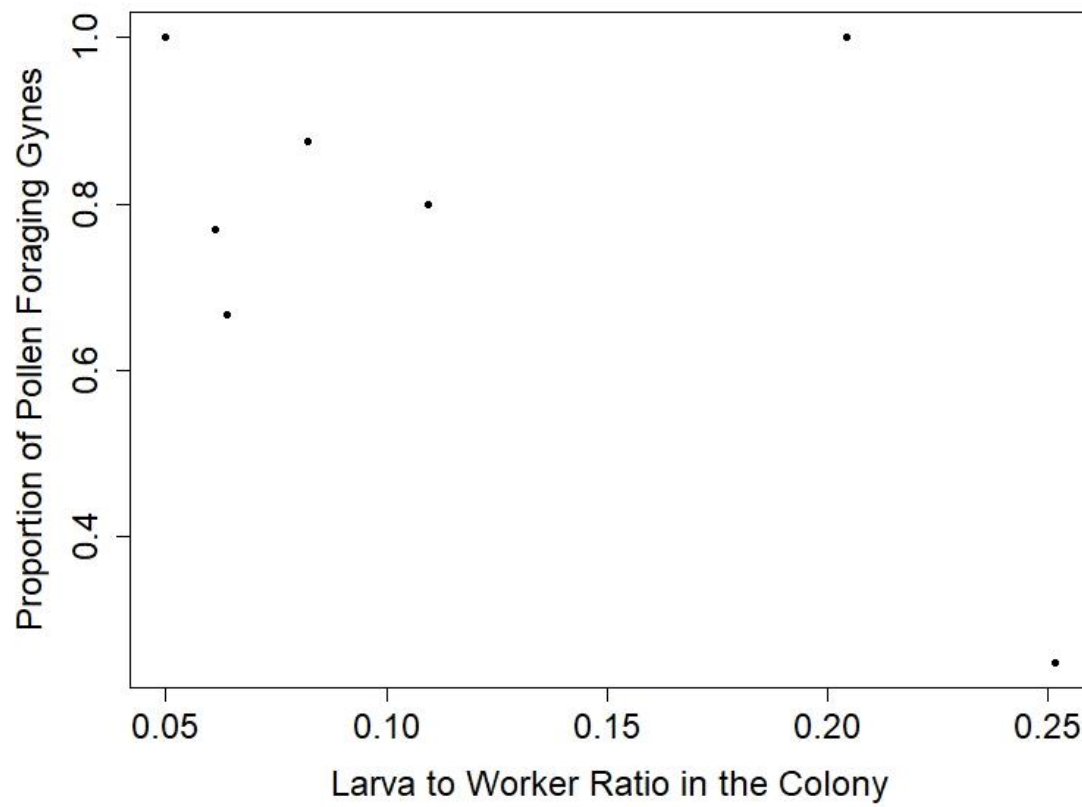
When comparing the state of each of the measured colonies, there was no correlation between the proportion of pollen foraging gynes and the total number of bumblebee workers found in the colony ( $r= 0.20$ ,  $n= 11$ ,  $p= 0.55$ ; Figure 6.6). There was no correlation between the proportion of pollen foraging gynes and the number of larvae in a colony ( $r=-0.62$ ,  $n=7$ ,  $p=0.14$ ; Figure 6.7). There was also no correlation between the proportion of pollen foraging gynes and the larva to worker ratio in a colony ( $r=-0.52$ ,  $n=7$ ,  $p=0.23$ ; Figure 6.8).



**Figure 6.6** | The proportion of pollen foraging gynes shown against the total number of bumblebee workers in their colony.  $N= 11$  colonies.



**Figure 6.7** | The proportion of pollen foraging gynes shown against the total number of larvae in their colony.  $N=7$  colonies.



**Figure 6.8** | The proportion of pollen foraging gynes shown against the larva to worker ratio in their colony.  $N=7$  colonies.

## 6.6 Discussion

In this experiment, the foraging behaviour of gynes in colonies subjected to different levels of pollen in their nest was investigated. For all the colonies tested, regardless of treatment, gynes were observed leaving the colony. Gynes were also observed returning to their colonies and, in all but three colonies, gynes returned with pollen loads in their corbiculae. As there was a statistically significant difference in the proportion of foraging gynes between Condition One and the control and Condition Two and the control (Figure 6.3), this suggests that the pollen availability during the experiment had a significant effect on the foraging behaviour observed.

During the experiment, gynes and workers were visually classified based on overall size differences. Following the completion of the experiment, the colonies were frozen and gynes and workers were weighed. A variety of different morphological traits were also measured. This was done in order to verify the ability of the experimenter to visually classify the castes during the experiment based on size. As a size bimodality between workers and gynes exists in *Bombus terrestris*, significant differences between the sizes of the visually classified bumblebees, especially for a variety of different traits, would lend support to the fact that the classification was correctly made. The visually classified bumblebees were found to differ significantly in size in all of the traits measured as well as in terms of weight (Figures 6.4.i-v and 6.5). In addition, the mean body lengths measured in this experiment fall within the range of those reported by Alford (1975): 20 to 23mm for gynes and 11 to 17 mm for workers.

Some overlap between the ranges of each measured trait were observed. This, however, is to be expected when individual traits are measured in isolation due to the inherent variation between individuals. Many dead bumblebees were collected within the margins of the nest box (R.Herascu, personal observation). Even though bumblebees were frozen and weighed at the end of the experiment, some of these bumblebees might have already been dead and decomposing prior to being frozen. This could have led them to be much lighter than bumblebees that had died upon freezing.

Gynes were observed returning with pollen in all experimental conditions and there was a significant difference found in the proportion of pollen foraging gynes

between the different experimental conditions (Figure 6.3). There was a significant difference in the proportion of gynes that foraged for pollen between Condition One and the control and between Condition Two and the control (Figure 6.3). There was no significant difference in the proportion of gynes that foraged for pollen between Condition One and Condition Two. This suggests that the foraging behaviour of gynes was influenced by the amount of pollen entering the colony throughout the testing days. Gynes were more likely to forage for pollen when the amount of pollen entering their colonies was restricted in some way. This suggests that gynes will forage and provision their maternal colonies when the pollen entering the colony does not meet the colony's nutritional needs. If the amount of pollen entering the colony was the only factor determining whether or not gynes forage for the colony, then it would be expected that the greatest proportion of foraging gynes would be found in Condition One, followed by Condition Two and finally by the control. This is because Condition One had the most restricted pollen flow, followed by Condition Two and finally the control, where the pollen entering the colony was not restricted in any way. This scenario, however, was not observed in this experiment. Rather, colonies in Condition Two had the greatest proportion of foraging gynes, followed by Condition One and finally the control. As there was no significant difference in the proportion of foraging gynes between Conditions One and Two, this suggests that, from a gyne's perspective, there was no significant difference in the amount of pollen entering the colony. In other words, the pollen that the workers were bringing back to the colonies in Condition Two may not have been enough to meet the colony's needs.

The fact that a greater proportion of gynes were observed foraging for pollen in Condition Two, even though workers in their colonies were allowed to provision the colonies with pollen, could be due to several reasons. It could be due to the fact that colonies in Condition Two had higher energy demands than colonies in Condition One. As such, even with the workers foraging, this was not enough to meet the needs of the colonies. A similar result could also occur if colonies in Condition One had greater pollen stores to fall back on than colonies in Condition Two. Even though all bumblebees were stripped of their pollen in Condition One, higher pollen stores in the colonies in this condition could have meant that not as many gynes needed to provision the colony to meet its energy needs. The high

proportion of gynes returning with foraged pollen in Condition Two could have also been due to the very small sample size in this condition. It is entirely possible that had more colonies been tested in this condition, a larger variation in the proportion of foraging gynes would have been observed. Future work could help to shed light on this result by establishing the pollen stores of each colony prior to testing and by testing a greater number of colonies in Condition Two.

In the control condition, some gynes were still observed foraging for pollen. This was the case even though the amount of pollen entering the colony in this condition was not restricted in any way. This may be due to the fact that the amount of pollen entering the colony was not sufficient to meet the colony's nutritional needs with only workers provisioning the colony. Future work could test this hypothesis by not restricting the pollen entering the colony in any way and supplementing the colonies pollen stores. This would allow us to establish whether there are any environmental conditions in which no gynes will forage and provision their colonies with pollen.

Previous observations have suggested that gynes only forage for their maternal colony when food stores are depleted and a large larva to worker ratio in the colony is reached. In a colony of *Bombus vosnesenskii*, Allen *et al.* (1978) observed that when the larva to worker ratio was 0.92, gynes did not forage for either pollen or nectar and only fed on the colony's food stores. Later in the colony life cycle, when the larva to worker ratio was 11.6, gynes were observed to be the primary providers of both pollen and nectar for the remaining brood of males. It is perhaps telling that in Allen *et al.*'s (1978) experiment the gynes were provisioning a brood that was entirely made up of males. By provisioning a brood of reproductives, potentially made up of both their nephews and their brothers, the gynes could have been increasing their overall inclusive fitness.

For the colonies in the current experiment where measurements were possible, there was no correlation found between the total number of workers in the colony and the proportion of foraging gynes (Figure 6.6), nor between the larva to worker ratio and the proportion of foraging gynes (Figure 6.8). The range in the larva to worker ratio in this experiment was also much lower than that observed by Allen *et al.* (1978) (0.05-0.25: Appendix F: Table F3). It should be stressed, however, that the number of workers used in this measurement were the total number of



workers found inside the colony upon being frozen, irrespective of whether they were dead or alive. As such, this is unlikely to be a true representation of the larva to worker ratio. The total number of workers found inside the colony, however, can give insights into how energy demanding the colony had previously been. There was also no correlation found between the total number of larvae inside the colony and the proportion of foraging gynes (Figure 6.7). As larva numbers in all colonies were low (range: 9-38: Appendix F; Table F3), future work is needed to fully investigate the relationship between a colony's state and the foraging behaviour of its gynes.

In Condition One and in the control condition, there was a large range observed in the proportion of gynes that foraged for pollen within each colony. This suggests that the experimental conditions did not have a uniform effect on gyne pollen foraging. Reasons other than the amount of pollen that was entering the colony throughout the experimental testing days may have been behind the behaviour observed. Such reasons may include physiological differences between the gynes themselves. In this experiment, the amount of pollen that was entering the colonies was only manipulated for the duration of the experiment. This amounted to a period of four days. Four days represents a relatively small proportion of the overall lifespan of a bumblebee colony. As such, it is possible that behavioural differences between the gynes were a result of events which occurred during their development, and before this experiment took place. During the development of a bumblebee colony, female larvae that are destined to be gynes spend a longer period of time as larval instars. They thus feed on larger amounts of pollen than female workers (Ribeiro *et al.*, 1999). As a result, it is entirely possible that historic deficiencies in a colony's pollen stores, particularly during periods of gyne larval development, may have negative consequences on gyne physiology. Specifically, gynes which experienced nutritional deficiencies during their larval development may have reduced fat bodies as adults.

The foraging behaviour of gynes which have reduced fat bodies seems to be different to that of gynes which have undergone normal development. Dissected gynes which have been observed foraging late in the season have been found to have reduced fat bodies (Alford, 1975) and to be frequently diseased (Skou *et al.*, 1963). Furthermore, Plath (1934) observed that gynes which foraged and provisioned their maternal colony had emerged from pupae at the very end of the

colony's life cycle. The end of a colony's life cycle is marked by a reduction in the number of workers. Such a reduction would result in a decreased amount of pollen and nectar entering the colony. As such, gynes which emerge at this time are likely to have experienced nutritional shortages during their larval development. It has also been observed that *Bombus terrestris* gynes with a wet weight of below 0.60 g prior to entering hibernation will not survive (Beekman, van Stratum & Lingeman, 1998) and that gynes with reduced fat bodies may not enter diapause at all (Fliszkiewicz & Wilkaniec, 2007). Nutritionally deficient gynes may thus attempt to increase their fat bodies above a certain threshold in order to increase their chances of surviving hibernation. They may do so while they are still in the maternal colony. This would allow them to take advantage of the benefits that the maternal colony offers, including shelter and a decreased risk of predation. The present experiment cannot tease apart whether some of the gynes that returned to their maternal colony with pollen, especially those in the control condition, were responding to the present nutritional needs of their colonies or whether they represented a particular subset of gynes, which had the abnormal physiological trait of reduced fat bodies. It might be possible to make this distinction in the future by investigating the feeding behaviour of gynes inside their colonies.

Rather than simply foraging for and consuming pollen during their foraging trips, returning to the maternal colony between these trips may confer certain benefits on gynes. Provisioning their maternal colony, which has the potential to benefit other gynes and workers, will ultimately increase the inclusive fitness of gynes (Ratnieks & Helanterä, 2009). Resting between foraging trips and potentially overnighing in the maternal colony also has the advantage of providing shelter, a thermoregulated environment and a decreased risk of predation. Due to the methodology used in this experiment, the sugar solution that accompanied the colonies from the manufacturer was also left inside each colony. As such, in this experiment, gynes would also have the added advantage of having access to a sugar source *ad libitum* inside the nest. The advantages that the colony may provide is also exemplified by the fact that Cumber (in Alford, 1975) found that up to 40 % of gynes present in a maternal colony were already fertilised. This suggests that gynes may return to their maternal colony under a variety of different circumstances.

As laboratory reared colonies, the fact that a subset of the colony's gynes may have been nutritionally deficient could point to the disadvantages of the widespread use of honeybee pollen as the protein source in bumblebee husbandry. Honeybee collected pollen is widely used both to feed bumblebee colonies in the laboratory (Dicks, Showler & Sutherland, 2010) and as a reward in many behavioural experiments (eg: Hagbery & Nieh, 2012; Konzmann & Lunau, 2014). Russel *et al.* (2017) point out that honeybee collected pollen is often adulterated with debris and up to 60% sugars (Russell & Papaj, 2016) and, as such, does not represent a realistic substitute for pollen collected by wild bumblebees. Furthermore, gynes produced from laboratory colonies that were fed dried and frozen honeybee pollen, rather than fresh and frozen honeybee pollen, were smaller, had lower biomass, had higher mortality and produced smaller colonies themselves (Ribeiro, Duchateau & Velthuis, 1996). Laboratory reared colonies are also usually fed a sugar solution rather than provided with flower nectar. Unlike sugar solution, flower nectar also contains amino acids, lipids, minerals and secondary plant compounds (Vaudo *et al.*, 2015) which are important for bee nutrition (Nicolson, 2011). Future work would help elucidate whether changes or supplementation in the diet of laboratory reared bumblebees would aid both workers and gynes and whether nutritional deficiencies have any impact on the conclusions drawn from current bumblebee research in which such bees are used. In the wild, if pollen deficiencies lead to an increase in the proportion of foraging gynes, then this could ultimately lead to negative consequences for both colony survival and propagation. As the proportion of foraging gynes increases, so too does the proportion of a colony's reproductives that are at an increased risk of predation prior to leaving their maternal colony. Such a trend could lead to an overall decrease in the number of subsequent colonies in the wild and points to the critical importance of adequate pollen resources throughout a colony's life cycle within the natural environment.

#### 6.6.1 Experimental Limitations and Improvements

Due to limitations in the testing schedule, the results of this experiment should be interpreted with caution. Although two different experimental sites were used, an unequal number of colonies and treatments were tested at each site. Although colonies undergoing the same treatment were run simultaneously, different treatments were not run in parallel. As a result, differences in the availability of

pollen in the landscape, due to both location and temporal differences, may have confounded the results. Future replication of this experiment should ensure that all treatments and controls are run in parallel and at the same experimental site. This will help to minimise environmental differences between treatments. In this experiment, there was no correlation found between the state of the colony, in terms of the larva to worker ratio, and the proportion of pollen foraging gynes. The state of the colony, however, could only be measured in seven of the 18 colonies tested. Although all the colonies were purchased from the same supplier and kept in similar laboratory conditions, this was not actively monitored as part of the experiment and differences in both nutritional state and brood were not accounted for prior to testing. Future replication of this experiment should closely monitor the state of the colony both before and after testing and strive to have colonies at similar nutritional states when testing begins. Visual inspection of the relationship between the larva to worker ratio and the proportion of foraging gynes also suggests a potential correlation between the two (Figure 6.8) and increasing the number of colonies used would help elucidate whether such a correlation exists.

In this experiment, both the amount of pollen that was entering a colony and the foraging behaviour of gynes were only tested for a period of four days. It is entirely possible that this was not enough time for gynes to respond to any changes in the perceived pollen state of the colony. As such, future work should explore the longer-term relationship that exists between a colony's nutritional state and the foraging behaviour of its gynes. Stripping the corbiculae of incoming foragers is one way of controlling the amount of pollen that enters a colony. Another, perhaps less intrusive method, would be to directly manipulate the amount of pollen stored within a colony. Future work could also explore this possibility.

## **6.7 Conclusion**

In this experiment, the foraging behaviour of bumblebee gynes prior to their mating and hibernation was investigated by manipulating the amount of pollen that entered the colony. In all three experimental conditions, a proportion of gynes were observed returning to their maternal colonies. Furthermore, in all three experimental conditions, gynes were also observed returning to their maternal colonies with foraged pollen. There was a significant difference found between

the proportion of foraging gynes in Condition One and the control and Condition Two and the control. There was no significant difference found between the proportion of foraging gynes in Condition One and Condition Two. There was no correlation found between the number of larvae in a colony and the proportion of foraging gynes. The results suggest that gynes can respond to pollen shortages in their colonies by foraging and provisioning the colony themselves. The fact that some gynes were observed foraging for pollen in the control condition may also suggest that some foraging gynes represent a specific subgroup which may have experienced historic pollen deficiencies. This points to the importance of adequate pollen resources throughout a colony's life cycle, both in laboratory reared bumblebees as well as those living in the wild.

## Chapter Seven

### General Discussion and Conclusions

The experiments presented in this thesis set out to explore the effect that the physical environment and individuals' experience have on bumblebee navigation and foraging behaviour. This concluding chapter will summarise the key findings of the previous five data chapters. Where appropriate, the results presented and the conclusions drawn will also be discussed in the context of practical applications for landscape management practices. The integration of findings will be discussed, as well as experimental limitations. Directions for future research, both for the experiments presented and for the field of bumblebee research overall, will be suggested.

#### 7.1 Key Findings

Focusing on a landscape feature, the experiments presented in Chapter Two investigated the effect of a hedgerow on the flight paths and foraging behaviour of *Bombus terrestris* workers in environments with mass flowering crops. In this experiment, the hedgerow did not significantly influence the flight paths of naïve bumblebees from colonies that were placed alongside it. Furthermore, the hedgerow did not have a significant effect on the pollen that bumblebees foraged for, both on their first and subsequent flights. This suggests that a single landscape feature, such as a hedgerow, does not seem to guide or restrict the navigation or foraging behaviour of *Bombus terrestris* workers in these particular rural settings.

Although the hedgerow in the experiment presented in Chapter Two did not seem to directly influence the flight paths and foraging choices of bumblebee workers, it may still have played a role in their navigation. It is highly likely that bumblebees were encoding the spatial relationship that exists between the hedgerow next to their nest and their nest during their orientation/learning flights. This hedgerow would have then aided bumblebees to pinpoint the location of their nest on their return flight. Bumblebees were also observed foraging for pollen from the flowering hedgerows despite having a mass flowering crop in the vicinity of their nests. Specifically, at Site Three, the majority of bumblebees foraged predominantly from the pollen found in the hedgerow plants. This result highlights

the importance of flowering hedgerows as a foraging resource for bumblebees. Unlike mass flowering crops, uncropped areas of farmland such as hedgerows and field margins can provide flowers throughout a bumblebee's life cycle (Corbet, 2000). Colony and gyne survival are also significantly increased in the presence of high-value forage, such as flowering hedgerows, if this habitat is found within 250-1000 m of a bumblebee colony (Carvell *et al.*, 2017). Foraging bumblebees and other insect pollinators will only derive maximum benefit from flowering hedgerows, however, if these resources are properly protected. From a landscape management perspective, the results presented in Chapter Two support hedgerow management guidelines which restrict the frequency of cutting. Taken together, the results presented in Chapter Two suggest that hedgerows can be used by bumblebees in different ways: as a local landmark in the context of navigation and as a valuable food source in the context of foraging.

In the experiments presented in Chapter Three, the role of experience was investigated by focusing on the pollen foraging behaviour of *Bombus terrestris* on their first five flights. To the extent of my knowledge, this is the largest experiment to date which has investigated this behaviour. Experience, in the form of the number of flights that individual bumblebees made, did not have a significant effect on the duration of their flights. Experience was found to have an effect on the amount of pollen foraged during the first five flights – with more experienced bumblebees collecting more pollen on a foraging trip. Throughout the experiments presented in Chapter Three, the behaviour of individual bumblebees was highly variable. Individual bumblebees varied in the duration of their flights as well as in the amount of pollen foraged, suggesting that workers adopted a variety of different foraging strategies. By adopting different foraging strategies at the level of the individual bumblebee, a colony can successfully exploit a variety of different landscapes, made up of different spatial arrangements and foraging resources.

Furthermore, in both the experiments outlined in Part A and Part B of Chapter Three, only a minority of bumblebees completed five flights outside their colonies throughout the experimental testing period. This result suggests that some foragers contribute disproportionately to the colony's foraging effort. Russel *et al.* (2017) also found that a small number of the bumblebee workers who forage were responsible for the majority of the colony's foraging trips. In their study, the

mean daily foraging flights varied nearly 40-fold among foragers and half of the colony's mean number of daily flights were performed by only 17.3% of foragers. Although it is well documented that the worker caste in a colony divides itself between bumblebees that forage and those that perform colony tasks; and that this particular division of labour is largely based on body size (Brian, 1954; Goulson, 2010); the results also suggest that a division is present within the foragers as well. Interestingly, the division among foragers in terms of the number of flights taken and their labour output seems to follow Pareto's principle. More than a century ago, Pareto observed that approximately 80% of the wealth in Italy was owned by 20% of the population (Pareto, 1897). Pareto's principle can be seen more generally that 70%, 80%, 90%, etc., of the effects can be due to 30%, 20%, 10%, etc., of the causes, respectively (Viswanathan *et al.*, 2011). This principle seems to fit with the natural world more widely. Pareto distributions have been found to best model a variety of natural phenomena. These include the intensity of 'starquakes' (when the crust of a neutron star undergoes a sudden adjustment; Garcia-Pelayo & Morley, 1993), atmospheric flow dynamics (Joshi & Selvam, 1999) and earthquake dynamics (Feder & Feder, 1991). In ecology, Pareto distributions have also accounted for the measured value of patches of vegetation (Hastings *et al.*, 1982), the biomass to size distributions in aquatic organisms (Vidando *et al.*, 1997), phytoplankton growth processes (Seuront & Mitchell, 2008) as well as ecosystem dynamics more broadly (Bak, Chen & Creutz, 1989). A Pareto distribution has even been found to best describe the number of birds observed in a long-term survey: a relatively small percentage of species accounted for a large percentage of the total observed bird population (Rispoli, *et al.*, 2014). In the experiment presented in Chapter Three, within the foragers of the colony, only a minority of bumblebees were found to contribute to the majority of the colony's foraging effort. The mechanisms behind the division of labour in a bumblebee colony and the factors which may govern it, however, are unknown. This may provide scope for future research.

In the experiment presented in Chapter Four, the homing success of bumblebee workers in urban and rural environments was investigated. Across all experiment sites, the proportion of released bumblebees that returned to their colony significantly decreased as the release distance increased. There was also a significant difference in the proportion of bumblebees that returned to their colony



between the two environment types. Bumblebees in the rural environments were significantly more likely to return to their colony and do so within the same day when compared to those in the urban environments. Due to the differences in the distribution of foraging resources around the colonies in each environment, it is likely that a larger proportion of bumblebees had to forage further away from their colony in the rural environments compared to the urban environments.

Although the differences in the distribution of resources around the colonies in each environment type can account for the results presented in Chapter Four, another explanation is also possible as to why bumblebees were more successful at homing in rural environments, as compared to urban, environments. Using their image matching systems, released bumblebees would have matched the views of their release site with memories of the surrounding environment. Whether or not a match occurs also depends on what aspects of the surrounding environment were first memorised. As previously discussed, during their orientation/learning flights at the nest, bumblebees encode the objects surrounding the nest and their spatial configuration with regards to the nest. These objects can then be used as local landmarks, allowing the bumblebee to pinpoint the location of their nest within the surrounding environment. Local landmarks are useful for finding exact locations over small scales. When travelling over large distances, however, the most useful guiding image would contain distant objects which remain relatively unchanged visually, when viewed from various local viewpoints.

In any landscape, the most distant and easiest to detect feature for insects is the skyline: the panoramic silhouette of terrestrial objects against the sky (Möller, 2002; Differt & Möller, 2015). Ants and honeybees have been shown to use the skyline panorama for navigation (Fukushi, 2001; Graham & Cheng, 2009; Town et al., 2017). If bumblebees also use the skyline panorama when navigating over large distances, then released bumblebees in the experiment presented in Chapter Four could have used this to guide them back to their colony. The two environment types used in the experiment presented in Chapter Four differed in the amount of large, nearby and visually obstructing structures that they contained. The urban environments, with higher clustering of man-made structures, had a much denser skyline panorama, which closely surrounded the bumblebees upon release. Due to a bumblebee's close proximity to this dense

skyline panorama, the appearance of the urban panoramas would have been highly contingent upon its current position within the urban landscapes. In other words, the bumblebee's visual perspective of the skyline panorama is relative to its position in that landscape and how close it is to those objects that make up the skyline panorama. The closer the bumblebee is to those objects, the less static that skyline panorama is when moving through that landscape. In this way, it is possible that the navigation of bumblebees in urban environments may have been impeded because they were unable to rely on a relatively stable panorama necessary for panoramic image matching. In order to gain a more navigationally useful panoramic image, bumblebees in the urban environments may have needed to fly above urban structures to glimpse a distant, and relatively more stable, horizon. If bumblebees do not normally fly at such heights, it is possible that such flying is very energetically costly and cannot be maintained for long periods of time. The skyline panorama in the rural environments was comparatively unobscured by large objects and was relatively distant when compared to urban environments. In contrast to urban environments, therefore, the skyline panorama would have been more stable and less contingent on the bumblebees' position within the rural environments. As such, rural environments would prove easier for bumblebees to navigate when compared to urban environments.

The experiment in Chapter Four also suggests that the habitual range of bumblebee extends to somewhere between 1000 m – 2500 m, at least in their early foraging career. This specific result can provide guidelines for landscape management initiatives which aim to re-link natural habitats in an effort to combat habitat fragmentation (see Chapter One: Section 1.1: Agricultural Intensification and Habitat Fragmentation). As a section of this network has been planned for the area in which the rural sites were located (the South West of England), it is hoped that these results can be directly implemented as a guideline of the maximum distance that the planned foraging environments should be placed.

In the experiment presented in Chapter Five, the effect of experience on homing ability was investigated. In this experiment, the amount of experience that bumblebees had prior to artificial displacement and release was manipulated. Bumblebees were able to take either one, two or five flights prior to release. As was the case in the experiment presented in Chapter Four, a homing paradigm

was used as a proxy for the information gathering and exploration that occurs during the first five flights. To the extent of my knowledge, this is the first experiment in which the homing paradigm was used to investigate bumblebees of differing and known experience. The number of previous flights taken did not have a significant effect on the proportion of bumblebees that returned from each release distance. Bumblebees released from 300 m, however, were more likely to return to their colony than bumblebees released from 1000 m. Both release distance and amount of experience had a significant effect on homing duration. Bumblebees released from 300 m were faster to return to their colonies than those released from 1000 m, as were more experienced bumblebees. Release distance and amount of experience also had a significant effect on whether bumblebees stayed out overnight before returning to their colony. Bumblebees released from 1000 m were more likely to stay out overnight before returning to their colony than those released from 300 m, as were bumblebees with less experience. Furthermore, release distance and amount of experience had a significant effect on whether bumblebees foraged for pollen before returning to their colony. Bumblebees released from 300 m were more likely to forage for pollen before returning to their colony than those released from 1000 m, as were bumblebees with more experience.

Before being artificially displaced and released, bumblebees were captured at the colony as they were preparing to leave it. Their primary motivation at that point would have been to forage for the colony. If this primary motivation had remained the same following artificial displacement and release, then it might be expected that returning bumblebees would have foraged before returning to the colony. But if a bumblebee found itself to be lost upon release, then its primary motivation would presumably be to return to the colony as soon as possible given its increased risk of exhaustion, predation and resultant stress. As such, it might be more reasonable to suggest that a bumblebee will only forage for the colony before returning to its nest if it is familiar with its location upon release. In such scenarios, its capture and transport by the experimenter could be perceived as a temporary anomaly during its foraging flight. It is possible, however, that such an explanation assigns concepts to bumblebees that they simply do not possess, such as a concept of being 'lost'. A more parsimonious explanation for this observed behaviour is that bumblebees are responding to a stressor. A

bumblebee that has been stressed during its capture, artificial displacement and release may behave differently to a bumblebee that has not been stressed during the experimental procedure. Even if the experimental procedure did not stress a captured bumblebee, the stressor may come through the form of being released into a novel environment. As such, stress may be the critical factor which changes the overall motivations of a bumblebee. In this experiment, a stressed bumblebee may have changed its primary motivation from foraging for the colony to returning to the colony as soon as possible. A non-stressed bumblebee may keep its primary motivation to forage for the colony upon release. The fact that experience significantly increased the likelihood that released bumblebees foraged for pollen before returning may simply be due to the fact that experienced bumblebees are less likely to get stressed than less experienced ones. Experienced bumblebees may be less likely to be stressed by their capture and be more likely to be familiar with their release location.

The experiment presented in Chapter Six focused on the foraging behaviour of *Bombus terrestris* gynes. Although gynes had been observed returning to their maternal colony with pollen in their corbiculae by several authors (Chapter Six: Section 6.2: Introduction), the exact cause of this behaviour remained unknown. To the extent of my knowledge, the experiment presented in Chapter Six is the first that aimed to investigate the underlying causes of this behaviour. The results of this experiment suggest that gynes respond to the amount of pollen that enters their colony and that they are more likely to forage for pollen when the amount of pollen entering their colony is limited. Interestingly, gyne foraging was observed in all experimental conditions, including the control. This result could suggest that even in the control condition, where the amount of pollen entering the colony was not manipulated, the amount of pollen entering the colony was still not meeting the colony's energetic needs. This could have then prompted certain gynes to forage for their colony. This result could also suggest that factors other than the amount of pollen entering the colony during the experiment induced certain gynes to provision their maternal colony. For example, the gynes that provisioned their colony in the control condition could represent a particular subset of gynes. This subset of gynes were most likely responding to historic nutritional deficiencies which had left them with reduced fat bodies.

The abundance of nutritionally appropriate foraging resources has been found to directly affect bumblebee colony growth and reproductive output (Persson & Smith, 2011; Carvell *et al.*, 2017; Vaidya, Fisher & Vandermeer, 2018; Vaudo *et al.*, 2018). As such, in environments which lack floral resources throughout the entire life cycle of a colony, gynes could be particularly susceptible. A lack of floral resources could increase the likelihood that gynes will forage for their maternal colony to increase the amount of pollen entering the colony. This will expose them to a higher risk of predation before they leave the colony to mate and hibernate. A lack of floral resources could also expose developing gynes to nutritional deficiencies and nutritionally deficient gynes are less likely to survive hibernation (Beekman, van Stratum & Lingeman, 1998; Fliszkiewicz & Wilkaniec, 2007). A high proportion of either foraging gynes or nutritionally deficient gynes in an environment could result in an overall decrease in the number of new bumblebee colonies that are established.

The experiment presented in Chapter Six focused on the foraging behaviour of bumblebee gynes. Landscape management initiatives aiming to preserve or even extend the distribution of bumblebee populations must consider the foraging and distribution behaviour of the colony's reproductive caste. As previously discussed, a decrease in the number of foraging gynes or an increase in the number of gynes that have adequate fat stores required to survive hibernation can lead to an overall increase in the number of subsequent colonies within a landscape. Ensuring that adequate food sources are available throughout a colony's lifetime may mitigate against the effects of foraging gynes or nutritional deficiencies in gynes. Furthermore, it is the gynes and, to a lesser extent, the males, that form the reproductive caste which directly influence the location of future colonies. It is only by gaining a detailed understanding of the effects that the physical environment has on the navigation and foraging behaviour of all the different castes of a bumblebee colony (workers and reproductives) that an effective conservation strategy can be achieved.

## **7.2 Integration of Findings**

The results of the experiments presented in this thesis advance our current knowledge of the effects that landscape structure and experience have on bumblebee navigation and foraging behaviour.

### 7.2.1 The Effects of Landscape Structures on Bumblebee Flights

The research presented in this thesis indicates that structural aspects of the landscape have some influence on bumblebee behaviour (Chapter Two and Chapter Four). These include both natural and man-made structures. In terms of natural structures, it was previously known that wild bumblebees were more likely to fly parallel to hedgerows the closer they were to them (Cranmer, McCollin & Ollerton, 2012). In our experiment (Chapter Two), hedgerows were not found to significantly influence the flight paths and foraging choices of naïve bumblebees. Coupled with our previous knowledge of how hedgerows affect wild bumblebees mid-flight, this suggests that bumblebees may respond to hedgerows in different ways depending on what stage of their flight they are in when they encounter them. This may also be the case for landscape structures more broadly.

Moving from a specific landscape structure to a broader landscape ‘type’, specific urban landscape structures had been previously investigated by Bhattacharya, Primack & Gerwein (2003), who studied the effects that roads and railways have on bumblebee flight and foraging behaviour. We compared the entire urban environment with the rural environment (Chapter Four). Our results contribute to our knowledge of bumblebee urban ecology by suggesting that the urban environment may prove challenging for bumblebees to navigate in. The results also suggest that the role of landscape structures might be modulated by factors external to the bumblebees, such as the physical characteristics of the structures themselves, as well as internal factors, such as the flight stage or experience level of the bumblebee.

### 7.2.2 Acquisition of Knowledge with Flight Experience

We have also gained insights into the knowledge acquisition that occurs during the first flights of bumblebees (Chapter Three & Chapter Five). Specifically, this includes what information is focused upon during these first flights outside the colony and how quickly this information is gained. The results suggest that during the first five flights of a bumblebee, foraging and navigational skills are acquired and improved upon. The results also suggest that the area around the colony is explored in a progressive manner. This is also evidenced in Chapter Four, where bumblebees were given five days of experience in their environment prior to release. Even after five days of experience, the proportion of returns was not

uniform across all release distances. Bumblebees were more likely to return to their colony the closer they were released from it, suggesting that not all bumblebees had explored up to 2500 m from their colony in every direction. Furthermore, the results also shed light on the speed at which navigation and foraging knowledge is gained. The average lifespan of a bumblebee worker is between two and three weeks (Rodd, Plowright & Owen, 1980). During that time, various studies have found that individual bumblebees take on average anything between 1.73 to 13 flights a day (Spaethe & Weidenmüller, 2002; Woodgate *et al.*, 2016; 2017). Based on these observations, many bumblebees will complete their first five flights during their first day of leaving the nest. If the bulk of a bumblebee's navigation and foraging skills are acquired within their first five flights outside their colony then this suggests that these skills are acquired at a very fast rate. The results of the experiments presented in this thesis also point to the fact that successful navigation of the environment in bumblebees is dependent on experience of that environment (Chapters Three, Four and Five).

### 7.2.3 Refining Models of Bumblebee Behaviour

The large sample sizes of bumblebees tested in the experiments presented in this thesis and the resulting conclusions drawn can help to inform models of bumblebee foraging behaviour. Predictive models of behaviour rely on a set of rules and assumptions which are based on data gathered from experimental results. Our current knowledge of bumblebee behaviour including flight paths taken, foraging ranges, trade-off with foraging requirements, colony energy demands and gyne foraging involvement (or lack thereof) are all used in such models. At present, the potential influence of landscape structures or type of environment are not incorporated into such models. Nor is the influence of experience in a bumblebee's first five flights on the duration of foraging trips or the weight of pollen foraged. The results of our experiments could be directly incorporated into such models to refine their outcomes. At present, the current models also do not incorporate the role that bumblebee gynes may play in provisioning their maternal colonies with pollen. The results of Chapter Six, as well as any future replications of this experiment, could also be incorporated into such models. Incorporating the results from this thesis into future behavioural models will increase their predictive power and aid them to more accurately predicting bumblebee behaviour and colony growth.

## 7.3 Experimental Limitations and Improvements

### 7.3.1 Individual Experiments

The results of the experiment presented in Chapter Two shed light on the effects that hedgerows have on the behaviour of naïve bumblebees when they first exit their colony. Hedgerows situated adjacent to the nest do not seem to influence the flight direction or foraging choices of bumblebees when they first exit their colonies. In order to determine the direction that bumblebees flew when exiting their colony, and thus determine whether the hedgerow influenced their flight paths, the vanishing bearings of a bumblebee were recorded. The vanishing bearings of a bumblebee are the compass bearing at the moment when the bumblebee vanishes from human sight. It is a technique that is commonly used in studies of insect orientation, especially with honeybees (Gould, 1986; Dyer, 1991, 1993; Dyer et al., 1993). This technique, however, relies entirely on human observers and how well these human observers can follow a bumblebee as it flies off within a landscape. In the experiment presented (Chapter Two), at least two human observers would watch a bumblebee and determine its vanishing bearings in an effort to avoid observer bias. In order for a vanishing bearing to be a sound indicator of what direction a bumblebee flies, it should really be taken once a bumblebee finishes its orientation/learning flight. In other words, once it has finished performing its arcing behaviour around the colony (Osborne *et al.*, 2013). This can pose problems for human observers as a bumblebee may have vanished from their sight, but it may not have actually finished performing its arcing behaviour. In such cases, the vanishing bearings observed will not be a true reflection of the compass bearing that the bumblebee chose to take following its orientation/learning flight. In this way, it is difficult to draw concrete conclusions from the results obtained with the use of vanishing bearings alone.

In flat and relatively featureless environments, technology such as harmonic radar tracking can be used as a viable alternative to the use of vanishing bearings. The use of this technology would allow experimenters to obtain the compass bearing that the bumblebee chooses to take following its orientation/learning flight without relying on human observation. Unfortunately, one of the radar's requirements at present is that it is operated in a landscape without physical obstructions due to signal interference (Goulson & Osborne,



2006). This requirement rules out the use of harmonic radar in landscapes with hedgerows. What future experiments can do to tackle the limitations of the use of vanishing bearings is to facilitate human observers in spotting and keeping track of a flying bumblebee. This could be done by making the hedgerow background or the bumblebee stand out. To simplify the hedgerow background, for example, a follow-up experiment could be performed in which an artificial 'hedgerow' is built and placed in a natural environment. Such artificial hedgerows, or 'linear features', have been built and used in previous experiments (eg. Dover & Fry, 2001; Cranmer, McCollin & Ollerton, 2012). This artificial hedgerow could mimic the hue and brightness of a real hedgerow but with a more uniform pattern in order to aid human observers. From feedback received from the human observers in the experiment presented in Chapter Two, the most common cause of losing a bumblebee from their sight was an inability to spot the bumblebee against the hedgerow's variable background (R.Herascu, personal observation). The bumblebee itself could be made easier to spot using powdered fluorescent dyes (as described and used in Martin *et al.*, 2006). Exiting bumblebees in Martin *et al.* (2006)'s novel marking system were marked with dye powder without the interference of human experimenters. Pilot laboratory experiments could also determine the optimal dye colour that would facilitate visual tracking of a bumblebee, both against a hedgerow background and against the landscape more generally. Follow-up experiments, using either an artificial hedgerow, dyed bumblebees, or both, should improve the reliability and use of vanishing bearings in experimental work until improved tracking technologies are developed.

It is also difficult to draw concrete conclusions on the effect that hedgerows have on the flight paths and foraging choices of naïve bumblebees without controlling for other potentially influencing factors. It is entirely possible that factors other than the hedgerow were influencing the flight paths and foraging choices of the naïve bumblebees in the experiments presented in Chapter Two. Although the effect of distance from the mass flowering crop was investigated, other potentially influencing factors include the nutritional needs of the colony; atmospheric conditions; the distribution of foraging resources around the colonies; the nutritional composition of the forage available; and the accessibility of pollen from the floral species available. Although it is extremely difficult to control for these factors when conducting field experiments, these factors could be monitored to

investigate their potential effects. Future experiments would also benefit from an increase in the number of experimental sites used and an increase in the number of bumblebee colonies used. This would help us to know the extent to which the results presented in Chapter Two are a true reflection of the effect of hedgerows on the behaviour of naïve bumblebees. The present experiments could also be extended to include different mass flowering crop species in order to investigate whether the attractiveness of the mass flowering crop also affects bumblebee behaviour. Furthermore, future investigations into the effects of distance from a mass flowering crop should include a variety of different distances, with the same distances used across all experimental sites.

The experiments presented in Chapter Three gave key insights into the navigation and foraging behaviour of bumblebees on their first five flights outside the colony. Like the experiments presented in Chapter Two, the experiments presented in Chapter Three would have benefited if additional potentially influencing factors had also been monitored. These include factors such as the nutritional demands of the colony, the nutritional composition of the pollen that was foraged for and the accessibility of the pollen that was foraged for. All three of these factors could have significantly influenced the type of pollen that was foraged for, the duration of foraging trips, and the weight of pollen foraged. Future work could investigate how these three factors influence bumblebee behaviour. The nutritional demands of the colony during the experiment could be established by monitoring its nectar and pollen stores, monitoring the number of larvae present, monitoring the number of workers present, and monitoring the presence and number of reproductives. The nutritional composition of the pollen that is foraged for could be established by analysing its protein, amino acid, lipid and fatty acid composition (eg. as described in DeGrandi-Hoffman *et al.*, 2018). Knowing both the nutritional demands of the colony and the nutritional composition of the pollen that is foraged for would reveal whether pollen foraging, even within the first flights of a forager's career, is directly linked to the nutritional demands of the colony. To the best of my knowledge, this latter point is currently unknown. The accessibility of pollen from the floral species that are foraged upon could be established by first identifying the pollen brought back to the colony, as was done in the experiments presented in Chapter Three. Once the pollen is identified, it can be traced back to the floral species. Samples of the flowers from

which the pollen originated could be taken from the experimental sites back to the laboratory. A complementary laboratory behavioural study could then be set-up in which the foraging time of naïve and experienced bumblebees on different floral species is investigated. Factors such as the distance from the colony to the floral array and the nutritional needs of the colony would be controlled for. This would allow us to isolate the effects that pollen accessibility may have on a bumblebee's pollen choice. It would also shed light on the effects that pollen accessibility may have on foraging trip durations. The experiments presented in Chapter Three also focused on the first five flights of bumblebee foragers. Future experiments could extend these investigations and look at a bumblebee's entire foraging career. This would shed light on the role of long-term experience on the navigation and foraging behaviour of bumblebees.

In Chapter Four, the differences in homing and overnight behaviour between the two environment types observed could have been due to both the distribution of forage around the colonies as well as differences in the structures of the two environment types. Although the present experiment could not disentangle the reasons why bumblebees in one environment type were more likely to return to their colonies compared to the other environment type, future work could strive to do this. In order to try to disentangle the effects of forage distribution and environment structure, a future experiment could first investigate the homing success of bumblebees in a relatively flat and featureless landscape. The distribution of forage could be artificially manipulated in such a landscape. This could be achieved by planting forage at set distances from the bumblebee colonies. This would allow the effects of the distribution of forage on homing success to be isolated from any potential effects of environment structure. Follow-up experiments could investigate how having foraging sources located at specific distances around the colonies may affect bumblebee homing. Having established the effects of forage distribution on homing success, the effects of environment structure could then be isolated. Homing experiments could be performed in urban environments which have very little forage available. This could be achieved by performing experiments at the end of the flowering season, when little forage is naturally available. The structure of urban environments is not uniform and will vary with factors such as the percentage cover of green spaces, the percentage cover of impervious surfaces or the height of the buildings within

it. An 'urbanisation' metric could be devised based on such factors (eg. Vaidya, Fisher & Vandermeer, 2018; Samuelson & Leadbeater, 2018; Samuelson *et al.*, 2018) and urban environments with different urbanisation scores could be selected. The results of homing experiments in urban environments with different urbanisation scores would shed light how the structure of an environment may affect the homing success of bumblebees within it. Such follow up experiments would reveal the exact causes which lie behind the results observed in the experiment presented in Chapter Four.

In the experiment presented in Chapter Five, the majority of released bumblebees were able to return to their colony when released from 1000 m if they had more than one flight of experience prior to release (Figure 5.3). This suggests that the majority of bumblebees were familiar with the area 1000 m from their colony after performing two flights outside the colony and that at least some bumblebees were also familiar with this area after performing a single flight outside the colony. The factors behind this exploratory behaviour are unknown and follow-up experiments are needed to shed light on this phenomenon. For example, the exploratory behaviour observed may be directly linked to the distribution of forage around the colony. The experiment presented in Chapter Five was conducted towards the end of the flowering season when few foraging resources were available in the landscape. It is entirely possible that in a landscape where adequate foraging resources were centred around 500 m from the colony, for example, that the majority of bumblebees would not explore further than 500 m after two flights. Because the experiment presented in Chapter Five was carried out at a single experimental site, it is difficult to generalise the results. Future work could build upon these results and investigate the relationship between a bumblebee's exploratory behaviour and the distribution of forage around the colony. This would also help to disentangle the effects that the distribution of forage around the colony and the structure of an environment have on the homing success of bumblebees. The exploratory behaviour of bumblebees would also affect their likelihood of staying out overnight as well as their foraging behaviour. Future work should strive to investigate the relationship that exists between the distribution of forage around the colony, the likelihood of staying out overnight and foraging behaviour. It is also important to note that both the homing experiments presented in Chapter Four and Five, as well as Goulson & Stout's (2001) homing

experiment, used a single species of bumblebee, *Bombus terrestris*. It is entirely possible that exploratory behaviour is directly linked to foraging range, which in turn has been suggested to be species dependent (Kreyer *et al.*, 2004; Westphal *et al.*, 2006; Greenleaf *et al.*, 2007). It is important that future homing experiments also use different bumblebee species to establish whether exploratory behaviour varies between different species of bumblebees.

The results of the experiment presented in Chapter Six suggest that that bumblebee gyne foraging is influenced by the amount of pollen that is entering the colony. The present experiment cannot, however, tease apart whether the results observed were entirely due to the amount of pollen that was entering the colony at the time of the experiment or whether the behaviour observed was also due to historic pollen shortages in the colony. The experiment also suffered from a very limited sample size, especially in Condition Two, where only three colonies were tested. Future replications of this experiment should increase the number of colonies used in each experimental condition and if possible, have an equal number of colonies tested in each experimental condition. In the present experiment, different treatments were not run in parallel. As a result, the bumblebees in the different treatments were potentially experiencing a different foraging environment, with different amounts and types of pollen available to them. Such different foraging environments could have influenced the amount and type of pollen that was entering the colony, which could have in turn influenced the foraging behaviour of gynes. Future replicates of this experiment should conduct the different experimental treatments in parallel in order to control for the effects of the foraging environment. Future replicates of this experiment should also try to disentangle the influences that the amount of pollen entering the colony during the experiment and the amount of pollen the colony had access to throughout its development have on gyne foraging. This could be done by beginning the experiment at the point where a bumblebee queen is laying her first brood of eggs. The nutritional needs of the colony could be monitored throughout the colony's development. The colony's pollen and nectar stores could be monitored throughout. Correlations could then be drawn between these stores and the number of larvae, workers and reproductives produced. This would allow conclusions to be drawn between the nutritional needs of the colony and its reproductive output. The foraging behaviour of gynes could then be observed and

any links with the nutritional needs of the colony or its reproductive output could be drawn. The experiment could then be expanded by subjecting different colonies to different feeding regimes from the point at which a bumblebee queen is laying her first brood of eggs. To control for the effects of nectar provisions, the amount of nectar could be kept constant throughout. In this way, the pollen feeding regime could be focused on. The amount and type of pollen, including pollen with different protein and lipid ratios, could be manipulated in order to investigate what effects this might have on gyne foraging. Furthermore, in the present experiment, not all gynes in a bumblebee colony were observed foraging for pollen. Some gynes remained in the colony throughout the experiment. It would be interesting to investigate whether physiological differences exist between the gynes which forage for pollen and those that did not. This could be done in future replicates of this experiment. This would help to pinpoint the reasons why bumblebee gynes may forage for pollen and return to their maternal colonies prior to mating and hibernation.

### 7.3.2 Overall

The methods used, and the limitations experienced, in the experiments presented in this thesis provide valuable insights for future bumblebee researchers. In all the experiments in this thesis, commercially reared *Bombus terrestris* colonies were used. Throughout the experiments, a substantial number of bumblebees did not return to their colonies. It was also observed that the activity levels of colonies differed widely. This was the case throughout all of the testing environments and in both years of testing. What can be concluded is that if researchers are to use commercially reared *Bombus terrestris* colonies for field work, they should not be surprised if high losses occur. In order to buffer against this scenario, either researchers should plan on using more colonies for each experiment or consider breeding colonies from wild-caught bumblebee queens. The potential pitfalls of using RFID technology for bumblebee field experiments was also observed. Although RFID technology did not pose problems in the experiment presented in Chapter Four, it did have many limitations in the experiment presented in Chapter Three: Part B. When RFID tagged bumblebees became experienced and moved through the RFID readers at a very fast pace, the readers failed to scan the tagged bumblebees. When this occurred, it resulted in a gap in the electronic record of activity which negatively impacted the entire activity record of a

bumblebee. In order to combat this problem in the future, a landing platform could be constructed, or the side entrance of the wooden bumblebee box could be used as the main exit and entry hole for the colony instead (Chapter Two: Figure 2.4). Before passing through the electronic readers, bumblebees would need to land and crawl through the electronic readers rather than fly straight through them. This would sufficiently slow them down for the electronic readers to scan them. This was not a problem when using RFID technology in the homing experiments presented in Chapters Four and Five. This is because in the homing experiments, once bumblebees were released, the wooden bee box was set to only allow bumblebees to enter their colony but not to exit it. In this scenario, even if the electronic reader did not scan a tagged bumblebee, the tagged bumblebee could still be found inside the colony once the experiment was complete. Due to the substantial current cost and potential pitfalls of using RFID technology, it is currently a viable option only when the use of human experimenters is not possible.

## **7.4 Future Work**

### **7.4.1 Investigating Landscape Features**

At present, little is known on the effects that landscape structures have on bumblebee flight and foraging behaviour. This is true for both natural as well as man-made structures. To my knowledge, the only studies which have previously investigated the effects of landscape structures have focused on forests (Kreyer *et al.*, 2003), hedgerows (Cranmer, McCollin & Ollerton, 2012) and roads and railways (Bhattacharya, Primark & Gerwein, 2003). The results presented in this thesis add to this very small number of studies. More work, however, must be done in investigating how individual structures, as well as clusters of structures, may affect bumblebee flight paths and foraging behaviour. A wide definition of structures could be adopted to also include man-made objects such as cars and motorways. Future work could begin with direct observations in the natural environment. The most straightforward way in which the flight paths and foraging choices of bumblebees could be visualised is using harmonic radar technology. In cases where harmonic radar technology is not possible, due to the interference caused by tall objects, proxy measurements will need to be used. Many of the possible proxy measurements that could be used were used in the experiments

presented in this thesis. These proxy measurements include homing experiments (eg. Chapters Four & Five) as well as experiments which identify the foraging areas of bumblebees by monitoring their pollen choices (eg. Chapters Two & Three). For example, harmonic radar tracking technology could help to investigate the effects that multi-lane motorways or water bodies of differing widths have on bumblebee flight and foraging behaviour. The investigations could also include large-scale experiments investigating the role of mountain ranges or valley systems. Such large-scale experiments could shed light on the limits of bumblebee flight. Following field experiments, complementary laboratory experiments could also be performed. Such experiments could investigate the features of specific structures that are affecting bumblebee flight. For example, laboratory experiments could focus on how structures of different heights affect bumblebee flight or how the surface of a structure may interact with a bumblebee's visual system to influence bumblebee flight. By coupling field experiments with laboratory experiments, future work could begin to untangle the underlying effects that landscape structures have on bumblebee flight and foraging behaviour.

#### 7.4.2 Variability Between Individuals

A key insight from the experiments presented is the fact that there was a large variability in individual bumblebee behaviour observed. Although this was not formally investigated in this thesis, there is evidence now emerging throughout studies of the social insects that some level of behavioural variability is also present among individuals within each caste (Pinter-Wollman, 2012; Walton & Toth, 2016). Within a bumblebee colony, it may prove beneficial for different workers to adopt different navigational and foraging strategies. This would mean that different environmental conditions would better suit the navigational and foraging strategies of different workers. For example, environments where foraging resources are scarce may be better suited to foragers who prioritise long foraging trips in which they explore the landscape for potential food sources. Environments where foraging resources are easily accessible and plentiful may be better suited to foragers who prioritise short but numerous foraging trips. Having both types of foragers in a colony may allow the colony as a whole to quickly respond to environmental change. Future research should begin to



investigate how behavioural variation at the individual level effects a colony's overall behaviour within an environment.

This line of research could also be expanded to investigate the specific factors that are driving the individual variation in behaviour observed. Future experiments could focus on investigating whether individual behaviour correlates with physiological factors such as body size and age or psychological factors such as learning speed or neophobia. This could be investigated with a series of laboratory experiments in which naïve bumblebees are first subjected to a battery of measurements and tests. In these tests, both physiological and psychological factors would be investigated. After a profile is achieved for each bumblebee, bumblebees could then be released and monitored as they forage within their natural environment. Observations would be made on behaviours such as flight duration, the number of foraging trips performed, and the weight of pollen foraged for. Correlates could then be drawn between the battery of measurements and tests taken and their observed behaviour in the natural environment. Such investigations would allow insights to be made into what individual differences exist in bumblebees and how these individual differences shape behaviour. Furthermore, the factors driving variation in individual behaviours could be investigated. For example, what effect does development have on behaviour and how does the rearing environment effect behaviour? Rearing conditions such as nutritional intake during the larval stage and rearing temperature are factors which have the potential to effect bumblebee development. This may in turn effect behaviour. For example, larval feeding has been found to correlate with adult body size in *Bombus impatiens* workers (Couvillon & Dornhaus, 2009). Larger foragers are known to be faster fliers (Spaethe, Tautz & Chittka, 2000), have better visual acuity (Spaethe & Weidenmüller, 2002) and carry heavier pollen loads in white corbiculae (Fisher, 1987).

Correlates could also be drawn between differences in internal anatomical structures such as the mushroom bodies and fat bodies of bumblebees and their behaviour. For example, a correlation has been found between the density of the synaptic complexes in a region of the mushroom bodies and visual discrimination (Li *et al.*, 2071). Furthermore, bumblebee gynes that were observed foraging late in the season were found to have reduced fat bodies (Alford, 1975). Differences in internal anatomical structures may also have a basis in differences in the

temporal or spatial patterns of gene expression. Lockett *et al.* (2016) have found a difference in the gene expression levels of aging-related genes between bumblebee workers and queens. Alaux *et al.* (2009) have also found that two different sub-species of honeybees differed in their expression of aggression-related genes and that this expression was subject to both inherited and environmental influences. Numerous studies using honeybees have found evidence to support the idea that changes in gene regulation can underlie the evolution of behavioural diversity (reviewed in Zayed & Robinson, 2012). This makes investigations into the correlates between gene expression and behavioural traits in bumblebees a very interesting route for future research.

In the results of the homing experiments presented, not all released bumblebees returned to their colony. This suggests that individual differences also exist in homing ability. These differences may have been due to individual differences in exploratory behaviour. The exact reasons behind this individual variability are unknown. Future work could investigate these reasons, focusing on correlations between exploratory behaviour and characteristics such as body size, age, and cognitive traits. Furthermore, factors such as the nutritional requirements of the colony may also influence the exploratory behaviour of individual foragers. Future experiments could monitor the nutritional needs of the colony in order to investigate the extent to which exploratory behaviour is influenced by individual differences or colony need.

#### 7.4.3 Bee Behaviour in Urban Environments

A key insight from the experiment presented in Chapter Four is the potentially negative effect that urban environments may have on bumblebee navigation. At present, there is mixed evidence on whether different pollinator species are negatively affected by increasing urbanisation. Bee species richness, for example, has been found to be higher in urban areas (Cane *et al.*, 2006; Baldock *et al.*, 2015; Sirohi *et al.*, 2015). This may be linked to the fact that agricultural landscapes have been associated with declines in bumblebee floral resources (Carvell *et al.*, 2006) and that urban areas can offer higher floral abundance and diversity in the garden and parks found within them (McFrederick & LeBuhn, 2006; Loram *et al.*, 2008). The nest densities of bumblebees specifically have been found to be higher in urban gardens compared to rural areas (Osborne *et*

*al.*, 2007). When investigating the reproductive success of *Bombus terrestris* colonies in both agricultural and urban environments, Samuelson *et al.* (2018) found that colonies in the urban environments reached higher peak size, had more food stores, encountered fewer parasite invasions and survived for longer than those in the agricultural environments. Contrasting results, however, have been observed and studies have also found negative impacts of urbanisation on bee abundance, diversity and parasitic load (Matteson, Ascher & Langellotto, 2008; Ahrné, Bengtsson & Elmqvist, 2009; Tonietto *et al.*, 2011; Goulson, Whitehorn & Fowley, 2012; Glaum *et al.*, 2017). Other studies have found no effect of urbanisation on bumblebee growth rate (Vaidya, Fisher & Vandermeer, 2018) or bee diversity and abundance more generally (Hostetler & McIntyre, 2001; Frankie *et al.*, 2005; Fetridge, Ascher & Langellotto, 2008). Overall, the effects of urbanisation on bumblebee abundance have been found to be species specific (Banaszak-Cibicka & Zmihorski, 2011).

For any pollinator, the urban environment is made up of a variety of different aspects. These aspects include factors such as forage availability, floral diversity, nesting sites, physical structures such as buildings and roadways, atmospheric pollutants, and competitive and predatory forces. It is clear that these aspects will affect different pollinator species in different ways. Understanding how these aspects interact and affect pollinators and bumblebees specifically is necessary if the full effects of urbanisation are to be understood. Current research is focusing on the effects that urbanisation has on bumblebee diversity, abundance and reproduction. As the results presented in Chapter Four suggest, future research should also focus on the effects that urbanisation can have on bumblebee behaviour. The experiment presented in Chapter Four can serve as a starting point into investigating the effects that man-made structures and artificial light sources have on bumblebee navigation and foraging behaviour. Such investigations could provide key contributions to the growing field of urban ecology.

Understanding the effects of urbanisation on bumblebee behaviour is also important in the context of urban bumblebee conservation. Green roofs, loosely defined as rooftops with varying depths of soil cover and extensive vegetation, have been proposed as a possible solution in combating the loss of green space in urban areas (Orbendorfer *et al.*, 2007; Braaker *et al.*, 2014). Bumblebees are

thought to be able to make use of green roofs as nesting and foraging sites and several studies have recoded their presence on green roofs (Colla, Willis & Packer, 2009; Tonietto *et al.*, 2011; Hofmann & Renner, 2017; Kratschmer, Kriechbaum & Pachinger, 2018). But as green roof research is a relatively new field of study (Blank *et al.*, 2013), many questions regarding the benefits of green roofs for the foraging and nesting ecology of bumblebees remain unanswered. As it is not currently known whether bumblebees fly above urban structures, it is equally unknown how accessible green roofs of varying heights are for different species of bumblebees. Given that green roofs have now become mandatory on new flat-topped buildings in many cities (Hofmann & Renner, 2017) and that some cities are even implementing green roofs on their bus shelters (Gemeente Utrecht, 2019), it is important to investigate whether such initiatives will actually benefit foraging and nesting bumblebees. The results of Chapter Four suggest that bumblebees may find it difficult to routinely fly above urban structures. As such, future work should investigate the heights at which different bumblebee species fly in the urban environment. This could then be linked to finding the optimal height that a green roof in a particular urban environment should be. In this way, experimental work could directly inform conservation policy.

#### 7.4.4 Bumblebee Navigation

In the results presented in Chapter Five, 38.7% of released bumblebees that only had a single experience flight prior to release returned to their colony when released from 1000 m. This suggests that on their first flight outside the colony, some bumblebees were exploring and learning characteristics of their environment up to 1000 m from their colony. It is already known that bumblebees undertake orientation/learning flights when they first leave their colony (please see Section 1.2.1: Learning Flights). Bumblebees also undertake orientation/learning flights when leaving a rewarding food source. Orientation/learning flights are characterised by arcing behaviour around a structure and are thought to allow a bumblebee to learn the properties of that structure in order to be able to return to it. When artificially displaced, bumblebees are also observed performing an arcing behaviour upon release (Goulson & Stout, 2001; R.Herascu, personal observation). Bumblebees tracked with harmonic radar have also been observed performing this behaviour on a wider scale during their first flights outside the colony (Osborne *et al.*, 2013).

Although highly theorised, it is not currently known how exactly bumblebees navigate within their environments (please see Section 1.4: Navigational Mechanisms). Likewise, it is not currently known what features of the environment bumblebees learn in order to navigate successfully. It is highly likely that a behaviour akin to the orientation/learning flights observed in proximity of the colony and rewarding food sources also occurs when features of the environment are learnt. These features might include the landscape panorama or even individual structures in the environment which may serve as landmarks, guiding bumblebees to a goal.

At present, the flight paths of bumblebees can be mapped using harmonic radar technology up to 1000 m (Osborne & Goulson, 2006). Future experiments could use this technology to find out whether specific flight patterns characterise the first flights of bumblebees. As the results presented in Chapters Three and Five suggest, the first flights are a period of large information gathering and learning in bumblebees. If specific flight patterns characterise this period, this could suggest that these flight patterns are undertaken when bumblebees learn about their environment. It may be the case that specific flight patterns will be linked to certain features of the environment. This would help to shed light on what features of the environment bumblebees use for navigation purposes. Harmonic radar tracking could also be used in homing studies, in order to visualise the flight patterns of displaced bumblebees. For example, the experiment presented in Chapter Five could be repeated but released bumblebees could be tracked with harmonic radar. This would have the added benefit of linking experience level with flight paths. A lack of resources in the experiment presented in Chapter Five limited bumblebees to undergoing one, two or five experience flights before release. A future replication of this experiment could have bumblebees additionally undergo three and four experience flights before release. This would allow us to see whether a threshold level of experience is reached after three or four flights. A future replication of the experiment presented in Chapter Five could also include displacing naïve but harmonic tagged bumblebees in order to compare their flight behaviour upon release. Such future experiments would contribute to our understanding of how bumblebees navigate, and by extension forage, within their environment.

#### 7.4.5 Behaviour of Reproductives

The experiment presented in Chapter Six focused on the pollen foraging behaviour of *Bombus terrestris* gynes. Although gynes had previously been observed foraging for pollen under particular circumstances (see Section 6.2: Introduction), this was the first study that tried to test the conditions under which gynes might forage for pollen. Although this experiment can be greatly improved in future replications, it still represents one of the few experimental studies that aimed to investigate the behaviour of the reproductive caste. In the field of bumblebee research, the vast majority of studies focus on the worker caste. It is entirely unknown whether the conclusions drawn from experiments conducted with the worker caste can also be applied to the reproductive caste.

At present, only a handful of studies have investigated the behaviour of the reproductive caste and how this may differ from the workers. The experiments presented in Chapters Two to Five could be reproduced using gynes instead. This would shed light on how landscape structures and experience effect gyne or male behaviour. It is easy to assume that if a behaviour is exhibited by all the castes, then little variation between the castes exists. Such an assumption would make any investigations into this potential variation unnecessary. Yet the workers, males, gynes and founding queen all play different roles in the colony's reproductive success and it should be expected that variation will not only exist but prove adaptive for the colony.

In the case of foraging behaviour, for example, *Bombus terrestris* queens were found to forage more cautiously but learn more quickly than workers in a laboratory associative task (Evans & Raine, 2014). For queens foraging in their natural environment, it is likely this has the effect of decreasing their predation risk but increasing their foraging efficiency, which would maximise their chances of establishing a colony. When extending this comparison to that of workers and males, Wolf & Chittka (2016) did not find significant differences in a laboratory associate task. This comparison, however, needs to be extended to include gynes as well.

Comparisons of caste behaviour must also extend beyond foraging behaviour. For example, the spatial exploration of the castes may also differ. Unlike workers, gynes will need to search for a mate, a hibernation site and a nest site after

hibernation. Whether this searching behaviour is different to the searching behaviour that gynes and workers undergo when foraging is currently unknown. It is entirely possible, for example, that a difference will exist between the two searching behaviours, and that gynes will undergo more expansive explorations of the landscape than workers. Such explorations could result in increases in spatial memory or quicker learning speeds for gynes. Future work could be extended to also include investigating differences in neural structures between the castes and finding whether correlations exist between behaviour and brain physiology. Males, on the other hand, leave their maternal colony at one point (Haas, 1976; Jennersten, Morse & O'Neil, 1991; Goulson, 2010) and must search for a mate and foraging sites. How this compares with the searching behaviour of gynes and workers remains currently unknown. Behavioural comparisons between the different bumblebee castes can prove to be a fruitful avenue of future research.

#### 7.4.6 Beyond *Bombus terrestris*

The experiments in this thesis shed light on the effects that landscape structures and experience have on the navigation and foraging behaviour of bumblebees. As all the experiments were performed using the bumblebee species *Bombus terrestris*, it is difficult to know how applicable the results are to different bumblebee species. This is not a limitation which is unique to the experiments in this thesis, but which characterises the field of bumblebee research as a whole. In the United Kingdom and Europe, the majority of experiments are conducted using *Bombus terrestris* while in North America, the majority of experiments are conducted using *Bombus impatiens*. Although we have a large body of research which has investigated the behaviour and ecology of these two species, we know very little of the approximately 248 other species of bumblebees (Goulson, 2010). Bumblebee body size and average colony size vary significantly between species (Benton, 2006) and bumblebee foraging range is also thought to be species specific (Greenleaf *et al.*, 2007). It is very likely that the results of the experiments presented in this thesis would be different if they were conducted with other species of bumblebees. The lack of knowledge that exists with regards to the majority of bumblebee species can pose challenges when conservation or policy initiatives look to existing research for guidance. For example, it would be difficult to devise and implement a successful conservation strategy that addresses the

needs of a variety of different bumblebee species when many aspects of their behaviour remain unknown. Future research should address this lack of knowledge by focusing on investigating the behaviour and ecology of different bumblebee species as well as any similarities and differences that exist between them.

One of the main reasons that the field of bumblebee research has been dominated by two model species has been the ease with which these bumblebees can be bought from commercial suppliers. Throughout the experiments in this thesis, the use of commercial bumblebee colonies presented its own challenges. In the experiments presented in Chapters Two and Three, a large number of bumblebees did not forage or did not return to their colony upon release. Activity levels also varied widely between colonies (R. Herascu, personal observation). In light of these observations and in an effort to combat the current reliance on these two model species of bumblebees, it would be beneficial for colonies to be bred from local, wild-caught bumblebee queens. Bumblebee queens could be caught in the spring following their hibernation and brought into the laboratory to be bred (eg. Samuelson *et al.*, 2018). This would allow a variety of different bumblebee species to be bred for experimental use. If the bumblebees are to be used for fieldwork experiments in the same environment in which the queen has been caught, this would eliminate the risks that are associated with using and introducing commercial bumblebees into an environment. These risks include hybridisation between commercial and wild populations (Ings, Raine & Chittka, 2005), the transmission of pathogens between commercial and wild populations (Colla *et al.*, 2006) and competition between commercial and wild populations (Ings, Ward & Chittka, 2006). The use of wild caught queens and the colonies that would be bred from them would also increase the ecological relevance of the experiments conducted. In the long term, the benefits of adopting such a breeding program would outweigh its increased costs. Future work should consider using wild-caught queens for both experimental and laboratory work and strive to use a variety of different bumblebee species.

#### 7.4.7 Collaborative Approaches

Studies which investigate aspects of the physical landscape are also difficult to generalise. In many cases, the aspects of the physical landscape that were under



investigation were unique to the experimental sites used. For example, in the experiment presented in Chapter Two, the effects of hedgerows on the flight paths and foraging behaviour of bumblebees was investigated. In this experiment, the hedgerows used were characteristic of lowland agricultural systems. It is difficult to generalise the results to agricultural systems which are made up of taller, denser hedgerows, such as those in upland, mountainous landscapes (eg. Campagne *et al.*, 2009). Similarly, the effects that roads have on bumblebee flight paths and foraging behaviour was investigated by Bhattacharya, Primack & Gerwein (2003) in Boston, United States. The road that was used in their experiment was a four lane, 14 m wide, multi-vehicle motorway. The results of their experiment provide valuable insights into the behaviour of bumblebees when faced with such a wide road but cannot be generalised to smaller inner-city roads or country lanes. When investigating the effects of landscape structures on bumblebee flight paths and foraging behaviour, experimenters should strive to maximise the number and type of experimental sites used. Access to resources, however, will always be a limiting factor. In an effort to maximise experimental sites while minimising costs, a collaborative approach between different research groups could be adopted. Research groups would need to adopt the same research question and follow an identical experimental protocol. Such an approach has been adopted by researchers who are investigating the effects that non-lethal doses of plant protection products have on honeybee behaviour (Fourrier *et al.*, 2017). Specifically, a homing 'ring test' was used with 11 voluntary research groups taking part. Such collaboration between research groups, following an identical experimental protocol, would greatly increase the number of experimental sites that would be used to answer a particular research question. This could greatly benefit our knowledge of the effects that different landscape structures have on bumblebee behaviour as well as our knowledge of the wider effects that different environments have on bumblebee behaviour. Future research in a variety of different fields could benefit from such collaborations.

## **7.5 Conclusion**

This thesis aimed to look at the effects that landscape structures and experience have on the navigation and foraging behaviour of bumblebees. The experiments presented in this thesis are amongst the first to investigate the effects that different environment types may have on bumblebee behaviour and are the first

to record in detail the foraging behaviour of hundreds of bumblebee workers throughout their first five flights outside their colony. The results of the experiments presented in this thesis suggest that both landscape structures and experience can have a significant effect on the navigation and foraging behaviour of bumblebees. Bumblebee foraging efficiency and homing ability were improved with experience. The likelihood of bumblebees staying out overnight before returning to their colony, a potentially risky behaviour, was also reduced with experience. The experiments presented in this thesis also revealed that individual bumblebee behaviour is far from uniform. Large variability was observed between individual bumblebees, in terms of their flight durations and weight of pollen foraged, as well as between an individual bumblebee's different flights. Different environment types were also found to significantly affect bumblebee homing, shedding light on the challenges that urban environments may pose for navigating bumblebees. This thesis also explored the behaviour of the reproductive caste, by focusing on the pollen foraging behaviour of bumblebee gynes. The amount of pollen entering the colony was found to have a significant effect on gyne foraging. Taken together, the results of the experiments presented in this thesis provide novel insights into key aspects of bumblebee behaviour. They also provide interesting avenues for future research, particularly in the growing field of urban ecology and in the exploration of individual differences. The results of the experiments presented in this thesis can also inform bumblebee conservation strategies, ensuring that such initiatives are supported by experimental research.

## **Appendix A: Supplementary Data and Information for Chapter Two**

### **A.1 Decision to Exclude Non-Comparable Data**

In this experiment at Site One, a second period of testing also took place between 23<sup>rd</sup>- 27<sup>th</sup> May 2016. On 23<sup>rd</sup> May 2016, the positions of the colonies were switched such that colony A was now on the side of the hedge facing away from the mass flowering crop and colony B was now on the side of the hedge facing towards the mass flowering crop. Bumblebees that had begun foraging for pollen when placed on one side of the hedge now had a new colony position. The effects of this switch on the subsequent foraging choices of these bumblebees, as well as their potential influence on workers in the colony that had not started foraging yet, could not be ascertained or isolated. As such, these effects would confound the potential effects of the hedgerow on the foraging choices and flight paths of workers. Although bumblebees do not seem to possess a direct communication system like the waggle dance of honeybees, workers do seem to be influenced by certain cues such as the odours present in the colony. For example, bumblebees leaving their colony strongly prefer the odour that was previously brought into the colony by a successful forager (Dornhaus & Chittka, 1999). Furthermore, successful foragers entering the colony distribute a pheromone signal which induces workers inside the colony to begin foraging (Dornhaus, Brockmann & Chittka, 2003). Pollen odour alone is enough to induce foraging (Kitaoka & Nieh, 2009). Workers in the colony also monitor the amount of nectar entering the colony in order to assess whether foraging is taking place (Dornhaus & Chittka, 2001). After the switch, the colonies may have been influenced by the foraging decisions of workers that had foraged from their previous location. Even when foraging from their new colony position, experienced foragers were observed returning to their old colony position. As such, the decision was made to exclude the data collected in the second part of testing at Site One as it was not obtained under similar experimental conditions. It should also be noted that such switches were not performed at Site Two or Site Three.

## A.2 Hedgerow Effects

**Table A.1. Number of released bumblebees by date from each of the ten colonies across all three experimental sites.**

	17/ 05/ 2016	19/ 05/ 2016	20/ 05/ 2016	16/ 08/ 2016	17/ 08/ 2016	18/ 08/ 2016	19/ 08/ 2016	24/ 04/ 2017	25/ 04/ 2017	26/ 04/ 2017	27/ 04/ 2017	28/ 04/ 2017	29/ 04/ 2017	02/ 05/ 2017	03/ 05/ 2017
<b>Site One Colonies</b>															
<b>A</b>	16	3	13	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
<b>B</b>	4	8	11	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
<b>Site Two Colonies</b>															
<b>C</b>	N/A	N/A	N/A	26	15	14	9	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
<b>D</b>	N/A	N/A	N/A	58	11	3	1	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
<b>E</b>	N/A	N/A	N/A	29	10	11	7	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
<b>F</b>	N/A	N/A	N/A	25	4	5	9	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
<b>Site Three Colonies</b>															
<b>G</b>	N/A	N/A	N/A	N/A	N/A	N/A	N/A	16	15	21	8	N/A	N/A	N/A	N/A
<b>H</b>	N/A	N/A	N/A	N/A	N/A	N/A	N/A	27	7	11	10	N/A	N/A	N/A	N/A
<b>I</b>	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	2	36	3	N/A
<b>J</b>	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	1	16	37	15

**Table A.2. Number of bumblebees which were released and then returned for each of the colonies across all three experimental sites, as well as the number of those which returned with pollen on their first flight and the numbers used in the ‘First Flight’ pollen analysis. The number of bumblebees that performed more than one flight outside the colony and the number of bumblebees which were used in the ‘Overall Flights’ pollen analysis is also shown.**

	Number of released bumble- bees	Number of returned bumble- bees	Number of bumble- bees that returned with pollen on their first flight	Number of bumble- bees that were used in the ‘First Flight’ pollen analysis	Number of bumble- bees that performed more than one flight	Number of bumble- bees that were used in the ‘Overall Flights’ pollen analysis <sup>11</sup>
<b>Site One Colonies</b>						
<b>A</b>	32	19	16	N/A	5	N/A
<b>B</b>	23	3	3	N/A	0	N/A
<b>Site Two Colonies</b>						
<b>C</b>	64	41	31	30	21	20
<b>D</b>	73	69	55	55	37	34
<b>E</b>	57	37	28	26	25	24
<b>F</b>	43	35	26	25	24	21
<b>Site Three Colonies</b>						
<b>G</b>	60	40	39	38	27	22
<b>H</b>	55	54	54	54	29	29
<b>I</b>	41	35	35	33	14	12
<b>J</b>	69	55	55	53	22	15

<sup>10</sup> As some bumblebees had an equal amount of pollen from different species and were thus excluded.

<sup>11</sup> As some bumblebees did not preferentially forage for a single type of pollen overall and were thus excluded.

### A.3 Distance Effects

**Table A.3. Number of released bumblebees by date from the additional colonies that were used at Site One and Site Three.**

	<b>24/ 05/ 2016</b>	<b>25/ 05/ 2016</b>	<b>26/ 05/ 2016</b>	<b>27/ 05/ 2016</b>	<b>28/ 04/ 2017</b>	<b>29/ 04/ 2017</b>	<b>02/ 05/ 2017</b>	<b>03/ 05/ 2017</b>
<b>Site One Colonies</b>								
<b>K</b>	32	0	18	2	N/A	N/A	N/A	N/A
<b>L</b>	N/A	13	3	18	N/A	N/A	N/A	N/A
<b>Site Three Colonies</b>								
<b>M</b>	N/A	N/A	N/A	N/A	5	19	32	0
<b>N</b>	N/A	N/A	N/A	N/A	N/A	N/A	16	3

**Table A.4. Number of bumblebees which were released and then returned for each additional colony at Site One and Site Three as well as the number of those which returned with pollen on their first flight and the numbers used in the ‘First Flight’ distance analysis. The number of bumblebees that performed more than one flight outside the colony and the number of bumblebees which were used in the ‘Overall Flights’ distance analysis is also shown.**

	Number of released bumble- bees	Number of returned bumble- bees	Number of bumble- bees that returned with pollen on their first flight	Number of bumble- bees that were used in the ‘First Flight’ distance analysis <sup>10</sup>	Number of bumble- bees that performed more than one flight outside the colony	Number of bumble- bees that were used in the ‘Overall Flights’ distance analysis <sup>1</sup>
<b>Site One Colonies</b>						
<b>K</b>	52	20	19	N/A	6	N/A
<b>L</b>	34	29	26	N/A	4	N/A
<b>Site Three Colonies</b>						
<b>M</b>	56	26	26	22	5	5
<b>N</b>	19	18	18	17	2	2

## Appendix B: Supplementary Data and Information for Chapter Three: Part A

### B.1 Flight Durations and Pollen Foraged

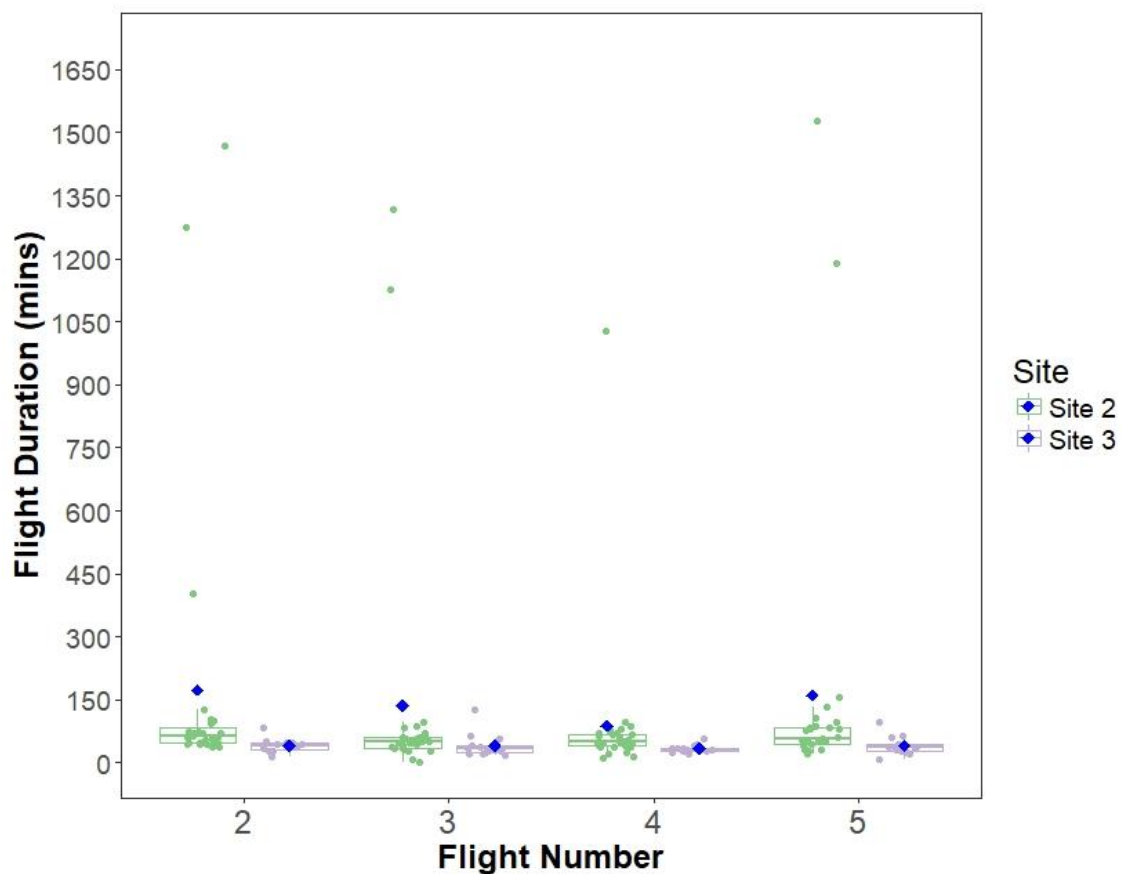
**Table B.1. Number of bumblebees which were released and then returned for each of the colonies across all three experimental sites, as well as the number of those which had completed five flights and for which a record of their flight durations and overnight flights exists. The number of bumblebees used in the ‘Duration’ analysis and the number for which a record of their pollen foraging throughout their first five flights exists is also shown.**

	Number of released bumblebees	Number of returned bumblebees	Number of bumblebees that completed five flights	Number of bumblebees that have a record of the duration of their flights <sup>12</sup>	Number of bumblebees with five flights that had an overnight flight	Number of bumblebees with five flights that were used in the ‘Duration’ Analysis	Number of bumblebees that have a record of their pollen foraging on their first five flights
<b>Site One Colonies</b>							
<b>A</b>	32	19	1	1	0	0	1
<b>B</b>	23	9	0	0	0	0	0
<b>Site Two Colonies</b>							
<b>C</b>	64	41	6	6	2	4	6
<b>D</b>	73	69	14	7	3	4	14
<b>E</b>	57	37	9	9	2	7	9
<b>F</b>	43	35	7	5	1	4	7
<b>Site Three Colonies</b>							
<b>G</b>	60	40	4	4	0	4	4
<b>H</b>	55	54	10	9	0	9	10
<b>I</b>	41	35	0	0	0	0	0
<b>J</b>	69	55	1	1	0	1	1

<sup>12</sup> Some bumblebees that had completed five flights did not have a complete record of their flights due to experimenter error.



## B.2 Second to Fifth Flight Durations Including Overnight Flights



**Figure B.1** | Box and whiskers plot of the flight duration (minutes) of all bumblebees that took five flights. The data includes bumblebees which also took an overnight flight. Individual data points are superimposed on the plots.  $N=41$  with  $n=27$  at Site Two and  $n=14$  at Site Three. Blue diamonds denote the mean. A small value of random noise was added to each data point for plotting purposes to aid visual representation.

### B.3 All Data Collected Throughout the Experimental Period

The analysis in this appendix was conducted in order to investigate whether limiting the analysis, to those bumbles which had a complete record of their first five flights, had biased the results. The dataset used in this appendix, explained below, was therefore less conservative.

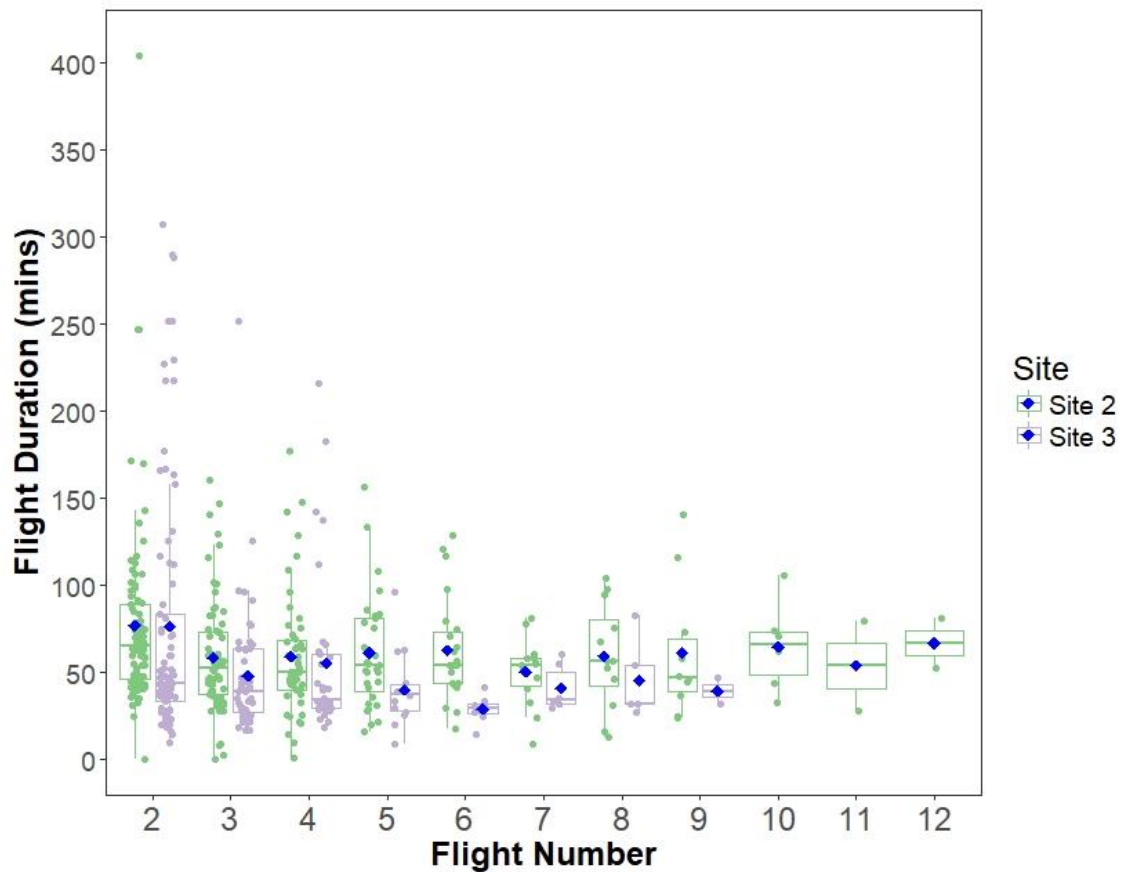
#### B.3.1 All Flight Durations Excluding Overnight Flights

Due to the way the experiment was conducted, there was not an accurate record for the duration of the majority of bumblebees' first flights. As such, only durations from the second flight onwards were analysed. In this case, all flight durations were analysed even if individual bumblebees differed in the number of flights that they took. Furthermore, the flight durations of individual bumblebees were included even if they did not have a complete record of their consecutive flights. Overnight flights (4.09% of total flights) were excluded in the following statistical analysis due to inaccuracies in their measurement and subsequent lack of convergence in the statistical models. A graph of the total flights, including the overnight flights, is shown for reference (Appendix B: Figure B.3).

To determine whether the flight number outside the colony had an effect on the flight duration, the relationship between the duration of each flight outside the colony and the number of flights taken was modelled using an LMM. As fixed effects, the experimental site and the flight number were entered into the model with an interaction term. As a random effect, there was an intercept for the individual bumblebee (as not all bumblebees included had a record of consecutive flights). Visual inspection was used to check residual plots for fit and homoscedasticity. P-values were obtained using the Satterthwaite's degrees of freedom method ('lmerTest' package; Kuznetsova, Brockhoff & Christensen, 2017).

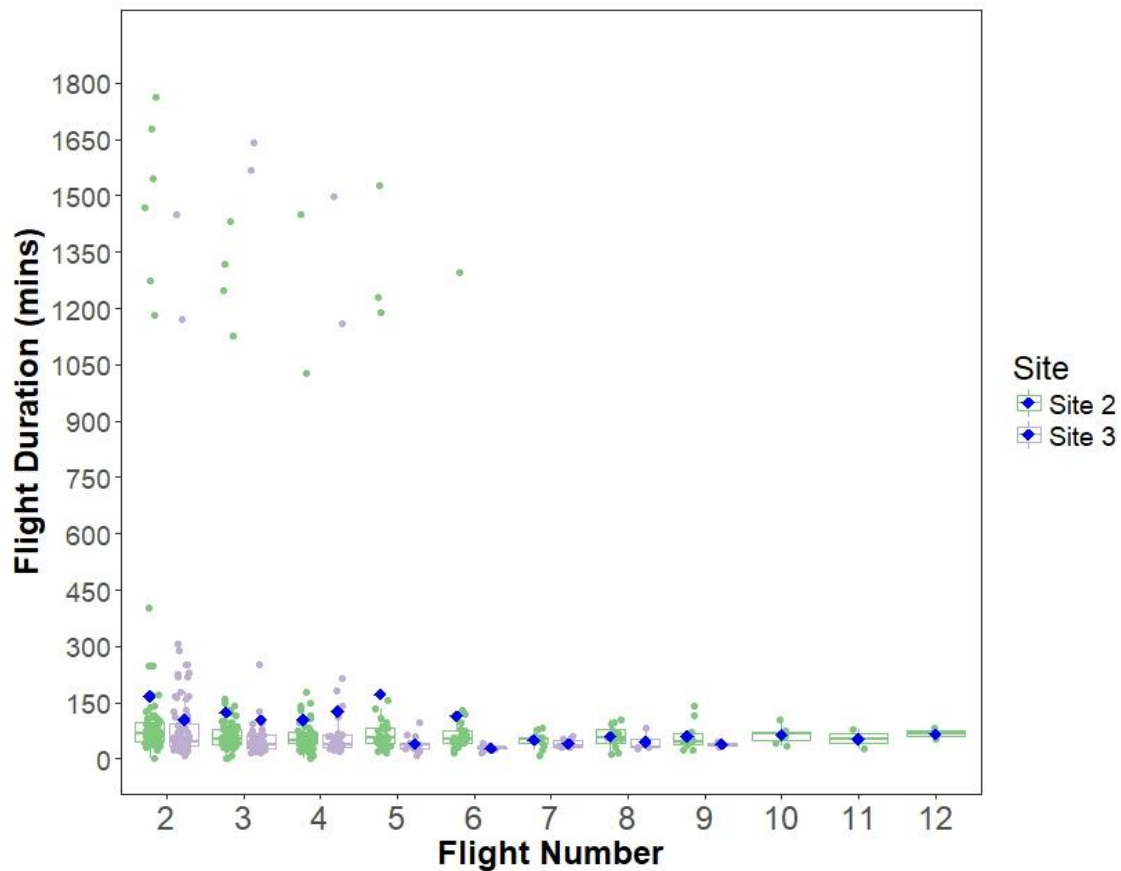
When comparing the flight duration of bumblebees throughout their second and subsequent flights outside the colony, there was no significant effect of the flight number on the duration of flights outside the colony (Table B.2; Figure B.2); and there was no significant effect of the experimental site on the flight duration (effect size: Table B.2; Figure B.2). There was also no significant interaction between the site and the flight number (Table B.2; Figure B.2).

These results mirror those observed in Part A.



**Figure B.2 |** Box and whiskers plot of the flight durations (minutes) for the second flight onwards for bumblebees tested at Site Two and Site Three. This does not include overnight flights.  $N=516$  total flights with  $n=308$  flights at Site Two and  $n=208$  flights at Site Three. Blue diamonds denote the mean. A small value of random noise was added to each data point for plotting purposes to aid visual representation.

### B.3.2 All Flight Durations Including Overnight Flights



**Figure B.3 |** Box and whiskers plot of the flight durations (minutes) for the second flight onwards for bumblebees tested at Site Two and Site Three. This includes overnight flights.  $N=538$  total flights with  $n=323$  flights at Site Two and  $n=215$  flights at Site Three. Blue diamonds denote the mean. A small value of random noise was added to each data point for plotting purposes to aid visual representation.

#### B.4. All Pollen Foraged Throughout the Experimental Period

In this case, the weight of all the pollen that was foraged throughout the experiment was analysed even if individual bumblebees differed in the number of flights that they took. The weight of all the pollen foraged was included even if there was not a complete record of each bumblebee's consecutive flights. This analysis was conducted in order to investigate whether limiting the analysis, to the first five flights of bumblebees, biased the results.

To determine whether the flight number outside the colony had an effect on pollen foraging, the relationship between the weight of pollen foraged on each flight and the number of flights taken was modelled using an LMM. As fixed effects, the experimental site and the flight number were inputted into the model with an interaction term. As random effects, the individual bumblebees were inputted as a random intercept (as a random slope model failed to converge). Post-hoc tests for estimated slopes for each site as well as differences between factors were also carried out ('emmeans' package; Lenth, 2019).

When comparing the weight of pollen foraged throughout all flights, there was a significant interaction between the site that the experiment took place and the flight number (Table B.2; Figure B.4). There was a significant effect of site on the weight of pollen foraged throughout all flights (Table B.2; Figure B.4). There was a significant effect of trip number on the weight of pollen foraged throughout all flights (Table B.2; Figure B.4).

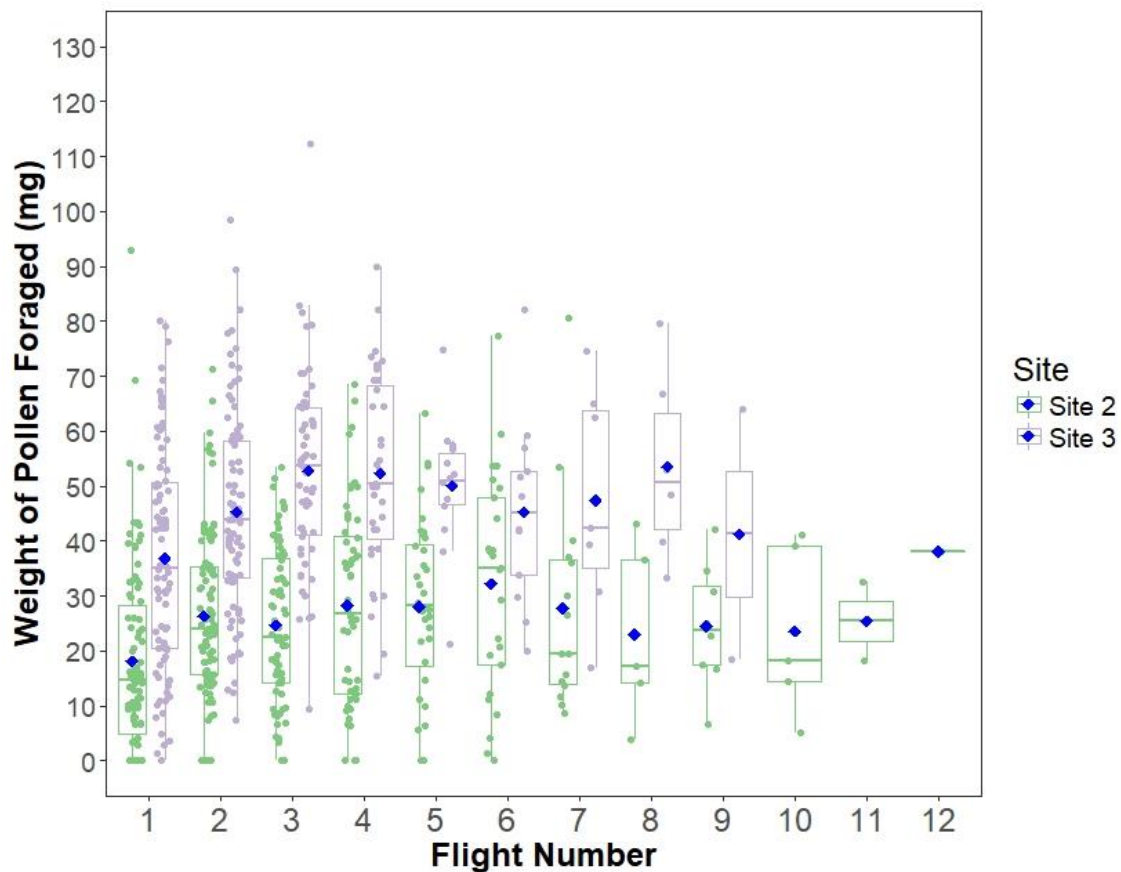
The weight of pollen foraged increased significantly at Site Three throughout all flights, but not at Site Two (Table B.3; Figure B.4; contrast estimate =  $2.32 \pm 0.95$ ,  $df = 701.00$ ,  $t\text{-ratio} = 2.45$ ,  $p = 0.015$ ).

**Table B.2. Model results from a linear mixed effects model testing a) the effect of the flight number and experimental site on the flight duration throughout all the flights performed during the sampling period and b) the effect of the flight number and experimental site on the weight of pollen foraged throughout all the flights performed during the sampling period. In both cases a random intercept model with individual bumblebee identity was used. Variables, effect sizes  $\pm$  standard error, degrees of freedom, *t*-values and *p*-values from the fitted model. Site Three was used as the reference level.**

<b>Variable(s)</b>	<b>Effect Size <math>\pm</math> Standard Error</b>	<b>Degrees of Freedom</b>	<b><i>t</i> Value</b>	<b><i>p</i> Value</b>
<b>a) Fight Duration</b>				
Site	-4.45 $\pm$ 7.67	348.83	-0.58	0.56
Flight Number	-4.01 $\pm$ 2.08	469.85	1.93	0.054
Flight Number * Site	2.53 $\pm$ 2.41	468.78	1.05	0.29
<b>b) Weight of Pollen Foraged</b>				
Site	-14.26 $\pm$ 3.33	548.40	-4.28	< 0.001
Flight Number	2.79 $\pm$ 0.78	691.85	3.57	0.0004
Flight Number*Site	-2.32 $\pm$ 0.95	700.58	-2.45	0.014

**Table B.3 Estimated slopes of the relationship between weight of pollen foraged and flight number for each site for all flights throughout the sampling period, degrees of freedom  $\pm$  standard error and upper and lower confidence intervals.**

<b>Site</b>	<b>Estimated Slope <math>\pm</math> Standard Error</b>	<b>Degrees of Freedom</b>	<b>Lower Confidence Interval</b>	<b>Upper Confidence Interval</b>
Site Two	0.47 $\pm$ 0.53	707.00	-0.58	4.34
Site Three	2.79 $\pm$ 0.79	694.00	1.25	1.52



**Figure B.4** | Box and whiskers plot of the weight of pollen foraged (mg) by bumblebees from their first flight onwards at Site Two and Site Three.  $N=711$  total pollen trips with  $n=407$  pollen trips at Site Two and  $n=304$  pollen trips at Site Three. Individual data points are superimposed on the plots. Blue diamonds denote the mean. A small value of random noise was added to each data point for plotting purposes to aid visual representation.



## Appendix C: Supplementary Data for Chapter Three: Part B

### C.1 RFID Flight Durations

**Table C.1. Number of released bumblebees by date from each colony at Site Two and Site Three for the RFID experiment.**

	<b>27/07/2016</b>	<b>28/07/2016</b>	<b>14/06/2017</b>
<b>Site Two Colonies</b>			
<b>1</b>	<b>70</b>	<b>56</b>	<b>N/A</b>
<b>2</b>	<b>17</b>	<b>29</b>	<b>N/A</b>
<b>3</b>	<b>15</b>	<b>35</b>	<b>N/A</b>
<b>4</b>	<b>36</b>	<b>69</b>	<b>N/A</b>
<b>Site Three Colonies</b>			
<b>5</b>	<b>N/A</b>	<b>N/A</b>	<b>54</b>
<b>6</b>	<b>N/A</b>	<b>N/A</b>	<b>100</b>
<b>7</b>	<b>N/A</b>	<b>N/A</b>	<b>44</b>

**Table C.2. Number of bumblebees which were released and then marked with an RFID tag on their return in each of the seven colonies across both experimental sites, as well as the number of those for which a complete record of their flight durations and overnight flights exists. The number of bumblebees used in the ‘Duration’ analysis is also shown.**

	<b>Number of bumblebees released</b>	<b>Number of bumblebees that returned and were RFID tagged</b>	<b>Number of bumblebees that have a record of the duration of their first five flights</b>	<b>Number of bumblebees with five flights that had an overnight flight</b>	<b>Number of bumblebees with five flights that were used in the ‘Duration’ Analysis</b>
<b>Site Two Colonies</b>					
<b>1</b>	126	59	15	3	12
<b>2</b>	46	16	1	0	1
<b>3</b>	50	30	5	0	5
<b>4</b>	105	48	11	0	11
<b>Site Three Colonies</b>					
<b>5</b>	154	49	0	N/A	0
<b>&amp; 6</b>					
<b>7</b>	44	26	1	0	0

## Appendix D: Supplementary Data for Chapter Four

**Table D.1. Number of RFID tagged bumblebees which were transported to each release location as well as the number tagged bumblebees that were subsequently used in the ‘Proportion of Returns’ analysis. The total number of released bumblebees that returned to their colony, electronically logged by the RFID equipment or later found inside the colony. Number of released bumblebees that returned to their colony and that were electronically logged. The number of returning bumblebees that were electronically logged and which stayed out overnight before returning is also shown.**

Site	Number of RFID tagged bumblebees Transported to each release location	Number of tagged bumblebees used in the ‘Proportion of Returns’ analysis <sup>13</sup>	Total number of released bumblebees that returned to their colony (electronically logged or found inside the colony)	Number of released bumblebees that returned to their colony and that were electronically logged <sup>14</sup>	Number of returning bumblebees that were electronically logged and which stayed out overnight before returning (separated by release distance)
RA	128	85	53	52	8 (1,5,2)
RB	150	143	101	87	21 (3,9,9)
UA	144	138	82	73	31 (8,11,12)
UB	128	115	47	29	12 (7,5,0)

<sup>13</sup> In some cases, tagged bumblebees died in transport while others did not display normal flying behaviour when released. In addition, due to experimental error, some bumblebees were released more than once.

<sup>14</sup> For 14.8% of returning bumblebees, the RFID equipment failed to log them and register their return even though they were later found inside the colony. If the RFID equipment failed to scan their return, these bumblebees did not have a record of the duration of their return flight.

**Table D.2. Number of RFID tagged bumblebees released from each cardinal point at each experimental site and for release distance. The number of bumblebees that returned is also shown.**

<b>Site</b>	<b>Distance (m)</b>	<b>Cardinal point</b>	<b>Number of RFID tagged bumblebees released</b>	<b>Number of released bumblebees that returned to their colony</b>
<b>RA</b>	300	East	3	3
	300	North	7	7
	300	South	12	12
	300	West	5	5
	1000	East	3	2
	1000	North	4	2
	1000	South	10	8
	1000	West	7	6
	2500	East	9	2
	2500	North	12	4
	2500	South	8	1
	2500	West	5	1
<b>RB</b>	300	East	13	11
	300	North	15	12
	300	South	15	13
	300	West	12	11
	1000	East	N/A	N/A
	1000	North	15	12
	1000	South	14	13
	1000	West	15	10
	2500	East	N/A	N/A
	2500	North	14	8
	2500	South	15	6
	2500	West	15	5
<b>UA</b>	300	East	11	10
	300	North	12	9

<b>Site</b>	<b>Distance (m)</b>	<b>Cardinal point</b>	<b>Number of RFID tagged bumblebees released</b>	<b>Number of released bumblebees that returned to their colony</b>
	300	South	10	8
	300	West	11	10
	1000	East	11	6
	1000	North	12	10
	1000	South	12	7
	1000	West	12	8
	2500	East	12	5
	2500	North	12	7
	2500	South	12	0
	2500	West	11	2
<b>UB</b>	300	East	10	5
	300	North	15	7
	300	South	9	8
	300	West	11	6
	1000	East	10	3
	1000	North	10	6
	1000	South	9	5
	1000	West	11	2
	2500	East	10	1
	2500	North	10	3
	2500	South	N/A	N/A
	2500	West	10	1

## Appendix E: Supplementary Data for Chapter Five

**Table E.1. Number of Tagged bumblebees which were released from each release location based on the number of flights that they experienced prior to release. The number of bumblebees that returned, the number of bumblebees that had record of returning, the number of bumblebees that stayed overnight before returning and the number of bumblebees that foraged for pollen before returning are also shown.**

Distance (m)	Cardinal point	Number of flights prior to release	Number of released bumble- bees	Number of returned bumble- bees	Number of returned bumble- bees with a record of the duration of their returning flight	Number of returning bumble- bees that stayed out overnight before returning	Number of returning bumble- bees that foraged for pollen before returning
300	East	1	15	13	13	3	5
300	East	2	14	12	12	0	3
300	East	5	13	13	13	0	11
1000	East	1	15	5	5	3	1
1000	East	2	14	5	5	2	1
1000	East	5	11	2	2	0	1
300	West	1	15	12	12	3	3
300	West	2	16	15	15	0	7
300	West	5	11	11	11	0	5
1000	West	1	16	7	7	3	3
1000	West	2	15	12	12	1	1
1000	West	5	9	9	9	1	2

**Table E.2. Number of released bumblebees at each release location and for each experimental date.**

<b>Date</b>	<b>300 m east</b>	<b>300 m west</b>	<b>1000 m east</b>	<b>1000 m west</b>
23/08/2017	N/A	15	N/A	N/A
24/08/2017	N/A	10	N/A	22
25/08/2017	7	9	N/A	17
26/08/2017	1	8	18	1
27/08/2017	18	N/A	13	N/A
28/08/2017	16	N/A	4	N/A
29/08/2017	N/A	N/A	5	N/A

## Appendix F: Supplementary Data for Chapter Six

### F.1 Experimental Testing Schedule, Proportion of Foraging Gynes and Colony Characteristics

**Table F.1. Experimental testing schedule for each colony showing the individual colonies, the condition they were assigned to, the dates that they were tested on as well as the experimental site at which testing took place.**

Colony	Condition	Dates	Site
1	1	21 <sup>st</sup> , 22 <sup>nd</sup> , 23 <sup>rd</sup> , 24 <sup>th</sup> June, 2016	Site A
2	1	3 <sup>rd</sup> , 4 <sup>th</sup> , 5 <sup>th</sup> , 8 <sup>th</sup> June 2016	Site B
3	1	11 <sup>th</sup> , 12 <sup>th</sup> , 13 <sup>th</sup> , 14 <sup>th</sup> June 2016	Site A
4	1	11 <sup>th</sup> , 12 <sup>th</sup> , 13 <sup>th</sup> , 14 <sup>th</sup> June 2016	Site A
5	1	11 <sup>th</sup> , 12 <sup>th</sup> , 13 <sup>th</sup> , 14 <sup>th</sup> June 2016	Site A
6	1	22 <sup>nd</sup> , 23 <sup>rd</sup> , 24 <sup>th</sup> June 2016	Site A
7	1	22 <sup>nd</sup> , 23 <sup>rd</sup> , 24 <sup>th</sup> June 2016	Site A
8	2	5 <sup>th</sup> , 6 <sup>th</sup> , 7 <sup>th</sup> , 8 <sup>th</sup> July 2016	Site B
9	2	1 <sup>st</sup> , 2 <sup>nd</sup> , 3 <sup>rd</sup> , 4 <sup>th</sup> June 2017	Site B
10	2	1 <sup>st</sup> , 2 <sup>nd</sup> , 3 <sup>rd</sup> , 4 <sup>th</sup> June 2017	Site B
11	3	15 <sup>th</sup> , 16 <sup>th</sup> , 17 <sup>th</sup> , 18 <sup>th</sup> September 2016	Site B
12	3	15 <sup>th</sup> , 16 <sup>th</sup> , 17 <sup>th</sup> , 18 <sup>th</sup> September 2016	Site B
13	3	14 <sup>th</sup> , 15 <sup>th</sup> , 16 <sup>th</sup> , 17 <sup>th</sup> September 2016	Site B
14	3	15 <sup>th</sup> , 16 <sup>th</sup> , 17 <sup>th</sup> , 18 <sup>th</sup> September 2016	Site B



Colony	Condition	Dates	Site
15	3	20 <sup>th</sup> , 21 <sup>st</sup> , 22 <sup>nd</sup> , 23 <sup>rd</sup> September 2016	Site B
16	3	20 <sup>th</sup> , 21 <sup>st</sup> , 22 <sup>nd</sup> , 23 <sup>rd</sup> September 2016	Site B
17	3	9 <sup>th</sup> , 10 <sup>th</sup> , 12 <sup>th</sup> , 13 <sup>th</sup> July 2017	Site B
18	3	9 <sup>th</sup> , 10 <sup>th</sup> , 12 <sup>th</sup> , 13 <sup>th</sup> July, 2017	Site B

**Table F.2.** The number of gynes that left the colony, the number of gynes that returned to the colony, the proportion of returning gynes, the number of gynes that returned with pollen loads and the proportion of returning gynes that returned with pollen loads for each colony and experimental site are shown. Whether or not gynes were present in the colony that did not leave during the experiment as well as the testing dates for each colony are also shown.

Colony	Condition	Number of gynes that left the colony	Number of gynes that returned at some point during the experiment	Proportion of gynes Returned	Number of gynes returned with pollen loads	proportion of returning gynes with pollen loads	Gynes present that did not leave the colony (where known)	Experimental date	Site
1	1	22	13	0.60	10	0.76	Yes	11-14/06/2016	Site A
2	1	5	3	0.6	2	0.67	Yes	3-5,8/06/2016	Site B
3	1	10	8	0.8	2	0.25	Yes	11-14/06/2016	Site A
4	1	11	6	0.55	6	1	N/A	11-14/06/2016	Site A
5	1	5	5	1	4	0.8	Yes	11-14/06/2016	Site A
6	1	15	8	0.53	7	0.87	Yes	22-24/06/2016	Site A
7	1	10	10	1	10	1	No	22-24/06/2016	Site A

Colony	Condition	Number of gynes that left the colony	Number of gynes that returned at some point during the experiment	Proportion of gynes returned	Number of gynes returned with pollen loads	Proportion of returning gynes with pollen loads	Gynes present that did not leave the colony (where known)	Experimental date	Site
8	2	10	4	0.4	4	1	Yes	5-8/07/2016	Site B
9	2	5	4	0.8	4	1	N/A	1-4/06/2017	Site B
10	2	3	2	0.67	2	1	N/A	1-4/06/2017	Site B
11	3	8	3	3.75	2	0.67	No	15-18/09/2016	Site B
12	3	4	1	0.25	0	0	Yes	15-18/09/2016	Site B
13	3	11	9	0.81	2	0.22	Yes	14-17/09/2016	Site B
14	3	1	1	1	1	1	N/A	15-18/09/2016	Site B
15	3	12	12	1	6	0.5	No	20-23/09/2016	Site B
16	3	5	5	1	3	0.6	N/A	20-23/09/2016	Site B
17	3	1	0	0	0	0	N/A	9-13,no11,2017	Site B
18	3	2	1	0.5	0	0	N/A	9-13,no11,2017	Site B

**Table F.3.** The weight of the colony, the total number of workers, gynes and larvae in the colony are shown. The proportion of returning gynes with pollen loads, the larva/worker ratio and the site are also shown.

Colony	Condition	Colony weight (g)	Total number of workers in the colony	Total number of gynes in the colony	Total number of larvae in the colony	Proportion of returning gynes with pollen loads	Larva/Worker ratio	Site
1	1	46.83	180	29	11	0.76	0.061	Site A
2	1	26.76	250	5	16	0.67	0.064	Site B
3	1	34.09	151	26	38	0.25	0.251	Site A
5	1	41.42	256	8	28	0.8	0.109	Site A
6	1	32.71	158	14	13	0.87	0.082	Site A
7	1	20.03	132	8	27	1	0.204	Site A
8	2	30.06	180	12	9	1	0.05	Site B
11	3	28.54	174	3	N/A	0.67	N/A	Site B
12	3	33.25	154	3	N/A	0	N/A	Site B
13	3	20.47	144	10	N/A	0.22	N/A	Site B
15	3	38.05	224	11	N/A	0.5	N/A	Site B

## F.2 Analysis Without The Outlier (Log(Body Weight) <1)

In order to determine the influence of the outlier ( $\log(\text{body weight}) < 1$ ), the analysis that was performed in Section 6.4.2: Confirming Caste Differentiation was also performed using a data set that excluded the outlier. An ANCOVA was used to investigate the relationship between each trait measured (body length, thorax width, wing bud distance, wing length and wingspan) and body weight for the visually assigned castes. The explanatory variables used were body weight (entered as a numeric variable) and caste, and the interaction term was included. Visual inspection was used to check residual plots for fit and homoscedasticity.

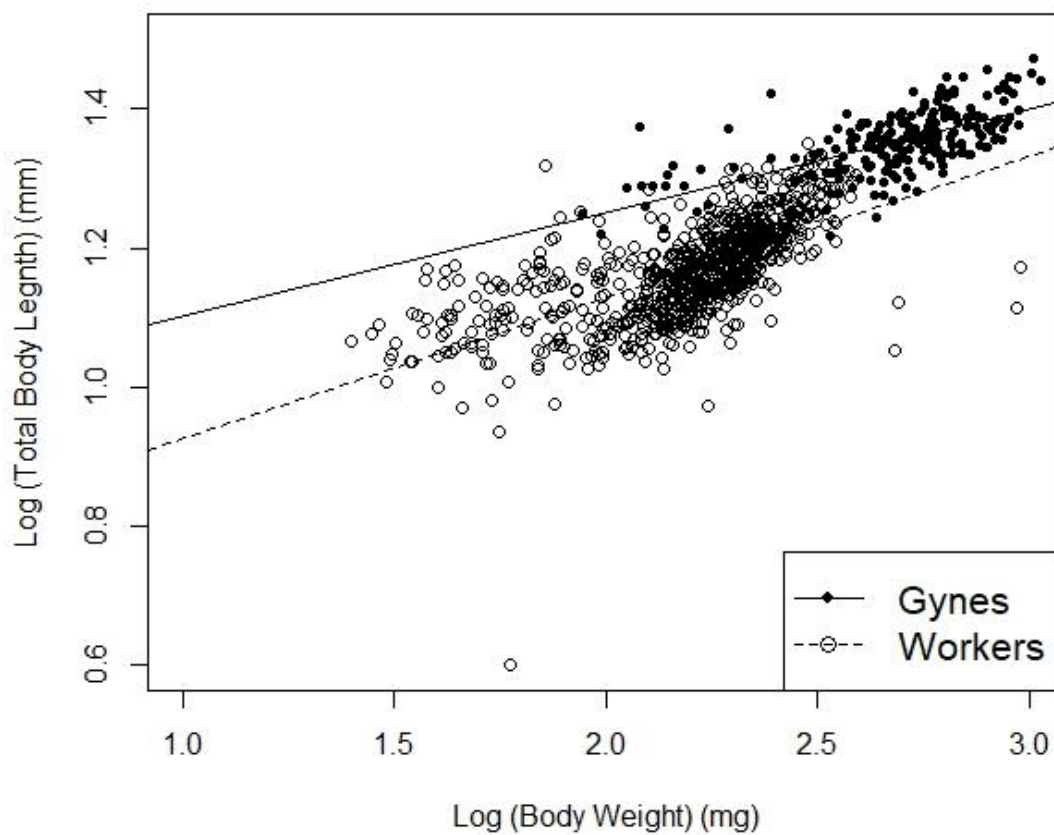
The following results and the conclusions that are derived from them are very similar to those with the outlier included (Section 6.5.2: Confirming Caste Differentiation).

Mirroring the results of the analysis with the outlier, the intercept for the slope for each caste was significantly different ('Caste' effect in Table F.4). For two traits (body length and wing bud distance), there was also a significant interaction between caste and body weight, suggesting the allometric scaling relationship of these traits also varied between the assigned castes.

**Table F.4. Model results from a linear model, ANCOVA, testing the relationship between the log a) body length, b) thorax width, c) wing bud distance, d) wing length and e) total wingspan and log body weight for each caste using a data set without the outlier ( $\log(\text{body weight}) < 1$ ). Variables, effect sizes  $\pm$  standard error,  $t$ -values and  $p$ -values from the fitted model. The ‘gyne’ caste was used as the reference level [‘Intercept’ values denote if the gyne intercept is significantly different from zero and ‘Caste’ values denote if the worker caste intercept differed significantly from the gyne caste intercept].**

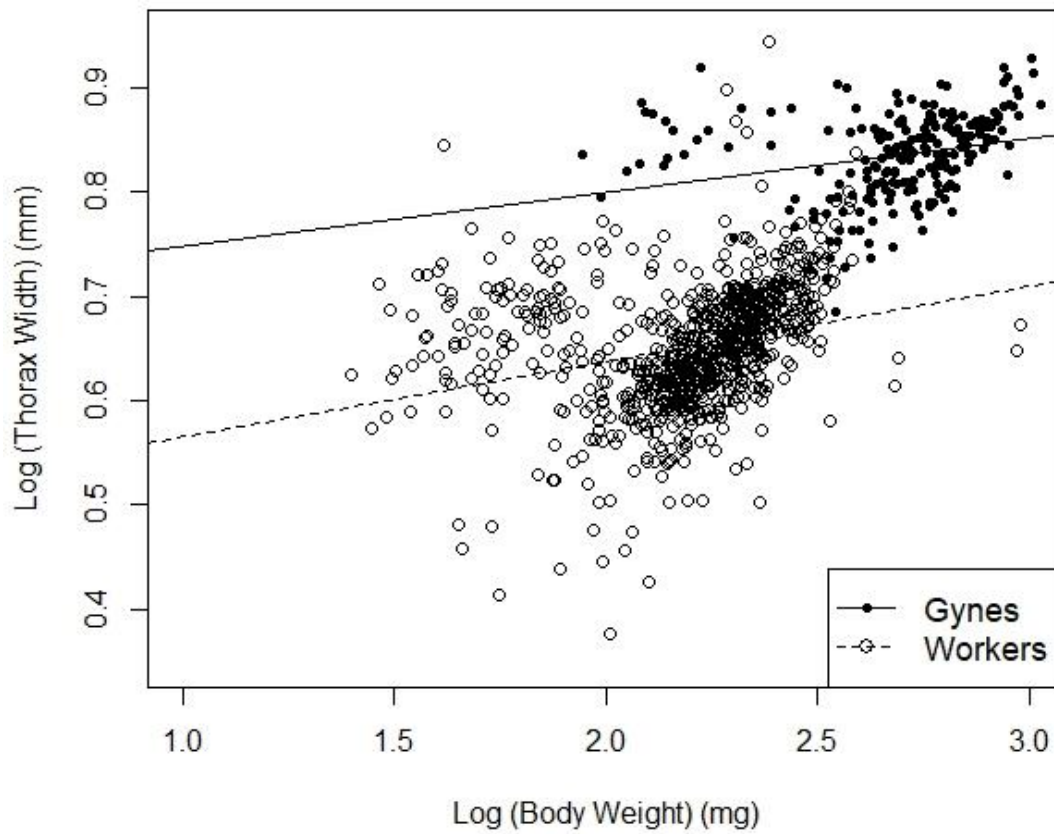
<b>Variable(s)</b>	<b>Effect Size <math>\pm</math> Standard Error</b>	<b><math>t</math> Value</b>	<b><math>p</math> Value</b>
a) Body Length			
Intercept	0.95 $\pm$ 0.04	21.43	<0.0001
Body Weight	0.15 $\pm$ 0.01	9.01	< 0.0001
Caste	-0.23 $\pm$ 0.05	-4.85	<0.0001
Body Weight * Caste	0.05 $\pm$ 0.02	2.97	0.003
b) Thorax Width			
Intercept	0.70 $\pm$ 0.05	14.35	<0.0001
Body Weight	0.05 $\pm$ 0.02	2.89	0.004
Caste	-0.20 $\pm$ 0.05	-3.87	0.0001
Body Weight * Caste	0.02 $\pm$ 0.02	1.00	0.32
c) Bud Distance			
Intercept	0.70 $\pm$ 0.06	11.19	<0.0001
Body Weight	0.04 $\pm$ 0.02	1.6	0.11
Caste	-0.31 $\pm$ 0.07	-4.67	<0.0001
Body Weight * Caste	0.06 $\pm$ 0.03	2.17	0.03
d) Wing Length			
Intercept	0.96 $\pm$ 0.05	19.82	<0.0001
Body Weight	0.08 $\pm$ 0.02	4.56	<0.0001

<b>Variable(s)</b>	<b>Effect Size <math>\pm</math> Standard Error</b>	<b>t Value</b>	<b>p Value</b>
Caste	-0.18 $\pm$ 0.05	-3.39	0.0007
Body Weight *	0.02 $\pm$ 0.02	1.09	0.28
Caste			
e) Total			
Wingspan			
Intercept	1.26 $\pm$ 0.05	26.03	<0.0001
Body Weight	0.08 $\pm$ 0.02	4.57	<0.0001
Caste	-0.17 $\pm$ 0.05	-3.39	0.0007
Body Weight *	0.02 $\pm$ 0.02	1.09	0.28
Caste			

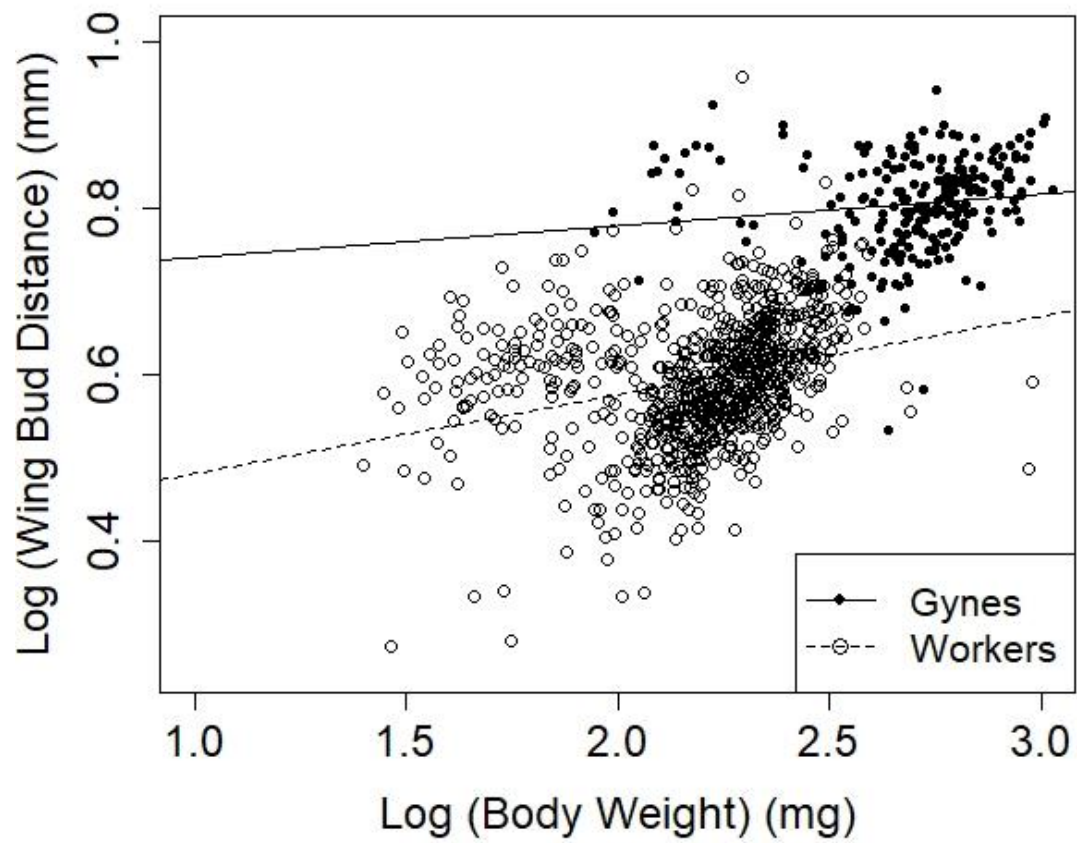


**Figure F.1.i** | Allometric (log-log) plot of total body length (mm) plotted against body weight (mg).  $n=221$  gynes and  $n=814$  workers taken from 16 colonies.

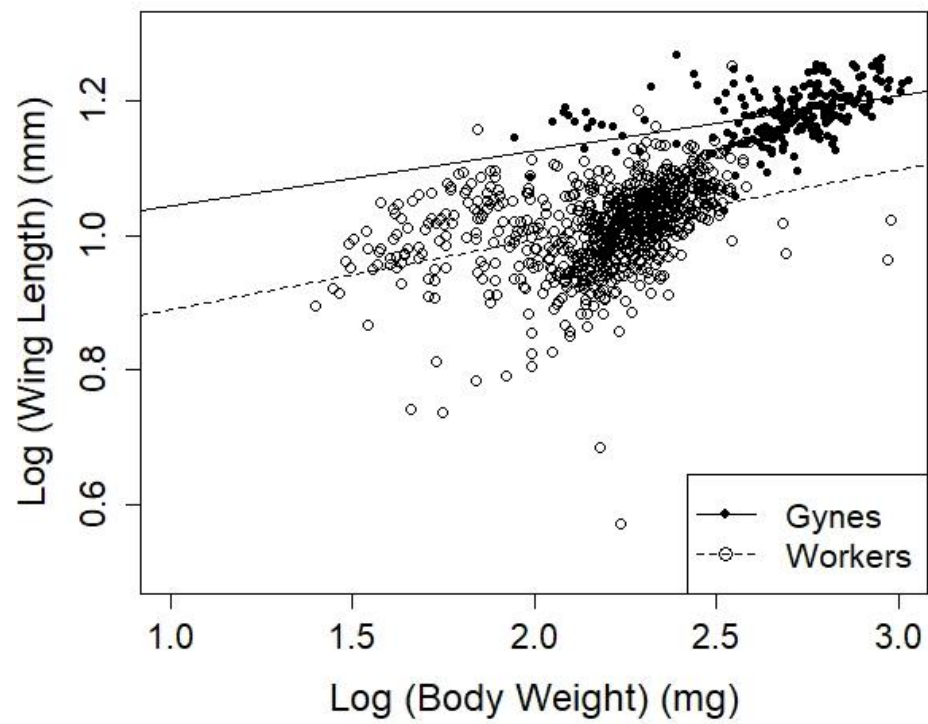




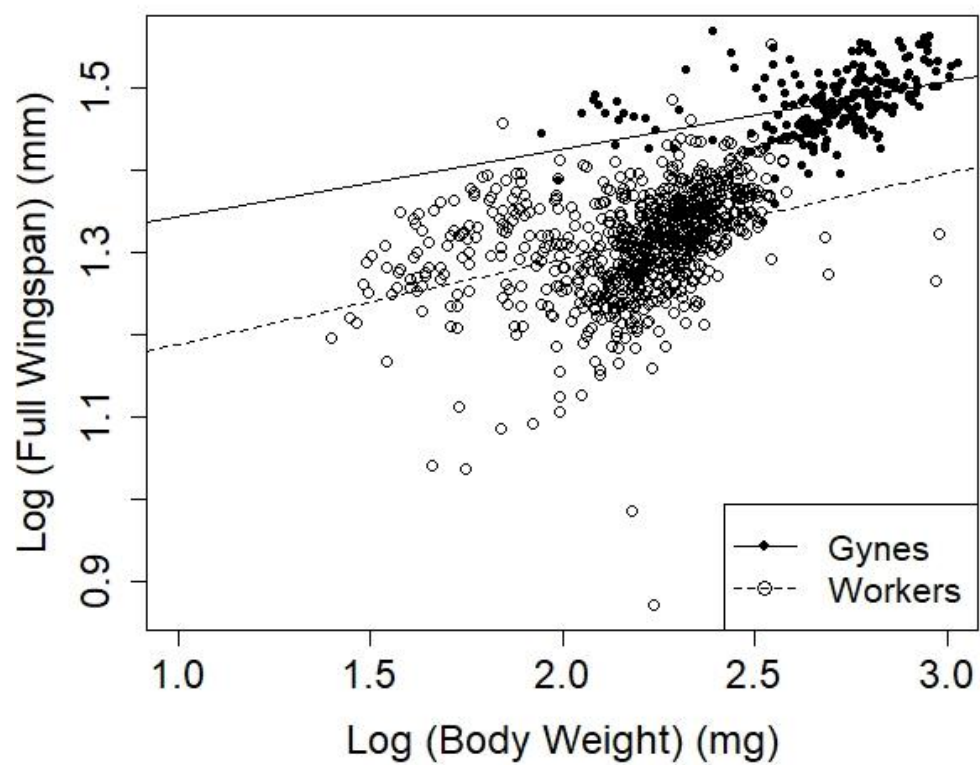
**Figure F.1.ii** | Allometric (log-log) plot of thorax width (mm) plotted against body weight (mg).  $n=221$  gynes and  $n=814$  workers taken from 16 colonies.



**Figure F.1.iii** | Allometric (log-log) plot of wing bud distance (mm) plotted against body weight (mg).  $n=221$  gynes and  $n=814$  workers taken from 16 colonies.



**Figure F.1.iv** | Allometric (log-log) plot of wing length (mm) plotted against body weight (mg).  $n=221$  gynes and  $n=814$  workers taken from 16 colonies.



**Figure F.1.v** | Allometric (log-log) plot of full wingspan (mm) plotted against body weight (mg).  $n=221$  gynes and  $n=814$  workers taken from 16 colonies.

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