The role of *Crithidia bombi* and commercial bumblebee colonies in pollination

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Abstract

Bumblebees provide crucial pollination services to crops and wild plants. They also play host to a variety of parasites. It is not known whether such parasites impact upon the pollination services that bees are providing. Commercially-reared bumblebee colonies, which are used around the globe to supplement crop pollination, have been shown to harbour high levels of parasites, which can spread to wild-bee populations. Despite such negative impacts, commercial bees are widely used on many different crop species. However, for many of these crops, the benefit commercial colonies provide has not been assessed. Furthermore, we do not have a full understanding of the role that commercial colonies play in the parasite epidemiology of wild bee populations. In this thesis I begin to fill these knowledge gaps.

My results show that the common bumblebee parasite *Crithidia bombi* did not affect the olfactory learning ability and foraging activity of the bumblebee *Bombus terrestris*. However, I was unable to conclude whether this parasite impacts upon pollination services. In addition, I demonstrated that commercial colonies improve the quality and value of a strawberry crop on a farm setting, but that this benefit is only observed at certain times of the year. These same colonies became infected with parasites likely to have been acquired from wild-bee populations, and the prevalence of these parasites was also found to vary temporally. Finally, I showed that altering the concentration and availability of the commercial bumblebees' nectar reservoir can significantly affect their foraging activity.

The results have important implications for the use and management of commercial bumblebees and could help reduce environmental damage caused by commercial colony use. I have also further increased our knowledge on the impacts of *C. bombi* on *B. terrestris*, and gained novel insights of the parasite prevalence in commercial colonies in a farm setting.

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Introduction

Ecosystem services and animal behaviour

Ecosystem services are the benefits humans obtain from ecosystems and their functioning (Costanza et al. 1997, 2017; Millennium Ecosystem Assessment (MA) 2005). Examples of such services include the provision of clean drinking water, flood regulation and pollination (Costanza et al. 1997, 2017; MA 2005). Recognition that the environment can provide human society with goods and services can be traced as far back as the ancient Greeks, who realised that forest cover plays an important role in the retention of soil (Fisher et al. 2009). However, it was only relatively recently, during the 1970/80s, that attempts began to be made to quantify the economic importance of these services (DeLorme et al. 1974; Westman 1977; Costanza et al. 1989). The modern concept of ecosystem services is now well established and they were a primary focus of the Millennium Ecosystem Assessment (MA 2005). Since then, the popularity of the concept has grown rapidly, with the number of journal articles addressing ecosystem services and attempting to place monetary value on them rising exponentially (Fisher et al. 2009). Today, ecosystem services are a major tool in global environmental policy, with the Intergovernmental Science-Policy Platform on Biodiversity and Ecosystem Services (IPBES) being the latest global scale assessment of ecosystem services currently ongoing (IPBES 2012; Costanza et al. 2017).

The provision of ecosystem services is based on ecosystem functioning, i.e., biotic and abiotic interactions (Naeem et al. 1994; Dobson et al. 2006; Tylianakis et al. 2010; Chapin et al. 2011; Valiente-Banuet et al. 2015). The nature of such biotic interactions is dependent on the functional traits displayed by the species involved (Hooper et al. 2005; Díaz et al. 2013; Cadotte 2017). A behavioural trait is one of many characteristics of a species that can be classified as a functional trait, and thus can impact upon ecosystem functioning (de Bello et al. 2010; Díaz et al. 2013). For example, herbivore foraging behaviour can alter plant abundances, which could have far-reaching effects on many trophic levels, ultimately altering ecosystem function. However, behavioural traits are not fixed and there can be much plasticity around behavioural traits between individuals of the same species (Dall et al. 2004), and the behavioural traits of one species can be manipulated by another (Moore

2013). This variation in behavioural traits can influence the properties and functioning of an ecosystem (Sih et al. 2012), and subsequently the ecosystem services supplied.

In addition, the regularity at which a behaviour is performed could also influence ecosystem function. This is dependent on how frequently individuals perform a particular behaviour, and on the total abundance of individuals that can perform the behaviour. Thus, if the abundance of a particular species falls to a low level, the interactions that it was involved in may become extinct, which will have impacts on ecosystem functioning and the subsequent services derived (Valiente-Banuet et al. 2015).

A classic example of an ecosystem service is animal-mediated pollination. This interaction between plant and pollinator is crucial for the maintenance of biodiversity, with an estimated 87.5% of flowering plants reliant to some extent on animal pollination for reproduction (Ollerton et al. 2011), and of vital importance for human food production and well-being (Klein et al. 2007; Eilers et al. 2011; Kleijn et al. 2015; Rader et al. 2016). This service can be provided by a wide variety of species including mammals (Fleming et al. 2009), birds (Stiles 1978) and reptiles (Traveset & Sáez 1997), but in most circumstances, it is carried out by insects (Ollerton 2017). Within insects, bees are considered one of the most important pollinator groups (Calderone 2012; Kleijn et al. 2015; Rader et al. 2016). This is partially down to the global management of relatively few bee species to provide pollination in agricultural systems. Honeybees (*Apis mellifera*) are the dominant managed pollinator globally (Klein et al. 2007; Calderone 2012), but commercially reared bumblebee colonies are becoming increasingly important and they are particularly effective pollinators of certain crop types (Stanghellini et al. 1997, 1998; Velthuis & van Doorn 2006; Zhang et al. 2015; Aizen et al. 2018).

Given how important insects, both wild and managed, are as pollinators, any alteration to their foraging behaviour could have major implications for the pollination services that they provide, consequently impacting upon plant reproduction and crop yields. Therefore, it is of key importance that we understand the drivers of insect foraging behaviour and how they might be influencing interactions between plant and pollinator.

Mechanisms by which parasites may alter ecosystem functioning

One such factor that can alter the behaviour of insects and many other organisms is parasitism. Parasites are ubiquitous in nature, with approximately 50% of all animal species thought to be parasitic (Poulin & Morand 2000; Dobson et al. 2008). There are numerous documented examples of parasitic infections altering the behaviour of their hosts (reviewed in Moore 2002, 2013; Lafferty & Shaw 2013), and these vary widely in the particular behaviour that is manipulated, and the extremity of the behavioural alteration (reviewed in Moore 2002, 2013). Such changes can alter ecosystem functioning as in Sato et al., (2012) where crickets (*Diestrammena* spp.) were manipulated by a nematomorph parasite to enter streams, in which the parasite can reproduce. The crickets entering the streams provided predatory fish with a significant alternative food source, which then lead to a cascading effect through numerous trophic levels and a subsequent change in ecosystem functioning. Given that parasites are able to alter ecosystem functioning, it is likely that they can also change the provision of ecosystem services, however this has not yet been demonstrated.

In addition to inducing behavioural change, parasites may also alter ecosystem functioning by inducing mortality in their host organisms. It is well established that parasites can increase the mortality of their hosts (Anderson & May 1978; Gulland 2008), and in some cases parasites have been associated with severe population declines and extinctions (Warner 1968; Daszak et al. 2000; Cameron et al. 2011). Such declines in species abundance lead to the reduction and even extinction of ecological interactions that the declining species was involved in, and subsequent alteration to ecosystem function and the ecosystem services provided (Valiente-Banuet et al. 2015). Thus, we may expect parasite-induced mortality, in addition to parasite-induced behavioural change, to have an impact upon ecosystem services.

Pollinating insects, as well as providing important ecosystem services, are known to play host to a variety of parasites (Schmid-Hempel 1998), and both behavioural change (reviewed in Gómez-Moracho et al. 2017) and increased mortality have been observed upon infection with these parasites (Altizer & Oberhauser 1999; Brown et al. 2000; Brown, Schmid-Hempel, et al. 2003; Higes et al. 2007; Otti & Schmid-Hempel 2007; Graystock, Meeus, et al. 2016). Thus, both potential

mechanisms by which parasitism may alter ecosystem function and services are active in pollinator populations. Parasite-induced behavioural changes that have been observed in pollinators that could impact upon pollination services include: reduced navigational ability, altered flower choices, impaired olfactory learning, impaired flight ability, impaired visual and motor learning skills, and precocious onset of foraging activity (Gómez-Moracho et al. 2017). For example, early work in this research area by Schmid-Hempel & Schmid-Hempel (1990) found that bumblebees infected with a conopid fly larva (Conopidae) were preferentially visiting a flower species with less complex morphology than uninfected bees. The authors suggest that if such parasites were present at a high prevalence, they could be affecting the plant community structure by increasing the reproductive success of the simpler species (Schmid-Hempel & Schmid-Hempel 1990). However, thus far it has only been speculated as to how such changes might affect pollination, and to my knowledge no experiments have attempted to address this knowledge gap.

For parasite-induced mortality the picture is clearer, as reduced pollinator abundance is likely to lead to fewer mutualistic plant-pollinator interactions occurring, and thus, reduced pollination. However, mortality effects vary widely depending on the parasite species and whether it is in combination with other stressors (Brown et al. 2000; Brown, Schmid-Hempel, et al. 2003; Rutrecht & Brown 2008; Nazzi et al. 2012; Aufauvre et al. 2012), meaning that predicting the extent to which a plant-pollinator interaction might decrease will be a complex task.

To the best of my knowledge, the only studies to have investigated the effects that parasites of pollinators may have on pollination services, have not found a causal link between the two. Gillespie and Adler (2013) observed a negative relationship between a common bumblebee parasite, *Nosema bombi*, and the pollination of bumblebee dependent plants, however, no clear trend was seen in the other bumblebee parasites that they detected. Another study found there was no relationship between pollination services and *Crithidia bombi* (another common bumblebee parasite) prevalence in a bumblebee population (Theodorou et al. 2016). A potential confounding factor here was that *C. bombi* prevalence was positively associated with bumblebee abundance, which itself was mediating the pollination success of plants (Theodorou et al. 2016). Thus, neither study can conclude whether or not parasitism was directly altering plant pollination.

The two studies mentioned used bumblebees as their focal species, and indeed most research on disease in pollinators has focussed on bees. This is partly due to their importance as wild and managed pollinators, as previously stated, and partly as they have been observed to be in decline and/or suffering from elevated levels of mortality (Williams 1982; Grixti et al. 2009; vanEngelsdorp & Meixner 2010; Cameron et al. 2011; Dupont et al. 2011; van der Zee et al. 2012). Parasites have been implicated as a potential cause of bumblebee declines in some areas of the world (Cameron et al. 2011; Meeus et al. 2011; Schmid-Hempel et al. 2014), yet despite this, no causal link has been made between the presence of parasites in bees and an alteration in the important pollination services that bees provide.

Commercial bumblebees

A major factor thought to be contributing to wild bumblebee decline in many areas, is the international trade in commercial bumblebee colonies (Colla et al. 2006; Meeus et al. 2011; Schmid-Hempel et al. 2014). This trade, started in the 1980s, involves the production of bumblebee colonies in rearing facilities, the colonies are then exported around the world including into areas where the commercial species is not native (Velthuis & van Doorn 2006). The colonies are then placed into glasshouse, polytunnel and open field crops with the purpose of supplementing pollination services to the crops to improve yield and quality (Stubbs & Drummond 2001; Morandin et al. 2001a, 2001b; Velthuis & van Doorn 2006; Zhang et al. 2015). However, it is common for commercial bumblebees to leave their target crop and forage elsewhere, and for wild bees to come onto crops where commercial bees are placed (Murray et al. 2013; Foulis & Goulson 2014). It is when this happens that problems may arise.

Three mechanisms have been identified by which the commercial trade of bumblebees may be contributing to wild bee declines. Firstly, it is possible for commercial bumblebees to mate with wild bumblebee species (Ings et al. 2005; Kanbe et al. 2008). If this process leads to the production of viable eggs and offspring, as may be the case when different subspecies of the same species mate, then it could be contributing to biodiversity homogenisation, as it may reduce the chance of future speciation between subspecies. As of yet there is no empirical evidence to support this. However, if the eggs produced from such mating events

are not viable, as has been observed in one case (Kondo et al. 2009), then the mating represents a serious threat to wild bumblebee populations as queens usually only mate once (Schmid-Hempel & Schmid-Hempel 2000).

Secondly, commercial bumblebees may be able to outcompete native species. When placed out in the field, commercial colonies foraged more efficiently and produced a greater number of gynes than native wild colonies (Ings et al. 2006), and in Japan, an invasive commercial *Bombus terrestris* population is thought to displace a native species via competition for nest sites (Inoue et al. 2008).

The third and most well studied negative impact that the trade in commercial bumblebees has on wild bee populations is pathogen transmission. Commercial colonies have repeatedly been shown to harbour pathogens (Whittington & Winston 2003; Otterstatter et al. 2005; Goka et al. 2006; Murray et al. 2013; Graystock et al. 2013), and when the colonies are placed into crops, these pathogens may spread to wild bee populations via shared use of flowers (Durrer & Schmid-Hempel 1994; Graystock et al. 2015). This process is known as pathogen 'spill-over' and there is strong evidence of it occurring in bumblebee populations (Colla et al. 2006; Murray et al. 2013; Graystock et al. 2014). Spill-over can be particularly damaging if the wild bee community has not encountered or coevolved with the pathogen species or strains that the commercial colonies are carrying. In such cases the pathogen may be more virulent than in coevolved populations (Imhoof & Schmid-Hempel 1998a; Thompson 2005). In other systems, spill-over of novel pathogens from managed populations to wild populations has been highly destructive (Daszak et al. 2000), for example, imported Asian cattle (*Bos indicus*) transmitted the rinderpest virus to African buffalo (*Syncerus caffer*), resulting in the loss of 90% of the buffalo population (Mack 1970).

A case study from South America illustrates well the negative impacts of the trade in commercial bumblebees (Schmid-Hempel et al. 2014). Here, *B. terrestris* was imported into Chile, where it is not a native species, for greenhouse pollination. It subsequently escaped from greenhouses and became established in the wild, where it has quickly spread into and across Argentina (Torretta et al. 2009; Montalva et al. 2011; Schmid-Hempel et al. 2014). Wherever *B. terrestris* spreads, the native bumblebee *Bombus dahlbomii* becomes locally extinct or persists at a greatly reduced abundance (Morales et al. 2013; Schmid-Hempel et al. 2014),

causing *B. dahlbomii* to now be considered an endangered species (Morales et al. 2016). The reasons for the severe decline of the native species have not yet been elucidated, but it is currently thought to be caused by a combination of competition with *B. terrestris*, and *B. terrestris* having brought with it novel pathogens which may have spread to the native bee species (Arbetman et al. 2013; Schmid-Hempel et al. 2014).

A final threat posed by commercial colonies to wild bee populations is pathogen 'spill-back'. This is closely related to spill-over and could occur when commercial colonies on crops acquire pathogens from the wild bee populations. The pathogens could then proliferate within commercial colonies, potentially aided by the high densities at which commercial colonies are deployed, eventually reaching sufficiently high prevalences where they may be a threat to wild bee populations by spilling back to them. The spill-back process has been observed in other systems and is thought to be a threat to native wildlife (Kelly et al. 2009; Nugent 2011). However, it has received little attention in commercial and wild bee populations, despite this system having all the necessary properties for the process to occur.

Regardless of whether spill-back does occur in this system, there is lots of evidence that commercial bumblebee colonies host a wide variety of parasites (Otterstatter et al. 2005; Colla et al. 2006; Whitehorn et al. 2013; Murray et al. 2013; Graystock et al. 2013). The gut trypanosome, *Crithidia bombi*, is among the most common of parasite species found in commercial colonies (Otterstatter et al. 2005; Whitehorn et al. 2013; Murray et al. 2013). In addition, infection with this parasite has been shown to reduce flower visitation rate and motor learning abilities in bumblebees (Gegear et al. 2005, 2006; Otterstatter et al. 2005); behaviours which could impact upon the pollination services bees provide. Given that commercial bumblebees are reared specifically for the provision of pollination services, it would be of great worth to the industry to gain an insight into whether these parasites are affecting the pollination services the colonies provide. Potentially parasites could be impairing the quality of the product that commercial rearing companies sell.

Are commercial bumblebees necessary?

Given the negative impacts of the trade in commercial bumblebees, it begs the questions of whether they are necessary, and if so, whether they can be used in a less environmentally damaging manner. Commercial bumblebees were first developed for use on greenhouse tomato crops, where they dramatically reduced labour costs, as people no longer had to hand pollinate the flowers (Velthuis & van Doorn 2006). Greenhouses are also difficult for wild pollinating insects to gain access to, meaning that commercial bumblebees are the only pollinator in this system. In scenarios such as this, commercial bumblebees are likely to be hugely beneficial to crop production. However, colonies are now regularly used in crops that are grown in open-ended and open-sided polytunnels, and in open fields (Velthuis & van Doorn 2006). In such cases wild pollinators, including bumblebees, can access the crop and provide pollination services, and wind pollination can also contribute. While there is no doubt that bumblebees are very good pollinators, it is not clear if commercial colonies provide a benefit to the crop in these cases, or if adequate pollination services are already being provided by the wild pollinator species assemblage. Assessments of whether commercial colonies are providing a beneficial pollination service to the various crop types in which they are used is necessary. This would allow for reasoned management decisions to be made that consider both the positive and negative aspects of commercial bumblebee colony use.

A mechanism that could potentially reduce the environmental damage caused by commercial colonies, and increase agricultural efficiency, is if the foraging workforce could be increased in size or encouraged to perform more regular foraging trips. This has been demonstrated to be possible, with the application of an artificial bumblebee foraging recruitment pheromone shown to increase nest traffic in commercial bumblebee colonies (Molet et al. 2009). Based on previous research (Dornhaus & Chittka 2005; Molet et al. 2008), there is the potential for similar increases in nest traffic to be achieved by altering the levels of nutrition that are directly available within commercial colony boxes. It is possible to alter nectar availability in commercial bumblebee colonies relatively simply, as they are shipped with a large supply of high sugar concentration nectar in a container directly underneath the colony box. Bees can access this nectar source without leaving the

nest. Removal of nectar stored in natural honey-pots within bumblebee colonies has been shown to increase foraging activity (Cartar 1992; Dornhaus & Chittka 2005; Molet et al. 2008), thus, dilution or removal of this nectar source may encourage increased foraging outside of the colony box and on the target crop. If a single colony were to forage more regularly, then fewer colonies may be necessary within a crop, potentially reducing both the environmental damage caused by commercial colonies, and the costs to the farmer.

Progress is being made within the commercial bumblebee industry to reduce the environmental impact of colonies. For example, some commercial suppliers now irradiate the pollen that they feed the colonies with during the rearing process (Graystock, Jones, et al. 2016), as this was previously shown to be a source of parasitic infection (Graystock et al. 2013). Furthermore, efforts are being made to commercially rear species that are local to the areas they are exported to, e.g., *Bombus atratus* is being reared for use in Argentina (Biobest 2012). Changes in legislation also reflect the growing realisation of the environmental damage that can be caused by the introduction of non-native species, with the UK now only allowing the importation of the native subspecies *B. terrestris audax* (DEFRA 2017). All of these measures represent progress in the industry, however, as I have highlighted, there are still many areas where improvements could be made.

Summary

Insects and especially bees provide crucial pollination services to wild plants and crops, but they also harbour a wide variety of parasites. We currently do not know how such parasites might be affecting the pollination services pollinators provide, even though such effects could have major implications for biodiversity maintenance and food security. Managed pollinator populations contribute significantly to such pollination services, but also play a large role in local parasite epidemiology, potentially contributing to wild pollinator decline. Given this, it is important that the use of managed pollinators is fully justified, with costs and benefits being fully analysed. However, we currently only know that commercial pollinators can be beneficial in greenhouse-based crops systems that are difficult for wild pollinators to access (Morandin et al. 2001a, 2001b; Roldán Serrano & Guerra-Sanz 2006). In open ended and open sided polytunnels, and open field

systems, which wild pollinators can access, the benefits that commercial pollinators provide is not well understood. In such systems, our knowledge of the role that commercial colonies play in crop pollination and in parasite epidemiology of wild bee populations is incomplete.

Whether parasites of pollinators affect their pollination services, is a question that could have implications for the commercial pollinator industry, and the use and management of commercial pollinators plays a large role in parasite impacts in wild bee populations. Thus, an integrated research approach to both of these aspects could prove particularly useful. In this thesis I bring together these research areas through a series of experiments, the results of which could have implications for the pollination of both wild flowers and crops, for wild bee health, and for commercial bee health and management.

Study system

The study system used throughout the experiments that make up this thesis consisted of: the pollinator *Bombus terrestris*, its gut parasite *Crithidia bombi*, and the crop plant strawberry (*Fragaria x ananassa* DUCH). In the following paragraphs, I explain why this system is highly relevant to the questions I wanted to answer to fill the knowledge gaps outlined in the Introduction.

Crithidia bombi

Crithidia bombi is a gut trypanosome and probably one of the most common parasites of bumblebees worldwide. *C. bombi* has been observed at prevalences of anywhere between 0-100% in wild populations, depending on the location and the time of year (Shykoff & Schmid-Hempel 1991; Korner & Schmid-Hempel 2005; Rutrecht & Brown 2008; Gillespie 2010; Whitehorn et al. 2011, 2013; Goulson et al. 2012; Popp et al. 2012; Jones & Brown 2014; Graystock et al. 2014). It is typically found at its lowest prevalence (0-25%) at the start of the year when queen bumblebees first emerge from hibernation (Rutrecht & Brown 2008; Jones & Brown 2014), then as colonies are established and bumblebee population density increases, greater rates of parasite transmission are enabled and thus prevalence increases. A peak prevalence is typically reached in the summer months, and although observations vary greatly, this normally lies within the range of 40-80%

(Shykoff & Schmid-Hempel 1991; Korner & Schmid-Hempel 2005; Gillespie 2010; Popp et al. 2012; Whitehorn et al. 2013). Importantly, it has also been found at high prevalences within commercial colonies, and there is evidence to suggest that it spills-over to wild bumblebee populations (Murray et al. 2013).

Regarding its fitness effects, *C. bombi* is known to significantly increase mortality in bees that are placed under starvation conditions and can reduce colony fitness by 40%, being particularly virulent during the most energetically stressful stages of the host's lifecycle (Brown et al. 2000; Brown, Schmid-Hempel, et al. 2003).

C. bombi has also been shown to impact upon the behaviour of its host, by impairing motor and visual learning ability and slowing foraging rate (Gegear et al. 2005, 2006; Otterstatter et al. 2005). These behavioural alterations have the potential to affect the pollination services infected bees provide. However, such effects have only been observed in *Bombus impatiens*, a common wild and managed bumblebee in North America. It is unknown whether *B. terrestris* is affected in the same way.

The combination of its high prevalence in wild and commercial bumblebees, and its mortality and behavioural effects, make *C. bombi* an ideal study species to examine if parasitism can alter the pollination services that bumblebees can provide.

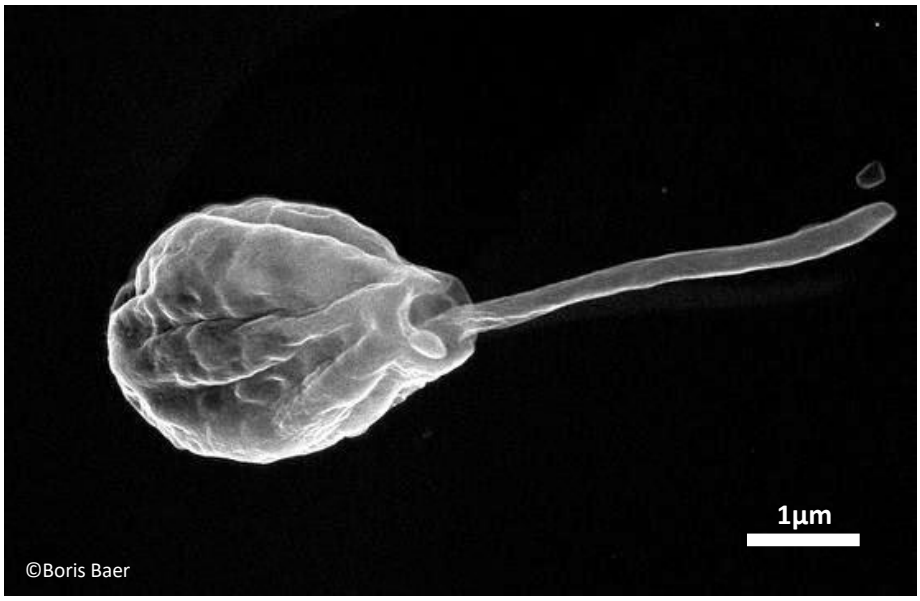


Figure 1. *Scanning electron microscopy image of* Crithidia bombi.

Bombus terrestris

Bombus terrestris is a very common bumblebee species in the West Palaearctic (Williams et al. 2012), where it is also commercially reared on a large scale for crop pollination purposes (Velthuis & van Doorn 2006). Its large range and commercial use make it a very important pollinator, however, this has also facilitated its invasion into several countries where it is not native, including New Zealand, Chile, Argentina, Japan, and Tasmania, Australia (Semmens et al. 1993; Matsumura et al. 2004; Schmid-Hempel et al. 2014).

B. terrestris has an annual life-cycle (Figure 2), with mated queens emerging from hibernation and establishing colonies in the spring. The queen will forage and provision the first batch of brood, but once the first workers emerge, she will no longer leave the nest. The queen continues to lay eggs while the workers collect nectar and pollen and tend to the brood. During summer, the colony will enter a sexual production phase, where males and new queens (gynes) are produced. The males and gynes mate, and then the mated gynes will find a suitable site to hibernate over the winter to come (Sladen 1912).

***Fragaria x ananassa* DUCH (strawberry)**

Strawberry is a hybrid species that is cultivated around the world for its fruit (FAOSTATS, 2018). Hundreds of commercial varieties exist that differ in size, shape, flavour and many other parameters. Strawberry flowers are hermaphroditic and self-fertile, and are thus able to set fruit without animal mediated pollination. However, bee pollination makes flowers more likely to be fully pollinated, i.e., it increase the likelihood of each pistil receiving a pollen grain from the stamens, which results in increased fruit yields and quality (Dimou et al. 2008; Klatt et al. 2014). Successful pollination leads to the development of fertilised achenes, which are then thought to release the phytohormone auxin which causes development of the receptacle (the strawberry fruit) (Wietzke et al. 2018). If the flower is not fully pollinated, i.e., not all pistils receive a pollen grain, it can lead to the development of deformed fruit, which have a reduced market value (European Commission 2011; Klatt et al. 2014). It is for this reason that commercial bumblebee colonies are regularly used to provide pollination services to strawberry crops.

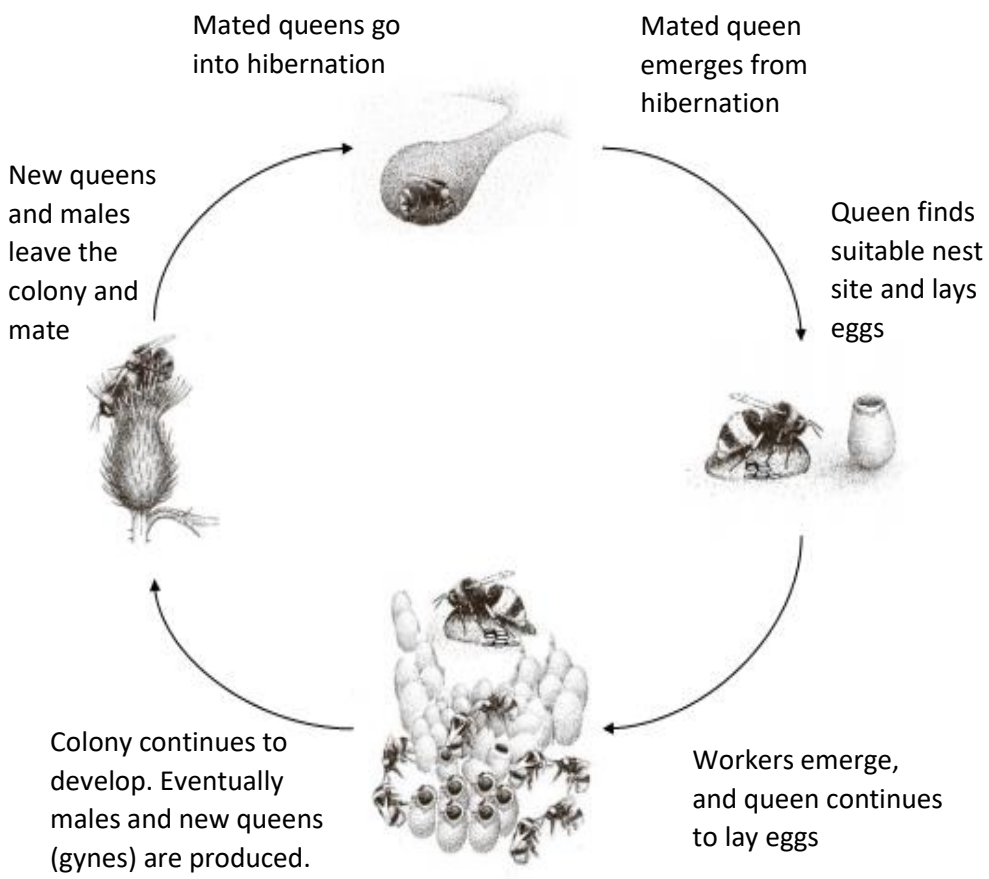


Figure 2. *Life cycle of* Bombus terrestris. *Figure has been adapted from Prŷs-Jones and Corbet (2011).*

Figure 3. Bombus terrestris *foraging on strawberry flower.*

Summary of research chapters

In the following five research chapters that make up this thesis, I aim to address the knowledge gaps highlighted in this introduction. A brief summary of the content of each chapter is given below:

Chapter One investigates whether the learning ability of *Bombus terrestris* (a common bumblebee species in the wild and also reared-extensively for commercial use) is affected by *Crithidia bombi* (a common parasite of both wild and commercial bumblebees around the globe). Learning is assessed using the proboscis extension reflex experimental methodology.

Chapter Two tests whether commercial *B. terrestris* colonies provide beneficial pollination services to two strawberry crops. Commercial colonies were placed into the crops and strawberries were picked and quality assessed.

Chapter Three examines the parasite diversity and prevalence within commercial colonies placed in two strawberry crops (the same commercial colonies as used in Chapter two), and assesses whether the parasite prevalence in these commercial colonies could pose a threat to wild bee health via spill-back.

Chapter Four investigates whether *C. bombi* effects the foraging activity and pollination ability of *B. terrestris* on strawberry plants. Parasitised and unparasitised colonies were paced into polytunnels. Foraging activity was observed and strawberries were picked and quality assessed.

Chapter Five tests if the presence and concentration of a nectar source available within commercial bumblebee colony boxes affected the number of bees entering and leaving the colony. Commercial colonies were placed out in the field, with a specific manipulation applied to their nectar source, and nest traffic was observed.

Chapter 1

Bumblebee olfactory learning affected by task allocation but not by a trypanosome parasite

This chapter was published in Scientific Reports in April 2018:

Martin CD, Fountain MT, Brown MJF *(2018) Bumblebee olfactory learning affected by task allocation but not by a trypanosome parasite. Scientific Reports 5809.*

Abstract

Parasites can induce behavioural changes in their host organisms. Several parasite species are known to infect bumblebees, an important group of pollinators. Task allocation within bumblebee colonies can also cause differences in behaviour. Thus, task allocation may lead to context-dependent impacts of parasites on host behaviour. This study uses *Bombus terrestris* and its gut trypanosome *Crithidia bombi*, to investigate the effects of parasitism, task allocation (foraging or nest-work) and their interactions, on olfactory learning. Prior to undergoing the olfactory learning task, bees were orally infected with a field-realistic dose of *C. bombi*, and observed to determine task allocation. Parasitism did not significantly affect olfactory learning, but task allocation did, with foragers being significantly more likely to learn than nest bees. There was no significant interaction between parasitism and task. These results suggest that *C. bombi* is unlikely to affect pollination services via changes in olfactory learning of its host if bees are under no environmental or nutritional stress. However, wild and commercial colonies are likely to face such stressors. Future studies in the field are needed to extrapolate the results to real world effects.

Introduction

Parasites are highly prevalent in ecosystems with approximately 50% of all animal species thought to be parasitic (Poulin & Morand 2000; Dobson et al. 2008). One way that parasites can impact hosts is through behavioural alteration (Moore 2002, 2013; Lafferty & Shaw 2013). Such parasite-induced behavioural changes may be manipulative and enhance the fitness or transmission of the parasite, but they can also be non-manipulative, benefitting host rather than parasite fitness (Moore 2013). Understanding such manipulations is increasingly important, as parasites have also been implicated in population declines of numerous taxa (Berger et al. 1998; Krkosek et al. 2007; Potts et al. 2010; Schmid-Hempel et al. 2014). Bumblebees are one such group; they host a wide variety of parasite species, and parasitic infections are thought to be one of the key drivers of their declines in Europe and the Americas (Schmid-Hempel 1998; Cameron et al. 2011, 2016; Meeus et al. 2011; Fürst et al. 2014; Schmid-Hempel et al. 2014).

A common parasite in many bumblebee species is the gut trypanosome *Crithidia bombi* (Lipa and Triggiani, 1980). This parasite is often found at prevalences of 10-30% in bumblebee populations, and has been recorded at prevalences as high as 80% at specific sites (Shykoff & Schmid-Hempel 1991; Gillespie 2010; Kissinger et al. 2011; Jones & Brown 2014; Malfi & Roulston 2014). *C. bombi* has also been introduced to South America via its host *Bombus terrestris*. Here, the parasite has spread rapidly, and is one of several potential causes for the decline of the native *Bombus dahlbomii* (Schmid-Hempel et al. 2014). *C. bombi* has been shown to affect host behaviour in several ways (Shykoff & Schmid-Hempel 1991; Gegear et al. 2005, 2006; Otterstatter et al. 2005). Experiments using artificial flowers have found motor-learning rate, learning based on colour cues, and foraging rate to be reduced in infected bumblebees (Gegear et al. 2005, 2006; Otterstatter et al. 2005). In addition, previous work observed a correlation between parasitism and a lower likelihood of pollen collection in the field (Shykoff & Schmid-Hempel 1991).

While the mechanism behind these changes is unknown, one potential explanation is that parasites activate the immune system of their hosts, which can subsequently interact with the nervous system (Mallon et al. 2003; Riddell & Mallon 2006; Alghamdi et al. 2008). *C. bombi* is known to activate the bumblebee immune system (Brown, Moret, et al. 2003; Riddell et al. 2011; Brunner et al. 2013; Deshwal & Mallon 2014), and both bumblebees and honeybees have been shown to display impaired olfactory learning when their immune systems are artificially activated (Mallon et al. 2003; Riddell & Mallon 2006). Thus, *C. bombi* infection may alter host behavior via activation of the immune system.

Context is key for understanding parasite impacts on host behaviour, and as *C. bombi* displays context-dependent virulence, with virulence increasing during periods of food stress or during energetically demanding stages of the host's life cycle (Brown et al. 2000; Brown, Schmid-Hempel, et al. 2003), behavioural impacts may also be modified by host context. Task allocation within bumblebee colonies provides differing contexts, with individual workers more regularly performing either energetically demanding foraging tasks outside the nest, or less energetically demanding tasks based inside the nest, such as brood care (Casey & Ellington 1989; Pouvreau 1989; Ellington et al. 1990). Furthermore, foraging activity has been shown to reduce immunocompetence in bumblebees (König & Schmid-Hempel 1995). The differing energy demands associated with performing these tasks, and

their trade-offs with immunocompetence, could be sufficient for context-dependent behavioural impacts to be induced by the parasite.

This study investigated the effect of *C. bombi* infection, task allocation, and their interactions on the olfactory learning of the common European bumblebee species *Bombus terrestris* L. (1758). I hypothesised that *C. bombi* would alter olfactory learning ability, potentially via interactions between the immune and nervous systems. I further predicted that this alteration would be dependent on task allocation within the bumblebee colony. Olfactory learning was assessed using the proboscis extension reflex (PER) experimental methodology, a classical conditioning procedure (Bitterman et al. 1983). During PER experimentation, bees undergo a series of trials where they can learn to associate an odour (conditioned stimulus) with a sugar solution reward (unconditioned stimulus) (Bitterman et al. 1983). During each trial whether or not a bee displayed a conditioned response was recorded.

Methodology

Queen collection

Wild *B. terrestris* queens were collected from Windsor Great Park, Surrey, UK (Latitude: 51.417677, longitude: -0.604263). Queens were collected between the 11th March and 7th April 2015. Collected bees had their faeces screened under a microscope at x400 magnification for the presence of *C. bombi*. Those individuals that did harbour the parasite (n=32) were placed into individual plastic nest boxes (W = 6.7, L = 12.7, D = 5cm) and provisioned with ad libitum pollen and nectar. When the first workers began to emerge, the colonies were transferred to larger plastic nest boxes (W = 22.5, L = 29, D = 13cm) where they were kept for the remainder of the experiment. These colonies did not forage outside of their colony box at any point during their life cycle, and they were not subjected to a regular light cycle. These colonies were then used as a source of parasitic cells for the inoculation of commercial colonies during experiments. The infectiveness of a parasite to its host can vary between different host populations (Imhoof & Schmid-Hempel 1998a; Yourth & Schmid-Hempel 2006). Thus, having several infected wild bees meant that there was a variety of different parasite strains, which increased

the likelihood that strains were present that could successfully infect commercial colonies. Furthermore, I used multiple strains to maximise the chances of observing broad impacts of the parasite in bumblebees, rather than strain specific effects.

Commercial colonies

Four commercial *B. terrestris audax* colonies were imported from Biobest (between November 2015 and January 2016) onto the Royal Holloway, University of London campus to be used for experimentation. Upon arrival 15 workers were removed from each colony and their faeces screened for the presence of parasites (Rutrecht & Brown 2008). All four colonies were uninfected and thus kept for the experiment. The queen was removed from each commercial colony, then each colony was split into four sub-colonies. Two of these sub-colonies consisted of 40 workers and half of the original brood each, these were the 'experimental' colonies and they were assigned a treatment (control or parasite) and placed into wooden nest boxes (W = 14, L = 24, D = 10cm). The remaining two sub-colonies were the 'stock' colonies. These were made up by splitting the bees remaining from the original commercial colonies after the set-up of the 'experimental' colonies. One of these sub-colonies was inoculated with *C. bombi* collected from faeces of bees from all of the wild colonies. The other colony was left uninfected as control stock. These stock colonies were used for any subsequent inoculations of their corresponding experimental colony (see figure 1 for overview of colony splitting process). This process allowed for filtration of all the *C. bombi* strains, so that only those strains infective to a particular commercial colony were used for subsequent infections.

In total, I had 8 experimental sub-colonies, 4 with the parasite treatment and 4 with control treatment, and I also had 8 stock sub-colonies, each one corresponding to an experimental sub-colony. This split colony design helped account for the large intercolony variation in learning ability that exists (Raine & Chittka 2008).

The wooden nest boxes containing the experimental sub-colonies were then connected to flight arenas (W =75, L = 100, D = 50cm) via a gated tunnel. Gravity feeders filled with 40% sugar solution were placed within the arenas to allow the bees to forage, and *ad libitum* pollen was provided to each colony directly into the nest box. The stock colonies were placed into plastic nest boxes and stored in a dark room with *ad libitum* pollen and nectar.

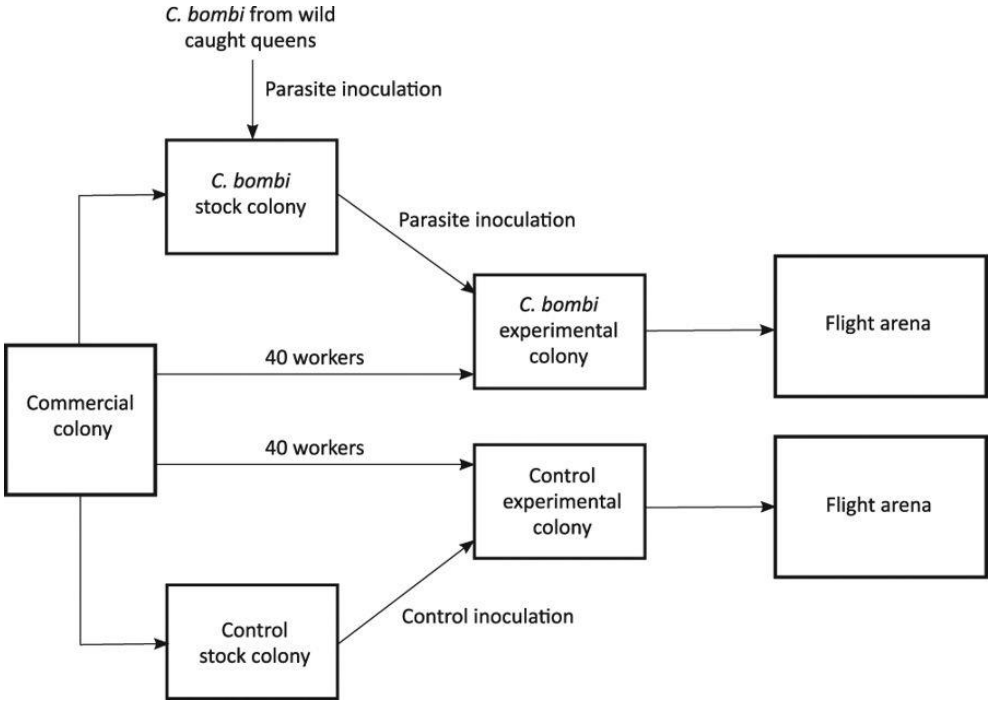


Figure 1. *Overview of the creation of 'experimental' and 'stock' colonies from a single commercial colony. The same process was repeated for 4 commercial colonies.*

Crithidia bombi purification and inoculation

Inocula were made by taking a minimum of 10 bees from the parasite or control stock colonies. The faeces of these bees were collected and then purified following the method used by Baron et al. (2014) modified from Cole (1970). The faeces were diluted with 0.9% Ringer's solution to make 1ml of total solution (dilution 1). The solution was centrifuged at 0.8G for two minutes, the supernatant was then removed and placed into another centrifuge tube (dilution 2), whilst the remaining pellet was diluted and re-suspended with another 1ml of Ringer's solution. This process was repeated until 8 dilutions had been prepared. Dilutions 4, 5, and 6 were taken and centrifuged at 8G for 1 minute, the supernatant removed, and the pellets mixed with 100µl of Ringer's solution. A small amount of the resulting solution was placed in a Neubauer chamber, allowing for the *C. bombi* cells to be counted and the concentration of the parasite in the solution to be calculated. The amount of solution that contained 10,000 parasite cells was calculated and this dose was diluted with 40% sugar solution to make a 20µl solution which was fed to individual bees. The same protocol was followed to make a control inoculum using the faeces

of bees from the control stock colonies. Any bee that did not consume the inoculum was not used for further experimentation.

Callow marking

Experimental sub-colonies that had been connected to flight arenas were observed every day for the emergence of callow workers. Workers were individually marked with uniquely numbered Opalith tags on the day they emerged, so the age of each marked bee was known. Marked bees were inoculated between the ages of 3 to 5 days using the method previously described. Bees were then left to harbour the parasite for a further 7 to 10 days post-inoculation. This time period was chosen as it has previously been shown that the parasite load 7 to 10 days post-inoculation is relatively high and remains stable (Logan et al. 2005). During the bee marking process, the flight arenas were observed in both the morning and afternoon. Bees could forage in flight arenas at all times, and any marked bee that was observed foraging on a nectar feeder was judged to be a forager, whilst bees never observed to forage were judged to be nest bees.

Olfactory learning

Olfactory learning was assessed using the proboscis extension reflex (PER) experimental methodology, where bees learn to associate an odour (conditioned stimulus) with a sugar solution reward (unconditioned stimulus) (Bitterman et al. 1983). This method has been used for over 50 years to test learning and memory in honeybees (*Apis mellifera*) with great success (Giurfa & Sandoz 2012), and has more recently been used successfully on bumblebees (Riveros & Gronenberg 2009; Smith & Raine 2014).

Between 13:00 and 15:00 on the afternoon before the PER experiment, marked workers 6-9 days post-inoculation (7-10 days on the following day of experimentation) were taken from the nest box and flight arena, placed on ice for approximately 5 minutes until quiescent, and then harnessed. The harness prevented the bee from flying and crawling, but allowed the bee to move its head. All workers were fed to satiety with 40% sugar solution two hours after harnessing, and were left upright in a container overnight.

The following morning between 08:00-09:00, bee responsiveness was tested by touching their antennae with a droplet of nectar solution. Those bees responding

with a proboscis extension were deemed to be sufficiently motivated to be used for behavioural assays, and were fed a small droplet of nectar solution to maintain motivation 15 minutes before the experiment. Those bees not responding were judged to be unmotivated, and were not used for behavioural assays.

The PER experiment itself was carried out between 09:00-12:30. During the experiment each harnessed bee was individually placed in an odour extraction hood. Air flow into the hood was controlled by a programmable logic controller computer. The air flow was directed onto the bee via an odour tube placed 3cm away from the bee. A piece of filter paper soaked in 4µl of lemon scented oil was placed inside the odour tube, and this filter paper was replaced every 20 trials to keep the intensity of the odour constant. The bees were exposed to 15 seconds of air flow in total, the first 5 seconds being clean air and the final 10 seconds being the odour. The reward was presented to the bee 6 seconds into the odour stimulus by touching its antenna with a 0.8µl droplet of 40% nectar solution using a Gilmont syringe. If presentation of the reward elicited a proboscis extension response then the bee was fed the nectar droplet, but if the bee did not respond to the reward then it did not receive any nectar. A conditioned response occurred when the bee extended its proboscis on exposure to the odour stimulus without needing presentation of the nectar solution on its antennae. In this case, the bee was fed the nectar droplet. Each bee underwent 15 odour exposures with a 12-minute interval between each exposure.

Three control trials were interspersed randomly within the final 10 odour exposures. During a control trial an unscented airflow was directed onto the bee for the duration of the trial, and no reward presentation occurred. These control trials were performed to check that bees were not becoming conditioned to the airflow rather than the scent. Any individual that appeared to be conditioned to the airflow, i.e., showed a conditioned response during a control trial, was removed from the analysis. Bees were also deemed unmotivated and excluded from the analysis if they did not respond to the nectar stimulus for 3 consecutive trials.

After completing the PER trials, bees were placed into a freezer at -20°C. The thorax width of each bee was recorded as a measure of bee body size, which in some cases has been shown to affect learning ability (Worden et al. 2005; Sommerlandt et al. 2014).

Parasite infection intensity

The parasite intensity was quantified for all bees from the parasite treatment following a similar methodology to Baer and Schmid-Hempel (Baer & Schmid-Hempel 2001). This was done by combining the hind-gut of a bee with 100µl of 0.9% ringers solution. The mixture was then ground-up in a 500µl reaction tube and mixed in a vortex mixer for 5 seconds. A *C. bombi* cell count was performed on 0.02µl of the gut solution using a Neubauer haemocytometer.

Data Analysis

All statistical analyses were carried out using 'R' programming software (R Core Team 2018). The total number of conditioned responses was analysed using a negative binomial generalised linear mixed effects models in the 'glmmADMB' package (Fournier et al. 2012; Skaug et al. 2016). Mixed effects Cox proportional hazards models, in the package 'coxme' (Cox 1972; Therneau 2015), were used to analyse the learning rate and final proportion of bees that displayed one or more conditioned responses. Mixed modelling techniques were used to allow for the colony each bee came from to be accounted for as a random effect. Co-variables used in the models included 'treatment', 'task (forager or nest)', 'thorax width', 'age', 'infection intensity', and the interaction term between 'treatment' and 'task'. These variables were chosen as I had *a priori* reasons to believe that they could affect learning or interact with the treatment to affect learning. There was no correlation between variables and models were not overdispersed. Validation of the Cox model was carried out to check it was not violating the proportional hazards assumption. Mixed effects models were validated by visual inspection of plots of the model residuals plotted against the fitted values.

Results

150 bees from 4 colonies were deemed motivated enough to undergo PER trials, with 80 of these being from the control treatment and 70 from the parasite treatment. 39 of the 150 were judged to be unmotivated (26 control bees and 13 parasite bees) during the trials after showing 3 consecutive non-responses to the sucrose solution. These bees were not included in further analyses. Of the 111 motivated bees, 57 were from the parasite treatment group and 54 from the

control group. 55 of the 111 (49.5%) bees showed at least one conditioned response. No bees responded to the control trials.

While a greater percentage of parasitized bees (54.4%) showed a conditioned response than control bees (44.4%) (Figure 2), treatment was not a significant covariate in the Cox regression analysis (Cox proportional hazards model: Hazard ratio (*HR*) = 1.13, *P* = 0.79). Similarly, although parasitised bees displayed, on average, a greater number of conditioned responses during the full duration of the trials, this was not significant (GLMM: *z* = 0.69, *P* = 0.49).

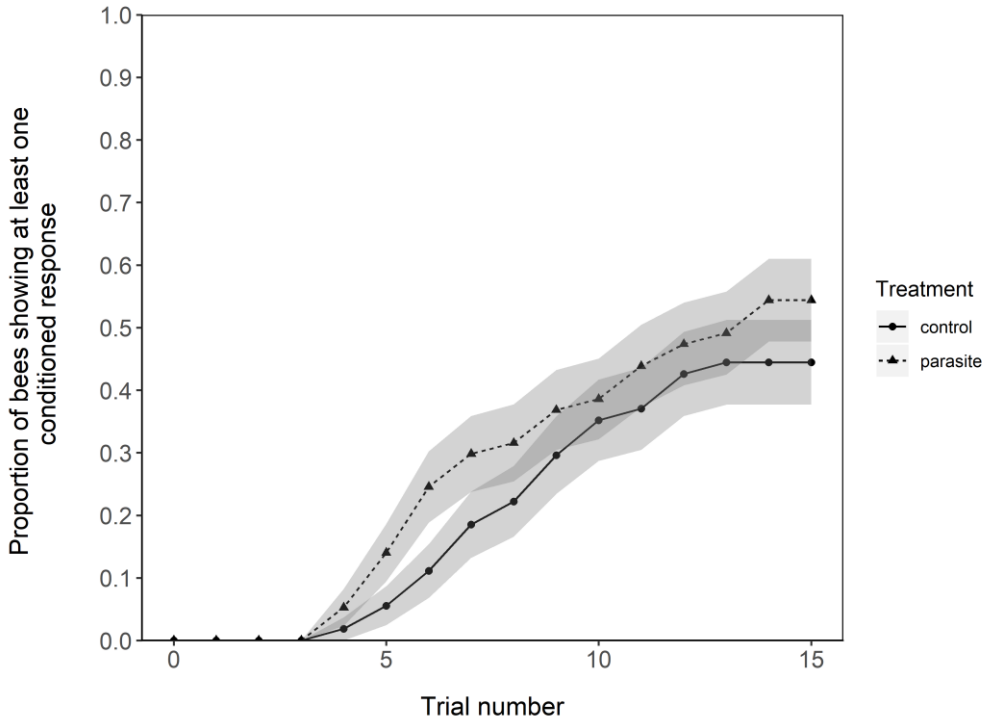


Figure 2. *Cumulative proportion of parasitised and control bees to have shown at least one conditioned response throughout the duration of the trials. Grey shaded area around lines represents ± the standard error of the mean.*

Infection intensity was also not a significant explanatory variable for either the likelihood of a bee showing one or more conditioned responses (Cox proportional hazards model: *HR* = 1.10, *P* = 0.25), or for the total number of conditioned responses a bee displayed (GLMM: *z* = 1.88, *P* = 0.06).

Of the 111 motivated bees, 31 were categorised as foragers, while the remaining 80 bees were categorised as nest bees. 64.5% of forager bees showed at least one conditioned response, whereas only 43.8% of the nest bees did (Figure 3), and the

task of the bee (forager or nest) was a significant predictor of learning in the Cox regression model (Cox proportional hazards model: *HR* = 2.50, *P* = 0.029).

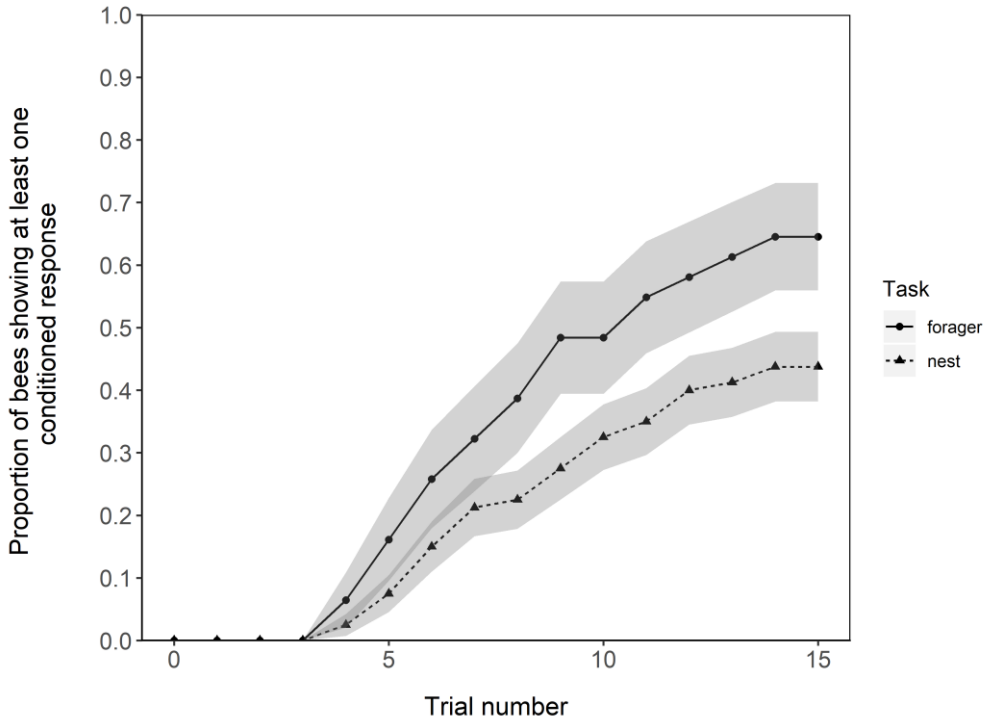


Figure 3. *Cumulative proportion of forager and nest bees to have shown at least one conditioned response throughout the duration of the trials. Grey shaded area around lines represents ± the standard error of the mean.*

Forager bees also displayed, on average, a greater number of conditioned responses than nest bees throughout the PER trials, but this was not a significant difference (GLMM: *z* = 1.55, *P* = 0.12).

The difference in the likelihood of a bee showing one or more conditioned responses was greater between the foragers and nest bees in the control group than in the parasitised group (Figure 4). In the control group 73.3% (11 out of 15) of the foragers showed at least one conditioned response, compared to 33.3% (13 out of 39) of the nest bees. In the parasitised group 56.3% (9 out of 16) of foragers and 53.7% (22 out of 41) of nest bees showed one or more conditioned responses. However, the hypothesised interaction between the treatment and the task that the bee performs (foraging or nest tasks) was not a significant explanatory variable in the Cox model output (Cox proportional hazards model: *HR* = 0.43, *P* = 0.14).

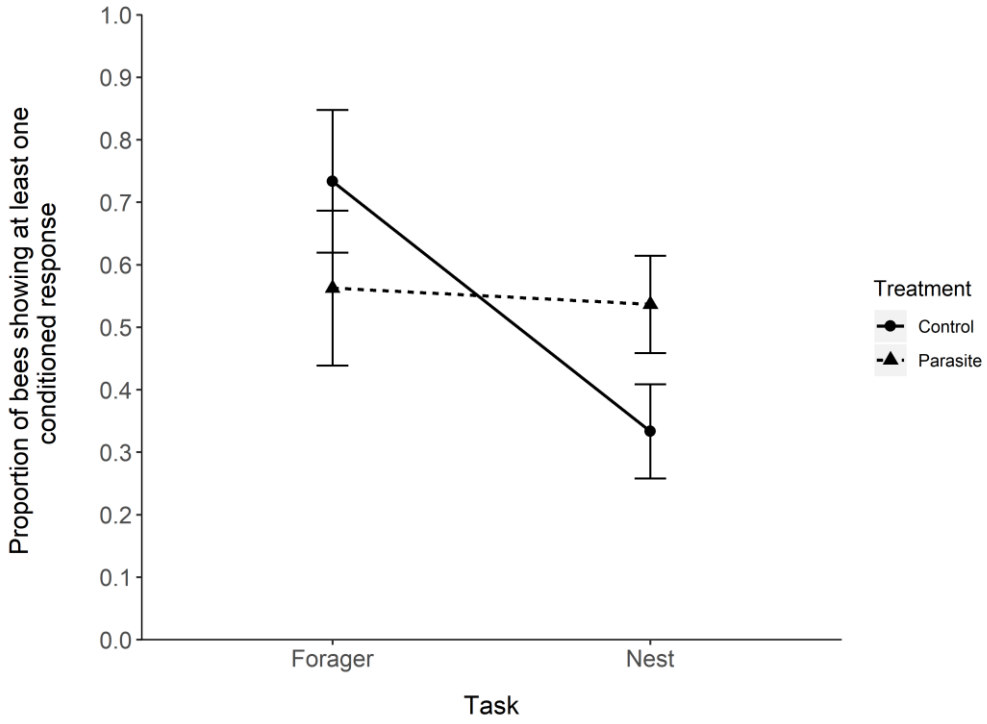


Figure 4. *Visualisation of the non-significant interaction (see results for statistics) between treatment and task, with proportion of bees that showed one or more conditioned responses as the response variable. Error bars represent ± standard error of the mean.*

The interaction term between treatment and task was also not a significant explanatory variable (GLMM: $z = -0.98$, $P = 0.33$) of the total number of conditioned responses that bees displayed. Here, the control and parasitised bees showed a very similar relationship for foragers and nest bees.

Discussion

In this experiment, infection with the parasite *C. bombi* had no significant effect on the olfactory learning ability of *B. terrestris*. However, the task the individual was allocated within the colony did affect the likelihood of the bee showing at least one conditioned response, with forager bees more likely to learn than nest bees. Interactions between task allocation and treatment were non-significant, contrasting with my initial hypothesis that task-dependent parasite-induced learning alterations could occur in this system.

It is surprising that I do not observe a significant effect in the parasite treatment group given that *C. bombi* is known to activate the immune system of *B. terrestris* (Brown, Moret, et al. 2003), and that activated bumblebee and honeybee immune systems can interact with the nervous system to cause impairments in cognitive function (Mallon et al. 2003; Riddell & Mallon 2006). However, in bumblebees, reduced learning ability as measured by PER methodologies was only observed in bees that were starved of pollen (Riddell & Mallon 2006), and pollen is crucial for proper functioning of the immune system (Brunner et al. 2014). During my experiment, bees had an *ad libitum* supply of pollen directly into the nest, so they were not in a state of nutritional stress. This could explain why parasitic infection had no effect on olfactory learning in this experiment, since bees may have had sufficient nutrition to support both functioning immune systems, and other physiological functions. However, it should be noted that *C. bombi* has been shown to impair learning ability in non-nutritionally stressed bees (Gegear et al. 2005, 2006; Otterstatter et al. 2005), although these studies did not use PER methodologies. Further work with nutritionally stressed bees is needed to clarify this relationship.

I found the infection intensity of *C. bombi* to have no significant effect on learning ability. This is contrary to other experiments that have found increasing *C. bombi* infection intensity to negatively impact learning ability (Gegear et al. 2005, 2006). These experiments did, however, use different experimental set-ups to test learning (i.e. not PER) and were performed on a different bumblebee species (*Bombus impatiens*), which could have contributed to the differing results. Furthermore, the number of cells in the *C. bombi* inoculum and the infection intensities observed in Gegear *et al.* (2006), one of the previously cited experiments, were much higher than in my experiment. This may further explain why, in contrast to the Gegear *et al.* (2006) study, no effect of infection intensity was observed in my experiment.

In this study foragers were more likely to show one or more conditioned responses than nest bees. Foraging is a complex task, requiring the individual to differentiate between the quality and quantity of a wide variety of potential forage resources. Given this, one might expect foragers to have increased cognitive function, and indeed in both ants and honeybees, individuals that forage have been shown to perform better at learning tasks than individuals based in the nest (Ray &

Ferneyhough 1999; Perez et al. 2013). Similar patterns have been observed in *B. terrestris* colonies when the queen is present, however, in contrast to my experiment, these patterns were no longer observed upon removal of the queen (Evans et al. 2016). In the bumblebee *Bombus occidentalis*, bees with more foraging experience were found to be better learners, and the foraging activity caused an increase in the size of the mushroom bodies, an area of the brain associated with learning and memory (Riveros & Gronenberg 2009, 2010). It is also possible that differential gene expression between task allocated bees could enhance the learning ability of foragers (Tobback et al. 2011). The results presented here on *B. terrestris* add further evidence to this pattern, strongly suggesting that task allocation can alter learning ability in social insects, and at least across bumblebee species.

The superior learning ability of foragers could alternatively be explained by the size of the individual. Bumblebees that more regularly perform foraging tasks are generally larger than their nest based counterparts (Goulson et al. 2002), and larger body size has been associated with increased learning ability (Worden et al. 2005; Riveros & Gronenberg 2009). However, in my experiment foragers did not have a larger body size, and body size was not a significant predictor of learning ability in any of the models.

Interaction terms between treatment and task were not significant predictors of either of the response variables analysed, suggesting that task-dependent parasite-induced learning alterations do not occur in this host-parasite system, or that I lacked sufficient power to detect such an effect. However, this result should be interpreted with caution as in my experimental set up bees could only forage in the flight arena (100 x 75 x 50cm), whereas in the wild foragers may travel hundreds or even thousands of metres in a single foraging bout (Darvill et al. 2004; Knight et al. 2005; Osborne et al. 2008; Redhead et al. 2016). This means that the energy demands of foraging in this experiment are greatly reduced compared to those of wild foragers, which subsequently could decrease the likelihood of observing any context dependent effects, as any trade-off between energy consumption for flight and for immune system upregulation is much less severe.

Further differences exist between the foraging arena and the wild environment in the prior conditioning that bees experience. In the foraging arena, it is necessary to

ensure that bees have not associated any odour with a reward prior to the PER protocol, but in the wild, foragers will have made associations between particular flower odours and the rewards that they can provide. Foraging from nectar feeders in the arena does, however, still require that bees use visual cues and learning in order to locate food, which does replicate the behaviour of wild foragers, albeit at a lower level of complexity.

It is possible that my observation and definition of forager and nest bees, led to the results being conservative, and the difference in learning ability between foragers and nest bees could in fact be greater. The observation protocol allowed me to be sure that the animals I labelled foragers had indeed foraged, but it is possible that a proportion of the bees that were judged to be nest bees left the nest and foraged at a time when there was no ongoing observation of the flight arenas. If this did occur, these individuals would have been included in the nest bee category, but they may have displayed the enhanced learning ability of a forager, which could dampen the effect of task allocation on learning that is being observed.

The results presented here provide evidence that *C. bombi* has no meaningful effect on *B. terrestris* olfactory learning, and that the task that the bee performs is a more important factor in predicting learning ability. It can be concluded that *C. bombi* is unlikely to affect pollination services via changes in olfactory learning of its host, at least in an environment where food is abundant, but it could still impair pollination services through previously described impacts on mortality and motor learning (Brown et al. 2000; Brown, Schmid-Hempel, et al. 2003; Gegear et al. 2005; Otterstatter et al. 2005). This experiment does not support the existence of task-dependent parasite-induced learning alterations, however their presence cannot be ruled out given the restricted foraging environment that bees were constrained to. Future research should focus on investigating cognitive function of parasitised bees in stressful conditions, or in a more field realistic situation where foraging is more energetically demanding and where food supply is not *ad libitum*.

Chapter 2

Seasonal differences in the effects of commercial bumblebees on fruit quality in strawberry crops

This chapter is currently under review at the journal Agriculture, Ecosystems and Environment.

Martin CD, Fountain MT, Brown MJF. *Seasonal differences in the effects of commercial bumblebees on fruit quality in strawberry crops. Agriculture, Ecosystems & Environment.*

Abstract

Colonies of commercially-reared honey bees and bumblebees, along with wild pollinators, significantly contribute to global food production by providing pollination services to crops. Commercial bumblebees are increasingly used on soft fruit crops, such as strawberry, an economically important crop globally. Despite the use of commercial bumblebees in strawberry crops, there is little quantitative evidence that they provide a benefit to farmers. Given the negative impacts that the importation of commercial colonies can have on wild bee populations, it is vital that the benefits of commercial bumblebees are quantified, so reasoned management decisions can be made that provide maximum benefit to both farmers and wild bees. In this study, commercial *Bombus terrestris audax* colonies were placed into June-bearer (flowering March-April) and everbearer (flowering May-June) strawberry polytunnels on a soft-fruit farm in the south east of England, and the colonies were opened and closed at weekly intervals. The flower-visiting assemblage inside polytunnels was quantified, and fruit was harvested and quality assessed. In the June-bearer crop, the presence of commercial bumblebees increased the amount of high commercial grade fruit by 17.5%. In contrast, no benefit of commercial bees on pollination or fruit quality was observed in the everbearer crop. The increase in quality of fruit in the June-bearer crop may be driven by the higher *B. terrestris audax* flower visitation rates seen in this crop. The number of flower visits by wild pollinators was not a well-supported predictor of strawberry quality, thus the benefit they provide in this system remains to be elucidated. The results presented here suggest that commercial bumblebees can greatly increase the quality and value of a strawberry crop. The improvements in quality of the June-bearer crop are estimated to be worth approximately £16 million to the total value of the UK strawberry crop. However, interactions between commercial bees, farm management practices and environmental factors may reduce their efficacy at certain times of the year. Furthermore, such factors may be different at other farm locations. Thus, careful consideration should be given before using commercial bumblebees on a crop.

Introduction

Entomophilous crop pollination is a valuable ecosystem service that contributes to human health, wellbeing, and global food security (Klein et al. 2007; Aizen et al. 2009). 75% of the 115 major global crop species depend to some degree on insect pollination that is provided by both wild and managed pollinators, and many of the most insect-dependent crops provide humans with valuable sources of micronutrients (Klein et al. 2007; Eilers et al. 2011; Wang & Ding 2012). Bees are one of the most important pollinator groups (Klein et al. 2007; Calderone 2012), partially due to their large scale management in order to support crop pollination. Globally, honeybees are the dominant managed pollinator (Klein et al. 2007; Calderone 2012), but managed bumblebees are of increasing importance, being superior pollinators in some crop types and possessing the ability to forage in cooler, windier weather (Berger et al., 1988; Goodell and Thomson, 2007; Stanghellini et al., 1998, 1997; Thomson and Goodell, 2002).

Bumblebees were first commercially produced in the mid 1980's, and colonies were used primarily in greenhouse tomato crops (Velthuis & van Doorn 2006). Prior to the introduction of commercial bumblebees, tomatoes had to be mechanically pollinated, so the use of bees drove down labour costs and also improved yield and quality of the fruit (van Ravestijn and Nederpel 1988; Velthuis and Van Doorn 2006). This success, combined with reports of pollinator declines and subsequent fears of pollen limitation in crops, has led to growth in the trade of commercial bumblebees (Potts et al. 2010, Lye et al. 2011). Colonies are now mass produced in several rearing facilities, and in 2006 it was reported that over 1 million colonies were shipped worldwide (Velthuis and van Doorn 2006). They are increasingly being used in crops other than tomatoes, some of which are grown in polytunnels and open fields (Velthuis and van Doorn 2006). In the UK, for example, around 15,000 colonies per year are used on soft fruit farms (Goulson 2009).

Despite the beneficial pollination services commercial bumblebees can provide, there are negative impacts associated with the trade in commercial bumblebees. Among these are competition with local species (Ings et al. 2006; Inoue et al. 2008) and disease spread (Colla et al. 2006; Goka et al. 2006; Graystock et al. 2013; Schmid-Hempel et al. 2014). Indeed, strong evidence of pathogen spill-over from commercial to wild bumblebee populations has been observed in both Europe and

the Americas (Colla et al. 2006; Murray et al. 2013), and in South America is thought to be a leading cause in the severe decline of a native bumblebee species (Schmid-Hempel et al. 2014).

Given the negative impacts associated with the commercial bumblebee trade, it is important that bumblebees are used responsibly, and only on crops for which there is supporting evidence of beneficial pollination services. Experiments investigating bumblebee pollination are often done in greenhouse crops, where wild pollinators have very limited or no access to the crop, and where wind pollination is minimal (Shipp et al. 1994; Dogterom et al. 1998; Zhang et al. 2015). Other studies have been done on a small scale, investigating pollen deposition during one or more flower visits, and it is unclear how the results extrapolate to the much larger scale of a farm (Thomson & Goodell 2002; Javorek et al. 2002). Some field trials have been done at a larger scale in polytunnel or open field crops, and have shown the addition of commercial bumblebees to increase yield and fruit set in blueberry (Stubbs & Drummond 2001; Desjardins & De Oliveira 2006), and increase yield in raspberry (Lye et al. 2011). A study on apple orchards in Israel suggested that not only does the addition of bumblebees directly increase the pollination of the crop, but that it can also alter the foraging behaviour of other pollinators on the crop, which then causes further alterations to pollination services (Sapir et al. 2017). However, there are many more polytunnel and open field crops in which commercial bumblebees are used, where their effectiveness has not been tested.

An example of this is strawberry (*Fragaria x ananassa* DUCH); this is a major soft fruit crop, worth £284 million in the UK in 2015 (DEFRA 2015). Several bee species, both managed and wild, have been shown to be effective pollinators of strawberry (Dimou et al. 2008; Klatt et al. 2014; Connelly et al. 2015). The proportion of the strawberry pollinator community that is made up of bumblebees varies. In some cases they only make up a small proportion of the community (Klatt et al. 2014; Ahrenfeldt et al. 2015), but in others, they are the dominant pollinator group (Wietzke et al. 2018), including in cases when commercial bumblebees are deployed (Feltham et al. 2015). The use of commercial bumblebees on strawberry farms in the UK is widespread, but the contribution of both commercial bumblebees and wild pollinators to strawberry pollination has not been investigated. Given how effective wild bees can be as pollinators of strawberry, it is possible that the addition of commercial bumblebees is providing little or no

benefit to the crop. In this case, the costs (both financial and environmental) of applying commercial bumblebees would outweigh the benefits. However, if wild pollinator populations are low at a particular time or location, and if certain very effective wild pollinators are not present in an area, then commercial colonies may provide an important pollination service to the crop justifying their use.

This study examined the contribution to crop pollination and fruit quality made by commercial bumblebees on a strawberry farm in the south east of England. Commercial colonies were placed into the crop and were or were not allowed to forage on the crop during specific time periods. Fruits that were pollinated during these time periods were picked and quality assessed to examine the impact commercial bees were having on fruit quality. The wild pollinator community was also surveyed to assess its potential for providing pollination services to the crop.

Methodology

Study species

Fragaria x ananassa DUCH (strawberry)

Strawberry is a hybrid species that is cultivated around the world for its fruit (FAOSTATS). Strawberry flowers are hermaphroditic and self-fertile, and are thus able to set fruit without animal mediated pollination. However, bee pollination increases the likelihood of each pistil receiving a pollen grain from the stamens, which results in increased fruit yields and quality (Dimou et al. 2008; Klatt et al. 2014). If the flower is not fully pollinated, i.e., not all pistils receive a pollen grain, it can lead to the development of deformed fruit, which have a reduced market value (European Commission 2011; Klatt et al. 2014). It is for this reason that commercial bumblebee colonies are regularly used to provide pollination services to strawberry crops.

In a polytunnel environment in the south of England, the lifespan of a strawberry flower is approximately 3-5 days (Whitehouse pers. comm.). After this time the petals begin to senesce and drop and the receptacle begins to form a fruit.

Bombus terrestris audax

The commercial bumblebees placed into the strawberry crop on the farm were *Bombus terrestris audax*. This *B. terrestris* subspecies is native to the British Isles (Rasmont et al. 2008), and is currently the only commercially produced bumblebee species used in the United Kingdom. Commercial colonies are typically placed into a crop when they reach a size of 50-100 individuals, although this can vary depending on the supplier. They are then estimated to be able to provide pollination services for the next 6-8 weeks.

Field site

The fieldwork was carried out at Kelsey Farms, an 80-hectare soft fruit farm in Kent in the South East of England (latitude: 51.288694, longitude: 1.183766). The landscape surrounding the farm is dominated by pasture and arable crops (predominantly cereal crops), with some small villages and patches of mixed deciduous woodland (see supplementary figure S1 and supplementary tables S1 and S2 for land use map and further details of the area surrounding the farm).

Two experiments were done on the farm, the first ran for 6 weeks from 21st March to 29th April 2016 and was done in two varieties ('Malling Centenary' and 'Flair') of June-bearing strawberries. The second ran for 8 weeks from 9th May to 1st July 2016 and was done in a single variety of everbearing strawberry. Due to proprietor constraints, the name of the everbearing variety cannot be released, thus from here on it is referred to as 'Proprietary variety 1'. June-bearing strawberries flower earlier in the season and have a shorter flowering period than everbearers, which can flower throughout the summer months. The strawberries were grown in irrigated coir grow bags on a table-top system in polytunnels. During the June-bearer experiment, the polythene at either end of each polytunnel was rolled up, meaning that the ends of the tunnels were open, allowing insects to enter and leave the tunnels. However, the polythene on the sides of the polytunnels was rolled down. During the everbearer experiment, both the ends and sides of the polytunnels were open. This setup was used for the entire strawberry crop on the study farm and is common practice for other UK grown polytunnel strawberry crops.

In the June-bearer experiment, 9 *B. terrestris audax* colonies were obtained from Biobest. The supplier states that these colonies arrive with approximately 60-80 bees. These colonies were spread across 3 fields (3 colonies in each field) on the farm. The 3 fields were 0.94, 1.41, and 1.26 hectares in size, and contained plants at a density of 47,000 plants/ha. The closest colonies in separate fields were separated by approximately 125m. Fruit were sampled from a sampling area around each colony (see supplementary material figure S2 for layout of sampling areas). Each sampling area consisted of the tunnel with the colony inside, and the two tunnels either side which did not contain colonies (see 'Strawberry marking and collection' for further details on fruit collection). Ideally, all sampling areas would have been the same size, but this was not possible as the polytunnels in the 3 fields varied in size. In the June-bearer experiment, the size of the sampling areas were between 0.16-0.21 ha, with the majority being between 0.16-0.19 ha. Thus, within each sampling area of 3 polytunnels, the colony densities were between 4.76-6.25 colonies/ha, with the majority being between 5.3-6.25 colonies/ha (see supplementary table S3 for colony densities in each sampling area). This is close to the 6 colonies/ha density recommended by the supplier, and similar to the densities used on other strawberry farms. In two of the sampling areas (sampling areas 3 and 6 on supplementary figure S2), only 2 tunnels were sampled, the tunnel containing the colony and one adjacent tunnel. This was done because these tunnels were much longer, and so to keep the colony density within the sampling area as close to 6 colonies/ha as possible, one fewer tunnel was sampled.

In the everbearer experiment 12 colonies were placed in 4 fields (3 colonies in each field). The 4 fields were 3.94, 1.64, 2.44, and 1.90 ha, and contained plants at a density of 53,000 plants/ha. In the everbearer experiment the size of the sampling areas were between 0.16-0.20 ha (see supplementary figure S3 for layout of sampling areas). The closest colonies in separate fields were separated by approximately 95m. 9 of the 12 sampling areas were between 0.160-0.165 ha and had colony density of 6.00-6.25 colonies/ha, however, one field had slightly longer polytunnels meaning that the 3 remaining sampling areas were 0.20 ha with a colony density of 5 colonies/ha (see supplementary table S3 for colony densities in each sampling area). Across June- and everbearers, colonies were placed in the centre of the polytunnels.

The fields were separated by >5m high alder hedge rows, which act as wind breaks to reduce damage to the crop and polytunnels. In order to have strawberries that were and were not pollinated by commercial bumblebees, the colonies were opened and closed on a weekly cycle for the duration of the experiment. When the colonies were open, both the commercial bees and wild pollinators were able to forage on and pollinate the strawberry crop, but when closed the bees had to remain in the nest, and the crop could only be pollinated by wild pollinators. This meant I had two groups of fruit at the end of the field experiment, one group which could have been pollinated by commercial bumblebees and another group which could not. Within fields, all colonies were kept on the same opening and closing cycle, but between fields colonies were on opposing cycles (i.e. in one field all the colonies would be closed, whilst at the same time in another field they would be open). When colonies transitioned from being open to closed, they were closed at nightfall to reduce the chance of trapping any workers outside the colony. Bees had access to sugar solution from a reservoir beneath the nest at all times, as this is standard practice used by commercial growers. During the weeks when colonies were closed, they were supplemented with approximately 20g of pollen (Biobest UK Ltd) to allow continued growth of the colony.

Strawberry marking and collection

10 recently opened strawberry flowers were marked in the sampling area around each colony every week. Flowers were marked two days into the treatment periods, so they could not have been visited the previous week as they were still closed. Flowers were judged to have recently opened if their anthers contained large amounts of bright yellow/orange pollen, there was no darkening or discolouration of the pollen or the petals, and the receptacle showed no signs of fruit formation (see Figure 1 for example of a recently opened flower). Marking flowers in this state meant they would be receptive during the time that commercial bumblebees were either able or not able to forage on them, but ensured they would not be receptive the following week when the state of the colony was reversed. 5 of the 10 marked flowers in each sampling area were evenly spaced along the same polytunnel as the commercial colony, and a further 5 were in the two tunnels either side of the tunnel containing the colony (3 marked in one tunnel and 2 in another). In the case of the two longer polytunnels in the June-bearer experiment (sampling areas 3 and 6 on supplementary figure S2) 5 flowers were marked in the same tunnel as the

commercial colony, and the other 5 were marked in one adjacent polytunnel. The flowers were marked with a small twist of coloured wire, different colours were used to represent different weeks of the experiment, and different weeks of the experiment were associated with times when colonies were open or closed.

Figure 1. *Recently opened strawberry flower with silver tag attached.*

Marked berries were picked just before they fully ripened to reduce the chance of farm employees harvesting them. Upon picking, the growth position (primary, secondary, tertiary, quaternary) of each fruit was noted following Darrow (1929). Noting the growth position is important because fruits from later growth positions are usually smaller in size. The distance of each fruit from the closest end of the polytunnel was also noted.

At the end of the experiment, there were a group of berries from times when colonies were open that could have been pollinated by commercial bees and wild pollinators, and a group of berries that could only have been pollinated by wild pollinators. Totals of 382 (207 and 175 from when colonies were open and closed respectively) and 826 (416 and 410 from when colonies were open and closed respectively) tagged fruit were picked from the June-bearer and everbearer experiment respectively. The growth positions of all berries were known. All fruits were stored at -20°C for later quality assessment (see 'Strawberry quality assessment' for details).

Recording flower visitation rate

Every week 30 minute transects were walked along the centre of each polytunnel that contained a colony, but not along the adjacent tunnels in the same sampling

area. Every individual insect that was observed visiting strawberry flowers within 1 metre either side of the transect line was recorded and identified into one of the categories defined in Table 1. If it was necessary to take a collection of an insect, the transect time was paused whilst the collection was done. Workers of *B. terrestris audax*, *B. lucorum, B. magnus* and *B. cryptarum* were grouped together due to the difficulty of reliably separating them in the field. A flower visit was defined as an individual being present on any part of the flower. The flower visitation rate was reported as visits hour^{-1}.

Table 1. *Taxonomic groups that all flower visitors were placed into. When possible, individuals were identified to lower levels within each category.*

Category	Description
Andrenidae	Hymenoptera the Andrenidae family
Apidae	Hymenoptera of the Apidae family. All bumblebees were identified to species level apart from workers of *B. terrestris* and *B. lucorum*
Coleoptera (other)	Coleoptera that were not be assigned to a family
Diptera (other)	Diptera that were not be assigned to a family
Formicidae	Hymenoptera of the Formicidae family
Lepidoptera	Individuals of the Lepidoptera order
Muscidae	Individuals of the Muscidae family
Anthomyiidae	Individuals of the Anthomyiidae subfamily of Muscidae
Nitidulidae	Coleoptera of the Nitidulidae family
Oedemeridae	Coleoptera of the Oedemeridae family
Stratiomyidae	Diptera of the Stratiomyidae family
Syrphidae	Diptera of the Syrphidae family
Unknown	Identity unknown

Dye dispensing boxes were attached to the entrance/exit of every commercial colony to make commercial *B. terrestris audax* and wild *B. terrestris audax* visually distinguishable. The design was adapted from Martin et al (2006). The dye dispensers worked by dispensing a small amount of non-toxic coloured powder dye onto the dorsal surface of the thorax of every bee that exited the colony. The coloured dye served to identify bees from commercial colonies during transect walks. The dye dispensing method was only used for the June-bearing experiment for two reasons. Firstly, it proved to be an unreliable method for distinguishing

between commercial and wild *B. terrestris audax*. Secondly, deposits of dye were found on the surface of a fruit, meaning I could not use this method in a commercial farm setting. Hence, I do not report results from this part of the experiment.

Colony activity and mass measures

Colony activity surveys were done during periods when the colonies were open (once every 2 weeks). The entrance/exit of the nest was observed for 15 minutes and every instance of a bee entering or exiting was recorded.

The mass of each colony was measured at the start of the experiment and during the time periods when they were closed (once every 2 weeks) when all the bees were present in the colony.

Strawberry quality assessment

Each fruit was assigned a classification ("extra class", "class 1", "class 2", or "class 3") based on deformations and areas of tightly clustered achenes (see Figure 2 for examples of strawberries from each commercial grade classification), and following EU marketing guidelines (European Commission 2011). Fruits are placed into 'extra class' if they are highly symmetrical and possess no deformations or clusters of achenes and are greater than 25mm in diameter. Class 3 fruits are highly asymmetrical and deformed, and have tightly clustered achenes. Classes 1 and 2 fall in between these two extremes, and must have a diameter of at least 18mm. Extra class and class 1 fruits are the highest commercial grades and consequently of the greatest market value, class 2 fruits have a reduced market value, and class 3 fruits are unmarketable. Although extra class and class 1 fruit can be separated as mentioned above, in practice they are often combined (Klatt et al. 2014).

The diameter of each fruit was measured to the nearest hundredth of a millimetre at its widest point with digital calipers (Mitutoyo Digimatic Caliper), and the mass of each fruit was measured to the nearest hundredth of a gram.

Finally, the fruit was placed in a food processor (Tefal Minipro 500W) and blended for 10-15 seconds with 100ml distilled water. Fertilised achenes are heavier than water and so sink to the bottom. In contrast, unfertilised achenes float at the surface (Klatt et al. 2014). This separation allows for a very direct measure of pollination success. A further 100-200ml of distilled water was added to the solution in order to create a greater degree of separation between the unfertilised

and fertilised achenes, and any achenes that were stuck to the lid, blades or sides of the food processor were washed back into the mixture. The unfertilised seeds were removed from the surface and counted. The water was then very slowly drained into another container, leaving the sunken fertilised seeds to be counted.

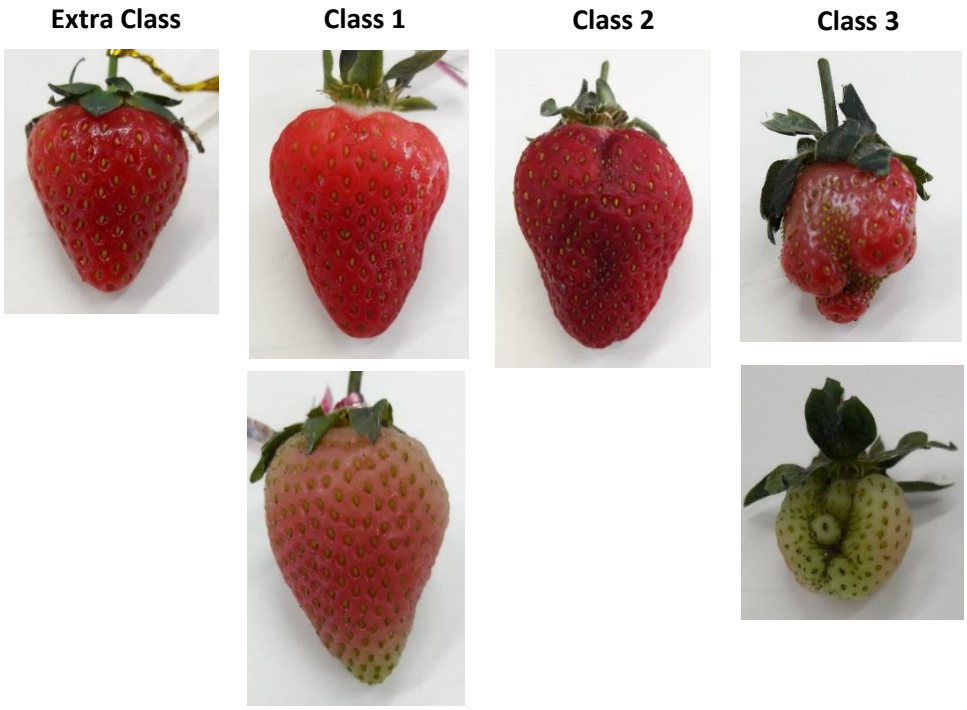


Figure 2. *Examples of strawberries from each commercial grade classification. Strawberry colour was not taken into account in the quality assessment, as berries often had to be harvested before fully ripened.*

Estimating fruit value

The value of the strawberries was calculated from the UK strawberry market value index for 2016, which gives approximations of the values of strawberries within each quality class. These values were applied to the proportions of strawberries from each quality class that were collected from the farm. A percentage change between the value of strawberries when colonies were open compared to when colonies were closed was calculated. This percentage increase was then applied to total value of the UK strawberry crop in 2015, which was £284.1 million (figures from 2016 are still listed as 'provisional' by DEFRA, and thus were not used). Of the total strawberry value, it was estimated that 55% (£156.26 million) of this value was from June-bearer varieties and 45% (£127.84 million) from everbearer varieties

(Richard Harnden pers comms.). The percentage changes in values for both everbearers and June-bearers were then subtracted from their respective total values to provide an estimate of the value that commercial bumblebees were providing to the crop.

This method to estimate the value does assume that all farmers in the UK are using commercial bumblebees on their crop, which is known not to be the case. However, the majority of farmers do use commercial bees in strawberry crops. It should also be noted that individual retailers have their own exact specifications for strawberry quality meaning that the boundaries between classifications could be slightly different from one retailer to the next. The monetary value calculated based on this method of estimation will also fluctuate from year to year based on the total value of the UK strawberry crop.

Bumblebee sampling and parasite screening (see Chapter 3 methodology page 101 for detailed description of bumblebee sampling and dissection protocol)

A sample of bumblebees were removed from each commercial colony during every week that they were in the strawberry crop for. All bee samples were then frozen and later dissected for the presence of parasites.

Statistical analyses

***Bombus terrestris audax* flower visitation**

All statistical analyses were done using 'R' programming software (R Core Team 2018). Generalised linear mixed effects models from the package 'lme4' (Bates et al. 2017), were used to analyse the number of *B. terrestris audax* flower visits in the June-bearer crop. Poisson error structures were used as the data were counts. In the everbearer crop, negative binomial generalised linear mixed effects models, from the package 'glmmADMB' (Skaug et al. 2016), were used to account for overdispersion. The covariables included in the models were 'colony status' (whether the colony was open or closed), and temperature. Humidity was measured and considered as a covariable, but it was omitted due to its high degree of collinearity with temperature. The random effects included the identity of the colony nested within the identity of the field, and both of these were crossed with the sampling week, to reflect that each field was repeatedly sampled each week.

All pollinator flower visitation

Negative binomial generalised linear mixed effects models, from the package 'glmmADMB' (Skaug et al. 2016), were used to analyse the number of wild pollinator visitation events and to investigate whether commercial *B. terrestris audax* presence or visitation abundance was influencing wild pollinator visitation. Negative binomial models were used to account for overdispersion. The covariables included in the models were 'colony status', 'number of *B. terrestris audax* flower visits' and temperature. The random effects structure was the same as in the *B. terrestris audax* flower visitation models.

Strawberry quality

Linear mixed effects models, from the package 'lme4' (Bates et al. 2017), were used to analyse the proportion of fertilised achenes per fruit, fruit mass and fruit diameter. Cumulative link mixed models, from the package 'Ordinal' (Christensen 2017), were used to analyse the strawberry classification variable, these models are suitable for handling ordinal response variables.

The covariables included in the models were 'colony status', the 'growth position' of the fruit (primary, secondary, tertiary or quaternary), the distance of the fruit from the end of the polytunnel, the Nitidulidae beetle abundance recorded from transects, and the wild pollinator abundance recorded from transects. Nitidulidae abundance was initially part of the wild pollinator abundance variable, but the beetles were so numerous that I treated them as a separate variable to investigate what, if any, effect they were having on strawberry quality. For the analysis of the June-bearing strawberries, the strawberry variety was also included as a covariable since two varieties were sampled. This was not necessary when analysing the everbearing strawberries as only one variety was sampled.

The random effects structures of all the strawberry quality models were the same. They all included the identity of the colony nested within the identity of the field, and both of these were crossed with the sampling week. For the June-bearer strawberry quality models, fitting a random slope and intercept for the strawberry variety was tested. This allowed for each variety of strawberry to respond differently to the presence of commercial bees. However, these models fitted less

well (ΔAICc > 2) than models with the simpler random effects structure, thus they were not used for further analyses.

For all response variables, candidate models were compared using an information theoretic approach. Candidate models included all possible combinations of covariables. A 'null model', which only included the intercept as a predictor, was also included in model comparison. The Akaike Information Criterion corrected for small sample sizes (AICc) was used to compare models, those with the lowest AICc were judged to be the best fitting (Johnson & Omland 2004). If several models were within two AICc units of the optimal model (model with lowest AICc), then parameter estimates were obtained by model averaging the best set of models (Δ2AICc set) using the 'MuMIn' package (Johnson & Omland 2004; Bartoń 2017). Models were validated by visual inspection of plots of the residuals plotted against the fitted values. Overdispersion and underdispersion were assessed by examining the ratio of the residual deviance to the residual degrees of freedom. Overdispersion was also tested using the R function 'overdisp_fun()'. Models were not over-dispersed and there was no collinearity between variables used.

The effects of parasitism

To assess whether parasitism of the commercial bumblebees had any effects on *B. terrestris* flower visitation and strawberry quality, separate analyses were performed on a subset of the data, only when colonies were open. When colonies were closed, commercial bumblebees were not visiting strawberry flowers or pollinating fruit regardless of their infection status, so this data was excluded. The models for *B. terrestris* visitation and strawberry quality measures took the same structures as those specified above, only with the prevalence of *Crithidia bombi* and *Apicystis bombi* included as covariables, and 'colony status' removed as the colonies were always open for the data used in these analyses. Model selection and validation was also performed in the same manner as specified above.

Colony mass and activity

The mass and foraging activity levels of colonies from the June- and everbearing experiments were aggregated for each experiment and compared using Wilcoxon-Mann-Whitney tests.

Results

In the June-bearer experiment, 27 hours of transect walks were completed during which 574 strawberry flower visits were observed. A total of 382 tagged fruit were recovered for quality assessment, 207 from when colonies were open and 175 from when colonies were closed. For the everbearer experiment, 48 hours of transect walks were completed during which 5176 flowers visits were observed. 826 tagged fruits were picked, 416 from when colonies were open and 410 from when colonies were closed.

Bombus terrestris audax **flower visitation**

In the June-bearing strawberry crop, the optimal model included only the colony status variable and no model averaging was required (Table 2), indicating that colony status was a good predictor of *B. terrestris audax* flower visitation. When colonies were open, *B. terrestris audax* visitation rate to strawberry flowers was higher than when colonies were closed (Figure 3; estimate = 1.43; 95% confidence intervals = 0.99 – 1.92). Models including temperature were not well supported, indicating that this variable was not an important predictor of strawberry flower visitation. However, this trend was not evident in the everbearing crop (Figure 4). Here, colony status was not a good predictor of flower visitation (ΔAICc to best model = 2.27), but temperature was (0.065; 0.0088 - 0.12), with more visits occurring at higher temperatures.

Table 2. *Candidate models used to investigate the effect of colony status and temperature* on B. terrestris *strawberry flower visitation in the June-bearer and everbearer crops. The null model included only the intercept as a predictor, but included the same random effects structure as all other candidate models. Models are presented from the optimal model with the lowest AICc to the model with the highest AICc at the bottom. The optimal model and those within <2ΔAICc are highlighted in bold. When more than 1 model is highlighted, model-averaging was performed to obtain estimates.*

Model	AICc	ΔAICc
June-bearers		
colony status	**237.50**	**0.00**
colony status + temperature	240.03	2.53
null	280.49	42.99
temperature	281.02	43.52
Everbearers		
temperature	**360.70**	**0.00**
colony status + temperature	362.97	2.27
null	363.33	2.63
colony status	365.53	4.83

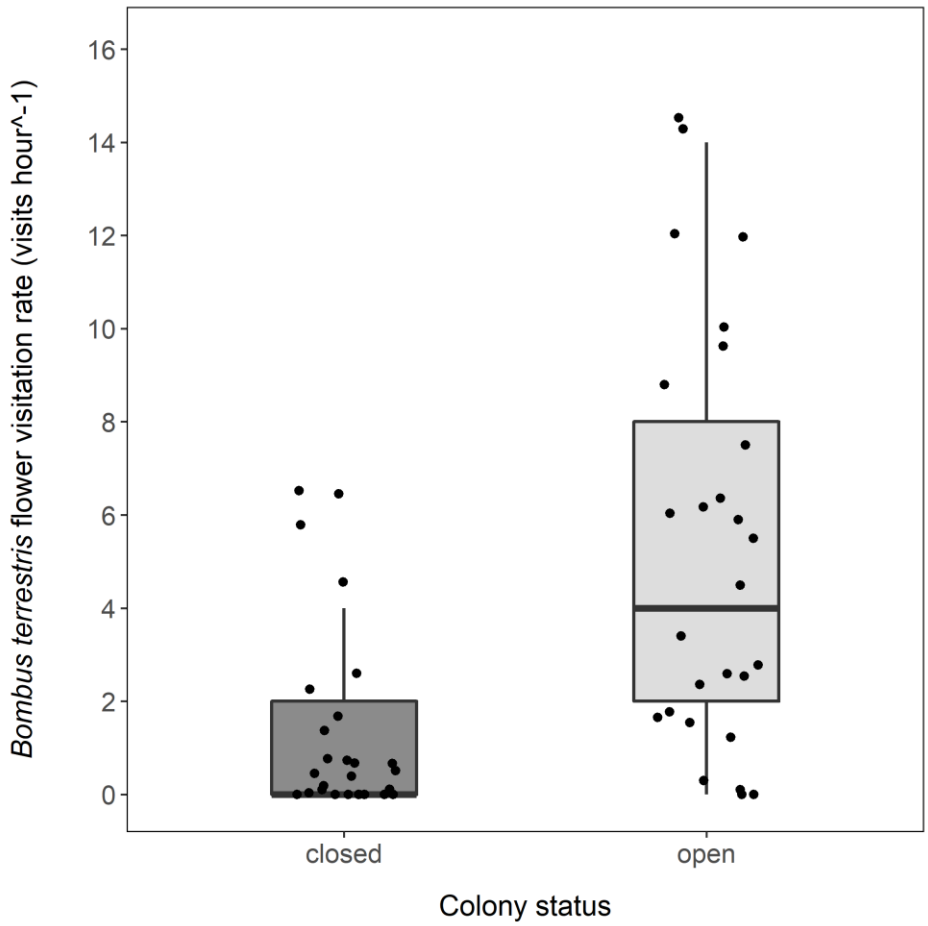


Figure 3. *The flower visitation rate (visits hour^{-1}) of* B. terrestris *on strawberry flowers in the June-bearing crop. The median (central horizontal line), quartiles (box), non-outlier ranges (vertical lines) and raw data (dots) are presented on the plot.*

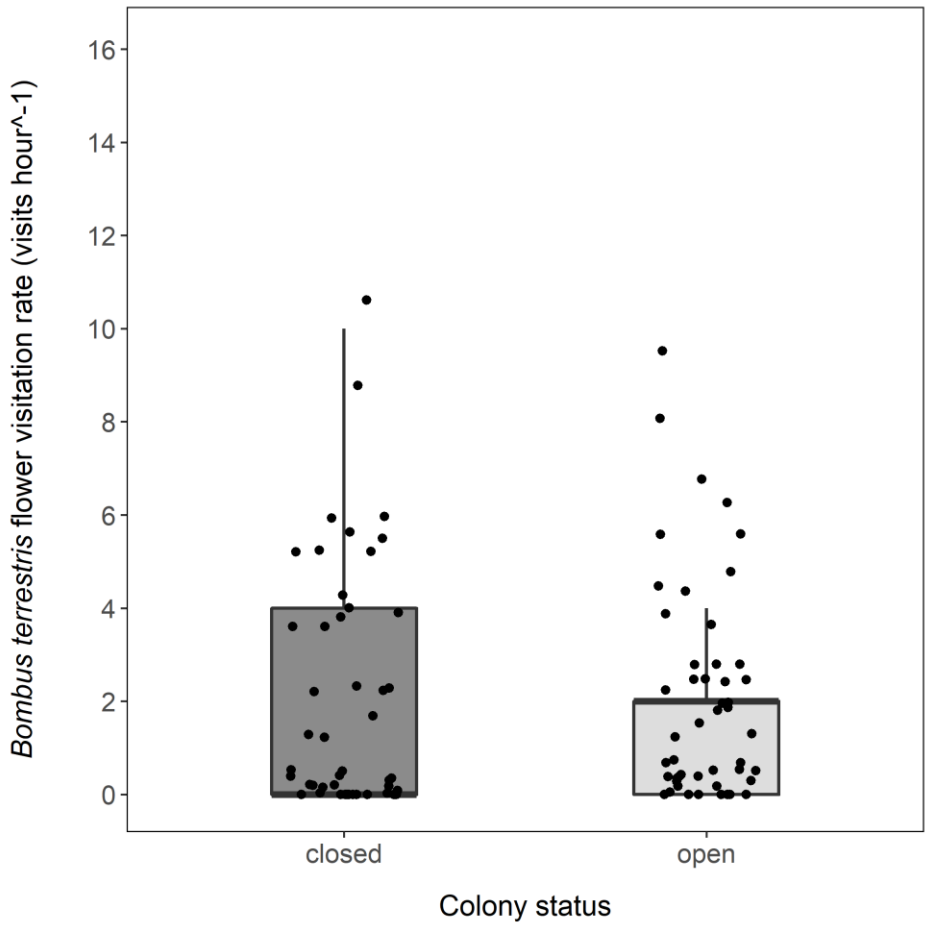


Figure 4. *The flower visitation rate (visits hour^{-1}) of* B. terrestris *on strawberry flowers in the everbearing crop. The median (central horizontal line), quartiles (box), non-outlier ranges (vertical lines) and raw data (dots) are presented on the plot.*

All pollinator visitation

In the June-bearers, a total of 574 strawberry flower visits were observed over the course of 27 hours of transects walks. Coleoptera of the family Nitidulidae were the most abundant flower visitors (n=325), followed by Muscoidea Anthomyiidae (n=103), and Apidae (n=94). Syrphidae (n=26) and 'other Diptera' (n=22) were scarcer. 'Other Muscidae' (n=2), Dermaptera (n=1), and Aphididae (n=1) were the least abundant flower visitors. The mean (± S.E.) wild pollinator visitation rate was (no. of wild pollinator visits = all flower visits – *B. terrestris audax* visits) 18.08 (± 2.66) visits/hour.

In the everbearers, 5176 flower visits were observed over 48 hours of transect walks. Again, Nitidulidae were by far the most abundant flower visitors (n=4378),

followed by Diptera (other) (n=231), Muscoidea Anthomyiidae (n=222), Apidae (n=135), Syrphidae (n=76), Empididae (n=63), Coleoptera (other) (n=45), Stratiomyidae (n=10), Formicidae (n=6), Lepidoptera (n=4), Muscidae (n=3), Andrenidae (n=1), Oedemeridae (n=1), and unknown (n=1). The mean (± S.E.) wild pollinator visitation rate was 105.92 (± 26.02) visits/hour.

B. terrestris audax visitation was not a good predictor of wild pollinator visitation in both the June- and everbearing crops, not featuring in the Δ2AICc model sets (June-bearer: ΔAICc to best model = 2.60, everbearer: ΔAICc to best model = 2.30). Colony status did feature in the Δ2AICc model sets as a predictor of wild pollinator visitation, but its effect was not strongly supported in either the June- or everbearing crops as parameter estimate confidence intervals crossed zero (June-bearer: 0.66; -0.41 – 1.73, everbearer: 0.15; -0.11 – 0.40).

Strawberry quality

Berry mass and diameter

In the June-bearer crop, model-averaged parameter estimates indicated that colony status was not a strong predictor of fruit mass or diameter (mass: ΔAICc to best model = 2.10, diameter: -0.12; -0.41 – 0.17). The growth position of the fruit received strong support as a predictor of both fruit mass and diameter. This variable appeared in all five of the Δ2AICc set of models. As expected, berries from secondary and tertiary growth positions were lighter and smaller than those from primary growth positions (**secondary**: mass: -0.21; -0.25 – -0.16, diameter: -1.00; -1.21 – -0.79, **tertiary**: mass: -0.39; -0.46 – -0.33, diameter: -1.98; -2.30 – -1.66). The variety of the fruit was also a strong predictor of mass and diameter (mass: -0.13; -0.23 – -0.037, diameter: -0.61; -0.91 – -0.30).

In the everbearer crop, colony status was not a good predictor of mass (mass: -0.034; -0.19 – 0.12), or diameter (ΔAICc to best model = 2.04). Growth position featured in all of the Δ2AICc models indicating that it was a good predictor of both fruit mass and size. Berries from secondary and tertiary growth positions were lighter and smaller (**secondary:** mass: -1.02; -1.20 – -0.84, diameter: -0.10; -0.13 – -0.071, **tertiary:** mass: -2.44; -2.79 – -2.08, diameter: -0.45; -0.51 – -0.38). Surprisingly, wild pollinator abundance had a negative effect on fruit mass and diameter (mass: -0.028; -0.047 – -0.0089, diameter: -0.0045; -0.0078 – -0.0011).

Pollen beetle abundance also had a negative effect on fruit mass (mass: -0.0016; -0.0030 – -0.00011).

Achene ratio

Colony status was not a strong predictor of the proportion of fertilised achenes on a fruit in the June-bearer crop (0.068; -0.016 – 0.15). Distance to the edge of the polytunnel was a well-supported predictor (-0.0041; -0.0068 – -0.0015), with the proportion of fertilised achenes increasing on fruits that were closer to the ends of the polytunnels. The variety of the fruit was also a good predictor (0.096; 0.011 – 0.18). In the everbearer crop, colony status was also not a well-supported predictor of the proportion of fertilised achenes (ΔAICc to best model = 2.03).

Strawberry Class

In the June-bearer crop, colony status was a well-supported predictor of fruit quality classification (-0.93; -1.64 – -0.21). Greater proportions of fruit of the highest commercial grades were found during periods when colonies were open (Figure 5). 17.5% more extra class and class 1 fruit were harvested from the open colony periods than from the closed. Variety was also a good predictor of strawberry class (1.51; 0.73 – 2.29). However, in the everbearer crop, colony status was not a well-supported predictor of strawberry quality (Figure 6), featuring in none of the model averaged Δ2AICc models (Table 3; ΔAICc to best model = 2.03). The only well-supported predictor was the growth position of the fruit, with fruit from secondary growth positions being of lower quality (-1.57; -2.00 – -1.13).

Table 3. *Model-averaged models* (*the optimal model and those models within <2ΔAICc*) *used to investigate the best predictors of fruit commercial grade in the June-bearer and everbearer crops. Each line of the table represents a unique model. + symbols indicate the inclusion of that covariate in the model. Models including all the predictor variables were tested. The null model included only the intercept as a predictor, but included the same random effects structure as all other candidate models. The covariable 'variety' was not included in everbearer models because only one variety of strawberry was sampled.*

colony status	growth position	distance to edge	beetle abundance	wild pollinator abundance	variety	AICc	ΔAICc
June-bearers							
+		+			+	509.60	0
+		+	+		+	509.98	0.38
+		+				511.55	1.95
+					+	511.57	1.97
Everbearers							
	+				na	817.80	0
	+	+			na	818.36	0.56
	+			+	na	818.92	1.12
	+	+		+	na	819.35	1.55
	+		+		na	819.56	1.76

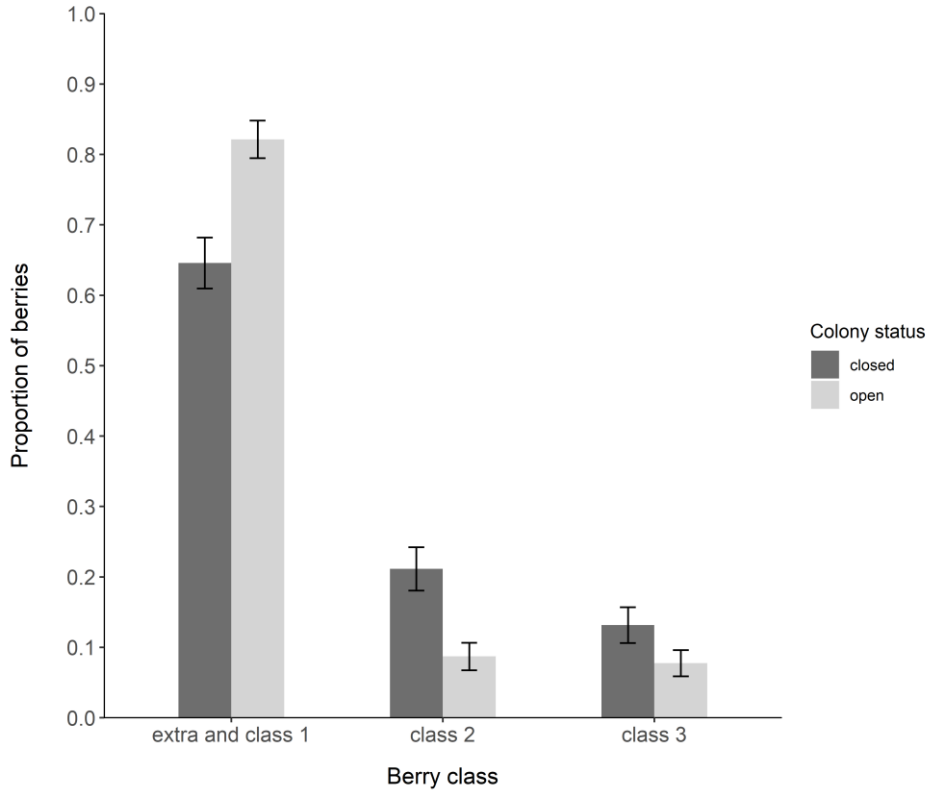


Figure 5. *Proportion of fruits within each commercial grade from each treatment in the June-bearing crop. Error bars represent ± standard error of the mean.*

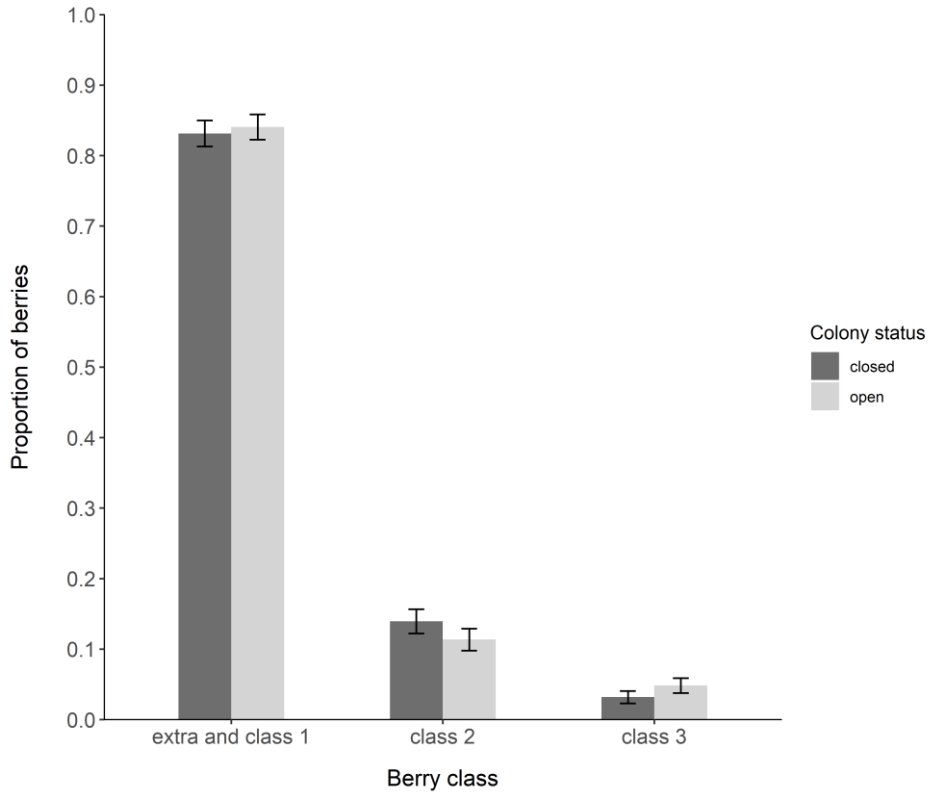


Figure 6. *Proportion of fruits within each commercial grade from each treatment in the everbearing crop. Error bars represent ± standard error of the mean.*

Colony mass and activity

Colony mass and activity did not differ between the colonies used in the June- and everbearer experiments (mass: $p = 0.81$, activity: $p = 0.53$).

Parasitism of colonies (see Chapter 3 for more detailed assessment of colony parasitism)

In the colonies placed into the June-bearing crop, *Apicystis bombi* was the only parasite detected. It was found in two bees from a single colony from the final sampling time point of the experiment. Parasites were more prevalent in the colonies in the everbearing strawberry crop. All 12 colonies became infected with at least one of *A. bombi*, *Crithidia bombi*, *Nosema bombi* or Conopid fly larvae (Conopidae) at some point during the experiment. *A. bombi* and *C. bombi* were by far the most prevalent, thus, the prevalence of each of these species was included in the everbearer strawberry quality and *B. terrestris* visitation models. Neither *A. bombi* nor *C. bombi* prevalence were well-supported predictors of any strawberry quality measure or *B. terrestris* visitation rate.

Discussion

To the best of my knowledge, this is the first study investigating the effect of commercial bumblebees on strawberry crop quality in a commercial farm setting. With commercial bumblebees being used on a variety of crop types, studies like this are essential to verify the assumed benefits that bees provide to the crop. The results indicate that the addition of commercial *B. terrestris audax* colonies to a June-bearing strawberry crop increases the percentage of high commercial grade (extra class and class 1) fruits by 17.5%. This improvement in quality is estimated to be worth approximately £16 million to the total value of the UK strawberry crop, based on the value of the crop during 2015 (Harnden pers. comm., 2018). However, in an everbearing strawberry crop, commercial bees provide no additional benefit to any aspect of fruit quality or yield.

One of the main drivers behind the observed difference in the effectiveness of commercial bumblebees in June- and everbearing strawberry crops, is likely to be the differing visitation rates of *B. terrestris audax* on strawberry flowers in these

two crops. In the June-bearer crop, there were considerably more *B. terrestris audax* visits when colonies were open, which suggests that these visits were being made by the commercial bees. This is important because previous studies have suggested that commercial bees may predominantly forage on alternative flowers to the target crop (Lye et al. 2011; Murray et al. 2013; Foulis & Goulson 2014). During the time periods when the colonies were closed, very few *B. terrestris audax* visits were observed in the June-bearers. Wild bumblebees are still establishing nests and in the early stages of colony development at this time of year, indeed 35.3% of the total flower visits made by wild bumblebees in this experiment were made by newly emerged queens. Consequently, it was not surprising to observe low numbers of wild *B. terrestris audax* in the June-bearer polytunnels.

In the everbearing crop, there was very little difference in the *B. terrestris audax* visitation rate between when colonies were open and closed. Even when colonies were open, the *B. terrestris audax* visitation rate to the crop was much lower (1.88 ± 0.34 visits/hour) than the equivalent periods in the June-bearer crop (5.26 ± 0.85 visits/hour). This could be because bees were leaving the crop to forage on other resources, which are likely to have been more abundant during the time of the everbearer experiment (May-June), than the June-bearer experiment (March-April). In addition, different strawberry varieties can vary in the sugar concentration of their nectar, and in the floral volatiles they produce, both of which could alter their attractiveness to pollinators (Abrol 1992; Klatt et al. 2013). Thus, it is possible that the everbearing variety used in this experiment was not highly attractive to bumblebees both wild and commercial. Furthermore, the wild *B. terrestris audax* population is likely to have been higher during the time of the everbearer experiment, as this time period coincides with when wild colonies are reaching their peak. This is likely to have contributed to the lack of difference seen between visitation rates when colonies were open and closed in the everbearer crop. This, in turn, could have contributed to commercial colonies having no impact on fruit quality in this crop. It is possible that deploying commercial bumblebees at a higher density in everbearing strawberry crops could increase the *B. terrestris* visitation rate and subsequent quality of the crop, but this remains to be tested.

Another factor that could have affected the results is that commercial bumblebees may have foraged on strawberry fields outside of the one in which the colony was placed i.e. bumblebees could have left a field in which all the colonies were open,

and foraged in a field in which the commercial colonies were closed, as some of the fields were within *B. terrestris audax* foraging range of each other. However, *B. terrestris audax* mainly fly at <3m above the ground (Osborne et al. 1999) and the windbreaking hedges separating all fields were >5m, which would have deterred bumblebees from crossing to adjacent fields. It is also not clear if *B. terrestris audax* would travel a greater distance (e.g. into a different field) to collect the same quality resource (strawberry). I believe that these factors reduce the likelihood that bumblebees will have foraged regularly in other strawberry fields. The results from the June-bearer experiment further support this, as I would not expect to see significant differences in visitation rate and strawberry quality between the open and closed colony treatments if commercial bumblebees were foraging in adjacent fields in large numbers.

The polytunnel architecture itself may also have influenced the results. Following standard practice, during the June-bearer experiment, the polythene on the sides of the tunnels was rolled down and only the ends of the tunnels were open, but in the everbearer polytunnels both the sides and ends were open. This may have had a large impact on the effectiveness of the commercial bumblebee colonies. For example, the June-bearer crop may have been more difficult both for wild pollinators to access and for commercial bees to leave (Ellis et al. 2017). If this were the case, the commercial bees may have been more likely to forage on the strawberry crop than on alternative foraging resources, thus providing a better pollination service to the crop. This situation could have been reversed in the everbearing crop, where the more open polytunnels may have allowed commercial bees to leave and wild pollinators to enter the crop more easily. This could have contributed to the commercial bees' apparent lack of effect in the everbearing crop, and suggests that it may be the interaction between crop management practices and commercial bumblebees, rather than the provision of commercial bumblebees alone, that drive potential pollination benefits.

A final factor that could have contributed to the colonies used in the June-bearer experiment being more beneficial to the crop could be the health of the colonies themselves. While colonies were parasite-free on arrival, parasite prevalence at the end of the experiment was much higher in the colonies from the everbearer experiment than those from the June-bearer experiment (Martin et al. unpublished data Chapter 3). Such parasitism could potentially have reduced the bees'

pollination ability by increasing host mortality (Brown et al. 2000; Otti & Schmid-Hempel 2007) or altering host behaviour (Gegear et al. 2006).

Despite the observed benefits provided by the commercial bees in the June-bearer crop, the *B. terrestris audax* visitation rate was low even when colonies were open (5.26 ± 0.85 visits/hour). Habitat management schemes have been shown to increase pollinator visitation on or around crops (Kleijn & Sutherland 2003; Blaauw & Isaacs 2014; Feltham et al. 2015; Campbell et al. 2017; Venturini et al. 2017), and it is possible that such schemes could reduce the need for commercial bumblebees, particularly if wild bumblebees benefitted from the management. However, evidence of habitat management schemes increasing the pollination services provided to a crop are rare (Blaauw & Isaacs 2014; Venturini et al. 2017), and the results presented here do not clarify the role that wild pollinators have in strawberry pollination on a farm setting. Thus, further research would be required to investigate the efficacy of habitat management schemes in improving pollination services to a strawberry crop, before this could be recommended as an alternative strategy to commercial bee use in June-bearing crops.

Our results provide no evidence that wild pollinators are improving the crop, which is in contrast to other studies (Greenleaf & Kremen 2006; Holzschuh et al. 2012; Garibaldi et al. 2013; Blaauw & Isaacs 2014). Wild pollinator flower visitation was not a well-supported predictor of fruit quality in the June-bearer crop. This could be explained by the relatively low numbers of wild pollinators present during this time of the year not being sufficient to impact any fruit pollination measures. In the everbearer crop, there was even a negative effect of wild pollinator flower visitation on fruit mass and diameter, despite there being much greater wild pollinator flower visitation rates than in the June-bearer crop. A factor contributing to this could be that the crop was not pollen limited for the duration of the everbearer experiment, even at times when wild pollinator abundances were at their lowest. If this was the case, then additional visits would not be providing any benefit to the crop, and could potentially even be decreasing the quality of the crop by overpollination (Velthuis and van Doorn 2006; Mommaerts, Put, and Smagghe 2011). Another possibility could be that the everbearer variety used in this experiment was not dependent on insect pollination to achieve good quality fruits and high yield. However, previous research suggests that even though strawberry varieties do differ in their levels of self-compatibility, the majority do depend on

insect pollination to maximise fruit yield and quality (Nye & Anderson 1974; Zebrowska 1998; Klatt et al. 2014; Hodgkiss et al. 2018). Thus, I believe that this explanation is unlikely to be a major contributor to the results I observed.

Of all the strawberry flower visitors, Coleoptera of the family Nitidulidae were by far the most abundant in the June-bearing and everbearing crops. Given their dominance in the system, I treated them as a separate covariable, to investigate whether their abundance was a predictor of strawberry pollination. The majority, if not all of these, were of the genus *Meligethes.* These are pollen and nectar feeding beetles, and there is contrasting evidence as to whether *Meligethes aeneus* provides pollination services or not on oilseed rape plants (Åhman et al. 2009; Chifflet et al. 2011). In this experiment, pollen beetle visitation did not positively affect any strawberry quality measure, suggesting they do not provide any pollination service to the crop.

It should be noted that my definition of flower visitor does not necessarily mean that the visitor was a pollinator of strawberry, and it does not take into account that different species are not equally effective at pollinating strawberry. These factors may have contributed to the apparent lack of effect that wild flower visitors were having on strawberry pollination and quality. However, the main focus of the study was to test the effectiveness of commercial bumblebees.

The lack of effect of commercial bumblebees on strawberry quality in the everbearer crop raises questions about commercial bumblebee utility in this crop system, especially considering the negative impacts associated with commercial colony importation. However, this experiment did not cover the entire flowering period of the everbearer crop, which can continue into the late summer months (September/October). It is possible that commercial bumblebees may become of more use later in the season, as wild pollinator abundance and alternate foraging resources decrease (Hallmann et al. 2017). Furthermore, other locations may have more depauperate pollinator communities than my selected study farm, and studies have shown that pollinator communities can drastically change from year to year (Kremen et al. 2002). In such cases, commercial bumblebees may be of use as an insurance policy for crop pollination. Further studies are required to investigate whether the effects observed here vary over space and time, but this study provides a detailed baseline from which to build further studies.

The weekly opening and closing of colonies could itself have impacted upon the foraging behaviour of the commercial bumblebees and their subsequent effectiveness at pollinating the crop. When colonies were closed, bumblebees may have become accustomed to feeding from the internal nectar reservoir that is supplied with all commercial colonies. Then when colonies were reopened, bees may have carried on preferentially foraging on the nectar reservoir rather than the crop, potentially reducing their effectiveness at pollinating the crop. However, another study that used the same opening and closing technique, also observed a positive impact of commercial bumblebee presence (Lye et al. 2011), which suggests that this technique does not disturb the foraging choices of commercial bumblebees. Furthermore, during periods of cold weather bumblebees may naturally remain within the colony regardless of whether it is open or closed, and thus, still have periods of time when they are solely foraging from the nectar reservoir.

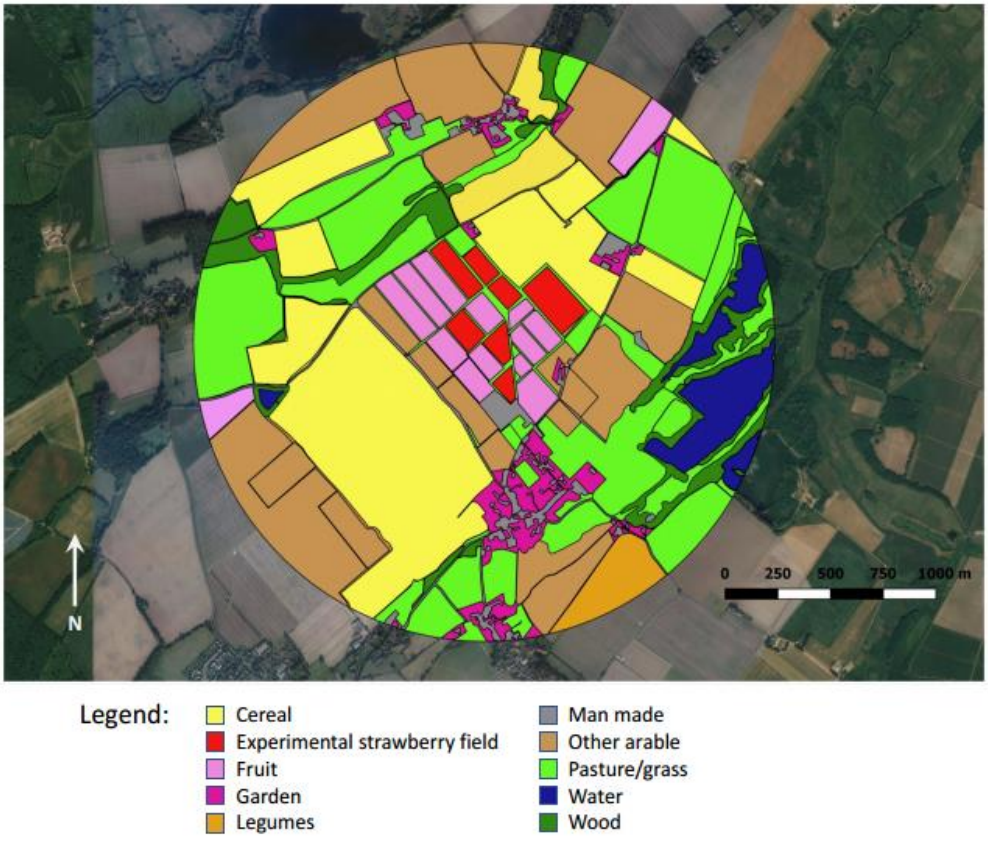
The positive effect of commercial bumblebees on fruit quality in the June-bearing crop is comparable to studies demonstrating benefits of commercial bees in other soft fruit crops (Stubbs & Drummond 2001; Lye et al. 2011). I believe the results observed in the June-bearer crop to be broadly generalisable across UK and Northern European strawberry crops grown in polytunnels. Most locations in these areas are unlikely to have strong wild pollinator populations during March and April when polytunnel-grown June-bearers are flowering (Hallmann et al. 2017), meaning that the addition of commercial bees to the crop during this time is likely to provide a similar benefit to that which was observed here. Given the clear utility of commercial bumblebees, greater emphasis should be placed on reducing the negative impacts associated with the trade in commercial colonies, so that they can be used with minimal risk to environmental health.

Conclusion

As commercial bumblebees are increasingly marketed for a broad range of crop types, studies like this one are essential to prove the bees are having a beneficial effect, and to inform growers of how to use commercial bumblebees in an environmentally responsible and cost-effective manner. A 17.5% increase in high commercial grade fruit represents a significant increase in the value of the strawberries produced, estimated to be worth approximately £16 million to the

total value of the UK strawberry crop in 2015. Thus, the results support the widespread use of commercial bumblebees in June-bearer crops on strawberry farms. Similar benefits are likely to be seen in other countries where similar growing systems are implemented. However, commercial bees placed in an everbearing crop appear to be providing no benefit to fruit quality, indicating that it may not be worth using them in this crop type. In the June-bearers, the increase in fruit quality is provided by a small number of commercial bumblebee flower visits; this raises the possibility that habitat management measures that increase wild bumblebee abundances could create a similar benefit to the crop. However, until such measures have been further researched and are proven to work, the results presented here suggest that commercial bumblebees are a valuable resource to strawberry growers, but that interactions between commercial bees, farm management practices and environmental factors should be carefully considered before deploying commercial bumblebees on a crop.

Supplementary material



Supplementary Figure S1. *Land use classification of the area surrounding the farm. The circular classified area has a diameter of 2.75km with the centre point being in the middle of the farm. This diameter was chosen as it included the land use up to 1km away from all the commercial bumblebee colonies. Definitions of each land use class and the area they occupy can be found in supplementary tables S2 and S1 respectively.*

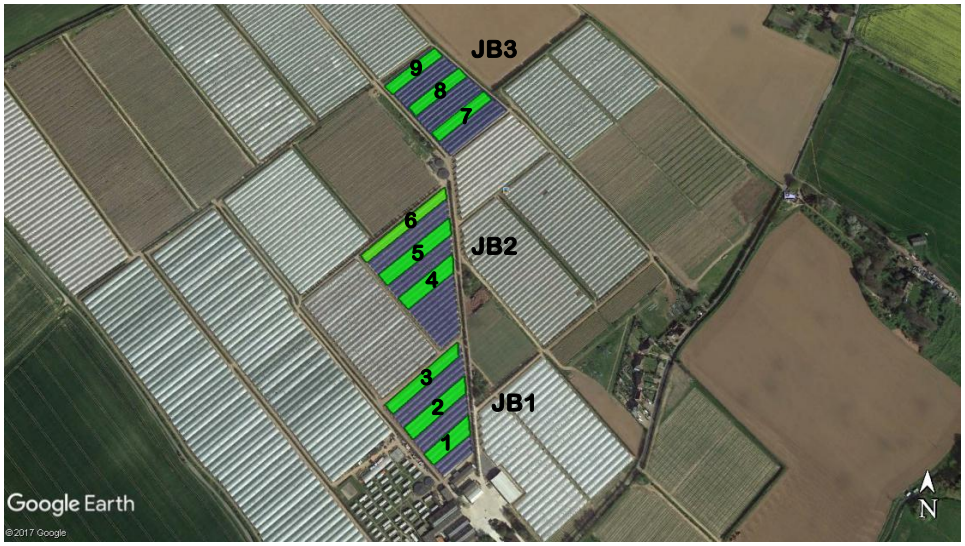
Supplementary Table S1. *The area covered (m^2) by each land use in a 2.75km diameter circle centred on the farm. This diameter was chosen as it includes the land cover within a 1km radius of every commercial bumblebee colony that was placed on the farm. Definitions of each land use class can be found in supplementary table S2.*

Land use	Area covered (m^2)
cereal	1473075
experimental strawberry fields	140113
fruit	313824
garden	216126
legumes	104329
man made	232891
other arable	1333681
pasture/grass	1544499
water	230598
wood	342082

Supplementary Table S2. *Definitions of each land use classification.*

Land use	Description
Cereal	Cereal crops (wheat, maize)
Experimental strawberry fields	Strawberry fields into which commercial colonies were placed
Fruit	Fruit crops (apple, blackcurrant, strawberry, raspberry)
Garden	Residential gardens
Legumes	Legume crops (field bean)
Man made	Roads and buildings
Other arable	Arable crops that were not identified
Pasture/grass	Grazing pasture and grassland areas that were not residential gardens
Water	Areas of water
Wood	Woodland (predominantly mixed deciduous)

Supplementary Figure S2. *Layout of colonies and fields in the June-bearer experiment. The fields sampled are shaded in blue. The green blocks indicate sampling areas; the polytunnels from which strawberries were sampled. Each green block consists of three tunnels: one central tunnel which contained the commercial bumblebee colony, and the two tunnels either side of this central tunnel. The northernmost sampling areas in the southernmost field and in the central field, only consist only of 2 polytunnels. This was done to take into account the differing sizes of the polytunnels (see Methodology for details).*



Supplementary Figure S3. *Layout of colonies and fields in the everbearer experiment. The fields sampled are shaded in red. The green blocks indicate sampling areas; the polytunnels from which strawberries were sampled. Each green block consists of three tunnels: one central tunnel which contained the commercial bumblebee colony, and the two tunnels either side of this central tunnel.*

Supplementary Table S3. *The strawberry variety grown, the size, and the commercial bumblebee colony density within each sampling area from which fruit were picked. The position of each sampling area can be seen in supplementary figures 2 and 3. Fields prefixed with 'JB' were sampled in the June-bearer experiment, and those prefixed with 'EV' are from the everbearer experiment.*

Sampling area	Field	Strawberry variety	Sampling area size (hectares)	Colony density within sampling area (colonies/ha)
1	JB1	Malling Centenary	0.16	6.25
2	JB1	Malling Centenary	0.185	5.405
3	JB1	Malling Centenary	0.168	5.952
4	JB2	Flair	0.167	5.988
5	JB2	Flair	0.21	4.762
6	JB2	Flair	0.188	5.319
7	JB3	Malling Centenary	0.181	5.525
8	JB3	Malling Centenary	0.18	5.556
9	JB3	Malling Centenary	0.178	5.618
10	EV1	Proprietary variety 1	0.165	6.061
11	EV1	Proprietary variety 1	0.16	6.25
12	EV1	Proprietary variety 1	0.16	6.25
13	EV2	Proprietary variety 1	0.165	6.061
14	EV2	Proprietary variety 1	0.164	6.105
15	EV2	Proprietary variety 1	0.164	6.105
16	EV3	Proprietary variety 1	0.163	6.154
17	EV3	Proprietary variety 1	0.163	6.154
18	EV3	Proprietary variety 1	0.163	6.154
19	EV4	Proprietary variety 1	0.2	5
20	EV4	Proprietary variety 1	0.2	5
21	EV4	Proprietary variety 1	0.2	5

Supplementary Table S4. *Model-averaged models* (*the optimal model and those models within <2ΔAICc*) *used to investigate the best predictors of strawberry mass, strawberry diameter, proportion of fertilised achenes, and strawberry class in the June-bearer strawberry crop. + symbols indicate the inclusion of that covariate in the model. Models including all combinations of the predictor variables were tested. The null model included only the intercept as a predictor, but included the same random effects structure as all other candidate models.*

beetle abundance	colony status	distance to edge	growth position	variety	wild pollinator abundance	AICc	ΔAICc
strawberry mass							
			+	+		2489.50	0.00
			+	+	+	2490.03	0.53
+			+	+		2490.60	1.10
			+			2491.04	1.54
+			+	+	+	2491.40	1.90
strawberry diameter							
			+	+		2316.80	0.00
			+	+	+	2317.73	0.93
+			+	+		2317.82	1.02
	+		+	+		2318.64	1.84
+	+		+	+		2318.72	1.92
achene ratio							
		+	+	+		1592.90	0.00
		+		+		1593.55	0.65
	+	+	+	+		1594.27	1.37
		+	+			1594.49	1.59
+		+	+	+		1594.78	1.88
strawberry class							
	+	+		+		509.60	0.00
+	+	+		+		509.98	0.38
	+	+				511.55	1.95
	+			+		511.57	1.97

Supplementary Table S5. *Model-averaged models* (*the optimal model and those models within <2ΔAICc*) *used to investigate the best predictors of strawberry mass, strawberry diameter, proportion of fertilised achenes, and strawberry class in the everbearer strawberry crop. + symbols indicate the inclusion of that covariate in the model. Models including all combinations of the predictor variables were tested. The null model included only the intercept as a predictor, but included the same random effects structure as all other candidate models.*

beetle abundance	colony status	distance to edge	growth position	wild pollinator abundance	AICc	ΔAICc
strawberry mass						
+		+	+	+	5826.90	0.00
+			+	+	5827.01	0.11
			+	+	5828.52	1.62
		+	+	+	5828.55	1.65
+	+	+	+	+	5828.57	1.67
+	+		+	+	5828.66	1.76
strawberry diameter						
+			+	+	5294.10	0.00
			+	+	5295.05	0.95
+		+	+	+	5295.42	1.32
achene ratio						
+			+		3142.90	0.00
+			+	+	3144.60	1.70
+		+	+		3144.62	1.72
strawberry class						
			+		817.80	0.00
		+	+		818.36	0.56
			+	+	818.92	1.12
		+	+	+	819.35	1.55
+			+		819.56	1.76

Chapter 3

The potential for parasite spill-back from commercial bumblebee colonies: a neglected threat to wild bees?

This chapter is currently in preparation for submission to Biological Conservation:

Martin CD, Fountain MT, Brown MJF. *The potential for parasite spill-back from commercial bumblebee colonies: a neglected threat to wild bees? Biological Conservation.*

Abstract

Commercially-reared bumblebee colonies provide pollination services to numerous crop species globally. However, these colonies have been shown to harbour parasites which can spill-over to, and potentially have negative impacts on, wild bee species. In contrast, the potential for parasites to spread from wild to commercial bumblebees, and the subsequent dynamics of these infections in commercial populations on a crop are not well known, despite possible implications for potential pathogen 'spill-back' to wild bumblebees and for crop pollination. To investigate this, I placed 9 commercial *Bombus terrestris audax* colonies into a June-bearing strawberry crop during March and April 2016, and a further 12 colonies into an everbearing strawberry crop during May and June 2016. Bumblebees were removed from each colony every week for their duration on the strawberry crops. These bees were dissected for the presence of parasites. No infection was detected in any of the colonies before they were placed on the crop. In the June-bearing crop, the only parasite detected was *Apicystis bombi* at a low prevalence (0.46%) at the end of the experimental period. Parasites were far more prevalent in colonies from the everbearing crop, with all colonies becoming infected at some point over the 8-week period. *A. bombi*, *Crithidia bombi*, *Nosema bombi* and conopid fly larvae were all detected. *C. bombi* and *A. bombi* were the most prevalent across all samples, at 10.44% and 4.21% respectively, and they reached their peak prevalences of 39.39% and 18.18% at the end of the experimental period. The *A. bombi* prevalence observed is greater than most other UK records, suggesting that commercial colonies could act as a source of *A. bombi* infection to wild bees. This is of concern as there is evidence that *A. bombi* is highly virulent. This is the first study to observe the dynamics of parasite infection in commercial colonies throughout their time on a crop. It clearly demonstrates that such colonies do acquire parasites from their environment, and that these infections can build-up to high levels, which subsequently could be a threat to wild bee populations via parasite spill-back.

Introduction

Commercial bumblebees have been produced and used for crop pollination since the 1980's (Velthuis & van Doorn 2006). They were first commercialised for the pollination of greenhouse tomato plants and the success of this has led to them being used on a wide variety of crop types (Velthuis & van Doorn 2006). The trade in commercial colonies is now a global industry with over 1 million colonies shipped around the world, and 40-50 colonies thousand imported to the UK on an annual basis (Velthuis & van Doorn 2006; Natural England 2009, 2012).

While commercial bumblebees provide significant pollination services to commercial crops (Morandin et al. 2001a, 2001b; Roldán Serrano & Guerra-Sanz 2006; Martin et al. unpublished data (Chapter 2)), there are, however, several drawbacks to their use. Firstly, commercial bumblebee species can breed with native species (Kanbe et al. 2008; Kondo et al. 2009), which could potentially contribute to biodiversity homogenisation (Meeus et al. 2011), although there is no evidence to support this. Secondly, commercial bumblebees are able to outcompete native species for resources (Ings et al. 2006; Inoue et al. 2008). Finally, commercial colonies have been shown to harbour high pathogen loads, and there is strong evidence that when commercial colonies are placed out in crops, these pathogens can spill-over to wild bee populations (Colla et al. 2006; Goka et al. 2006; Murray et al. 2013; Graystock et al. 2013). The most likely mechanisms of transmission are via the flowers of the target crop which both commercial and wild bees may visit, and via the well-documented occurrence of commercial bumblebees leaving the target crop and foraging on alternative resources where wild bees also forage (Murray et al. 2013; Foulis & Goulson 2014). Such pathogen spill-over is thought to be one of the contributing factors to declines in bumblebee species that have been observed in several areas around the world (Cameron et al. 2011, 2016; Meeus et al. 2011; Schmid-Hempel et al. 2014).

Pathogen spill-over from commercial bumblebee colonies to wild bees has been relatively well studied (see references above), but little consideration has been given to pathogen transmission in the other direction in this system. Commercial bumblebee colonies can occur at high densities on farms (Whitehorn et al. 2013). If these colonies then become infected with a parasite from an external source, subsequent transmission of the parasite between colonies could occur rapidly,

leading to high parasite prevalences in commercial bee populations on farms. These populations could then act as sources for further infection of wild bees. This process is known as 'spill-back' (Kelly et al. 2009). Such spill-back has been documented in other systems and is thought to have the potential to negatively impact upon indigenous wildlife populations (Kelly et al. 2009; Nugent 2011). The potential for spill-back to occur in a commercial bumblebee-wild bee system is not known. Given that several bumblebee parasites are known to reduce the fitness and alter the behaviour of their hosts (Brown et al. 2000; Brown, Schmid-Hempel, et al. 2003; Gegear et al. 2006; Otti & Schmid-Hempel 2007; Graystock, Meeus, et al. 2016), not only could spill-back be harmful to native bee populations, but it could also negatively impact upon commercial colonies with subsequent implications for crop pollination and agricultural outputs.

In addition to their high density, another factor that might enhance the susceptibility of commercial bumblebees to spill-back is the large geographic scale over which this trade occurs. Colonies are often shipped hundreds or thousands of miles to reach their target crop (Velthuis & van Doorn 2006), meaning that they may not have previously encountered the parasite species and strains that the local bee populations are carrying. Novel strains from different geographical areas can induce higher mortality than strains that the population has co-evolved with (Imhoof & Schmid-Hempel 1998a), resulting in higher impacts on commercial colonies than those faced by the native bee population.

The time of year in which commercial bumblebees are deployed on a crop is also likely to have a major effect on the likelihood of colonies spreading parasites to, or picking up parasites from, wild bee populations. Crops grown in polytunnels and greenhouses can have extended flowering periods, including at times when wild bumblebees are not abundant. Commercial bumblebees deployed at these times are less likely to come into contact with wild bees or flowers recently visited by wild bees, and are thus less likely to transmit or become infected with pathogens.

In order to assess the potential for pathogen spill-back to occur in commercial bees, we first need to know the pathogen dynamics in these colonies when they are placed into crop systems. Studies have shown that commercial colonies can carry parasites (Whitehorn et al. 2013; Murray et al. 2013; Graystock et al. 2013; Sachman-Ruiz et al. 2015), but we currently know little about the dynamics of

parasite prevalence in commercial colonies during their full time on a crop. Some evidence suggested that parasite prevalences can become very high in these systems (Whitehorn et al. 2013), in which case commercial colonies could pose a threat to wild bees. However, it is not known if commercial colonies regularly pick up parasitic infection from wild bees, and whether these parasites are able to develop high prevalences in commercial bumblebee populations in a commercial setting.

In this study, I screened commercial bumblebees from colonies on a strawberry farm for parasites throughout a 4-month period of the growing season to address the following questions: 1) do commercial bumblebee colonies acquire parasites from wild populations? 2) If so, do these infections develop to prevalences that could pose a risk to wild bees via spill-back? 3) And does the time of year that commercial bumblebees are deployed affect their likelihood of acquiring a parasitic infection and developing high prevalence infections?

Methodology

The colonies sampled during this experiment were the same as those used in Chapter 2. Thus, the location of fieldwork, the spatial arrangement of the colonies, and the time periods they were in the strawberry crop for, were the same as stated in the methodology of the previous chapter. The colonies were also subject to the same cycle of opening and closing as in Chapter 2. The mean density of the colonies was however different, due to differences in the sampling protocol between the experiments. The sampling areas from which strawberries were sampled in Chapter 2 were not relevant for this chapter, thus the colony densities were taken over whole fields. In the June-bearing strawberry crop, the mean density of colonies across the 3 fields was 2.83 colonies hectare^{-1}, and in the everbearing strawberry crop the mean density across the 4 fields was 1.52 colonies hectare^{-1}. The colonies in the June-bearing crop remained there for 6 weeks, and the colonies in the everbearing crop for 8 weeks. These durations are both within the time frame for commercial colony use as recommended by the supplier (6-8 weeks).

Bumblebee sampling

Bumblebees were removed from commercial colonies using metallic forceps. The forceps were submerged in alcohol and flamed between sampling from different colonies to avoid transmission of pathogens via this route. 10 bees were removed from each colony immediately after colonies were delivered to the field site, and before colonies were opened, so there was no possibility that an infection could have been picked up from an external source at this sampling time point. After this initial sample, bees were positioned in the strawberry crop and sampled at the end of each week of the experiment. Initially, 10 bees from each colony were to be removed each week, but after the first week of the June-bearer experiment this was reduced to 5. This was done as the colonies I was sampling from were also being used in a pollination experiment, so I did not want to remove a large proportion of their workforce. Upon removal from the colonies, all bees were placed into plastic vials and temporarily stored, for between one to four days, in a freezer at approximately -15°C, before being transferred to a -80°C freezer for longer term storage. At the end of the final week of both experiments, colonies were closed at nightfall, to ensure almost all workers were inside, and placed into a -20°C freezer. Once the bees were freeze killed, a further 10 bees from each colony were collected and placed into the -80°C freezer for later dissection. During certain weeks of both experiments, the planned number of bees could not be sampled from some of the colonies, this was particularly evident towards the end of the everbearer experiment and was due to colonies beginning to naturally expire.

Dissection

The abdomens of all sampled bumblebees were dissected and examined for the presence of conopid fly larvae (Conopidae*)* and tracheal mites (*Locustacarus buchneri*). The hind gut, Malpighian tubules and fat body were removed from the abdomen and individually examined under a phase-contrast microscope at x400 magnification for the presence of the parasites *Crithidia bombi*, *Nosema bombi* and *Apicystis bombi* (Rutrecht & Brown 2008).

Statistical analyses

Statistical analyses were performed using R programming software (R Core Team 2018). Overall parasite prevalence (the prevalence of all parasite species), *C. bombi* prevalence and *A. bombi* prevalence were all analysed using binomial generalised linear mixed effects models in the package 'lme4' (Bates et al. 2017). Calendar week was fitted as a fixed effect, and colony identity was fitted as a random effect. For the overall parasite prevalence model 'sampling event' was fitted as an observation-level random effect to account for overdispersion (Harrison 2015). The proportion of colonies infected with all parasites, and individually with *C. bombi* and *A. bombi* were analysed using binomial generalised linear models, with calendar week as the only fixed effect. Models were validated by visual inspection of the residuals plotted against the fitted values. Models were not overdispersed.

The overall parasite prevalences in the colonies from the June- and everbearing crops were compared using a two-sample z-test for equality of proportions.

95% binomial proportion confidence intervals were calculated around all prevalence estimates using the Clopper-Pearson 'exact' method (Clopper & Pearson 1934), and these intervals are presented in Figures 1, 2 and 3 of this chapter.

Results

Parasite prevalence

A total of 438 bumblebees were dissected from the nine colonies placed in the June-bearing strawberry crop (see table 1A for number of bees dissected each week) and the overall parasite prevalence (prevalence of all parasite species combined) in these colonies for the duration of the experiment was 0.46% (95% confidence interval (CI): 0.055 – 1.64%). The only parasite observed was *A. bombi*, which was detected in two bees from a single colony from the final sampling time point of the experiment.

594 bumblebees were dissected from the twelve colonies placed in the everbearing crop. Towards the end of the sampling period the planned number of bees could not be sampled due to colonies beginning to naturally expire (see Table 1B for

number of bees dissected each week). During week 25, colony EV3 was not sampled due to a sampling error made by the experimenter. The overall parasite prevalence for the duration of the experiment was 14.14% (95% CI: 11.44 – 17.21%), which was significantly higher than the overall prevalence from the June-bearer colonies (z-test for equality of proportions: $X^2 = 60.03$, $p < 0.05$). *C. bombi* was the most prevalent parasite throughout the experiment at 10.44% (95% CI: 8.10 – 13.18%), followed by *A. bombi* at 4.21% (95% CI: 2.74 – 6.15%). Eleven colonies became infected with *C. bombi* at some point during the experiment with six of these colonies also acquiring *A. bombi*. Only one colony was solely infected with *A. bombi* (Table 1B). Conopid fly larvae (Conopidae) were only detected in three bees from three colonies from the final week of the experiment. *Nosema bombi* was detected in only one bee from calendar week 19. Conopid fly larvae and *N. bombi* were not analysed further because of these low prevalences.

Table 1. *Infection dynamics of* Crithidia bombi and Apicystis bombi *in each colony used in the* ***(A)*** *June-bearing strawberry crop and* ***(B)*** *everbearing strawberry crop. Small numbers indicate the number of bees that were dissected from each colony at each time point. The black boxes, indicating that no bees were sampled, were caused by the colonies having expired (week 17 and 26) or by experimental error (week 25).*

A

	Calendar week						
Colony	**11**	**12**	**13**	**14**	**15**	**16**	**17**
JB1	10	10	5	5	5	5	10
JB2	10	10	5	5	5	5	10
JB3	10	10	5	5	5	5	10
JB4	10	10	5	5	5	5	
JB5	8	10	5	5	5	5	10
JB6	10	10	5	5	5	5	10
JB7	10	10	5	5	5	5	10
JB8	10	10	5	5	5	5	10
JB9	10	10	5	5	5	5	10
Total	**88**	**90**	**45**	**45**	**45**	**45**	**90**

B

	Calendar week								
Colony	**18**	**19**	**20**	**21**	**22**	**23**	**24**	**25**	**26**
EV1	10	5	5	4	5	5	5	2	1
EV2	10	5	5	5	5	5	5	5	10
EV3	10	5	5	5	5	5	5		4
EV4	10	5	5	5	5	5	5	5	1
EV5	10	5	5	5	5	5	5	5	10
EV6	10	5	5	5	5	5	5	5	10
EV7	10	5	5	5	5	5	5	5	2
EV8	10	5	5	5	5	5	5	5	
EV9	10	5	5	5	5	5	5	5	7
EV10	10	5	5	5	5	5	5	5	1
EV11	10	5	5	5	5	5	5	5	10
EV12	10	5	2	5	5	5	5	5	10
Total	**120**	**60**	**57**	**59**	**60**	**60**	**60**	**52**	**66**

Infection status

Crithidia bombi

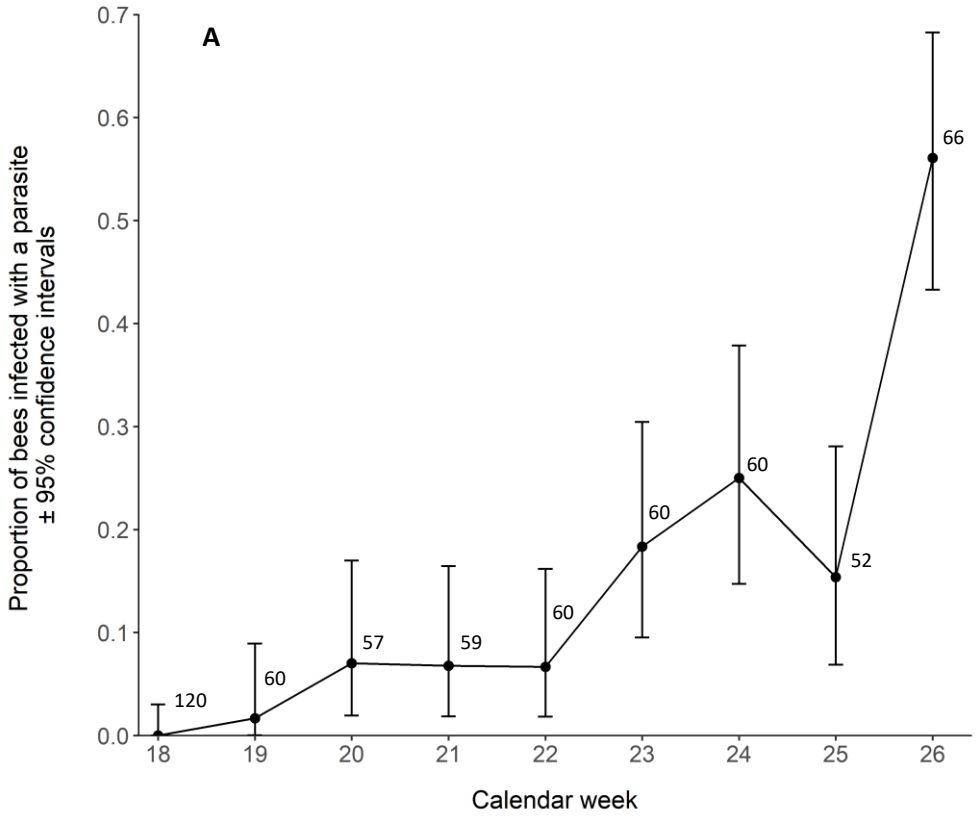
Apicystis bombi

Co-infection

No *A. bombi* or *C. bombi* detected

No data

Overall parasite prevalence increased during the everbearer experiment and a particularly large increase in prevalence occurred during the final week of the experiment (Figure 1A), where a peak prevalence of 56.06% (95% CI: 43.30 – 68.26%) was reached. The proportion of colonies infected by a parasite also increased throughout the experiment, and by the final week 90.91% (95% CI: 58.72 – 99.77%) of colonies were infected by at least one parasite species (Figure 1B). The calendar week was a significant predictor of overall parasite prevalence (GLMM: $z = 6.40$, $p < 0.05$), as well as the proportion of colonies infected by a parasite (GLM: $z = 4.42$, $p < 0.05$) in the everbearing crop.



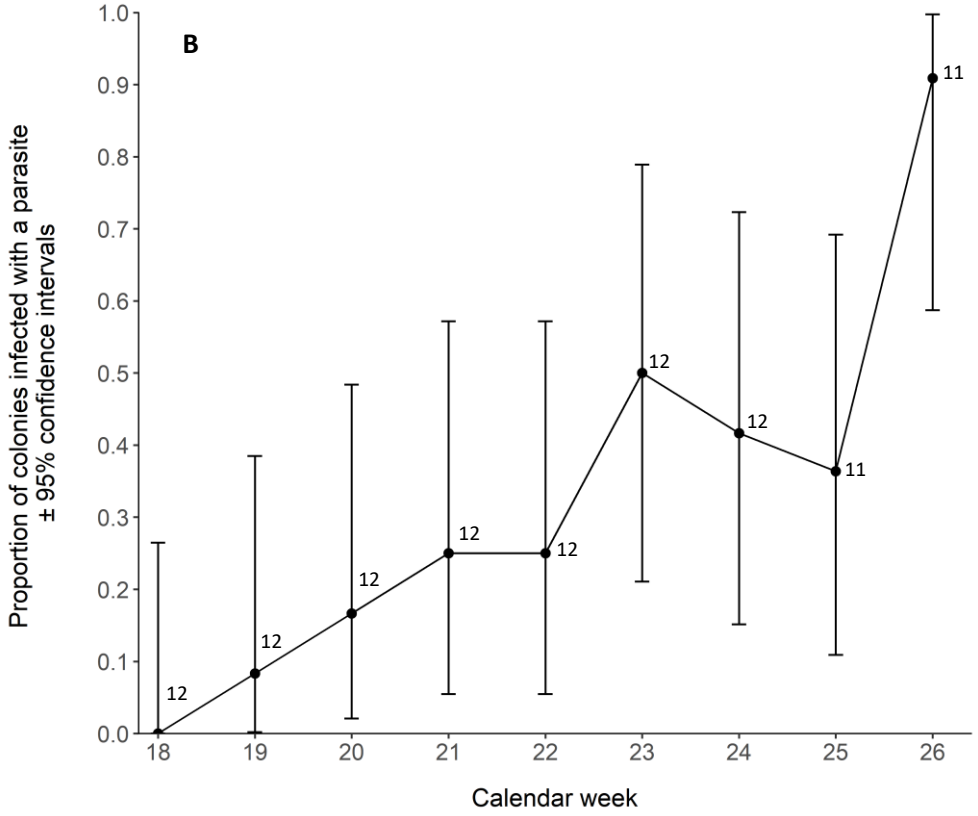
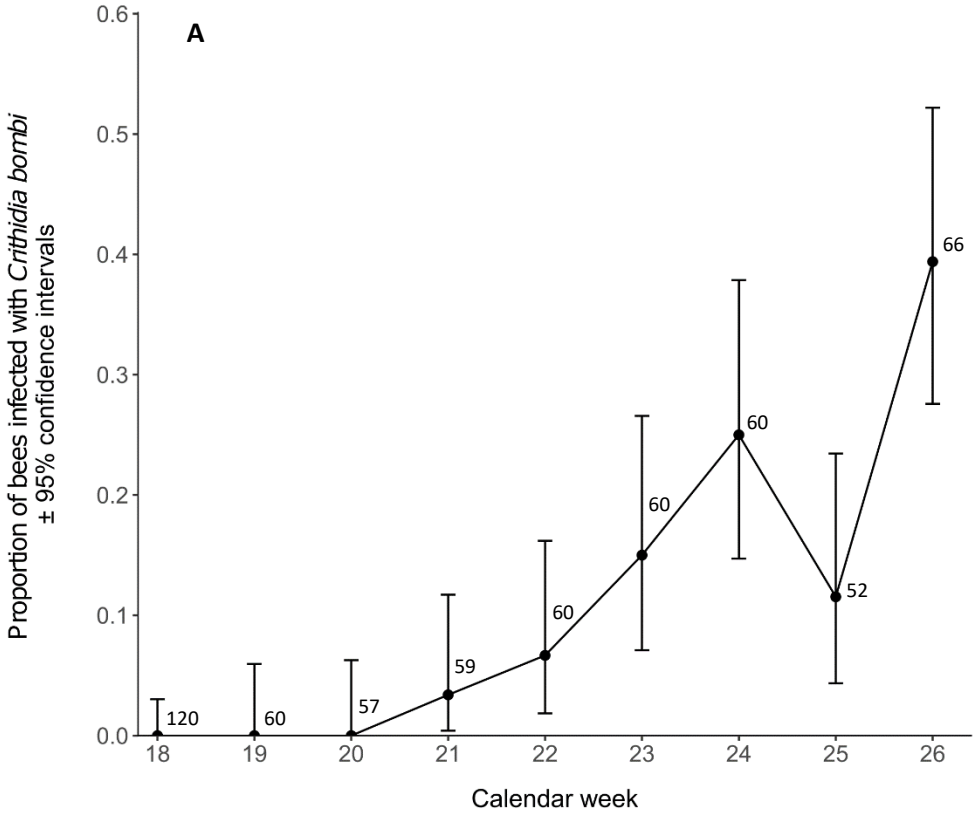


Figure 1. (A) *The proportion of* B. terrestris *from the commercial colonies placed in the everbearing strawberry crop that were infected with at least one parasite species. The numbers next to the points indicate the number of bees dissected.* **(B)** *The proportion of colonies placed in the everbearing strawberry crop that were infected with at least one parasite species. The numbers next to the points indicate the number of colonies sampled. On both figures the bars represent ± 95% binomial proportion confidence intervals.*

C. bombi prevalence across all samples was 10.44% (95% CI: 8.10 – 13.18%), but it had a pronounced temporal dynamic, and reached a high of 39.39% (95% CI: 27.58 – 52.19%) during the final week of the experiment, after displaying a general trend of increasing prevalence during the experiment (Figure 2A). The proportion of colonies infected by *C. bombi* also increased throughout the experiment (Figure 2B). By the final week 72.73% (95% CI: 39.03 – 93.98%) of colonies were infected with the parasite. The calendar week was a significant predictor of both *C. bombi* prevalence (GLMM: $z = 7.37$, $p < 0.05$) and the proportion of colonies infected by *C. bombi* (GLM: $z = 4.30$, $p < 0.05$).


A
Proportion of bees infected with Crithidia bombi
± 95% confidence intervals
0.0
0.1
0.2
0.3
0.4
0.5
0.6
120
60
57
59
60
60
60
52
66
18
19
20
21
22
23
24
25
26
Calendar week

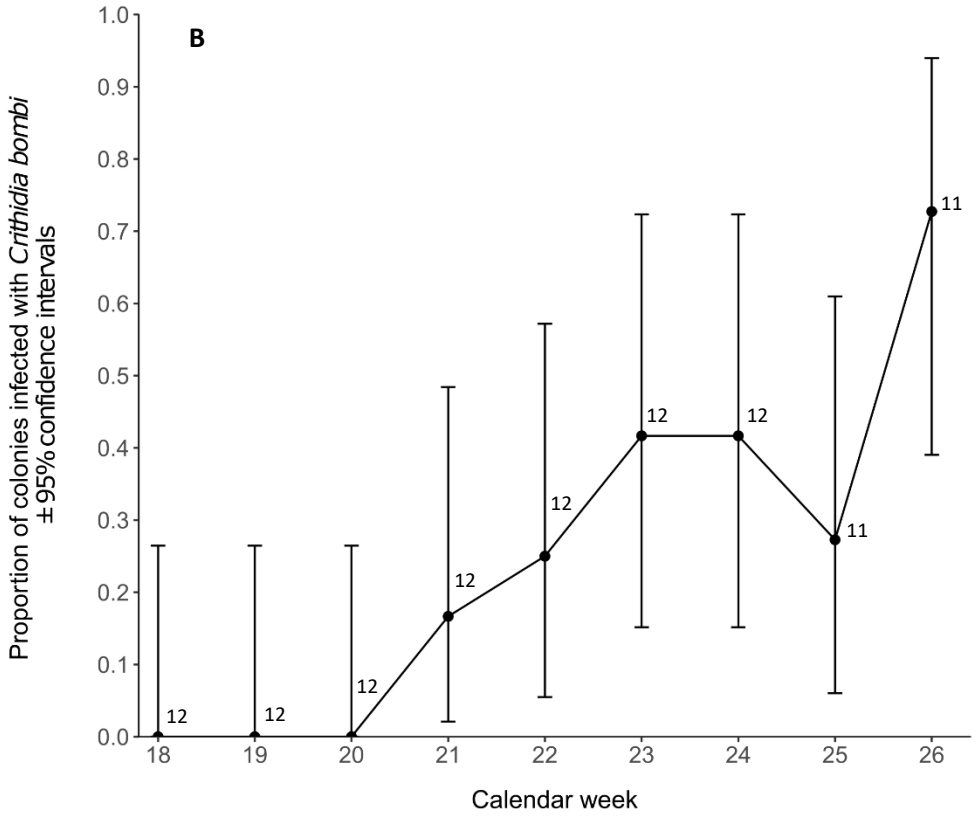
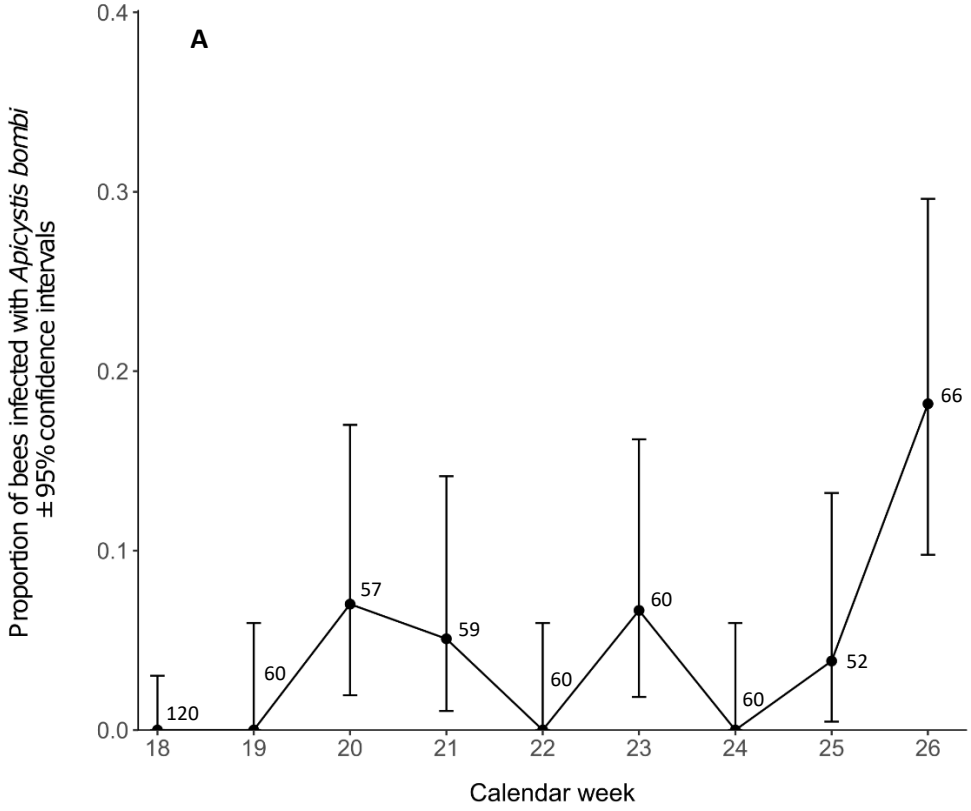

B
Proportion of colonies infected with Crithidia bombi
± 95% confidence intervals
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0
12
12
12
12
12
12
12
11
11
18
19
20
21
22
23
24
25
26
Calendar week

Figure 2. (A) *The proportion of* B. terrestris *from the commercial colonies placed in the everbearing strawberry crop that were infected with* C. bombi*. The numbers next to the points indicate the number of bees dissected.* **(B)** *The proportion of colonies placed in the everbearing strawberry crop that were infected with* C. bombi*. The numbers next to the points indicate the number of colonies sampled. On both figures the bars represent ± 95% binomial proportion confidence intervals.*

A. bombi was detected at a prevalence of 4.21% (95% CI: 2.74 – 6.15%) across all samples from the colonies placed in the everbearing crop. It reached a peak prevalence of 18.18% (95% CI: 9.76 – 29.61%) in the final week of the experiment (Figure 3A). The proportion of colonies infected with *A. bombi* displayed a trend for increasing throughout the experiment, with a peak of 36.36% (95% CI: 10.93 – 69.21%) colonies being infected by the end of the experiment (Figure 3B). The calendar week was again a significant predictor of *A. bombi* prevalence (GLMM: z = 3.87, $p < 0.05$) but not of the proportion of colonies infected by *A. bombi* (GLM: z = 1.92, $p > 0.05$).



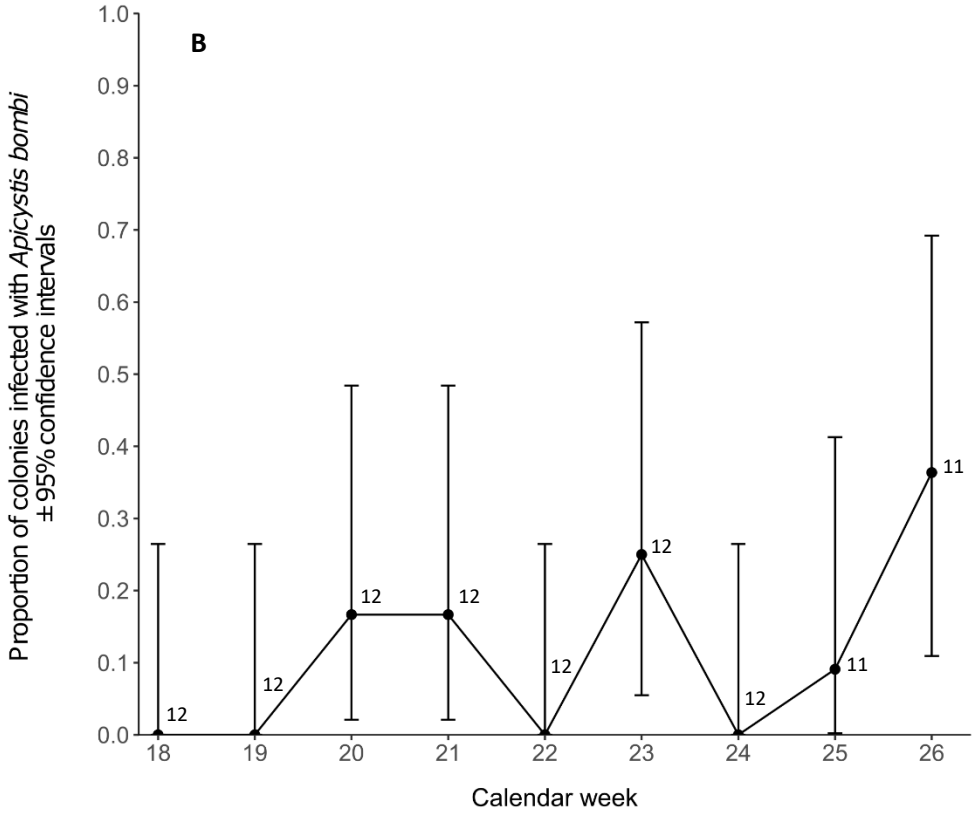


Figure 3. (A) *The proportion of* B. terrestris *from the commercial colonies placed in the everbearing strawberry crop that were infected with* A. bombi*. The numbers next to the points indicate the number of bees dissected.* **(B)** *The proportion of colonies placed in the everbearing strawberry crop that were infected with* A. bombi*. The numbers next to the points indicate the number of colonies sampled. On both figures the bars represent ± 95% binomial proportion confidence intervals.*

Discussion

In this study, I demonstrated that commercial *Bombus terrestris audax* colonies placed into a strawberry crop became infected with parasites that were likely to have been acquired from wild bee populations, and that these parasites increased in prevalence in the commercial bee population through May and June. In the case of *A. bombi*, the peak prevalence reached could pose a hazard to wild bee populations if it were to spill-back to these populations. However, the presence and prevalence of parasites were highly dependent on the time of year that the commercial colonies were deployed, with colonies that were deployed earlier in the growing season (March) being significantly less likely to become infected.

Of the colonies deployed in March on the June-bearing strawberry crop, none were found to harbour any parasites before they were placed into the crop. The same pattern was found in the colonies deployed in May on the everbearer crop. This suggests that commercial production companies have been successful in eliminating a range of micro- and macro-parasites from their colonies (Graystock et al. 2013), reducing the potential for parasite spill-over into wild bees (Daszak et al. 2000). The initial parasite screening of commercial colonies from Chapters 1 and 4 also support this suggestion, as no infections were found.

The only parasite observed in the June-bearer colonies was *A. bombi*, which was detected in two bumblebees from a single colony at the final sampling time point. These very low observed parasite prevalences are most likely due to the time of year that these colonies were deployed. In the south east of England during March and April, queen bumblebees are still emerging from hibernation and if colonies have been established, they are in the early stages of development with only very small numbers of foraging workers (Sladen 1912). This means bee abundance in the environment is low, and thus, the chances of parasite transmission between bees is greatly reduced. Furthermore, *C. bombi* prevalences are generally much lower in the early stages of the colony life cycle, usually reaching peak levels in the summer months (Imhoof & Schmid-Hempel 1999; Gillespie 2010; Popp et al. 2012). Thus, in addition to reduced contact with other bees, there is less chance that any bees that are contacted are carrying an infection. Together, these biological patterns are likely to explain the almost zero levels of parasitism I observed in the commercial colonies placed in the crop during March and April, and are indicative of an almost complete lack of parasite spill-back. Therefore, commercial bumblebees deployed in the UK during this time period are unlikely to acquire parasites from wild bees and are subsequently unlikely to pose a threat to wild bees via parasite spill-back.

However, I observed much higher parasite prevalences in the colonies placed into the everbearing strawberry crop during the months of May and June. Parasites were detected in all twelve colonies from at least one sampling time point of the experiment. The demonstrated absence of parasites before they were placed in the field suggests that all the parasites they did pick up are highly likely to be from wild bees. The calendar week was a strong predictor of parasite prevalence with a clear increase in prevalence during the time that the colonies were in the crop. A peak parasite prevalence of 56.06% was reached at the final sampling time point, with

10 out of 11 colonies (one colony could not be sampled at the final time point, but this colony had been infected with *C. bombi* for the previous 4 weeks) being infected with either *C. bombi* or *A. bombi*. Individually, *C. bombi* and *A. bombi* both reached their peak prevalences during the final week too. For *C. bombi* the general increase in prevalence during the late spring and early summer is comparable to patterns in wild bumblebee populations, which are thought to be due to the growth in bumblebee populations causing an increase in the rate of transmission of the parasite (Imhoof & Schmid-Hempel 1999; Gillespie 2010; Popp et al. 2012). During the final week of the experiment, *C. bombi* displayed a marked increase in prevalence of 27.86% reaching its peak prevalence of 39.39% and infecting 8 out of 11 colonies. Although dramatic, such an increase in a short space of time is not unprecedented, an even greater increase over a similar length of time (10 days) has been recorded in another *C. bombi* infected bumblebee population (Korner & Schmid-Hempel 2005). A study by Whitehorn et al. (2013) observed a similar pattern of *C. bombi* prevalence in *B. terrestris* populations on soft fruit farms that deployed commercial bumblebees. They also observed a marked increase in prevalence late in the season, however, they could not be sure whether the bees they sampled were commercial or wild, which is essential for assessing the potential for parasite spill-back. The prevalence of *C. bombi* recorded for the whole everbearer experiment was 10.44%; this is lower than most *C. bombi* prevalences recorded in wild UK bumblebee populations (Whitehorn et al. 2011, 2013; Goulson et al. 2012; Graystock et al. 2014) and in populations from Europe and North America (Shykoff & Schmid-Hempel 1991; Gillespie 2010; Popp et al. 2012). Some studies have found prevalences similar to or lower than those found here, but these studies sampled newly emerged queens in spring (Rutrecht & Brown 2008; Jones & Brown 2014), a time when *C. bombi* is normally at its lowest prevalence. The peak prevalence of 39.39% is closer to the prevalences previously recorded for *C. bombi* in summer, but still lower than many. Thus, at these commercial colony densities, *C. bombi* transmission from commercial bumblebees is unlikely to pose an additional risk to wild populations through spill-back, as artificially high levels of prevalence were not reached.

However, the prevalence of *A. bombi* at the final sampling point may be more of a cause for concern. At 18.18%, its prevalence was higher than most other records from wild populations in the UK and Ireland (Rutrecht & Brown 2008; Goulson et

al. 2012; Jones & Brown 2014). One study from the UK did find higher prevalences than 18.18% (Graystock et al. 2014), but this study only used molecular screening to detect parasites and thus cannot be certain that all detected parasites were true infections rather than parasite vectoring. In my study, on the other hand, I can be sure of the infection status of individual bees. As there is evidence that *A. bombi* may be highly virulent (Rutrecht & Brown 2008; Jones & Brown 2014; Graystock, Meeus, et al. 2016), it is of concern that this parasite can be acquired by commercial colonies and proliferate to a high prevalence within them. Subsequent spill-back of *A. bombi* to wild bumblebees could pose a threat to their populations, and future studies should assess this potential threat.

Within individual colonies, *A. bombi* was detected sporadically; after its first detection in a colony it was often not detected again for several weeks or at all. One explanation for this is that the colonies are acquiring the infection and then quickly clearing it, however, not enough is known about the epidemiology of *A. bombi* infections in bumblebee colonies to comment on the likelihood of such an occurrence. Another possible explanation is that *A. bombi* is generally found at low prevalences in the UK (see previous references), meaning that I was unlikely to detect it within a colony every week given the sample sizes available. *C. bombi* was also detected sporadically within colonies, although not to the same extent as *A. bombi*. Bumblebees can clear *C. bombi* infections, albeit under artificial conditions (Imhoof & Schmid-Hempel 1998b), but it is likely that the number of bees being sampled was the major contributor to this detection pattern, particularly in the weeks preceding the final week, where its population prevalence rarely exceeded 20%. That the more prevalent parasite was less sporadic in its detection also supports the idea that *A. bombi* was not cleared, and remained present in colonies after its initial detection.

As well as posing a risk to wild bee populations, the parasite prevalence observed in the commercial colonies could be reducing their effectiveness at pollinating crops. Both *C. bombi* and *A. bombi* are known to have negative effects on *B. terrestris* fitness (Brown et al. 2000; Brown, Schmid-Hempel, et al. 2003; Rutrecht & Brown 2008; Jones & Brown 2014; Graystock, Meeus, et al. 2016), which could cause the commercial colonies infected with them to be smaller in size, resulting in fewer foraging bees and consequently a possible reduction in the pollination services that colonies provide. There is also evidence that *C. bombi* infection can

alter the behaviour of bumblebees in ways that would further reduce the pollinating ability of the bees (Gegear et al. 2005, 2006; Otterstatter et al. 2005; but see Martin et al. 2018). Thus, the infection levels that I observed could potentially have a negative effect on crop pollination, but this remains to be tested.

As the colonies used in this experiment were also part of another experiment (see Chapter 2), there are two factors that may be causing these results to be conservative. The first of these is that across the June-bearer and everbearer fields, the commercial colonies were at densities of 2.83 and 1.52 colonies hectare^{-1} respectively, which is lower than the recommendation from the suppliers of at least 6 colonies hectare^{-1} for strawberry crops. Given that transmission of *C. bombi* is density-dependent, the lower colony density is likely to reduce parasite transmission, meaning that parasites are likely to reach lower prevalences than they would in denser bumblebee populations, and take longer to reach peak prevalence. The second factor that could cause my results to be conservative is the opening and closing schedule that each colony was exposed to. Every colony was closed for half of the time that it was in the field. During the closed periods, all the bumblebees were contained within their colony and no bee could leave or enter, meaning inter-colony parasite transmission was not possible during this time. Intra-colony transmission may have increased during the time when colonies were closed. As foragers were not able to leave the colony at any time, the density of bees within the colony will have been higher than when colonies were open, leading to possible increased parasite transmission. However, I believe that if there was an effect it would have been marginal, because even when colonies were open foragers naturally returned to the colony during the night, thus a bee density comparable to that when the colonies are closed is achieved for a prolonged period of time every night. Based on this, I believe intra-colony transmission is not likely to be vastly different between when colonies were open and closed, and that the decrease in inter-colony transmission caused by the treatment is likely to have had a greater effect on my results than a possible minor increase in intra-colony transmission.

Despite these caveats, I clearly show that commercial bumblebee populations do pick up infections, most likely from wild bees, in a commercial farm setting, and that these infections can reach prevalences similar to or, in the case of *A. bombi*, greater than those found in the wild. This suggests that there is potential for

parasite spill-back to occur from commercial colonies to wild bees, which could have detrimental impacts on wild bee populations. I also show the importance of time of year on the prevalence of parasites in commercial bee colonies, information that is useful for lessening any potential environmental damage from commercial colonies. It is also noteworthy that none of the 21 colonies that were used had any parasites before they were placed into the crop. Previous research had found commercial colonies to contain a wide variety of parasites (Graystock et al. 2013), so this result suggests that improvements have been made in the commercial rearing facilities to reduce parasite loads, in response to new regulation (Department for Environment, Food and Rural Affairs 2018). Further studies on farms in different locations, on different crops and with different bee densities are necessary to fully understand the potential of commercial colonies as threats to wild bees via parasite spill-back, but this study represents an important step in gaining this understanding.

Chapter 4

Does *Crithidia bombi* infection impact upon bumblebee pollination ability?

Abstract

Parasites can alter the mortality and behaviour of their host organisms. In doing this they can potentially affect interspecific interactions that their host is involved in. Pollination is an interaction of crucial importance for the maintenance of biodiversity and human well-being. Pollinators are known to host a variety of parasites, but whether these parasites can impact upon the pollination services that pollinators provide is not known. To test this, commercial *Bombus terrestris audax* colonies were inoculated with the common bumblebee parasite, *Crithidia bombi*, or a control inoculum. Colonies were then placed into polytunnels containing strawberry plants. Flower visitation and activity of the bumblebees was observed, and fruits pollinated by the bumblebees were picked and quality assessed. No differences were found in the foraging rate and colony activity between parasitised and unparasitised colonies. This may be because the bumblebees were not nutritionally or energetically stressed during the experiment, enabling them to compensate for any possible effects of *C. bombi*. Strawberries pollinated by parasitised and control bees were not significantly different from each other, but they both were of significantly worse quality and had proportionally fewer fertilised seeds than the negative control bagged flower treatment that received no bumblebee visitation. One possible cause of this is that the density of bees may have been too high in the polytunnels causing the non-bagged strawberry flowers to be overpollinated. While *C. bombi* did not affect the flower visitation rate or the activity of commercial bumblebees in a small-scale foraging environment when bees were not subject to other stressors, it remains difficult to draw conclusions about parasite impacts on pollination services because of the probable overpollination that occurred in this experiment.

Introduction

Parasites are pervasive throughout nature and make up a large proportion of biodiversity (Dobson et al. 2008). They can induce mortality and behavioural change in their host organisms (Anderson & May 1978; Moore 2002, 2013; Lafferty & Shaw 2013), and in doing so they can potentially affect species-species interactions that their host is involved in, which can have subsequent effects on ecosystem functioning (Lefèvre et al. 2009; Sato et al. 2012).

Animal-mediated pollination is an interaction between a pollinator and a plant. This interaction plays a crucial role in the maintenance of biodiversity, with an estimated 87.5% of flowering plants at least partially reliant on animal pollination for reproduction (Ollerton et al. 2011). Animal-mediated pollination also significantly contributes to global food production (Klein et al. 2007; Kleijn et al. 2015; Rader et al. 2016), with crops that are highly dependent on pollination providing humans with valuable micronutrients (Eilers et al. 2011). Pollinators are predominantly insects and, unsurprisingly, they are known to host a range of parasite species (Schmid-Hempel 1998; Evison et al. 2012; Fürst et al. 2014). However, it is not known if these pathogens can alter the interaction between plant and pollinator, which could have subsequent implications for pollination services.

There are two mechanisms by which parasites of pollinators could alter plant-pollinator interactions. The first is via mortality effects of the parasite on its host. If a pathogen increases the mortality of its host, then the host population will be decreased by the presence of the pathogen and subsequently there would be fewer pollinators in the environment. Thus, one would expect fewer plant-pollinator interactions to occur, which could potentially have negative impacts upon pollination services. In bees, an important group of pollinators (Kleijn et al. 2015; Rader et al. 2016), there is certainly the potential for this mechanism to be having an effect, as numerous parasites have been shown to increase host mortality levels (Brown et al. 2000; Brown, Schmid-Hempel, et al. 2003; Higes et al. 2007; Otti & Schmid-Hempel 2007; Rutrecht & Brown 2009; Graystock, Meeus, et al. 2016). Furthermore, in social bees, parasitism can also reduce colony growth, resulting in a decrease in colony fitness (Brown, Schmid-Hempel, et al. 2003; Rutrecht & Brown 2009), which in the long-term may decrease the bee population, and thus pollination services.

The second mechanism is via parasite induced behavioural change in the host organism. Again, in pollinators there are many examples of this occurring (Schmid-Hempel & Schmid-Hempel 1990; Gómez-Moracho et al. 2017) and reported change to behaviours such as foraging and learning could directly affect plant-pollinator interactions and impact pollination services. Together, these two mechanisms may have contributed to the negative relationship observed between *Nosema bombi* parasitism and pollination of bumblebee dependent plants (Gillespie & Adler 2013). In contrast, another observational study found no correlation between *Crithidia*

bombi prevalence and pollination services, but here *C. bombi* prevalence was positively associated with bumblebee abundance, which made detecting an effect of *C. bombi* on pollination difficult (Theodorou et al. 2016). To my knowledge, no study has yet detected a pollinator pathogen having a causal effect on a plant-pollinator interaction and subsequently impacting upon pollination services.

To fill this important gap in our knowledge, I conducted an experiment using the host-parasite-plant system of *Bombus terrestris audax*, *Crithidia bombi* and strawberry (*Fragaria* x *ananassa* DUCH). This system is well suited to answering this question for several reasons. The pollinator *B. terrestris* is a common bumblebee species in the wild in Europe and Asia (Williams et al. 2012), where it is also commercially reared and used for crop pollination (Velthuis & van Doorn 2006). It also plays host to several parasite species, the most common of which is *C. bombi*. This parasite is regularly found in the wild at prevalences between 10-30% and can reach prevalences as high as 70-100% in some populations (Shykoff & Schmid-Hempel 1991; Gillespie 2010; Whitehorn et al. 2011; Goulson et al. 2012; Popp et al. 2012). It is also known to regularly infect commercial bumblebee colonies, either pre- or post-placement in agricultural systems (Whitehorn et al. 2013; Graystock et al. 2013; Martin et al. unpublished data Chapter 3). Furthermore, *C. bombi* increases *B. terrestris* mortality under stressful conditions (Brown et al. 2000) and has been shown to reduce foraging rate and motor and colour learning in another bumblebee species (Gegear et al. 2005, 2006; Otterstatter et al. 2005), behaviours that are highly relevant for the delivery of pollination services. Strawberry is an important soft fruit crop grown in many countries (FAOSTATS). Pollination by honeybees, solitary bees and bumblebees has been shown to improve crop yield and quality (Dimou et al. 2008; Klatt et al. 2014; Martin et al. unpublished data), and commercial bumblebee colonies are widely used on this crop (Velthuis & van Doorn 2006).

I hypothesised that *C. bombi* would alter interactions between *B. terrestris* and strawberry plants with subsequent impacts on the pollination of the fruit. Given the ubiquity of this host-parasite system, if *C. bombi* was found to influence the pollination services bumblebees are providing, the implications would be far reaching for both wild and agricultural ecosystems.

Methodology

The fieldwork was carried out at NIAB EMR horticultural research station located in Kent in the South East of England. 10 small polytunnels (12 x 1.5 m) were each planted with 200 strawberry plants (variety 'Flamenco') in April 2017. The plants were planted in 1 m peat grow bags (Agrii), at a density of 10 plants per bag. The polytunnels were surrounded by insect netting so no pollinators from the external environment could gain access to the strawberry plants. The polytunnels were all situated in one field (latitude: 51.172087, longitude: 0.271804) and exposed to the same environmental conditions. Before bee observations began each polytunnel was assigned to a treatment (parasite or control), and each tunnel remained in the same treatment group for the duration of the experiment to reduce contamination risk between parasite and control treatments.

Queen collection

The *B. terrestris* queen collection, parasite screening and colony rearing were done by a similar methodology as outlined in the 'queen collection' section of the methodology of Chapter 1 (page 42). The only differences were that during this experiment the queens were collected during February and March of 2017, and the total number of *C. bombi* infected colonies that were reared for inoculations was 14.

Commercial colony inoculation

40 *B. terrestris audax* colonies were ordered from Biobest over the course of 5 weeks (10 colonies per week with a 1-week gap in the middle). Upon arrival, the colonies were placed into a dark room, and inspected for the presence of a queen and healthy brood. The faeces of 5-10 workers from each colony were screened for the presence of *C. bombi*, *Apicystis bombi* and *Nosema bombi*. No infections were found so all colonies were deemed acceptable for use in the experiment. Colonies were then reduced to a size of 40 or 20 workers and the queen. The first 20 colonies used during the first two weeks of the experiment were reduced to 40 workers in size, but for the final 20 colonies used during the last two weeks the number of workers was reduced to 20, because the bee density was observed to be too high in the polytunnels.

The colonies were inoculated with *C. bombi* 4 days after arrival. Inocula were made by taking a minimum of 14 bees (one bee from each colony) from the wild *C. bombi* infected colonies. The faeces of these bees were collected and then purified following the method used by Baron et al. (2014) modified from Cole (1970), and outlined in the methodology section of Chapter 1.

After purification, a quantity of parasite solution containing 400,000 parasite cells was combined with 400 µl of 40% sugar solution and placed onto a petri dish. The petri dish with the solution was then placed into a plastic box containing 40 worker bees that had previously been removed from a commercial colony and starved for 2-3 hours. When a bee was observed consuming the parasite solution, it was allowed to continue for 10 seconds. 10 seconds is approximately the time taken for a bee to consume 10 µl of nectar solution (personal observation) which contains 10,000 parasite cells; this is within the range of what bees are exposed to if they consume food contaminated with *C. bombi* infected faeces (Logan et al. 2005; Ruiz-González & Brown 2006). The bee then had its thorax marked with paint to indicate that it had been inoculated, and it was subsequently returned to its original colony box. This process continued until 30-40 bees had drunk from the parasite solution. Ideally, the same number of bees from each colony would have been inoculated, however not all bees were motivated to drink from the parasite solution.

Bees in the control treatment went through the same starvation and paint marking process, but no control inoculum was produced for them to consume.

After the inoculation, bees remained in their colony boxes in the dark room for 10 days with *ad libitum* nectar and pollen. This time period was selected as peak *C. bombi* levels are observed in bee faeces 11-14 days post inoculation (Logan et al. 2005). During this time, workers were removed from colonies to maintain the number of bees at 40 or 20 bees in each colony. Bees without paint markings (i.e. bees that had not been inoculated) were preferentially removed, so that each colony was predominantly made up of inoculated bees that had acquired the infection at the same time and thus had harboured the parasite for the same amount of time. The amount of brood was also equalised between colonies so that each colony would have similar food requirements when placed into the polytunnels. All removed workers and brood were placed into a freezer at -20°C.

Field observations

Colonies were placed into polytunnels 10 days after inoculation took place, meaning colonies would be in the polytunnels during the period where *C. bombi* levels are at their highest (Logan et al. 2005). One polytunnel housed one colony for one week, thus 10 colonies could be observed in one week. All 40 colonies were observed during a 5-week period from 8th June – 13th July 2017 (during one week no observations occurred).

Transects, flower visitation and colony activity

Flower visitation observations were taken where the observer stood at a fixed point in the polytunnel. The number of receptive flowers was counted in either a 3 m^2 or 4 m^2 area and the number of flower visitation events that occurred in this area during a 10-minute period was recorded. The size of the area was not always kept constant as the visibility of flowers changed with the density of the strawberry plant foliage. Recordings were done at least two times a week in each polytunnel, and from this, a visitation rate per flower per minute was calculated.

10-minute transects were walked down each tunnel at least two times a week. Every flower visit that took place 1 m either side of the observer was recorded. A flower visit was defined as an individual being present on any part of the flower.

Colony activity surveys were done for each colony at least two times a week. The entrance/exit of the colony was observed for 10 minutes and every instance of a bee entering or exiting was recorded.

Strawberry marking and collection

10 receptive strawberry flowers were marked in each polytunnel each week (definition of receptive flower is the same as that given in Chapter 2). The flowers were marked with a small twist of coloured wire, different colours were used to represent different weeks of the experiment. A further 5 flowers in each polytunnel were covered with pollination bags to stop bees from visiting the flowers. Pollination bags were made using bridal veil, and were attached to the flower with string tied around the stem (Figure 1).

Figure 1. *Bagged strawberry flowers*.

Marked and bagged berries were picked, once ripe, or occasionally just before ripening due to time constraints. Upon picking, the growth position (primary, secondary, tertiary, quaternary) of each fruit was noted following Darrow (1929). Noting the growth position is important as it can have an impact on the quality of the fruit. The berries were then stored at -20°C for further quality analyses.

Unmarked berries were picked and disposed of before ripening to reduce risk of disease outbreak in the polytunnels, and to encourage further flowering of the plants. The plants were not treated with any chemical before or during the time when the bees were in the tunnels.

Strawberry quality assessment

The methods used for strawberry quality assessment were identical to those outlined in the 'strawberry quality assessment' section of the methodology in Chapter 2 (page 68).

Parasite infection intensity

The doors to colonies were closed at nightfall on the final day of their week-long duration in the polytunnels. Closing them at night ensured that all workers would be inside the colony. Colonies were then removed from the tunnels and then frozen at -20°C. 10 dead bees were then removed from each of the parasite treatment colonies and dissected to quantify parasite infection intensity. The protocol for quantifying infection intensity was identical to that used in Chapter 1.

Statistical analyses

Flower visitation and colony activity

All statistical analyses were carried out using 'R' programming software (R Core Team 2018). Stationary flower visitation was analysed using linear mixed effects models in the 'nlme' package (Pinheiro et al. 2017). Heteroscedasticity was present in this dataset as the variance in visitation rate increased at higher temperatures. Mixed models can be created that account for heteroscedasticity in this package.

Flower visitation from transects was analysed with a generalised linear mixed effects model with Poisson error structure from the 'lme4' package (Bates et al. 2017). Colony activity (the number of bees entering and leaving the colony) was analysed with a negative binomial generalised linear mixed effects model from the 'glmmADMB' package (Skaug et al. 2016).

The visitation and colony activity models all contained 'treatment', 'infection intensity' and 'temperature' in their fixed effects structures. 'Humidity' was left out of the fixed effects component due to its strong collinearity with temperature. The random effects structure for these models included the 'colony ID' nested within 'polytunnel ID'.

Strawberry quality

Four different response variables were examined for the strawberry quality analysis, these were strawberry mass (g), strawberry diameter (mm), strawberry commercial grade classification (extra class – class 3), and fertilised:unfertilised achene ratio. The fertilised:unfertilised achene ratio variable was produced by dividing the number of fertilised achenes by the number of unfertilised achenes. Berries that have a higher proportion of fertilised achenes will have a higher value for this variable. A higher proportion of fertilised achenes suggests that the flower was more fully pollinated.

Achene ratio and strawberry mass were analysed with linear mixed effects models in the 'lme4' package (Bates et al. 2017). The achene ratio variable was Box-Cox transformed ($\lambda = 0.02$) to achieve a normal distribution of the residuals. Strawberry diameter was analysed using a generalised linear mixed effects model with negative binomial error structure in the 'glmmADMB' package (Skaug et al. 2016) to account for overdispersion of the data. Strawberry classification was analysed with

cumulative link mixed models using the 'ordinal' package (Christensen 2017). The random effects structure for these models included the 'colony ID' nested within 'polytunnel ID'. The fixed effects in the strawberry quality models were 'treatment' (parasitised or unparasitised bee colony), the growth position of the fruit (1-4) and the infection intensity.

Secondary analysis

Based on the results of the previously outlined data analysis, a secondary analysis was conducted on all the strawberry quality measures (mass (g), diameter (mm), quality classification (extra class – class 3) and fertilised:unfertilised achene ratio). The models used in this analysis had the same random effects structure as those previously outlined, only with the additional inclusion of 'number of bees' as a fixed effect. This either took the value of 20 or 40 depending on how many bees were present in the colony.

The secondary analysis was performed to investigate whether there was any evidence for overpollination occurring. Overpollination can happen when flowers receive too many visits and become damaged, which can cause fruit to be deformed (Velthuis & van Doorn 2006; Mommaerts et al. 2011; Sáez et al. 2014). Results from the initial analysis suggested that overpollination might have occurred in this experiment. Including the number of bees as a fixed effect in the models helped to identify whether overpollination was occurring. If a lower number of bees was having a positive effect on fruit quality, it would provide evidence that overpollination was occurring.

Results

Parasite inoculation and infection intensity

Dissections revealed that all colonies inoculated with *C. bombi* were still infected at the end of their time in the polytunnels. The mean (± S.E.) infection intensity in the hind gut was 2779.25 (± 241.47) cells μl^{-1}.

Flower visitation

13.5 hours of stationary flower visitation data were collected over the course of 81 observation periods across the 40 colonies. A flower visitation rate was calculated

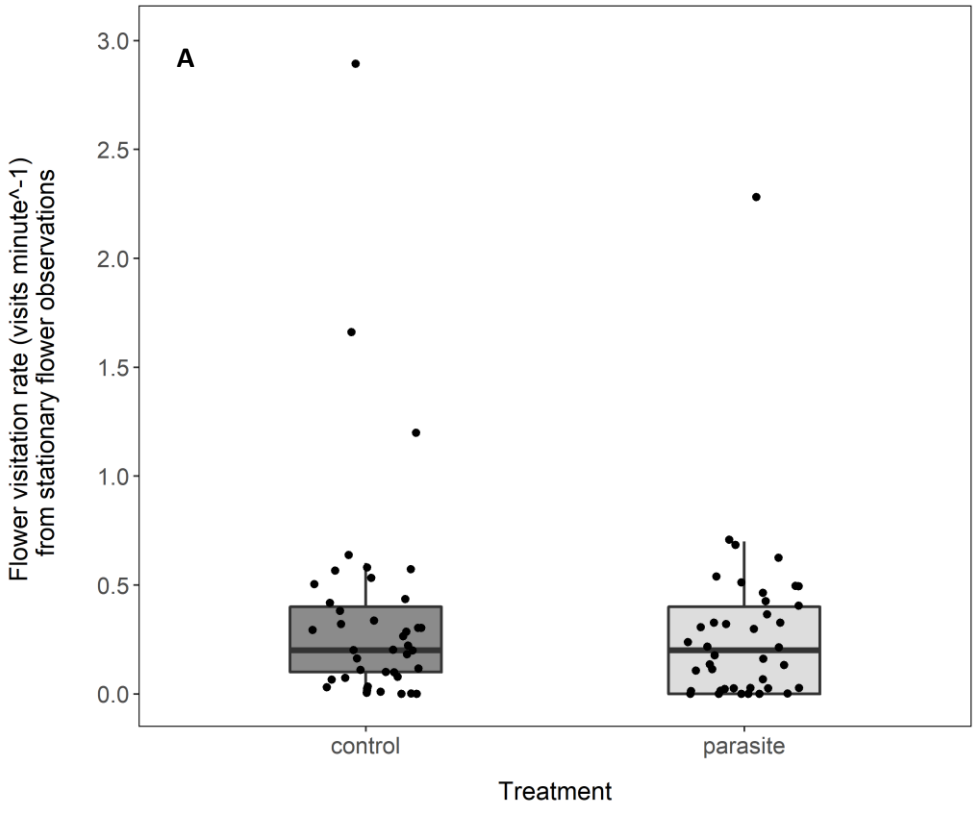
that took into account the number of flowers in the observed area. The parasite treatment did not have a significant effect on the flower visitation rate of colonies (LME: $t = 0.12$, $p = 0.91$; Figure 2A), and neither did the infection intensity (LME: $t = -0.41$, $p = 0.68$). Temperature had a significant positive effect on flower visitation rate, with higher visitation rates being observed at higher temperatures (LME: $t = 2.87$, $p = 0.0064$).

Transect flower visitation

A further 13.5 hours of flower visitation data were collected during 81 transect walks. Here, neither the parasite treatment (GLMM: $z = 0.23$, $p = 0.82$; Figure 2B), the infection intensity (GLMM: $z = 0.97$, $p = 0.33$) nor the temperature (GLMM: $z = 1.21$, $p = 0.23$) had significant effects on the number of flower visitation events observed.

Colony activity

Nest entrances were also observed for 13.5 hours. Parasite treatment (GLMM: $z = 1.40$, $p = 0.16$; Figure 2C) and infection intensity (GLMM: $z = -0.96$, $p = 0.34$) both had no significant effect on colony activity, while temperature had a significant positive effect on colony activity (GLMM: $z = 2.26$, $p = 0.024$).



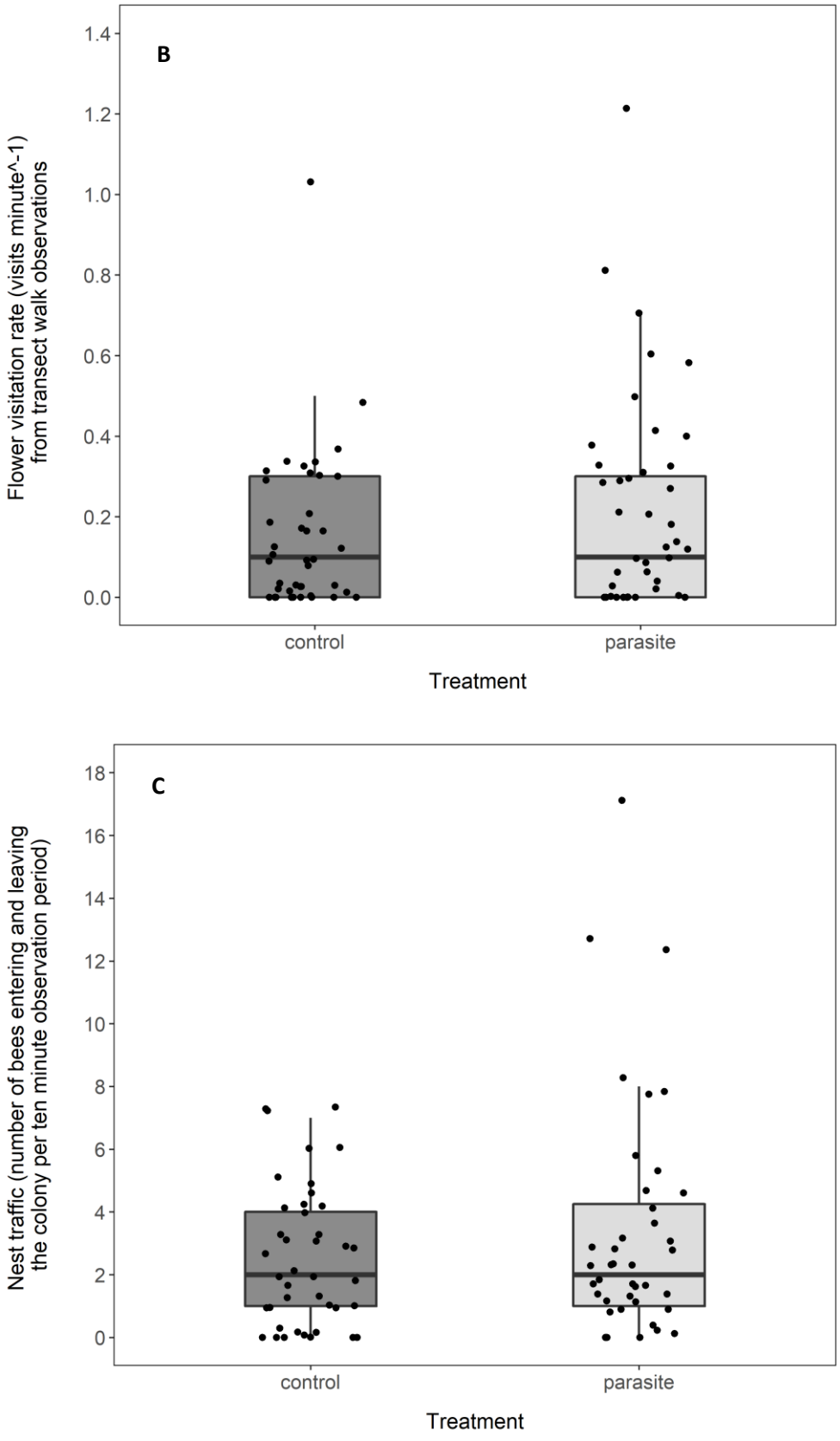


Figure 2. ***(A)*** *Strawberry flower visitation rate (visits minute^{-1}) by* Bombus terrestris audax *from control or* Crithidia bombi *infected colonies in 10-minute observation*

*periods (data from stationary observations); (**B**) strawberry flower visitation rate (visits minute^{-1}) by* B. terrestris *from control or* C. bombi *infected colonies in 10-minute observation periods (data from transect walks); (**C**) number of* B. terrestris *from control or* C. bombi *infected colonies observed entering or leaving the colony in 10-minute observation periods. The median (central horizontal line), quartiles (box), non-outlier ranges (vertical lines) and raw data (dots) are presented on the plots.*

Strawberry quality

A total of 534 berries were harvested from the experiment; 175 from the parasite treatment, 162 from the control treatment, and 197 from the bagged treatment. The planned 600 fruits were not reached as some marked fruits were not recovered, and on week 2 of the experiment there were not enough receptive flowers in the polytunnels to tag the full quota of flowers.

Achene ratio

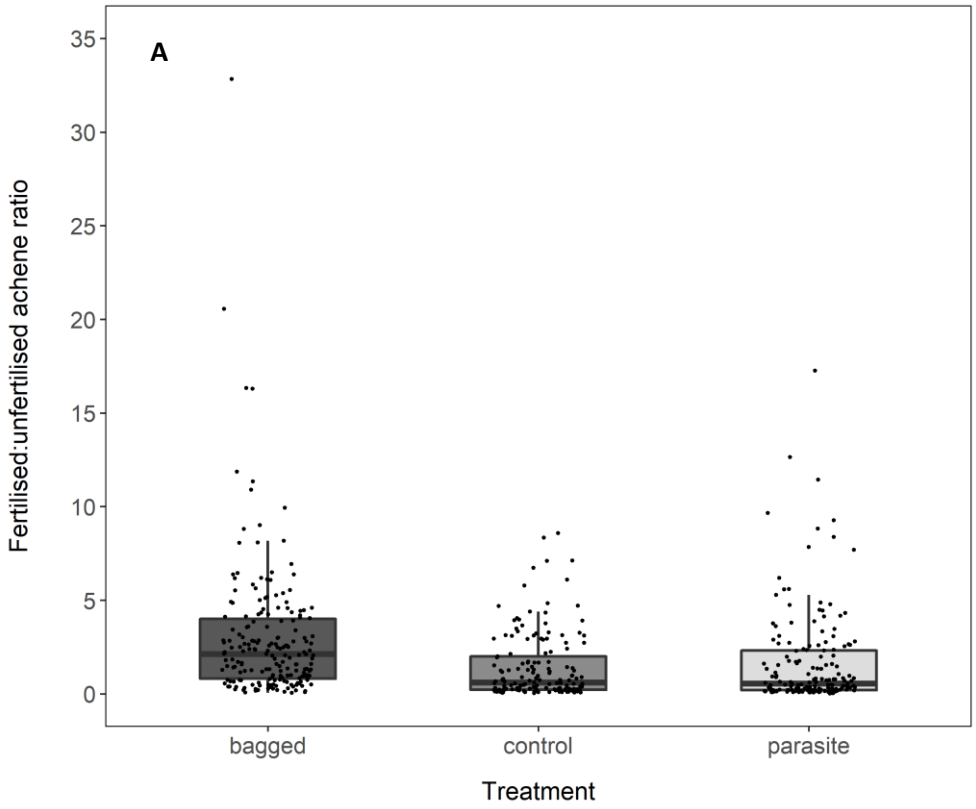
Berries pollinated by bees from both control (LMER: t = -6.17, p = 1.46e^{-9}) and parasitised (LMER: t = -6.63, p = 8.98e^{-11}) colonies had significantly lower proportions of fertilised achenes when compared to berries from the bagged flower treatment (Figure 3A). The proportion of fertilised achenes was not significantly different between the parasite and control treatments (LMER: z = -0.009, p = 0.99) and the intensity of infection did not have a significant effect (LMER: t = 0.027, p = 0.98).

Strawberry mass

The mass of strawberries from the control and parasite treatments were not significantly different from the bagged treatment (LMER: control: t = 1.24, p = 0.22; parasite: t = -1.07, p = 0.28) or from each other (LMER: t = 1.78, p = 0.18; Figure 3B). Infection intensity had no effect on mass (LMER: t = 1.77, p = 0.099), but fruits from secondary and tertiary growth positions had significantly less mass than those from the primary growth position (LMER: secondary growth position: t = -8.82, p = 2e^{-16}; tertiary growth position: t = -8.89, p = 2e^{-16}).

Strawberry diameter

The diameter of strawberries from the control and parasite treatments were also not significantly different from the bagged treatment (LMER: control: t = 1.16, p = 0.25; parasite: t = -0.93, p = 0.35) or from each other (LMER: t = 1.61, p = 0.25; Figure 3C). Infection intensity had no effect on diameter (LMER: t = 1.70, p = 0.11). The growth position of the fruit had a similar effect on diameter as it had on mass, with fruits from secondary and tertiary growth positions having significantly smaller diameters than those from the primary growth position (LMER: secondary growth position: t = -9.93, $p = 2e^{-16}$; tertiary growth position: t = -10.03, $p = 2e^{-16}$).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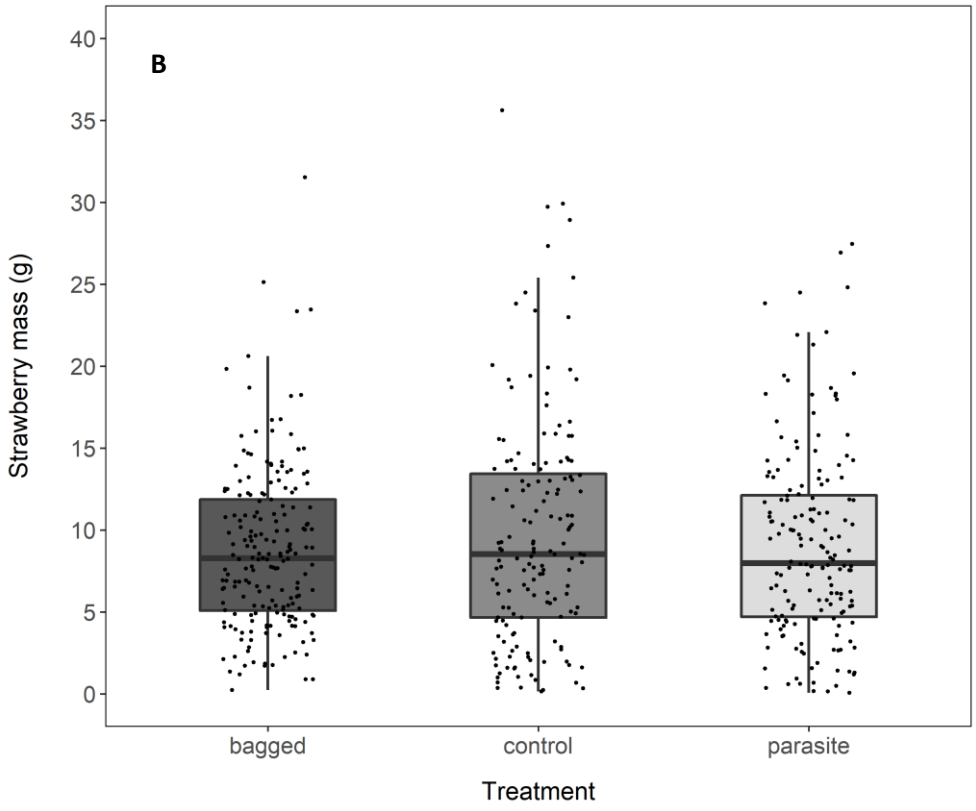
B
Strawberry mass (g)
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Treatment

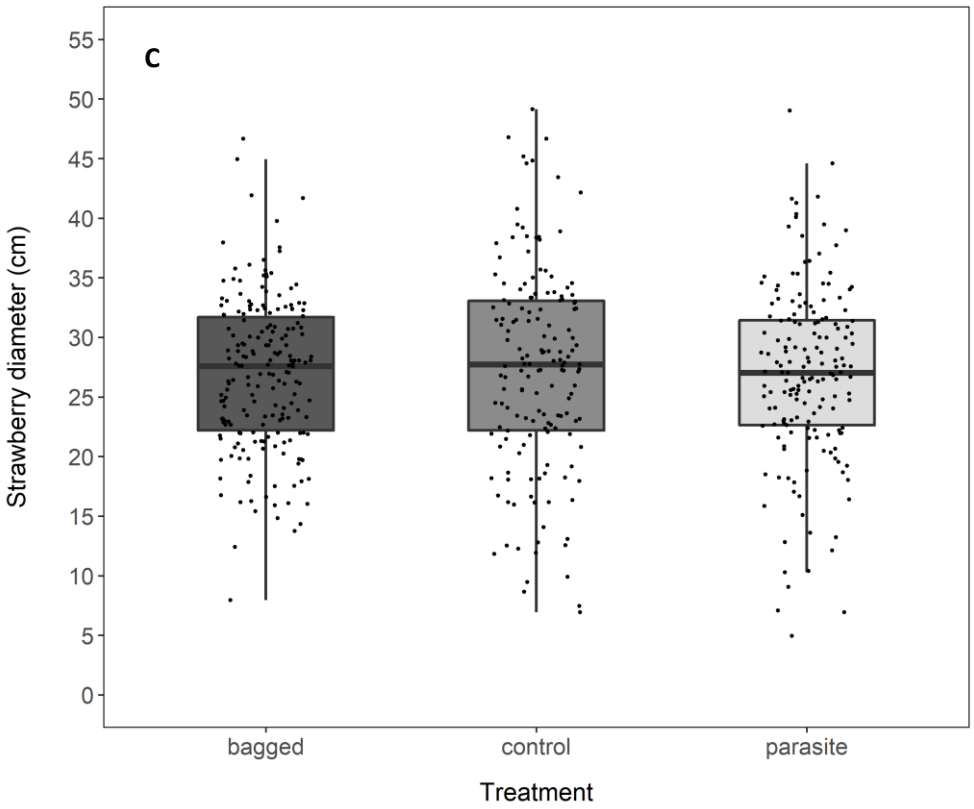

Figure 3. *(**A**) Fertilised:unfertilised achene ratio; (**B**) mass; and (**C**) diameter of strawberries from the three treatment groups. In the bagged treatment, flowers were covered with a fine mesh, so they did not receive any insect visitation. In the control treatment, flowers were visited solely by unparasitised* Bombus terrestris*. In the parasite treatment, flowers were visited solely by* B. terrestris *from colonies infected with* Crithidia bombi*. The median (central horizontal line), quartiles (box), non-outlier ranges (vertical lines) and raw data (dots) are presented on the plots.*

Strawberry class

The control and parasite treatment both had a significant negative effect on berry class when compared to the bagged treatment (CLMM: control: z = -2.89, p = 3.86e^{-3}; parasite: z = -5.25, p = 1.52e^{-7}; Figure 4). There was no significant difference between control and parasite treatments (CLMM: z = 1.69, p = 0.21) and neither growth position of the fruit nor infection intensity had a significant effect (growth position: CLMM: z = -1.72, p = 0.086; infection intensity: CLMM: z = 1.36, p = 0.17).

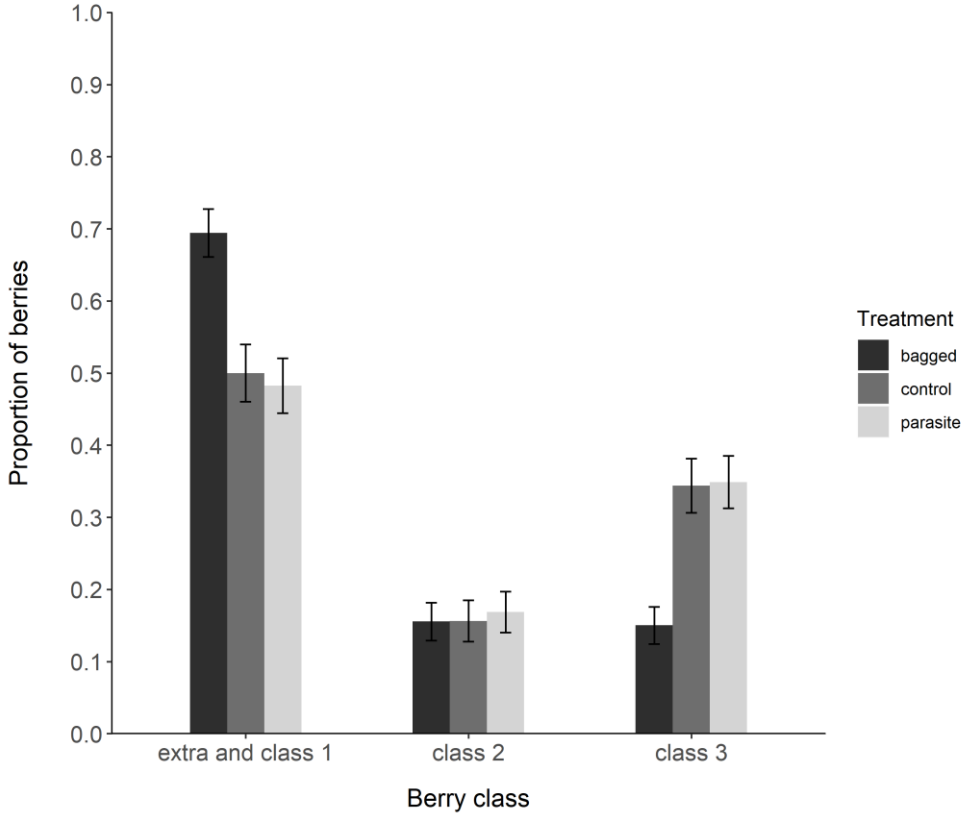

Figure 4. *Proportion of berries from each treatment in each shape classification. Error bars represent ± standard error of the mean. In the bagged treatment, flowers were covered with a fine mesh, so they did not receive any insect visitation. In the control treatment, flowers were visited solely by unparasitised* Bombus terrestris*. In the parasite treatment, flowers were visited solely by* B. terrestris *from colonies infected with* Crithidia bombi*. Extra and class 1 are the highest quality fruits and class 3 are the lowest, with class 2 falling between these two extremes.*

Secondary analysis

The number of bees did not have a significant effect on any strawberry quality measure, but for each measure, there was a trend for an increased number of bees to have a negative effect (Table 1).

Table 1. *Model outputs for the 'number of bees' covariable for each strawberry measure that was analysed.*

Response variable	Model coefficient for 'number of bees' covariate	Standard error	Z-/T-value	P-value
Strawberry mass	-2.74E-04	9.04E-03	-0.03	0.98
Strawberry diameter	-0.02	-0.05	-0.45	0.66
Strawberry achene ratio	-2.38E-04	2.29E-04	-1.04	0.31
Strawberry commercial class	-0.037	0.023	-1.61	0.11

Discussion

In this experiment, infection with *C. bombi* had no effect on the flower visitation rate or colony activity of commercial *B. terrestris* colonies. Pollination by both infected and uninfected bees had a negative effect on the proportion of fertilised achenes on each strawberry, and on fruit quality, when compared to the negative control treatment. This result is surprising, as the negative control treatment, which involved bagging individual flowers to prevent bee visitation, was expected to produce the most poorly pollinated and thus lowest quality fruits. There was no difference in the quality, size and proportion of fertilised achenes between fruits pollinated by parasitised and unparasitised bees. However, the unexpected result of the negative control makes interpretation of this result difficult, meaning that I cannot conclude whether *C. bombi* infection impacts upon the pollination services bumblebees provide.

What can be concluded is that in the environment and the one-week time-frame used in this experiment, *C. bombi* does not affect the colony activity or flower visitation rate of *B. terrestris*. Given that *C. bombi* is highly prevalent in commercial bumblebee systems (Whitehorn *et al.*, 2013; Martin et al. unpublished data Chapter 3) and wild populations (Shykoff & Schmid-Hempel 1991; Gillespie 2010; Kissinger et al. 2011; Jones & Brown 2014; Malfi & Roulston 2014), it is significant that it does not appear to affect nest traffic or foraging rate in this experimental set up. These results suggest that pollination services may not be heavily impacted by *C. bombi* via reductions in colony activity and flower visitation. There are several possible reasons why *C. bombi* did not affect these measures. Firstly, it is likely that *C. bombi*

was not inducing mortality in its host in this experimental setup, meaning that the number of bees available to leave the colony and visit flowers was the same as in the control colonies. *C. bombi* can be highly virulent when hosts are under nutritional stress or at an energetically demanding stage of their life cycle (Brown et al. 2000; Brown, Schmid-Hempel, et al. 2003), however, the environment in which the bees were placed during this experiment is unlikely to have caused nutritional stress or excessive energetic expenditure. The polytunnels provided a very small foraging environment compared to the wild foraging range of *B. terrestris* (Darvill et al. 2004; Knight et al. 2005; Osborne et al. 2008; Redhead et al. 2016), which means energy expenditure is likely to have been low. Furthermore, commercial bumblebee colonies contain an internal artificial nectar reservoir which bees can drink from at any time. These reservoirs remained accessible for the duration of the experiment to replicate the conditions on commercial farms and this meant that bees were unlikely to have been lacking nectar during the experiment. Along with nectar, the other key nutritional source for bumblebees is pollen (Sladen 1912). Colonies were not supplemented with additional pollen during their time in the polytunnels, but they were provided with pollen during the period after their arrival from the supplier up until they were placed in the tunnels, and once in the tunnels, bumblebees were observed foraging for pollen from the strawberry flowers. Pollen is important for bumblebee immune system functioning (Brunner et al. 2014), and is thus likely to play a role in moderating the impacts of *C. bombi* infection. The availability of both pollen and nectar, combined with the low energy requirements of the foraging environment in my experiment, may have reduced the mortality and behavioural deficits that *C. bombi* can cause to negligible levels. Thus, the populations of control and parasite treatment colonies are likely to have remained similar throughout the experiment, which would reduce the expectation of seeing differences in colony activity and flower visitation rate.

The other mechanism I hypothesised by which *C. bombi* could alter plant-bumblebee interactions was through inducing behavioural change in its host. Previous research demonstrated that *C. bombi* infection slowed flower visitation and learning rate in unstressed host bumblebees and that such impairments were greater when the parasite was present at a higher intensity (Gegear et al. 2005; Otterstatter et al. 2005). However, no evidence of these effects were apparent in my results, with the parasite treatment and the infection intensity having no effect

on flower visitation rate. The cited experiments used a different bumblebee species (*Bombus impatiens*) on an array of artificial flowers in an indoor laboratory setting, and the infection intensities were greater than in my experiment. It is possible that these differences caused the contrasting results.

A final consideration for the lack of difference seen between parasite and control treatments is that *C. bombi* does not affect colony activity and flower visitation rate in *B. terrestris* independent of nutritional and energetic state of the bumblebees. However, I believe this to be unlikely considering the research highlighted in the previous two paragraphs.

That bumblebee visitation, both by parasitised and unparasitised colonies, negatively affected fruit pollination and quality compared to the negative control bagged flower treatment was unexpected. Bagging each flower was designed to stop bee visits, with the aim of making these flowers less well pollinated than the parasite and control treatments. Hence, fewer fertilised achenes and subsequently worse quality fruit were expected from this treatment group. However, this was not the case, with the opposite trend being observed and bagged fruits having a significantly higher proportion of fertilised achenes and being of significantly higher class. The reason behind this result is unclear, but the most likely explanation is that the flowers were overpollinated. This typically occurs when the bee density is too high for the number of flowers that are available, leading to individual flowers being repeatedly visited, and many attempts being made to release pollen and extract nectar (Velthuis & van Doorn 2006). In these repeated attempts bumblebees can bite the stigma and stamens of flowers. Damage to the stigmas is likely to reduce ovule fertilisation, which would then lead to fewer fertilised achenes and consequently to the development of deformed fruit. Overpollination is known to occur in strawberry and raspberry and it would explain the results observed here given the high density of bumblebees present in the tunnels (Velthuis & van Doorn 2006; Mommaerts et al. 2011; Sáez et al. 2014).

In this experimental setup, bees were stocked in the polytunnels at a higher rate than is recommended by the commercial suppliers. This was done as the colonies were only in the tunnels for one week and it can take the bees 2-3 days to settle and begin foraging regularly, but it is possible that the bees were still at too high density. Even when only 20 bees were present in the colonies the symptoms of

overpollination still occurred, although to a lesser degree than when 40 bees were present. In addition, there was a negative trend between the number of bees in the colony and the size, quality and pollination of the fruit (Table 1). If this experiment were to be repeated, there are two strategies that may improve the methodology to avoid the overpollination problem occurring again. One would be to reduce the number of bumblebees in each colony further, to between 5 and 10 workers per colony. Second would be to reduce the amount of time that colonies were present in the polytunnel to 4 or 5 days. Both of these should reduce the number of visitations that each flower is receiving and thus reduce overpollination.

Across all the strawberry quality data (fertilised achene ratio, mass, diameter and class) there was no significant difference between the parasite and control treatments. However, if overpollination did occur in this experiment, then these results are difficult to interpret, and no conclusions can be drawn about whether *C. bombi* does affect strawberry quality. Nevertheless, given that foraging rates and colony activity were not affected by the parasite treatment, it is possible that pollination would also not be affected by *C. bombi* in a scenario where overpollination was not occurring.

Conclusion

Given the ubiquity of pollinator parasites and the importance of pollination for both biodiversity maintenance and human wellbeing, it is vital that we increase our understanding of how and if parasites of pollinators affect pollinator-plant interactions and subsequent pollination services. This study represents a first step in gaining this understanding. The results presented here suggest that *C. bombi* infection does not affect the flower visitation rate or nest entrance activity of commercial *B. terrestris* colonies. This is suggestive of *C. bombi* not affecting the pollination services, but this cannot be confirmed with the current dataset. However, the knowledge that *C. bombi* does not affect foraging rate and nest entrance activity is valuable information, particularly for farmers of crops in which commercial colonies are used, where any impairment to foraging could reduce the value of each colony. The results I present here may also be useful in guiding future experiments that are much needed in this research area.

Chapter 5

Busier bees: increasing nest traffic in commercial bumblebee colonies

This chapter has been accepted for publication and is in the final stages of editing at the Journal of Pollination Ecology:

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Abstract

Commercially-reared bumblebee colonies contribute to the pollination of crops globally. If the efficiency of commercial colonies at providing pollination services could be increased, it would have implications for agricultural outputs. Commercial colonies are sold with an internal nectar reservoir on which bees can forage from within the nest. Nectar stores in naturally-produced nectar pots of colonies can affect forager recruitment and activity outside the nest. Thus, it is possible that artificial nectar reservoirs could impact the foraging activity of colonies. To investigate this, commercial *Bombus terrestris audax* colonies were placed in a university parkland campus. Colonies were split into three treatment groups: those with (1) access to an unaltered nectar reservoir; (2) access to a diluted reservoir; and (3) no reservoir access. Foraging observations were made for all colonies over a 19-day period. The mass of each colony was measured and demographic data were collected. Colonies with diluted reservoirs had 131% and 39% more bees entering and leaving than colonies with no reservoir access and unaltered reservoirs respectively. Both treatments with access to a nectar reservoir gained more mass, had a higher proportion of pollen foraging bees, and had more workers, males, larvae and pupae, than colonies with no access to a reservoir. These results demonstrate that manipulating the availability and concentration of internal nectar reservoirs of commercial *B. terrestris* colonies significantly affects the number of bees entering and leaving the colony. Dilution of the nectar reservoir could be a strategy for increasing the pollination services commercial colonies provide to crops. Further research in commercial crops is required before such a strategy could be implemented on farms.

Introduction

Bees are one of the most important pollinator groups, contributing to global food production, and human health and wellbeing (Klein et al. 2007; Aizen et al. 2009; Kleijn et al. 2015; Rader et al. 2016). A significant proportion of crop pollination services are provided by populations of managed honey-bees and commercial bumblebees, which increase yield and quality of crops, and provide a buffer against the declines of many wild pollinator species (Klein et al. 2007; Calderone 2012; Klatt

et al. 2014). Honeybees are the dominant managed pollinator globally, but commercially reared bumblebees are increasingly used as they are superior pollinators for certain crop types and environments (Willmer et al. 1994; Stanghellini et al. 1998; Zhang et al. 2015).

Despite the widespread use of commercial bumblebees, little research has investigated how to optimise their pollination services. One obvious driver of pollination services is the number of bees that leave a colony to visit the crop. If the rate at which bees leave their colony to forage on the crop could be enhanced, the pollination services each colony provides to the crop could potentially be improved. In addition to enhancing crop productivity, this could reduce the number of commercial colonies that are required in crops, meaning growers could save money purchasing fewer colonies, and reduce the negative impacts that commercial bumblebee colonies may have on wild bee populations (Inoue et al. 2008; Meeus et al. 2011; Schmid-Hempel et al. 2014).

One area of commercial colony design that could be altered to induce changes in foraging is the internal nectar reservoir. All commercial colonies are supplied with an internal nectar reservoir filled with an artificial nectar solution located underneath the plastic nest box containing the colony. If the cap of the nectar reservoir is removed (as is standard practice for colonies placed among crops), then bees can access nectar in the reservoir from inside the nest via a cotton wick (Biobest 2017). The reservoir was initially introduced to stop commercial bumblebees from starving, because they were used predominantly to pollinate tomato, whose flowers do not provide nectar (Velthuis & van Doorn 2006). However, commercial bumblebees are increasingly being used on a variety of other crops, many of which do provide a source of floral nectar (Velthuis & van Doorn 2006). On such crops, the utility of the commercial colony nectar reservoir is less obvious, although when colonies are placed outside, the reservoir may help to sustain them during periods of poor weather. However, such nectar reservoirs may also act to alter the foraging activity of bees, and thus their effectiveness as pollinators. If a source of high quality nectar within the nest alters the motivation of bees to forage, this could affect the yield, quality and value of the crop.

There are several possible mechanisms by which nectar reservoirs in commercial colonies could influence foraging activity. Given that the nectar reservoir is

accessible at all times, it may be possible for colonies to keep all of their naturally-constructed wax nectar pots full, meaning that demand for nectar is low, which is likely to reduce the foraging activity of the colony (Cartar 1992; Pelletier & McNeil 2004; Dornhaus & Chittka 2005; Molet et al. 2008). However, if not all the nectar pots are full and demand for nectar is high, a sudden influx of nectar to the nectar pots from the nectar reservoir could stimulate more foragers to search for nectar (Dornhaus & Chittka 2001, 2005). Thus, there are potential mechanisms by which the reservoir could both increase and decrease foraging activity of commercial bees.

Furthermore, the concentration of the nectar within the reservoir may also impact the number of foragers recruited from the colony. When a forager returns to the colony with nectar of a high sugar concentration, it is more likely to perform longer excitatory runs and spend more time running quickly around the nest (Dornhaus & Chittka 2005; Nguyen & Nieh 2012). This activity, combined with the release of a pheromone, alerts nest mates of nectar resources (Dornhaus & Chittka 1999, 2001; Dornhaus et al. 2003), and doing it for a longer period, at a higher speed, and over a longer distance is thought to recruit a larger number of foragers (Dornhaus & Chittka 2005; Nguyen & Nieh 2012). Indeed, studies have shown colony activity to increase more when bees return with high quality nectar compared to lower quality nectar (Dornhaus & Chittka 2005; Nguyen & Nieh 2012). In addition, if high and low quality nectar are injected directly into honeypots, then the high quality nectar stimulates more activity from the colony than the low quality nectar (Dornhaus & Chittka 2005). This suggests that in order to maximise the number of foragers in a colony, higher quality nectar should be used in the reservoir, however it is not known how high quality nectar available directly in the nest will influence foraging outside the nest. It could be that foraging for high quality nectar from the reservoir only stimulates further foraging from the reservoir, rather than stimulating foraging outside the nest, and it is foraging outside the nest that is required for crop pollination.

Finally, both nectar and pollen are required by colonies, and their availability has been demonstrated to affect colony development (Cartar & Dill 1991; Pelletier & McNeil 2003). Thus, the presence and concentration of the nectar reservoir could also impact colony development. Access to the nectar reservoir may give workers of the colony more energy to enable pollen foraging bouts. In addition, the nectar

reservoir may provide all the nectar that a colony requires, meaning that the colony is able to increase the proportion of foragers, and thus, collect more pollen. Both of these factors could subsequently allow the colony to raise more worker offspring and produce larger colonies with more foragers to pollinate a crop. In contrast, the opposite may be true for colonies with no access to a nectar reservoir.

It is clear that there are numerous possibilities for how the nectar reservoir could affect commercial bumblebee foraging activity, with different mechanisms acting in opposing directions. With the aim of clarifying what affect the nectar reservoir has, this study addresses the following questions: 1) does altering the availability and concentration of the internal nectar reservoir of commercial *Bombus terrestris audax* colonies affect the number of bees entering and leaving colonies (hereafter referred to as 'nest traffic')? 2) And do the same nectar reservoir alterations affect the maintenance and development of the colony (i.e. the mass of the colony, the number of workers, males, pupae and larvae produced by the colony)? Finally, I discuss the possible implications of the results for the pollination services provided by commercial bumblebees.

Methodology

Twenty-one *B. terrestris audax* colonies were purchased from Biobest (Belgium). Upon arrival each colony was removed from its outer cardboard box and its mass was measured three times to generate a mean mass, and then returned to its box. Colonies were randomly assigned to one of three treatments (seven colonies in each treatment). In the control treatment, the caps were removed from the nectar reservoirs allowing bees access to the nectar, and the content of the reservoirs was left unaltered (remaining at 60% w/w sugar concentration), as would be the case for standard deployment of commercial colonies in a crop. The sugar water in Biobest nectar reservoir consists primarily of fructose, glucose and sucrose with potassium sorbate and citric acid added as preservatives (Biobest personal communication, 2018). In the diluted treatment, half of the contents (700 ml) of each nectar reservoir was removed and then replaced with the same amount of distilled water, to make a solution of 40% w/w sugar concentration. The diluted reservoir was shaken vigorously to ensure a fully mixed solution, and again the caps of the reservoirs were removed. In the final treatment, the nectar reservoirs were

left unaltered and with their caps on, meaning bees could not access the contents of the reservoir. After the treatments had been applied, the sugar concentration of each reservoir was measured from a 1 ml sample using a hand-held refractometer (Bellingham & Stanley), and the mass of each reservoir was measured three times, so that consumption of the contents by the colonies could be estimated for the duration of the experiment. Reservoirs were then placed underneath their associated colonies, as recommended by the supplier.

On the same day, after treatments had been applied, colonies were placed into field boxes (details below) positioned in a clearing on the Royal Holloway University of London campus (latitude: 51.424643, longitude: -0.563490). The campus is a mix of florally rich borders, meadows, and woodland, surrounded by suburban areas containing gardens, habitats which are known to provide significant foraging resources for bumblebees (Baldock et al. 2015). The boxes were positioned in triplets around the clearing. Within a triplet, one colony from each of the three treatments were placed 3-6 metres apart from each other. Triplets were separated from other triplets by 10-20 m. The boxes were positioned so the entrances to the colonies were all south facing. Field boxes were sturdy plastic boxes (W 67 x L 127 x D 50 cm; Allied Plastics, Kingston, UK) lined with insulation and pegged to the ground. Lids to the boxes were held secure with a ratchet. The boxes protected colonies from the rain and disturbance by wildlife. The field boxes were connected to the colony boxes with a transparent plastic tube through which bees could enter and exit the colony. The end of the tube was wrapped in black tape to make it easier for returning bees to find the colony entrance.

Foraging observations

A day after colonies were placed outside, foraging observations began. Nineteen days of foraging observations were carried out, from 21 July – 8 August 2017, this was 34-45% of the 6-8 week time period that colonies are recommended to be used for in commercial crops. Seven colonies were observed each day between 09:00 and 12:30, so all 21 colonies were observed once over a 3-day period. Over the whole 19-day sampling period, each colony was observed 6 times.

To ensure that colonies from a given treatment were not all observed at a similar time of day, which could bias the results, colonies from each treatment group were observed one after the other, e.g. a colony from the control treatment would be

observed, followed by a colony from the diluted treatment, followed by a colony from the undiluted treatment. The order of selection of each colony from its treatment group was done randomly.

Each colony observation period lasted 30 minutes. The observer positioned themselves so the nest entrance was visible, and every incidence of a bee entering or leaving the colony was recorded. It is possible that the same individual bee was counted more than once in a single observation, but since bees were not marked I cannot quantify this. For returning bees it was also noted if the bee was carrying pollen in its corbiculae or not. Temperature was recorded at the start of each observation. If it was raining no observations were taken.

At the end of the 19-day observation period, all colonies were closed at nightfall, to ensure that most bees were inside their respective colonies. The colonies were then removed from field boxes and placed into a freezer at -20°C. The mass of each reservoir was measured three times and its sugar concentration measured to see if any changes in mass and concentration occurred during the trial.

Colony demographics

After the colonies had been freeze-killed, their masses were measured three times, and the numbers of workers, males, gynes (female reproductive offspring), larvae, and pupae were counted for each colony. Whether the founding queen was present was also noted.

Statistical analyses

All statistical analyses were performed using 'R' programming software version 3.5.1 (R Core Team 2018).

Nest traffic

Nest traffic (the total number of bees entering and leaving the colony in each 30 minute observation window) was analysed using a negative binomial generalised linear mixed effect model (GLMM), using the R package 'glmmADMB' (Fournier et al. 2012; Skaug et al. 2016). The covariables included in the model were 'treatment' and 'temperature'. The random effects structure contained the 3-day sampling period that observations occurred in, and the colony identity nested within the triad of colonies.

To examine the differences between the treatment groups, post-hoc Tukey pairwise comparisons were performed using the package 'emmeans' (Lenth 2018). The same post-hoc test was applied to all the following models.

Proportion of pollen foragers

For each treatment, the proportion of returning bees that were carrying pollen loads was analysed. A zero-inflated binomial generalised linear mixed effects model (GLMM) was fitted with the package 'glmmADMB' (Fournier et al. 2012; Skaug et al. 2016) was used to analyse this variable. The covariables and random effects structure were identical to those in the nest traffic model, and the same post-hoc analysis was performed.

Colony and reservoir mass change

The changes in mass of the colonies and nectar reservoirs that occurred during the 19-day observation period were calculated by subtracting their end mass from their start mass. The mass change variables were then analysed using generalised linear models (GLM). The response variable 'colony mass change' was Box-Cox transformed ($\lambda = 0.22$) to meet the assumptions of the statistical method, and the covariables used were 'treatment' and 'colony starting mass'.

For the 'reservoir mass change' variable, no transformation was necessary, and the covariables used were 'treatment' and 'nest mass change'. Reservoir mass change was used as an approximation of how much reservoir nectar colonies had consumed.

Number of workers, males, larvae and pupae

The number of workers counted from the frozen colonies after the 19-day observation period was analysed using a generalised linear model (GLM) with a Poisson error structure. The response variable was Box-Cox transformed ($\lambda = 0.67$) to meet the assumptions of the statistical method. The covariables used in the model were 'treatment' and 'nest starting mass'.

The number of males, larvae and pupae were all analysed using negative binomial generalised linear mixed effects models (GLMM). All these models had 'treatment' and 'nest starting mass' as covariables and included 'colony identity' nested within 'triad' as a random effect.

Models that were fitted using the glmmADMB package were validated by visual inspection of plots of the residuals plotted against the fitted values, and QQ-plots. Normality of the residuals was also formally tested using Shapiro-Wilk tests. All other models were validated using functions within the 'DHARMa' package (Hartig 2018). Normality of residuals, dispersion and zero-inflation were all formally tested for using this package.

Number of gynes

The counts for gyne production were low, with many colonies not producing any at all, thus no statistical analyses were carried out on these data.

Results

Over the 19-day observation period each colony was observed 6 times; a total of 63 hours of colony observations.

Nest traffic

The diluted reservoir treatment had 39% more nest traffic than the control colonies and 131% more than the closed reservoir colonies (GLMM: control: $Z = -4.45$, $P < 0.05$; closed: $Z = 7.58$, $P < 0.05$; Figure 1).

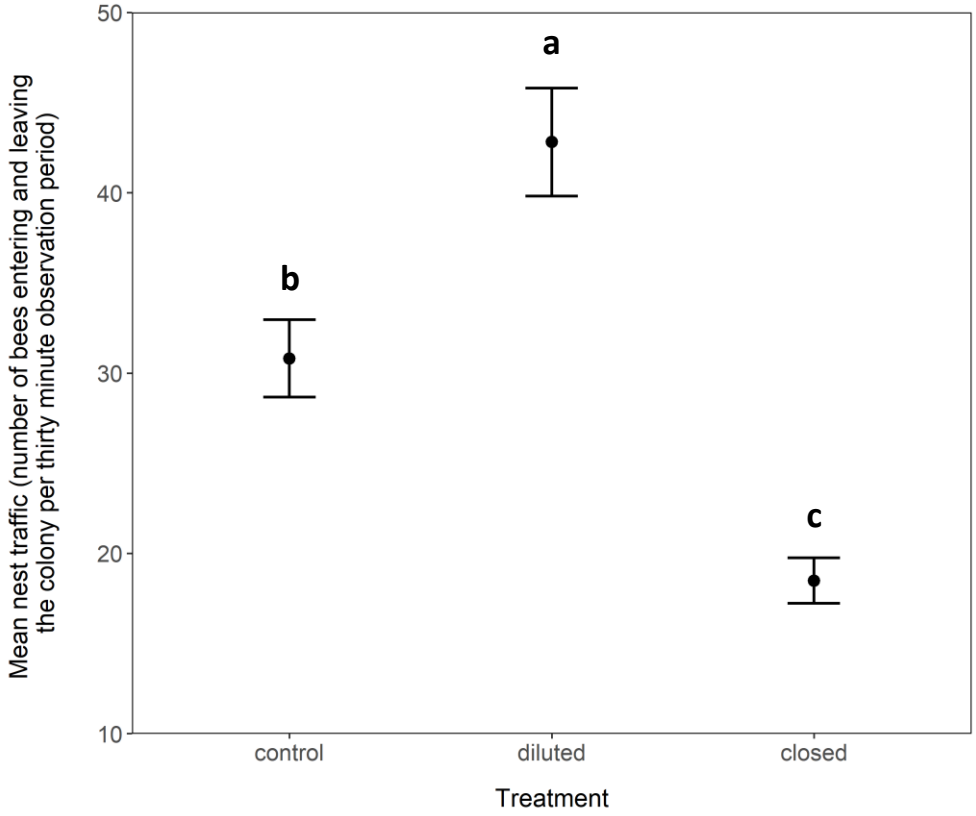


Figure 1. *Mean nest traffic (number of* B. terrestris *entering and leaving the colony) in the 30-minute colony observation periods for colonies within the three treatment groups. Circles indicate back-transformed least square means ± standard error. Means sharing the same letter are not significantly different from each other at the .05 significance level. Control treatment colonies (n=7) had an unaltered internal nectar reservoir (60% sugar concentration), diluted treatment (n=7) had a diluted reservoir (40% sugar concentration), and the closed treatment (n=7) had no access to an internal nectar reservoir.*

Proportion of pollen foragers

Colonies from the diluted treatment had a significantly higher proportion of pollen foragers than colonies from the closed reservoir treatment (GLMM: $Z = 2.65$, $P < 0.05$; Figure 2). Control colonies did not significantly differ in the proportion of pollen foragers compared to diluted and closed reservoir colony treatments (GLMM: diluted: $Z = -1.62$, $P > 0.05$; closed: $Z = 2.26$, $P > 0.05$).

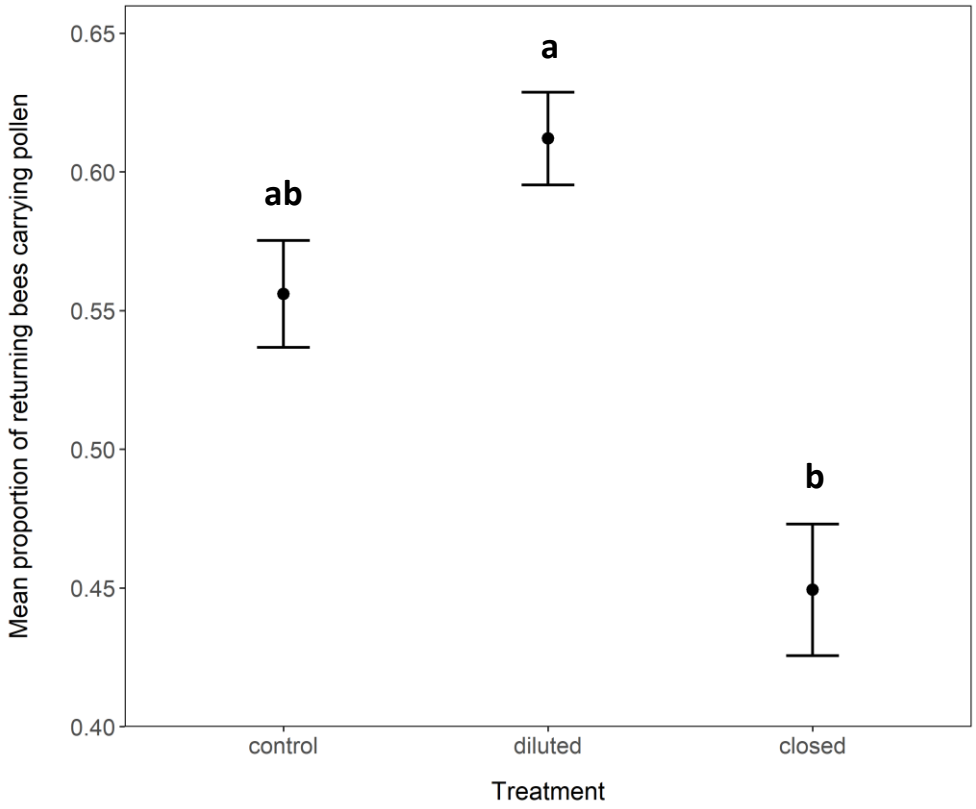


Figure 2. *Mean proportion of* B. terrestris *returning to the colony with pollen from each treatment group in the 30-minute colony observation periods. Circles indicate back-transformed least square means ± standard error. Means sharing the same letter are not significantly different from each other at the .05 significance level. Control treatment colonies (n=7) had an unaltered internal nectar reservoir (60% sugar concentration), diluted treatment (n=7) had a diluted reservoir (40% sugar concentration), and the closed treatment (n=7) had no access to an internal nectar reservoir.*

Colony and reservoir mass change

Colonies from all treatments gained mass, however colonies from the closed reservoir treatment gained significantly less mass than the control and diluted colonies (GLM: control: $Z = 5.72$, $P < 0.05$; diluted: $Z = 3.39$, $P < 0.05$; Figure 3). The starting mass of each colony had a significant positive effect on the colony mass change (GLM: $T = 2.71$, $P < 0.05$).

There was no effect of the diluted treatment on the nectar reservoir mass change (GLM: $T = -0.99$, $P = 0.35$). Colonies that gained the most mass also had the greatest reduction in nectar reservoir mass (GLM: $T = 2.24$, $P = 0.047$).

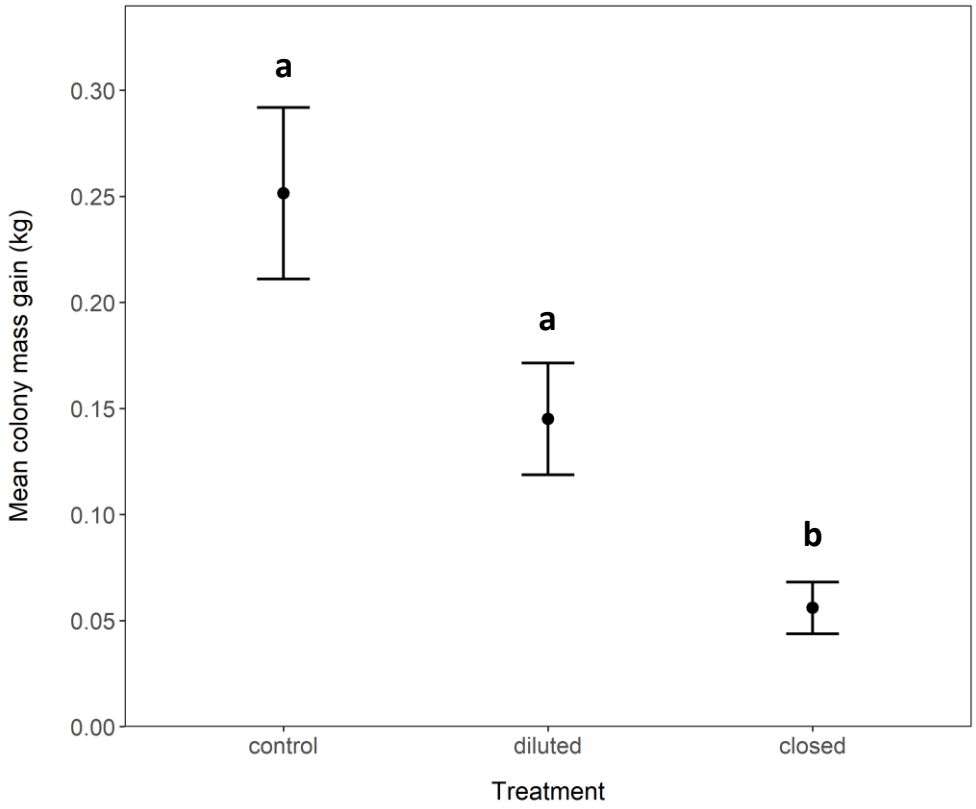
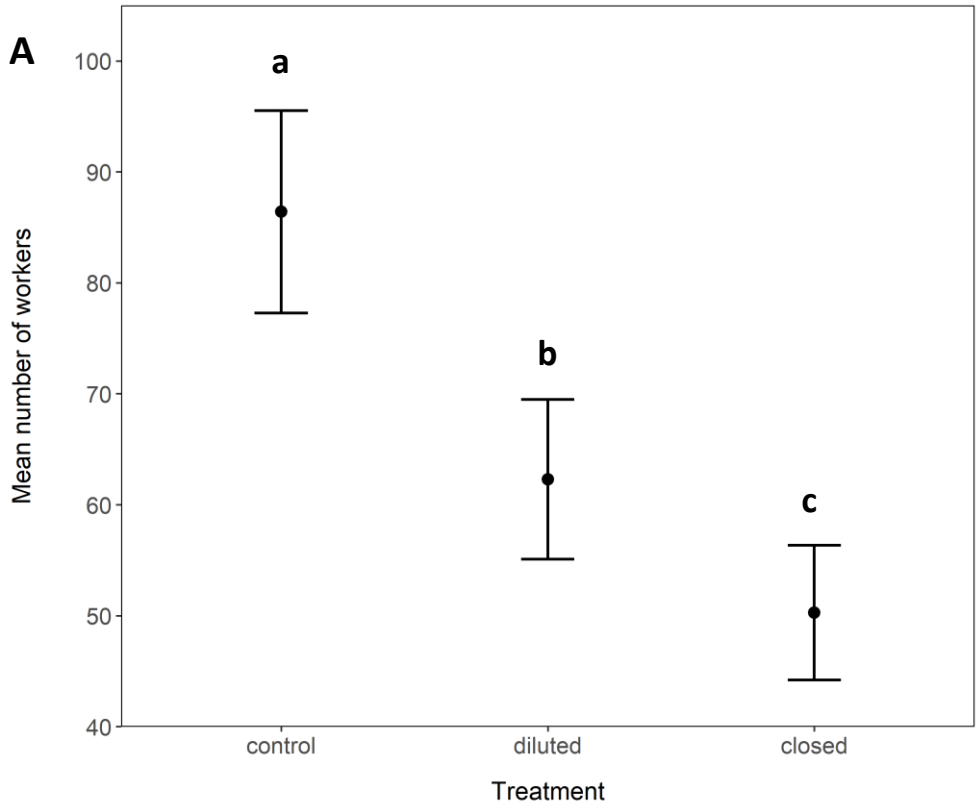
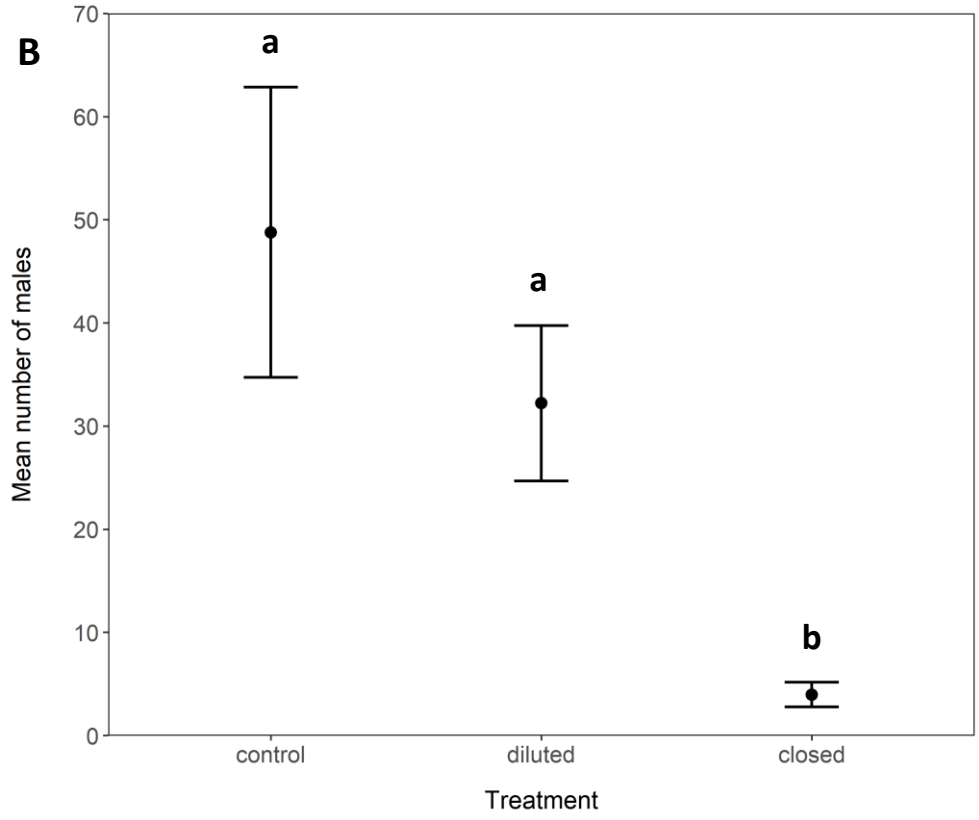


Figure 3. *Mean mass gain of* B. terrestris *colonies from each treatment group. Circles indicate back-transformed least square means ± standard error. Means sharing the same letter are not significantly different from each other at the .05 significance level. Control treatment colonies (n=7) had an unaltered internal nectar reservoir (60% sugar concentration), diluted treatment (n=7) had a diluted reservoir (40% sugar concentration), and the closed treatment (n=7) had no access to an internal nectar reservoir.*

Colony demographics

Closed reservoir colonies had significantly fewer workers, males, pupae and larvae than control colonies (GLM: workers: $Z = 8.74$, $P < 0.05$; GLMM: males: $Z = 5.31$, $P < 0.05$; pupae: $Z = 7.70$, $P < 0.05$; larvae: $Z = 4.53$, $P < 0.05$; Figure 4) and diluted colonies (GLM: workers: $Z = 8.41$, $P < 0.05$; GLMM: males: $Z = 3.11$, $P < 0.05$; pupae: $Z = 5.11$, $P < 0.05$; larvae: $Z = 4.40$, $P < 0.05$; Figure 4) after 19 days in the field. In addition, diluted reservoir colonies had significantly fewer workers than control colonies (GLM: $Z = 8.80$, $P < 0.05$). There were no other significant differences between the treatment groups.


A
Mean number of workers
100
90
80
70
60
50
40
a
b
c
control
diluted
closed
Treatment


B
Mean number of males
70
60
50
40
30
20
10
0
a
a
b
control
diluted
closed
Treatment

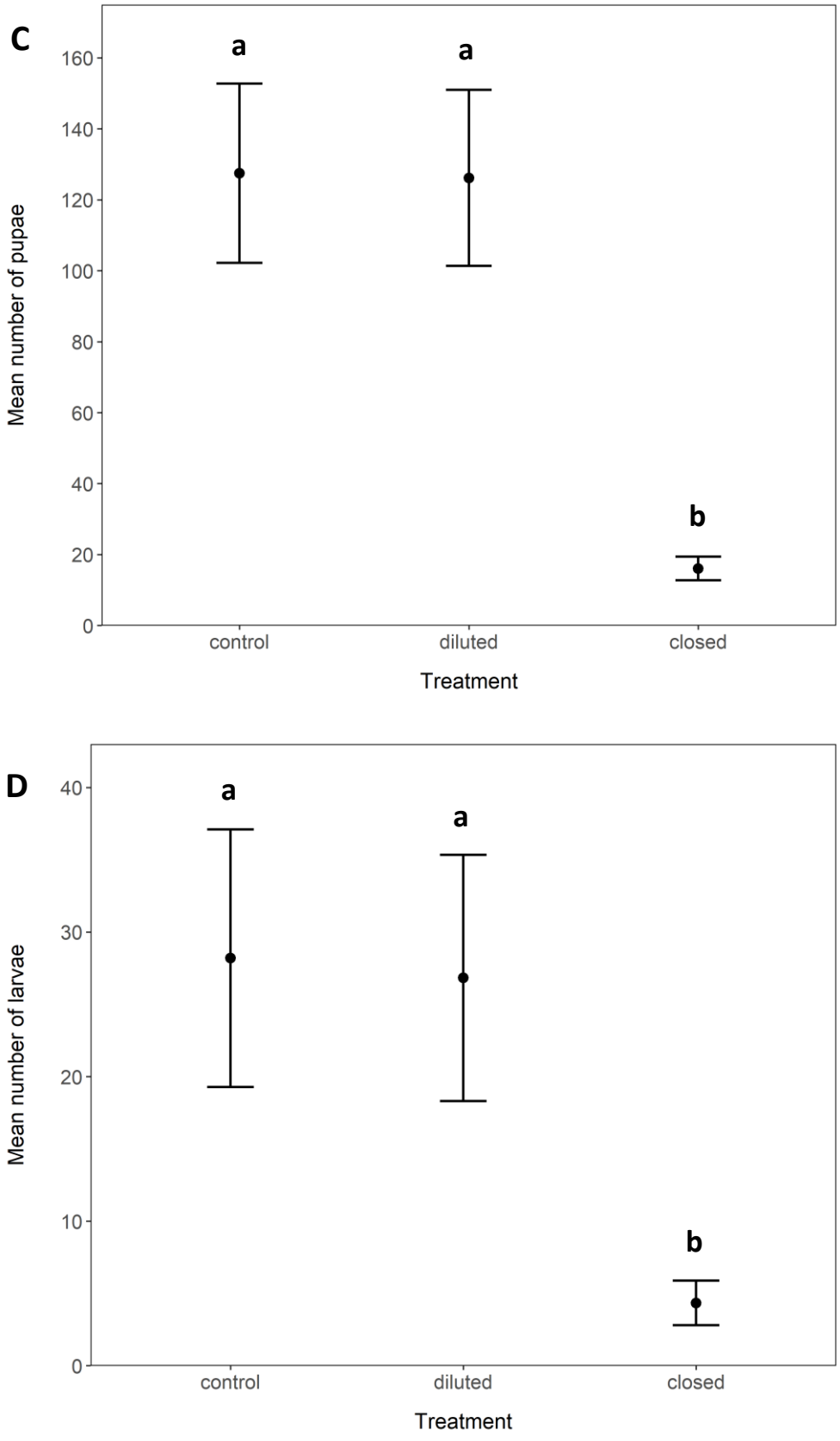


Figure 4. *Mean number of* B. terrestris *(**A**) workers, (**B**) males, (**C**) pupae, and (**D**) larvae from each treatment group found in colonies after 19 days in the field. Circles indicate back-transformed least square means ± standard error. Means sharing the*

same letter are not significantly different from each other at the .05 significance level. Control treatment colonies (n=7) had an unaltered internal nectar reservoir (60% sugar concentration), diluted treatment (n=7) had a diluted reservoir (40% sugar concentration), and the closed treatment (n=7) had no access to an internal nectar reservoir.

Number of gynes

A mean (± S.E.) of 0.43 (± 0.20) gynes were found in control colonies, 6.14 (± 2.68) were found in diluted treatment colonies, and none were found in closed reservoir colonies.

Nectar reservoir concentration

The sugar concentration of the nectar reservoirs were the same at the end of the experiment as they were at the beginning.

Discussion

The results clearly demonstrate that manipulating both the availability and concentration of the internal nectar reservoirs of commercial *B. terrestris audax* colonies can significantly affect the number of bees entering and leaving the colony. Colonies with a diluted nectar reservoir had 39% and 131% more nest traffic than control and closed reservoir colonies respectively. This suggests that there is potential for the pollination services provided by commercial bumblebees to be improved with such a dilution.

The higher levels of nest traffic observed from the diluted reservoir treatment colonies compared to the undiluted control treatment may have been caused by bumblebees' preference for higher concentration nectar (Cnaani et al. 2006; Bailes et al. 2018). Nectar of 40% sugar concentration, as in the nectar reservoirs of colonies from the diluted treatment, is of a higher concentration than the nectar provided by a range of floral resources (Wolff 2006; Fowler et al. 2016), but it is still possible to find higher concentration nectar in the natural environment (Chalcoff et al. 2006). In contrast, it is unlikely that there will be nectar in the environment with a higher concentration than 60% (Chalcoff et al. 2006; Knopper et al. 2016;

Fowler et al. 2016). In addition, nectar with sugar concentrations of over 60% starts to become highly viscous which slows down the rate at which bumblebees can imbibe it, thus slowing the rate of energy intake and potentially making it a less attractive nectar source (Nardone et al. 2013; Bailes et al. 2018). Given that bumblebee foraging behaviour must have evolved against a background of natural diversity in nectar concentrations, it is possible that bumblebees from the diluted treatment had more motivation to forage outside the nest than bees from the undiluted treatment. Furthermore, the nectar reservoirs from the control treatment lost on average more mass than those from the diluted treatment, though not significantly so, adding further evidence that the commercial bumblebees preferred the 60% over the 40% sugar concentration nectar. In addition, both treatments with access to a reservoir had more workers, males, larvae and pupae than closed reservoir colonies. Such increases will have further increased the nectar demand of the colonies. However, diluted colonies could not gain as much nutrition from their reservoirs as undiluted colonies, and so had to forage more, potentially outside of the nest to acquire nectar of higher sugar concentration.

If the potential for better quality foraging resources outside the nest was the only factor influencing the activity of the colonies, then I would expect the closed reservoir treatment to show the highest activity levels, but this was not the case. The control and diluted treatment (i.e. the colonies with access to nectar) both had significantly higher nest traffic counts than the closed reservoir treatment. Here, I hypothesise some possible mechanisms that may lie behind this result. Firstly, both treatments with access to nectar gained significantly more mass and contained more workers at the end of the 19-day sampling period than the colonies without access. This suggests that these colonies grew to a larger size, and thus were able to recruit more foragers, resulting in the elevated levels of nest traffic. Nectar and pollen are critical in the development of a bumblebee colony (Sladen 1912), and colonies with access to nectar also had a significantly higher proportion of pollen foragers, which is likely to have helped enable their growth. This increased pollen foraging may itself have been enabled by the nectar reservoir giving bees more energy to forage more frequently and for longer periods of time, and by providing them with a source of nectar with which to form corbicular pollen loads. In addition, higher numbers of males, gynes and pupae were recorded in colonies with access

to a reservoir, suggesting that these colonies were better able to maintain the eggs and larvae that were present when the colonies were placed in the field. Higher larvae numbers were also observed from these colonies, indicating that they had enhanced levels of development. This is in agreement with literature showing the importance of nectar and pollen for colony development, size, and reproductive success (Cartar & Dill 1991; Pelletier & McNeil 2003).

Secondly, bees from the closed reservoir treatment may have lacked energy to perform regular and long foraging trips, reducing the chances of their foraging trips being successful. Even colonies with a high demand for nectar have low activity levels unless foragers are returning and providing a nectar influx into the nest (Dornhaus & Chittka 2005). It has been suggested that this could be a strategy for energy conservation, so foraging trips are only initiated if they have a high chance of being successful (Dornhaus & Chittka 2005), and it is likely to have contributed to closed reservoir treatment colonies having much reduced nest traffic.

A final possible mechanism is that in the colonies that had nectar reservoir access, regular influxes of nectar from the nectar reservoir could have stimulated foraging (Dornhaus & Chittka 2005). Since bumblebees do not communicate the location of forage resources to other nest mates, the stimulated foraging may have occurred outside the nest, rather than on the nectar reservoir, resulting in more nest traffic. However, I believe that this mechanism is unlikely, as returning foragers do communicate the odour of foraging resources (Dornhaus & Chittka 1999). Thus, it is possible that this mechanism could have resulted in more foraging from the reservoir.

The results I present seemingly contrast with other similar studies from the literature (Cartar 1992; Pelletier & McNeil 2004). Cartar (1992) found that depletion of nectar stores from bumblebee colonies increased nest traffic, however, the depletion was immediate and nest traffic was monitored for 1.5 hours very shortly after the depletion. If the colony had remained in a nectar depleted state for a longer period (1-2 days), nest traffic might have decreased to much lower levels similar to those seen in the depletion treatment in Dornhaus and Chittka (2005) and in the closed reservoir treatment in this experiment. In a longer-term study, Pelletier & McNeil (2004) found that bumblebee colonies provided with ad libitum nectar and supplementary pollen within the nest foraged less than those that were

not supplemented. In their study, foraging observations only began one month after the nutritional manipulations were implemented, meaning there was no overlap with the time period of my study. It is possible that I might have seen an increase in nest traffic from the closed reservoir colonies had I left them in the field for a longer time. However, I think this is very unlikely given that these colonies had significantly lower numbers of workers, pupae and larvae than colonies with access to a nectar reservoir, and so would be unable to greatly increase their foraging workforce. I believe the habitat the colonies were placed in may have been more nectar rich in the Pelletier & McNeil (2004) study compared to my study, thus allowing non-supplemented colonies to forage and develop well.

A factor that could have affected the results is nectar robbing between colonies, especially given the proximity of colonies to each other. For example, colonies from the diluted reservoir treatment may have robbed colonies from the undiluted control treatment for their higher quality nectar, resulting in more nest traffic from the diluted reservoir colonies. In response to this, more bees from the undiluted control colonies may have remained in the nest to defend against robbers. Such an effect could explain the differences observed in nest traffic between diluted and undiluted reservoir colonies. However, although drifting between bumblebee colonies is known to occur (Birmingham & Winston 2004; Zanette et al. 2014), it is not known how much of this drifting is for nectar robbing purposes. Thus, I do not know the magnitude of the effect that robbing may have had on my results. Quantification of nectar robbing would be something to consider in future experiments.

The results presented here suggest that diluting nectar reservoirs of commercial *B. terrestris audax* colonies can increase the number of foragers, which could subsequently improve the pollination services provided to a crop. However, a potential disadvantage to diluting the nectar reservoir could be a reduction in longevity of the nectar, as microorganisms are better able to survive at lower sugar concentrations. In this experiment, no fungal growths were observed with the naked eye in any of the nectar reservoirs after 19 days in the field. However, suppliers recommend that commercial colonies be left out in the target crop for 6-8 weeks (approximately 2-3 times the length of this experiment). After this length of time, it is possible that the reservoirs could be contaminated with fungus, in which case such contamination is likely to be more severe in diluted reservoirs with

a lower sugar concentration. It is possible that contamination of the reservoir may cause bees to become averse to this nectar source, and bees feeding from it may suffer negative health consequences leading to a possible reduction in the size of the colony, its foraging activity, and subsequently the pollination services it is providing. However, nectar contaminated with yeast has been shown to be preferentially selected by bumblebees over non-contaminated nectar (Herrera et al. 2013; Schaeffer et al. 2017), and no detrimental fitness effects have been detected. The preferential selection of yeast contaminated nectar may even suggest a positive fitness effect of yeast containing nectar, but this has not been demonstrated. What effect microorganism growth in commercial colony nectar reservoirs may have on commercial colonies remains to be tested, but it is potentially a complex issue.

Whilst this study clearly demonstrates that manipulations of the nectar reservoir of commercial *B. terrestris audax* can significantly alter nest traffic, further research is required to investigate what effect these alterations might have on the pollination of crops. An obvious follow-up to this study would be to apply nectar reservoir dilution treatments to commercial bumblebee colonies in different cropping systems. This would clarify whether the trends observed here are replicated in a very different foraging environment, and if they are, whether higher nest traffic does translate into better pollination services, crop quality and yield. Effects of dilution could vary in different crops depending on the volume and sugar concentration of the nectar that they produce. For example, tomato crops produce no nectar and can be grown in closed glasshouses from which bees may not be able to escape, thus, the nectar reservoir is their only source of nectar. In this case, dilution of the reservoir would be reducing the total sugar content of the only available nectar source, which could negatively affect the development of the colony. However, several other crop types for which commercial bumblebees are used do produce nectar. It is on such crops that I would recommend testing the effect of reservoir dilution on colony nest traffic and crop pollination. In addition, the spatial arrangement of cropping systems is likely to affect the accessibility of alternate foraging resources in the surrounding landscape to the commercial bumblebees, which could also be a factor in the effect that nectar reservoir dilution has on commercial colonies.

Another factor that should be considered in future experiments is how the effect

of the treatments I applied to colonies might vary over longer time periods than three weeks. It seems likely that over a longer duration the effects that the treatments have on colony demographics and development may play a greater role in determining the amount of foraging activity. For example, colonies that have more larvae and pupae at 3-weeks, are likely to have more of workers at 6 weeks, as the larvae and pupae have developed and emerged as adults. In this experiment, diluted and undiluted reservoirs had similar numbers of pupae and larvae, and significantly more pupae and larvae than closed reservoir treatments, suggesting that the trends seen in this study may have continued. However, a study conducted over the recommended pollination lifespan of commercial colonies of 6-8 weeks would be required to fully test whether the effect of the treatments changes temporally.

The results of this study could have applied value to the field of bumblebee research itself. Commercial colonies are regularly used for research purposes, and experimenters may wish to maximise nest traffic from colonies to allow greater volumes of data to be collected. Here, I demonstrated a method of increasing such nest traffic. Furthermore, studies investigating aspects of commercial colony development and reproduction will want to consider what impact use of the internal nectar reservoir may have on their results.

As it stands, this study shows that there is potential for the foraging activity of commercial bumblebee colonies to be significantly increased with a simple manipulation. If this trend can be replicated across different crop types in agricultural settings, then pollination efficiency may increase, and fewer colonies may be required to meet pollination demands, making farming more cost efficient and reducing the potential for environmental damage caused by commercial bumblebee colonies (Inoue et al. 2008; Meeus et al. 2011; Schmid-Hempel et al. 2014).

Chapter 6

Discussion

Key findings

Effects of *Crithidia bombi* on bumblebee behaviour and pollination

Bumblebees are important wild and commercial pollinators (Fijen et al. 2018), particularly in temperate regions of the world, and they play host to a variety of parasites, which have previously been shown to alter their behaviour and survival (Schmid-Hempel & Schmid-Hempel 1990; Schmid-Hempel 1998; Brown, Schmid-Hempel, et al. 2003; Rutrecht & Brown 2008). However, it is currently unknown whether such parasites can alter the pollination services that bumblebees provide.

The experiments reported in Chapters 1 and 4 of this thesis attempted to fill this knowledge gap. I found that the common bumblebee parasite *Crithidia bombi* did not alter the olfactory learning behaviour of the important bumblebee pollinator *Bombus terrestris* (Chapter 1). Furthermore, in a polytunnel setting, *C. bombi* did not have any effect on the number of bees entering and exiting the nest, or on the flower visitation rate of the bees (Chapter 4). There was no evidence of previously reported learning deficits and reduced flower visitation rate displayed by *C. bombi*-infected bumblebees (Gegear et al. 2005, 2006; Otterstatter et al. 2005). Together, these results suggest that *C. bombi* may not alter the pollination services that bees provide via behavioural changes. However, there are other behavioural facets that remain to be tested that could impact upon the pollination services bumblebees provide. For example, parasites of honeybees have been shown to impair the host's homing ability (Wolf et al. 2014), potentially resulting in a reduced colony workforce. Such effects have not been tested for in bumblebees, but they could be impairing colony fitness and subsequent pollination services.

The lack of difference in the flower visitation and nest traffic between the *C. bombi*-infected and control colonies may also be indicative of *C. bombi* not exerting large mortality impacts in the infected colonies. This is possible as bumblebees were unlikely to have been nutritionally or energetically stressed in the polytunnel environment, which are the conditions required for *C. bombi* to be most virulent (Brown et al. 2000; Brown, Schmid-Hempel, et al. 2003).

Despite the lack of evidence for either parasite-induced behavioural alterations or mortality, I cannot definitively say whether *C. bombi* affected pollination services. This is because of the possible overpollination that occurred in the experimental set up in Chapter 4 that caused the treatments to display an unexpected pattern when compared to the negative control. Thus, it remains to be tested what effect *C. bombi* has on bumblebee pollination, but the results from Chapter 4 are valuable for informing future methodologies attempting to answer this question (see 'Further research' section of the discussion for more details of such methodologies).

Commercial bumblebee effectiveness and parasite dynamics within colonies

During the thesis introduction and the introduction of Chapter 2, I highlighted the great need to test the effectiveness of commercial bumblebee colonies at providing pollination services in crops. I followed this up by testing how effective commercial bumblebees are in a strawberry crop in Chapter 2. The results provided valuable information as to commercial bumblebee effectiveness and further demonstrated why such experiments are necessary. I found that commercial *B. terrestris* colonies can be beneficial by providing pollination services that increase the quality and value of a strawberry crop. However, this beneficial effect was only evident during the early part of the strawberry growing season (March-April) in the UK. During May and June, commercial colonies no longer provided a benefit to the crop. This experiment clearly demonstrated that colonies cannot be assumed to improve crop yield and quality, and that ecological factors along with farm management practices must be considered when deploying colonies.

As well as being ineffective at increasing the quality of the crop, the colonies used in the later flowering everbearer crop harboured parasites at potentially hazardous prevalences by the end of their time in the crop (Chapter 3). The parasites were likely to have been acquired from wild bees and, at the prevalences observed in the commercial colonies, could pose a threat to the same wild bee communities via spill-back.

Finally, in Chapter 5, I suggest a potential manipulation that could increase the efficiency of commercial bumblebee colonies. By diluting the internal nectar reservoirs that all commercial colonies are shipped with, the nest traffic of colonies was significantly increased. An increase in nest traffic is suggestive of a higher rate

of foraging trips, which could lead to improved pollination services being provided. If individual commercial colonies could be made more effective, fewer may be required for agricultural pollination, potentially reducing the environmental damage caused by colonies.

Are commercial bumblebees necessary in strawberry and other crops?

Performing and discussing the experiments that make up this thesis, has given me new insights into the issues surrounding commercial bumblebee colony use, and so I come back to the question initially raised in the introduction of whether commercial bumblebees are necessary in strawberry and other non-greenhouse crops.

In one crop system I have demonstrated both positive and negative aspects of their use. The positive impact was that commercial colonies improved the quality and value of a June-bearing strawberry crop. The negative impacts were that they provided no benefit to a later flowering everbearer strawberry crop, and the colonies used in this system harboured parasites that reached a prevalence level where they could potentially pose a threat to wild bees via spill-back. I provide evidence both in support of and against commercial colony use, highlighting the complexity of the issue.

In addition to these negative impacts, there are those which I have highlighted previously in this thesis. These include, pathogen spillover from commercial to wild bees, and competition between commercial and wild bees (Colla et al. 2006; Ings et al. 2006; Murray et al. 2013). However, there are further environmental impacts associated with the production and distribution of colonies that are rarely considered in the scientific literature. For example, large amounts of resources and energy are likely required to mass produce bumblebee colonies. Rearing facilities need to be maintained at optimum temperatures, plastic and cardboard must be used to house the colonies, and extensive transportation networks are required to distribute colonies around the world. Thus, any reduction in commercial bumblebee colony use would not only reduce the environmental impact caused by the bees themselves, but also the impact of the production process.

I believe that in an ideal situation, commercial bumblebee colonies would not be necessary outside of closed greenhouse growing systems that wild pollinators cannot access. Sufficient pollination services to crops would be provided by a rich diversity of wild pollinators. However, the intensive agricultural systems that are currently used globally are not good for supporting wild pollinator communities (Kremen et al. 2002; Samuelson et al. 2018). Such systems are associated with habitat destruction, loss of floral resources, and increased pesticide use, all of which are thought to be major causes of pollinator declines (Ollerton et al. 2014; Goulson et al. 2015). In the face of increasing food demand from a growing human population, the intensity of agricultural systems seems unlikely to reduce. Although there is hope that practices may become more pollinator friendly, with bee harming pesticides being banned in some areas (e.g., ban on the outdoor use of three major neonicotinoid pesticides in The European Union), and increased emphasis placed on environmentally friendly farming methods being reflected in policy (e.g., agri-environment schemes providing funding to farmers that manage their land in a manner that supports biodiversity). However, whether such policies increase wild pollinator numbers and pollination services to crops to the extent that commercial pollinators are no longer required is not known.

Thus, I believe that commercial bumblebee colonies can play a valuable role in our current agricultural systems if the following criteria are met:

- Commercial colonies are known to benefit the yield and/or quality of the crop in which they are used.
- The pollinating effectiveness of the wild pollinator community and whether the crop is pollen limited has been assessed.
- Colonies can be guaranteed to be pathogen-free upon delivery (my experiments show that progress has been made in reducing parasite loads, but viruses were not screened for, and these are important to consider).
- The commercial species that is being used is native to the area.
- The colonies are efficient and used in the most environmentally friendly manner possible (see below for management recommendations).

Ultimately, other than in closed greenhouse systems or areas where wild pollinators are naturally absent, we should strive for all our pollination requirements to be met by a diverse wild pollinator community. However, if

commercial bumblebees can meet all the above criteria, they can enable agriculture to be more efficient in a way that minimises environmental damage. More efficient agricultural production will be crucial in providing food security to the human population, while also allowing land to be spared for conservation purposes.

Commercial bumblebee management recommendations

Based on the results from Chapters 2 and 3 reported in this thesis, I propose some provisional management recommendations that could potentially reduce the environmental impact that commercial bumblebee colonies are having. Further research is required to confirm whether these suggestions would be useful on a large scale.

My first suggestion is that commercial bumblebee colonies be deployed on crops for a period that is less than the currently recommended maximum of 8 weeks. This could have several potential benefits. Firstly, it could reduce the number of males that escape into the wild. After 8 weeks in the field, commercial *B. terrestris* colonies are well into the sexual production phase of the colony cycle (Martin personal observation), and it is highly likely that some males will already have escaped into the wild. However, earlier removal of colonies will reduce the regularity of this occurrence, lessening the chance of hybridisation between commercial and wild bees occurring, and potentially reducing competition between commercial and wild species by decreasing the influx of commercial bees into the environment.

Imposing a shorter time limit may also help to reduce parasite spill-back from commercial colonies. In Chapter 3, I report that parasite prevalence in the commercial colonies dramatically increased between week 7 and 8. Thus, by removing them at an earlier time, there is a greater chance that parasite prevalences will not yet have built up to potentially hazardous levels, and so the threat of spill-back could be significantly reduced.

A counter argument to a reduction in the time that commercial colonies can spend in the field, may be that the colonies could still provide valuable pollination services after 6 weeks. Whilst this may be true for some colonies, the data collected for

Chapter 3 demonstrated that over 50% (7/12) of colonies had fewer than 10 worker bees remaining after 8 weeks in the crop. These colonies had clearly declined massively from their peak populations and were close to complete expiry. At such a size, these colonies were likely providing only a minimal pollination service, if any at all, so removal of colonies one or two weeks earlier is unlikely to result in a large loss of pollination services, and could reduce the potential health hazard to wild bees. However, it should be noted that the longevity and strength of commercial colonies could also be dependent on the crop into which they are placed and the nutrition that it provides.

My second suggestion is that consideration be given to the time of year and the wild pollinator community where colonies are deployed. My results demonstrated that the time of year can have strong effects on both the effectiveness of commercial colonies at providing pollination services (Chapter 2), and on the prevalence of parasites within the colonies (Chapter 3). The abundance of wild pollinators is also highly seasonal and, despite not being a significant predictor of strawberry quality in Chapter 2, has previously been shown to be important for crop pollination (Garibaldi et al. 2013).

Based on an experiment on a single farm, in one crop species, I cannot definitively say that commercial colonies should not be used during May and June. However, this study clearly highlights that it is not always certain that commercial colonies will provide benefits to a crop. Ecological factors and farm management practices, that vary with time of year, could be having an impact on the effectiveness of commercial bumblebees. Such factors include:

- The wild pollinator community and the pollination services it can provide at different times of year. The wild pollinator community itself may be influenced by farm management practices, such as the adoption of agri-environment schemes.
- Whether the crop is pollen limited and if pollen limitation displays temporal patterns throughout the year.
- How much the crop relies on insect pollination for fruit/seed set.
- Whether the crop is attractive to bumblebees.
- The polytunnel architecture, i.e., whether the sides of polytunnels are rolled up may affect pollinator dispersal in the crop. The polytunnel

architecture itself depends upon climatic conditions, which display a temporal pattern.

An in depth understanding of such factors is required for more detailed commercial bumblebee management recommendations to be determined.

Further research

Completion of the experiments that make up this thesis has highlighted several other research avenues.

The value of commercial bees in other crop types

Chapter 2 of this thesis is the first stage of what could be a large research undertaking. I have provided a first step in understanding the contribution that commercial bumblebees provide in a strawberry crop in the UK. However, strawberry is grown around the globe, and differing environmental factors could influence the effect that commercial bees are having. Further replication of an experiment similar to that which was conducted in Chapter 2 is required to gain a full understanding of the contribution that commercial bumblebees provide to the crop.

Additionally, there are many more crop types on which commercial bumblebees are used. I would suggest that any crop that uses commercial bumblebees and is not grown in a closed system (i.e. the crop can be accessed and pollinated by wild pollinators) would be a good target on which to test the effectiveness of commercial bumblebees. As I have previously stated, it is essential that we know the true value of the pollination services commercial bumblebees provide, both from the farmers perspective, to judge whether they are worth the monetary expense, and from a management and conservation perspective so their benefits can be compared to the environmental risks of using them.

However, it may be unrealistic to test commercial bumblebees on the diversity of crop types and in the various geographical areas in which they are used. A simpler and more widely applicable method could be to test pollen limitation in crops, and use this as a guideline as to whether commercial bumblebees should be deployed. A subset of crop flowers could be exposed to one of three treatments. One

treatment (negative control) would cover the flower in a mesh bag to stop it receiving any insect pollination. A second treatment would have the flower left open and able to receive visits by the wild pollinator community. The final treatment would be a hand pollinated flower (positive control), which is assumed to be fully pollinated. The seed set and/or fruit quality of the open pollinated treatment could then be compared to the negative and positive control, and an assessment of pollen limitation made. If a crop is found to be pollen limited, it is more likely that the addition of commercial bumblebees would provide a benefit to the crop. This method may be more applicable and repeatable at a broader scale, compared to that used in Chapter 2.

Increasing commercial bumblebee effectiveness

Linked to the research of Chapter 2, the results from Chapter 5, suggest a possible mechanism to increase the effectiveness of individual commercial colonies. By diluting the internal nectar reservoir of commercial bumblebees, the nest traffic and the proportion of bees collecting pollen increased. Both of these factors could contribute to increased pollination services provided by colonies. Again, this experiment represents the first step in what could potentially be a useful strategy to increase commercial colony effectiveness. The next steps would involve using the same internal nectar reservoir dilution technique with commercial colonies in a commercial crop setting. This would allow us to observe whether the patterns seen in Chapter 5 still hold in a completely different foraging environment, and if this was the case, whether these patterns lead to increased yield and/or quality of the crop to be pollinated.

A further commercial colony research area, highlighted in Chapter 4, is optimisation of the density at which commercial colonies are deployed in commercial crops. This chapter highlighted the significant decreases in fruit quality that can be caused by there being bumblebees at too high densities in a crop, causing the crop to be overpollinated. Commercial suppliers do recommend densities at which to deploy bumblebees on certain crops, but realistically the actual pollination requirement for the crop will vary depending on environmental factors, such as the wild pollinator species assemblage and the relative pollinating ability of each species within an assemblage. Greater optimisation of the densities could provide a benefit to both growers and wild bee populations.

The maximum necessary colony density required for a crop could be estimated by deploying commercial colonies, each containing different numbers of bees, in separate individual closed polytunnels containing the target crop. The optimum number of bees necessary per unit area or per flower could then be estimated by examining which polytunnels had the crop with the best yield and/or quality. This estimate of the maximum density of commercial bees required could then be reduced depending upon wild pollinator visitation to the target crop in a farm setting.

Is pathogen spill-back a threat to wild bees?

In Chapter 3, I demonstrate that there is potential for commercial colonies to acquire parasites from wild bees, and for these parasites to build up to prevalences that could pose a threat to wild bees via spill-back. However, I was not able to examine whether spill-back actually occurs, and if it does, whether it could be damaging to the health and populations of wild bees. It seems likely that spill-back is occurring, as the same processes that lead to commercial colonies picking up infections from wild bees (e.g. flower sharing), could also lead to transmission in the other direction. Whether this is a threat to wild bees is more difficult to speculate and would require data on the extent to which spill-back is occurring.

To test whether spill-back is occurring, commercial colonies inoculated with a particular parasite strain could be placed into a crop system. The wild bumblebee community in the area could then be sampled and molecularly screened to see whether this unique strain is found to be infecting them. Detection of the strain would indicate spill-back was occurring. However, on ethical grounds, I would advise that such an experiment not be performed, as this may introduce new pathogens to wild bee populations. Alternatively, modelling techniques could be used to shed light on this issue.

Does parasitism affect pollination services?

The experiment reported in Chapter 4 was unable to determine whether *C. bombi* does impact upon the pollination services that *B. terrestris* provides. Although, along with Chapter 1, it provided some evidence that *C. bombi* may not be affecting pollination-relevant behaviours, such as olfactory learning and flower visitation rate. However, the knowledge gap of whether parasitism of pollinators can affect

pollination services still remains. If I were to repeat the experiment of Chapter 4, the bumblebee colonies would contain far fewer workers and they would be left in the polytunnels for a shorter period of time, to avoid the problems caused by the probable overpollination that was occurring.

In such an experiment, a stronger effect may be more likely if a different, more virulent bumblebee parasite were to be used, such as *Nosema bombi* or *Apicystis bombi*. However, I believe that answering this question with *C. bombi* should be a priority. Given its high prevalence both in the wild and in commercial colonies, if it were found to have an effect, this could have large consequences for ecosystem functioning and services.

Concluding remarks

The experiments highlighted in this discussion would increase our knowledge of the pollination services that commercial colonies provide and whether these services can be improved. Furthermore, they would provide us with further insight into pathogen infection dynamics within commercial bumblebee colonies and populations, and importantly allow us to understand what effects such pathogens may have on the pollination of crops. Ultimately, this research could help to increase agricultural outputs at a lower environmental cost, factors which may be critical in providing food security to the human population, and conserving biodiversity. Outside of agricultural systems, understanding the role that parasites of pollinators play in pollination will provide us with invaluable fundamental knowledge of ecosystem functioning. Knowledge which could prove vital in protecting such systems.

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