Parasite Insight: *Apicystis bombi*

Prevalence of the under-reported parasite *Apicystis bombi* across natural barriers and life stages in pollinator hosts.

Submitted by Meriel Autumn Joy Anderson to the University of Exeter as a thesis for the degree of Masters by Research in Biological Sciences In July 2019

Signature: ……..

Abstract

Annually, bees contribute over US$298 billion to the world economy, mostly through their ecosystem service of pollination. Several anthropogenic effects are reducing global wild and managed bee species populations, such as climate change and agricultural land use. There is an urgent need to fully understand the causes of these declines in order to find lasting solutions. One important factor in these declines caused by these anthropogenic effects may be emerging bee pathogens. Bee pathogens can easily jump when transmitted oral-fecally between species due to shared food resources and the commercial movement of honeybee and bumblebee hives. Bees typically have less resistance to emerging pathogens which they have not evolved alongside of, compared to established pathogens, potentially making emerging pathogens a major contributor to bee declines. This thesis studies the spread of the largely under-researched neogregarine parasite *Apicystis bombi*, which has been shown to have lethal and sub-lethal effects on bumblebees.

Island biogeography predicts that islands have a lower prevalence of pathogens compared to nearby mainland. Chapter 2 compares the prevalence of *A. bombi* in the UK and France across island and mainland sites in foragers of two bumblebee species, *Bombus terrestris* and *Bombus pascuorum*, and the honeybee, *Apis mellifera*. The island prevalence of *A. bombi* in *B. terrestris* (41%±3 s.e.) and *B. pascuorum* (30%±3) was significantly lower than nearby mainland sites (65%±7 and 65%±9), despite having similar climates. This suggests that natural barriers significantly slows the spread of pathogens. However, in *A. mellifera* the island prevalence (65%±5) of *A. bombi* was as high as the mainland prevalence (63%±5). A possible explanation is that the commercial transportation of honeybees bypasses natural barriers, spreading pathogens.

The distribution of *A. bombi* in *A. mellifera* hives has never been reported before. Chapter 3 investigates the prevalence of *A. bombi* across *A. mellifera* capped larvae, nurses and foragers. All three life stages contained *A. bombi*, although larvae (59%±10) and foragers (63%±15) had a significantly higher prevalence than nurses (23%±12), confirming that *A. bombi* can be spread throughout the hive either by contaminated pollen or faeces. The *A. bombi* prevalences found here are much higher than previously reported, in both *A. mellifera* and *Bombus* species. Such high prevalence is cause for concern, and could be a contributing factor in

global bee declines.

Dedication

Thank you Parpar for encouraging me to love the bees, if it wasn't for you I wouldn't be researching them. And thank you mum for all your supportive words and for checking my grammar.

Lena you have been a wonderful supervisor for the last 3 years. Thank you for all your support and letting me research something that really interests me.

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Introduction

1.1 Importance of bees

Many plant species rely on animals for pollination, including 78% of temperate plant species and 94% of tropical plant species (Ollerton *et al*, 2011). Of the world's food crops, 35% require animal pollinators, and 70% of those that do not require it gain additional benefits from animal pollinators (Klein *et al*, 2007), as the presence of pollinators increases the fruit and seed production of self- and wind-fertilised plants (Klein *et al*, 2003; Morandin and Winston 2005; Fijen *et al*, 2018). The productivity of certain fruit, seed and nut crops can decrease by 90% in the absence of animal pollinators (Southwick and Southwick 1992). Worldwide, bees are the most economically important pollinators (Blacquière *et al*, 2012), accounting for nearly 75% of the world's food crop pollination (Grozinger and Flenniken 2019). Here when discussing bees this includes a wide range of species including solitary and social wild bees and managed bees. However, bumblebees and honeybees are experiencing high annual mortality rates around the world (Potts *et al*, 2010a; Potts *et al*, 2010b; Brodschneider *et al*, 2018).

Bee species have undergone substantial range reductions and extinctions in the past 50 years (Kosior *et al*, 2007; Goulson *et al*, 2015). These declines are primarily driven by habitat loss and declines in floral abundance and diversity due to farming intensification (Goulson *et al*, 2008; Marshman *et al*, 2019). Urbanized areas can be beneficial to pollinators due to their high density of flowers, however this may facilitate increased contact rates and in turn increases pathogen transmission (Goulson *et al*, 2012). Emerging infectious diseases play an important role in bee declines (Williams and Osborne 2009; Graystock *et al*, 2013a).

1.2 Emerging diseases

Emerging infectious diseases (EIDs) are infectious diseases that are increasing in reports due to either their appearance in a new host, or a change in the cur-

rent host epidemiology (Woolhouse and Dye 2001). The emergence of these infectious diseases are largely driven by socio-economic, environmental and ecological factors (Jones *et al*, 2008). There are three classifications for the main sources of these EIDs; (i) spill-over from domestic animals to local wild populations; (ii) host or parasite translocations through human intervention; and (iii) no obvious human or domestic animal association (Daszak *et al*, 2000). Emerging diseases that can infect human populations, such as the mosquito-borne Zika virus (Weaver *et al*, 2018) tend to get more publicity compared to EIDs that infect other organisms such as insects. However EIDs in insect populations are a major threat, which is especially clear in globally important pollinator species (Manley *et al*, 2015). Due to globalisation and the trans-continental trade of commercial bees and equipment, parasites and pathogens have been spreading into new areas at an ever-increasing rate, spilling-over and infecting the native bees (Brown 2004; Mutinelli 2011).

1.2.1 Spillover and host jumping

Pathogen spillover occurs when a pathogen in a host reservoir population comes into contact with and is transmitted to a new host population of the same or different species (Flanagan *et al*, 2012). In spillover, these pathogens are not well adapted to the new host so they are not able to efficiently transmit between individuals in the new host population (Flanagan *et al*, 2012). Host jumping occurs when the pathogen changes (usually genetically), becomes well adapted and is able to efficiently transmit among the new host population (Flanagan *et al*, 2012).

Bee pathogens can thoroughly infest commercial honeybee and bumblebee hives because bees are bred in facilities with ad libitum food, promoting host survival and breeding despite high parasite loads (Brown *et al*, 2000). A constant supply of hosts can lead to the evolution of higher virulence (Ebert 1998); conditions in commercial bee species may thus promote increased virulence because

of the easy transmission of parasites between commercial hive neighbours and their successive generations. Such highly virulent parasites may then be readily transmitted into the local wild bee populations through shared floral resources (Meesus *et al* 2011). An example of pathogen spillover is documented in the spread of the bumblebee gut parasite *Crithidia bombi* (Otterstatter and Thomson 2008). Native bees have a much higher prevalence of *C. bombi* when they live in close proximity to a commercial hive (<2km). Otterstatter and Thomson (2008) also found that native species that occupied niches which overlapped heavily with commercial *B. impatiens* experienced higher rates of infection, in accordance with experiments demonstrating transmission via contaminated flowers (Durrer and Schmid-Hempel 1994; Graystock *et al*, 2015).

For host jumping to occur, the new host must be suitable for the pathogen to infect and reproduce within (Woolhouse *et al*, 2005). An example of successful host jumping is the microsporidian parasite *Nosema ceranae* jumping hosts from the Asian honeybee (*Apis ceranae*) to the Western honeybee (*Apis mellifera*), and potentially to wild bumblebees (Graystock *et al*, 2013b). When infected with *N. ceranae*, the *A. mellifera* immune response is much lower than that of *A. ceranae* (Sinpoo *et al*, 2018). This low immune response allows *N. ceranae* to replicate and transmit more effectively leading to a higher fitness of *N. ceranae*. This makes *A. mellifera* a potentially better host allowing *N. ceranae* to now spread all over the world contributing to the collapse of bee colonies (Higes *et al*, 2008; Higes *et al*, 2009).

1.3 Threats to bees

1.3.1 Pathogens

New pathogens of bees are being discovered at an increasing rate, and infections may have a major role in the decline of wild bee populations (Colla *et al*, 2006;

Goulson *et al*, 2008). Natural populations of pollinators are usually infected by multiple parasite species (Rutrecht and Brown 2008). There is limited literature available on coinfections between gut parasites, however there are some studies on the potential for antagonistic competition between congeneric parasites who share hosts (Solter *et al*, 2002; Milbrath *et al*, 2015). Inter-specific competition between parasite species is likely to affect their prevalence and potential virulence. A recent study in *A. mellifera* found that the effect of competition depended on the order of parasite infection, the first parasite, *N. ceranae*, inhibiting the growth of the second parasite, N. apis (Natsopoulou *et al*, 2015).

Bees are infected by a wide range of gut parasites (Graystock *et al*, 2015). *Nosema sp*ecies infect honeybees and bumblebees, and has been known to reduce functional fitness of males and females (Imhoof and Schmid-Hempel, 1999). *Crithidia bombi* can increase mortality rates in bumblebees, and its virulence appears to be condition dependent (Brown *et al*, 2000). *Apicystis bombi* is a parasite of many bumblebee species, and causes sucrose sensitivity and a lower lipid/body mass ratio (Graystock *et al*, 2016b). It is unknown how many insect pollinator species *A. bombi* infects and the true reach of this parasite. All of these gut parasites have negative effects on their host, however bees are exposed to a combination of simultaneous stressors in the natural environment (Vanbergen 2013). Pesticides and parasites are a major interacting threat and are probably implicated in many colony collapses (Goulson *et al*, 2015; vanEngeldorp *et al*, 2009). It has been shown that adult honeybees infected with the gut parasite *Nosema ceranae* and exposed to a pesticide have a much higher mortality rate than if just infected with *N. ceranae* (Doublet *et al*, 2015).

The ectoparasitic mite, *Varroa destructor*, is a specialist parasite of honeybees. It jumped hosts from the Asian honeybee (*Apis ceranae*) to the European honeybee (*Apis mellifera*) in the 1940s (vanEngelsdorp and Meixner 2010). *Varroa* feeds on the fat body and hemolymph of the honeybees (Ramsey *et al*, 2019) whist also transmitting pathogens to the larvae/pupa that are sealed in a brood

cell. As the pupa have no defences against *Varroa*, it can cause death, reduced lifespan or impairment of cognitive abilities (Kralj *et al*, 2007; Rosenkranz *et al*, 2010). *Varroa* carries the Deformed Wing Virus-complex (DWV A and B) (Genersch *et al*, 2006; Grozinger and Flenniken 2019), and can facilitate the spread of DWV in bees (Wilfert *et al*, 2016). *Varroa* is known to increase mortality of bee colonies over the winter mainly through the spread of DWV (Highfield *et al*, 2009; Genersch *et al*, 2010; Martin *et al*, 2012; Zhao *et al*, 2019). *Varroa* might also be able to transmit bacteria as well as many honeybee viruses (Kanbar and Engels 2003; Vanikova *et al*, 2015).

1.3.2 Farming threats

Intensive agriculture poses many threats to bees, which can also increase the likelihood of EID emergence. Nomadic commercial beekeepers transport huge numbers of colonies (>100) on trucks all over large continents to pollinate agricultural lands and find flowering plants to feed their bees (Phillips 2014). These unnatural movements allow for a rapid spread of pathogens to native pollinators and other commercial hives (Gordon *et al*, 2014). This threat to bee health requires more research attention to better understand the pathogen transmission pathways.

Anthropogenic land use changes can promote EID outbreaks by modifying the infection transmission pathways (Patz *et al*, 2004). Intensive farming practices such as monocultures reduce floral variety and fragment the natural habitat, threatening local pollinator diversity (Goulson 2003; Goulson *et al*, 2005). This negatively affects crop yields, as crops with either specific or generalized pollinators both benefit from a high pollinator diversity (Steffan-Dewenter *et al*, 2005; Babu 2018). Even in self-fertilising highland coffee and canola, an increased diversity of pollinator species improves fruit production (Klein *et al*, 2003; Morandin and Winston 2005). Decreased pollination in plant species also has a negative

impact on the animal species that depend on these plants for food or shelter.

In addition, pesticides such as neonicotinoids damage pollinator populations. These pesticides are synthetic neurotoxins that disrupt the nervous system of pests to lethal effect (Moffat at al, 2016). Studies have shown that neonicotinoids have detrimental effects on bees, such as reduced production of brood and reduced food consumption (Mommaerts *et al*, 2010; Laycock *et al*, 2012), reduced growth rate and queen production (Gill *et al*, 2012; Whitehorn *et al*, 2012). In honeybees it has been shown that sub-lethal concentrations in surrounding areas can drastically reduce honey production (Chambers *et al*, 2019). The accumulation and contamination of neonicotinoids in the global environment is likely to increase, as their use increases around the world, except for Europe (Blacquière *et al*, 2012; Schaafsma *et al*, 2019).

As well as pesticides, antibiotics are regularly used on crops and are introduced into the environment. In apiculture, antibiotics are used to manage bees' bacterial pathogens, such as the protection of larvae from contracting foulbrood (*Paenibacillus larvae*) (Evans and Schwarz 2011; Hamdi *et al*, 2011). However this use of antibiotics can have lasting negative effects on the gut microbiota of the honeybees (Raymann *et al*, 2017). Invertebrates exposed to these antibiotics have their natural microbiota altered (Martinez 2009), changing the gut microbiome's proportion and species composition (Dethlefsen and Relman 2011; Modi *et al*, 2014). The gut microbiome is crucial to the host's immune system and ability to fight pathogens and can increase disease resistance by providing colonization resistance (Kwong *et al*, 2017; Mockler *et al*, 2018). Honeybees and bumblebees pass their gut microbiome faecal-orally to nest mates, which is an important defence against gut parasites (Koch and Schmid-Hempel 2011; Ribière *et al*, 2018). The gut microbiome in bees is specialised, and can increase resistance to the bee gut parasites such as *Crithidia bombi* (Koch and Schmid-Hempel 2012; Praet *et al*, 2018). Altering honeybees' gut microbiota with antibiotics may be a measure for protecting them against bacterial parasites, but it opens the door for other

pathogens. This highlights the importance of healthy gut bacteria in pollinators, raising concerns over the use of antibiotics in farming practices.

1.4 *Apicystis bombi*

1.4.1 Background

Initially described as *Mattesia bombi* (Liu 1974), Lipa and Triggiani re-classified *Apicystis bombi* to the new genus of *Apicystis* in 1996 due to its morphology and life cycle. *A. bombi* is a faecal orally transmitted neogregarine parasite of adult bees (SchmidHempel 1995), which resides primarily in the fat body tissues (Lipa and Triggiani 1996). The sporozoites of *A. bombi* emerge in the intestine, penetrate through the midgut wall, and replicate in the fat body cells (Lipa and Triggiani 1996). *A. bombi* is considered to be a low prevalence parasite of *Bombus* species (Plischuk *et al*, 2011; see table 1.2), but molecular screening suggests a higher prevalence (> 25%) of the parasite (Graystock *et al*, 2013b; Graystock *et al*, 2014). *A. bombi* is thought to originally be a parasite of bumblebees that spills over into honeybees and other pollinators (Lipa and Triggiani 1996), and is therefore likely to be a translocation EID due to global transportation of the host *B. terrestris*.

1.4.2 Prevalence and distribution of *A. bombi*

Studies are documenting the global spread of *A. bombi* in bumblebee populations (table 1.2). However, the literature is sparse in documenting the rise of *A. bombi* in honeybees. Even though it has been demonstrated that *A. bombi* can transfer from bumblebees to *Apis mellifera* via faeces and contaminated pollen (Graystock *et al*, 2013b), it has not been shown whether *A. bombi* can reproduce in *A. mellifera*. Just like the multihost parasite *Nosema ceranae* (Plischuk *et al*, 2009), it is thought that *A. bombi* can infect many pollinator species.

The invasion of non-native bee species has been shown to cause the infection

Table 1.1: Results from Plischuk *et al*, 2017a; percentage of prevalence of *Apicystis bombi* in two study species (*Bombus terrestris* and *Apis mellifera*) in North West Patagonia, Argentina.

Species/Year	2008	2009	2010	2014	2015
B. terrestris	3.6	12.1	14.0	11.1	1.2
A. mellifera	7.6	13.6	3.9	17.2	14.3

Table 1.2: Results from previous studies on the prevalence of *Apicystis bombi* around the world. Prevalence (Prev) is measured as a percentage of the sample taken in each study. The studies are ordered by year due to the changes in methodology over the years. The first 4 studies and Jones and Brown (2014) used microscopy as the detection method, the other 6 studies used PCR, using general gregerine primers.

Host species	Year	Country	Prev (%)	Source
B. terrestris	1988	Italy	4	Lipa and Triggiani 1992
B. hortorum	1988	Italy	10	Lipa and Triggiani 1992
A. mellifera	1990	Finland	3	Lipa and Triggiani 1992
Bombus species	2005	Canada	1.8	Colla *et al*, 2006
Bombus species	2011	England	30	Graystock *et al*, 2013
B. hypnorum	2011	England	18	Jones and Brown 2014
A. mellifera	2012	Japan	3	Morimoto *et al*, 2013
A. m. intermissa	2013	Algeria	2	Menail *et al*, 2016
B. atratus	2013	Colombia	63	Gamboa *et al*, 2015
B. pascuorum	2013	Netherlands	48	Piot *et al*, 2019
Meliponini species	NA	Brazil	3	Nunes-Silva *et al*, 2016

of native bees by introduced parasites (Plischuk and Lange 2009; Kojima *et al*, 2011). This is due to processes such as pathogen spillover and host jumping. This became a major problem in South America, due to the commercial importation of the non-native *B. terrestris* (Torretta *et al*, 2006; Plischuk and Lange 2009). In samples taken from before and after a *B. terrestris* introduction in Argentina, *A. bombi* was not present in the area until after *B. terrestris* had been introduced. *A. bombi* was later found in all three tested native species (Arbetman *et al*, 2013). Table 1.1 shows the fluctuations in prevalence in Argentina, which could be due to many factors such as managed bee importation fluctuations or high mortality rates of individuals carrying *A. bombi*. South American isolates of *A. bombi* share the same haplotype as European isolates, consistent with the hypothesis that this parasite was introduced during the invasion of *B. terrestris* from Europe. The study

by Plischuk *et al*, (2017a) shows the importance of controlling imported bees in areas where they are non-native.

A. bombi has been found in invasive *A. mellifera* in Japan, but not in the native *Apis ceranae japonica* (Japanese honeybee) (Morimoto *et al*, 2013). However, a previous study found that the *A. mellifera* invasion has increased the presence of honeybee viruses in the local *A. ceranae japonica* (Kojima *et al*, 2011), so *A. bombi* transferring to native bees is a possibility, as witnessed in Argentina (Arbetman *et al*, 2013). *A. bombi* has also been detected in *Apis mellifera intermissa* in Africa (Menail *et al*, 2016). These results reflect worldwide dispersion of *A. bombi* in honeybees, but *A. bombi*'s effects on and virulence towards honeybees are not well understood.

The prevalence of *A. bombi* is also affected by the landscape. Large flower fields are a hotspot for bees, and therefore may facilitate the transference of pathogens such as *A. bombi*. A nearby semi-natural habitat can decrease this transference, as it spreads out the concentration of bees, limiting their interaction (Piot *et al*, 2019). The presence of *Apis* species also seems to increase the observed prevalence of *A. bombi* (Piot *et al*, 2019). The prevalence of *A. bombi* in this study was 48.8% in *B. pascuorum*, which is relatively high compared to other studies (see table 1.2).

It is difficult to test from previous work on *A. bombi* (table 1.2) if PCR is definitively better than microscopy alone due to confounding factors such as different host species and different sampling times and locations. However the table does point towards a higher detection rate with PCR compared to microscopy. When comparing the Jones and Brown (2014) and Graystock *et al*, (2013) both data sets were collected in England, however the Jones and Brown (2014) study only using microscopy detected a lower prevalence compared to the Graystock *et al*, (2013) study which used PCR detection. Goulson *et al*, (2017) mentioned that studies using only microscopy tended to report lower prevalences compared to studies using PCR. This demonstrates the need for PCR techniques in detecting

A. bombi, using specific primers.

1.4.3 Virulence and effects

A. bombi has been found to be more virulent than previously thought. It is a sub-lethal and lethal parasite that causes sucrose sensitivity and a lower lipid/body mass ratio in bumblebees (Rutrecht and Brown 2008). The parasite replicates in the fat body tissues, and the destruction of this essential tissue can negatively affect the health of the insect host (Rutrecht and Brown 2008; Arrese and Soulages 2010). *A. bombi* reduces the lifespan of the solitary bee *Osmia bicornis* (Tian *et al*, 2018). In a study by Graystock *et al*, (2016b), *Bombus terrestris* exposed to *A. bombi* had a mortality rate of 22%.

Individual pollinators can contract multiple parasite species from their natural environment. The virulence of *A. bombi* can be affected by other pathogens in the environment. Founding *Bombus hypnorum* queens infected with *A. bombi* and *Sphaerularia bombi* had a significantly lower life span and a decreased chance of founding a colony, compared to when they were just infected with *A. bombi* (Jones and Brown 2014). This is supported by Graystock *et al* (2016b), who found that *B. terrestris* suffered significantly higher mortality rates when infected with both *A. bombi* and DWV than when just infected by one of the pathogens.

1.4.4 Microbiota

In *B. terrestris*, there is a clear inverse relationship between the presence of *A. bombi* and the abundance and diversity of the host's gut microbiota (Parmentier *et al*, 2018). This could be because *A. bombi* might change the microbiota environment when infecting its host, improving its own chances of survival and colonization. As the parasite migrates from the gut into the fat body via the gut wall, it may create opportunities for bacteria to translocate from one tissue to another. Alternatively, this relationship could reflect that a diverse gut microbiota is a hos-

tile environment for *A. bombi*, and so the parasite is unable to colonise individuals with diverse gut flora. Either way, the microbial structure is an influential element for the success of infection by pathogens, as has been demonstrated in the bumblebee parasite *Crithidia bombi* and *Paenibacillus larvae* in honeybees (Koch and SchmidHempel 2011; Vasquez *et al*, 2012). This could have major implications in areas where antibiotics are used on crops, as the interaction between the host and its microbiota is critical to nutrient provisioning, immune responses, and pathogen protection (Koch and SchmidHempel 2011; Engel and Moran 2013).

1.4.5 Consequences

Overall, *A. bombi* has the potential to be a highly virulent, multi-host parasite, and could be a contributing factor to global pollinator declines. Due to viable infective oocysts of *A. bombi* being found in pollen collected by honeybees (Graystock *et al*, 2016a: Pereira *et al*, 2019), it is likely that *A. bombi* could spread to other pollinator species through honeybees dispersing *A. bombi*, contaminating shared flower resources. It is possible that *A. bombi* cannot replicate in *A. mellifera*, as it has been observed that the oocysts are non-viable or immature in some *A. mellifera* gut samples (pers comm Santiago Plischuk), however a more recent study has found mature oocysts in *A. mellifera* samples (Schilz *et al*, 2019). If *A. bombi* becomes viable in *A. mellifera*, then this parasite could further contribute to colony collapses around the world, lowering pollination and honey production, causing damage to the economy.

1.4.6 Preliminary work on *A. bombi*

Two recent field studies (Pascall *et al*, in prep, Manley *et al*, in prep) indicate that *A. bombi* could be highly prevalent both in bumblebees and honeybees in the UK. Pascall *et al*, studied the effect of pesticide exposure on parasite accumulation in *B. terrestris*. 20 parasite-free colonies of *B. terrestris* were reared in the lab-

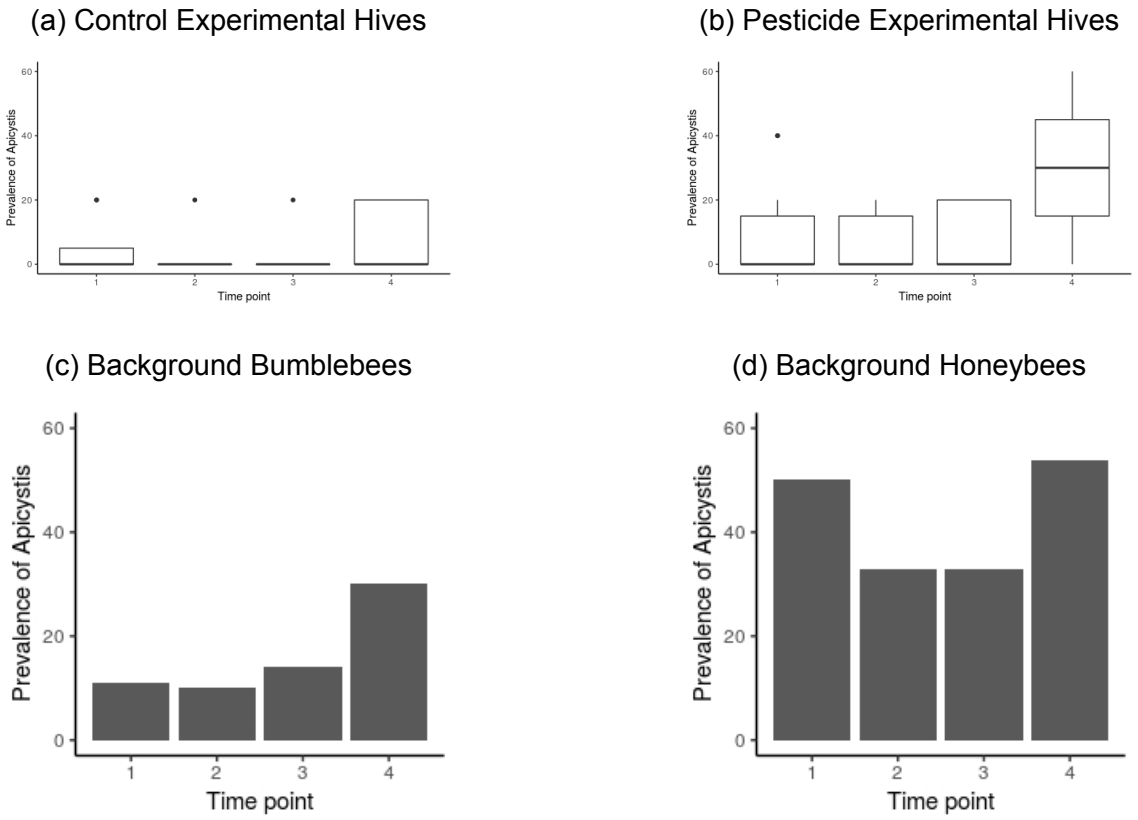

Figure 1.1: Mean prevalence of *Apicystis bombi* in experimental hives, control *B. terrestris* (a) and pesticide exposed *B. terrestris* (b), background bumblebee (c) and honeybee (d) populations surrounding the experimental hives over the course of 8 weeks. Each time point is two weeks apart. Pascall *et al*, in prep

oratory, and half were exposed to the pesticide clothianidin. Colonies were then placed in semi urban field sites for 8 weeks, and the accumulation of gut parasites was recorded throughout this time. This study showed that throughout the summer the experimental *B. terrestris* hives steadily increased in *A. bombi* prevalence, and by the eighth week in the field the parasite reached 30% prevalence in pesticide exposed colonies and 12% in control colonies (figure 1.1). This large difference in *A. bombi* prevalence shows that stressors such as pesticides acting on bee populations could have negative implications on pathogen acquisition. In the wild bumblebee population in this study, the average *A. bombi* prevalence increased from 10% to 30% over the two months (June - July). In foraging honeybees the prevalence was between 33-54%. No other studies have ever reported such a high prevalence of *A. bombi* in honeybees. Due to this high prevalence further research was carried out to confirm the prevalence of this parasite in UK populations.

Data from Manley *et al* (in prep) illustrates the wide variety of host species infected with *A. bombi* as this study drew on over 8,000 pollinator samples taken from a range of species from 10 sites in the South of England. *A. bombi* prevalence in pollinators varied drastically between species, with prevalence being highest in *A. mellifera* (52%±17s.e.). This high prevalence confirmed the findings in the previous study (Pascall *et al*, in prep) and the need for further investigation of *A. bombi* in *A. mellifera*. Within bumblebee species, average prevalence varied between 14%±3 to 40%±7. *A. bombi* was also found in some pollinating fly species such as yellow dung fly (*Scathophaga stercoraria*) and hover-fly species (*Syrphidae*) with average prevalence of 50%±4 and 20%±9 respectively. In solitary bees average prevalence was 16%±11 and 16%±7 for *Andrena* species and *Anthophora plumipes*, and 21%±12 for *Lasioglossum malachurum*. This was the first study to find *A. bombi* in species other than bees and shows the possibility of this parasite having wide impacts on pollinator communities.

Manley *et al*, (in prep) also tested how prevalence varied through time (fig-

ure 1.2). *A. bombi* prevalence increased from early spring to summer, and then dropped back down again in early spring the next year. This could be because when the bumblebee hives die in the winter, the parasite also diminishes in prevalence. More work is needed to track *A. bombi* prevalence in pollinator species throughout the year to see how the prevalence is affected by the decline of bumblebees in the autumn months.

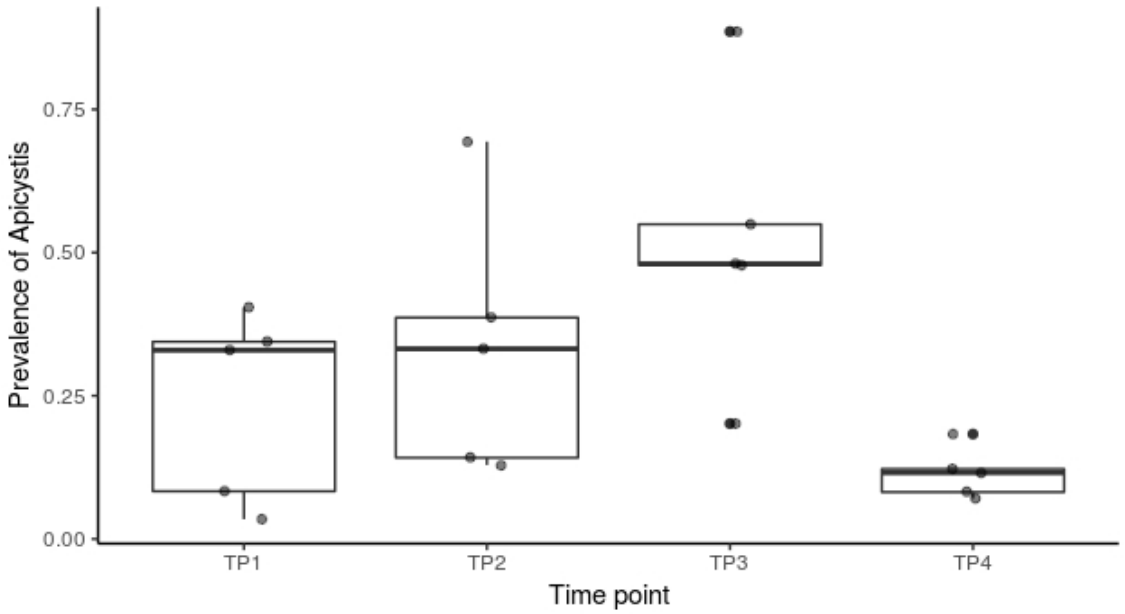

Figure 1.2: The median prevalence of *Apicystis bombi* sampled over four time points (TP1 Early Spring 2016; TP2 Late Spring 2016; TP3 Summer 2016 and TP4 Spring 2017), averages from *A. mellifera* and the four most common UK *Bombus* species (*B. terrestris*, *B. lapidarius*, *B. pascuorum* and *B. hortorum*) have been compiled together. Box = 25th and 75th percentiles, bars = min and max values. Data is from Manley *et al* in prep.

1.5 Aims of this thesis

It is becoming increasingly clear that *A. bombi* could reduce bee health and pose an additional threat to bee populations. To increase our understanding of *A. bombi* prevalence and transmission potential within and between bee species, I firstly studied the prevalence of this parasite in island and mainland populations of wild bumblebees and managed honeybees (Chapter 2). To investigate the potential

for transmission within honeybees, I studied prevalence within honeybee hives (Chapter 3).

Chapter two will focus on the prevalence of *A. bombi* on island sites compared to mainland sites, within different host species. The natural barrier of the ocean between island and mainland sites should impede the spread of pathogens from the mainland, and so island populations should exhibit less infections. It is expected that different species of bumblebees will have differing prevalences of *A. bombi*, due to traits that give the species different resistances as found in previous studies (Jones and Brown 2014, see table 1.2).

Due to the high prevalence of *A. bombi* found in foraging honeybees, chapter three will concentrate on the prevalence of *A. bombi* in *Apis mellifera* hives. Investigating if *A. bombi* can be transmitted to other members within the hive who do not have direct contact with infected bumblebee species. If *A. bombi* is found within other hive members this would show that the parasite can be transmitted via forager honeybees, through factors such as contaminated food or faeces.

Chapter 2

The prevalence of *Apicystis bombi* across island and mainland sites in three study host species; *Bombus terrestris*, *Bombus pascuorum* and *Apis mellifera*.

Abstract

In general, islands tend to have fewer pathogens than nearby mainland sites, because the sea acts as a natural barrier preventing pathogen spread. However we are unsure if this is true of bee pathogens as human agricultural practices in managed pollinators might help pathogens jump these natural barriers. To confirm whether this holds true for bee pathogens, this chapter quantified the prevalence of *A. bombi* in *Bombus terrestris*, *Bombus pascuorum* and *Apis mellifera* across island and mainland sites around the UK and France. The study sites comprised of five mainland sites, and seven island sites which were within 130km of the nearest mainland. The prevalence of *A. bombi* in *B. terrestris* and *B. pascuorum* populations was significantly lower in island populations (41%±3 s.e. and 30%±3) compared to mainland populations (65%±7 and 65%±9), suggesting that natural barriers slow the spread of bee parasites. However, *A. bombi* prevalence in *A. mellifera* is similar in both island (65%±5) and mainland (63%±5) populations. This similarity in *A. bombi* prevalence across island and mainland populations can likely be explained by the global transportation of commercially valuable *A. mellifera* as crop pollinators and producers of honey. In this study we find higher prevalence of *A. bombi* across host species than previous studies in any bee species, and we see the difference in spatial dynamics in the prevalence of *A. bombi* across the host species. While *A. bombi* has negative effects on bumblebee hosts, the impacts of infection on honeybees are unknown, raising the question of whether the high *A. bombi* prevalence is of concern in these key pollinators.

2.1 Introduction

Theory predicts that islands have fewer pathogens compared to mainland areas with the same climate (Spurgin *et al*, 2012; Cashdan 2014; Millins *et al*, 2018). This is likely because islands are isolated by natural barriers, preventing pathogens originating from the mainland infecting island populations (Millins *et al*, 2018). Islands that are more isolated tend to contain fewer pathogens than less isolated islands, indicating that population, colonization and extinction are important in determining pathogen distribution (Spurgin *et al*, 2012). Human movement and trade helps pathogens spread across natural barriers. Islands with low human traffic have no detection or lower prevalences of Chytrid fungus compared to high traffic islands and nearby mainland sites (Stockwell *et al*, 2015). As a result, globalisation is facilitating the global spread of pathogens, and known diseases are emerging in new locations at an alarming rate (Tatem *et al*, 2006; O'dowd 2007).

Island geography also affects the spread of pathogens, for instance, pathogen species richness and prevalence increases with island size (Lindström *et al*, 2004; Bell *et al*, 2005; Orrock *et al*, 2011). On small islands, endemic species tend to have small population sizes and constricted distribution ranges (Pimm *et al*, 1988; Harris and Pimm 2008), and are therefore more likely to be driven to extinction by introduced pathogens (McCallum and Dobson 1995). Islands that are more isolated (due to increased distance from the nearest mainland) are more difficult for alien pathogen species to invade, and so are usually associated with a lower pathogen species richness (Jean *et al*, 2016). Additionally, the more isolated an island is, the greater the impact a new invading pathogen is likely to have (D'Antonio and Dudley 1995). This is because organisms native to isolated islands tend to be more immunologically naïve at an evolutionary level, and are particularly vulnerable to introduced pathogens (van Riper *et al*, 1986; Manne *et al*, 1999).

Humans that colonize islands not only act as vectors for pathogens, carrying

them on their domesticated livestock (Milberg and Tyrberg 1993; Wikelski *et al*, 2004; Gottdenker *et al*, 2005), but human activity on the island can also facilitate the rapid propagation of the pathogens they introduce. Grazing livestock and changes in land use, such as replacing natural landscapes with agricultural lands, can have drastic impacts on the natural landscape, which can exacerbate the spread of emerging infectious diseases (EIDs) (Carrete *et al*, 2009; McFarlane *et al*, 2013).

One way humans spread bee pathogens is through the increased trade of bee colonies for commercial pollination. This trade is facilitating the spread and introduction of bee pathogens around the world (De la Rúa *et al*, 2009; Genersch 2010; Forsgren *et al*, 2018), devastating immunologically naïve populations (Meeus *et al*, 2011). Pathogens of bees such as *Nosema sp*ecies and *Varroa* mites have been spread around the globe at an industrial scale (Chen *et al*, 2008; Wilfert *et al*, 2016) through anthropogenic means (Owen 2017).

It is particularly important that we understand how human activities promote the spread of diseases in bees as some population declines can be attributed to the introduction of new pathogens (Forsgren *et al*, 2018). Honeybees and bumblebees share a range of harmful pathogens (Furst *et al*, 2014), and the spillover of shared pathogens from commercial bee hives is likely to be a contributing factor to the recent and rapid decline of pollinators (Otterstatter and Thomson 2008; Manley *et al*, 2015). Deformed wing virus (DWV) is associated with high winter mortality of *Apis mellifera* colonies (Highfield *et al*, 2009). The spread of DWV has been caused by global trade (Wilfert *et al*, 2016). The prevalence of DWV and the gut parasite *Nosema ceranae* does not differ between island and mainland populations (Manley *et al*, 2019). This could be because both island and mainland sites have high rates of importing and exporting honeybee colonies.

Here, we investigated the bumblebee pathogen *Apicystis bombi*. *A. bombi* is a neogregarine parasite originally found in the fat tissue and gut of bumblebees (Lipa and Triggiani 1996). Recently, it has also been recorded in honeybees and

many solitary bee species (Plischuk *et al*, 2017a; Ravoet *et al* 2014), and in some pollinating fly species such as *Scathophaga stercoraria* and *Syrphidae* (Manley *et al*, in prep). To test whether island populations show reduced parasite prevalence as expected by island biogeography, we measured the prevalence of *A. bombi* in honeybees and two bumblebee species collected from 5 populations in mainland France and England and 7 populations on islands off the coast of these two countries.

2.2 Methods

2.2.1 Sample sites

Samples were taken from 12 island and mainland sites in England and France; island were located between 40-130km from the nearest mainland (see figure 2.1 and table 2.1). The sites included seven islands (Isle of Man (Douglas), Scilly Isles (St Mary's), Ushant and Alderney (St Anne), (Guernsey (St Peter Port), Jersey (St Helier) and Belle Ile (Le Palais) and five mainland sites (Liverpool, Penryn, Cherbourg, Le Conquet and Quiberon). Data collection took place in June and July 2015.

2.2.2 Sample collection

Approximately 30 *A. mellifera*, 30 *B. pascuorum* and 60 *B. terrestris* / *lucorum* individuals from each site were collected (see table 2.1). *B. terrestris* / *lucorum* individuals were grouped together as they are cryptic species and cannot be identified apart without molecular screening. Bees were collected from a 1km^2 area within each site whilst foraging on flowers. Bees at each site were caught on the same day. Bees were only collected on days with a minimum temperature of 15°C and less than 80% cloud cover. Individual collecting tubes were used and samples were kept on ice, before sacrificing and storing them at -190°C in a dry

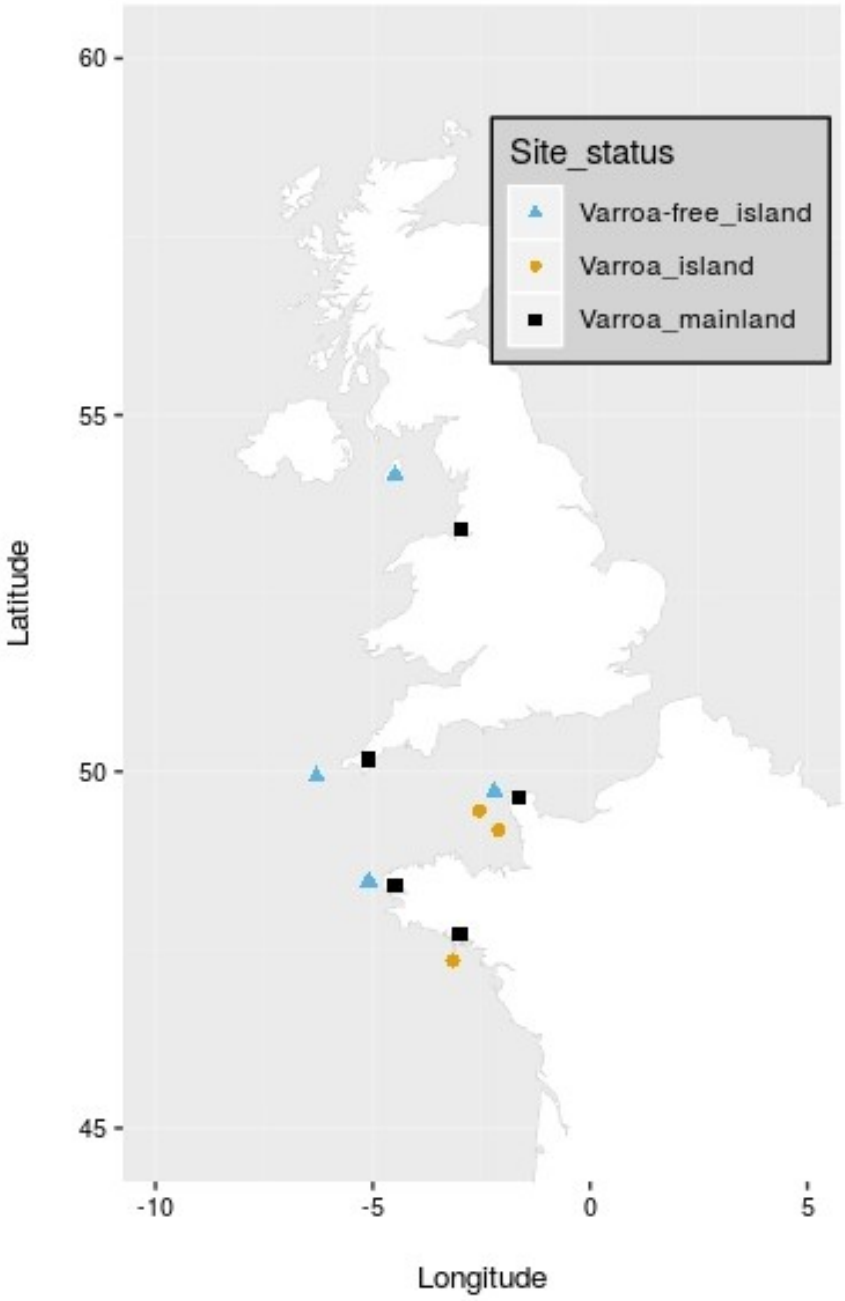

Figure 2.1: Map of study sites across the UK, France and Channel Islands. Marks with black squares are mainland (all of which are *Varroa* positive), marks with blue triangles are *Varroa* free islands, and marks with orange circles are *Varroa* positive islands.

Table 2.1: Total number of each bee species collected at each site.

Location/species	*A. mellifera*	*B. terrestris*	*B. lucorum*	*B. pascuorum*
Guernsey(St Peter Port)	22	45	15	32
Jersey(St Helier)	30	59	1	33
Alderney(St Anne)	30	57	3	30
Cherbourg(marina)	30	61	3	30
Ushant	30	13	2	29
Le Conquet, Brittany	33	59	2	19
Quiberon, Brittany	30	59	1	19
Belle Ile(Le Palais)	29	59	0	1
Penryn(University)	30	56	5	30
Isles of Scilly(St Mary's)	30	60	0	0
Liverpool	29	59	0	29
Isle of Man(Douglas)	32	53	5	29

shipper on the day of collection. For Belle Ile and Jersey, bees were sacrificed and stored in the dry shipper within 48 hours of collection. All samples were then stored at -80°C upon return to the laboratory.

2.2.3 RNA isolation and RT-PCR

The gut from each individual was removed and macerated individually in 200µl of insect ringer solution. For individual RNA extractions, half the head and thorax of individuals (bisected laterally) and 80µl of the gut solution described above were pooled and extracted using Trizol© (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. Samples were homogenised with glass beads in 1.3ml Trizol© in a tissue-lyser. RNA was separated using bromo-chloropropane and precipitated in isopropanol. The RNA was washed with 75% ethanol and re-suspended in 400µl diethylpyrocarbonate (DEPC)-treated water. 2µl of RNA was converted into first-strand cDNA using GoScript™ Reverse Transcriptase, according to the manufacturer's instructions (Promega), using random hexamer primers and RNasin® to prevent RNA degradation. Honeybee RNA was eluted in 100µl of RNase-free water and bumblebee RNA in 400µl to allow for variation in RNA pellet size between the species due to the bumblebee RNA pellets being bigger than the honeybee RNA pellets (Manley *et al*, 2019).

2.2.4 Parasite detection

To determine *A. bombi* prevalence, cDNA also used for viral screening was diluted 1:10 prior to PCR. We designed species specific primers for *A. bombi* - A.bombi.18S.F and A.bombi.18S.R (table 2.2) – which unlike primers used in previous studies (Meeus *et al*, 2010; Marharramov *et al*, 2013) do not amplify other neogregarine species such as *Mattesia* species. These primers amplify a fragment of the 18S rRNA gene and can be used to amplify both cDNA and DNA samples. The PCR reaction mix contained 4µl of 5x GoTaq® Flexi buffer, 2µl of MgCl2, 4µl of dNTPs, 1µl of the forward and reverse primers, 0.2µl of GoTaq® Flexi DNA polymerase and 2µl of template DNA. The total reaction volume was 20µl made up with Milliq H2O. PCRs were performed in a thermocycler with an initial denaturing step of 94°C for 2 minutes, followed by 35 cycles of 30sec at 94°C, 30sec at 60.7°C, 60sec at 72°C, with a final extension step of 3 minutes at 72°C. The amplified products were then visualised with UV transilluminator on a 1.5% gel stained with RedSafe, running at 110V with a 50bp ladder. The positive bands were expected at 392bp.

Differentiation between the *B. terrestris*/*lucorum* species complex was performed via a DNA length polymorphism in the mitochondrial IGS region, using the primer pair BBMI_IGSF1 and BBM1_IGSR1 (pers comm Regula Schmid-Hempel: table 2.2).

2.2.5 Sequencing

17 positive samples were randomly chosen from different sites and different species to verify that the new *A. bombi* primers were parasite specific in different host species. Samples were amplified using PCR and the A.bombi.18S primers. Samples were then PCR purified using the Promega PCR purification kits and sent to Eurofins for DNA sequencing. Sequences are on Genbank under the references MK491513 - MK491529. Sequences were individually manually inspected

Table 2.2: Primers used for the differentiation of *Bombus terrestris* and *Bombus lucorum* samples, and for the detection of *Apicystis bombi*.

Target	Primer	Sequence	Amplification program	Amplicon (bp)
B. terrestris/*lucorum* complex	BBM1-IGSF-1 BBM1-IGS-R	GGAG-CAATAATTTCAATAAATAG. AARTTCAAAG-CAC-TAATCTGC.	95°C for 1 min; 38 cycles (95°C for 15s, 55°C for 15s, 72°C for 45s); 72°C for 7 min	ter: 180 luc: 210
Apicystis bombi	*A. bombi*. 18S.F *A. bombi*. 18S.R	TGATC-CATAATAATT TTGT-GAATCGCG. AGTGCTAT-GTTTGTTTT-TAACGACA.	94°C for 2 min; 35 cycles (94°C for 30s, 60.7°C for 30s, 72°C for 60s); 72°C for 3 min	392

in Geneious® (v.6.8)(https://www.geneious.com); each sequence matched that of the known *A. bombi* sequence with no variation between samples extracted from honeybees and bumblebees. The sequences were also significantly different from *Mattesia* sequences according to the BLAST threshold.

2.2.6 Statistical analysis

All statistical analyses were carried out in R (v3.4.1). *B. lucorum* samples were excluded from prevalence analyses because of low sample size (table 2.1). To examine whether *A. bombi* prevalence was affected by host species, *Varroa*-presence and island/mainland location, generalised linear mixed models (GLMMs) were used with binomial error distribution and logit link function, using the lme4 package (v1.1-18) (Bates and Sarkar 2006). Full models included three-way interactions between the fixed effects *Varroa*-presence, host species (a factor with three levels: *A. mellifera*, *B. terrestris* and *B. pascuorum*) and island/mainland location (a factor with two levels: island and mainland), with sunshine hour duration as an additional fixed effect; field site and individual were included as random ef-

fects (individual was added to account for over-dispersion in the model (Harrison 2014)). Sunshine hours provided a proxy for favourable disease transmission conditions (Fürst *et al*, 2014) and were calculated as the mean sunshine hours from monthly data between March and July 2015 collected from MET office data and Meteo France (http://www.meteofrance.com/climat/france).

True prevalence with 95% confidence intervals was calculated to account for assay efficiency and sensitivity, which was conservatively set at 95% (Reiczigel *et al*, 2010) using R library epiR v.0.9-97 and the function epi.prev. Within the package, confidence intervals are calculated based on methods in Blaker (2000).

Backwards model simplification was used to find the minimum adequate model (MAM). The MAM was found through removal of non-significant terms and comparison of models using ANOVA, if the simplified model was not significantly different at $p > 0.05$ the term was removed from the model. To assess the full effect of the test predictors the MAM was compared with the null model (which only included random effects) using ANOVA. Residual plots were examined to assess model fit.

2.3 Results

An analysis was performed on the prevalence of *A. bombi* across 12 sites and 3 host species: *A. mellifera*, *B. pascuorum*, and *B. terrestris* (figure 2.2, figure 2.3). *A. bombi* prevalence in island populations was 65%±4.9 (n=208) for *A. mellifera*, 30%±3 (n=168) for *B. pascuorum* and 41%±3.4 (n=347) for *B. terrestris*. *A. bombi* prevalence in mainland populations was 63%±5.4 (n=149) for *A. mellifera*, 65%±8.6 (n=105) for *B. pascuorum*, and 65%±6.8 (n=293) for *B. terrestris*. The true prevalence of *A. bombi* across these sites for each species is shown in table 2.3, true prevalence takes into account for the change in variance that arises from imperfect test sensitivity and specificity (Reiczigel *et al*, 2010).

In a GLMM analysis, *A. bombi* prevalence is significantly affected by island/main-

Table 2.3: True prevalence of *Apicystis bombi* within different species at either mainland or island sites detected using PCR (primer details table 2.2). True prevalence was calculated using the function epi.prev within epiR in R. *B. lucorum* is excluded from prevalence analyses because of low sample size.

Species	Island/Mainland	True prevalence measurements (%)		
		True Prevalence	Lower	Upper
Apis mellifera	Island (n=208)	68	60	75
	Mainland (n=149)	64	55	72
Bombus terrestris	Island (n=346)	41	36	48
	Mainland (n=294)	67	60	73
Bombus pascuorum	Island (n=168)	28	20	36
	Mainland (n=105)	63	52	74

land location (estimate ± s.e. of the fixed factor 'island' in the model = -0.78 ± 0.41, p = 0.04, table 2.4, prevalence is always reports with ± s.e.). There was a significant interaction between species and location, affecting *A. bombi* prevalence (ANOVA: $X^2 = 8.71_2$, p = 0.01). This suggests that the three host species have a different relationship to *A. bombi* prevalence depending on whether populations are located on an island or the mainland, as shown in fig 2.3 and illustrated in fig 2.2. This significant interaction reflects a higher proportion of *A. bombi* prevalence in mainland *Bombus* populations than island *Bombus* populations, while this difference is not found in *Apis* populations (figure 2.3).

In mainland populations, prevalence was approximately 65% for all species (*A. mellifera*: 63%±5.4 (n=149), *B. pascuorum*: 65%±8.6 (n=105), *B. terrestris*: 65%±6.8 (n=293). These three mainland prevalences are not significantly different (test of proportions; $X^2 = 1.88_2$, p = 0.39).

Island *A. mellifera* maintained similar levels of prevalence (65%±4.9 (n=208)) to mainland populations of the same species. However, island prevalence in *B. pascuorum* decreased to 30%±3 (n=168), a significant difference from mainland *B. pascuorum* (test of proportions; $X^2 = 26.1$, $p < 0.001$). Similarly, prevalence in *B. terrestris* decreased to 41%±3.4 (n=347), a significant difference from mainland *B. terrestris* (test of proportions; $X^2 = 57.24$, $p < 0.001$). The prevalence of *A. bombi* in island *A. mellifera* is significantly higher than island *bombus* species (test of

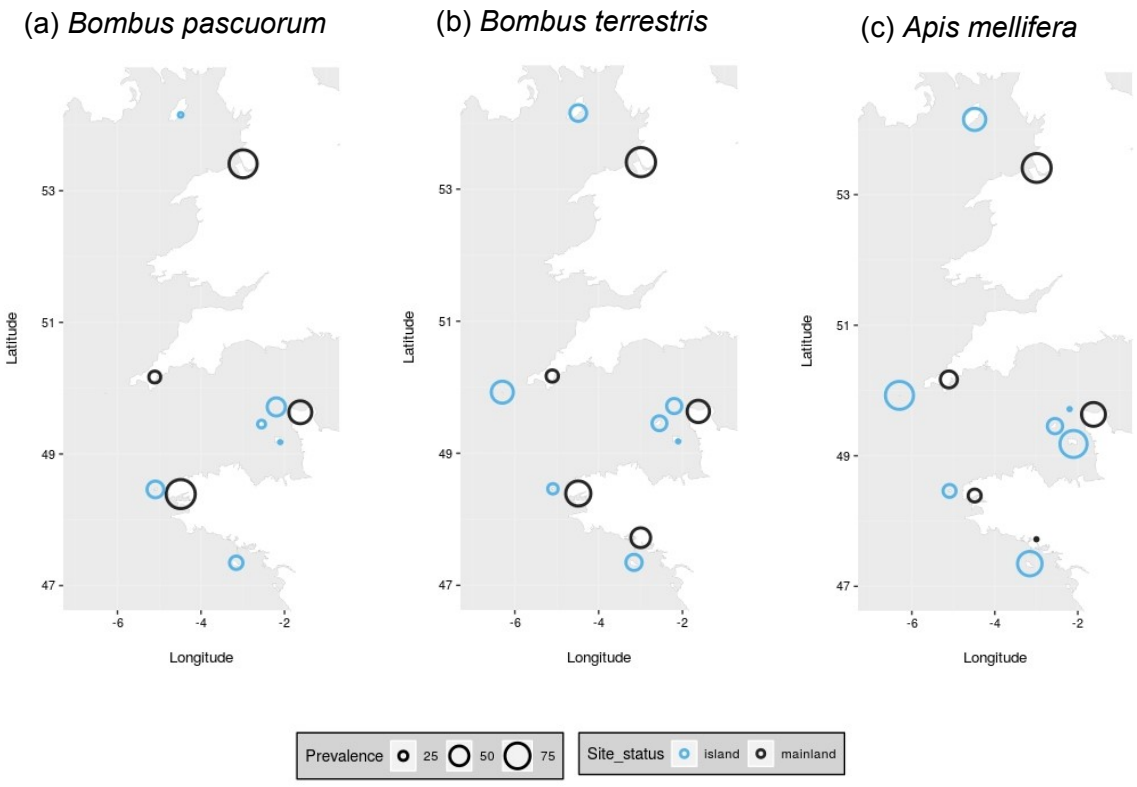

Figure 2.2: Prevalence of *Apicystis bombi* mapped by location, host species (*A. mellifera*, *B. pascuorum* and *B. terrestris*) and island/mainland status. Island sites are blue, mainland sites are black. Prevalence at each site is shown by size of circle, the larger the circle the higher the prevalence.

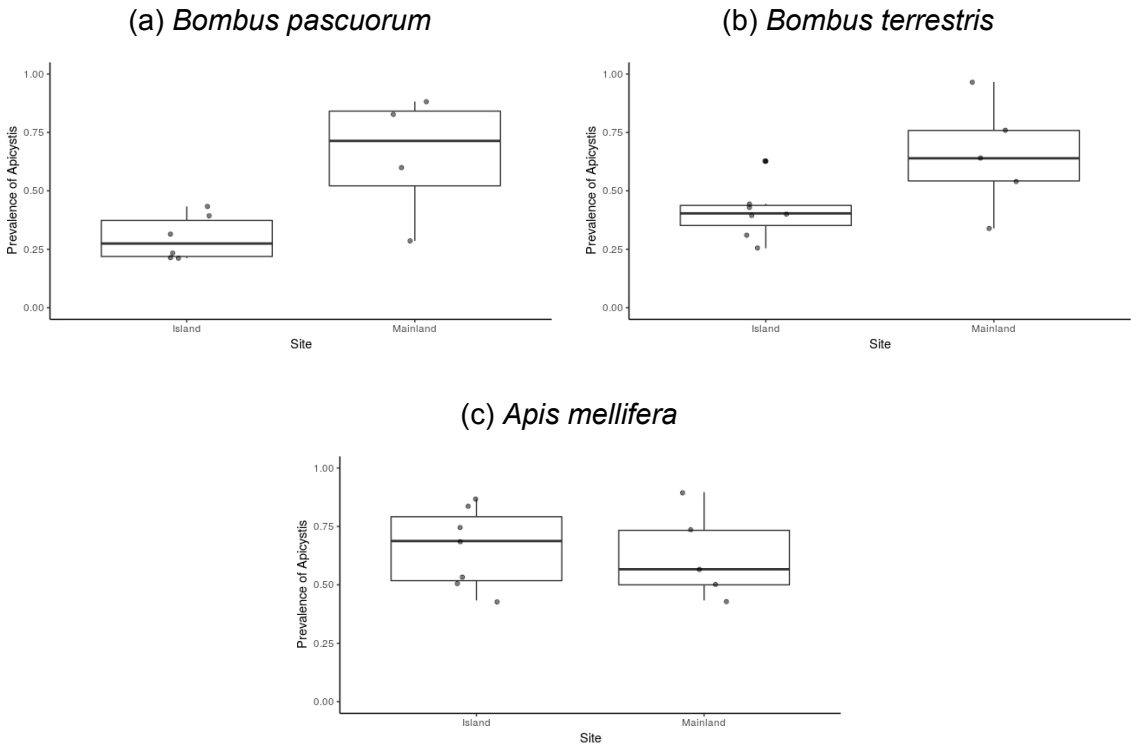

Figure 2.3: Prevalence of *A. bombi* in the three host species at island or mainland sites.

proportions; X^2 = 10.94, $p < 0.001$).

As expected, *Varroa* presence was not a significant predictor of *A. bombi* prevalence (GLMM: estimate ± s.e. of the fixed factor '*Varroa* presence' in the model = -0.237 ± 0.51, $p = 0.642$). *Varroa* presence also had no significant contribution to the GLMM model, (anova: X^2 = 0.215_1, $p = 0.643$) and was therefore removed from the model. Sunshine hours did not significantly contribute to the model either and was removed (anova: X^2 = 0.3972_1, $p = 0.5285$).

The full model for *A. bombi* prevalence fitted the data significantly better than the null model with only random factors included (anova: X^2 = 20.33_5, $p < 0.001$, table 2.4).

Table 2.4: To better understand the interaction between location and species, island and mainland populations were analysed separately. Minimum Adequate Model explaining *A. bombi* prevalence using GLMMs with binomial error structure and logit function. Top model is with the interaction and bottom model is without.

Parameters(predictors)	Estimate	Std. Error	z value	P-value
(Intercept)	0.611	0.368	1.661	0.097
B. pascuorum	-0.096	0.406	-0.235	0.814
B. terrestris	0.169	0.342	0.495	0.621
island/mainland	0.101	0.481	0.210	0.834
B. pascuorum:island/mainland	-1.373	0.523	-2.625	0.009
B. terrestris:island/mainland	-1.249	0.448	-2.785	0.005
(Intercept)	1.137	0.352	3.235	0.001
B. pascuorum	-0.934	0.296	-3.150	0.002
B. terrestris	-0.568	0.266	-2.135	0.033
island/mainland	-0.775	0.409	-1.895	0.043

2.4 Discussion

In this study we compared the prevalence of *A. bombi* in island and mainland sites, to test the hypothesis that sites with a large natural barrier have lower pathogen prevalences. Prevalence of *A. bombi* was significantly lower in island populations of *B. terrestris* (41%±3.4) and *B. pascuorum* (30%±3) compared to mainland populations (65%±6.8 and 65%±8.6) (figure 2.3). However, *A. bombi* prevalence does not significantly differ between island (65%±4.9) and mainland (63%±5.4) populations in the host species *Apis mellifera*. Across the mainland sites the average prevalence of *A. bombi* were not significantly different between the host species, varying from 63-65%. On island sites *A. mellifera* had a significantly higher prevalence of *A. bombi* compared to the *Bombus* species.

The prevalences of *A. bombi* we find in this study are much higher than those previously reported in the literature (table 1.2), with record highs being reported in *B. pascuorum* at around 48% in the Netherlands (Piot *et al*, 2019). The high prevalences we find in *A. mellifera* have never been reported before, with the highest previous records only reaching 17% (Plischuk *et al*, 2017a). In fact, prevalence of this parasite is thought to be so low that it is seldom screened for in bee gut parasite studies. However, these results show that prevalence is much higher than

previously thought, especially in *A. mellifera*. The sequenced region of 18S DNA of *A. bombi* was identical across pollinator hosts and sites, showing that they are carrying the same haplotype of *A. bombi*.

While we detected *A. bombi* at a particularly high prevalence in *A. mellifera*, it remains unclear if *A. mellifera* is a suitable host for *A. bombi*. *A. mellifera* may only be filling the role of a vector for *A. bombi* (Graystock *et al*, 2015). For example, previous work has shown that while *A. mellifera* may play a role as a passive vector, it is not a suitable host for the bumblebee parasite *Crithidia bombi* (Ruiz-Gonzalez and Brown 2006). However, Piot *et al* (2019) found that *A. mellifera* presence was an important contributing factor to *A. bombi* prevalence in *Bombus* species, supporting the idea that *A. mellifera* is an efficient vector species.

In bumblebees *A. bombi* infection causes sucrose sensitivity and reduced fat body size (Graystock *et al*, 2016b). When infected with other pathogens alongside *A. bombi*, bumblebees exhibit higher mortality rates (Graystock *et al*, 2016b). However, it is unknown if *A. bombi* has negative fitness costs for honeybees. To determine whether *A. bombi* can infect honeybees and whether these infections cause negative fitness effects needs to be addressed by experimental infection assays.

The large bodies of water between islands and the mainland may prevent pollinators from spreading disease from the mainland to the islands. We find general support for the hypothesis that water acts as a natural barrier protecting island bee populations from pathogens, but there is some evidence that human activities can help pathogens jump these natural barriers. We find an island effect for the wild *Bombus* species, with island populations exhibiting a significantly lower prevalence of *A. bombi* than mainland populations. However *A. mellifera* populations did not show this pattern. The high prevalence of *A. bombi* in *A. mellifera* in both mainland and island populations could be due to anthropogenic movement of *A. mellifera*. *A. bombi* is not the only bee pathogen that does not show an island effect in the host *A. mellifera*, it has also been shown in a few other pathogens

(Manley *et al*, 2019).

Commercially traded *A. mellifera* colonies are not screened for *A. bombi* as it is not considered as a serious affliction for honeybees. However, the trade of *A. mellifera* could facilitate the spread of *A. bombi* to more *A. mellifera* hives around the world and potentially spill back over into the surrounding bumblebee species. The global prevalence of *A. bombi* as reported in the literature appears to be increasing (table 1.2), which could be due to either an actual increased prevalence or a higher rate of successful detection due to improved screening protocols, moving from microscopy to specific molecular detection.

The high prevalences of *A. bombi* reported in *A. mellifera* pose new research questions which require attention. Are honeybee foragers only carrying *A. bombi* or are they spreading the parasite within their hives? How is the prevalence of *A. bombi* in an *A. mellifera* hive distributed, and can *A. bombi* infect and replicate within *A. mellifera*? Additional research is needed to prevent the further spread of *A. bombi* in honeybees and bumblebees.

Chapter 3

The prevalence of *Apicystis bombi* in three different life stages of *Apis mellifera*.

Abstract

Honeybees are the most managed pollinator species, and are experiencing ever increasing annual mortality rates. This is due to many interacting factors such as climate change, pesticides and pathogens. One factor that may play an important causal role in these declines is parasitism. Emerging parasites pose a particular challenge to bee species as they can be spread all over the world infecting native populations of bees. Previous research revealed very high prevalences of the potentially harmful parasite *Apicystis bombi* in *Apis mellifera* foragers. However it is not clear whether *A. mellifera* is acting as a host or a vector. To answer this this question, we examined whether *A. bombi* is potentially being spread by infected honeybees to other hive members. Capped larvae, adult nurse bees and adult foragers were sampled from 14 hives around West Cornwall (UK), and screened for the parasite A. bombi. *A. bombi* had high, and previously unreported, prevalences in larvae (59%±10 s.e.), with significantly lower prevalences in nurse bees (23%±12), and increased prevalences in forager bees (63%±15). The lower prevalences in the nurse bees could be due to high mortality rates in infected larvae. An alternative explanation is that the parasites are expelled with the gut lining as the larvae pupates. The high prevalence in foragers is most likely caused by these bees becoming infested with *A. bombi* as they explore their surrounding environment. The high prevalence in larvae suggests that honeybees can indeed transmit the parasite to conspecifics.

3.1 Introduction

Honeybees have three castes (queen, worker and drone) that all go through 4 distinctly different developmental stages during their holometabolous life cycle; egg, larvae, pupa and adult (Winston 1987). The honeybee life cycle begins when an egg is laid by the queen in a brood cell, where it develops into a larvae. Larvae undergo 5 moults, during which the gut linings are shed each time (Winston 1987). The larvae are then enclosed inside their brood cell by adult worker bees to pupate, a lifestage usually referred to as capped brood. During the pupal stage the larval midgut is completely restructured around the larval gut as the pupae metamorphose into adult bees (Braun *et al*, 2010). Adult worker bees then emerge and stay in the hive, tending to the developing brood for around 2-3 weeks. These bees are referred to as nurse bees. After the first few weeks in the hive, the nurse bees transition to foragers and leave the hive to forage for nectar and pollen. These bees are referred to as foragers (Winston 1987). This variation within hive tasks as a function of age is referred to as age-polyethism. A consequence of age-polyethism is that honeybees may be exposed to different pathogens as they grow older, and that their physiological potential to become infected and pathogens may change with age.

Developmental life stages in insects can differ drastically in their physiology and chemistry, creating different environments for pathogens. Holometabolous insects have distinctly different larval and adult stages, linked by total metamorphosis (Anderson 1972). Some pathogens can maintain infections through multiple life stages, being acquired during one stage and transmitted through the moult and metamorphosis to the next life stage. This is called transstadial transmission (Reisen 2002). A pathogen that can infect the larval stage may not be able to infect the adult stage.

Ticks vector the bacterium *Borrelia burgdorferi*. During a tick's early life it acquires the pathogen, which travels to the salivary gland where it stays while the

tick moults. The bacterium needs to survive moulting events to be passed onto subsequent hosts (Kurtenbach *et al*, 2006). These stages do not differ greatly between each moult in ticks and other arachnid vectors, However in holometabolous insects the developmental stages vary in morphology considerably. Transstadial transmission of pathogens is not well recorded for hosts that undergo complete metamorphosis.

There are a few examples of pathogens being able to transstadially transmit in holometabolous insects (Agnew *et al*, 1999; Brown *et al*, 2018; Duneau and Lazzaro 2018), such as the deformed wing virus (DWV) in bees (Annoscia *et al*, 2018). In this case, bee pupae become infected with DWV, and the virus is able to pass into the adult stage, sometimes causing wing deformities (Schroeder and Martin 2012).

However there are also studies showing the transstadial blockage of a pathogen passing from the larval stage to the adult stage (Washburn *et al*, 1995; Braun *et al*, 2010; Davies *et al*, 2017). An example of this is *Paenibacillus larvae* (American foulbrood) which can only infect honeybee larvae (Wilson 1971). Brown *et al* (2018) showed that the prevalence and intensity of an infection differs with developmental stage and age. As the insect moulted and metamorphosed the prevalence of infection decreased, suggesting that transstadial transmission is rare.

The larvae of honeybees are well equipped to cope with infections as they are exposed to pathogens constantly through feeding or external injury (Randolt *et al*, 2008). In contrast, the following developmental stage, pupa, are less prepared for infections. Before the larvae are encased in the brood cell they defecate, this typically removes any ingested intestinal pathogens (Gilliam and Prest 1987). The larvae are then in the protected environment of the brood cell to pupate, where pathogens usually cannot enter. Due to the high costs of metamorphosis, the immune defences of capped brood are lower than that of uncapped brood (Laughton *et al*, 2011). In a study where the pupae of honeybees were infected with *E. coli*,

they were incapable of activating an immune response, whereas uncapped larvae could (Gätschenberger *et al*, 2013).

Honeybee immune defenses generally increase over age, younger honeybee larvae (24 hours old) are more susceptible to infections from *P. larvae* than older larvae (72 hours old) (Brødsgaard *et al*, 1998). Larvae have lower immunity than adult bees (Wilson-Rich, *et al* 2008). Younger adult bees (nurse bees) have lower immunocompetence than older foraging bees (Laughton *et al*, 2011). These differences may be explained by foraging bees potential need for stronger immune system, as they are constantly exposed to pathogens while foraging on flowers.

It is important that we understand honeybee pathogens because these insects essential for crop pollination are at high risk of infection with emerging diseases. In some countries, beekeepers transport their bees thousands of miles specifically to pollinate certain crops (Phillips 2014). This increased anthropogenic movement of bees increases the exposure of colonies to diseases (Goulson and Hughes 2015). Honeybees are constantly interacting with different pathogens from other pollinators due to shared flowers (Graystock *et al*, 2015). It has been shown that some honeybee pathogens spill-over to *Bombus* species hosts (Genersch *et al*, 2006; Plischuk *et al*, 2009; Fürst *et al*, 2014; Manley *et al*, 2019), however there are fewer documented cases of *Bombus* pathogens spilling-over to honeybee hosts. Nonetheless one *Bombus* parasite seems to be doing this. *Apicystis bombi* is highly prevalent in *Bombus* species in the UK and it has only recently been found to be prevalent in *A. mellifera* (Graystock *et al*, 2013; Chapter 1 results). Due to the seemingly increasing high prevalences and spread of *A. bombi* in *A. mellifera* samples it is possible that *A. mellifera* is a spill-over host (Plischuk *et al*, 2011).

Pollen collected by honeybees is a probable source of infective pathogens. Graystock *et al* (2016a) found a diverse range of pathogens in the pollen collected by honeybees, all of which could still infect *B. terrestris* workers. After treating the pollen with gamma irradiation most pathogens could not infect *B. terrestris*, however *A. bombi* was the exception (Graystock *et al*, 2016a). Viable infective

oocysts of *A. bombi* have been found in honeybee collected pollen (Pereira *et al*, 2019; Graystock *et al*, 2016a). Pereira and colleagues (2019) found 53% of honeybee collected pollen was positive for *A. bombi*. Commercially sold pollen could become a possible mechanism for the spread of *A. bombi* even if the pollen has been cleaned using radiation.

A. bombi has only recently been detected in South America, Africa and Asia in honeybees (table 1.2). In Argentina the prevalence of *A. bombi* in *A. mellifera* is increasing, in the span of 8 years the prevalence has doubled, from 7% to 14% (Plischuk *et al*, 2017a). Mature *A. bombi* oocysts were found in *A. mellifera* in Poland (Schulz *et al*, 2019) possibly demonstrating that *A. bombi* can reproduce in *A. mellifera*. *A. mellifera* may experience *A. bombi* jumping host due to their close proximity to the natural *Bombus* hosts.

After finding *A. bombi* in a large percentage of the samples of *A. mellifera* in chapter 2, it was important to investigate whether *A. bombi* could be found within a hive and not just foraging *A. mellifera*. We investigated whether the foragers could potentially pass *A. bombi* to nest mates. There are currently no studies on the distribution of *A. bombi* in the different life stages of *A. mellifera* within a hive.

In this study, capped larvae, nurse bees and foragers were sampled from 14 hives from 4 apiaries in West Cornwall (UK). We expected that *A. mellifera* life stages would differ in *A. bombi* prevalence. Given age-associated changes in immune function, we expected that the larvae will have high levels of *A. bombi* due to contaminated food sources. Capped brood should be able to remove the parasite through defecation, however if the parasite had moved out of the intestinal tract and into another part of the body then high prevalences would be observed. If *A. bombi* cannot transstadially transmit or causes high mortality, then it would be expected that the nurse bees would show much lower prevalences of *A. bombi* compared to the larvae. Due to foragers sharing flower sources with *Bombus* species, the natural *A. bombi* host, it is expected that they will have similar *A. bombi* prevalences to the background *Bombus* species.

3.2 Methods

3.2.1 Sample sites

To measure the prevalence of *A. bombi* in managed *Apis mellifera* colonies, 4 apiaries in West Cornwall (UK) were used. 14 colonies were selected from the apiaries (figure 3.1), only strong colonies that had been established for over a year were chosen. Only apiaries using the same maintenance chemicals (Oxalic acid and Apiguard) were selected so as not to bias the results. All apiaries were at least 4.5 miles from each other with a maximum distance of 12 miles, reducing any risks of the bees from one apiary coming into contact with bees from another apiary (Danner *et al*, 2016).

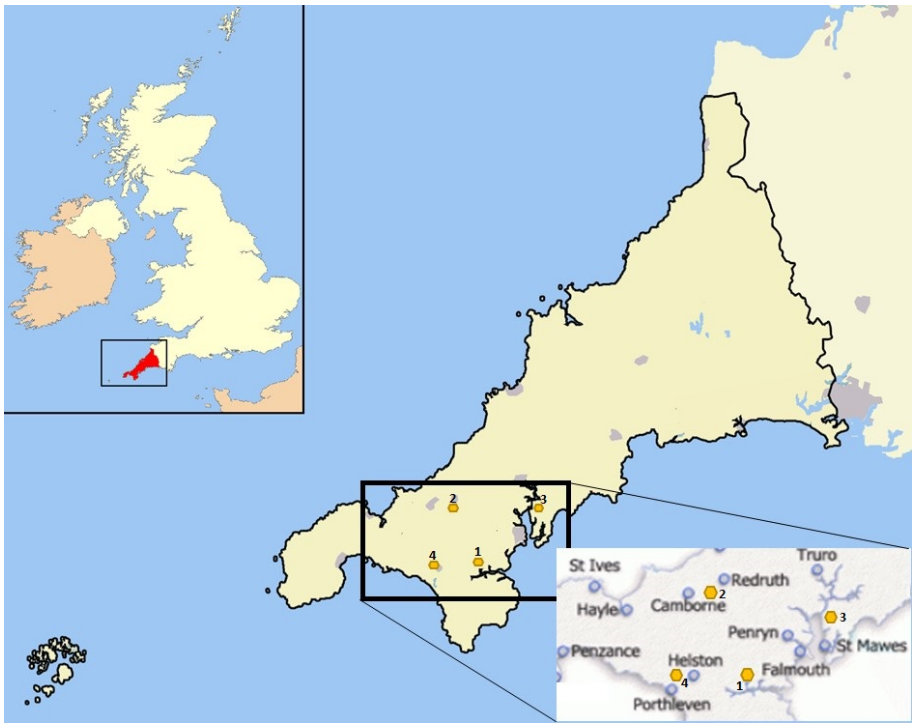

Figure 3.1: Map of apiaries in West Cornwall, UK, where *Apis mellifera* samples were collected. Yellow hexagons represent the location of each apiary, numbers next to the yellow hexagons refer to the apiary identity. Pale blue circles are the locations of Cornish towns.

3.2.2 Sample collection

To test for a difference in prevalence across three distinct honeybee life stages (capped larvae, nurse and forager), 30 individuals from each stage from each hive were collected (foragers=420, nurse=420, capped larvae=408) (see table 3.1). To get a rough proxy of the background prevalence of *A. bombi*, opportunistically sampled foraging bumblebee workers were taken, according to the local species composition (refer to supplementary material 5.1). 20-30 bumblebee individuals from the surrounding area (<0.5mile radius) were collected at three of the four apiaries (apiary 1 n = 30, apiary 3 n = 20, apiary 4 n = 30); because of poor weather conditions, it was not possible to collect bumblebees at apiary 2. Bumblebees were caught on nearby flowers, individually stored in plastic containers.

Table 3.1: Total number of samples collected from each hive for each life stage. Prevalence of *A. bombi* as a percentage for each of the life stages within each hive is also shown.

Hive	Apiary	Number of samples			Prevalence (%)		
Identity	Identity	Larvae	Nurse	Forager	Larvae	Nurse	Forager
1	1	20	30	30	75	3	10
2	1	28	30	30	32	7	23
3	2	30	30	30	43	17	57
4	2	30	30	30	77	27	90
5	2	30	30	30	70	47	83
6	2	30	30	30	53	67	43
7	3	30	30	30	70	23	70
8	3	30	30	30	60	3	83
9	3	30	30	30	43	3	77
10	4	30	30	30	40	23	90
11	4	30	30	30	47	7	37
12	4	30	30	30	53	3	60
13	4	30	30	30	83	50	60
14	4	30	30	30	87	37	93

A custom made insect hoover was used to remove adult honeybees from the hive. Foragers were caught coming back to the hive by blocking the entrance and any honeybees crawling/flying around the entrance were collected. To collect nurse bees, frames were removed from the hive, and given 2-3 sudden shakes to remove the males and foragers (Pickard and Kither 1983). Once nurses and for-

agers were collected, they were anaesthetized with a short puff of CO_2 and transferred to small plastic boxes corresponding to their hive and life stage. Between each hive collection, the collection capsules were changed, between each nurse and forager collection within the same hive the collection capsules were cleaned with 100% ethanol. Larvae in capped brood cells were collected by using forceps to open individual brood cells and remove the larvae, they were then placed in individual 1.5ml collection tubes. Samples were transferred to the laboratory in a cool bag, where they were immediately sacrificed at -80°C.

3.2.3 Extractions

Gut extraction

The gut and abdominal fat body were extracted from each bee and deposited into a 1.5ml tube with 100µl of Phosphate-buffered saline and crushed with a pestle to release parasite DNA from the tissues.

DNA extraction

20µl of homogenized gut, 100µl of 10% Chelex-100 Resin and 2µl of proteinase K (20 mg/ml) was pipetted into each well of a deep-well plate. The plate was vortexed thoroughly for 1 minute. The plate was then placed in the 56°C water bath for 1 hour, with a 30 second vortex in-between. The plate was then put into the 95°C water bath for 15 minutes to deactivate the proteinase K. The plate was centrifuged for 30 seconds and kept at -20°C.

3.2.4 *Apicystis bombi* detection

A. bombi was detected by PCR using the oligonucleotide primers A.bombi.18S.F and A.bombi.18S.R in table 2.2. For the detection of *A. bombi*, each PCR reaction mix contained 4µl of 5x GoTaq® Flexi buffer; 2µl of MGCl2 (25mM); 4µl

of dNTPs (2mM); 1µl of the forward and reverse primers; 0.2µl of GoTaq® Flexi DNA polymerase and 2µl of template. All reaction mixtures were made up to 20µl volume with ddH2O. Amplification was carried out on an Applied Biosystems Veriti Thermocycler and steps for the thermocycler sequence is shown in table 2.2. PCR products were resolved on a 1.5% agarose gel stained with redsafe at 110V for 35 minutes, and photographed with a UV transilluminator with a 50 base pair ladder. Primers were species specific as verified by Sanger sequencing (Chapter 2).

3.2.5 Statistical analysis

All statistical analyses were carried out in R (v3.4.1). To examine if *A. bombi* prevalence was affected by honeybee life stage and apiary, generalised linear mixed models (GLMMs) were used with binomial error distribution and logit link function, using the lme4 package (v1.1-18) (Bates and Sarkar 2006). Full models included two-way interactions between the fixed effects apiary (n=4) and honeybee life stage (a factor with three levels: capped larvae, nurse and forager), hive identity (n=14) and individual were included as random effects (individual was added to account for over-dispersion in the model (Harrison 2014)).

Backwards model simplification was used to identify the minimum adequate model (MAM). The MAM was found through removal of non-significant terms and comparison of models using anova – if the simplified model was not significantly different at $p > 0.05$ the term was removed from the model. To assess the full effect of the test predictors the MAM was compared with the null model (which only included random effects) using anova. Residual plots were examined to assess model fit.

True prevalence with 95% confidence intervals was calculated to account for assay efficiency and sensitivity, which was conservatively set at 95% (Reiczigel *et al*. 2010) using R library epiR v.0.9-97 and the function epi.prev. Within the

package, confidence intervals are calculated based on methods in Blaker (2000).

3.3 Results

A. bombi prevalence in honeybees across 14 hives within 4 different apiaries was analysed to test if prevalence differed according to life stage, illustrated by fig 3.2. Note that the background bumblebee prevalence is not significantly different from the forager prevalence (test of proportions: X^2 = 1.28, *p* = 0.26). The true prevalence of *A. bombi* across these sites for each lifestage is shown in table 3.2, this takes into account for the change in variance that arises from imperfect test sensitivity and specificity (Reiczigel *et al*, 2010).

Table 3.2: True prevalence (prev) of *Apicystis bombi* within different life stages in each hive, detected using PCR (primer details table 2.2). True prevalence was calculated using the function epi.prev within epiR in R.

Hive	Larvae True Prev (%)			Nurse True Prev (%)			Forager True Prev (%)		
Identity	Prev	Upper	Lower	Prev	Upper	Lower	Prev	Upper	Lower
1	77.8	52.6	89.6	0	0	12.5	5.6	0	23.3
2	30.2	13.3	52.1	1.9	0	17.7	20.4	6.3	40.5
3	42.6	23.3	63.4	13	2	32.8	57.4	36.6	63.4
4	79.6	59.5	93.8	24.1	9	44.3	94.4	76.7	100
5	72.2	51.9	88	46.3	27.2	67.2	87	67.2	98
6	53.7	32.8	72.8	68.5	48.1	86.1	42.6	23.3	63.4
7	72.2	51.9	88	20.4	6.3	40.5	72.2	51.9	88
8	61.1	40.5	79.3	0	0	12.5	87	67.2	98
9	42.6	23.3	63.4	0	0	12.5	79.6	59.5	93.8
10	38.9	20.7	59.5	20.4	6.3	40.5	94.4	76.7	100
11	46.3	27.2	67.2	1.9	0	17.7	35.2	17.7	55.7
12	53.7	32.8	72.8	0	0	12.5	61.1	40.5	79.3
13	87	67.2	98	50	30.5	69.6	61.1	40.5	79.3
14	90.7	72.8	100	35.2	17.7	55.7	98.2	82.3	100

Honeybee life stage significantly influenced *A. bombi* prevalence (table 3.3) and was a significant contributor to the model (anova: X^2 = 29.3922, $p < 0.001$). Apiary was also a significant contributor to the model (anova: X^2 = 8.19993, *p* = 0.04206). There is evidence that the honeybees at different apiaries may have different distributions of *A. bombi* infection across life stages, as the interaction

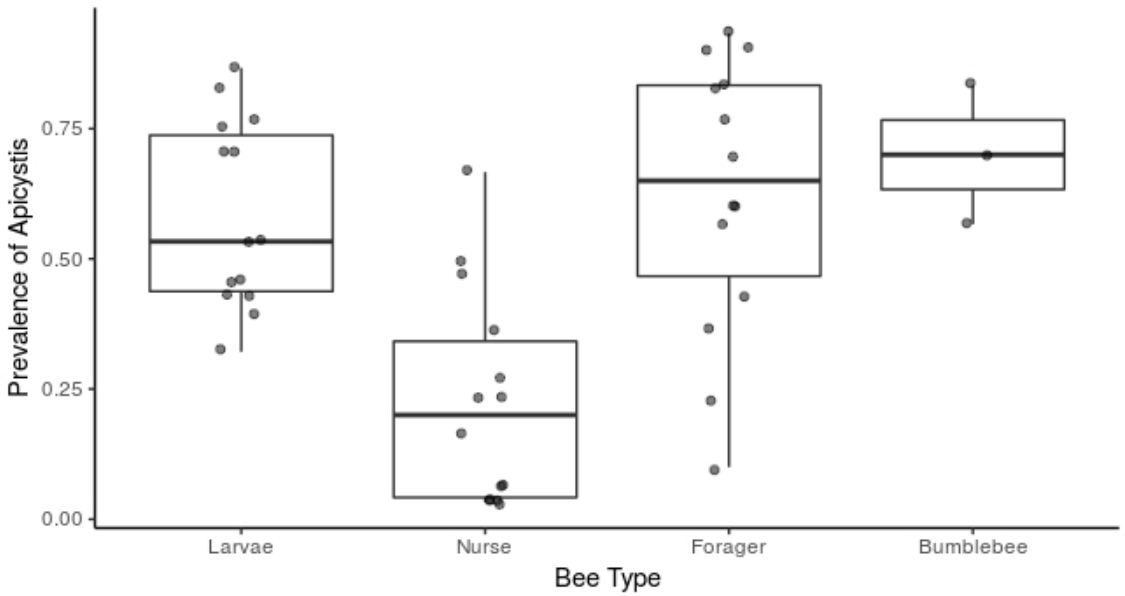

Figure 3.2: Prevalence of *Apicystis bombi* in three life stages of honeybee and the background *Bombus* populations.

between honeybee life stage and apiary also significantly contributed to the model (anova: X^2 = 13.2816, p = 0.03878). The full model for *A. bombi* prevalence (table 3.3) fitted the data significantly better than the null model with only random factors included (anova: X^2 = 49_{11}, $p < 0.001$).

Table 3.3: Minimum Adequate Model explaining *A. bombi* prevalence within the three different honeybee life stages using GLMMs with binomial error structure and logit function.

Parameters(predictors)	Estimate	Std. Error	z value	P-value
(Intercept)	1.2337	0.5071	2.433	0.014976
life stage larvae	-1.1152	0.6004	-1.858	0.063234
life stage nurse	-3.6707	0.6873	-5.341	9.25e-08
apiary2	-2.9242	0.8218	-3.558	0.000373
apiary3	-0.3433	0.6692	-0.513	0.607991
apiary4	-0.3249	0.6419	-0.506	0.612751
life stage larvae:apiary2	2.9210	0.9826	2.973	0.002951
life stage nurse:apiary2	2.2806	1.1621	1.962	0.049713
life stage larvae:apiary3	0.7023	0.7963	0.882	0.377798
life stage nurse:apiary3	2.2806	0.8634	2.641	0.008260
life stage larvae:apiary4	0.7912	0.7647	1.035	0.300843
life stage nurse:apiary4	1.3850	0.8421	1.645	0.100047

Overall interaction of life stages

The differences of *A. bombi* prevalence in larval, nurse and forager life stages in association with apiary are shown in figure 3.3. For 3 out of the 4 apiaries nurse bees had a significantly lower prevalence of *A. bombi* than the larval and forager life stages (test of proportions using Benjamini-Hochberg correction (Benjamini and Hochberg 1995); Apiary 2: X^2 = 10.42, p = 0.001, X^2 = 19.38, $p < 0.001$. Apiary 3: X^2 = 43.74, $p < 0.001$, X^2 = 78.76, $p < 0.001$. Apiary 4: X^2 = 42.65, $p < 0.001$, X^2 = 56.70, $p < 0.001$). Apiary 1 was the exception to this, the forager prevalence was not significantly different from the nurse prevalence, both having a low prevalence (test of proportions; X^2 = 3.11, p = 0.078). The capped larvae from apiary 1 did follow the trend of the other apiaries with the larvae having a significantly higher prevalence than the nurse bees (test of proportions; X^2 = 26.45, $p < 0.001$).

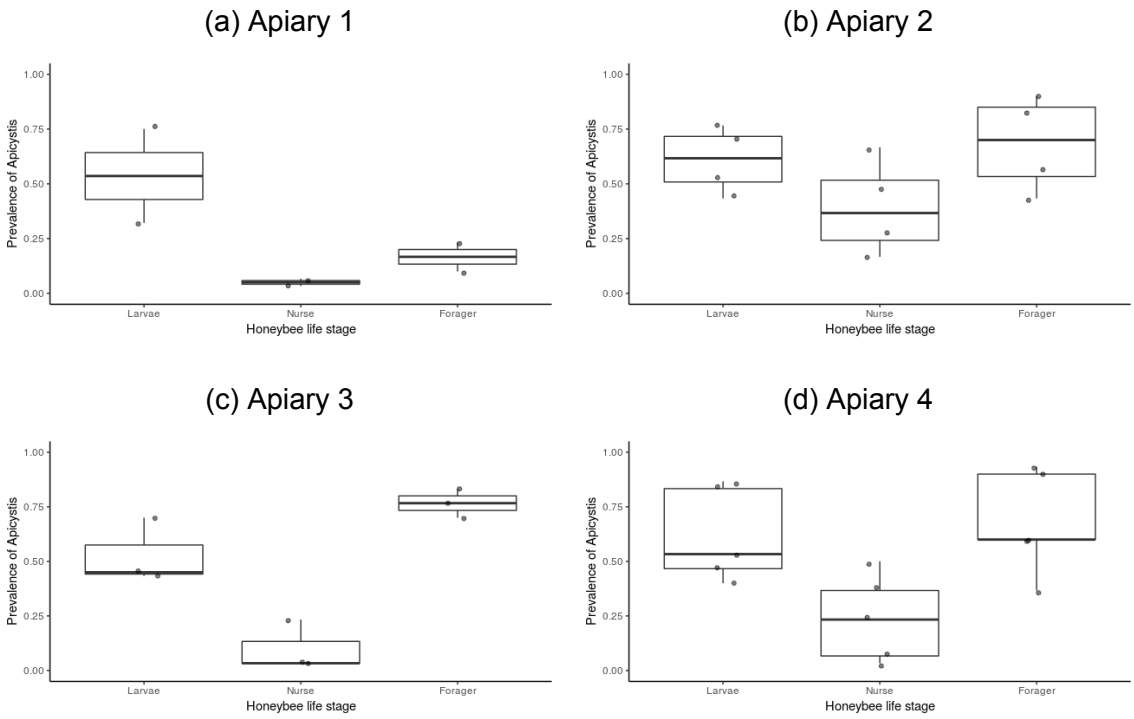

Figure 3.3: The prevalence of *Apicystis bombi* at each apiary for each of the honeybee life stages. For sample sizes refer to 3.1

The mean prevalence of *A. bombi* in capped larvae was 54%±21.4 (n=48),

61%±7.6 (n=120), 53%±8.6 (n=90) and 62%±9.6 (n=150) for apiaries 1, 2, 3, and 4 respectively. These prevalences were not significantly different from one another (test of proportions; X^2 = 2.38_3, *p* = 0.5).

The mean prevalence of *A. bombi* in nurse bees was 5%±1.7 (n=60), 39%±11 (n=120), 10%±6.7 (n=90) and 24%±8.8 (n=150) for apiaries 1, 2, 3 and 4 respectively. The prevalences in apiary 1 and 3 were not significantly different from each other (test of proportions; X^2 = 0.64, *p* = 0.42), whereas the prevalences in apiary 2 and 4 were significantly different from each other and apiaries 1 and 3 (test of proportions; X^2 = 37.77_3, $p < 0.001$).

The mean prevalence of *A. bombi* in foragers was 17%±6.7 (n=60), 68%±11 (n=120), 77%±3.8 (n=90) and 68%±10.5 (n=150) for apiaries 1, 2, 3 and 4 respectively. The prevalence in apiary 1 was significantly lower than the other three apiaries (test of proportions; X^2 = 65.24_3, $p < 0.001$). The prevalences in apiaries 2, 3, and 4 did not significantly differ from each other (test of proportions; X^2 = 2.35_3, *p* = 0.31).

3.4 Discussion

The prevalence of *Apicystis bombi* differs in the three life stages of the honeybee *Apis mellifera*. Prevalence in the first life stage (capped larvae) was similar across all the apiaries with a mean prevalence of 58%±4.8 (mean ± s.e.). In all apiaries the next life stage (nurses) was significantly lower than the capped larvae life stage, with a mean prevalence of 23%±5.5. The last life stage (forager) was significantly higher than in nurse bees and tended to be slightly higher than the larval stage in all but one of the apiaries (63%±7). The average background prevalence in *Bombus* species (70%±3.6) was similar to that of the *A. mellifera* forager average prevalence.

This work highlights the possible role of the life cycle of a host in the evolution of host-parasite interactions. Larvae of bees are non-motile, this means that

pathogens infecting just larvae are unlikely to disperse to other colonies. Some pathogens such as *P. larvae* are able to vertically transmit through adult bees taking spores back to their colonies (Lindström *et al*, 2008). In the natural world the dispersal rate of this pathogen would be low, however due to anthropogenic factors colonies are in closer contact to each other, increasing dispersal (Fries *et al*, 2006). Pathogens of and vectored by adult flying insects tend to have a higher dispersal capability due to the distances these insects can travel (El-Hamalawi 2008; Martini *et al*, 2015). If a pathogen has the ability to stay within a host throughout the larval stage and into the adult stage then it might increase its dispersal capability.

In this study capped larvae are significantly more infected than nurse bees. Other research has shown that the capped larvae have much lower immune defences compared to nurse bees (Laughton *et al*, 2011). This lowered immune system could be the reason for the very high prevalence. The significantly lower prevalence of *A. bombi* in the nurse bees compared to the capped larvae could be due to *A. bombi* causing high mortality rates so only capped larvae with no or small parasite loads survive to adulthood. However this is unlikely as there was no evidence of mass larval mortality in any of the sampled hives. The more likely answer is the larvae are shedding the parasite while pupating (Davies *et al*, 2017). This would need to be proven with further research. The large prevalence range within the different apiaries for the nurse prevalence could be due to differing immune responses within each hive. Some hives might be able to mount a stronger immune response compared to other hives.

Even though it has been shown that foraging bees tend to have a higher level of immune defence than nurse bees (Laughton *et al*, 2011), the foraging bees had a significantly higher prevalence of *A. bombi* compared to the nurse bees. The higher prevalence in foragers could be due to the foragers being in constant contact with *A. bombi* spores while foraging on flowers (Graystock *et al*, 2015). Even though foragers have a more active immune system (Laughton *et al*, 2011),

it is possible that *A. bombi* itself does not elicit an immune response.

The foragers in apiary 1 had a significantly lower prevalence of *A. bombi* compared to the other apiaries. This is despite the background prevalence in *Bombus* species being higher than around the other apiaries. This lower prevalence could be the result of genetic factors. These bees might have a particular strain of gut microbiota that helps protect them from *A. bombi* (Parmentier *et al*, 2018) or a genetically different innate immune system to the foragers from the other apiaries (Wilson-Rich *et al*, 2008). In addition to an innate immune system, bees have many different defence mechanisms against pathogens, such as removal of infected individuals from the hive, avoidance of infected individuals and grooming (Kurze *et al*, 2016). This social immunity contributes to the fitness of the hive. Hygienic behaviours such as diseased brood removal have a genetic basis (Conte *et al*, 2011) and the genes for these behaviours are favoured by selection (Harpur *et al*, 2019).

For a pathogen to survive metamorphosis and reach the host's adult stage, it must not kill the host during its development. In free flying insects such as bees, pathogens can be dispersed further in the adult stages compared to the larval life stages (El-Hamalawi 2008). It is advantageous to the pathogen for their dispersal if they can survive through to the adult stage of the host. Selection should favour low virulence in the larval host until they reach the adult host stage. However pathogens that are certain to be destroyed during metamorphosis might be more virulent during the larval stage. The spore-forming bacteria *Paenibacillus larvae* is an example of this pathogen life history. Only young honeybee larvae are susceptible to *P. larvae* (Yue *et al*, 2008). The pathogen is highly virulent and causes high mortality in larvae (Brødsgaard *et al*, 1998), and does not persist through to the honeybee adult stage. *A. bombi* could be using a similar life-history strategy. Either *A. bombi* could be causing high larval mortality rates or the larvae could be shedding the parasite when pupating.To determine whether the reduced prevalence in the nurse life stage is caused by differential mortality of the host or

shedding of the parasite, experimental infection studies will need to be conducted in honeybee larvae. This will also shed light on whether *A. bombi* affects honeybee fitness and hive stability

Here I show that *A. bombi* has a higher prevalence within *A. mellifera* than ever published before. *A. bombi* is found within individuals in the hive that do not have direct contact with the outside environment (including bumblebees that are infected with *A. bombi*). This proves that honeybee foragers can bring *A. bombi* into the hive and pass the parasite on to nestmates. We can see that the distribution of *A. bombi* within an *A. mellifera* hive is different across three of the developmental stages, with capped larvae and foragers showing high prevalences and nurse bees showing a significantly lower prevalence. More research is needed to find out if *A. bombi* can replicate in *A. mellifera*. There is also a need to find out if *A. bombi* is causing high mortality rates in *A. mellifera* larvae or if the larvae are shedding the parasite during pupation.

Chapter 4

Conclusion

Worldwide, bees are the most economically important pollinators (Blacquière *et al*, 2012), accounting for nearly 75% of the world's food crop pollination (Grozinger and Flenniken 2019), yet are suffering from devastating declines (Potts *et al*, 2010a; Potts *et al*, 2010b; Brodschneider *et al*, 2018). These declines can have terrible effects on plants that we are dependent on. We do not fully understand the reasons for these declines, but pathogens clearly play a role. For bee conservation, it is important to know how these pathogens are being transported around the world, and if natural barriers like the ocean can stop them from spreading. In my thesis I investigated the prevalence of *Apicystis bombi* in pollinators. This parasite has lethal and sublethal effects on bumblebees. *A. bombi* is considered to be a low prevalence parasite of *Bombus* species (Plischuk *et al*, 2011), but molecular screening suggests a higher prevalence (> 25%) (Graystock *et al*, 2013b; Graystock *et al*, 2014). It is important to gain a better understanding of *A. bombi* as it could be contributing to global bee declines. Below , I discuss the key insights arising from my research and discuss how they might impact pollinator health and contribute to our understanding of pollinator declines.

It is essential that we understand the geographic distribution of *A. bombi* to find out if it is able to cross natural barriers such as the ocean. This will help us identify key populations which are vulnerable to its potential spread. This in turn could help inform the creation of stricter trade regulations, slowing the spread of this parasite. It is known that isolated islands contain fewer pathogens compared to nearby mainlands (Spurgin *et al*, 2012). However, bee pathogens often traverse geographic barriers through commercial apiculture, which facilitates the spread of bee diseases globally. We do not know how natural barriers affect the distribution of *A. bombi* or if they affect different host species differently. In Chapter 2, I investigated whether this held true for the parasite *A. bombi*. To do this, I sampled three pollinator species (*Bombus terrestris*, *Bombus pascuorum* and *Apis mellifera*) across mainland and island sites in the UK and France, and measured the prevalence of *A. bombi*. I found significantly lower prevalences of *A. bombi*

in the island populations compared to the mainland populations for the *Bombus* species. However, this was not true for *A. mellifera*, where both the island and mainland populations had relatively high prevalences of *A. bombi*. This research in Chapter 2 clearly illustrates an island effect on the prevalence of *A. bombi* for *Bombus* species but not for *A. mellifera*. This result raises the question of why the island effect does not occur for *A. mellifera*. One hypothesis is that trade of the commercially valuable *A. mellifera* opens pathogen pathways, facilitating the spread of pathogens and causing high prevalences in both island and mainland populations (Fürst *et al*, 2014; Wilfert *et al*, 2016). A comparison of the number of commercial hives each island imports yearly would allow for an analysis of whether parasite prevalence is higher on islands with a higher trade rate. This could possibly support the hypothesis that increased trade increases the spread *A. bombi*.

A. bombi is known to be lethal to bumblebees, but its effects on honeybees are yet to be studied and are not well understood. *B. terrestris* mortality associated with Deformed Wind Virus (DWV) is known to increase in the presence of *A. bombi* (Graystock *et al*, 2016b). As *A. mellifera* exhibit DWV at higher rates than *Bombus* species (Fürst *et al*, 2014), it is highly possible that *A. bombi* can have colony-destroying effects on *A. mellifera*. After finding such a high prevalence of *A. bombi* in A . mellifera foragers in Chapter 2, I thought it was important to investigate if *A. bombi* can transfer to nest-mates throughout the forager's hive, as this would support the theory that *A. bombi* can infect *A. mellifera* . If *A. bombi* is only found in the foragers, it could be that the presence of *A. bombi* is due to accidental contamination from pollen sources. If the parasite is not found in other nestmates, this would imply that *A. bombi* is not capable of replicating and spreading through an *A. mellifera* hive, and is therefore not a natural parasite of *A. mellifera*. I measured the prevalence of *A. bombi* in *A. mellifera* hives across three different life stages, and found *A. bombi* in all three. I found a significantly lower prevalence of *A. bombi* in *A. mellifera* nurse bees compared to the capped larvae and for-

agers. This could be because the process of pupation is an effective parasite control measure, or because only non-infected larvae survive to adulthood. The latter is less likely, as no high larval mortality was observed in any of the hives. My results show unprecedented high prevalences of *A. bombi* across these important pollinators, and its potential to infect *A. mellifera*. The high prevalence of *A. bombi* in *A. mellifera* larvae is cause for concern, and further study is needed to determine whether this parasite effects larval mortality. Previous research has completely underestimated the prevalence of this potentially harmful parasite to these globally vital pollinators, and this under reporting may be due to the use of non-specific primers.

When breeding domesticated insects to be transported for purposes such as pollination and biocontrol of pests, pathogen dispersal by the domesticated insects should be considered. Native bee populations are experiencing parasitic pressure from bee species that are commercially reared in pathogen-dense environments and then imported (Graystock *et al*, 2013). To prevent their extinction, efforts to conserve these wild bees and maintain pockets where they can continue to exist must be supported. But given the ever increasing rate of the commercial bee trade (Aizen and Harder 2009), the increased risk of an unchecked *A. bombi* epidemic cannot be ignored. A possible course of action would be to enforce strict parasite screening as part of global bee trade regulations. Specific primers, successfully used as an accurate identifier of *A. bombi* during my research, could be used to reliably screen traded domesticated insects for *A. bombi* to prevent global outbreaks. When first discovered, *A. bombi* was found in *Bombus* species and one *A. mellifera* sample (Lipa and Triggiani 1996). Since then, studies of *A. bombi* in *A. mellifera* have been sparse, and only in the last few years has focus returned to *A. mellifera* (table 1.2). This recent literature and my own research report the increase of *A. bombi* in *A. mellifera* populations. The high prevalences found in my research indicate that *A. bombi* has always been parasite of *A. mellifera*, challenging the theory that *A. bombi* is only a parasite of *Bombus* species.

I found evidence of *A. bombi* presence in individuals of every level of the *A. mellifera* life cycle, likely transferred through faeces or contaminated pollen. This contaminated pollen includes commercially sold pollen which has been found to carry *A. bombi* along with many other pathogens (Graystock *et al*, 2016a: Pereira *et al*, 2019). To better understand the implications of high *A. bombi* prevalence in *A. mellifera* hives, we need more research into the possible harmful effects of an *A. bombi* infection. Since their evolution 160 million years ago (Cappellari *et al*, 2013), the relationship between flowering plants and eusocial pollinators has become a vital part of the planet's ecosystem, with a majority of terrestrial organisms relying on it directly or indirectly. Efforts to maintain this ecosystem service must be supported by those in power, as the environmental and economic benefits are incalculable.

Chapter 5

Supplementary material

Table 5.1: Number of each bumblebee species at each apiary and number of infected with *Apicystis bombi* from chapter 3 to show what each background bumblebee group at each apiary comprised of.

Apiary Identity	Species	Total	*A. bombi* Positive
1	*B. hortorum*	2	2
1	*B. pascuorum*	26	22
1	*B. pratorum*	1	1
1	*B. terrestris* / *lucorum*	1	0
3	*B. lapidarius*	2	1
3	*B. pascuorum*	6	9
3	*B. sylvestris*	1	1
3	*B. terrestris* / *lucorum*	6	8
4	*B. hortorum*	4	4
4	*B. pascuorum*	25	13
4	*B. terrestris* / *lucorum*	1	0

Chapter 6

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