Investigating epidemiological questions using *Crithidia bombi* and *Bombus terrestris* as a model system

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Declaration of Authorship I Hannah S. Wolmuth-Gordon hereby declare that this thesis and the work presented in it is entirely my own. Where I have consulted the work of others, this is always clearly stated.

Signed: Hannah S. Wolmuth-Gordon

Date: 4/10/2023

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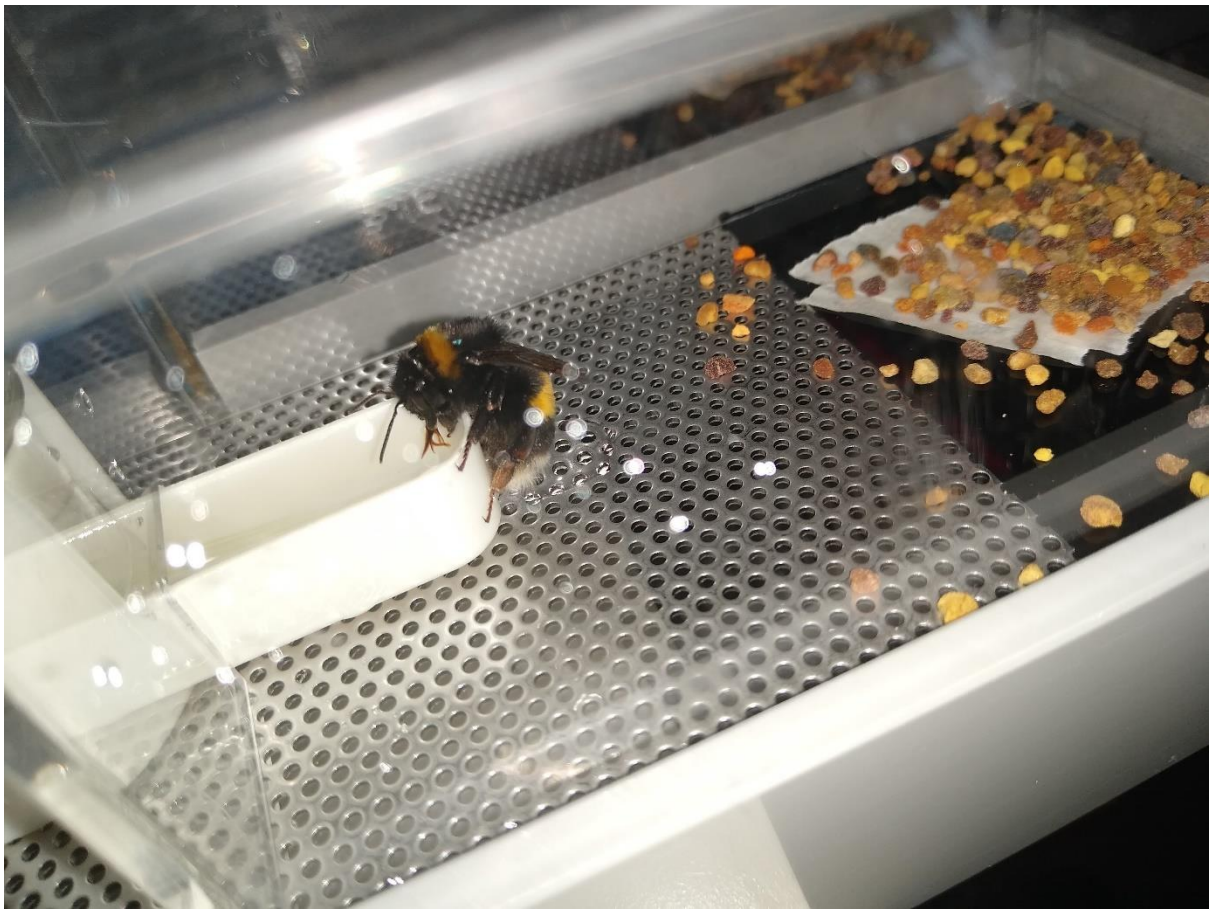
Finally, my PhD would not have been possible without the bees I sadly sacrificed in my experiments (such as bee 174 below). Thank you, I learnt a lot and hopefully the knowledge we gained makes the sacrifice worthwhile.



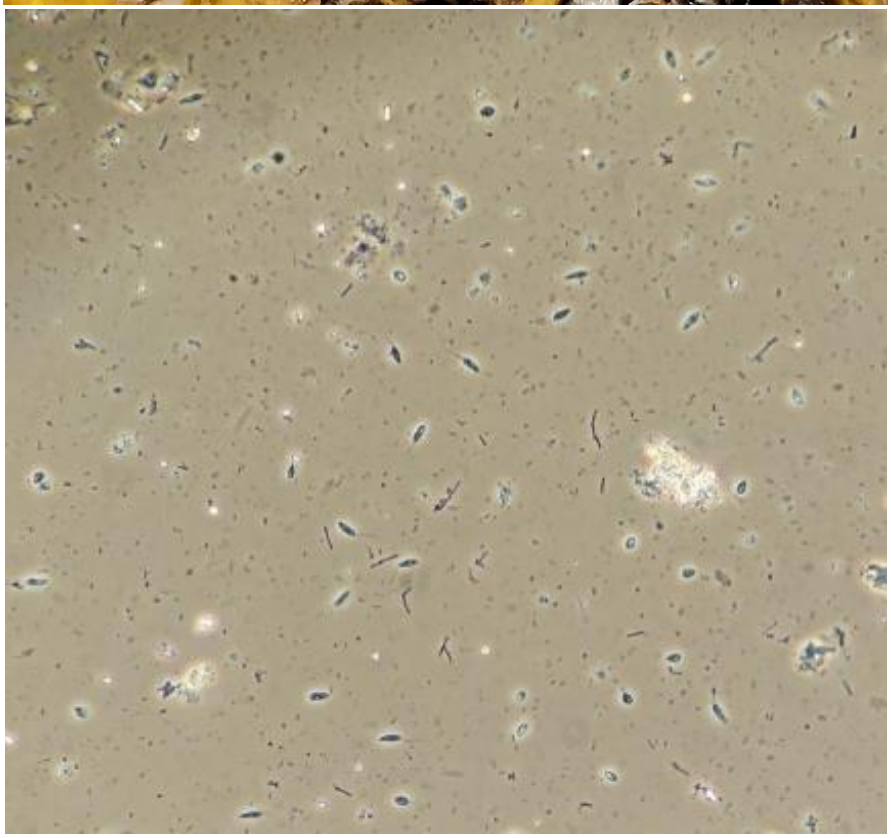
Bee 174 (used in Chapter 3) was a notably photogenic individual.

Abstract

The bumblebee gut parasite, *Crithidia bombi*, is widespread and prevalent in the field. It is spread faecal-orally between colonies via the shared use of flowers when foraging. Its interaction with the buff-tailed bumblebee, *Bombus terrestris,* is also an established epidemiological model and well-suited to laboratory experiments. This thesis uses this interaction, between *B. terrestris* and *C. bombi,* to explore epidemiology questions, specifically focussing on two themes. Firstly, the effects of structure and demography of social populations on parasite transmission and secondly the role of climate on parasite transmission. Investigating the factors shaping the transmission of *C. bombi* in bumblebee populations can help us to further understand wild bumblebee health, whilst also testing hypotheses applicable to parasite transmission in a range of social populations.

In the introductory chapter I review the general epidemiological literature, including key themes such as parasite transmission within and between populations, within host dynamics, the invertebrate immune system and the effects of anthropogenic change on parasite transmission. This is followed by a review and analysis of the current literature on the interaction between *C. bombi* and *Bombus* spp.. Chapters 2 and 3 investigate the effects of host population structure and demography on *C. bombi* transmission. Chapter 2 tests this at the individual level, looking at the effect of age on bumblebee susceptibility to *C. bombi* infection. A component of the immune system is measured to find out whether this varies between individual bumblebees of different ages. In contrast, the third chapter focusses on the whole colony and investigates whether bumblebee colony demography and structure affect within colony transmission.

Chapters 4 and 5 investigate the effect of climate on parasite transmission. The fourth chapter consists of a broad meta-analysis and tests whether climate (temperature, precipitation and humidity) affect parasite prevalence in terrestrial animal hosts. The fifth chapter looks at whether exposing the parasite to a range of temperatures affects its infectivity to bumblebee hosts. Chapter 6 compares two commonly used methods of measuring *C. bombi* infection in bumblebees, to find out whether they produce similar estimates of infection. The final discussion chapter explores the key findings and themes of the thesis as a whole and discusses their implications for bumblebee health and the epidemiology of parasite transmission in social populations.



To aid the imagination, here are photographs of the study system used throughout my PhD. A laboratory colony of *Bombus terrestris audax* (above) and the gut parasite, *Crithidia bombi* (below), viewed at X400 under a phase contrast microscope.

Table of contents

Chapter 1

Introduction....16

Acknowledgements....3

Abstract....5

Table of figures....9

Table of tables....14

1.1 General host-parasite epidemiology....17

1.2 Bumblebees and *Crithidia bombi* as a model system for epidemiology....28

1.3 Limitations using bumblebees and *Crithidia bombi* as an epidemiological model....41

1.4 Future Directions....42

2.1 *Crithidia bombi* purification....45

2.2 *Crithidia bombi* inoculation....46

2.3 Measuring *C. bombi* infection via faecal sampling....47

2.4 Measuring *C. bombi* infection via gut dissection....49

Abstract....52

3.1 Introduction....53

3.2 Methods....55

3.3 Results....60

3.4 Discussion....65

Abstract....70

4.1 Introduction....71

4.2 Methods....74

4.3 Results....80

4.4 Discussion....87

Abstract....93

5.1 Introduction....94

5.2 Methods....98

5.3 Results....104

5.4 Discussion....120

Abstract....126

6.1 Introduction....127

6.2 Methods....130

6.3 Results....135

6.4 Discussion....140

Abstract....146

7.1 Introduction....147

7.2 Methods 148
7.3 Results 150
7.4 Discussion 152
8.1 Overview 158
8.2 Summary of findings 159
8.3 Key themes and implications 160
8.4 Future directions for research 169
8.5 Conclusions 170
Searches 180
References 212
Appendices 168
References 209

Table of figures

Chapter 2

Figure 1 Triangulation protocol for purifying *Crithidia bombi* faeces, modified from Cole (1970).

Figure 2 Bees in nicot cages attached to 2ml syringes with masking tape.

Figure 3 Method of screening faecal samples for *C. bombi.*

Figure 4 Measuring infection intensity of *C. bombi* under a phase contrast microscope at X400 magnification using an improved Neubauer chamber haemocytometer showing:

a) a higher infection intensity

b) a lower infection intensity.

Figure 5 a) Inside a *B. terrestris* abdomen under a dissecting microscope at X10 magnification. The ventral side of the abdomen has been peeled back and pinned exposing the crop and guts.

b) The crop, mid- and hindguts of *B. terrestris.*

Chapter 3

Figure 1 The infection intensity of *C. bombi* in bees inoculated at four different ages.

Figure 2 a) Boxplot showing abaecin standardised gene expression in *B. terrestris* of four different ages 18 hours after inoculation with *C. bombi.*

b) Boxplot showing defensin standardised gene expression in *B. terrestris* of four different ages 18 hours after inoculation with *C. bombi.*

Figure 3 a) Boxplot showing abaecin standardised gene expression in *B. terrestris* from five different colonies 18 hours after inoculation with *C. bombi.*

b) Boxplot showing defensin standardised gene expression in *B. terrestris* from five different colonies 18 hours after inoculation with *C. bombi.*

Fig. A1.1 Photographs of callow workers.

Fig. A1.2 Reference gene expression across age treatment groups of the genes:

a) RPL13

b) AK

c) RPS18.

Fig. A1.3 Boxplot showing standardised defensin gene expression of bees of four different ages, 18 hours after inoculation with *C. bombi*

Chapter 4

Figure 1 The annual lifecycle of *B. terrestris* showing the three stages of the ergonomic growth phase (early, middle and late) that colonies were inoculated.

Figure 2 Predicted timeline of infection based on data from Logan et al. (2005).

Figure 3 The percentage prevalence of *C. bombi* through time of colonies infected at different points during the ergonomic growth stage of their lifecycle for:

a) early colonies

b) middle colonies

c) late colonies.

Figure 4 a) The percentage prevalence of all 18 colonies across treatments seven days after infection

b) Same as a), excluding two early colonies not carried through the whole experiment

c) The estimated R_0 (mean number of individuals one infected individual infects) when all 18 colonies were infected at different points during their ergonomic growth stage

d) Same as c), excluding two early colonies not carried through whole of experiment.

Figure 5 The number of workers in each colony over time from colony arrival (week 1) to end of colony lifecycle for:

a) early colonies

b) middle colonies

c) late colonies.

Chapter 5

Figure 1 Examples of the effects of temperature, precipitation and humidity on parasite development, parasite transmission and host condition, that can in turn affect parasite prevalence.

Figure 2 Number of studies retrieved and removed at each stage of screening, shown as a PRISMA flow diagram.

Figure 3 Distribution of field studies included in this meta-analysis showing:

a) the frequency of studies

b) the frequency of effect sizes.

Figure 4 Orchard plot showing results from the random effects three-level model of the effect of temperature on prevalence using data from permutation one.

Figure 5 Scatterplot of the difference in temperature between each field site or experimental treatment and the transformed log odds ratio of infected compared to uninfected in each location or treatment group. Figure:

a) shows all the data

b) excludes one outlier

c) excludes one outlier and removes 95% prediction intervals to highlight the prediction line.

Figure 6 Orchard plots showing results from the random effects three-level model of the effect of temperature on prevalence including moderators using data from permutation one for the following moderators:

a) methodological approach

b) host trophic level

c) parasite lifecycle and whether the parasite has free-living stages

d) parasite taxonomy

e) host taxonomy

f) whether a host or vector was infected.

Figure 7 Orchard plot showing results from the random effects three-level model of the effect of precipitation on prevalence using data from permutation one.

Figure 8 Scatterplot of the difference in precipitation between each field site and the transformed log odds ratio of infected compared to uninfected in each location.

Figure 9 Orchard plots showing results from the random effects three-level model of the effect of precipitation on prevalence using data from permutation one and including the following moderators:

a) host trophic level

b) whether the parasite was an ectoparasite or endoparasite.

Figure 10 Orchard plot showing results from the random effects three-level model of the effect of temperature and precipitation on prevalence using data from permutation one.

Figure 11 Orchard plot showing results from the random effects three-level model of the effect of temperature and precipitation on prevalence using data from permutation one when the following moderators were included:

a) whether the parasite has free-living stages

b) host taxonomic group

c) host trophic level.

Chapter 6

Figure 1 Photograph of *C. bombi* cells at X400 magnification using a phase contrast microscope showing

a) healthy *C. bombi* cells

b) unhealthy *C. bombi* cells.

Figure 2 The estimated percentage of the number of unhealthy *C. bombi* cells in 1µL of inoculum following incubation at five temperatures for 10 and 60 minutes.

Figure 3 The prevalence of infection of *C. bombi* in *B. terrestris audax* one week after infection. Prior to inoculation, *C. bombi* was exposed to five temperatures for 10 and 60 minutes.

Figure 4 Boxplot showing the infection intensity (cells per µL) of *C. bombi* in *B. terrestris audax* one week after infection showing:

a) the mean infection intensity

b) the raw data.

Figure 5 The prevalence of infected *B. terrestris audax* with unhealthy *C. bombi* cells one week after being infected with *C. bombi.*

Fig. A4.1 Photograph showing *Bombus terrestris audax* housing in nicot cages (Becky's bees, UK).

Fig. A4.2 Prevalence of *C. bombi* in bees one week after pilot inoculation of 15,000 cells.

Fig. A4.3 Boxplot showing the intensity of infection of *C. bombi* in bees one week after a pilot inoculation of 15,000 cells.

Fig. A4.4 Prevalence of *C. bombi* in bees one week after pilot inoculation of 8,000 cells.

Fig. A4.5 Boxplot showing the infection intensity of *C. bombi* in bees one week after a pilot inoculation of 8,000 cells.

Fig. A4.6 The prevalence of infection of *C. bombi* in individuals one week after infection with 8,000 cells.

Fig. A4.7 Boxplot showing the infection intensity (cells per µL) of *C. bombi* in individuals one week after infection with a dose of 8,000 cells showing:

a) the means

b) the raw data.

Fig. A4.8 Prevalence of *C. bombi* in individuals from each colony after inoculation with 20,000 cells.

Fig. A4.9 Mean body mass of bees from each colony before inoculation with *C. bombi*

Fig. A4.10 Residual plots for checking assumptions using the package "DHARMa" (Hartig, 2022) showing results from:

a) the full model

b) the reduced model without colony.

Chapter 7

Figure 1 The infection intensity of *C. bombi* one week after bees were given a standardised dose when infection intensity was measured using one of two methods.

Figure 2 The infection intensity of *C. bombi* in *B. terrestris* in different colonies one week after a standardised inoculation dose when infection intensity was measured using one of two methods.

Fig. A5.1 Photograph of housing of *Bombus terrestris audax* in nicot cages (Becky's bees, UK)

Table of tables

Chapter 3

Table 1 Primer sequences used in the qPCR.

Table 2 GLMM model results testing the effect of bumblebee age on *C. bombi* infection intensity.

Table 3 Tukey post hoc results from GLMM model testing the effects of bumblebee age on *C. bombi* infection intensity

Table A1.1 Sample for measuring infection intensity divided by age treatment and colony of origin.

Table A1.2. Samples collected and lost during the measuring infection experiment.

Table A1.3. Sample for measuring abaecin and defensin gene expression by age treatment and colony of origin.

Chapter 4

Table A2.1. The inoculation process for each colony.

Table A2.2 Number of infected individuals when R_0 was estimated seven days after inoculation of the colony.

Chapter 5

Table 1 Eligibility criteria used to screen studies, defined by criteria for the population, the intervention, the comparison and the outcome.

Table 2 The number of studies and comparisons within each climate variable.

Table 3 Model results from three-level random effects models when temperature and precipitation difference were included as continuous moderators separately and together.

Table A3.1 Three-level random effects model results for all permutations from the temperature dataset.

Table A3.2 Results from subgroup analysis of the temperature dataset permutation one.

Table A3.3 Three-level random effects model results of all the permutations of the precipitation datasets.

Table A3.4 Results from moderator analysis of the precipitation dataset using a random effects three-level model and data from permutation one.

Table A3.5 Results of three-level random effects models from all permutations of the combined temperature and precipitation dataset.

Table A3.6 Results from subgroup analysis using a three-level random effects model and the combined temperature and precipitation dataset using data from permutation one.

Chapter 6

Table 1 Criteria for identifying healthy compared to unhealthy *C. bombi* cells based on observations from pilot experiments.
Table A4.1 Blocking design for the order of incubation duration and temperatures tested on each day to prevent order effects.
Table A4.2 Number of bees lost from the sample in pilot experiment 1 and the reason why.
Table A4.3 Number of bees lost from the sample in pilot 2 and the reason why.
Table A4.4 Number of bees lost from the sample in experiment 1 and the reason why.

Chapter 7

Table A5.1 Comparison of gut methods used in previous studies.
Table A5.2 Comparison infection prevalence and intensity estimates from studies that use the gut and faecal sampling method.

Chapter 1
Introduction

1.1 General host-parasite epidemiology

1.11 Introduction

Parasitism is a ubiquitous threat to host fitness, with a range of possible outcomes from asymptomatic infection (Imwong et al., 2016) to high fitness costs, such as reproductive incompatibility (Werren et al., 1995) and host castration (Lafferty & Kuris, 2009). The epidemiology of host-parasite interactions can be explored at multiple scales, ranging from infection at the level of the individual to parasite transmission between species. Across these scales, host-parasite interactions vary with factors such as genotype and the environment (e.g. Sadd, 2011). Furthermore, anthropogenic factors, for example, urbanisation, are changing the distributions and densities of host communities and thus, host interactions with their parasites (e.g. Goulson et al., 2012). In addition, global trade and travel are moving parasites and their vectors large distances across the world (Tatem et al., 2006). This introduces novel parasites to susceptible hosts, who have not coevolved with the parasite, increasing the risk of epidemics and pandemics. Consequently, understanding individual infections, within-host dynamics and transmission within and between species is vital to preventing and controlling disease in wildlife and humans. Here, I will review the current literature on the epidemiology of host-parasite infections, followed by a review of the literature that has used *Crithidia bombi* and *Bombus* spp. as an epidemiological model, in order to identify questions for future research to address.

1.12 Parasite infection

When hosts are exposed to or infected by parasites, defence mechanisms can act to reduce the fitness costs of infection. There are many forms of defence, including behavioural and physiological, and these often incur costs of their own (e.g. Moret & Schmid-Hempel, 2000). Thus, the evolution and employment of these mechanisms should maximise their benefits and reduce their costs. The following section will focus on the defence mechanisms in invertebrates since these have the most relevance to my research. These mechanisms can be subdivided into pre-infection and post-infection defences. Pre-infection defences reduce the likelihood of parasite exposure. Examples of behavioural pre-infection defences include diel vertical migration that reduces exposure to parasites in *Daphnia magna* (Decaestecker et al., 2002). Social insects also exhibit many behavioural adaptations, including the separation of waste and guarding at nest entrances, that minimise the uptake of parasites into the nest (Oi & Pereira, 1993; Breed et al., 2004). In addition, insects display physiological defence mechanisms, such as density-dependant prophylaxis, which increases their defence when in high

densities (Barnes & Siva-Jothy, 2000; Ruiz-González et al., 2009; Wilson & Cotter, 2009). Furthermore, physical barriers, such as the arthropod exoskeleton (Moret & Moreau, 2012) and mucus in the gut (Zeng et al., 2022) reduce the likelihood of parasitism. Some species may also take up defensive compounds, for example, antibacterial resin by wood ants (Chapuisat et al., 2007) which can minimise parasitism. Following infection, post-infection defences attempt to clear the infection. Behavioural examples include inducing fever or chilling in bees (Müller & Schmid-Hempel, 1993; Starks et al., 2000) and locusts (Elliot et al., 2002; Sangbaramou et al., 2018). However, it is the immune response that represents the core physiological post-infection defence mechanism.

The majority of our understanding of the insect immune system comes from studies on *Drosophila* spp. and *Anopheles* spp. (Strand, 2008), and to a lesser extent the beetles, *Tenebrio molitor* and *Tribolium castaneum* (Takov et al., 2022). However, conclusions based on these models may not be applicable across all insects. This is because the number of immune genes in insects is highly variable (Evans et al., 2006) and different insects possess different immune response pathways (e.g. Gerardo et al., 2010) and therefore, insect immune systems are very diverse. Invertebrates possess an innate immune system which can be subdivided into the humoral and cellular defences. Humoral defences consist of soluble molecules and cellular components are controlled by haemocytes. In insects, the innate response consists of three interlinked components: proteolytic cascades, the cellular response, and the synthesis of antimicrobial peptides (AMPs) (Hoffmann et al., 1996). The phenoloxidase cascade is a major defence mechanism, triggering melanisation-encapsulation and nodulation, which defend against large parasites, such as parasitoids (Söderhäll & Cerenius, 1998; Cerenius & Söderhäll, 2004). This response results in the deposition of melanin at the site of intrusion, which discourages the parasite's growth (Cerenius & Söderhäll, 2004). Furthermore, the cascade stimulates phagocytosis (Strand, 2008), in which parasitic cells are engulfed. The haemocyte cell types involved in phagocytosis differ between species (Meister, 2004; Jiravanichpaisal et al., 2006). The humoral response consists of the rapid synthesis of AMPs. These have low specificity and are secreted in the haemolymph where they act in combination to kill parasites (Hoffmann et al., 1996; Meister et al., 1997; Lemaitre & Hoffmann, 2007). The Toll pathway, that responds to fungi and bacteria, and the Imd pathway, that responds to Gram-negative bacteria, are regulated by microRNAs (Lu & Chtarbanova, 2022), and both pathways play a vital role in activating AMPs (Lemaitre et al., 1995; Meister et al., 1997). The Toll (Xi et al., 2008; Ferreira et al., 2014) and Imd (Costa et al., 2009) pathways are also stimulated by some viral infections, whilst other viruses stimulate the Jak-STAT signal pathway (Dostert et al., 2005; West & Silverman, 2018). In addition, anti-viral RNAi, which targets and degrades specific viral RNA (Ding, 2010), has been identified in *Drosophila* spp. and *Anopheles* spp. (Keene et al., 2004; Galiana-Arnoux

et al., 2006; Wang et al., 2006). Another anti-viral response in insects is programmed cell death, that prevents viral replication by removing viral infected cells (e.g. Liu et al., 2013)

There is increasing evidence that the invertebrate immune system acts adaptively. Previous encounter with a parasite, immune priming, has been shown to enhance immunity upon secondary infection (e.g. Little & Kraaijeveld, 2004; Rahman et al., 2004; Eleftherianos et al., 2006; Sharrock & Sun, 2020). Moreover, this response can be specific to parasite species (Sadd & Schmid-Hempel, 2006; Pham et al., 2007) and strain (Roth & Kurtz, 2009). Priming varies between populations (Khan et al., 2016a) and lasts for varying time periods (Moret & Siva-Jothy, 2003; Pham et al., 2007), with some exhibiting persistence across life stages (Thomas & Rudolf, 2010). In addition, transgenerational priming, when immunity is transferred to offspring, occurs in many insects (Moret & Schmid-Hempel, 2001; Little et al., 2003; Schulz et al., 2019). Modelling has shown that the transfer of immunity to offspring should be more common in longer-lived invertebrate species, since they are more likely to encounter the same parasite again, and those with lower dispersal, due to the coevolution of sympatric parasites to their hosts (Pigeault et al., 2016). This phenomenon may be particularly important in social insects, in which parasite transmission is facilitated by the high relatedness and density of individuals (Sadd et al., 2005; Sadd & Schmid-Hempel, 2006).

1.13 Within-host dynamics

Following infection, microparasites must sufficiently multiply and macroparasites must mature, to be transmitted to the next host. Often, higher parasite populations in hosts increase transmission (Mackinnon & Read, 2004; Matthews et al., 2006). However, transmission can also be altered by parasite virulence, here defined as harm to the host (Ebert, 1994; Levin & Bull, 1994). Parasite reproduction and thus, the within-host dynamics, impact the rate of transmission and virulence. The course of infection by microparasites is characterised by rapid multiplication, often followed by periodic fluctuations in the population size (Hetzel & Anderson, 1996; Hoshen et al., 2000; Vizoso & Ebert, 2004). Few studies have observed the within-host dynamics of non-human diseases. Studies on malaria (e.g. Taylor et al., 1997) and trypanosomiasis (e.g. Seed & Sechelski, 1988; Tyler et al., 2001) constitute the majority of empirical studies, along with computer simulations (eg. Frank, 1996; Mandal et al., 2011; Sun et al., 2022). For example, the course of infection by the trypanosome, *Blastocrithidia triatomae*, has been observed in *Triatoma* spp., in which six days post-feeding the within-host populations of *B. triatomae* fell, followed by a rise in population which peaked 15 days post-feeding (Kollien & Schaub, 2002).

Parasite reproduction depends on several factors, including the success of the host's immune response (Haydon et al., 2003; Tschirren et al., 2007) and the infection dose (Ebert et al., 2000; Williams, 2001; De Roode et al., 2009;). Clearly, these factors are unlikely to be constant across hosts. Indeed, host condition (Altizer & Oberhauser, 1999; Tschirren et al., 2007), host genotype (Hamilton et al., 2005; Hall & Ebert, 2012) and differences in exposure (Woolhouse et al., 1997) interact with these factors (Druilhe et al., 2002). Furthermore, within-host dynamics are highly influenced by whether single or multiple parasite strains are present. In single infections, parasites maximise host exploitation without compromising higher host mortality (Bremermann & Pickering, 1983; Antia et al., 1994). However, in multiple infections, there is competition between strains and it is often assumed that strains with the highest growth rate and hence, virulence, will outcompete less virulent strains. This is true in some cases (e.g. Ben-Ami et al., 2008), whereas in other cases, strains do not compete within the host (e.g. Ikeda et al., 2003; Mouton et al., 2003), or a less virulent strain persists (e.g. Berchieri & Barrow, 1990). It is debatable whether faster compared to slower growing strains are more virulent. For example, there are examples of less virulent, faster replicating strains persisting in mixed infections (e.g. Gower & Webster, 2005). Alternatively, there is evidence that infection method, such as via inhalation, ingestion or via the skin, influences virulence (Leggett et al., 2017). Furthermore, in mixed infections, recombination between coinfecting strains can occur, resulting in novel genotypes. In trypanosomatids experimental observations of recombination have been observed (Jenni et al., 1986; MacLeod et al., 2005; Akopyants et al., 2009) and this may lead to the production of more virulent strains (Scholtissek et al., 1979; Grigg et al., 2001; Goodhead et al., 2013).

1.13 Parasite transmission within populations

Hosts are temporary islands for parasites (Ewald, 1983) and thus, effective transmission is vital for parasite survival. Transmission can occur horizontally, between members of the same generation, or vertically, between different generations. Vertical transmission plays a fundamental role in parasite transmission in insects and occurs in many host-parasite systems (De Roode et al., 2009; Vilaplana et al., 2010; Arismendi et al., 2020). As previously mentioned, due to their high densities and relatedness, social insects provide a highly favourable environment for parasite transmission. The following two paragraphs will review the types of transmission that are of relevance to social insects (Shykoff & Schmid-Hempel, 1991a; Sadd et al., 2005; Sadd & Schmid-Hempel, 2006). In social insects, horizontal transmission can occur within a colony, between members of the same generation, and between colonies in a population (Cremer et al., 2018). Many bee and wasp parasites are transmitted horizontally though the faecal-oral route (Boomsma et al., 2005). Transmission within a colony occurs through contact with infected individuals or with contaminated nest material (Cremer et al., 2007;

Rutrecht & Brown, 2008b). Social insects exhibit a wide variety of post-infection defences which prevent the establishment and spread of parasites around the nest (Cremer et al., 2007). For example, in some social insect colonies there is reduced contact between infected workers and the queen (Wang & Mofller, 1970).

Colony demography can affect parasite transmission, for example, there is evidence that larger social insect colonies exhibit higher rates of parasitism (Strassmann, 1981; Müller & Schmid-Hempel, 1992). The critical population size determines the length of time a parasite persists in a population; below this threshold the parasite will go extinct (Anderson & May, 1981). One would expect that in larger colonies this threshold is more likely to be surpassed. Larger colonies are also often denser and therefore, the rate of transmission can be faster (Naug & Camazine, 2002). However, this is not a consistent pattern, since MacFarlane & Pengelly (1974) found a negative correlation between colony size of *Bombus* spp. and parasitism. The size of the colony is dynamic and can change throughout the colony lifecycle (eg. Cameron, 1989). Therefore, susceptibility to parasitism may change if there is a relationship between colony size and susceptibility. The proportion of workers compared to reproductives in a colony also changes as the lifecycle progresses. This demography can also affect within colony transmission. For example, foragers are more likely to introduce parasites into the colony due to exposure to the outside environment, whilst, individuals with other roles in the colony may be more likely to transmit parasites within the colony, for example, *Vairmorpha bombi* is more likely to be transmitted by larvae (Rutrecht & Brown, 2008b).

Intercolony transmission, within a population, can occur indirectly or directly. Drifting, when an individual resides in a non-natal nest, and robbing, when an individual takes resources, such as nectar, from a neighbouring nest, are direct transmission mechanisms (Free, 1958; Boomsma et al., 2005; Oliveira et al., 2016; Peck & Seeley, 2019) and occur in ants (Hölldobler, 1986; Breed et al., 2012), wasps (Kasuya, 1981; Sumner et al., 2007; Oliveira et al., 2016) and bees (Free, 1958; Bordier et al., 2017; Peck & Seeley, 2019). Robbing facilitates bidirectional transmission. However, this may also occur in drifting if the individual returns to their natal nest (Cremer et al., 2018). Parasites can be transmitted indirectly through contact with parasite cells on resources, which are visited by many colonies, such as flowers. Indeed, flowers can act as transmission hubs for numerous parasites (Durrer & Schmid-Hempel, 1994; Graystock et al., 2015; McFrederick et al., 2017). In social insects, vertical transmission, when parasites are transmitted to the next generation, can happen from founding queens to offspring, or horizontally from workers rearing new queens. For example, in honeybees, swarming facilitates vertical transmission (e.g. Fries et al., 2006)

The transmission route can be a key driver in the evolution of parasite virulence (Ebert & Herre, 1996). Theory predicts that recurring horizontal transmission increases parasite virulence (Lipsitch et al., 1996), since after host death the parasite can continue to survive on another host. In contrast, vertical transmission will select for lower virulence (Lipsitch et al., 1996), since parasite transmission depends upon host reproduction, hence, host survival and condition are paramount. Reduced virulence may take the form of lower within-host replication or localised distribution (e.g. Dunn et al., 2001). These theories are supported by multiple empirical studies (e.g. Bull et al., 1991; Herre, 1993; Messenger et al., 1999; Stewart et al., 2005). In vertical transmission, there may be sex-specific virulence as there is only selection for low virulence in the sex that transmits the infection (Bandi et al., 2001). However, in reality vertically transmitted parasites cannot solely rely on vertical transmission, due to the risk of host line extinction (Lipstich et al., 1995; Schmid-Hempel, 2011). Consequently, many parasites are transmitted horizontally and vertically (Knell & Webberley, 2004), which reduces virulence. Furthermore, different infection routes may be favoured in different environments (e.g. Zilio et al., 2018). Often vertical transmission is favoured when the number of susceptible hosts is low and horizontal transmission when the number of susceptible hosts is high (Lipsitch et al., 1996; Berngruber et al., 2013).

1.14 Parasite transmission between populations

In spatially and temporally heterogeneous environments hosts and parasites will adapt to their local environment (Gandon & Michalakis, 2002). Parasites track locally abundant host genotypes (Lively & Dybdahl, 2000) and it is argued that due to their short generation time, large populations and higher mutation rates, parasites possess a higher ability to adapt to their hosts than vice-a-versa (Kaltz & Shykoff, 1998). Parasite resistance is selected for in hosts, which imposes selection for increased parasite infectivity, resulting in antagonistic coevolution (e.g. Clayton et al., 1999; Buckling & Rainey, 2002). Antagonistic coevolution is a driving force in the population dynamics of hosts and their parasites (Thompson, 1998) and in the evolution of virulence (Ebert & Hamilton, 1996). Ebert (1994) proposed that parasite infectivity and virulence in sympatric compared to allopatric hosts is often higher (e.g. Lively, 1989; Failloux et al., 1995). However, this pattern is not universal, as indicated by a meta-analysis of 54 studies (Greischar & Koskella, 2007). Local adaptation to sympatric hosts can be impeded when levels of host dispersal are high (eg. Johnson et al., 2021). Furthermore, some parasites can be maladapted to their sympatric hosts (e.g. Kaltz et al., 1999; Oppliger et al., 1999), and exhibit lower infectivity in sympatric compared to allopatric populations. This may be a result of a time lag between the parasite's allelic frequencies, which track the host allelic frequencies, and the host's

allelic frequencies (Gandon et al., 2008). There is a strong selection pressure for parasites to infect host genotypes with the highest frequency. However, it is not possible for parasites to immediately track changes in host genotype, resulting in oscillating frequency-dependent selection (eg. Nee, 1989; Dwyer et al., 1990; Lively, 1996).

Local maladaptation can also be explained by the geographical mosaic theory of coevolution (Nuismer et al., 1999; Thompson, 1999a). The geographical mosaic theory of coevolution states that the dynamics of coevolutionary interactions, such as those between hosts and parasites, are a result of a combination of three processes (Thompson, 1994; Thompson, 1997). Firstly, spatially heterogeneous landscapes, with contrasting abiotic and biotic conditions, impose differing selection pressures on hosts and their parasites (Thompson, 1999b). Thus, selection favours varying traits across the landscape, creating a selection mosaic (e.g. Kraaijeveld & Godfray, 1999). One condition that may vary across a landscape is resource availability (e.g. Forde et al., 2004). Secondly, these landscapes produce coevolutionary "hot spots", in which coevolving hosts and parasite undergo reciprocal coevolution, and "cold spots", in which one or none of the interacting species are affected by interspecific selection (Gomulkiewicz et al., 2000). These may occur along a gradient or in matrices of cold and hot patches (Thompson, 1999). Thirdly, the genetic background of a host-parasite interaction shapes its outcome. Local extinctions, genetic drift, genetic diversity and gene flow between populations, such as through host or parasite migration, affect local coevolutionary dynamics (Thompson, 1999b; Gandon & Michalakis, 2002; Morgan et al., 2005). Gene flow between populations was originally predicted to hinder parasite local adaptation to their hosts, through homogenising populations and introducing non-adapted genotypes (Slatkin, 1987). Indeed, even low levels of host or parasite migration can decrease the parasite or host local adaptation respectively (Gandon et al., 1996). However, when the local selection pressure is very high, parasite and host local adaptation can occur in the face of gene flow (e.g. Dybdahl & Lively, 1996). In addition, whether the outcome of gene flow is beneficial is random and therefore, in open populations the extent of local adaptation varies temporally and spatially (e.g. Forde et al., 2004). The combination of selection mosaics, hot and cold spots and varying genetic backgrounds results in a geographical mosaic of coevolution (Thompson, 1994), across which different selection pressures result in different outcomes.

Due to local host adaptation, parasite transmission between host populations can have harmful impacts on the host. This is highlighted by pathogen spill-over, in which parasites are transmitted from reservoir populations, which are often domesticated and more susceptible to disease, to sympatric populations (Daszak et al., 2000). There are many examples of this, particularly in mammals (eg. Petch

et al., 2022), which has resulted in the decline of wild populations (Kat et al., 1996; McVicar, 1997; Power & Mitchell, 2004). Spillover has also been identified from farmed salmon to wild populations (Jones et al., 2014). In addition, prevalence in wild populations can be increased through parasite spill-back, in which parasites are transmitted from wild to commercial populations. For example, parasite prevalence can reach extremely high levels in commercial bee colonies, due to living conditions and this 'spills back', causing unusually high prevalence in wild populations (Kelly et al., 2009; Graystock et al., 2016).

1.15 Interspecific transmission of parasites

Interspecific transmission of parasites between hosts can occur in multiple contexts. Firstly, parasites may have one (monoxenous) or several (heteroxenous) hosts in their lifecycle; for example, the lifecycle of some helminths consist of four obligate host species (Cribb et al., 2003). In heteroxenous lifecycles, parasites are only able to sexually reproduce in the final host of the lifecycle, the definitive hosts and therefore, interspecific transmission is needed to complete their lifecycle. Parasites with heteroxenous lifecycles often manipulate their hosts in order to facilitate the interspecific transmission between hosts (Poulin, 2010). For example, rodents infected with *Toxoplasma gondii* are attracted to cat urine and lose their fear of cats (Berdoy et al., 2000; Vyas et al., 2007).

Secondly, many parasites have multiple definitive hosts and thus, are able to infect and replicate within multiple host species. The number of host species a parasite is able to infect dictates whether it is classed as a specialist or generalist. Generalist parasites are less susceptible to extinction, since they are not solely dependent on one host species (Timms & Read, 1999). On the contrary, generalism imposes costs, such as reduced transmission in novel hosts and suboptimal fitness in the original host (Benmayor et al., 2009), resulting in a trade-off between host range and replication within a specific host. The close balance between the costs and benefits of generalism is emphasised by the fact that closely related parasites, such as *Trypanosome brucei* and *T. b. gambiensis*, exhibit contrasting host ranges (Woolhouse et al., 2001). How specialist or generalist a parasite is will influence the likelihood of a parasite spilling over from one species to another. There are many examples of interspecific pathogen spillover, for example honeybees have been shown to transmit a range of parasites to other species (Nanetti et al., 2021). Since generalism affects within host replication, generalism can affect parasite virulence. It is predicted that specialist parasites will replicate faster and be more transmissible, compared to generalist parasites (Leggett et al., 2013). Indeed, this is supported by experimental evidence. For example, specialist RNA plant viruses exhibit higher virulence compared

to generalist RNA plant viruses (Agudelo-Romero & Elena, 2008). On the other hand, a novel host may not increase parasite fitness. Consequently, there will be no limitations on the parasite's virulence and virulence will be extremely high, such as the infection of humans by *Echinococcus multilocularis* (Woolhouse et al., 2001). Another factor to consider, is that generalist parasites are more likely to be in hosts with multiple infections. Within host selection can be higher in multiple infections and lead to the evolution of higher virulence (Leggett et al., 2013).

The ability of a parasite to infect multiple species partially depends on its transmission route. Transmission may be direct, through close contact, indirect, through contaminated environments, or via a vector here, defined as an organism which carries a parasite between species (Wilson et al., 2017). Parasites that are transmitted directly may have less opportunity for interspecific transmission compared with parasites that are transmitted indirectly (Woolhouse et al., 2001). Factors, such as host and vector distribution and abundance (Lord et al., 1996), and host behaviour (Hide, 1999), determine whether vector-borne parasites are transmitted between species. Thus, another context of interspecific transmission is the transmission of vector-borne parasites. Vector-borne diseases, notably those transmitted by arthropods, such as dengue virus, Chagas disease and malaria, are responsible for 20% of human deaths (Wilson et al., 2017). Vectors can be divided into mechanical vectors, such as horseflies, which carry the parasite on their mouthpart of body surface, and biological vectors, such as mosquitoes, in which the parasite undergoes development or replication prior to transmission (Sarwar, 2015). Vectors may also play a role in interspecific transmission, through passing the parasite through their gut without becoming infected (Graystock et al., 2015). These dispersal mechanisms are particularly important in faecal-oral transmission and when closely related species are in close contact. For example, the sharing of flower resources by multiple pollinator species (Graystock et al., 2015), which is 'the rule' in the field (Memmott, 1999).

Ewald (1995) argued that vector-borne parasites are more virulent to their hosts than directly transmitted parasites. This is because the parasite is still transmitted even if the host exhibits low activity or is immobile, whereas low virulence is predicted in their vectors as they are vital for transmission (Elliot et al., 2003; de Angeli Dutra et al., 2022). Higher virulence in hosts also reduces host defensive behaviours towards vectors and therefore, increases the probability of transmission (Ewald, 1995). This contrasts to directly transmitted parasites, in which host immobility due to high parasite virulence would impose the cost of reduced transmission. However, modelling has emphasised that variation in host immunisation (Gandon, 2004) and phenotypic or genetic variations in the host and parasite can alter the relationship between transmission and virulence (Alizon & van

Baalen, 2005). It has also been suggested that higher inoculum size in vector-borne parasites may explain their high virulence, due to higher parasite density early in the infection (Day, 2002). On the other hand, there is evidence to disprove this hypothesis (e.g. Froissart et al., 2010), emphasising the complexity of the evolution of virulence in vector-borne parasites.

1.16 Anthropogenic change and host-parasite epidemiology

In recent years humans have altered the epidemiology of many host-parasite interactions. Urbanisation is one anthropogenic factor that is rapidly changing and can influence the interactions between parasites and their hosts. In the last century, 20% of the world's population inhabited cities, whereas currently, 55% live in cities (UN-Habitat, 2012, United Nations, 2019). This is predicted to increase over the next 30 years, particularly in Africa and Asia (United Nations, 2014). Evidently, this will impact human-parasite interactions, however, many other host-parasite interactions will also be affected as urban landscapes expand. It is often observed that parasite burdens differ in urban compared to rural landscapes (King et al., 2007; Hamer & McDonnell, 2008; Delgado-V. & French, 2012; DeVore et al., 2020; Łoś et al., 2020). The effect of urban compared to rural landscapes on transmission varies with each host-parasite interaction. For example, urban landscapes may interrupt the parasite's lifecycle, through reduced availability of intermediate hosts or vectors (e.g. Calegaro-Marques & Amato, 2014). Urbanisation has many impacts including altering host densities, stress and behaviour (DeVore et al., 2020). These can affect parasite transmission and host susceptibility, leading to higher (e.g. Goulson et al., 2012) or lower (e.g. King et al., 2007; DeVore et al., 2020; Łoś et al., 2020) parasite prevalence in urban landscapes. In addition, rapid urbanisation can cause closer relationships between humans and wildlife, particularly on the outskirts of cities, which increases the risk of transmission of zoonotic diseases, and host switching from animals to humans. This is highlighted by the fact that 60% of the 335 identified emerging diseases from 1940 to 2004 were zoonotic (Jones et al., 2008). Further knowledge of the impacts of urbanisation on host-parasite interactions can help mitigate this. For example, through the implementation of control measures and surveillance (Neiderud, 2015).

Another anthropogenic factor with a substantial impact on host parasite epidemiology is globalisation; the movement of people and objects around the world. Indeed, international travel has escalated in recent years, with international tourist arrivals rising from 25 million in 1950 to 1,087 million in 2013 (Neiderud, 2015). Travel, migration and trade are large drivers of the spread of parasites across the world. For example, Chagas disease, which is endemic to South America, has spread to many other places, notably North America and Spain (Gascon et al., 2010). In new environments, parasites will

encounter novel hosts that have not coevolved with the parasite. Thus, they will not possess specific defences against the parasite (Purkiss & Lach, 2019). This is illustrated by the globalisation of commercial bee rearing that has translocated and introduced non-native hosts across the world. For example, native bumblebees in South America have been shown to be infected with the honeybee parasite, *Nosema ceranae* (Plischuk et al., 2009), due to the use of commercial honeybees for pollination.

Finally, human activities, such as deforestation and the use of fossil fuels, have driven increased levels of atmospheric greenhouse gases, which is causing climate change and global warming. Atmospheric levels of carbon dioxide, the predominant greenhouse gas, are predicted to rise further over this century, increasing the temperature by 1.5°C globally over the next 20 years (Pörtner et al., 2022). Higher temperatures will affect the geographic distribution of hosts and their parasites, as their ranges expand into previously unsuitable environments (Epstein et al., 1998; Jones, 2016). New interactions may emerge due to a new availability of 'reservoir' hosts (Dobson & Foufopoulos, 2001; Haydon et al., 2002; Brooks & Hoberg, 2007). Similarly, increased range of a parasite's vector can enable the expansion of a parasite's geographical range (eg. Lourenço & Recker, 2014; Venturi et al., 2017). This is shown by the expansion of the climatic zone suitable for ticks in Europe (Medlock et al., 2013). Shorter winters have led to an increase in tick-borne diseases, such as *Babesia canis* (Halos et al., 2014). In addition to distribution changes, temperature can affect many aspects of parasite transmission. When considering the parasite, temperature can alter the rate of development, survival and infectivity (Leathwick, 2013; Barber et al., 2016; Kalinda et al., 2017) and different stages of their lifecycle may be differentially affected by temperature (e.g. Studer et al., 2010). Parasites with free-living stages and indirect transmission modes may be particularly vulnerable to changes in temperature, due to higher environmental exposure (eg. O'Connor et al., 2006; Morgan & van Dijk, 2012). In addition, parasites with poikilothermic hosts may be disproportionately affected, as host body temperature varies with the environment. There is also evidence that parasite virulence changes with temperature, for example, *Pasteuria ramosa,* a bacteria that parasitizes *Daphnia magna,* castrates a higher percentage of hosts at increased temperatures (Mitchell et al., 2005). Changes in parasite virulence could be a result of changes to the parasite, however, it could also be due to changes in host susceptibility. Indeed, temperature can alter host susceptibility directly via the immune response (Adamo & Lovett, 2011; Murdock et al., 2012) or indirectly, for example through changes to host growth and thus, host density leading to changes in the rates of transmission (Burdon & Chilvers, 1982). Hence, when investigating the impact of global warming and climate change on host-parasite

interactions, it is paramount to consider the impact on both the host and the parasite and all stages of transmission and establishment in the host.

1.2 Bumblebees and *Crithidia bombi* as a model system for epidemiology

1.21 Introduction

The flagellated trypanosome, *Crithidia bombi*, (Gorbunov, 1987; Lipa & Triggiani, 1988) is a common gut parasite of *Bombus* spp. and has become a model system for investigating host-parasite epidemiology (Schmid-Hempel et al., 2019). Since its discovery, two new, cryptic species have been identified, *C. expoeki* (Schmid-Hempel & Tognazzo, 2010) and *C. mexicana* (Gallot-Lavallée et al., 2016), the latter of which appears to be confined to Mexico and the former more widespread (Schmid-Hempel & Tognazzo, 2010; Gallot-Lavallée et al., 2016; Tripodi et al., 2018). *Crithidia bombi* has a monoxenous life cycle, consisting of three morphologically distinct life stages; the promastigote, choanomastigote and amastigote (Logan et al., 2005). Following ingestion, *C. bombi* attaches to the gut wall where it multiplies and releases propagules in the faeces, which are unable to survive outside the host for prolonged periods (Schmid-Hempel & Schmid-Hempel, 1993; Figueroa et al., 2019). The genomes of *C. bombi* and *C. expoeki* have been sequenced revealing that large sections, particularly those involved with host interactions, such as cell surface molecules, are under strong selection pressure (Schmid-Hempel et al., 2018). Increasing our understanding of this model system is important for understanding both bumblebees, which are vital pollinators, and host-parasite interactions at multiple scales.

1.22 Prevalence of *C. bombi*

Crithidia bombi is widespread across Europe (Shykoff & Schmid-Hempel, 1991a; Rutrecht & Brown, 2008a; Salathé & Schmid-Hempel, 2011; Popp et al., 2012; Jabal-Uriel et al., 2017; Votavová et al., 2022) and North America (Gillespie, 2010; Kissinger et al., 2011; Cordes et al., 2012; Tripodi et al., 2014; Averill et al., 2021; Jones et al., 2022). Prevalence in Europe is highly variable within and

between populations, with average prevalence ranging from 15.7% in Spain (Jabal-Uriel et al., 2017) to 23% in Ireland (Rutrecht & Brown, 2008a). Similarly, in North America 24% of workers sampled in 2006 in West Massachusetts were infected (Gillespie, 2010), whereas Cordes et al. (2012) found that mean prevalence was 2.7% across the US with high variability among species and sites. For example, prevalence was 32% in a Californian population of *B. mixtus* compared to 0% in many other populations. Furthermore, *C. bombi* has been observed in Mexico (Gallot-Lavallée et al., 2016), Chile and Argentina (Schmid-Hempel et al., 2014). This may have been due to the introduction of *B. terrestris* and *B. ruderatus* for commercial pollination, although, there is recent evidence that *C. bombi* was present prior to this (Revainera et al., 2020). Average prevalence in southern South America is 14% however, prevalence of up to 80% has been observed in Central Chile in *B. terrestris* (Schmid-Hempel et al., 2014; Fernández et al., 2020). There is evidence that this may be increasing (Arismendi et al., 2021). More recently, *C. bombi* has been identified in Australasia, with a high prevalence in New Zealand (Felden et al., 2022) however, surveys in Tasmania have not detected *C. bombi* (Allen et al., 2007).

Prevalence varies across several parameters. There is evidence that prevalence is correlated with latitude, with increased *C. bombi* prevalence in *B. vosnesenskii* at higher latitudes (Ivers & Jha, 2023). In the northern hemisphere, prevalence peaks in June and July (e.g. Parsche & Lattorff, 2018;) and falls as the summer progresses (Popp et al., 2012; Parsche & Lattorff, 2018). Popp et al. (2012) found that prevalence rose from 14.8% in June to 77.7% in July. This pattern appears to be consistent across *Bombus* species (Popp et al., 2012). However, the prevalence of *C. bombi* differs between species (Shykoff & Schmid-Hempel, 1991a; Gillespie, 2010; Cordes et al., 2012; Whitehorn et al., 2013; Graystock et al., 2020; Votavová et al., 2022; Tommasi et al., 2023), for example, *B. terrestris* and *B. lapidarius* often exhibit high prevalence (Shykoff & Schmid-Hempel, 1991a; Popp et al., 2012). Differences in prevalence between species can also be influenced by species rarity, with rarer species less likely to be infected compared to common species (Malfi & Roulston, 2014). However, this pattern does not always hold true, for example, *B. impatiens* is the most common bumblebee species in Massachusetts yet it exhibits lower prevalence compared to rarer species, such as *B. bimaculatus*, *B. perplexus* and *B. vagans* (Averill et al., 2021).

As the seasonal pattern would suggest, prevalence in spring queens is lower compared to workers (Shykoff & Schmid-Hempel, 1991a). This pattern could partly be explained by the lower susceptibility of queens to infection compared to workers in a laboratory setting (Ruiz-González et al., 2022). This may be due to higher levels of baseline humoral cell-free anti-bacterial activity compared to workers,

however, this was not the case in infected queens (Ruiz-González et al., 2022). In contrast, there is opposing evidence as to whether sex affects the likelihood of infection. Multiple studies have found higher infection prevalence in workers compared to males (Shykoff & Schmid-Hempel, 1991a; Malfi & Roulston, 2014; Theodorou et al., 2016; Parsche & Lattorff, 2018; Votavová et al., 2022). However, Gillespie (2010) found no difference between infection in males and workers, although, this may have been a product of their low sample of males compared to females. Susceptibility of males and females to *C. bombi* appears to be equal when tested experimentally (Ruiz-González & Brown, 2006b). Thus, if sex does impact infection risk, it may be a product of their behavioural role in the colony as workers are exposed to intra-colonial transmission throughout their lives, whereas males leave the colony after approximately three to four days (Free & Butler, 1959) .

In addition, landscape differences affect *C. bombi* prevalence. Higher prevalence has been observed in urban compared to rural landscapes (Goulson et al., 2012; Theodorou et al., 2016; Ivers et al., 2022). It is unclear whether this effect is indirect, and a result of increased abundance in urban areas (Theodorou et al., 2016). Higher *C. bombi* prevalence has also been observed in forest compared to more urbanised valley landscapes in Pennsylvania (Gratton et al., 2023), whereas in solitary bees (*Xylocopa augusti, Eucera fervens* and *Lasioglossum* spp.) higher prevalence has been observed in agricultural areas compared to nature reserves (Fernandez De Landa et al., 2023). Garden management also influences the prevalence of *C. bombi,* highlighted by the link between the proportion of mulch cover and *C. bombi* prevalence (Ivers et al., 2022). In addition, modelling (Otterstatter & Thomson, 2008) and field surveys (Colla et al., 2006; Otterstatter & Thomson, 2008; Graystock et al., 2014) indicate that prevalence is higher near greenhouses that use commercial bumblebees. *Crithidia bombi* can reach high prevalence in commercial colonies and a lack of measures preventing commercial bumblebees from escaping greenhouses causes *C. bombi* to spillover from commercial to wild populations through the shared use of flowers (Whittington et al., 2004; Murray et al., 2013). Furthermore, the transmission of *C. bombi* from wild to parasite-free commercial colonies put in the field suggests that spill-back could occur (Hicks et al., 2018; Martin et al., 2021; Pereira et al., 2021), from wild bumblebees to commercial colonies. Pathogen spillover is not limited to commercial bumblebee colonies, with increased prevalence also observed in bumblebees in close proximity to honeybee apiaries. However, limited sites have been surveyed (Graystock et al., 2014) and the extent of pathogen spillover and commercial farm permeability may vary with location. For example, pathogen spillover has not been observed in Scotland, where some wild populations have exhibited higher prevalence than commercial populations in some species (Whitehorn et al., 2013).

1.23 Transmission of *C. bombi*

Crithidia bombi is transmitted horizontally and vertically. Horizontal transmission can occur between colonies and between individuals within colonies. *Crithidia bombi* is transmitted between colonies when workers forage. Workers acquire the infection when foraging on contaminated flowers and ingest *C. bombi* cells left by an infected individual on the inflorescence (Durrer & Schmid-Hempel, 1994; Adler et al., 2018; Figueroa et al., 2019; Pinilla-Gallego et al., 2022). Between colony transmission is not affected by colony density in an area (Bailes et al., 2020), however, Bailes et al. (2020) compared a limited range of colony densities. Thus, manipulations of both colony density and size, across a greater range of densities is required to confirm this. Transmission rate via flowers in the field is high (Imhoof & Schmid-Hempel, 1999) and *C. bombi* is most infectious soon after cells have been shed (Schmid-Hempel et al., 1999). Due to shared floral resources transmission can occur between *Bombus* species (Ruiz-González et al., 2012; Hicks et al., 2018) and a range of *C. bombi* strains can infect several species. It was recently discovered that *C. bombi* can infect and replicate in two solitary bee species, *Osmia lignaria* and *Megachile rotundata* (Figueroa et al., 2021). Sampling of *Osmia* spp. in the field indicates that cross-infection between *Bombus* spp. and *Osmia* spp. occurs frequently (Lim et al., 2023). Figueroa et al. (2021) found that following ingestion of a standardised inoculum, a higher percentage of *O. lignaria* compared to *M. rotundata* became infected. Parasite replication was also higher in *O. lignaria* (Figueroa et al., 2021), suggesting that certain species may be more susceptible to infection and act as sources of infection compared to other species. Indeed, there is evidence that some species, such as *B. lapidarius,* are more likely to transmit *C. bombi* compared to others (Ruiz-González et al., 2012). Furthermore, despite the fact that cross infection between *Apis mellifera* and *Bombus* spp. cannot occur (Ruiz-González & Brown, 2006a; Schmid-Hempel & Tognazzo, 2010), *A. mellifera* can act as a mechanical vector for *C. bombi* (Ruiz-González & Brown, 2006a). This is also the case for the flower fly, *Eristalis tenax* (Davis et al., 2021). Transmission between *Bombus* sp. and honeybees can be high, shown by Bartolomé et al. (2020) and Pislak Ocepek et al. (2021), who identified *C. bombi* prevalence of 53.8% and 75% in honeybee colonies respectively. However, *C. bombi* presence was measured using PCR of whole abdomens. Therefore, this prevalence measure may include *C. bombi* from the exoskeleton and could be inflated.

The inflorescence morphology affects the probability of transmission and infection intensity (Durrer & Schmid-Hempel, 1994; Adler et al., 2018). Within a plant species, linear compared to spiral inflorescence arrangements may increase transmission risk, as a result of bumblebees skipping flowers when arranged in a spiral. However, this pattern was only tested in one species (Durrer & Schmid-Hempel, 1994) and manipulation of inflorescence orientation in another species had no effect on *C.*

bombi transmission (Van Wyk et al., 2023). Manipulation of some floral traits has been shown to affect transmission, for example trimming the corolla lip on the inflorescence lowers the probability of infection and infection intensity (Van Wyk et al., 2023). Furthermore, Adler et al. (2018) found plant species and species traits, such as corolla size and shape, predicted infection intensity, but not transmission risk. In this experiment the inoculum was inserted into the corolla tube. In contrast, in the field, bumblebees will defecate across the inflorescence and the location of defecation influences the survival of *C. bombi* and the likelihood of transmission (Figueroa et al., 2019; Pinilla-Gallego et al., 2022). Evidence suggests that *C. bombi* can be transmitted via contaminated nectar (Durrer & Schmid-Hempel, 1994; Figueroa et al., 2019), however, there is some conflict over this (Cisarovsky & Schmid-Hempel, 2014). Bees are more likely to defecate on different floral structures across plant species. For example, the likelihood of defecation on *Monarda didyma* is highest inside the flower, but highest on the leaves and bracts of *Lythrum salicaria* (Figueroa et al., 2019). Due to the combined effects of *C. bombi* survival, defecation likelihood and defecation location, shorter and wider flowers lead to higher *C. bombi* transmission (Pinilla-Gallego et al., 2022). Consequently, floral landscape species composition is likely to impact transmission and hence, prevalence, however, this is yet to be investigated.

Transmission within a colony occurs through contact with faeces and contaminated surfaces in the nest. A higher initial number of infected individuals results in faster spread of *C. bombi* and increased infection intensity in individuals (Pinilla-Gallego et al., 2020). Horizontal transmission between queens and workers, and between workers, within colonies has been reconstructed using network analysis. This revealed that role and task allocation does not affect the risk of transmission once a colony has been infected. Rather, denser networks result in faster transmission (Otterstatter & Thomson, 2007). Contact frequency as opposed to duration is most important in explaining within colony transmission (Sah et al., 2021). Furthermore, transmission can occur between castes. Although larvae do not exhibit signs of *C. bombi* infection, they can transmit the parasite to workers when feeding (Folly et al., 2017). Likewise, workers who have acquired the infection whilst foraging can infect new sexuals and other workers (Ulrich & Schmid-Hempel, 2015).

1.24 The immune response of *Bombus* spp. to *C. bombi*

Crithidia bombi infection elicits an immune response that varies through time, between individuals and environments. Brown, Moret & Schmid-Hempel (2003) found that despite inhabiting the gut, *C. bombi* caused an increase in prophenoloxidase in the haemocoel. However, it is likely this was a type 1 error, since several other studies have found no difference in phenoloxidase activity in infected

compared to control individuals (Otterstatter & Thomson, 2006; Ruiz-González et al., 2012; Fowler et al., 2022). Analysis of gene expression in infected individuals has identified three temporal phases in the immune response to *C. bombi,* consisting of distinct gene expression. For example, peroxidase expression peaks 4-5 hours after infection, whereas antimicrobial peptide (AMP) expression, such as that of defensin and abaecin, increase 12 hours after infection (Riddell et al., 2011). Seven days after inoculation, the haemolymph of infected compared to uninfected bees is less likely to produce zones of inhibition in assays of antibacterial activity, which suggests that at this time-point infected bees have a lower baseline level of AMP expression in the haemolymph. Perhaps there is a trade-off in AMP expression either in different areas of the body or temporally (Fowler, Sadd, et al., 2022). Interestingly, there is no difference in the haemolymph proteome after 48 hours in infected and uninfected bees (Askri et al., 2023). Increased AMP expression following *C. bombi* infection is supported by multiple studies (Riddell et al., 2009; Brunner et al., 2014) and their importance is highlighted by Deshwal and Mallon (2014) who found that *B. terrestris* individuals with knocked out defensin and abaecin exhibited higher levels of *C. bombi* infection. This indicates that AMPs play a vital role in the immune defence against *C. bombi*, as has been found for trypanosomes infecting other insects (McGwire & Kulkarni, 2010). This study only tested one colony. However, the role of AMPs in the defence against *C. bombi* has been confirmed by Marxer et al. (2016), who found that AMPs are used in varying combinations and concentrations against *C. bombi.*

The immune response can vary depending on the *C. bombi* strain, with some clones producing AMP gene expression similar to that from bacteria and others producing small changes in gene expression (Barribeau & Schmid-Hempel, 2013). In addition to strain variation, host differences cause variation in the immune response (Riddell et al., 2009). Host variation occurs at both the colony (Mallon et al., 2003; Schlüns et al., 2010) and individual level (Allander & Schmid-Hempel, 2000; Riddell et al., 2009; Schlüns et al., 2010; Riddell et al., 2011). For example, colonies vary in their encapsulation response (Mallon et al., 2003) and individuals vary in their melanisation ability (Allander & Schmid-Hempel, 2000) and AMP expression (Riddell et al., 2011).

Variation in the immune response reflects individual and colony condition. The immune response is energetically costly and therefore, there are trade-offs associated with it that impact the host-parasite interaction. Trade-offs occur within the immune system, between the specific response to *C. bombi* and the non-specific response (Mallon et al., 2003). Trade-offs also occur between the immune response to *C. bombi* and other tasks, such as foraging (Doums & Schmid-Hempel, 2000). These are more evident in colonies infected with *C. bombi* in the field compared to the laboratory due to the

presence of additional stressors, such as limited food availability, that affects immune gene expression (Brunner et al., 2014). This is contradicted by Brown, Moret & Schmid-Hempel (2003) who found that phenoloxidase activity, anti-bacterial activity and haemocytes were not affected by pollen deprivation. Three colonies were used in the experiment, and one colony exhibited reduced phenoloxidase activity compared to controls when starved of pollen. Thus, the effects of stress, such as food deprivation, on the immune response may vary between colonies and depend upon the colony genotype and condition (Brown, Moret, & Schmid-Hempel, 2003).

1.25 Within-host dynamics of *C. bombi* in *Bombus* spp.

Hosts become infectious from two days post-infection, when *C. bombi* cells are shed in the faeces (Schmid-Hempel & Schmid-Hempel, 1993; Imhoof & Schmid-Hempel, 1998a). Over the following 10 days, the number of cells shed increases and then plateaus (Imhoof & Schmid-Hempel, 1998a; Logan et al., 2005; Otterstatter & Thomson, 2006). This initial increase is positively and negatively correlated with dose and host body size respectively (Otterstatter & Thomson, 2006). However, bees given the same dose demonstrate a range of faecal cell counts (Logan et al., 2005), suggesting that within-host dynamics rather than inoculation dose impact infectivity and transmission. Indeed, these can be influenced by the infecting *C. bombi* strains, as some strains replicate faster than others (Schmid-Hempel et al., 1999) or are more infective (Barribeau et al., 2014). On the other hand, colony genotype may play a larger role in determining infection success and transmission than strain characteristics (Imhoof & Schmid-Hempel, 1998a).

Furthermore, within-host dynamics depend on whether the infection consists of a single strain or multiple strains. Mixed genotype infections are common in the field (Schmid-Hempel & Reber-Funk, 2004; Salathé & Schmid-Hempel, 2011; Tognazzo et al., 2012) with estimates of 44% of bees harbouring mixed infections (Salathé & Schmid-Hempel, 2011). For multiple strains to successfully infect a host, infection needs to occur simultaneously or within a short time period (Ulrich & Schmid-Hempel, 2015). When infected with multiple strains, it is unlikely for all strains to persist, usually some are eliminated (Ulrich et al., 2011; Ulrich & Schmid-Hempel, 2015). The strain diversity in queens and workers varies, with evidence of higher strain diversity in workers compared to queens (Ulrich et al., 2011). However, in Ulrich et al. (2011), all queens were naturally infected, whereas workers were infected experimentally. In contrast, Tognazzo et al. (2012) found higher strain variation in queens compared to workers, with queens harbouring up to 29 genotypes. Moreover, the probability of transmission to queens is higher in mixed infections (Ulrich et al., 2011; Ulrich & Schmid-Hempel, 2015). Ulrich & Schmid-Hempel (2015) found a correlation between strain growth and success in

mixed but not single infection treatments. Hence, within-host competition between strains may select for faster growth and perhaps higher virulence (Ulrich & Schmid-Hempel, 2015). Mixed infections typically consist of several common genotypes and many rare genotypes (Tognazzo et al., 2012). Recombination can occur between coinfecting strains (Schmid-Hempel et al., 2011; Tognazzo et al., 2012) and the genome of rarer genotypes often consists of elements from the genome of common strains. Recombinant genotypes are more common in infected queens compared to workers, due to their longer lifespan (Tognazzo et al., 2012).

1.26 Resistance and susceptibility of *Bombus* spp. to *C. bombi*

Differences in susceptibility to *C. bombi* occur at the individual and colony scales. At the individual level, larger bees consistently exhibit lower susceptibility (Otterstatter & Thomson, 2006; Adler et al., 2018; Palmer-Young, Raffel, et al., 2018; Van Wyk et al., 2021) perhaps due to their increased phenoloxidase activity (Whitehorn et al., 2011). Despite their lower susceptibility, larger bees are more likely to transmit *C. bombi* due to larger defecation volumes (Van Wyk et al., 2021). Furthermore, there is a genotypic basis to susceptibility with some colonies exhibiting susceptibility to a higher diversity of strains compared to others (Baer & Schmid-Hempel, 2003; Cisarovsky et al., 2012). There is some evidence that variation in infection intensity could be explained by genetic variation in the surface mucoglycoprotein, *mucin,* which could be involved in the adhesion of *C. bombi* to the gut wall (Barribeau et al., 2022). Colonies may also vary in their immune response, as colonies exhibit differences in phenoloxidase activity (Ruiz-González et al., 2022). Furthermore, susceptibility to a high diversity of *C. bombi* strains is associated with increased infection intensity (Schmid-Hempel et al., 1999). The infectivity of *C. bombi* differs between strains (Mallon et al., 2003) and different strains induce contrasting gene expression patterns in the host (Barribeau et al., 2014). Thus, this system demonstrates host-parasite genotype-genotype interactions, which influence infection success (Mallon et al., 2003). Colony heterozygosity is negatively associated with prevalence and infection intensity (Shykoff & Schmid-Hempel, 1991c; Baer & Schmid-Hempel, 2001, 2003; Whitehorn et al., 2011). Several quantitative trait loci (QTL) have been linked to infection intensity (Wilfert et al., 2007). Furthermore, hosts display differential expression of immune-related genes (Schlüns et al., 2010; Brunner et al., 2013). These differences may cause variation in host susceptibility, as gene expression patterns vary according to host and parasite genotype, in addition to predicting the likelihood of infection and intensity (Barribeau et al., 2014).

There is some evidence that hosts exhibit behavioural avoidance of contaminated flowers, which would decrease susceptibility. Higher visitation frequency of non-contaminated compared to contaminated flowers suggests hosts can recognise contaminated flowers. However, the same proportion of time was spent foraging on each, weakening this claim (Fouks & Lattorff, 2011). Visual cues and social learning do not play a role in behavioural avoidance (Fouks & Lattorff, 2013; Fouks et al., 2019). Rather, odour cues are the most probable mechanism for behavioural avoidance of *C. bombi.* All these studies have been conducted using artificial flowers consisting of Eppendorf tubes containing sugar water and *C. bombi* (Fouks & Lattorff, 2011, 2013; Fouks et al., 2019) and therefore, the field relevance of these results is debatable.

The host gut microbiome also plays a large role in susceptibility to *C. bombi* infection. The gut microbiome is acquired in the nest through contact with faeces (Koch & Schmid-Hempel, 2011). Faecal transplant experiments have highlighted that faeces source and hence, gut microbiota, predict infection outcome (Koch & Schmid-Hempel, 2012). There is evidence that the gut microbiome explains a higher proportion of variation in infection intensity than host genotype (Koch & Schmid-Hempel, 2012; Mockler et al., 2018), however, only one *C. bombi* strain was used by Mockler et al. (2018). Furthermore, host genotype may play a role in determining which microbiota become established (Koch & Schmid-Hempel, 2012). Microbiome diversity has been positively associated with *C. bombi* resistance (Koch & Schmid-Hempel, 2012; Mockler et al., 2018), however, high microbiome diversity has also been linked to increased susceptibility to *C. bombi* infection (Näpflin & Schmid-Hempel, 2018; Felden et al., 2022). In addition, specific species are associated with increased resistance, notably *Lactobacillus* spp. and *Gilliamella* spp. (Koch & Schmid-Hempel, 2012; Näpflin & Schmid-Hempel, 2016; Mockler et al., 2018). *Lactobacillius bombicola* isolated from wild *B. lapidarius* inhibits *C. bombi* growth completely, due to high acidity (Palmer-Young, Raffel, et al., 2019). Additionally, there is conflicting evidence on whether landscape differences impact the composition of hosts' gut microbiome. Alpha diversity estimates for *Bombus* spp. gut microbiota do not differ between semi-natural and agricultural habitats (Cariveau et al., 2014). However, Bosmans et al. (2018) found a higher abundance of core gut bacteria, such as *Gilliamella* spp., in bees inhabiting urban compared to forest habitats. This was observed in queens and therefore, it is uncertain whether workers show a similar pattern.

Secondary metabolites, which are unessential compounds produced by the plant and are found in nectar, can substantially increase resistance to *C. bombi.* For example, anabasine can reduce *C. bombi* infection intensity (Anthony et al., 2015; Palmer-Young et al., 2017) by up to 65% (Richardson et al.,

2015). Nicotine (Richardson et al., 2015), thymol (Palmer-Young et al., 2016) and callunene (Koch et al., 2019) also reduce *C. bombi* cell counts. The effectiveness of secondary metabolites vary with concentration (Biller et al., 2015; Richardson et al., 2015; Palmer-Young et al., 2016; Koch et al., 2019). For instance, jessamine reduces infection intensity at the highest naturally occurring concentration (Manson et al., 2010). However, in the field it is unlikely bees will be exposed to the maximum concentration, as their nectar source varies. Moreover, *C. bombi* strains differ in their susceptibility (Palmer-Young et al., 2016) and *C. bombi* can develop resistance to secondary metabolites under repeated exposure (Palmer-Young et al., 2017; Palmer-Young, Calhoun et al., 2018). There is a range of mechanisms through which secondary metabolites reduce *C. bombi* infection. Callunene lowers *C. bombi* infectivity through flagellum removal (Koch et al., 2019), whereas, eugenol affects cell size (Palmer-Young, Calhoun et al., 2018). The timing of host exposure to infection relative to secondary metabolite ingestion also impacts their effectiveness. For example, if ingested following infection, some compounds are ineffective at reducing infection (Koch et al., 2019). Furthermore, chemical changes following ingestion can increase the effectiveness of secondary metabolites ingested in nectar. Chemical changes may be induced by the host, as is the case for tiliaside from linden trees, or the gut microbiome, which occurs for unedone; a compound from strawberry tree nectar. This evidence emphasises that the interaction between secondary metabolites and *C. bombi* depend on the host genotype and gene expression, in addition to their gut microbiome (Koch et al., 2022). In spite of their benefits, secondary metabolites can have negative side effects, such as reduced colony productivity (Palmer-Young et al., 2017) and increased worker mortality (Giacomini et al., 2018). These costs could be reflected by host preference for alkaloid-free nectar (Palmer-Young et al., 2017). *In vitro* experiments do not detect the costs of these compounds or their interactions with other gut compounds (Palmer-Young et al., 2016, 2018). Therefore, further investigation into the costs of secondary metabolites is required *in vivo* to sufficiently evaluate their costs and benefits.

In contrast to some secondary metabolites, infection is supressed by sunflower pollen when ingested after infection (LoCascio, Pasquale, et al., 2019). Sunflower pollen reduces *C. bombi* infection prevalence and intensity (Giacomini et al., 2018; LoCascio, Aguirre, et al., 2019; LoCascio, Pasquale, et al., 2019; Giacamini et al., 2021; Fowler, Giacomini, et al., 2022; Fowler, Sadd, et al., 2022). For example, colonies fed a diet of 50% sunflower pollen exhibited prevalence which was fourfold lower than wildflower diets (Giacomini et al., 2021). The effect of sunflower pollen on *C. bombi* infection is also highlighted by the negative correlation between the area of sunflower planted and *C. bombi* intensity (Giacomini et al., 2018; Malfi et al., 2023). There is no evidence for self-medication, as infected bees show no preference for sunflower pollen (Vanderplanck et al., 2023). Furthermore, it

appears that *C. bombi* does not develop resistance to sunflower pollen (Giacomini et al., 2021), however, the effectiveness of sunflower pollen on reducing infection is weaker in wild compared to commercial *B. impatiens* colonies and varies between species. The majority of studies have tested the effects of sunflower pollen on *C. bombi* in *B. impatiens,* whereas studies on other host species, such as *B. bimaculatus, B. vagans* (Fowler, Giacomini, et al., 2022) and *B. terrestris* (Gekière et al., 2022), have found variable effects of sunflower pollen on *C. bombi* infection. The mechanism behind this reduction in infection appears not to be mediated by the immune response (Fowler, Sadd, et al., 2022) or phytochemical compounds (Adler et al., 2020; Figueroa et al., 2023). Resistance may be because sunflower pollen induces faster excretion, since bees fed sunflower pollen excrete larger volumes and excrete more frequently (Giacomini et al., 2022). Sunflower pollen has large spines on its surfaces called exines and when bees are fed only sunflower exines *C. bombi* infection intensity is reduced, indicating that exines are responsible for the effect of sunflower pollen on *C. bombi* infection (Figueroa et al., 2023). Exines may prevent *C. bombi* adhesion to the gut wall, but this has not been confirmed. Pollen from other plants in the Asteraceae family, with larger exines, also lower *C. bombi* infection (Figueroa et al., 2023). Despite the benefits of sunflower pollen it can have negative impacts on colony health, for example, lower brood mass (Gekière et al., 2022; Vanderplanck et al., 2023). This is likely because it is relatively low in protein and is deficient in several amino acids that are required for bee development (Nicolson & Human, 2013; Yang et al., 2013). However, sunflower abundance is positively associated with queen abundance (Malfi et al., 2023). This positive effect on colony health could be explained by pollen diets consisting of pollen from a range of species. Indeed, a pollen diet of 50% sunflower pollen yields the same resistance to *C. bombi* as a diet of 100% sunflower pollen (Giacomini et al., 2021) and therefore, combining sunflower pollen with other wildflower could alleviate the costs of sunflower pollen. For example, colonies fed a diet of 50% sunflower pollen and 50% sunflower pollen exhibit slightly lower colony weights compared to colonies fed 100% wildflower pollen, but pollen diet does not affect the number and size of workers produced (Giacomini et al., 2021).

1.27 Host specificity and population structure of *C. bombi*

The degree of *C. bombi* host specificity affects the spatial and temporal genetic structure of *C. bombi. Crithidia bombi* populations are separated by colony (Schmid-Hempel & Reber-Funk, 2004; Salathé & Schmid-Hempel, 2011), indicating that *C. bombi* specialises on single colonies (Ruiz-González et al., 2012). This is supported by reduced infection success in unrelated colonies following serial-passage through a single colony (Yourth & Schmid-Hempel, 2006). However, Yourth et al. (2008) found that one occurrence of within-colony transmission does not increase *C. bombi* host specialisation. Perhaps

more transmission events are required for specialisation or these results were a product of the strains used in each treatment (Yourth et al., 2008). The driving factor of *C. bombi* population structuring may vary according to the prevalence of *C. bombi* in the population. Salathé & Schmid-Hempel (2011) suggested that when prevalence is high, host species and niche overlap influence population structure, whereas, when prevalence is low, niche overlap plays a dominant role. This relationship may explain the contradicting results of Erler et al. (2012) and Bartolomé et al. (2022), and Ruiz-González et al. (2012) regarding the structuring of *C. bombi* populations according to host species. Ruiz-González et al. (2012) found *C. bombi* populations were structured according to host species. This is supported by laboratory serial-passage experiments through *M. rotunda* and *B. impatiens* (Pinilla-Gallego & Irwin, 2022). Alternating hosts decreased the probability of infection in the next host, however, serial passage through *M. rotunda* increased the probability of infection in the next host suggesting that *C. bombi* adapts to its host species. However, repeated transmission between *B. impatiens* decreased the probability of infection in the next *B. impatiens* host, indicating that this may not always occur. Furthermore, both Erler et al. (2012) and Bartolomé et al. (2022), found no evidence of local adaptation of *C. bombi* to host species, suggesting that frequent pollinator interactions aids mixing.

Crithidia bombi populations are also structured according to geographical region (Salathé & Schmid-Hempel, 2011; Gerasimov et al., 2019). For example, Alaskan populations are distinctly separate and more clonal compared to European populations (Gerasimov et al., 2019). This may reflect the lower density and thus, niche overlap of hosts. Different habitat structures, such as geographical barriers, impact the population structure of *C. bombi* (Imhoof & Schmid-Hempel, 1998b; Salathé & Schmid-Hempel, 2011). In addition, host specificity varies with geographical scale. Imhoof and Schmid-Hempel (1998b) observed that allopatric compared to sympatric *C. bombi* interactions resulted in more virulent infections. This pattern was observed between populations in three regions of Switzerland that were at least 20km apart. However, this pattern was not observed within these regions, on a smaller geographical scale.

The temporal genetic structure of *C. bombi* varies within and between years. The summer can be divided into two periods. The first is characterised by increasing prevalence and thus, interspecific and intraspecific transmission leading to high competition and selection between *C. bombi* genotypes (Popp et al., 2012). The second is characterised by lower prevalence but high genetic recombination between genotypes (Popp et al., 2012). Population dynamics also vary between years, with population

structures differing within one geographical region across years (Schmid-Hempel & Reber-Funk, 2004; Salathé & Schmid-Hempel, 2011; Erler et al., 2012; Ruiz-González et al., 2012).

1.28 Effects of *C. bombi* on *Bombus* spp. fitness

Crithidia bombi affects the fitness and survival of its hosts and these effects interact with the environment and host genotype. When queens are infected they exhibit reduced colony founding and increased weight loss during hibernation (Brown, Schmid-Hempel & Schmid-Hempel, 2003; Baron et al., 2017). In addition, infection reduces oocyte size (Shykoff & Schmid-Hempel, 1991b), with fitness of infected queens reduced by 40% (Brown, Schmid-Hempel & Schmid-Hempel, 2003). There is correlational evidence that *C. bombi* alters dynamics within the nest, by suppressing ovary production in workers. This may increase gyne production due to increased worker co-operation (Shykoff & Schmid-Hempel, 1991b). However, Brown, Schmid-Hempel & Schmid-Hempel (2003) concluded that *C. bombi* infection had no effect on the timing of worker reproduction, suggesting that this correlational result was due to alternative factors. This is also supported by Straw & Brown (2021) who found no effects of *C. bombi* on worker reproduction. In addition, *C. bombi* impacts the foraging efficiency of workers. Infected workers have reduced flower handling ability (Gegear et al., 2005; Otterstatter et al., 2005; Otterstatter & Thomson, 2006) and thus, carry 20% less pollen than uninfected workers (Shykoff & Schmid-Hempel, 1991a). There is a negative correlation between infection intensity and flower handling ability (Gegear et al., 2005; Otterstatter & Thomson, 2006). However, both studies (Gegear et al., 2005; Otterstatter et al., 2005) used natural infections, rather than artificial infections using a standardised inoculum. Therefore, the results are correlative. It is possible that hosts who are more likely to become infected also exhibit reduced flower handling ability. Reduced flower handling could be explained by slower learning (Gegear et al., 2006). Martin et al. (2018) found that infection did not predict learning ability, rather task allocation (nurse vs. forager) predicted infection. This result may be because bees were not experiencing additional stress. Alternatively, the result may be due to the nature of the experiment, which was a Proboscis Extension Reflex (PER) experiment, compared to foraging experiments. In addition, there is evidence that infected bees spend less time foraging on plants that have been damaged by herbivores, indicating that *C. bombi* infection could exacerbate the effects of herbivory on crops (Aguirre & Adler, 2022). The reason for this is unknown, it could be because infected bees have a lower tolerance to secondary compounds produced following herbivory, or infected bees have a reduced ability to collect pollen from damaged flowers.

The effects of *C. bombi* on host fitness interact with the environment. When food stressed, *C. bombi* can lower survival by 1.5 times compared to uninfected individuals (Brown et al., 2000). In addition, pollen affects the development of *C. bombi* infection, with bees fed more pollen demonstrating higher infection intensity and altered order of appearance of transmission stages (Logan et al., 2005). In the field, bees are also exposed to pesticides. Both *C. bombi* and neonicotinoids reduce worker survival separately, yet in combination no further impact on survival has been observed (Fauser et al., 2017). In the field, queens may hibernate for six to nine months, whereas Fauser et al. (2007) used a three month hibernation period, which may have alleviated harmful effects. However, others have also concluded that pesticides do not interact with the effects of *C. bombi* to reduce learning (Vaughan et al., 2022), survival and changes to infection (Baron et al., 2014, 2017; Straw & Brown, 2021; Siviter et al., 2022). Furthermore, when *B. terrestris* are exposed to the pesticide glyphosate and *C. bombi* there is no effect on the gut microbiome composition, although it is unknown whether bacteria functioning, for example, metabolism remains unchanged (Straw et al., 2023). The neonicotinoid, thiamethoxam, can increase infection intensities, however, only at lethal doses (Siviter et al., 2022). In addition to pesticides, farmers may apply antibiotics to their crops. When bees are given varying field-realistic doses of the antibiotic oxytetracycline, the probability of *C. bombi* infection increases following inoculation, potentially due to gut dysbiosis (Marceau et al., 2021). Finally, over the following decades, global warming is likely to incur additional stress on bumblebees and alter their interaction with *C. bombi*. The optimum growth conditions for *C. bombi in vitro* varies between strains, and peaks at 33.7-34.4°C and is inhibited above 37.9°C (Palmer-Young, Raffel, et al., 2018; Palmer-Young et al., 2021). The optimum conditions for *C. bombi* growth *in vitro* do not correlate with the optimum conditions for the parasite *in vivo*. For example, infection intensities in *B. impatiens* were 81% higher in hosts incubated at 21°C compared to 37°C. However, it is unclear whether host environmental temperature affects *C. bombi* as Tobin et al. (2019) found that incubating hosts at 21 and 29°C had no effect on the prevalence or infection intensity of *C. bombi*.

1.3 Limitations using bumblebees and *Crithidia bombi* as an epidemiological model

There are some limitations of using the interaction between bumblebees and *C. bombi* as an epidemiological model and thus, applying findings within this system to other social species. Firstly,

the relatedness between workers in a colony is higher than in populations of other social species because bumblebees are haplodiploid (Hamilton, 1964, 1972). Higher relatedness between individuals can increase the likelihood of parasite transmission through a population (Sadd et al., 2005; Sadd & Schmid-Hempel, 2006). Therefore, parasite transmission within this system may be higher compared to in social species without haplodiploidy. Furthermore, in a bumblebee colony there is one reproducing queen and therefore, workers in the colony are not subject to trade-offs between reproduction and other factors, such as the immune system. Differences in these trade-offs likely have implications on the selection pressures on queens compared to workers, that would not occur in social populations in which all individuals reproduce.

This epidemiological model is well-suited to testing the effects of host population demography on parasite transmission because of demography changes throughout the colony lifecycle. At the start of their lifecycle, colonies consist of a small number of workers with a low average age and as the lifecycle progresses the colony increases in size to up to 150 workers with a higher average age. Being able to experimentally test the effects of host population demography on parasite transmission in the lab is a relatively unique advantage. However, it is not possible to separate the effects of the age of individuals and the population size on transmission, because throughout the colony lifecycle these are correlated.

1.4 Future Directions

In conclusion, the *C. bombi* and *Bombus* spp. model system can aid our understanding of key epidemiological questions, in addition to furthering our knowledge of the causes of bumblebee decline and aiding their conservation. There are many unanswered questions in this system, for example, how does the intracolony immune response vary throughout the colony lifecycle and when does intercolony transmission need to occur to maximise transmission to the following generation? The former, would shed light on the effects of colony demography on within colony transmission. The latter, on horizontal versus vertical transmission which will have impacts on parasite fitness and virulence. Furthermore, we know that certain floral traits and species affect transmission, however, it is unclear whether this can have a largescale effect on transmission and whether the plant species composition of a landscape can affect transmission. Anthropogenic changes, including global warming and urbanisation, will have a large impact on many host-parasite interactions, including human, plant and animal diseases. It will be important to understand these impacts as temperatures continues to

increase and urban areas expand. For example, increased temperatures may alter *C. bombi* survival on flowers, the speed of its lifecycle or host fitness. Host resistance to infection may also be altered by changes in gut microbiota at higher temperatures. Finally, it is important to consider the effects of global changes in combination. For example, what are the effects of urbanisation on *C. bombi* transmission and how does transmission across different landscapes interact with global changes in temperature. It is likely that these effects will vary spatially and temporally, for example, effects may vary between different climates. Understanding these effects will increase our knowledge of this system but also aid insight into the effects of global change on many other host-parasite interactions.

Chapter 2
General methods

2.1 *Crithidia bombi* purification

Two colonies (Agralan, UK) infected with *Crithidia bombi* were consistently maintained in the lab. *Crithidia bombi* originated from infected post-hibernation spring queens collected at Windsor Great Park (Surrey, UK) in March 2021 and March 2022. Colonies were kept under red light at approximately 25°C and ambient humidity. They were fed via a reservoir of sterile sugar solution (50% concentration) and honeybee collected pollen (Agralan, UK). Workers were removed from infected colonies and placed in specimen tubes. The number of workers removed depended on the number of *C. bombi* cells required for the inoculation that day. One 10µL microcapillary tube was put into each tube to remove the faeces. When bees did not defecate, the tube was gently shaken to agitate the bee and encourage defecation. All of the faeces were combined into one 1.5ml Eppendorf, labelled 'tube 1' for purification. Purification was necessary to remove contaminants, such as pollen and other excreted cells. The following purification method was modified from the Cole (1970) "triangulation" protocol (Baron et al., 2014).

1ml of 25% Ringer solution (Oxoid, Thermo Scientific, UK) was added to tube 1 and mixed (all solutions were mixed via pipetting). Tube 1 was centrifuged at 68g for two minutes at 21°C (spin 1, Fig. 1). The supernatant was removed and transferred to tube 2. 1ml of 25% Ringer was added to the pellet in tube 1 and mixed, and tubes 1 and 2 were centrifuged at 68g for two minutes (spin 2, Fig. 1). The supernatant from tube 2 was transferred to tube 3 and the supernatant from tube 1 into tube 2. 1ml of 25% Ringer was added to tube 1 and mixed (spin 3, Fig. 1). This process was repeated eight times until the supernatant from tube 7 had been transferred into tube 8.

The middle tubes (4, 5 and 6) were spun at 6,797g for one minute at 21°C. These tubes contained the highest concentration of *C. bombi* cells, since heavier contaminants were collected in tubes 1-3 and lighter contaminants in tubes 7 and 8. This yielded a pellet of *C. bombi* cells in each tube. 200µL of 25% Ringer solution was added to tube 4 and mixed to dissolve the pellet. The entire solution in tube 4 was added to 5 and mixed and repeated to tube 6. This resulted in a 200µL solution of purified and concentrated *C. bombi* cells in tube 6. To measure the concentration of *C. bombi* cells in tube 6 the solution was vortexed and the number of cells in 10µL was counted using an improved Neubauer chamber haemocytometer at X400 magnification under a phase contrast microscope (Nikon Eclipse 50i).

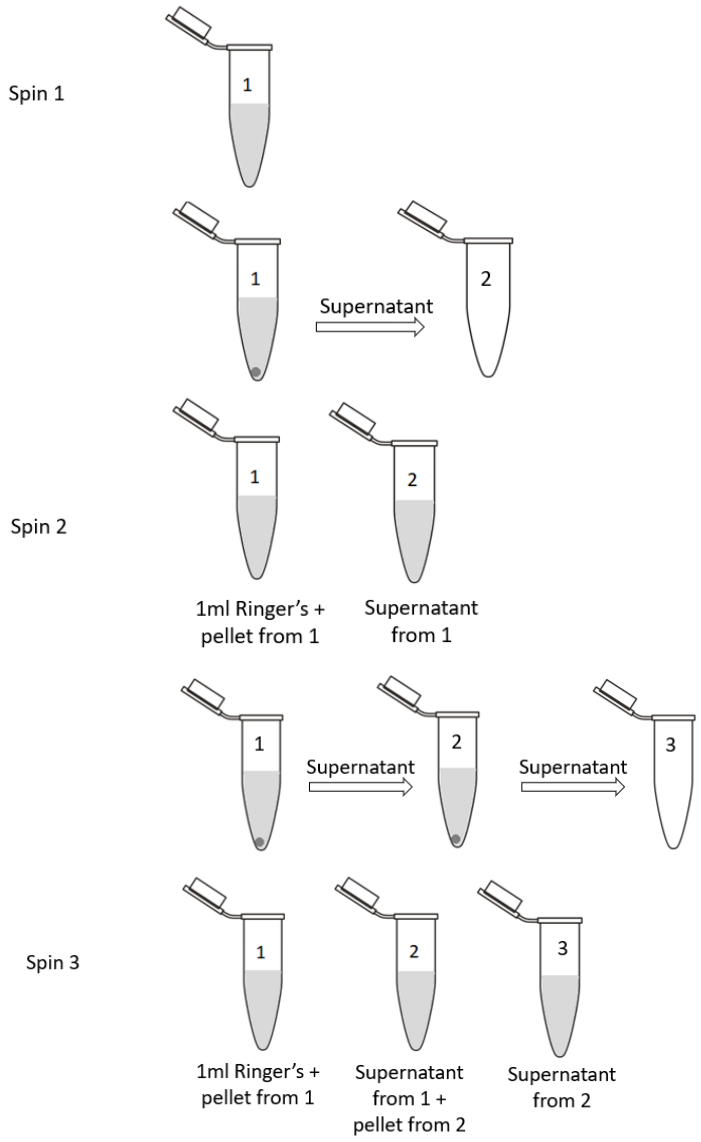


Fig. 1 Triangulation protocol for purifying *Crithidia bombi* faeces, modified from Cole (1970).

2.2 *Crithidia bombi* inoculation

Bees were removed from their colonies, placed individually in labelled nicot cages (Becky's Bees, UK), which are cylindrical containers adapted from hair rollers to house bees. To increase consumption of the inoculum, bees were starved for two hours prior to inoculation. *Crithidia bombi* was purified and mixed with sterile sugar solution (50% concentration) to yield a stock solution with a concentration that would yield the desired dose in 30µL. Each individual was given a 30µL droplet of inoculum in a

2ml syringe. 30µL was chosen as it is likely that an individual bee will drink this volume in four hours. The syringe had the end clipped off to enable the bees' proboscis to reach the inoculum. 30µL was pipetted into the end of the syringe and the syringe was attached to the base of the nicot cage with masking tape (Fig. 2, adapted from the ecotoxicology testing protocol OECD 247). Bees were left to drink the inoculum for four hours. After four hours the syringe was checked to ensure all of the inoculum had been drunk. If all or some of the inoculum remained the individual was discarded from the experiment as they had not consumed the desired *C. bombi* dose.

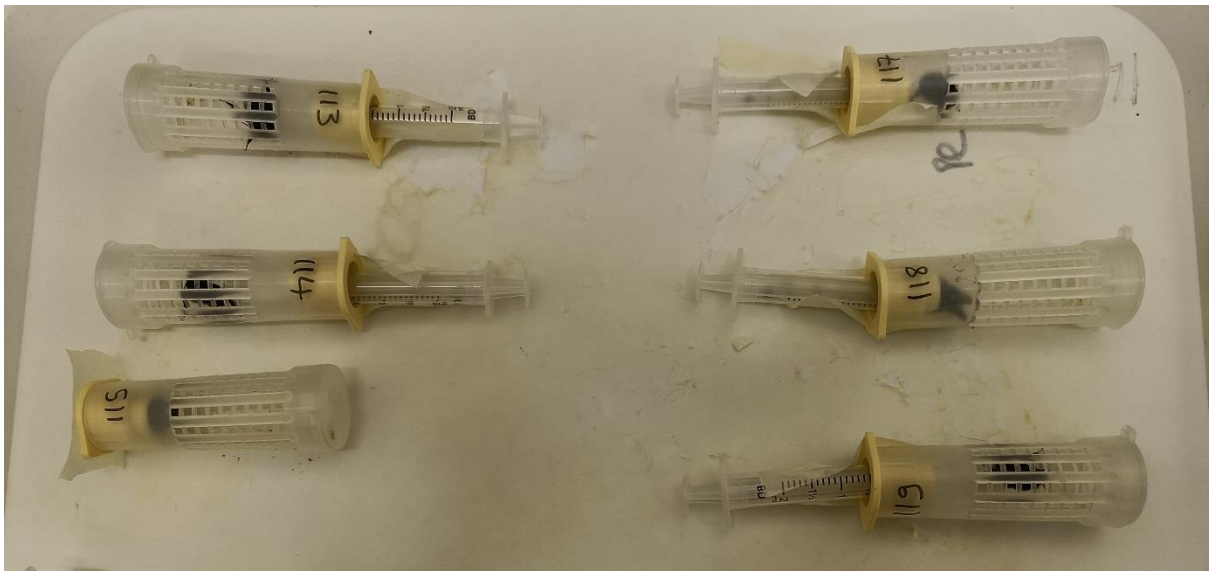


Fig. 2 Bees in nicot cages attached to 2ml syringes with masking tape. Syringes contain 30µL of *C. bombi* inoculum.

2.3 Measuring *C. bombi* infection via faecal sampling

Bees were put in individual, labelled specimen tubes with a 10µL microcapillary tube to collect faeces. When bees did not defecate the tube was shaken gently to encourage defecation. To measure prevalence of infection (defined as the number of infected hosts out of the total sampled) the faeces was viewed on a microscope slide under a phase contrast microscope at X400 magnification. If *C. bombi* cells were not identified immediately, the slide was viewed in a clockwise direction along three sides (Fig. 3). Following this, if no cells were identified the individual was recorded as 'uninfected'. It was not possible to view the entire slide due to time constraints and this method enabled sampling of a wide range of the slide.

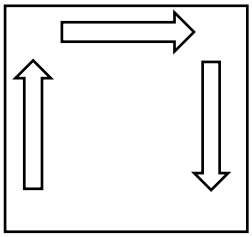


Fig. 3 Method of screening faecal samples for *C. bombi.* The square represents the slide and the arrows the direction of movement of the phase contrast microscope at X400 magnification.

To measure infection intensity (defined as the number of cells per µL of faeces), the faeces was viewed on an improved Neubauer chamber haemocytometer at X400 magnification under a phase contrast microscope. The number of cells in the first 4 x 4 square was counted and every 4 x 4 square along the diagonal to yield five counts of which the mean was taken (Fig. 4a, b). To calculate the number of cells per µL this mean value was multiplied by 250.

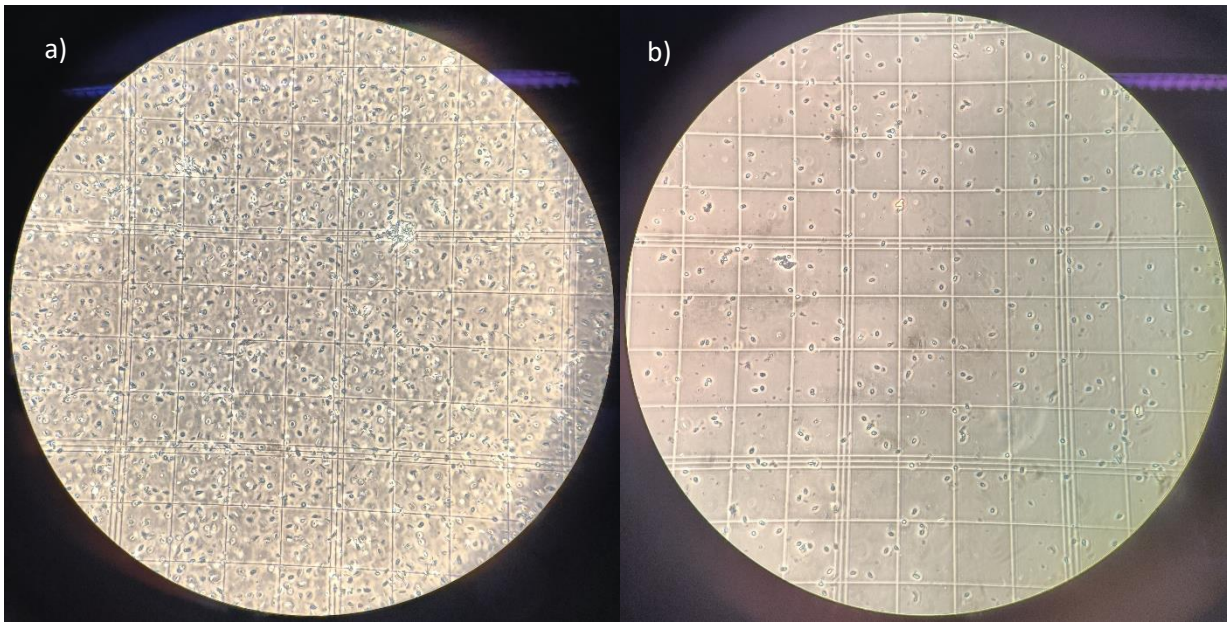


Fig. 4 Measuring infection intensity of *C. bombi* under a phase contrast microscope at X400 magnification using an improved Neubauer chamber haemocytometer. **a)** shows a higher infection intensity compared to **b).**

2.4 Measuring *C. bombi* infection via gut dissection

Crithidia bombi infection was measured in frozen bees via gut dissection. This method was used when it was not possible to measure infection on the day due to time constraints. Bees were frozen at -80°C in 1.5ml eppendorfs. To dissect, the heads and thorax were removed and the abdomen pinned onto a wax plate dorsal side down. The wax plate was put under a dissecting microscope at X10 magnification. One pin was placed at the centre top of the abdomen being careful not to pierce the gut. The abdomen was extended using forceps and pinned in the second to last abdomen segment. The abdomen cuticle was cut along each side of the abdomen, starting at the thorax intersection. The ventral side of the cuticle was peeled down and pinned (Fig. 5a). 1-2 small drops of 25% Ringer solution were pipetted onto the abdominal cavity to make the organs float and aid gut removal. The entire gut (crop, midgut and hindgut) was removed (Fig. 5b). The crop was removed as *C. bombi* in the crop would have recently been consumed and therefore, not yet infected and established within the individual. The mid- and hindguts were put in a 2ml Eppendorf with 300µL 25% Ringer solution and ground vigorously with a pestle for 30 seconds to release the *C. bombi* cells. The mixture was vortexed for five seconds and 10µL was viewed on a microscope slide or improved Neubauer chamber haemocytometer to measure infection prevalence or intensity respectively as described above.

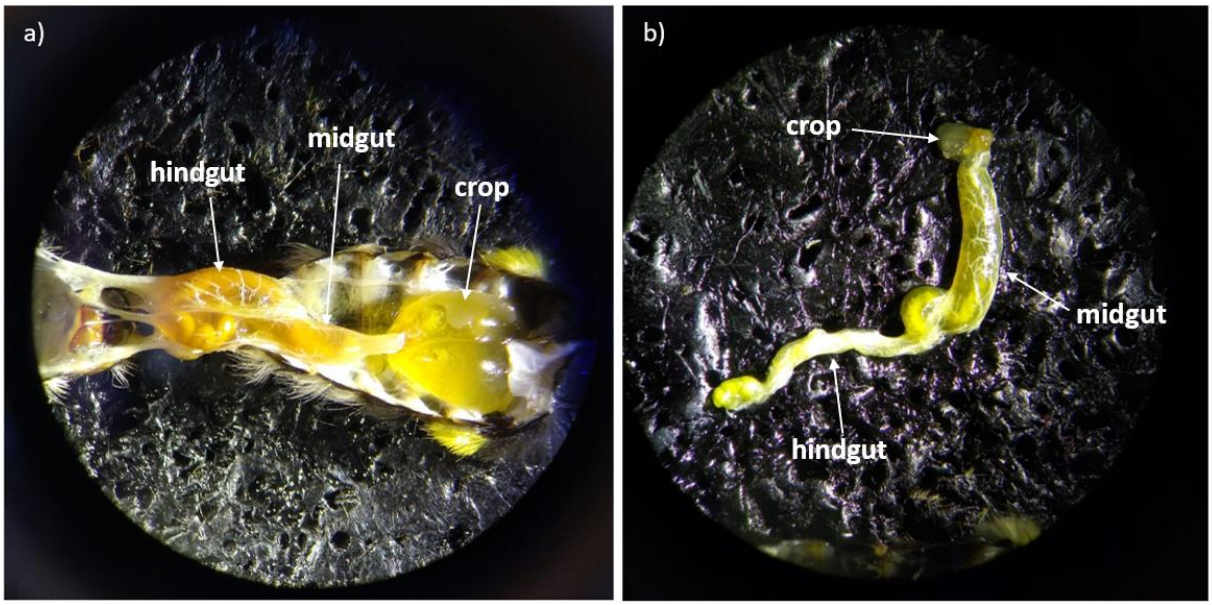


Fig. 5 a) Inside a *B. terrestris* abdomen under a dissecting microscope at X10 magnification. The ventral side of the abdomen has been peeled back and pinned exposing the crop and guts. **b)** The crop, mid- and hindguts of *B. terrestris.*

Note on measuring infection blind to treatment

I conducted experiments alone and therefore, it was not possible to be 100% blind to the treatment being counted. However, all bees were given individual ID numbers. Due to the large number of bees being screened each day, when I measured infection (usually one week later) it was essentially impossible to remember which ID number was from each treatment and therefore, in most cases measuring infection was effectively, but not formally blind.

Chapter 3
Newly emerged bumblebees are highly susceptible to gut parasite infection

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Author contributions:

HSWG: Conceptualisation, experimental design, data acquisition, data analysis, manuscript drafting, manuscript editing.
KN: qPCR experimental design, qPCR advice.
MFJB: Conceptualisation, manuscript editing.

Abstract

One factor that can affect infection susceptibility is host age. Susceptibility varies with age in many ways, for example, susceptibility may increase with age due to senescence, or decrease with age due to immune system maturation. If certain ages are more susceptible to infection, populations with contrasting demographics will exhibit differing disease prevalence. I use the bumblebee, *Bombus terrestris*, and its interaction with the gut trypanosome *Crithidia bombi* as a model system to investigate age-related susceptibility in a social insect. *Crithidia bombi* is a widespread and prevalent parasite of bumblebees that is spread between colonies via faeces on flowers when foraging. In the field, *Bombus* spp. live for approximately three weeks. Here, I inoculated bumblebees at 0, 7, 14 and 21 days of age and measured their infection after one week. I also measured the level of gene expression of two antimicrobial peptides important in the defence against *C. bombi* in bumblebees. I found that younger bumblebees are more susceptible to *C. bombi.* Specifically, individuals inoculated on their first day of emergence had significantly higher infection intensities seven days later compared to those inoculated at 7 and 21 days of age. In contrast, the gene expression of abaecin and defensin, did not significantly vary with age. These results suggest that age does affect susceptibility to *C. bombi* infection in *B. terrestris*. The higher susceptibility of callows may have implications for the susceptibility of colonies at different stages of their lifecycle, due to the contrasting age demography of workers.

3.1 Introduction

Susceptibility to parasite infection can vary with age, and has been observed across many animal taxa, including invertebrates (reviewed by Ben-Ami, 2019). Infection susceptibility can vary with age in a variety of ways, for example susceptibility may increase, decrease or follow cyclic patterns with age. One factor that can increase susceptibility to infection with age is senescence, which is defined as the increased rate of mortality with age due to a decline in an organism's functioning (Kirkwood & Holliday, 1979; Stanley, 2012). Senescence can impact infection susceptibility by lowering the efficacy of the immune system (immunosenescence). This occurs in many organisms (reviewed by Peters et al., 2019), including well-studied groups like humans (e.g. Beharka et al., 2001; Goldstein, 2012) and birds (Haussmann et al., 2005). Although less studied, there is also increasing evidence that immunosenescence occurs in invertebrates (reviewed by Stanley, 2012). For example, older crickets, *Gryllus assimilis,* have increased susceptibility to infection and lower haemocyte counts (Park et al., 2011). Furthermore, susceptibility to infection in red flour beetles (Khan et al., 2016b) and honeybees (Amdam et al., 2005; Roberts & Hughes, 2014) has been shown to rise with age.

Alternatively, young organisms may be more susceptible to parasite infection and susceptibility to infection may decrease with age. This is observed for some infections in humans, for example children are more likely to develop an infection when exposed to both malaria (Baird, 1998) and cholera (Deen et al., 2008). In some cases, higher susceptibility to infection in young organisms can be explained by the development of the immune system. For example, newborn babies are more susceptible to some infections because components of the immune system, such as the complement, have not yet fully established (McGreal et al., 2012; Kollmann et al., 2017). On the other hand, susceptibility may not follow a linear pattern with age, rather hosts may exhibit periodic increases in infection susceptibility. This can be seen in some invertebrates that are more susceptible to infection following moulting (eg. Corteel et al., 2009).

The effect of age on host susceptibility to infection may lead to population-level effects. Host populations often consist of a wide variety of different ages, the structure of which is spatially and temporally dynamic. If certain ages are more susceptible to infection, populations with contrasting demographics, such as same-age cohorts versus a mixture of ages, will exhibit differing disease prevalence (Ben-Ami, 2019). Consequently, the relationship between age and disease susceptibility is

critical for understanding the dynamics of parasite transmission in a host population and thus, can help to predict the trajectory and impact of a disease (eg. Woolhouse & Hargrove, 1998).

Here, I use the bumblebee, *Bombus terrestris*, and its interaction with the gut trypanosome *Crithidia bombi* as a model system to investigate age-related susceptibility in a social insect. *Bombus terrestris* are annual, eusocial insects and colonies consist of one singly-mated queen and her offspring (Schmid-Hempel & Schmid-Hempel, 2000). Workers help raise their sisters through brood care, foraging and guarding the nest and therefore, their fitness depends upon the success of the colony. In the field, *Bombus* spp. workers live for approximately three weeks (Brian, 1952; Rodd et al., 1980; Cartar, 1992). *Crithidia bombi,* a highly prevalent parasite of bumblebees (Shykoff & Schmid-Hempel, 1991a; Rutrecht & Brown, 2008a; Gillespie, 2010; Popp et al., 2012), is transmitted between colonies via faeces on flowers (Durrer & Schmid-Hempel, 1994; Graystock et al., 2015; Adler et al., 2018; Figueroa et al., 2019; Pinilla-Gallego et al., 2022) and within colonies through contact with infected individuals and contaminated nest material (Schmid-Hempel & Schmid-Hempel, 1993; Otterstatter & Thomson, 2007; Sah et al., 2021).

There is some evidence that populations with a higher mean age exhibit increased *C. bombi* prevalence (Whitehorn et al., 2011). However, age was not directly manipulated in this study, rather wing wear was used as a proxy for age. This is problematic as wing wear is confounded by activity levels, with individuals partaking in more foraging exhibiting increased wing wear (Foster & Cartar, 2011). However, if older individuals are more susceptible to *C. bombi,* this may be explained by immunosenescence. Doums et al. (2002) found a reduction in melanisation and encapsulation of a foreign object in older bumblebee workers and phenoloxidase activity has been shown to decline with age (Whitehorn et al., 2011). Whilst initial work suggested that *C. bombi* might elicit phenoloxidase activity (Brown, Moret et al., 2003), since then studies have repeatedly shown that *C. bombi* infection success is related to the expression of anti-microbial peptide (AMP) genes (Riddell et al., 2011; Brunner et al., 2013; Deshwal & Mallon, 2014). Whether the expression of these immune genes declines with age remains unknown.

However, susceptibility in this system is not solely determined by the host immune system. In fact, changes to the gut microbiota through an individual's life may also affect host susceptibility to *C. bombi* infection. A host's microbiome is established through contact with faeces in the colony (Koch & Schmid-Hempel, 2011) and its constitution is an important predictor of *C. bombi* infection intensity (Koch & Schmid-Hempel, 2012), with certain species, such as *Lactobacillus* spp. and *Gilliamella* spp.,

conferring reduced susceptibility. Callows emerge without a gut microbiome (Hakim et al., 2009; Kapheim et al., 2021; Hammer et al., 2023) and acquire the microbiome through contact with their colony (Koch & Schmid-Hempel, 2011). The gut microbiome takes time to establish and increase in diversity, for example, the relative abundance of *Gilliamella* spp. increases with age (Hammer et al., 2023). Consequently, younger bees could be more susceptible to *C. bombi* infection compared to older bees, reversing the expectation from immunosenescence alone.

As *B. terrestris* colonies grow, they are characterised by contrasting population demographics. At the beginning of the lifecycle, there is a higher number of younger workers compared to the end, when the colony consists of a larger population of older workers. Elucidating the relationship between age and *C. bombi* susceptibility may therefore shed light on the spread and impact of *C. bombi* infection at different points in the colony lifecycle. In addition, if certain ages are more susceptible to infection, this relationship could highlight disease reservoirs in a population. Here, I investigated the susceptibility of *B. terrestris* workers between 0-21 days old to *C. bombi* infection. I inoculated workers with a standardised inoculum, allowed the infection to develop for one week and measured the infection intensity through faecal sampling. I also used qPCR to measure the impact of infection on AMP expression, as these genes are involved in host defence against *C. bombi* (Deshwal & Mallon, 2014; Marxer et al., 2016). If age does affect infection susceptibility, I hypothesise that infection intensity will be highest in the youngest and oldest individuals. In addition, I predict that AMP expression will be lowest in the youngest and oldest individuals. This is because in the youngest individuals the immune system may not have fully established and in older individuals the immune system may be less effective due to immunosenescence.

3.2 Methods

3.21 Experimental organisms

Bumblebees

Five commercial colonies of *Bombus terrestris audax,* with approximately 50 workers, were purchased from Agralan (UK). All bees were housed under red light, 25°C and ambient humidity throughout the experiment. Faecal samples of 15 bees per colony were screened for *Crithidia bombi, Vairimorpha* (formerly *Nosema*) spp. and *Apicystis bombi* using a phase contrast microscope (Nikon Eclipse 50i) at

X400 magnification (Rutrecht & Brown, 2009). None of the samples contained these parasites. Colonies were provided with honeybee collected pollen (Agralan, UK) and sterile sugar solution (50% concentration) *ad-libitum.*

Crithidia bombi and inoculation protocol

One additional colony (Agralan, UK) was used as *C. bombi* stock for inoculations. This colony was infected with *C. bombi* originating from post-hibernation spring queens collected at Windsor Great Park (Surrey, UK) in March 2021. On the day of inoculation, faecal samples from 15-20 workers were purified using a modified triangulation protocol developed by Cole (1970) (see General Methods 2.1). Bees were starved for two hours and then fed a standardised inoculum of 12,000 cells mixed with sterile 50% sugar solution in a 30μl droplet. This dose is field-realistic (Schmid-Hempel & Schmid-Hempel, 1993) and has a high chance of leading to infection (Ruiz-González & Brown, 2006b). Bees from the experimental colonies were inoculated using the protocol in General methods 2.2. Following inoculation, bees were housed individually in transparent plastic cages (12 x 7 x 5.5cm) for one week. 50% sterile sugar solution and honeybee collected pollen (Agralan, UK) were supplied *ad-libitum,* since restricted access to pollen reduces longevity (Smeets & Duchateau, 2003).

3.22 Experimental design

Treatment groups

To test the impacts of worker age on susceptibility to infection, I inoculated workers at four different ages: 0 days, 7 days, 14 days and 21 days old. These ages were chosen to represent a range of ages over the average worker life in the field, which is approximately three weeks for *Bombus* spp. (Brian, 1952; Rodd et al., 1980) and to allow the inoculation to develop into a full infection, which takes 7-10 days (Schmid-Hempel & Schmid-Hempel, 1993; Imhoof & Schmid-Hempel, 1998a; Logan et al., 2005; Otterstatter & Thomson, 2006). Within each age group there were two treatment groups. To assess the impact of age on infection, one group was screened for infection through faecal sampling. In the second group, AMP expression was measured using qPCR to test the effect of age on the immune response, resulting in a 4 x 2 factorial design.

Obtaining bumblebee samples

Colonies were checked three times per day for callow workers. Callow workers were removed within 24 hours of emergence. They were identified by their greyish legs, white stripes, ruffled fur, sluggish

and clumsy behaviour, curved wings, little wing movement and low levels of aggression or resistance when handled (HWG pers. obs.; O'Donnell et al., 2000; see Fig. A1.1).

Callows were marked with a coloured spot that corresponded to their colony of origin and were randomly assigned to one of the eight treatment groups: 0 day faecal screening, 7 day faecal screening, 14 day faecal screening, 21 day faecal screening, 0 day qPCR, 7 day qPCR, 14 day qPCR and 21 day qPCR. These groups contained 2-10 individuals and were housed together until inoculation. Individuals were inoculated when they reached the age assigned to their group. Groups were housed in wooden boxes (21 x 12 x 10cm) containing cat litter to prevent faeces accumulating. They were provided with honeybee collected pollen (Agralan, UK) and sterile sugar solution (50% concentration) *ad-libitum.* Individuals assigned to the 0 day faecal screening and 0 day qPCR treatments were inoculated immediately and therefore, were not housed in groups. The date that callows were removed from their colony was recorded, since colony age may affect the immune response of workers (Moret & Schmid-Hempel, 2009).

Measuring the infection outcome

Measuring infection intensity

Seven days post-inoculation faecal samples were taken from those bees assigned to the faecal sampling treatment groups. Infection intensity was measured using the protocol from General methods 2.3. A digital calliper (Mitutoyo) was used to measure the thorax width at the wing intersection. Each individual was measured three times and the mean calculated. This measure was used as a proxy for bee size, since size affects infection intensity of *C. bombi* (Otterstatter & Thomson, 2006).

Measuring the immune response

Individuals assigned to qPCR groups were snap frozen 18 hours post-inoculation in liquid nitrogen and stored at -80°C. Eighteen hours was chosen due to practical constraints and previous work showing that AMP expression remains elevated 18 hours after inoculation (Riddell et al., 2011; Brunner et al., 2013). The gene expression of the AMPs abaecin and defensin were chosen because previous work has shown that they are upregulated following *C. bombi* inoculation (Barribeau & Schmid-Hempel, 2013; Brunner et al., 2013; Riddell et al., 2014) and are involved in the immune response against *C. bombi* (Deshwal & Mallon, 2014).

Total RNA extraction

Whole abdomens were ground in liquid nitrogen and approximately half was used to extract Total RNA using 0.5ml TRIzol reagent (Invitrogen) following the manufacturers protocol. Total RNA was further purified twice using 2M lithium chloride and finally dissolved in 50 uL of nuclease-free water. RNA quality was checked spectrophotometically using nanodrop (Thermofisher Scientific) and RNA gel electrophoresis.

Primer design

Primers for qPCR were designed using the PrimerQuest tool and checked using Geneious ver.8.1.9. based on the mRNA sequences of *B. terrestris* which were (RPL13: FN391387.1., Arginine kinase: AF492888, RPS18: XM_048411652.1, RPS6: XM_012314237.3, abaecin: XM_003394653.4, defensin: FJ161700.1). Primer sequences are given in Table 1.

Table 1 Primer sequences used in the qPCR. RPL13, Arginine kinase and RPS18 were used as reference genes. Abaecin and defensin AMPs were chosen because they are upregulated following *C. bombi* inoculation and are involved in the immune response against *C. bombi.*

Gene	Forward primer	Reverse primer	Annealing temperature (°C)
RPL13	GGTGATGCTACTGAAGAAGAAATG	AGAAATGACACGGGCCTTAG	60
Arginine kinase	TCTAGCACTTTGTCTGGCTTAG	AGTGGTCGTCGATCAGTTTC	60
RPS18	AAGGTGTTGGTCGTCGTTAC	CATTCTCCAGCACGCTTATCT	64
RPS6	ATGTCGTTCGTATCTCGGGC	CGCTCACCATCACGTCTAGG	66
Abaecin	GAAGGAACAAGTTGTGGAGAGA	GGTCGTGGCGGATTATATGG	64
Defensin	GCTCTTCTCTTTGTGGCTGTA	TCGCAGGTCACTCTTCTTTG	60

qPCR

RNA samples were treated with RQ1 RNase-Free DNase (Promega). First strand cDNA was synthesised from 500ng total RNA in a volume of 20µL using random pentadecamer primers and SuperScript III 3 Reverse Transcriptase (Invitrogen). No genomic DNA contamination in the prepared cDNA was confirmed by PCR using primers for RPS6 which span an intron. The qPCR reaction in 10µL contained cDNA prepared from 2.5ng total RNA, 400 nM of forward and reverse primers and 4µL iTaq™ Universal SYBR® Green Supermix (BioRad). qPCR was performed using a CFX 96 thermal cycler (Bio-Rad), using

the following programme: 95°C, 15min, then 49 cycles of 95°C, 15s, 60/64/66°C (Table 1), 30s and 72°C, 30s. Melt curve analysis was conducted to check for primer dimer amplification. The mean Cq value of technical replicates was calculated, and gene expression was normalised against the mean of the three reference genes, RPS18, AK and RPL13 by subtracting the mean Cq values of the reference genes from each target gene Cq value to produce ΔCq. Standardised gene expression was calculated using $2^{-\Delta CT}$. Two best stably expressed reference genes (AK and RPL13) were selected out of 4 tested reference genes (reference gene expression across treatments is shown in Fig. A1.2).

3.23 Data analysis

Statistical analyses were conducted in R version 4.1.0 (R Core Team, 2021). Figures were produced using the package 'ggplot2' (Wickham, 2016).

Throughout analyses age was analysed as a categorical variable and model assumptions were tested using the 'DHARMa' package (Hartig, 2022). The 'drop1()' function from the package lme4 (Bates *et al*., 2015) was used to assess the significance of model components using a likelihood ratio Chi-squared test. To test how age affected infection intensity (number of *C. bombi* cells per μl) the glmmTMB package (Brooks *et al.*, 2017) was used to conduct a mixed effects model with a negative binomial error distribution. The model included age as a fixed effect and colony as a random effect. Bee size, and the date the individual was removed from their colony of origin were covariates and included as fixed effects. Since this model did not account for multiple comparisons, a Tukey post-hoc test was used to test for significance between age levels using the package 'emmeans' (Lenth, 2022). When plotting the results, the variance within each age group appeared to change with age and therefore, we tested whether there were significant differences in the coefficient of variation of each level of age using the asymptotic test in the 'cvequality' package (Marwick, & Krishnamoorthy, 2019).

The sample sizes for each treatment group in the qPCR were low (Table A1.3). Therefore, a power analysis was conducted using the package 'pwr' (Champerly et al., 2017) and function 'pwr.f2.test' to find out the sample size required to detect a difference in infection intensity with age if the model explained 30% and 85% of the variation in infection intensity with 85% power. These values were chosen to obtain sample size estimates for a wide range of age effects on infection intensity.

To test how age affected abaecin and defensin gene expression, separate general linear models with a Gamma error distribution were used including standardised AMP expression as the response variable, and treatment and colony as fixed factors. Colony was not included as a random effect

because there were not a sufficient number of samples of each colony within each age treatment group (Gelman & Hill, 2006; Arnqvist, 2020). A Gamma error distribution was used due to a high level of overdispersion. Tukey post-hoc tests were conducted using the package 'emmeans' (Lenth, 2022) if fixed factors were significant.

3.3 Results

3.31 Effect of age on infection intensity

A total of 103 callows from five colonies were collected for use in the faecal screening experiment. They were collected on seven different dates from 29/04/2021 – 10/05/2021. The final sample sizes for the 0, 7, 14 and 21-day age treatment were 27, 30, 27 and 19 respectively (see Table A1.1). Sample sizes varied between treatment groups and colonies due to variation in size and production of callows across colonies, the random allocation of callows to age treatment groups and the loss of samples due to the reasons detailed in Table A1.2.

All individuals inoculated became infected. Age significantly affected infection intensity ($X^2_3 = 11.139$, $p = 0.011$). Tukey's HSD test for multiple comparisons found that the estimated mean infection intensity of 0 day old individuals (40,325 cells/µl, 95% C.I. = [22,104, 73,567]) was significantly higher than that of 7 day old individuals (13,910 cells/µl, 95% C.I. = [8,427, 22,961], $p = 0.0486$) and 21-day individuals (9,732 cells/µl, 95% C.I. = [5,130, 18,462], $p = 0.009$, Fig. 1). There was no significant difference between 0 and 14 days (14 days: 19,896 cells/µl, 95% C.I. = [11,725, 33,761]; $p = 0.227$), 7 and 14 days ($p = 0.751$), 7 and 21 days ($p = 0.789$) or 14 and 21 days ($p = 0.278$). Size was not a significant predictor of infection intensity ($X^2_1 = 1.067$, $p = 0.301$). There was no significant difference in infection intensity between bees collected on different dates ($X^2_6 = 5.633$, $p = 0.466$; Fig. 1).

Table 2 GLMM model results testing the effect of bumblebee age on *C. bombi* infection intensity. * denotes significant *p* values.

Factor	**Degrees of freedom**	**X^2**	***p* value**
Age	3	11.139	0.011*
Bee size	1	1.067	0.301
Collection date	6	5.633	0.466

Table 3 Tukey post hoc results from GLMM model testing the effects of bumblebee age on *C. bombi* infection intensity. ab shows significant differences after Tukey post hoc tests.

Age (days)	Degrees of freedom	Estimate	Standard error	95% confidence intervals
0	90	40,325[a]	12,203	22,104, 73,567
7	90	13,910[b]	3,509	8,427, 22,961
14	90	19,917[ab]	5,303	11,736, 33,802
21	90	9,732[b]	3,137	5,130, 18462

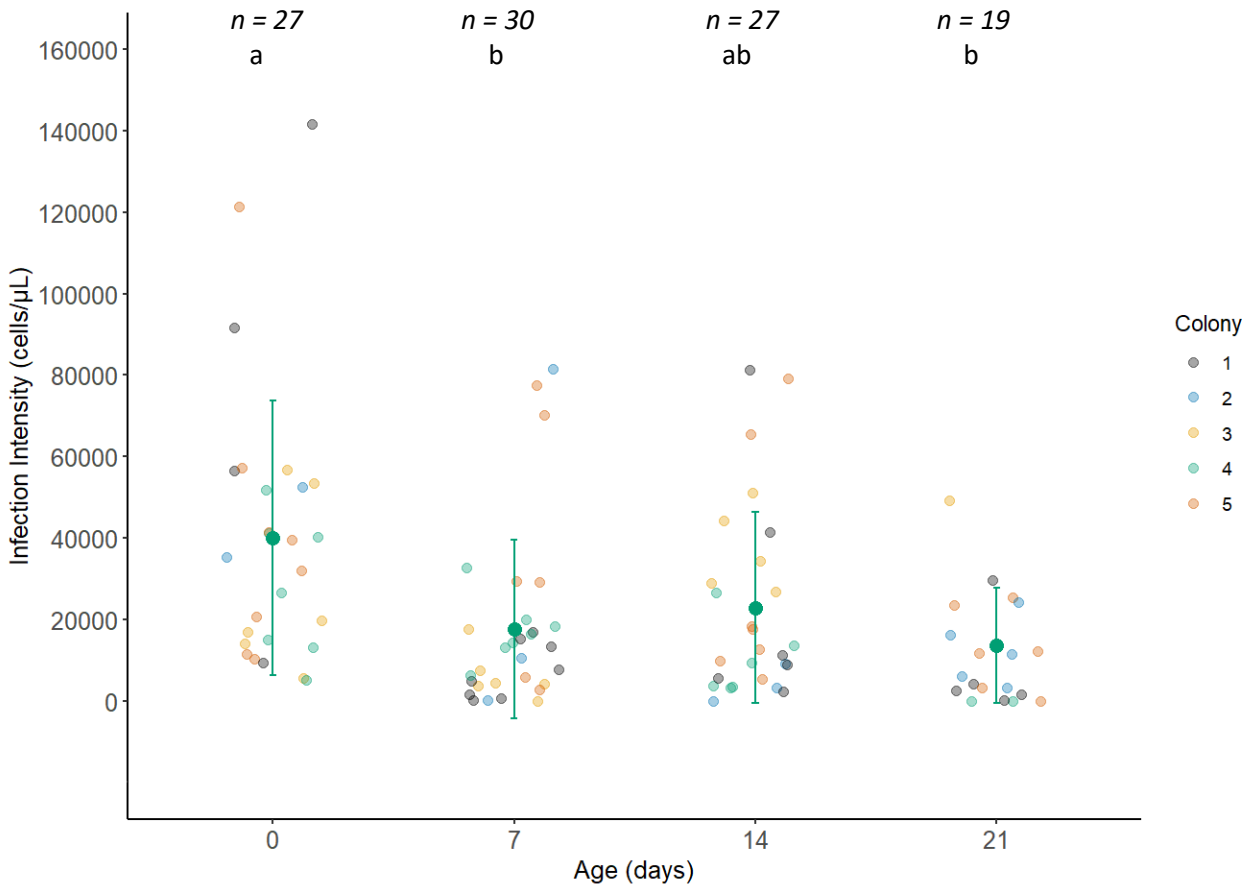
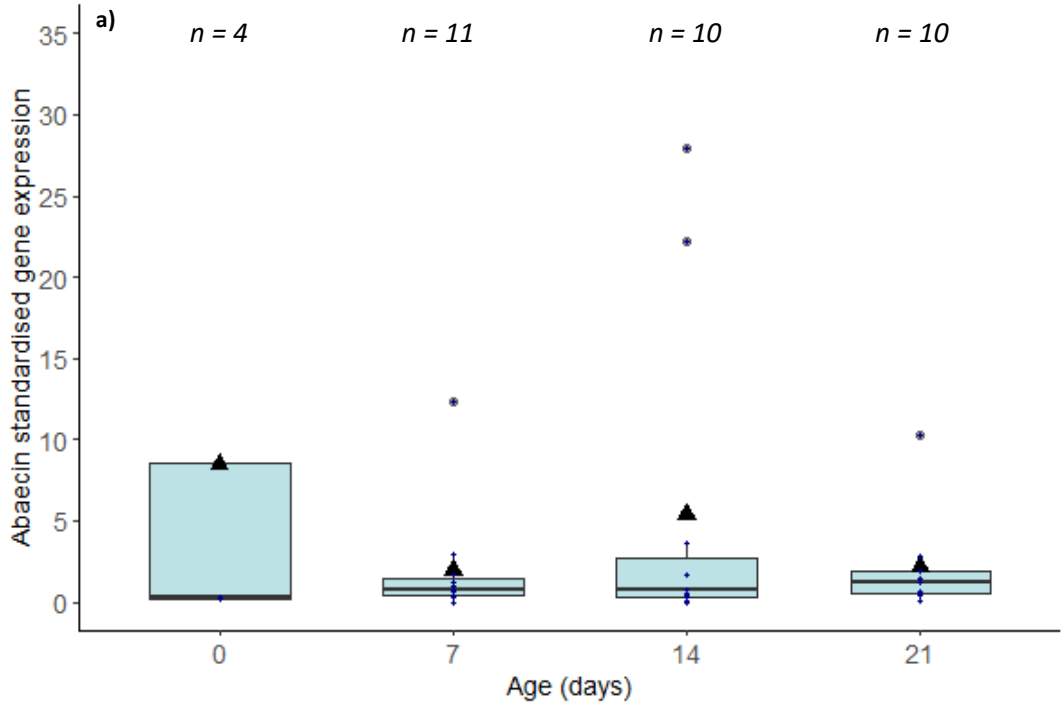


Fig. 1 The infection intensity of *C. bombi* in bees inoculated at four different ages. The mean of the raw data is shown by the large circular datapoint, the error bars show the standard deviations and the smaller circular datapoints show the raw data. Colour of datapoint denotes colony of origin. Sample sizes are shown above the datapoints. a and b show significant differences after Tukey post hoc tests.

3.32 Effect of age on AMP expression

The final sample sizes used in qPCR for the 0, 7, 14 and 21 day age treatment were 5, 11, 11 and 10 respectively (see Table A1.3). Sample sizes varied between groups due to variation in colony size, the production of callows across colonies and the random allocation of callows to age treatment groups. Sample sizes across treatments per colony are shown in Table S2. Power analysis showed if age predicted 30% of the variation in infection intensity, a sample size of 26 per group is needed and for 85% a sample size of 6. Consequently, the following analyses should be interpreted with caution, as our sample sizes are likely not large enough to detect anything but a very large difference in AMP expression.

In two samples (one from 0 days and one from 14 days) abaecin was not amplified and these were excluded from subsequent analysis. Age did not affect abaecin expression ($X^2_3 = 80.660$, $p = 0.264$; Fig. 2a). In contrast, colony significantly affected abaecin expression ($X^2_4 = 116$, $p < 0.001$; Fig. 3a). When adjusted for multiple comparisons, Tukey-post hoc tests found no pairwise significant differences in abaecin expression between colonies.



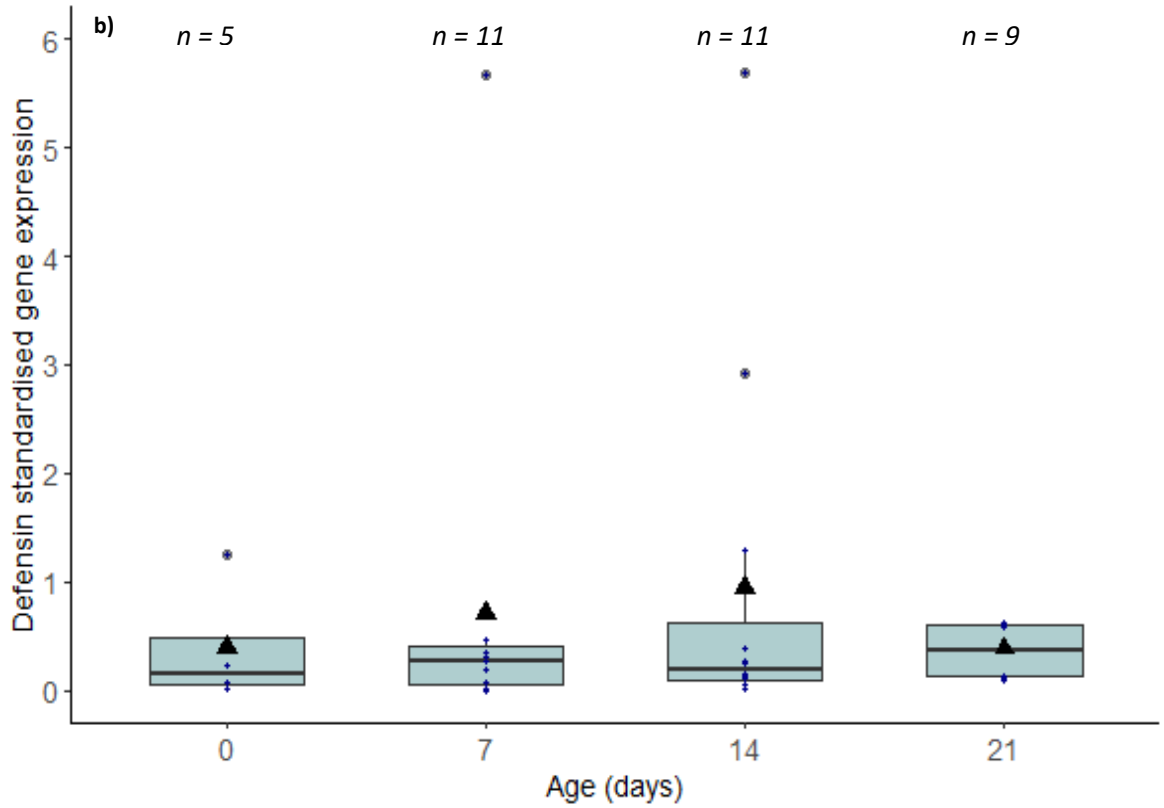


Fig. 2 a) Boxplot showing abaecin standardised gene expression in *B. terrestris* of four different ages 18 hours after inoculation with *C. bombi.* Abaecin gene expression is standardised against two reference genes, AK and RPL13. The triangle shows the mean, the large circular datapoints show the outliers and smaller blue circular datapoints show the raw datapoints. In two samples abaecin was not amplified and therefore, these were excluded (one from the 0 day and from 14 day treatment groups) **b)** same as a) for defensin gene expression. One extreme outlier has been excluded from the 0 day treatment group.

There was one large outlier in the defensin gene expression dataset (from the 0 day treatment group; Fig. A1.3). Analyses were conducted with and without this outlier resulting in the same qualitative outcome. Age did not significantly affect defensin gene expression (all data: $X^2_3 = 69.43$, $p = 0.0719$; without outlier: $X^2_3 = 59.0$, $p = 0.944$; Fig. 2b). However, like abaecin, defensin expression significantly varied with colony (all data: $X^2_3 = 109$, $p < 0.001$; without outlier: $X^2_3 = 91.5$, $p < 0.001$; Fig. 3b). When adjusted for multiple comparisons, Tukey-post hoc tests found no pairwise significant differences in defensin expression between colonies.

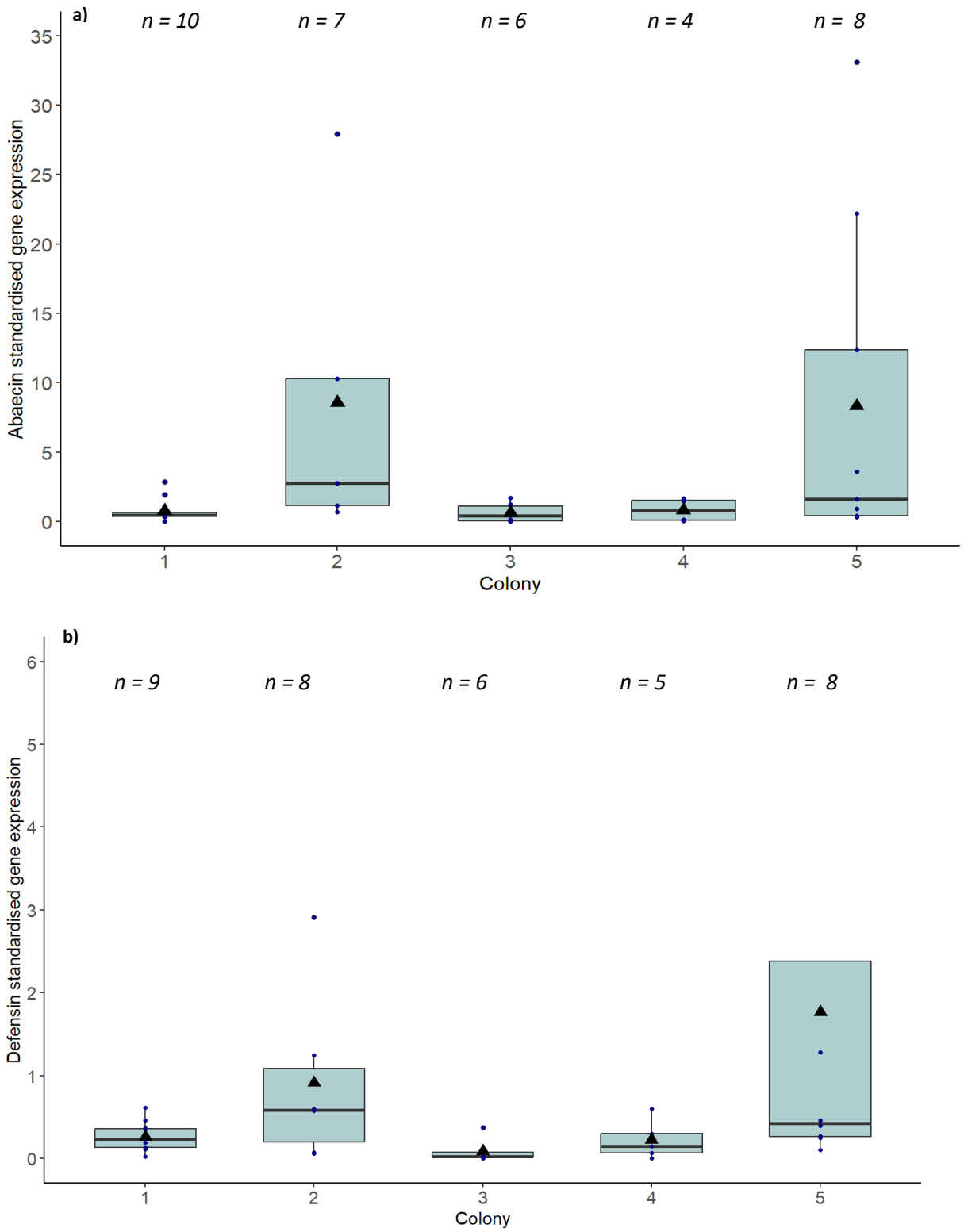


Fig. 3 a) Boxplot showing abaecin standardised gene expression in *B. terrestris* from five different colonies 18 hours after inoculation with *C. bombi.* Large circular datapoints show outliers and small circular datapoints show raw datapoints. Sample sizes are shown above the boxplots. Two samples (one from colony 2 and one from colony 4) were excluded as abaecin was not amplified. **b)** Same as a), for defensin gene expression. One extreme outlier from colony 1 was excluded.

3.4 Discussion

Here we show that younger bumblebees are more susceptible to infection by a trypanosome parasite than their older siblings. On average, individuals inoculated at 0 days of age had an infection intensity seven days later of 40,325 cells/µl, which was more than double the infection intensity of individuals inoculated when they were 7 days old, and more than quadruple the infection intensity of individuals inoculated at 21 days of age. In contrast, the gene expression of two AMPs known to protect against *Crithidia bombi,* abaecin and defensin, did not significantly vary with age, however these results are inconclusive since power analysis showed that our sample sizes were only able to detect an extremely large age effect.

We found that 0 day old individuals, or callows, were most susceptible to *C. bombi* infection, confirming our hypothesis that susceptibility would be highest in younger individuals. There was larger variation in infection intensity in younger age groups, however, testing for differences in the coefficient of variation showed this was not significant. Unlike our hypothesis, susceptibility was not higher in the oldest individuals. Despite infection intensity not being significantly different between those inoculated on day 0 and 14, the mean infection intensity of those inoculated at 7, 14 and 21 days old are very similar and this was likely due to high variation in day 14. The high susceptibility of callows to infection may reflect a broader increased susceptibility to gut parasites in young bees, as two day old *B. terrestris* are also more susceptible to another gut parasite, *Vairimorpha* (*Nosema) bombi* (Rutrecht et al., 2007) compared to 10 day old individuals. A reduced immune response might explain such higher susceptibility, however, we found no age-related difference in the expression of either abaecin or defensin after inoculation (which also argues against immunosenescence in the expression of these immune genes). It is unclear whether this finding is a result of an actual lack of difference in gene expression or experimental limitations. Our power analyses suggests that we only have a sufficient sample size to detect a very large difference in AMP expression, specifically our sample size could detect a significant difference if age explained over 85% of the variation in infection intensity. Furthermore, we measured AMP expression at a single time point post-infection and therefore, we could not detect whether temporal AMP expression varied with age. Furthermore, we cannot rule out the role of AMPs in the susceptibility of callows because lower AMP expression has previously been observed in younger honeybees and bumblebees (Hammer et al., 2023; Lourenço et al., 2019). Hammer et al. (2023) found abaecin and defensin gene expression was significantly lower in the hindgut of individuals aged 0-1 days compared to 3-19 days old. This study measured baseline

expression, whereas we measured expression after an immune-challenge (in this case, inoculation with *C. bombi*). Furthermore, gene expression was only measured in the hindgut. These differences in methodology may limit the comparability of our results, but this previous study provides some evidence that reduced abaecin and defensin expression could explain the increased susceptibility of callows to *C. bombi.* in our experiment.

It is important to acknowledge that we only measured the expression of two immune-related genes when, in reality, the immune system is very complex and many genes are likely upregulated following infection. It is possible that following *C. bombi* inoculation, the expression of abaecin and defensin do not vary with age but the expression of other immune-related genes, not measured here, do vary. For example, peroxidase, which produces reactive oxidative species (ROS), is upregulated early in *C. bombi* infection (Riddell et al., 2011). Indeed, Hammer et al. (2023) found that the expression of dual oxidase, which generates ROS, increases as bumblebees mature, suggesting that other aspects of the immune response, not measured here, may vary with age.

The gut microbiome provides another explanation for the increased susceptibility of callows to infection. Callows emerge without a gut microbiome (Hakim et al., 2009; Kapheim et al., 2021) and 24 hours post-emergence gut bacteria in the mid- and hindgut of bumblebees are scarce and exhibit reduced diversity (Hammer et al., 2023). Post-emergence, bumblebee gut bacteria exhibit logistic growth that stabilises after four days (Hammer et al., 2023). The gut microbiome is a vital predictor of *C. bombi* infection intensity (Koch & Schmid-Hempel, 2011, 2012) and individuals without a gut microbiome have exhibited increased susceptibility to *C. bombi* (Koch & Schmid-Hempel, 2011). Furthermore, the abundance of *Gilliamella,* which confers resistance to *C. bombi* (Cariveau et al., 2014; Mockler et al., 2018)*,* significantly increases with age (Hammer et al., 2023). Combined, this indicates that *a* reduced gut microbiome in callows could contribute to the higher susceptibility of callows to *C. bombi.*

It is possible that the housing conditions throughout the experiment could have affected our results. Following removal from colonies callows were housed in multi-colony groups consisting of only workers. Workers start developing ovaries when housed in worker-only groups (Bloch & Hefetz, 1999) which could divert resources away from the immune system. Indeed, a trade-off between the immune response and energy expended on foraging has been observed (Doums & Schmid-Hempel, 2000). Furthermore, workers were housed in mixed colony groups for varying lengths of time depending on

their age treatment. However, if there is an effect of housing conditions on the immune response, we would expect to see an increase in infection intensity with age, which was not observed.

The increased susceptibility of callows to *C. bombi* infection has implications for the susceptibility of the whole colony to infection. If callows develop higher infection intensities, *C. bombi* will be transmitted faster through the colony, because of an increased concentration of propagules in the faeces. Consequently, *C. bombi* might be more likely to be transmitted through the colony during the early compared to late stages of the colony lifecycle, when the average age of workers is lower. The susceptibility of the colony may also peak and trough in accordance with the emergence of cohorts of callows. However, when considering the implications of our findings on within colony transmission, callow behaviour in the colony needs to be considered. In larger colonies, callows spend the majority of their time hidden underneath pupae and often do not feed in the first hours after emergence (HWG pers. obs). Consequently, in spite of their high susceptibility to infection at 0 days of age, their initial low exposure in the colony may reduce the likelihood, or magnitude, of peaks in infection during the emergence of cohorts of callows, particularly in larger, older colonies. Previously, callows have been used in studies of *C. bombi* epidemiology, due to their lack of infection (Shykoff & Schmid-Hempel, 1991b; Cisarovsky et al., 2012). Hence, the increased susceptibility we show here is particularly interesting in this host-parasite system. Importantly, our results show, that if inoculating individuals less than one week old, results may not be applicable to adult bees due to differences in infection susceptibility.

The lack of a colony effect on infection intensity in our experiment is surprising, given previous studies which show that this host parasite system exhibits specific host-parasite genotype-genotype interactions (Baer & Schmid-Hempel, 2003; Cisarovsky et al., 2012; Barribeau & Schmid-Hempel, 2013). In contrast, abaecin and defensin expression did vary between colonies. Previous work has shown that different colonies produce contrasting patterns in AMP expression in response to both different *C. bombi* strains and immune challenges, for example inoculation with *C. bombi* compared to bacteria (Barribeau & Schmid-Hempel, 2013). Furthermore, Riddell et al. (2009) found that different *C. bombi* strains produced contrasting defensin, but not abaecin, expression across different colonies. The lack of relationship in our study between colony variation in immune gene expression and parasite susceptibility suggests that the gut microbiome of the colony may play a role in determining the infection outcome (Koch & Schmid-Hempel, 2012).

Overall, these results suggest that age does affect susceptibility to *C. bombi* . infection in *B. terrestris*, with callows exhibiting higher infection intensities than other age treatment groups. We found no difference in AMP expression, however, our low sample size reduced our power to detect an effect of age on AMP expression. Therefore, we cannot disregard the role of AMPs in the susceptibility of callows to *C. bombi* infection. Higher susceptibility of callows may have implications for the susceptibility of colonies at different stages of their lifecycle, due to the contrasting age demography of workers in the colony. However, the size of this impact is unclear because the risk of infection for callows in the colony may be relatively low due to their behaviour and therefore, exposure to *C. bombi* within the colony.

Chapter 4
Timing is critical: the timing of exposure determines a parasite's spread in a social host

Hannah S Wolmuth-Gordon & Mark JF Brown

I am conducting further collaborative work on this chapter before I publish it.

Author contributions:
HSWG: Conceptualisation, experimental design, data acquisition, manuscript drafting, manuscript editing.
MFJB: Conceptualisation, manuscript editing.

Abstract

The speed that a disease spreads through social insect colonies dictates its impact on the colony. Transmission may depend on demographic factors, such as colony size. The interaction between the eusocial bumblebee, *Bombus terrestris*, and its gut parasite, *Crithidia bombi*, is an ideal model system to test the effects of population demographics on parasite transmission as during their annual lifecycle, colonies pass through several life stages. Here, I investigated whether the timing of exposure to a parasite influences its transmission through a colony. Colonies were inoculated at the start, in the middle and at the end of their ergonomic growth stage. The basic reproduction number (R_o) was estimated as 8.48 (±6.38) and did not differ between lifecycle stages. For the remainder of their lifecycle, colonies were screened biweekly to determine infection prevalence. Colonies exposed to the parasite earlier in their lifecycle experienced a higher, and more prolonged period of transmission. Surprisingly, by the end of their lifecycle parasitaemia had died out in 88% of colonies. At this stage, males were screened to determine the impact of the epidemic on transmission to the sexual caste and potentially the following generation. These results not only provide insight into disease dynamics in this important model system but also shed light on disease transmission in populations of different sizes and structures. This understanding could aid the control and help predict the impact of epidemics on populations of many social species.

4.1 Introduction

Parasites pose a threat to host populations of all organisms, from plants (reviewed by Ristaino et al., 2021) to humans (reviewed by Sampath et al., 2021). For example, Devil Facial Tumour Disease in Tasmanian devils has caused population declines of 80% in some regions of Tasmania (Hawkins et al., 2006). Similarly, chytrid fungi have drastically affected amphibian populations globally (Fisher & Garner, 2020). The spread and impact of parasites such as these can be affected by host population variability. Some ways that populations can vary include differences in population size, structure, or density. These differences within and between populations have a large effect on the dynamics of a disease. For example, population size affects the transmission of Covid-19 (Jahangiri et al., 2020; Zhang et al., 2021) and has also been shown to affect parasite transmission in many other host-parasite interactions (eg. Jennersten et al., 1983; Vilaplana et al., 2010; Zhou et al., 2017; Dalziel et al., 2018). Likewise, population density (Dekesel, 1993; McCallum et al., 2001) and demography (Acosta-Jamett et al., 2010; Geard et al., 2015; Dowd et al., 2020) can also influence parasite transmission.

Demographic composition can differ substantially between populations and is often temporally dynamic within a population. The proportion of individuals of different ages and sex are two demographic factors that can vary in a population, and also affect an individual's susceptibility to infection. For example, children are more susceptible to cholera infection (Deen et al., 2008) and many invertebrates exhibit changes to disease susceptibility with age (reviewed by Ben-Ami, 2019). Furthermore, certain demographic groups may be more likely to transmit the parasite, as is the case with bumblebee larvae and *Vairimorpha* (*Nosema) bombi* (eg. Rutrecht & Brown, 2008b). Evidently, investigating the role of host population demography is vital to our understanding of parasite transmission and assessing impacts of the parasite in social populations. This may also enable the implementation of control strategies to limit the impact of the parasite on a population (eg. McCann et al., 2020).

To assess the impact of demographic composition on the epidemiology of a disease, it is useful to quantify transmission. The dynamics of parasite transmission can be described using models that compartmentalise hosts in a population into susceptible, infected and removed individuals (SIR) (Anderson & May, 1981). SIR models incorporate the term 'the basic reproductive rate of the parasite' (R_0), which defines the mean number of secondary infections caused by one infected individual when all individuals are susceptible (Anderson & May, 1992). R_0 estimates parasite transmissibility and when

$R_0 > 1$ a parasite will invade a host population (Diekmann et al., 1990). Thus, estimating R_0 in populations with contrasting demographics enables transmission to be compared between or within populations.

Social insect colonies are ideal systems to study the transmission of parasites in host populations, as each colony is representative of one population and there are frequent social interactions between individuals. One example of the effect of colony demography on infection susceptibility is that smaller honeybee colonies with a lower proportion of adults have an increase susceptibility to chalkbrood disease (Koenig et al., 1987; Spivak & Gilliam, 1993). This could be because larger colonies are stronger, less stressed and have more nurses maintaining the condition of the larvae. Due to the method of transmission of the fungus, it is likely an example of changes in susceptibility rather than changes to intracolony transmission. Colonies of varying sizes and demographics may also have different network structures, which has been shown to affect the intracolony parasite transmission of directly transmitted parasites. For example, trophallactic transmission within honeybee colonies is affected by the degree of clustering in the social network (Naug, 2008). Network structure can vary with the demography of the colony, as an individual's age is often associated with its role within the colony (Oster & Wilson, 1978). Furthermore, within colony transmission can be altered by small changes in parasite epidemiology. This was demonstrated by Naug & Smith (2007) who found trophallactic parasite transmission through a colony changed according to the length of the infectious period.

The relationship between the social insect *Bombus terrestris* and its gut parasite, *Crithidia bombi,* is a well-established model for investigating the epidemiology of host-parasite interactions (Schmid-Hempel et al., 2019). *Bombus terrestris* colonies produce up to 300 workers under laboratory conditions (Zhang et al., 2018) and have a singly-mated queen (Schmid-Hempel & Schmid-Hempel, 2000). *Bombus terrestris* colonies pass through a cycle of different stages over one year. During this annual lifecycle, colonies pass through several distinct lifecycle stages with varying colony sizes, ages of individuals and numbers of reproductives. At the beginning of their lifecycle, colonies consist of one queen and a small number of young workers. Over the following weeks, the population of workers gradually increases and peaks after approximately 10-12 weeks (Cameron, 1989), with a concomitant increase in the average age of workers. This increase in colony size is ergonomic growth phase of the colony lifecycle. Towards the end of their lifecycle, sexual reproduction begins, and males and new queens are produced. This repeatable variation in population size and demography enables the role of these factors on transmission to be investigated.

Crithidia bombi is a gut parasite of bumblebees that is transmitted through contact with contaminated faeces and colony material (Schmid-Hempel & Schmid-Hempel, 1993; Folly et al., 2017). The parasite is introduced to the colony when a colony is founded by an infected queen (Otterstatter & Thomson, 2007) or by workers infected when foraging on flowers (Durrer & Schmid-Hempel, 1994; Graystock et al., 2015; Adler et al., 2018; Figueroa et al., 2019; Pinilla-Gallego et al., 2022). The parasite can subsequently be transmitted from workers to new sexuals within the colony (Ulrich & Schmid-Hempel, 2015). Our knowledge of *C. bombi* transmission within colonies is very limited, particularly in relation to colony demography. Otterstatter & Thomson (2007) found that behavioural role in a colony does not affect the probability of infection from an infected forager. Furthermore, network density, but not population size, affected the transmission of *C. bombi*, due to the frequency of social interactions. This was further supported by network modelling, which demonstrated that the frequency, but not duration, of contact between individuals best explained within colony transmission (Sah et al., 2021). In addition, a higher initial prevalence has been shown to increase the speed of *C. bombi* transmission in a colony and the infection intensity in infected individuals (Pinilla-Gallego et al., 2020). When measuring prevalence in the field, repeated sampling of the same areas and thus, presumably colonies, illustrates that *C. bombi* prevalence increases as the spring and summer progress. This aligns with the ergonomic growth phase of bumblebees; when the colonies are increasing in size (Popp et al., 2012). Therefore, the rate of transmission must increase through the summer, however, it is unknown whether this results in or is a result of, higher intracolony transmission.

Here, I use this model system to investigate whether host demography affects parasite transmission through a population. I infected colonies at the start, in the middle and at the end of their ergonomic growth stage. R_0 was estimated and transmission between workers was measured for the remainder of their lifecycle. At the end of their lifecycle infection in males was measured to determine the impact of the epidemic on transmission to the sexual individuals and potentially the following generation. I hypothesised that R_0 would be lower when colonies were infected earlier, since R_0 would be constrained by the small population. In addition, I predicted that prevalence would be higher in earlier infected colonies because their smaller population size and thus, higher frequency of contact with the infected individual and contaminated colony material. Finally, I hypothesised that prevalence in males would be higher in early infected colonies, as *C. bombi* would have been transmitted through the colony for a longer time period. Results from this work provide insight into disease dynamics in this important model system and also shed light on disease transmission in populations of different sizes

and structures. Such understanding is essential for predicting the impact of epidemics on populations of social species.

4.2 Methods

4.21 Experimental organisms

Bumblebees

18 commercial colonies of *Bombus terrestris audax,* with 6-8 workers, were purchased from Agralan (UK). All bees were housed under red light, 25°C and ambient humidity throughout the experiment. Faecal samples of five bees per colony were screened for *Crithidia bombi, Vairimorpha* (*Nosema*) spp. and *Apicystis* spp*.* using a phase contrast microscope (Nikon Eclipse 50i) at X400 magnification (Rutrecht & Brown, 2009). None of the samples contained these parasites. Colonies were provided with honeybee collected pollen (Agralan, UK) and sterile sugar solution (50% concentration) *ad-libitum.*

Colonies were housed in two interconnecting boxes (29 x 22.5 x 13cm). Boxes were connected by a 10cm transparent tube. Cardboard ramps wrapped in duct tape (for strength) led up to the connecting tube in both boxes. The 'colony box' contained the brood, queen and workers and the 'foraging' box contained pollen and a reservoir of sugar solution. A layer of cat litter (Catsan non-clumping hygiene litter) was put along the bottom of the foraging box to prevent faeces accumulating (Pinilla-Gallego et al., 2020). Foraging boxes were replaced approximately every three to four weeks when the cat litter was saturated to prevent faeces and mould build-up. The separate foraging box made the set-up more field-realistic since foragers had to leave the colony to collect pollen and sugar.

Crithidia bombi

Four colonies (Agralan, UK) were used as *C. bombi* stock. Two of these were used in the first half of the experiment and two in the second half due to the initial colonies reaching the end of their lifecycle. These colonies were inoculated with *C. bombi* originating from post-hibernation spring queens collected at Windsor Great Park (Surrey, UK) in March 2021. The second cohort of colonies were inoculated using *C. bombi* from the first two colonies.

4.22 Experimental design

Treatment groups

To test the impact of the timing of infection on the transmission of *C. bombi,* six colonies were inoculated at three different time points during the ergonomic growth phase of their lifecycle (Fig. 1). Thus, 18 colonies were inoculated in total. In treatment one, the colonies were inoculated on the week of arrival; when the colony consisted of 6-8 workers. In treatment two, the colonies were inoculated when the colony reached 45 workers. This time point was chosen as it was approximately halfway through the lifecycle (HWG, pers. obs.). Colonies in treatment three were inoculated when either the colony reached 80 workers or when the first male was produced, whichever occurred first. These timepoints signified the end of the ergonomic growth stage and the beginning of the sexual reproduction stage.

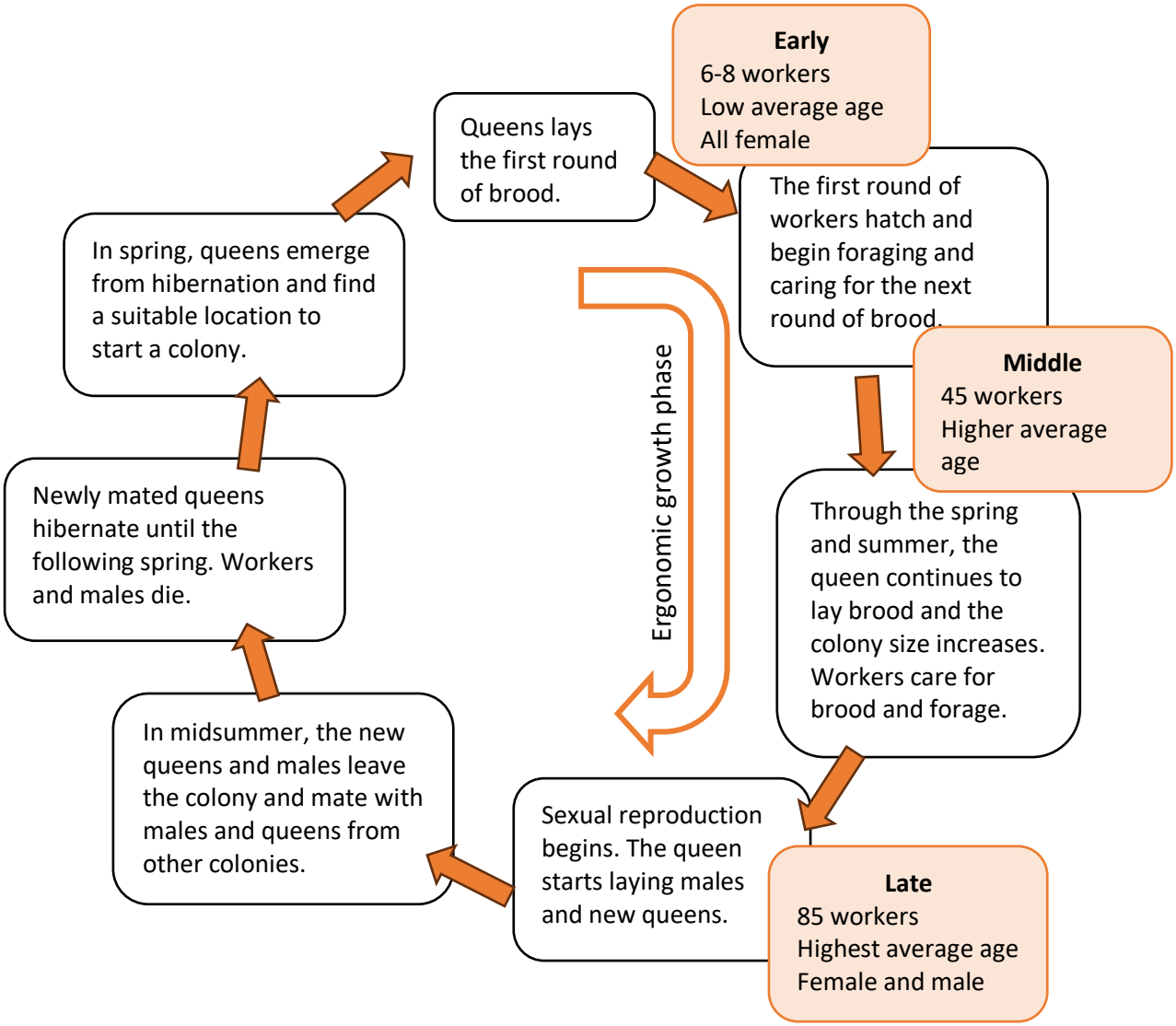


Fig. 1 The annual lifecycle of *B. terrestris* showing the three stages of the ergonomic growth phase (early, middle and late) that colonies were inoculated.

Inoculation protocol

Colonies were inoculated by introducing one infected individual into the colony, hereafter referred to as the 'marked individual'. This enabled estimation of R_0, the number of individuals one individual infects (Anderson & May, 1982), and comparison of R_0 across treatment groups. The marked individual was chosen by removing six workers from the foraging box. If after five minutes of observation there were less than six workers in the foraging box, the remaining individuals were selected from the colony box. These workers were labelled 1-6 and two were randomly selected using a random number generator. Two individuals per colony were inoculated to protect against the likelihood of an individual not drinking the inoculum. The unselected workers were returned to the foraging box. This ensured the marked individual was randomly selected and replicated a forager introducing *C. bombi* into a colony from the field. Inoculated individuals were marked with a coloured spot so that they could be identified when returned to their colony. They were put in separate, labelled nicot cages (Becky's bees, UK), which are cylindrical containers adapted from hair rollers to house bees. Bees were starved for two hours prior to inoculation.

On the day of inoculation, faecal samples were obtained from 20 workers from two colonies. This was purified using a modified triangulation protocol developed by Cole (1970) (see General methods 2.1). Bees were given a standardised dose of 25,000 cells (as was done by Logan et al. (2005)). This dose was chosen to maximise the chance of infection, whilst being field realistic (Logan et al., 2005). Bees were inoculated using the protocol in General methods 2.2. If one individual did not drink the entire droplet after four hours the individual was discarded and the individual that drank the droplet was returned to their colony (n = 10). If both individuals from the same colony drank the inoculum one was randomly selected to return to the colony and the other discarded (n = 12). If neither individual from a colony drank the inoculum, both individuals were discarded, and the inoculation protocol was repeated the following day (n = 1). Individuals that drank the inoculum were returned to their foraging box. Four days after inoculation, marked individuals were removed from their colony. Their faeces were screened using a phase contrast microscope at X400 magnification to check if they were infected as infected individuals will beginning shedding cells four days after infection (Logan et al., 2005). If the individual was infected they were returned to the colony. If the individual was not infected, or died before screening, the marked individual was discarded and the inoculation repeated as soon as possible (n = 7; n = 2 respectively).

4.23 Measuring *C. bombi* transmission

Estimating R_0

One week after inoculation, 20 individuals per colony were removed, avoiding callows and marked individuals (newly hatched workers who are too young to have become infected). Some young colonies had fewer than 20 workers and therefore, all workers were sampled in stages to minimise disruption to the colonies. Individuals were removed from the colony box to ensure the sample contained a mix of workers that care for brood (and therefore, have high contact with infected individuals and contaminated nest material) and foragers (which will have a lower contact rate with infected individuals and contaminated nest material) and therefore, was representative of the colony as a whole. Faecal samples were screened for infection using a phase contrast microscope at X400 magnification (see General methods 2.2). Presence or absence of infection was recorded. Individuals were weighed (Ohaus, Scout, STX123), since bee size affects *C. bombi* infection intensity (Otterstatter & Thomson, 2006). Bees were weighed whilst in pre-weighed vials to the nearest milligram. One week was chosen as the timepoint at which to estimate R_0 based on a predicted timeline of infection using data from Logan et al. (2005) (Fig. 2), which suggests that on day seven 95% of secondary infected individuals will be shedding cells.

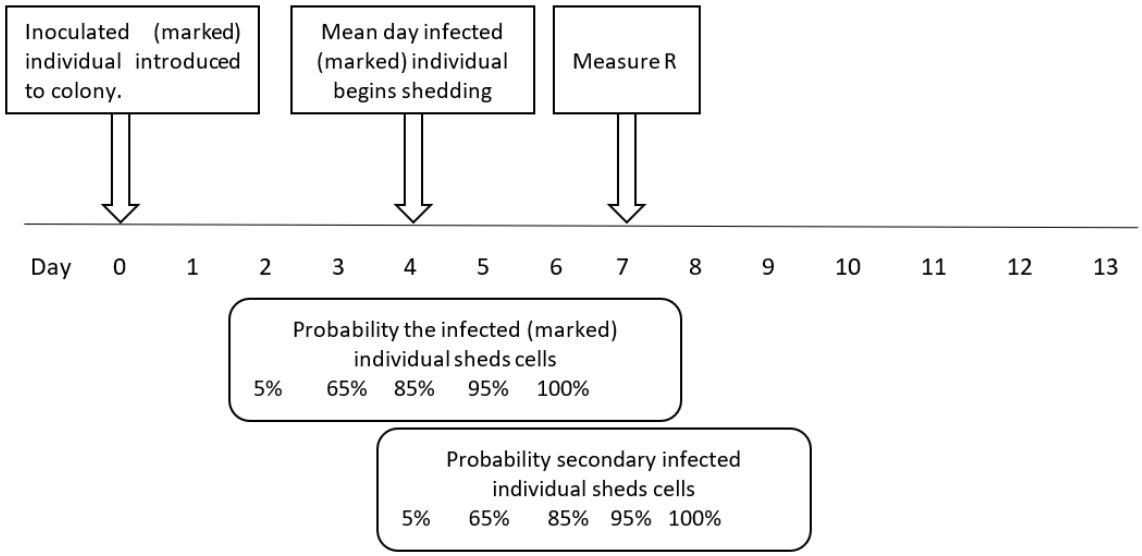


Fig 2. Predicted timeline of infection based on data from Logan et al. (2005).

Measuring prevalence over time

Twice per week after estimating R_0, 10 workers per colony were screened for infection, avoiding callows and the marked individual, using a phase contrast microscope at X400 magnification and weighed to the nearest milligram.

Measuring colony growth

Once per week the numbers of workers in all colonies were counted. Counts were conducted under white light for maximum visibility. A clicker was used and the colony was scanned from left to right. Counts informed inoculation dates as previously described. Once per week, when the number of workers surpassed 15, colonies were culled by 10%. The percentage of the colony culled was rounded down to the nearest ten. For example, if the colony count was 66 then six individuals were culled. This enabled colonies to grow, whilst mimicking the death rate of foragers in the field (Brian, 1952; Rodd et al., 1980; Goldblatt & Fell, 2011). Bees were randomly selected from the foraging box, to ensure foragers were culled.

Measuring prevalence in males

Colonies were observed twice per week to identify the date when male production began. Four days after a male was first observed in the colony, males were removed twice per week at 3-4 day intervals and frozen at -80°C. Callow males were not removed, since males usually spend 3-4 days in the colony prior to leaving to mate and they would not have had field-realistic exposure to *C. bombi* in the colony (Free & Butler, 1959). Prevalence of infection in males was measured using the protocol in General methods 2.4.

Terminating colonies

Measuring the transmission of *C. bombi* in a colony ceased when either 36 males had been collected from the colony or at least 12 weeks has passed since the colony arrived, whichever occurred first. A sample size of 36 males was predetermined through power analysis.

4.24 Data analysis

Statistical analyses were conducted in R version 4.1.0 (R Core Team, 2021). All figures were created using the ggplot() function from the ggplot2 package (Wickham, 2016).

1. Does the probability of an individual being infected change depending on the lifecycle stage when a colony is infected?

To test how the likelihood of an individual being infected (presence or absence) varied with treatment over time the package lme4 (Bates *et al*., 2015) was used to construct a mixed effects model with a binomial error distribution and logit link. The full model included treatment and day since inoculation as fixed effects and their interaction. Bee mass (g) was included as a covariate and colony as a random effect. The 'drop1()' function from the package lme4 (Bates *et al*., 2015) was used to assess the significance of these model components using a likelihood ratio Chi-squared test. These values and AIC values were used to compare the reduced and full models. To account for multiple comparisons between treatments I used a Tukey post-hoc test. This model was also rerun on data from the first 28 days of screening when all colonies were alive, to ensure that results from the full analysis were not just a product of an imbalance across treatments of the length of the sampling period.

2. Does the percentage prevalence at seven days differ depending on when during the lifecycle a colony is infected?

To find out if percentage prevalence at seven days depended on when the colony was infected the package lme4 (Bates *et al*., 2015) was used to build a generalised linear mixed model with a binomial error distribution and logit link. The full model included treatment as a fixed factor and colony as a random effect. A Tukey post-hoc test was used to compare the multiple levels of treatment and account for multiple comparisons. The model included colony size at seven days as a weight and was run using both a dataset that included all 18 colonies and a subsidiary dataset from which two treatment 1 colonies that were discontinued (see above) were excluded (hereafter referred to as 'all data' and 'subset of data' respectively).

3. Does the estimate of R_0 vary depending on when during the lifecycle a colony is infected?

To assess whether treatment affected the estimate of R_0 a Kruskal-Wallis test was used. This is because R_0 is bounded by the colony size and therefore, it can only robustly be modelled as a proportion (see above). The Kruskal-Wallis test does not have the assumption of non-bounded values. To estimate R_0 the percentage infected at seven days was multiplied by the colony count at seven days. R_0 was used as the response variable and treatment as the predictor. Analysis was conducted on all data and the subset of data.

4. Does the timing of infection during a colony's lifecycle affect colony growth?

To investigate whether the timing of infection affected colony growth a mixed effects model with a negative binomial error distribution with a log link function was conducted using lme4 and the glmer.nb() function (Bates *et al*., 2015). The full model included colony counts as a response variable. Treatment, week since colony arrived and their interaction were included as fixed factors and colony as a random factor. The 'drop1()' function from the package lme4 (Bates *et al*., 2015) was used to assess the significance of these model components using a likelihood ratio Chi-squared test.

5. Does the timing of infection during a colony's lifecycle affect the prevalence of infection in males?

Prevalence data from male screening was not included in analysis, since the prevalence was extremely low across all treatment groups.

4.3 Results

4.31 Prevalence sampling

Prevalence was measured for the entire lifecycle of 16 colonies in total (four colonies in treatment one and six each in treatments two and three). In treatment one, one colony died prematurely. In this colony and one other, the first inoculated individual died before screening and a new individual was inoculated (see Table A2.1). I was unsure whether the first marked individuals were infective prior to death and therefore, these colonies were excluded from the rest of the experiment. This resulted in a smaller sample size than originally planned. In early colonies prevalence was measured for 9-13 weeks following inoculation, in middle colonies, 5-8 weeks and in late colonies, 4-6 weeks. The inoculation process for each colony is outlined in Table A2.1.

1. The probability of an individual being infected depended upon when during the lifecycle its colony was infected

The full model had the best fit and was used as the final model. Individuals in colonies infected early in the ergonomic growth phase of their lifecycle were significantly more likely to be infected compared to those infected in the middle (GLMM: z = -2.356, p = 0.0185) and at the end of this lifecycle phase

(GLMM: z = -2.466, p = 0.0137). This remained significant after a Tukey's HSD for multiple comparisons. The mean prevalence of individuals infected early in the ergonomic growth stage was 32.6% (95% C.I. [18.27, 58.27]), which was significantly higher than that of colonies infected in the middle (3.58%, 95% C.I. [1.86, 6.79], $p < 0.001$) and end of this lifecycle stage (0.12%, 95% C.I. [0.013, 6.79], $p < 0.001$). Those infected in the middle were also more likely to be infected than those infected at the end of the ergonomic growth phase (p = 0.013). As expected, day since inoculation and bee mass significantly affected infection likelihood, (GLMM: z = -9.968, $p < 0.001$, GLMM: z = -2.053, p = 0.04 respectively). The interaction between treatment and time since inoculation significantly affected infection likelihood (χ^2_2 = 16.762, $p < 0.001$). Figure 3 shows that infection transmission persisted the longest in those infected early in the growth phase, whilst the infection was less prolonged with a lower percentage prevalence when colonies were infected in the middle. Colonies infected late in the growth phase exhibited the shortest period of infection with the lowest prevalence and sharpest decline in prevalence. Five out of six colonies exhibited 0% prevalence 14 days after inoculation. Prevalence of infection for the first 28 days after inoculation, when all colonies were alive (see Table A2.1), was best explained by a model including day screened and the interaction between lifecycle stage at inoculation and time since inoculation. Treatment reduced model fit and therefore, was removed from the model (χ^2_2 = 1.570, p = 0.456). The interaction term significantly affected infection (χ^2_2 = 37.962, $p < 0.001$). Over the first 28 days of screening, prevalence was relatively stable in early infected colonies, whereas in middle infected colonies prevalence gradually fell and in late infected colonies prevalence steeply decreased to 0% from 7-14 days. Day since inoculation and mass did not significantly affect infection (GLMM: z = -0.875, p = 0.382) and (GLMM: z = 1.186, p = 0.055).

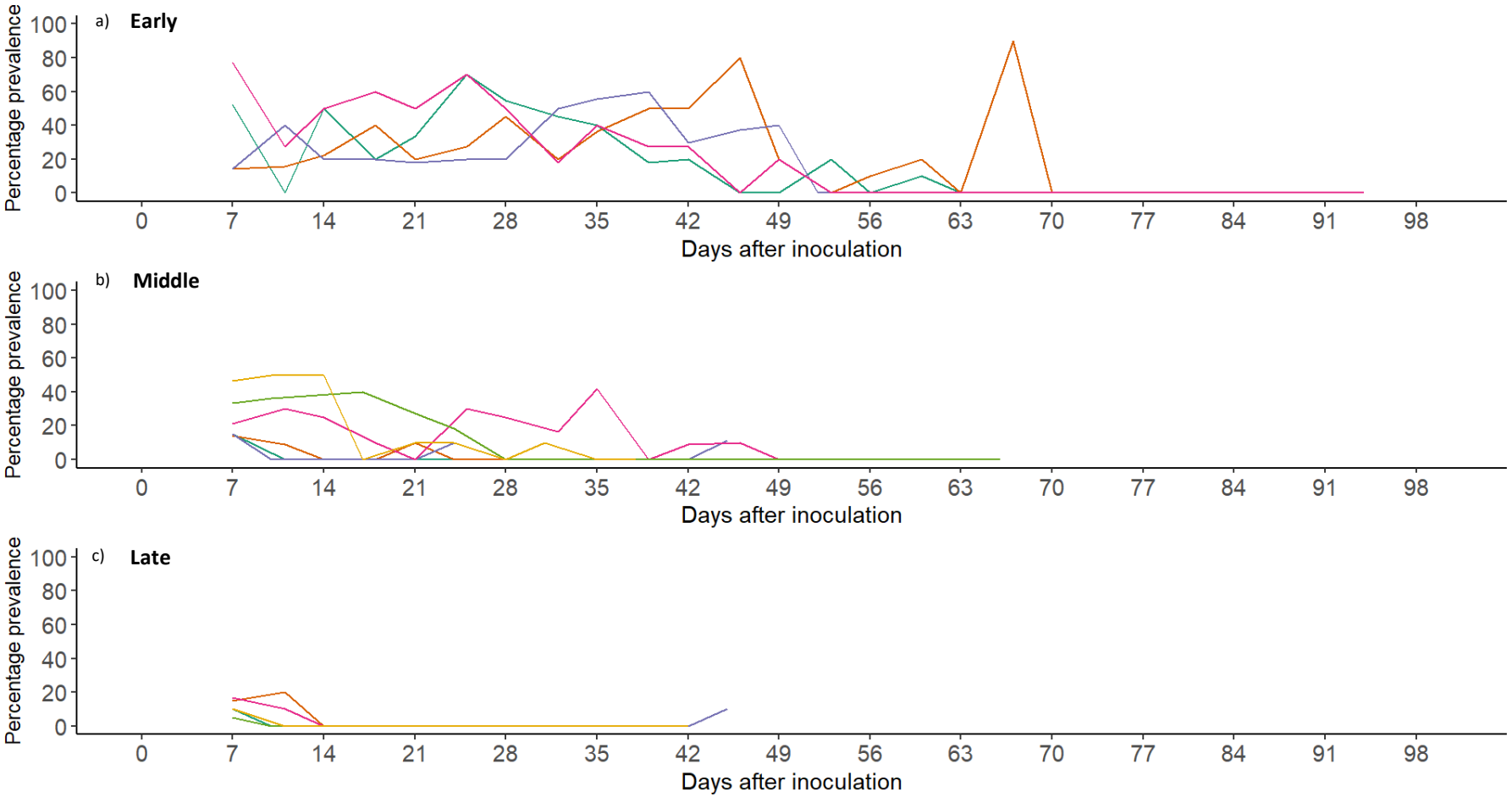


Fig. 3 The percentage prevalence of *C. bombi* through time of colonies infected at different points during the ergonomic growth stage of their lifecycle. Colonies were inoculated on day 0 and screening commenced on day seven. Each line represents one colony. **a)** Early infected colonies were inoculated at the start of the growth stage when the colony consisted of 6-8 workers. **b)** Middle infected colonies were inoculated in the middle when colonies had 45 workers. **c)** Late infected colonies were inoculated at the end of the growth phase when colonies had 80 workers or had started sexual reproduction.

2. Percentage prevalence after seven days was higher in colonies infected at the start compared to the end of their lifecycle

When in the ergonomic growth phase the colony was inoculated significantly affected the percentage prevalence after seven days (all data: $\chi^2_2 = 7.552$, $p = 0.0229$; subset: $\chi^2_2 = 11.306$, $p = 0.00351$; Fig. 4a, b). Colonies infected at early in the ergonomic growth phase exhibited significantly higher prevalence compared to colonies infected at late in the ergonomic growth phase (all data: 4.295%, SE = 1.62%, $p = 0.007$; subset: 7.4%, SE = 1.63%, $p = 0.001$). Furthermore, colonies infected in the middle had higher prevalence compared to colonies infected late in the ergonomic growth phase (all data: 3%, SE = 1.54%, $p = 0.0287$; subset: 3.04%, SE = 1.63%, $p = 0.0107$; Fig. 4b, c). There was no significant difference in percentage prevalence between colonies infected at the start and the middle of the ergonomic growth phase (all data: 1.43%, SE = 1.62%, $p = 0.74$; subset: 2.43%, SE = 1.63%, $p = 0.165$).

3. Estimate of R_0 did not differ depending on when during the lifecycle a colony was infected

When in the ergonomic growth phase did not significantly affect R_0 (all data: $\chi^2_2 = 3.31$, $p = 0.191$; subset: $\chi^2_2 = 0.9$, $p = 0.637$, Fig. 4c, d, Table A3.2). The average R_0 across all colonies was estimated as 8.48 (±6.36) when all data was included and 9.36 (±6.21) when only colonies that were carried forward for the entire experiment were included.

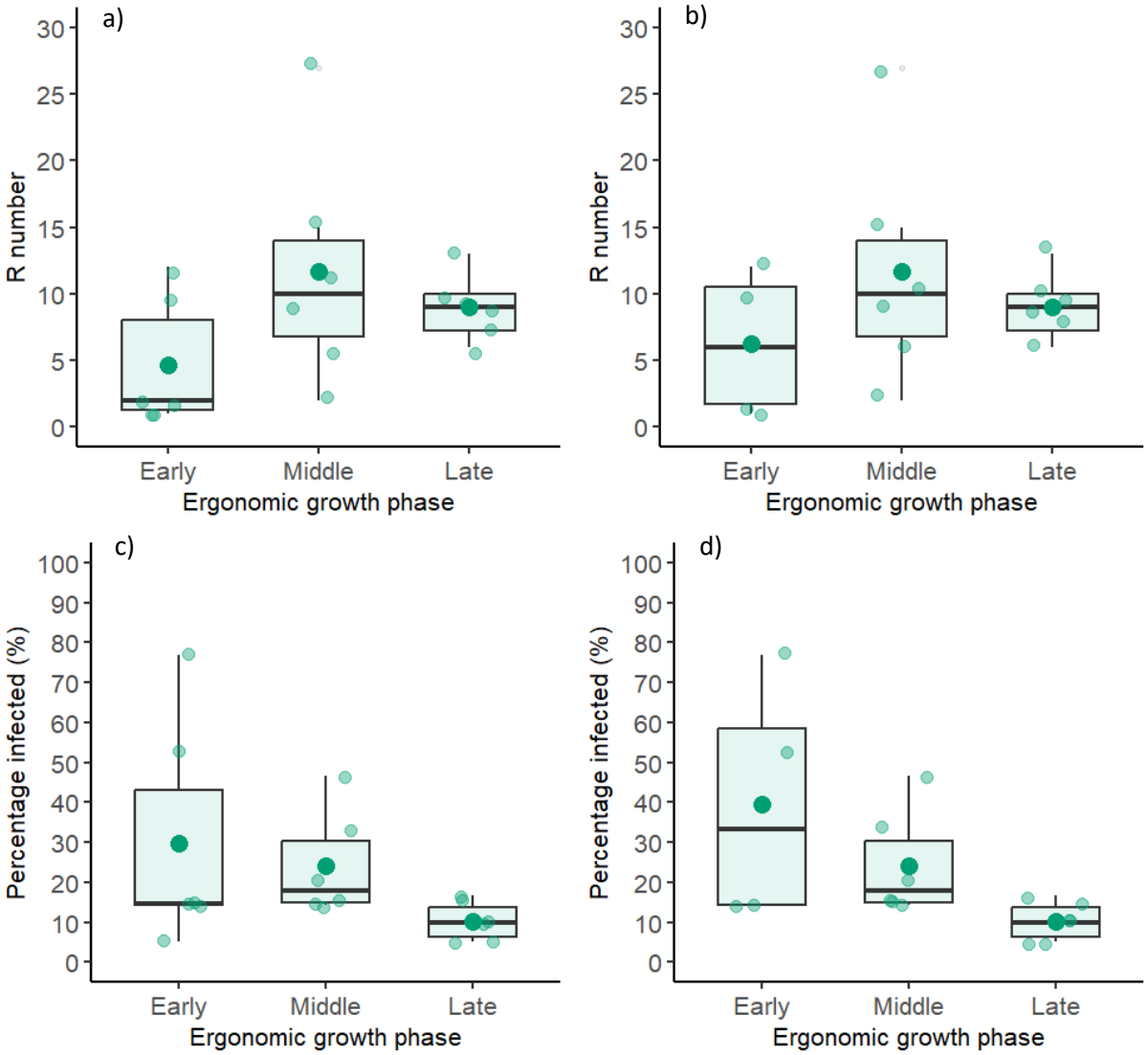


Fig. 4 In all boxplots the mean is indicated by the large dark green datapoint. Early colonies were infected at the start of the ergonomic growth phase when the colony consisted of six to eight workers, middle colonies in the middle when colonies had 45 workers and late colonies at the end when colonies had 80 workers or had started sexual reproduction. **a)** The percentage prevalence of all 18 colonies seven days after infection. **b)** Same as a), excluding two treatment one colonies not carried through the whole experiment. **c)** The estimated R_0 (mean number of individuals one infected individual infects) when all 18 colonies were infected at different points during their ergonomic growth stage. **d)** Same as c), excluding two early colonies not carried through whole of experiment.

4. When the lifecycle the colony was inoculated interacted with the week of colony arrival to affect colony growth

When in the lifecycle the colony was infected did not affect colony growth (χ^2_2 = 0.494, p = 0.781), but there was a significant interaction between when the colony was infected and the week since the colony arrived (χ^2_2 = 13.985, p < 0.001, Fig. 4). Generally, colonies infected later in their lifecycle exhibited larger and steeper colony growth and peaked at approximately 11 weeks. Growth in colonies infected at early in the lifecycle was slower and more varied (Fig. 5a).

5. When the lifecycle the colony was inoculated did not affect the prevalence of infection in males produced at the end of the lifecycle

A total of 414 males were produced in total across all colonies, 75 in early colonies, 109 in middle and 226 in late colonies. When colonies produced more than 30 males only 30 were dissected. Hence, 52 were dissected in early colonies, 120 in middle colonies and 163 in late colonies. Infection prevalence was very low and approximately equally distributed across all colonies. Prevalence was 1.95% in early colonies (n = 1), 1.67% in middle colonies (n = 2) and 1.23% in late colonies (n = 2). Eleven of 16 colonies produced no infected males.

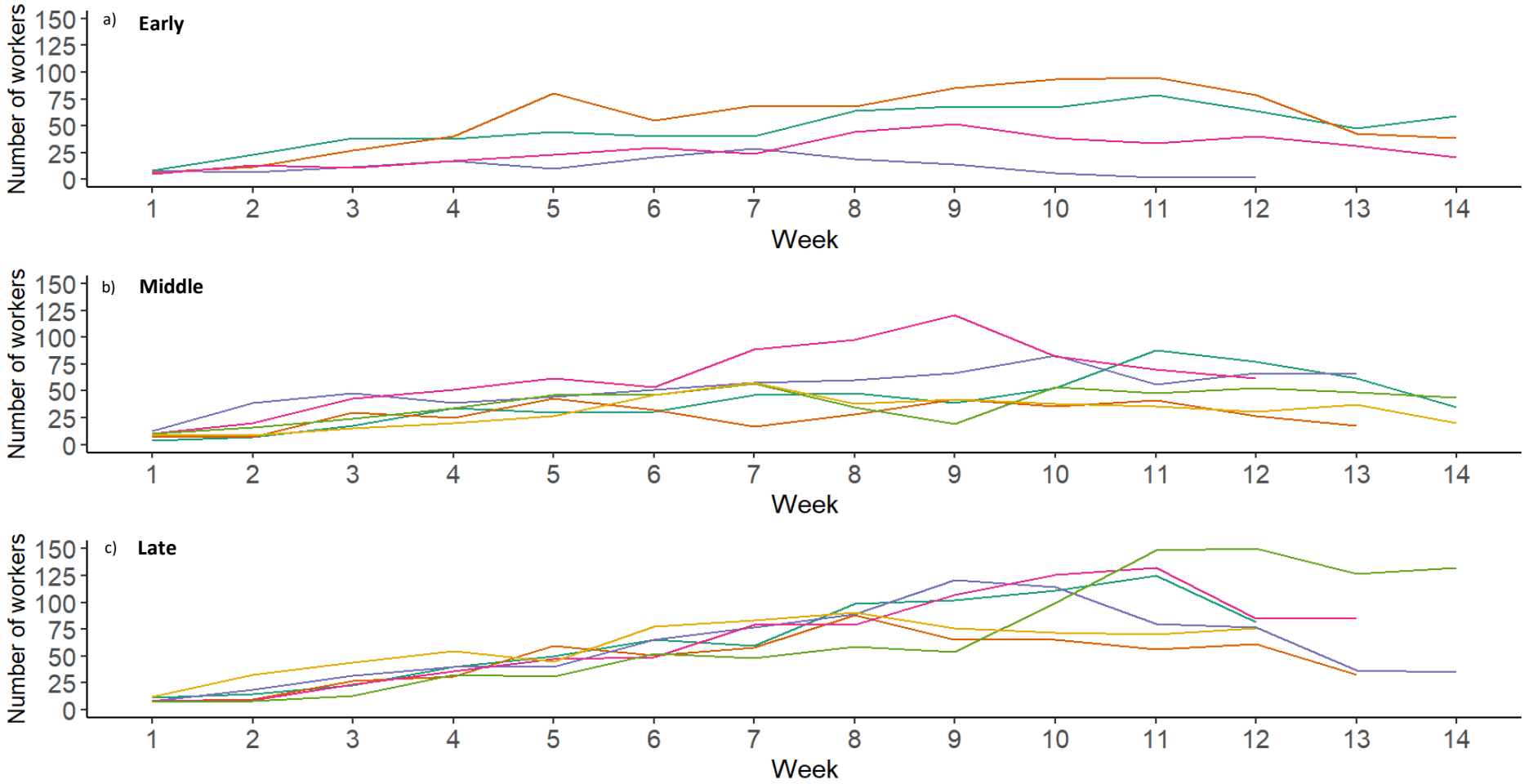


Fig. 5 The number of workers in each colony over time from colony arrival (week 1) to the end of the colony lifecycle. Each line represents one colony. **a)** Early colonies were infected at the start of the ergonomic growth phase when the colony consisted of 6-8 workers, **b)** Middle colonies in the middle of the ergonomic growth phase when colonies had 45 workers and **c)** Late colonies at the end of the ergonomic growth phase when colonies had 80 workers or had started sexual reproduction.

4.4 Discussion

Here, I have shown that the timing of infection in the colony lifecycle and thus, the colony demography of *B. terrestris*, affects within colony transmission of *C. bombi*. Individuals in colonies infected at the early in the ergonomic growth phase exhibited a higher likelihood of infection throughout the colony lifecycle and this decreased the later colonies were inoculated. In addition, the duration of the infection at the colony-level fell the later colonies were infected. In contrast, the estimated R_0 and prevalence after seven days did not vary with the timing of infection. Across all colonies, transmission to males was very low.

In this model system, transmission depends upon the rate of contact between susceptible individuals (the number of which varies with the birth and death rate within the colony) and infected individuals (the number of which varies with the death rate and clearance rate), and also susceptible individuals and contaminated nest material. The clearance rate of *C. bombi* in *Bombus* spp. is approximately 66% over the average lifespan of a bee in the field (22 days) (Imhoof & Schmid-Hempel, 1998a). However, to my knowledge this is the only evidence of clearance and in this study the possibility of reinfection was minimised because the bees were kept in clean vials and faeces were routinely removed. Whereas in my experiment and in colonies in the field, reinfection following clearance is highly likely because of contact with infected individuals and contaminated nest material. Colonies infected earlier were smaller and therefore, the higher prevalence and duration of infection in these colonies could be explained by an increased contact frequency between infected and susceptible individuals (Sah et al., 2021). In smaller populations there is less of a buffer between infected and susceptible individuals and therefore, they are more likely to come into contact. There is some evidence of reduced parasitism in larger groups in other systems, for example, larger groups exhibit reduced parasitism from mobile parasites, such as flies (Mooring & Hart, 1992; Côté & Poulin, 1995). However, in contrast to my results the prevalence of a range of faecal-oral transmitted parasites transmission has been shown to increase with group size (Côté & Poulin, 1995). Furthermore, the effect of colony size on *C. bombi* transmission may have implications for interspecific transmission. The size of colonies across *Bombus* spp. vary, for example, *B. terrestris* colonies have larger colonies compared to *B. ruderatus* and *B. lucorum* (Falk & Lewington, 2019). Species with smaller colonies sizes may be more susceptible to *C. bombi* due to increased transmission and thus, they may require a lower infection dose or number of infections to cause an epidemic. This could mean that some species are more likely to act as sources or reservoirs of *C. bombi* (Parsche & Lattorff, 2018).

In addition to differences in population size, the colonies at the three lifecycle stages consisted of different proportions of individuals of different ages. The average age of individuals in colonies at the early in the ergonomic growth phase was, by definition, lower compared to the average age of individuals in the middle and end of their ergonomic growth stage. Age can affect infection susceptibility, due to a range of factors, such as changes to physiology (eg. Corteel et al., 2009; Garbutt & Little, 2017) or the immune response (eg. Amdam et al., 2005; Haussmann et al., 2005; Khan et al., 2016). In bumblebees, there is limited knowledge of the effects of age on the immune response. Some evidence suggests that as individuals age the efficacy of their immune response declines (Doums et al., 2002; Whitehorn et al., 2011), whereas the expression of antimicrobial peptides involved in *C. bombi* defence appear not to vary with bumblebee age (see Chapter 2). Therefore, it is unclear whether differences in age demography through the lifecycle contributed to differences in *C. bombi* transmission. Furthermore, the sex demography of the colony changes through the ergonomic growth phase as males are only produced at the end of this phase. Whilst a number of studies have found that prevalence is lower in males compared to workers in the field (Shykoff & Schmid-Hempel, 1991; Parsche & Lattorff, 2018), when individuals are given a standardised dose males and workers do not differ in their susceptibility to infection (Ruiz-González & Brown, 2006b). Consequently, it is unlikely that the increase in males produced at the end of the lifecycle reduced transmission. An alternative explanation for the reduced prevalence in colonies infected at the end of their ergonomic growth stage might be that males were getting infected and thus, diluting the number of *C. bombi* cells available to infect workers. However, I do not believe that this is likely, as the prevalence in males across all colonies was extremely low and therefore, transmission to males was very rare.

It is clear that even in colonies with high initial prevalence, transmission was not high enough for the parasite to cause a persistent epidemic throughout the lifecycle. The absence of a persistent epidemic suggests that the death rate and clearance rate of infected individuals was higher than the rate of contact between susceptible and infected individuals. One could argue that the absence of infection at the end of the lifecycle of the majority of colonies could be a product of the sampling method, as low levels of infection may not have been detected. However, the repeated, random sampling combined with the final screening of males makes this unlikely. The lack of sustained transmission is surprising, since individuals are infectious for the entirety of their infection and even if clearance of the infection is possible (Imhoof & Schmid-Hempel, 1998a) reinfection is highly likely as discussed above. My results differ to those of Naug & Smith (2007) who found that a longer infectious period increased transmission within honeybee colonies, whereas a reduced infectious period confined the infection to the periphery of the colony. My contrasting results could be because honeybees and

bumblebees differ in their social organisation (eg. Van Doorn, 1986; Johnson, 2010). One reason why parasites fail to persist in a host population is due to a lack of susceptible hosts (Bartlett, 1957). However, here this was not the case, as when prevalence was falling the colonies were growing and the number of susceptible individuals increasing. An alternative explanation for the absence of a sustained epidemic, is that R_0 was below one and therefore, too low for *C. bombi* transmission to persist indefinitely. Yet, my estimations of R_0, at least at the start of the epidemics, indicate that R_0 was above one.

The absence of a prolonged epidemic in the majority of colonies suggests that in the field, the persistence of *C. bombi* must rely on multiple infections across the lifespan of the colony. The high prevalence of *C. bombi* in many areas in midsummer, for example 77.7% (Popp et al., 2012), indicates that this is extremely probable. This is further confirmed by the fact that when Imhoof & Schmid-Hempel (1999) put uninfected colonies in the field, all colonies became infected after 9.5 days. When colonies are founded by an infected queen, multiple infections are not required to cause sustained, high-levels of transmission (Otterstatter & Thomson, 2007). This is likely a result of extremely high contact frequency between the infected queen and the first brood of workers, as only the queen rears these workers (Sah et al., 2021). In addition, queens are larger than workers and larger bees are more likely to transmit *C. bombi,* since they shed a larger number of propagules in their faeces (Van Wyk et al., 2021).

The significant effect of timing of infection on percentage prevalence after seven days but not on R_0 shows that when colony size is accounted for, the timing of infection affects the transmission of the parasite after one week. The higher prevalence in colonies infected earlier could be explained by the increased contact rate between the marked and susceptible individuals as previously discussed. I predicted that R_0 would be higher in colonies infected later because R_0 would not be constrained by the size of the colony, however, this was not observed. This could be because R_0 is affected by a wide range of factors in addition to demography (reviewed by Delamater et al., 2019), that can cause large variation in R_0, for example, R_0 for measles varies between 2.7 and 303.3 (Guerra et al., 2017). Whilst I controlled a large number of confounding variables (e.g. all colonies arrived with the same number of workers and were housed under the same conditions) R_0 could still vary between colonies due to differences in colony susceptibility to the *C. bombi* strains used in the inoculum (Baer & Schmid-Hempel, 2003; Cisarovsky et al., 2012). Indeed, Otterstatter & Thomson (2007) found there was high variability in the transmission of *C. bombi* from infected queens to the first brood of workers. This possibility is also supported by the fact that marked individuals from some colonies failed to get

infected after the first inoculation, emphasising the differences in susceptibility between colonies. Furthermore, there is some evidence that the spatial organisation of a bumblebee colony is non-random, with 12% of individuals adhering to particular spatial zones (Comte et al., 2020). Consequently, in my experiment, the spatial zones of inoculated individuals may have varied in size across colonies, leading to variation in the area contaminated by faeces. For individuals with larger spatial zones, this could have increased the chance of transmission to a larger number of susceptible individuals.

Alternatively, the lack of difference in the estimated R_0 between early and late infected colonies could be a result of experimental limitations. The estimated R_0 was an approximate measure based on the average time it takes for an individual to become infectious. For example, some inoculated individuals may have shed propagules after two days and others after seven. Therefore, in this system there is high variability in the timing of secondary infections that makes detecting differences in the estimated R_0 between treatment groups very difficult, particularly when the number of colonies is relatively low. Furthermore, individuals were randomly selected to be inoculated and therefore, varied in size. Larger bees are more likely to transmit *C. bombi* due to larger defecation volumes and consequently colonies with a larger marked individual may have had a higher R_0 (Van Wyk et al., 2021). Combined, these experimental limitations likely contributed to the wide variation in R_0 within treatment groups. Consequently, it remains unclear whether colony demography affects R_0.

The difference in colony growth between colonies infected at different stages over time may have been a result of the infection. *Crithidia bombi* can reduce colony growth at the start of the lifecycle (Shykoff & Schmid-Hempel, 1991) and the early infected colonies were infected after the emergence of the first brood. An alternative explanation is that these colonies experienced the most disruption, as workers were removed biweekly from the start of their development causing stress and disruption to brood care and foraging. This stress could also have contributed to the reduction in males produced from colonies infected earlier in their lifecycle. However, I cannot distinguish between the effect of the parasite, experimental manipulation and the possibility that weaker colonies were coincidently assigned to particular treatment groups. There is a higher chance that weaker colonies were assigned to treatment one because it consisted of a lower sample size.

The limited transmission to males was predictable given the low prevalence of *C. bombi* at the end of the lifecycle in all colonies. Even in colonies still infected with *C. bombi*, the likelihood of encountering an infected individual or *C. bombi* propagules in the colony would likely have been very low over the

three to four days males spent there after eclosion. This would have been compounded by the fact that males were callows for most of this period. Callows spend a large proportion of time hidden under the brood with little contact with other individuals in the colony (HWG, pers. obvs.), that further reduces the likelihood of becoming infected when prevalence is low. Nevertheless, the fact that some males were infected indicates that within colony transmission to males must occur in the field, particularly in colonies with a high prevalence. I know that infected males are found in the field (Shykoff & Schmid-Hempel, 1991; Murray et al., 2013; Parsche & Lattorff, 2018), however, infection within the colony has not previously been demonstrated. Infected males could transmit *C. bombi* to queens during mating, as infected individuals often have propagules on their surface. Thus, it is feasible that males could contribute, through horizontal transmission during mating, to the ultimate vertical transmission of *C. bombi.*

In conclusion, I have shown that the colony demography of *B. terrestris* does affect the intracolony transmission of *C. bombi.* When colonies are infected earlier in their lifecycle, the infection is more likely to persist, however, a single infection is unlikely to cause a sustained epidemic. Rather, multiple infections are required for *C. bombi* persistence and vertical transmission to the next generation. These results can be applied to directly transmitted parasites in other social populations. They highlight that even for parasites with a long infectious period, a large number of susceptible individuals may not be sufficient to cause a sustained period of transmission. Consequently, in some cases smaller populations may be more susceptible to epidemics despite having a smaller pool of susceptible hosts. These findings inform our ability to predict the trajectory and outcome of directly transmitted parasites in social populations of varying sizes and structures.

Chapter 5
Large variation in the effect of climate on parasite prevalence in terrestrial animals: a meta-analysis

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Further analysis is being conducted prior to publication

Author contributions:
HSWG: Conceptualisation, experimental design, data acquisition, data analysis, manuscript drafting, manuscript editing.
JK: Experimental design, data analysis advice, manuscript editing.
MFJB: Experimental design, manuscript editing.

Abstract

Climate can vary spatially and temporally and is becoming increasingly unpredictable due to climate change. Climate can have a large impact on host-parasite interactions and investigating its effect is vital for understanding both current and future parasite distribution and epidemiology. I conducted a meta-analysis of 54 studies to determine how temperature, precipitation and humidity affect parasite prevalence in terrestrial animals. To my knowledge, this is the first quantitative synthesis to assess the overarching patterns in parasite prevalence across contrasting climates in terrestrial animals. I found that the effect of temperature and precipitation on parasite prevalence varies widely, with no overall effect of temperature or precipitation on prevalence. I found that the difference in temperature between field sites and experimental treatments may affect the likelihood of observing trends in prevalence, which has implications for the interpretation of results from previous studies, and the design of future studies. I found no difference in the effect of precipitation on endoparasite compared to ectoparasite prevalence and parasite lifecycle did not affect the relationship between temperature and parasite prevalence. Although primary studies had limited scope across some host and parasite taxa, I found no consistent taxonomic patterns. Importantly, I reveal large gaps in the literature. Focussing future research in these areas will help to confirm whether the general trends identified here hold true or whether certain types of host-parasite interactions are more sensitive to climate, with obvious implications for conservation.

5.1 Introduction

Climate plays a major role in shaping biotic interactions, from predator-prey (Laws, 2017) to host-parasite (Wu et al., 2016). Climate can vary spatially, between geographical areas, but also temporally across different seasons. Climate is also likely to change dramatically and often unpredictably, across the world in the future, driven by anthropogenic climate change (Pörtner et al., 2022). Given that climate change is already having a marked effect on terrestrial populations and communities (Parmesan, 2006; Thakur et al., 2022), it is important to understand how climatic factors will change and the implications of this for biotic interactions. Global temperatures are due to rise by 1.5°C over the next 20 years (Pörtner et al., 2022) and extreme heatwaves are becoming increasingly common (Coumou et al., 2013). Changes to precipitation are more difficult to predict, due to their interaction with land-use changes (eg. Zhou et al., 2010). However, it is generally agreed that the likelihood of extreme precipitation events and droughts will increase, as is already being observed (Pörtner et al., 2022). Climate models also predict that over land, relative humidity will fall (O'Gorman & Muller, 2010; Byrne & O'Gorman, 2013).

Due to the importance of parasites in ecological communities, understanding the effect of climate on host-parasite interactions is vital for both understanding their current distribution and epidemiology, and predicting how this will change under future climate scenarios. Parasites, hosts, vectors and transmission are all sensitive to climate and consequently, changes in climate can have a large impact on epidemiology. Here, I focus on the impact of climate on parasite prevalence, defined as the number of infected hosts out of the total sampled. Parasite prevalence can depend on parasite transmission, parasite development and host condition, all of which can be affected by climate (Fig. 1). Parasite growth and development may be higher at increased temperatures (Carrington et al., 2013; Leathwick, 2013; Portier et al., 2013), for example, *Escherichia coli* grows faster at higher temperatures (Freeman et al., 2009). Relative humidity can affect insect vector survival (Lyons et al., 2014) and thus, vector-borne transmission (Patz et al., 2003; Lega et al., 2017). In addition, airborne transmission can change with relative humidity (reviewed by La et al., 2022). Precipitation affects the survival of soil transmitted helminth eggs in the environment (Weaver et al., 2010) and the availability of breeding sites of mosquitoes (Patz et al., 2003; Gage et al., 2008). Climate may alter host body condition via changes in resource quality or quantity (eg. Marshal et al., 2008) and temperature can also affect the functioning of the immune system (Linder et al., 2008; Adamo & Lovett, 2011; Murdock et al., 2012). Whilst the impacts of individual climatic variables are important, understanding how combinations of them affect

host-parasite interactions has more realistic applications. This is emphasised by the fact that drought increases the availability of breeding sites for *Culex* mosquitoes, which vector dengue virus, while high temperatures increase the rate of development of dengue virus within mosquitoes. Thus, dry and hot climates together may result in high transmission (Epstein, 2001).

In the 1990s, multiple researchers observed that parasite prevalence was increasing in a wide array of species, and in particular, the prevalence of vector and waterborne pathogens (eg. Shope, 1991; Martens et al., 1995; Patz et al., 1996). This increase correlated with rising mean global temperatures, due to climate change, and led to the proposal of the 'warmer sicker world' hypothesis' (Harvell et al., 2002). This hypothesis predicts an increase in disease prevalence as global mean temperatures rise. Whilst there is some evidence of increased parasite prevalence at higher temperatures (eg. Garamszegi, 2011; Ruiz-Moreno et al., 2012), this hypothesis has received considerable criticism due to oversimplification (Hall et al., 2006; Lafferty, 2009; Altizer et al., 2013). This is partly because warming will also alter other climate variables, such as precipitation, humidity and drought. Although less is known about the effects of these, understanding their impact is necessary to predict future patterns in prevalence (Altizer et al., 2013).

Indeed, the impact of climate on host-parasite interactions is not easy to predict, partly because the intrinsic features of host-parasite interactions may affect the likelihood of climate driving their prevalence. For example, the prevalence of parasites with free-living stages might be more affected by climate than that of parasites without free living stages, due to the environmental exposure of free-living stages (Morgan, 2008; Morgan & van Dijk, 2012; Khadijah et al., 2013). Parasites with an arthropod vector may also be particularly sensitive to climate, because arthropods are poikilotherms and thus, their temperature fluctuates with the environment (Watts et al., 1987; Liu-Helmersson et al., 2014). Likewise, ectoparasites experience a higher level of environmental exposure compared to endoparasites and therefore, might have increased sensitivity to climate changes. Furthermore, host trophic level may influence prevalence, as carnivore diets include the intermediate hosts of a range of parasites. Consequently, carnivores may be exposed to a wider variety of parasites compared to herbivores, leading to higher parasite prevalence in carnivores. This effect can be seen by the fact that carnivores harbour a larger diversity of parasites than herbivores, although this is not universally the case (Watve & Sukumar, 1995). In addition, carnivores and herbivores may be differentially affected by the effects of climate on food availability. Climate can affect the availability of food, which may have consequences for host body condition and therefore, parasite susceptibility (Burger et al., 2012; Fancourt et al., 2018; Mann et al., 2019; Helman et al., 2022). In addition to biological factors, the methodological approach of a study could affect whether an effect of climate on prevalence is

detected. For example, it is difficult to control extraneous variables in field surveys and therefore, the effects of extraneous variables on prevalence may obscure the effects of climate on prevalence. In contrast, laboratory studies can control extraneous variables and consequently, they may be more likely to detect an effect of climate on prevalence.

Here, I conduct a meta-analysis to investigate whether there is an overall effect of temperature, precipitation or humidity on parasite prevalence in terrestrial animals. In addition, I test whether (i) the difference in prevalence between contrasting climates correlates with the magnitude of difference in climatic condition, (ii) the prevalence of ectoparasites is more affected by climate than that of endoparasites and (iii) the prevalence of parasites with free-living stages and vector-borne parasites is more affected by climate than parasites with direct, single host lifecycles. I also investigate whether the effect of climate varies with host and parasite taxonomy. In addition, I test whether the methodological approach, such as laboratory experiment or field survey, influences prevalence estimates.

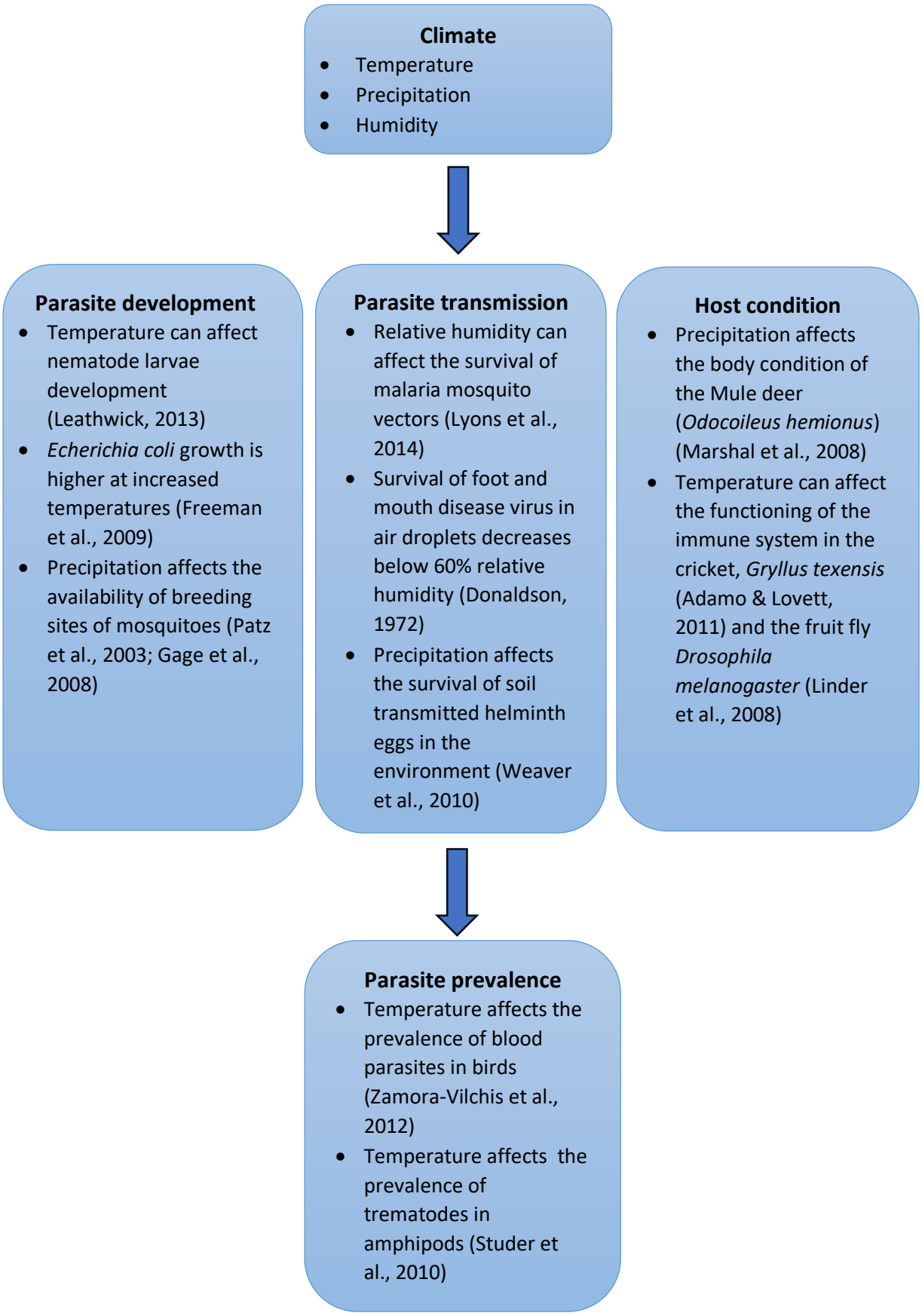


Fig. 1 Examples of the effects of temperature, precipitation and humidity on parasite development, parasite transmission and host condition that in turn, can affect parasite prevalence.

5.2 Methods

5.21 Literature searches

Searches were conducted in February 2021. Initially, the Web of Science Core collection database was searched using an advanced search of titles, abstracts and key words. No limits on publication dates were specified and thus, studies spanned 1970 to 2021. Studies were limited to those written in English, and to articles and early access papers. The search terms 'parasite', 'host susceptibility' and 'climate change' were used in combination. Multiple synonyms were used for each search term, in addition to truncations to pick up plurals and other wording variations.

The search string used for Web of Science Core Collection was TS=(parasit* or cestod* or helminth* or nematod* or tapeworm* or virus* or viral or acanthocephala* or copepod* or bacteria* or monogenea* or protozoa* or trematod* or digenea* or ectoparasit* or endoparasit* or pathogen* or flea* or tick* or mite* or leech* or nematomorph* or fungus or fungi or chytrid* or microsporidia* or trypanosome* or fungal) AND TS=("host susceptib*" or "host vulnerab*" or "disease vulnerab*" or "disease susceptib*" or "host* resistan*" or "disease resistan*" or prevalence or "disease level" or "disease abundan*" or "disease outcome*" or "disease intensit*" or "level of disease" or "degree of disease" or "pathogen intensit*" or "parasite intensity*" or "pathogen level*" or "level of pathogen*" or "pathogen abundan*" or "abundance of pathogen*" or "pathogen load" or "disease load" or "disease burden" or "parasite burden" or "pathogen burden") AND TS=("climate change" or "global warming" or "climat* varia*" or temperature* or precipitation or rain or rainfall or "water availability" or "water defic*" or "moisture defic*" or heat or thermal or humid* or cold* or chill* or altitud* or "elevation gradient" or latitud* or warm* or "ephemeral water bod*" or "temporary water bod*"). Supplementary searches were conducted in Scopus, PubMed and Google Scholar. In addition, Ethos, Base and OpenGray were searched to include sources of grey literature. Search strings were adapted to meet the criteria of other databases and these modified search strings are provided in Appendix 3.

5.22 Data screening

These searches returned 18,174 results and 12,995 after duplicate removal. Studies were screened by title, abstract and then full text using the Cadima synthesis tool (Kohl et al., 2018). The numbers of studies removed at each screening stage is shown in Fig. 2. Five full text papers could not be found, despite authors being contacted. Inclusion criteria were used to screen studies to ensure they were

relevant and contained suitable data (Table 1). Criteria were designed based on the population, intervention, comparison and outcome in accordance with the Collaboration for Environmental Evidence guidelines (Pullin et al., 2018). When data were missing authors were contacted when possible.

5.23 Critical Appraisal

The Collaboration for Environmental Evidence critical appraisal tool was used to assess the validity of studies (Konno et al., 2020). Studies were ranked as low, medium or high validity based on these criteria. A subset of 10 studies were critically appraised by two people (HSWG and MJFB) and the results cross-referenced to ensure the criteria were being applied consistently.

Table 1 Eligibility criteria used to screen studies, defined by criteria for the population, the intervention, the comparison and the outcome. Studies that were included met all criteria.

Population: **Terrestrial animal hosts or a vector**	**Intervention:** **Climate (temperature, precipitation or humidity)**	**Comparison:** **Parasite prevalence in multiple field locations or experimental treatments**	**Outcome:** **Parasite prevalence**
• Study records the total number of hosts or vectors sampled for prevalence in each location or experimental group. • Number of hosts or vectors sampled must be more than 1. • Hosts include terrestrial hosts and vectors of a parasite.	• The temperature, precipitation or humidity data at each site must have been measured or extracted by the authors. • Excluding seasonal data from a single location. • Differences in microclimate (defined as climate differences in a small local area) were excluded. For example, a sunny compared to a shaded area, that hosts can move between, based on their biology. Differences in the wider climate (defined by two separate locations where it is not possible or much harder for hosts to move between, based on their biology) were included.	• At least two climate conditions are compared, or combination of conditions are compared in two different places with different climatic conditions, including laboratory conditions. • The same host and parasite interaction must be studied in two or more climate conditions.	• Parasite is defined as an organism living in or on another organism and obtaining food or resources from the host (parasitoids excluded). • Parasite prevalence is defined as the number of infected hosts out of the total sampled. • Data for prevalence must be available for each location of differing climate.

5.24 Data Extraction

Data was extracted into separate excel datasets depending on which climatic conditions the study investigated. For example, one database contained studies that measured the effect of temperature and another contained studies that investigated both temperature and precipitation (combinations of climatic variables are shown in Table 2). Each row in the dataset consisted of a comparison of one host-parasite interaction in two or more locations or experimental treatments. When one study sampled prevalence of several host-parasite interactions, these were entered as separate rows. Different studies compared prevalence in a different number of locations or experimental treatments. For each location or experimental treatment where prevalence was measured, the temperature, precipitation or humidity measurement, prevalence and sample size were extracted. Therefore, the number of columns for each row varied depending on the number of locations of experimental treatments prevalence was measured in. For each row entry, the following data were also recorded:

- Host taxonomic group, hosts were classified into mammal, reptile, amphibian, arthropod, bird, mollusc or annelid
- Host trophic level, hosts were categorized into herbivore, omnivore or carnivore
- Whether prevalence in the host or vector was being measured
- Parasite taxonomic group, parasites were classified into bacterium, fungus, virus, helminth, protozoa, flea (*Siphonaptera*), mite (*Acariformes* and *Parasitiformes*), louse (*Phthiraptera*) or fly (*Diptera*)
- Parasite lifecycles were categorized into single host lifecycle, multiple host lifecycle or vector-borne lifecycle
- Parasite lifecycles were categorized as having free-living stages or not
- Parasites were classified as ectoparasite or endoparasite
- The methodological approach was categorized as a field survey, field experiment or laboratory experiment

There were not sufficient numbers of studies or comparisons to allow inclusion of effects of humidity (alone and in combination with temperature and precipitation) into the further analysis (Table 2). Therefore, only temperature, precipitation and the combined temperature and precipitation dataset were used for data analysis.

Table 2 The number of studies and comparisons within each climate variable. Climate variables marked with a (*) were used in further data analysis.

Climate variable	Number of studies	Number of comparisons
Temperature*	21	62
Precipitation*	4	26
Humidity	1	5
Temperature & precipitation*	21	60
Temperature & humidity	3	12
Precipitation & humidity	0	0
Temperature, precipitation & humidity	4	8

Studies compared prevalence in a highly variable number of field locations or experimental treatments, for example, some measured prevalence in two field sites, whereas other studies measured prevalence in 10. This made it difficult to select comparators for each climatic condition. To deal with this issue, for studies with more than two climatic conditions I randomly selected a comparator for each condition and therefore, for every row two locations or experimental treatments with differing climatic conditions were compared. To test the effects of random comparator selection on the results, 10 randomly generated permutations were computed for the temperature, precipitation, and combined temperature and precipitation datasets, resulting in 30 datasets. 10 permutations were chosen because across all climatic variables, 94-96% of studies compared prevalence in less than 10 locations or experimental treatments. I did not create datasets using locations or treatments with the minimum and maximum temperature or precipitation because (i) there were some cases where multiple locations had the highest temperature or precipitation, (ii) this could have biased my analyses towards finding effects, and (iii) I wanted to use as much of the data as possible.

5.25 Meta-analysis

Analyses were conducted in R version 4.2.0 (Team, 2022) using RStudio "Prairie Trillium" (Team, 2022) and the package metafor (Viechtbauer, 2010). Figures were generated using the packages ggplot2 (Wickham, 2016) and orchaRd (Nakagawa et al., 2019). Effect sizes were calculated as the log odds ratios and associated variance of the number of infected compared to uninfected hosts in location/treatment 1 compared to location/treatment 2 (Wilson & Mason, 2017). These were calculated using the function escalc() and its default parameters. Ratios were calculated such that

location/treatment 1 always had the lower temperature or precipitation. For example, if one location had a temperature of 23°C and another 30°C, the location of temperature 23°C would be location 1. The difference in temperature or precipitation for each pairwise comparison was calculated as temperature of location 2 - location 1 and therefore, was always positive. For the combined temperature and precipitation dataset, location/treatment 1 had the lowest temperature. Temperature difference between the sites was always positive as before. Precipitation difference was calculated as location 2 – 1 and was not always positive.

For the temperature, precipitation and combined temperature and precipitation datasets, the first permutation dataset was analysed initially. Selecting permutation one did not bias results as each permutation was created randomly as described above. The rma.mv function was used to construct a three-level random effects model using restricted maximum likelihood estimation in order to assess the overall mean effect of the climatic factor on prevalence. Study and effect size ID were included as random effects with effect size ID nested within study, since there were some studies with multiple effect sizes and therefore, these effect sizes were not independent. A t-distribution was used to reduce the chance of a false significant result (Assink & Wibbelink, 2016). In a three-level model effect sizes can vary due to sampling variation (level 1), variation within studies (level 2) and variation between studies (level 3). A one-tailed log-likelihood ratio test was used to compare the three-level model with a two-level model that did not include variance within studies (level 2 variance was constrained to 0). The three-level model was also compared to a model that assumed independent effect sizes (level 3 variance was constrained to 0), to test whether between study variability was significant (Assink & Wibbelink, 2016). As part of sensitivity analysis, for each dataset, the three-level model was repeated for each of the nine other permutations, to test whether different permutations yielded similar model results.

When datasets contained studies with a range of critical appraisal bias ratings (see above), sensitivity analysis was performed, by repeating the three-level model excluding the studies rated as highly biased. Sensitivity analysis was also conducted to test whether including only studies that measured prevalence in two field sites or experimental treatments produced the same model results as using only studies that measured the prevalence in three or more field sites or experimental treatments.

To analyse potential sources of variation in effects of climate on parasitism prevalence, moderator analyses were performed when sampling variance (level 1) was below 75% of the total variation (Schmidt & Hunter, 2015). Temperature or precipitation difference between sites or treatments were

included as continuous moderators. Host taxonomy, host trophic level, parasite taxonomy, whether prevalence was measured in a host or vector, parasite lifecycle, whether the parasite lifecycle had free-living stages, whether the parasite was an ectoparasite or endoparasite and the methodological approach were included as moderators in separate multilevel models. Categorical moderators were only included when each level of the moderator contained at least four effect sizes.

Not all hypotheses regarding categorical moderators could be tested due to insufficient data (see Appendix 3). Whether parasites had free-living stages and their lifecycle were combined into one moderator with the following categories: single host lifecycle no free-living stages, single host lifecycle with free-living stages and vector-borne lifecycle. This was because by definition, there were no effect sizes with both vector-borne lifecycles and free-living stages and therefore, it would not have been possible to separate the effects of these two moderators.

5.26 Publication bias

I tested for publication bias by using an Egger's regression test modified for multilevel models. The three-level model was altered to include effect size variance of the odds ratio as a moderator, following Habeck & Schultz (2015). The test shows that there is publication bias when the relationship between the precision and size of studies is asymmetrical. This is the case when the intercept coefficient is significantly different from zero at the 0.1 level (Egger et al., 1997).

5.3 Results

5.31 Overview of studies included

My search returned 18,174 studies and after screening I included 54 studies for further analyses (Fig. 2, see Appendix 3 for a list of all studies). Excluding laboratory studies, studies sampled prevalence in 27 countries, spanning all continents apart from Antarctica (Fig. 3). Australasia and North America had the lowest number of studies with four each and Europe the most with 14 studies.

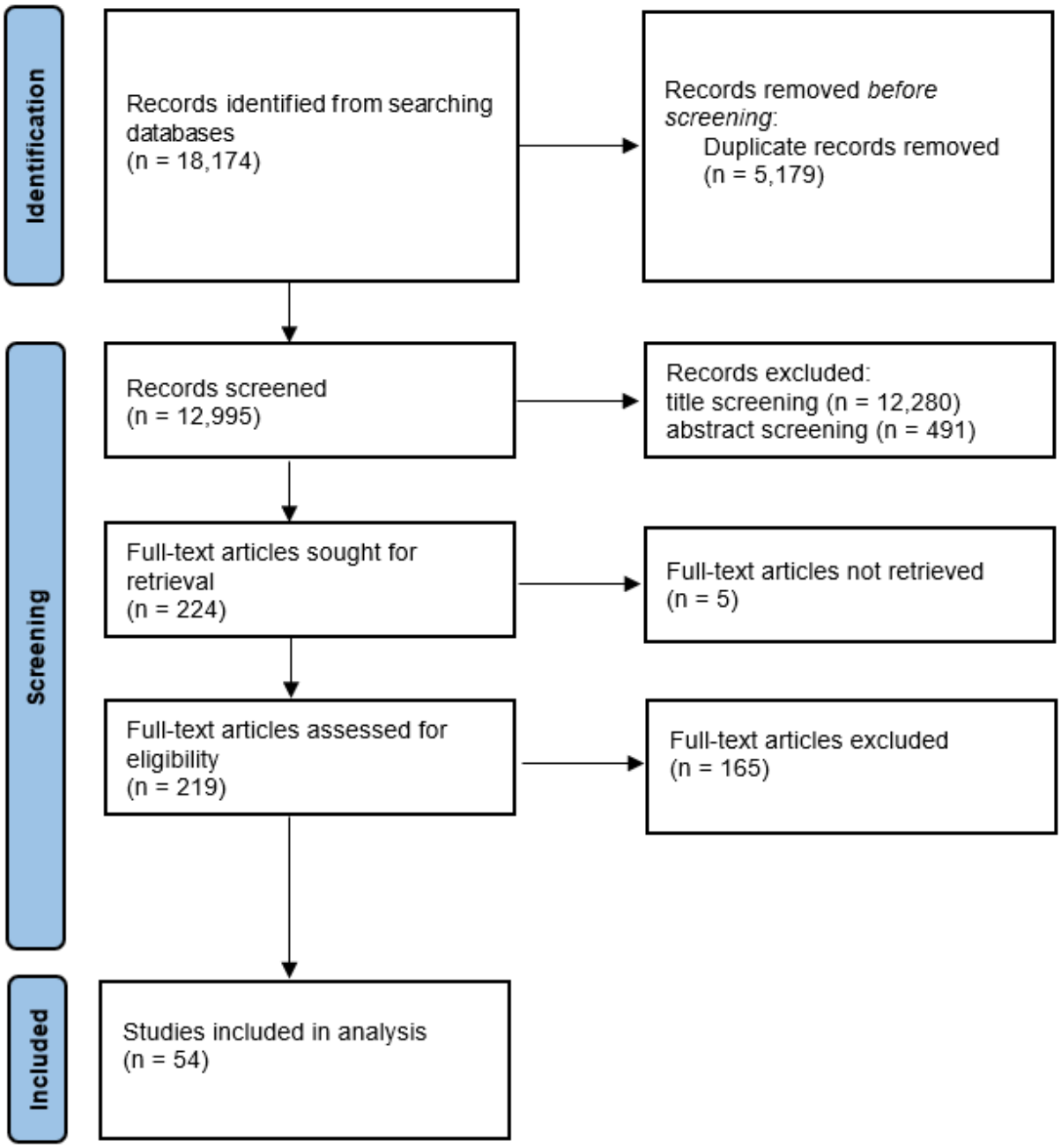


Fig. 2 Number of studies retrieved and removed at each stage of screening, shown as a PRISMA flow diagram.

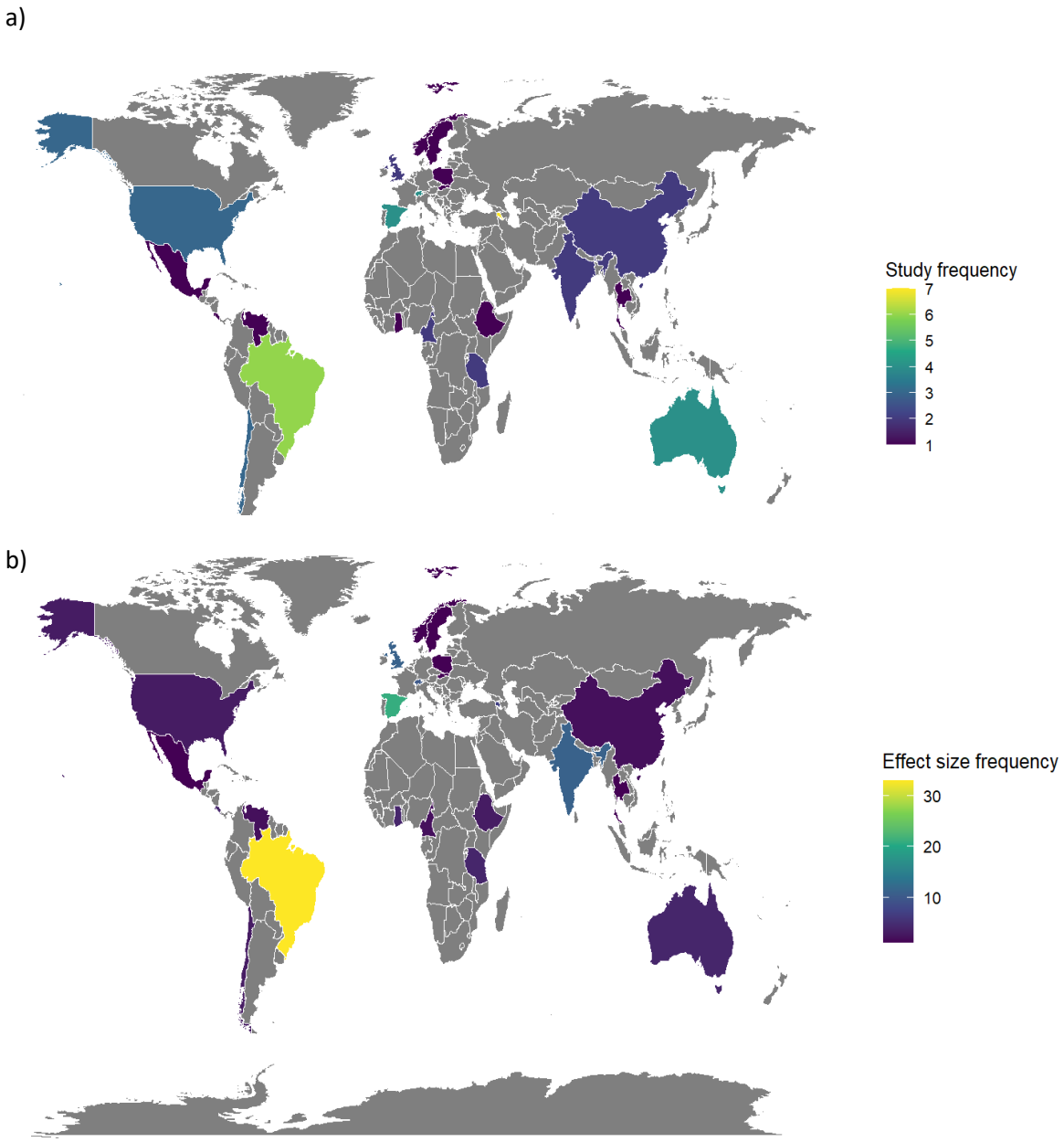


Fig. 3 a) Distribution of field studies included in this meta-analysis. Shaded countries are where hosts were sampled for parasite prevalence. The frequency is shown by the colour shading. **b)** Same as a), for the number of effect sizes from field studies in each country. Some studies measured the prevalence of multiple parasites or the prevalence of the same parasite in multiple hosts and these were analysed as separate effect sizes.

5.32 Effect of temperature on parasite prevalence

21 studies including 62 comparisons investigated the effect of temperature on prevalence of infection. The three-level random effects model showed that overall an increase in temperature does not significantly affect the risk of parasite infection (transformed OR = 1.05, 95% CI = 0.61, 1.82, $p = 0.852$; Fig. 4). Estimated variance was $\tau^2_{Level\ 3} = 0.655$ and $\tau^2_{Level\ 2} = 1.539$. 26.93% of the total variation was explained by between study heterogeneity and 63.25% by within study heterogeneity. Within study variation was not significant ($\chi_2 = 2.638$, $p = 0.052$). The two-level model that assumed independent effect sizes was a significantly better fit ($\chi_2 = 201.296$, $p < 0.001$). However, the full model was used for subsequent analyses to account for the lack of independence of effect sizes within studies. Moderator analyses were performed since 9.81% of total heterogeneity was attributed to sampling variance (see below).

Sensitivity analysis for studies critically appraised as low, medium or high validity was not needed, since all studies were rated as medium validity. Furthermore, the three-level model was repeated excluding the five studies that had only two comparisons, with the same qualitative result (transformed OR = 1.2, 95% = 0.72, 1.998, $p = 0.464$, $\tau^2_{Level\ 3} = 0.29$, $\tau^2_{Level\ 2} = 1.55$). Repeating the three-level model for the other permutations yielded similar results to permutation one reinforcing the result that temperature did not affect infection prevalence (Table A3.1). Permutation one was used for further analyses.

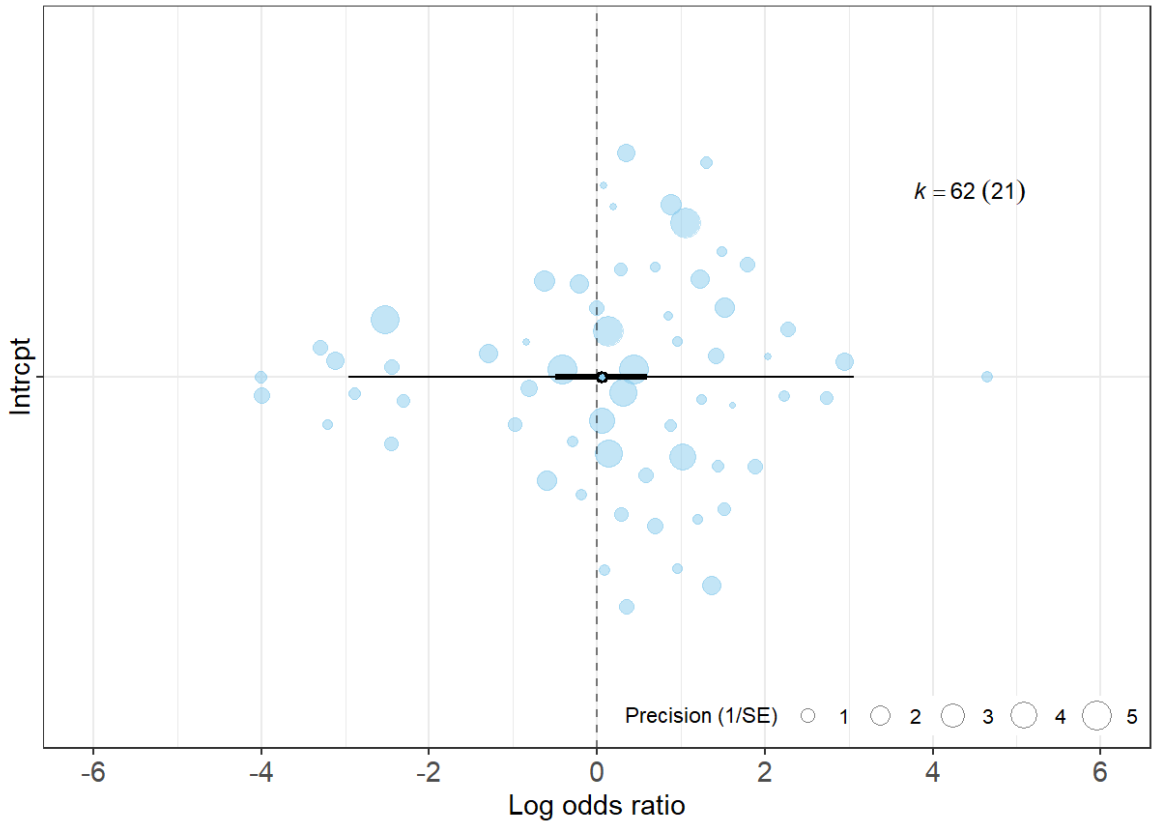
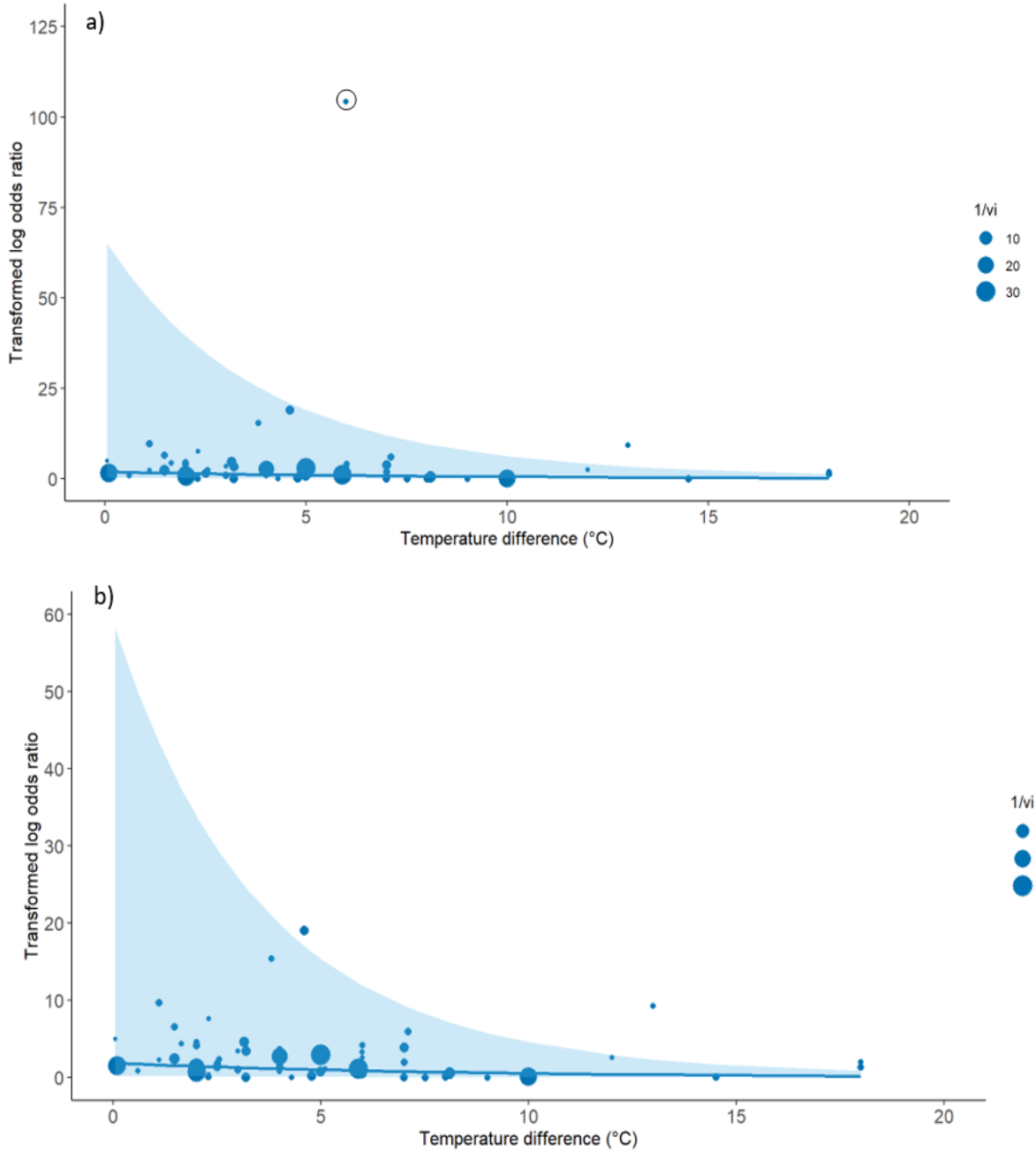


Fig. 4 Orchard plot showing results from the random effects three-level model of the effect of temperature on prevalence using data from permutation one. K shows the number of effect sizes and the number of studies that data were extracted from is shown in brackets. Thick bars are 95% confidence intervals and thin bars are 95% prediction intervals. When 95% confidence intervals do not overlap with zero the effect is significant.

The difference in temperature between two sites or experimental groups was included as a continuous moderator ($F_{(1, 60)} = 3.63$, $p = 0.0615$) and did not significantly affect the likelihood of observing higher parasite prevalence at higher temperatures (transformed OR = 0.517, $t_{(62)} = -1.91$, $p = 0.0615$; Fig. 5a). However, analysis of Figure 5a identified one outlier, which was effect size one from study 37. When the three-level model including temperature difference was repeated without this outlier, temperature difference significantly affected the prevalence of infection ($F_{(1, 59)} = 4.536$, $p = 0.0374$). The positive effect of temperature increase on prevalence of infection decreased with an increase in temperature differences between the locations (transformed OR = 0.89, $t_{(61)} = -2.13$, $p = 0.0374$; Fig. 5b, c).


a)
125
100
75
50
25
0
Transformed log odds ratio
0
5
10
15
20
Temperature difference (°C)
1/vi
10
20
30
b)
60
50
40
30
20
10
0
Transformed log odds ratio
0
5
10
15
20
Temperature difference (°C)
1/vi
10
20
30

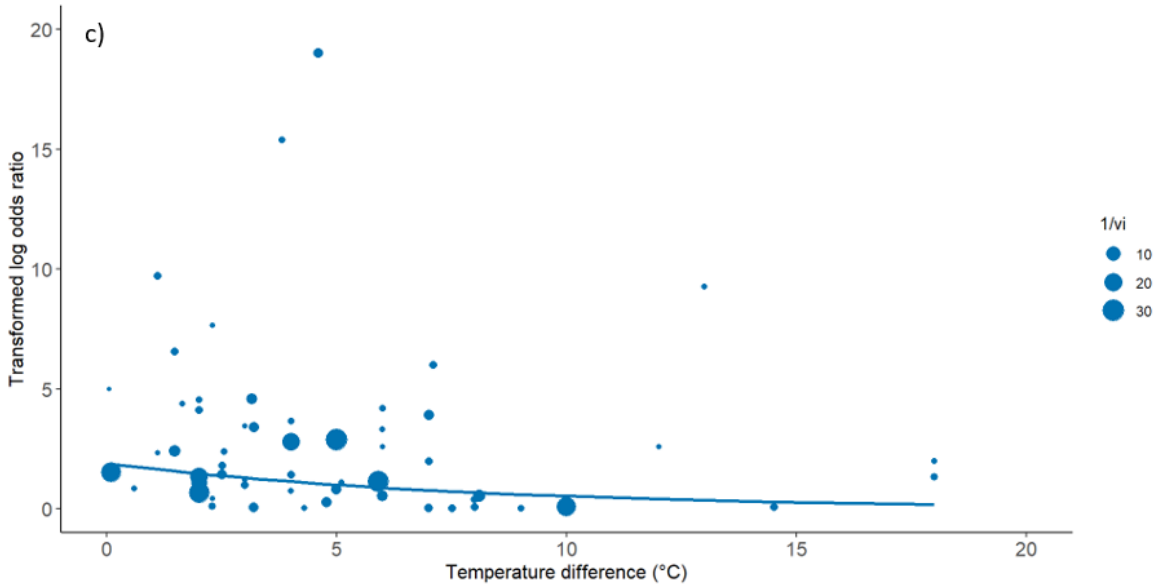
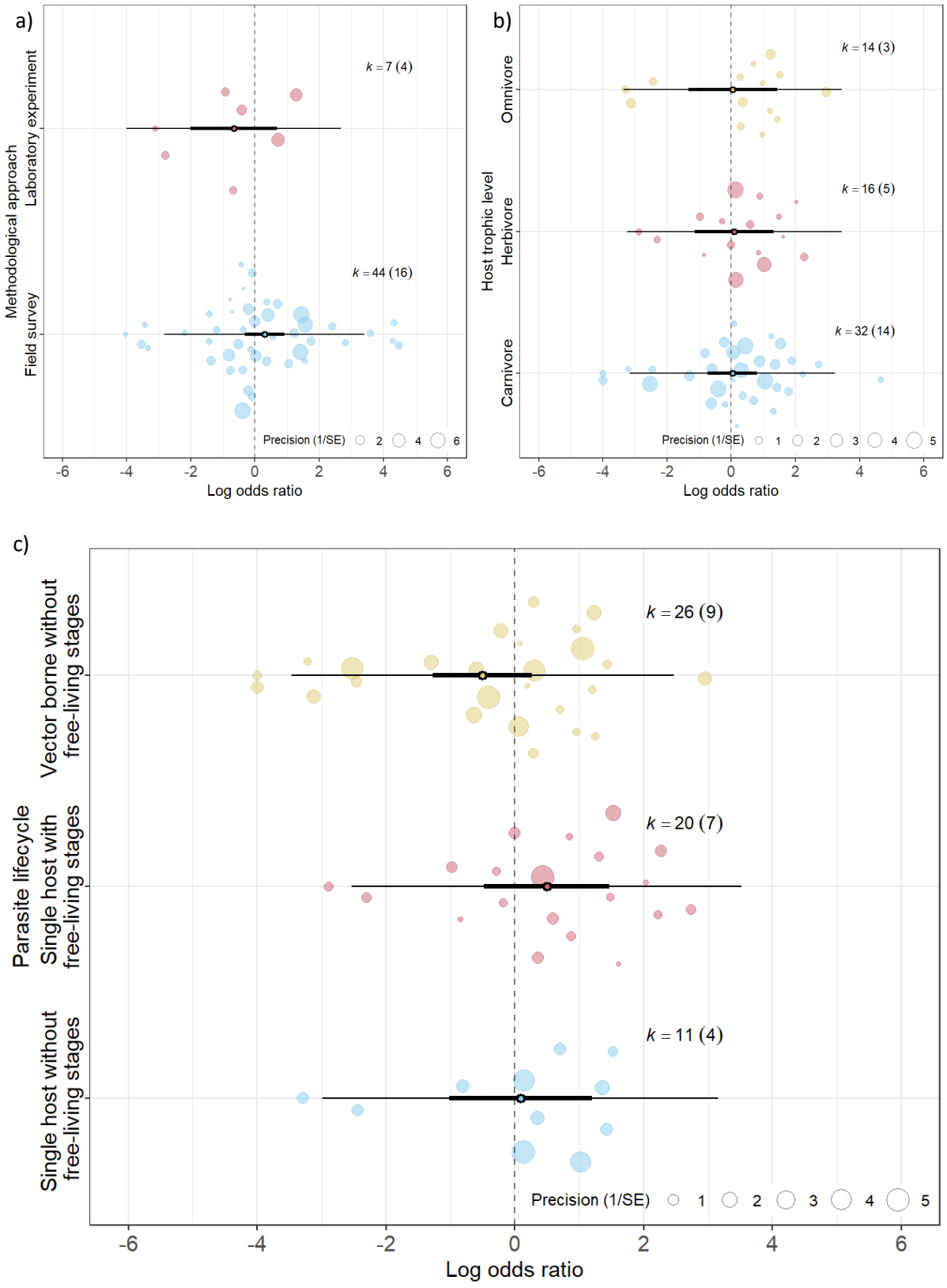


Fig. 5 a) Scatterplot of the difference in temperature between each field site or experimental treatment and the transformed log odds ratio of infected compared to uninfected in each location or treatment group. The transformed log odds ratio is the likelihood of having a higher parasite prevalence at higher temperatures. Each point is an effect size, weighted by 1/effect size variance. Line shows the predicted values from the three-level model including temperature difference as a moderator and data from permutation one. Shaded area shows 95% prediction intervals. **b)** as plot a), excluding the outlier labelled with a circle. **c).** Same as b) not including 95% prediction intervals to highlight the prediction line.

Methodological approach did not have a significant effect on the relationship between the likelihood of infection and temperature ($F_{(1, 58)} = 1.51$, $p = 0.132$; Fig. 6a, Table A3.2). Similarly, host trophic level did not affect the relationship between the likelihood of infection and temperature ($F_{(2, 59)} = 0.003$, $p = 0.997$; Fig.6b, Table A3.2). There were no significant differences in effects of temperature on prevalence of parasitism by parasites with free-living stages and single host lifecycles, parasites with single host lifecycles and no free-living stages, and vector-borne lifecycles ($F_{(2, 54)} = 1.341$, $p = 0.270$; Fig 6c, Table A3.2). Host and parasite taxonomy did not significantly influence the effects of temperature on parasite prevalence (host taxonomy: $F_{(3, 52)} = 1.780$, $p = 0.163$; Fig. 6d, Table A3.2, parasite taxonomy: $F_{(4, 56)} = 0.821$, $p = 0.457$; Fig. 6e, Table A3.2). Finally, whether a host or vector was infected did not affect the relationship between the likelihood of infection and temperature ($F_{(1, 60)} = 2.95$, $p = 0.091$; Fig. 6f, Table A3.2).


a)
Methodological approach
Laboratory experiment
Field survey
k = 7 (4)
k = 44 (16)
Precision (1/SE) 2 4 6
-6 -4 -2 0 2 4 6
Log odds ratio
b)
Host trophic level
Omnivore
Herbivore
Carnivore
k = 14 (3)
k = 16 (5)
k = 32 (14)
Precision (1/SE) 1 2 3 4 5
-6 -4 -2 0 2 4 6
Log odds ratio
c)
Parasite lifecycle
Vector borne without free-living stages
Single host with free-living stages
Single host without free-living stages
k = 26 (9)
k = 20 (7)
k = 11 (4)
Precision (1/SE) 1 2 3 4 5
-6 -4 -2 0 2 4 6
Log odds ratio

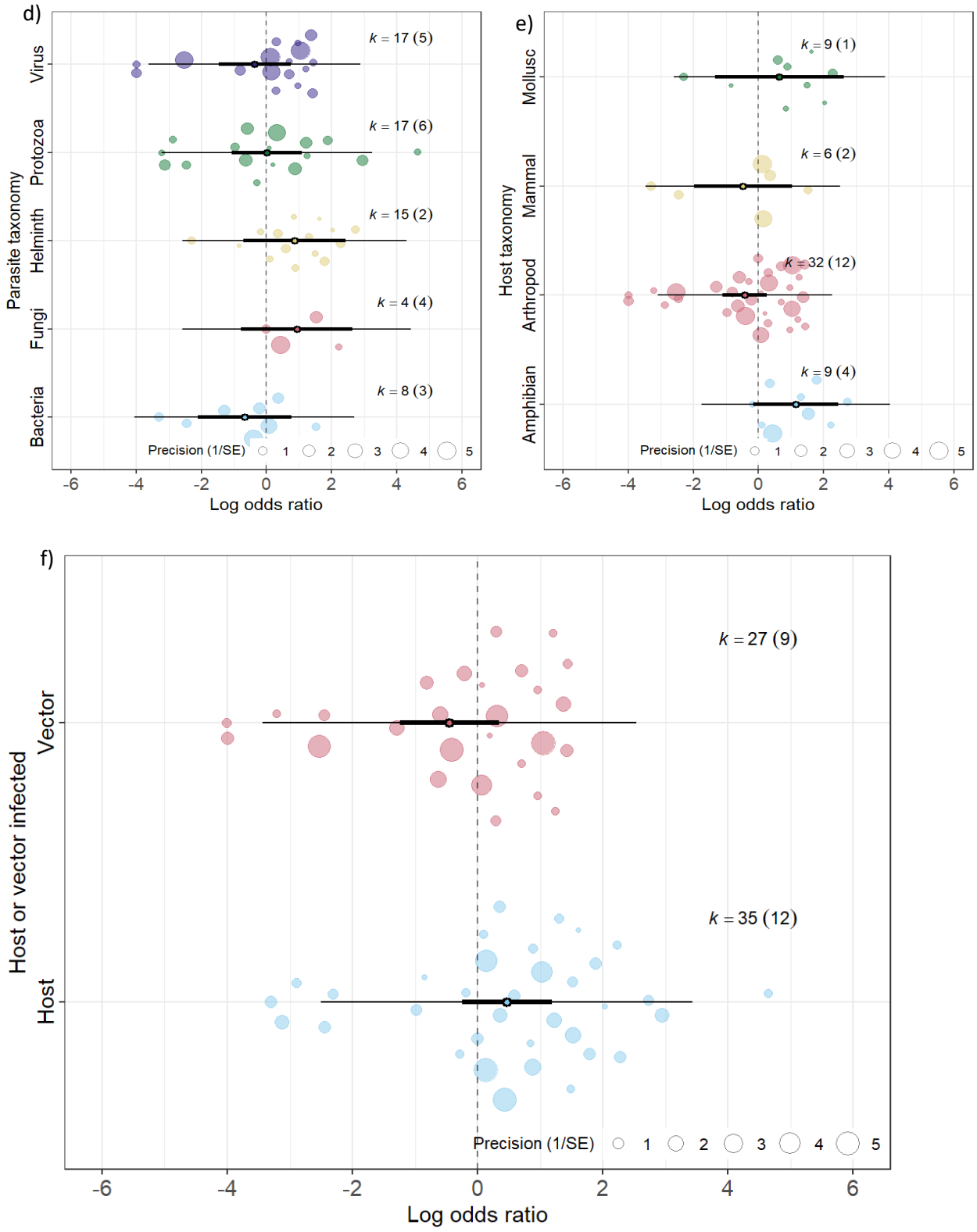


Fig. 6 Orchard plots showing the results from the three-level random effects model on the effect of temperature on prevalence including moderators using data from permutation one. K shows the number of effect sizes and the number of studies that data were extracted from is shown in brackets. Thick bars are 95% confidence intervals and thin bars are 95% prediction intervals. When 95%

confidence intervals do not overlap with zero the effect is significant. Moderators included were **a)** methodological approach **b)** host trophic level **c)** parasite lifecycle and whether the parasite has free-living stages **d)** parasite taxonomy, **e)** host taxonomy and **f)** whether a host or vector was infected.

5.33 Effect of precipitation on parasite prevalence

Four studies with 25 comparisons investigated the effect of precipitation on prevalence of infection. Overall, precipitation did not significantly affect prevalence (transformed OR = 1.65, 95% = 0.71, 3.83, $p = 0.233$; Fig. 7). Estimated variance was $\tau^2_{Level\ 3} = 0.39$ and $\tau^2_{Level\ 2} = 0.64$ and therefore, 31.4% of the total variation was explained by between study heterogeneity and 51.4% by within study heterogeneity. Comparing this model to a two-level model showed that within study variation was not significant ($\chi^2 = 0.125$, $p = 0.724$). A reduced model, that assumed independent effect sizes, was a significantly better fit ($\chi^2 = 25.744$, $p < 0.001$). As before, the full three-level model was used to account for the lack of independence of effect sizes within studies. Moderator analyses were performed because 17.19% of total heterogeneity was attributed to sampling variance (see below).

Sensitivity analysis for studies critically appraised as low, medium or high validity was not needed, since all studies were rated as medium validity. Furthermore, the three-level model gave qualitatively similar results when repeated excluding the seven studies that had only two comparisons (transformed OR = 0.97, 95% = 0.58, 1.63, $p = 0.905$, $\tau^2_{Level\ 3} = 0$, $\tau^2_{Level\ 2} = 0.43$). Repeating the three-level model using data from the other nine permutations largely also supported the conclusion that precipitation does not significantly affect prevalence (Table A3.3). However, in the analysis of the permutation nine dataset, precipitation significantly affected prevalence (transformed OR = 1.7, 95% = 1.001, 2.87, $p = 0.047$), with a higher chance of infection at higher levels of precipitation. When the Bonferroni correction was applied to account for the multiple testing of each permutation, the effect was no longer significant at the 0.005 level (0.05/10).

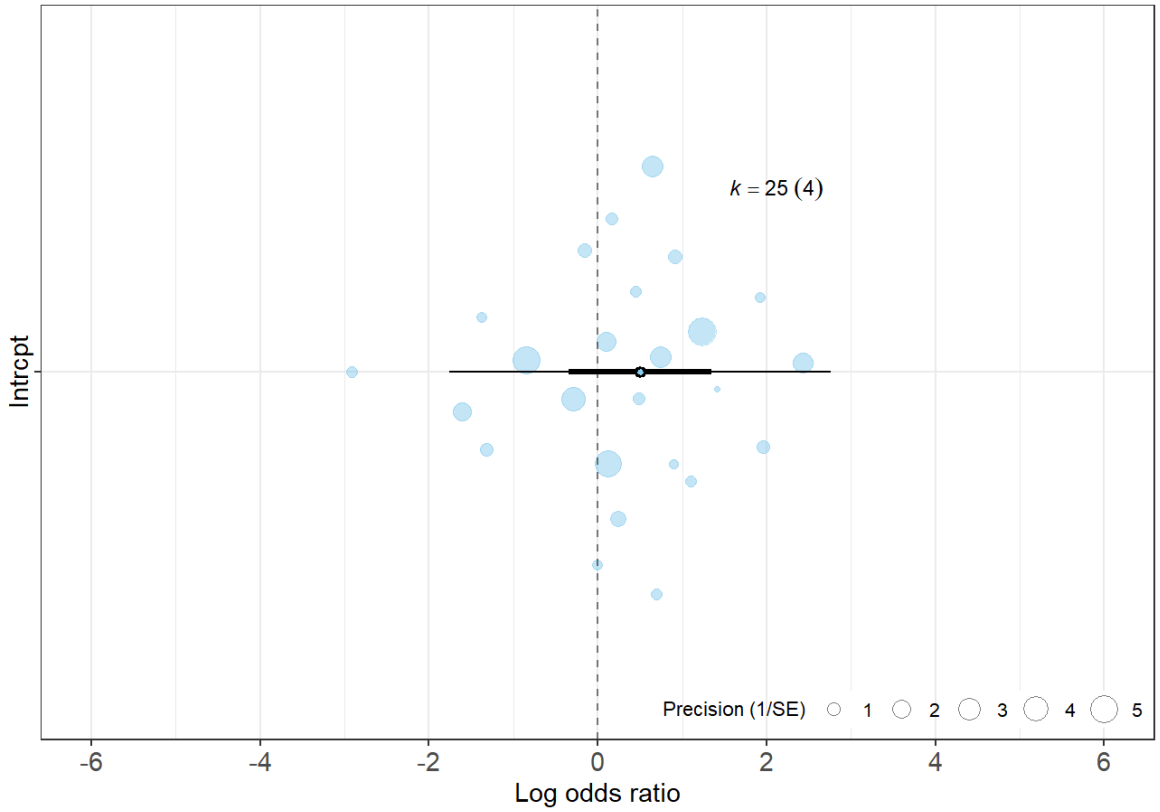


Fig. 7 Orchard plot showing results from the three-level random effects model of the effect of precipitation on prevalence using data from permutation one. K shows the number of effect sizes and the number of studies that data were extracted from is shown in brackets. Thick bars are 95% confidence intervals and thin bars are 95% prediction intervals. When 95% confidence intervals do not overlap with zero the effect is significant.

Precipitation difference between field sites significantly affected the relationship between precipitation and prevalence of infection ($F_{(1, 23)}$ = 9.135, p = 0.006). The slight positive effect of precipitation on prevalence increased with differences in precipitation between site (OR = 1.0003, $t_{(25)}$ = 3.022, p = 0.006; Fig. 8). However, when three outliers with precipitation differences over 4000mm were excluded from analysis, precipitation difference was no longer a significant moderator ($F_{(1, 20)}$ = 3.096, p = 0.0938) and higher prevalence at higher precipitation levels were not more likely to be observed at higher precipitation differences (1.000, $t_{(20)}$ = 1.76, p = 0.0938).

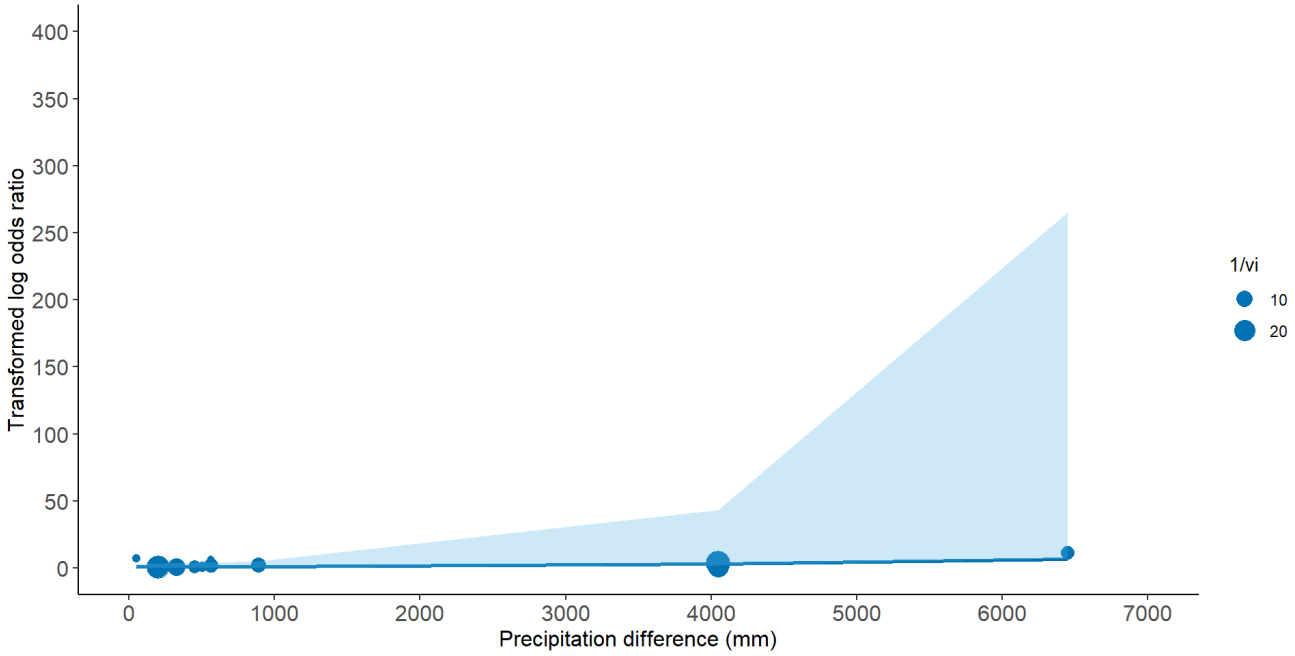


Fig. 8 Scatterplot of the difference in precipitation between each field site and the transformed log odds ratio of infected compared to uninfected in each location. The transformed log odds ratio is the likelihood of having a higher parasite prevalence at higher precipitation levels. Each point is an effect size, weighted by 1/effect size variance. The line shows predicted values from a three-level random effects model including precipitation difference as a moderator and data from permutation one. Shaded area shows 95% prediction intervals.

Host trophic level did not alter the effect of precipitation on parasite prevalence ($F_{(1, 22)} = 0.105$, $p = 0.749$; Fig. 9a; Table A3.4). Similarly, whether a parasite was an endoparasite or ectoparasite did not affect the relationship between parasite prevalence and precipitation ($F_{(1, 23)} = 0.437$, $p = 0.515$; Fig. 9b; Table A3.4).

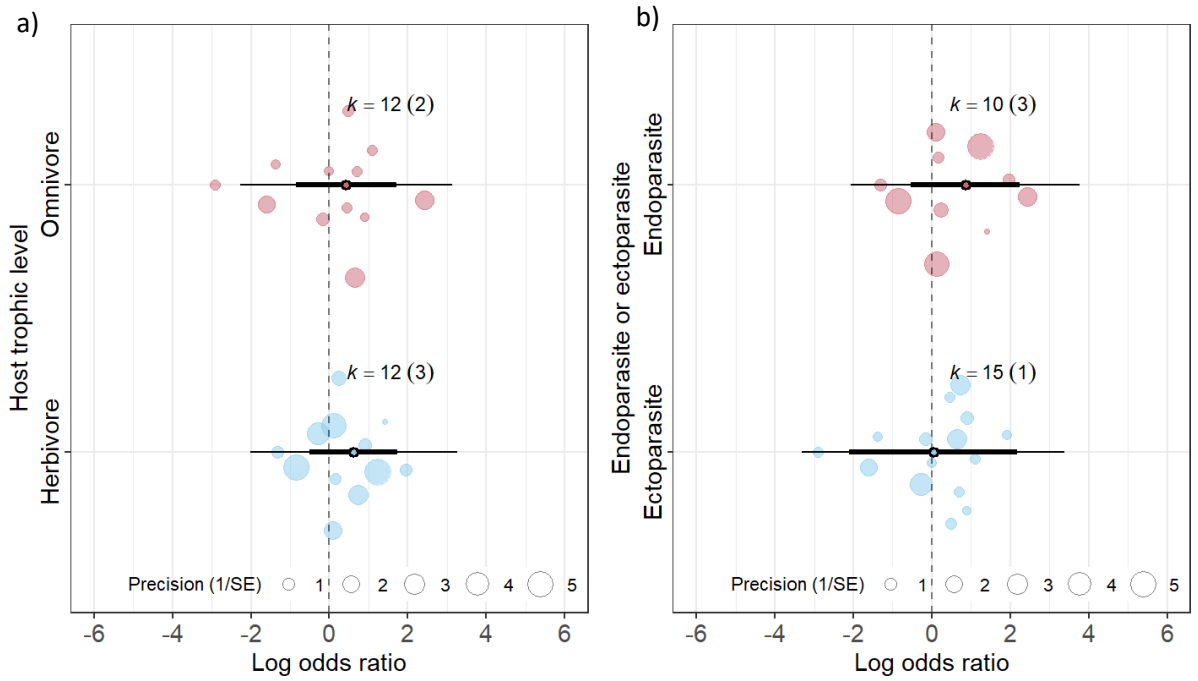


Fig. 9 Orchard plots showing the results from the random effects three-level model of the effect of precipitation on prevalence including moderators using data from permutation one. K shows the number of effect sizes and the number of studies that data were extracted from is shown in brackets. Thick bars are 95% confidence intervals and thin bars are 95% prediction intervals. When 95% confidence intervals do not overlap with zero the effect is significant. Moderators included were **a)** host trophic level and **b)** whether the parasite was an ectoparasite or endoparasite.

5.34 Combined effects of temperature and precipitation on parasite prevalence

20 studies with 51 effect sizes investigated effects of both temperature and precipitation. The original three-level model tested just the effect of temperature on parasite prevalence, while temperature and precipitation difference were tested by including moderators. The three-level model demonstrated that an increase in temperature does not significantly affect the overall odds risk of parasite infection, mirroring results from the analysis of just temperature studies (see above) (transformed OR = 1.13, 95% = 0.65, 1.96, $p = 0.649$; Fig. 10). Estimated variance was $\tau^2_{Level\ 3} = 0.32$ and $\tau^2_{Level\ 2} = 1.99$ and thus, 12.84% of the total variation was explained by between study heterogeneity and 80.76% by within study heterogeneity. Within study variation was not significant ($\chi^2 = 0.33$, $p = 0.057$) and a reduced two-level model was a significantly better fit ($\chi^2 = 54.84$, $p < .001$), however as described previously I proceeded with a three-level model to account for multiple comparisons within

one study. Repeating the three-level model for the other permutations yielded similar results to permutation one (Table A3.5).

34 effect sizes were categorised as medium biased and 17 as highly biased, therefore a sensitivity analysis was conducted that excluded the effect sizes categorized as highly biased. The pooled log odds ratio when these effect sizes were removed was estimated as transformed OR = 0.88 and again was non-significant (95% = 0.52, p = 0.614). The full model was also repeated excluding the 17 effect sizes that had two comparisons, but with the same qualitative result (transformed OR = 0.86, 95% = 0.46, 1.62, p = 0.63). 6.39% of variance was attributed to sampling variance, which confirmed the need to perform moderator analyses.

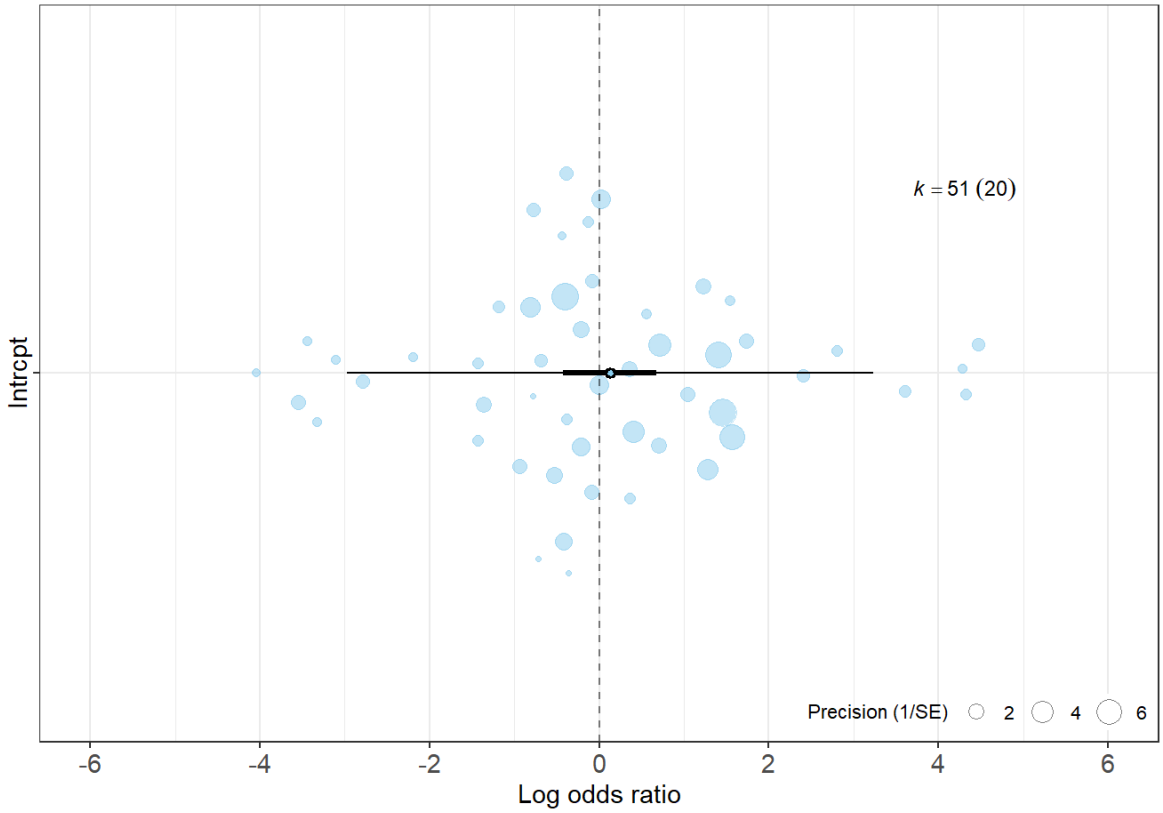
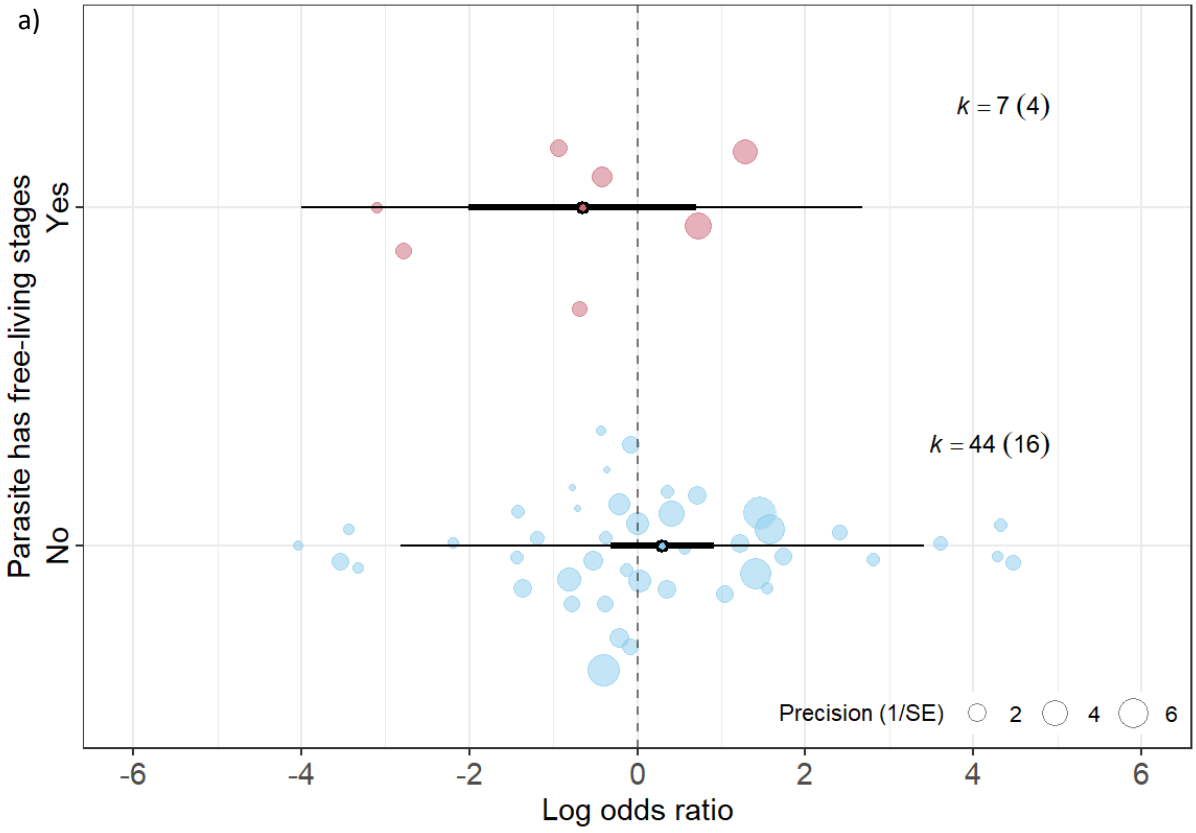


Fig. 10 Orchard plot showing results from the random effects three-level model of the effect of temperature and precipitation on prevalence using data from permutation one. K shows the number of effect sizes and the number of studies that data were extracted from is shown in brackets. Thick bars are 95% confidence intervals and thin bars are 95% prediction intervals. When 95% confidence intervals do not overlap with zero the effect is significant.

Table 3 Model results from three-level random effects models when temperature and precipitation difference were included as continuous moderators separately and together.

	Beta coefficient	$T_{(51)}$	*p*
Temperature difference	0.367	-1.445	0.155
Precipitation difference	0.141	-0.844	0.403
Temperature and	0.368	-1.31	0.195
precipitation difference	0.514	-0.724	0.473

Temperature difference and precipitation difference between sites did not significantly influence the effects of climate on parasite prevalence (Table 3). Similarly, whether the parasite has free-living stages did not affect the relationship between temperature and precipitation and parasite prevalence ($F_{1, 49}$ = 1.656, *p* = 0.204; Fig. 11a, Table A3.6). Finally, host taxonomic group and host trophic level did not change the effect of temperature and precipitation on parasite prevalence ($F_{1, 47}$ = 0.343, *p* = 0.561, Fig. 11b; $F_{2, 48}$ = 0.006, *p* = 0.994; Fig. 11c, Table A3.6).



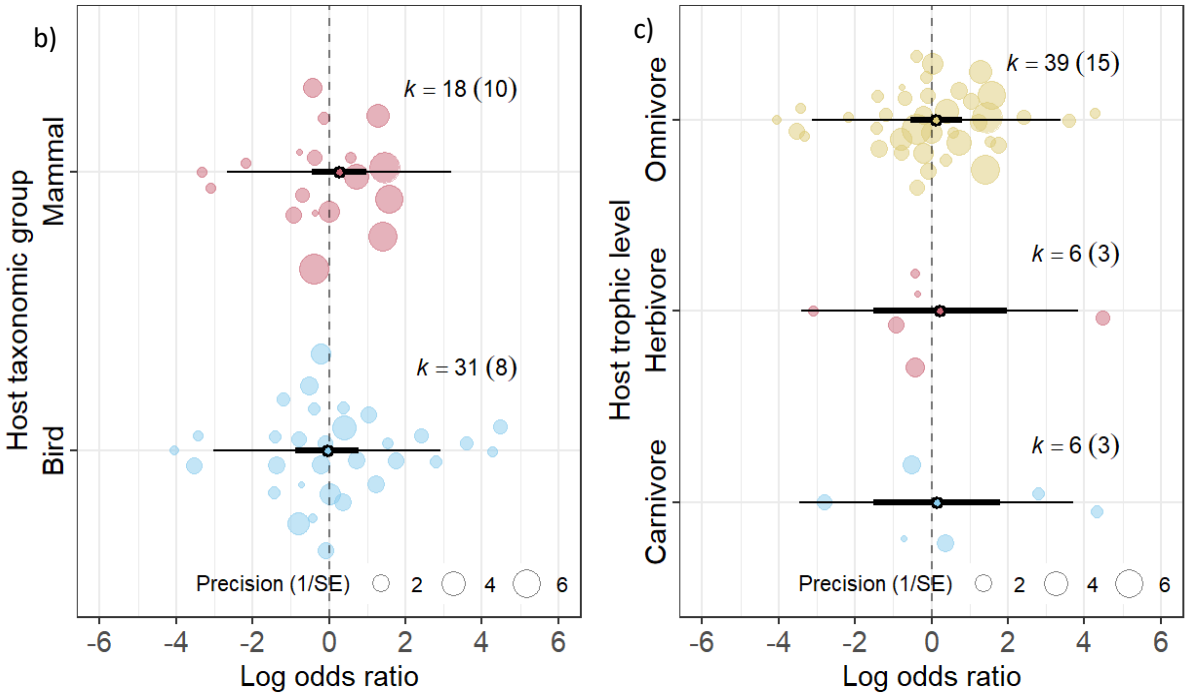


Fig. 11 Orchard plot showing results from the random effects three-level model of the effect of temperature and precipitation on prevalence using data from permutation one when moderators were included. K shows the number of effect sizes and the number of studies that data were extracted from is shown in brackets. Thick bars are 95% confidence intervals and thin bars are 95% prediction intervals. When 95% confidence intervals do not overlap with zero the effect is significant. Moderators included were **a)** whether the parasite has free-living stages, **b)** host taxonomic group and **c)** host trophic level.

5.35 Publication bias

Publication bias was not identified in the temperature (p = 0.727), precipitation (p = 0.404) or combined temperature and precipitation dataset (p = 0.115), because the intercept coefficient was not significantly different from zero at the 0.1 level.

5.4 Discussion

To my knowledge, this is the first quantitative synthesis to assess the overarching patterns in parasite prevalence across contrasting climates in terrestrial animals. I found that the effect of temperature and precipitation on parasite prevalence vary widely, with no overall effect of temperature or precipitation on prevalence of infection. However, the magnitude of temperature and precipitation difference between field sites or experimental treatment groups may impact the likelihood of observing a relationship between these climate parameters and parasite prevalence. I found no difference in the effect of precipitation on endoparasite compared to ectoparasite prevalence. Furthermore, parasites with single host lifecycles and free-living stages or vector-borne lifecycles were not more sensitive to changes in temperature compared to parasites without free-living stages and single host lifecycles. Although studies had limited scope across host and parasite taxa, I found no consistent taxonomic patterns.

The large variation in the effect of temperature on parasite prevalence provides evidence against the simple and controversial 'warmer sicker world' hypothesis that was previously proposed as an effect of future climate change (eg. Shope, 1991; Martens et al., 1995; Patz et al., 1996). Here, by quantifying the net effect of increased temperature on parasite prevalence, for a range of host and parasite taxa across the world, I provide quantitative evidence to support the view that the relationship between climate change and parasite prevalence is more complex than previously realised (Hall et al., 2006; Lafferty, 2009; Altizer et al., 2013). It was originally hypothesised that prevalence would increase in a warmer world due to higher rates of biochemical reactions and thus, parasite development, growth, or infectivity. Whilst this holds true in some cases (eg. Epstein, 2001; Vale & Little, 2009; Paull et al., 2012; Leathwick, 2013), higher reaction rates also come at the cost of higher food requirements. Therefore, not all parasites will benefit from higher temperatures and the prevalence of some may fall. Likewise, a "warmer sicker world" was predicted due to the expansion in the geographical range of parasites and their vectors. This can be further understood by considering the relationship between temperature and organism performance. This relationship is not linear, rather performance typically exhibits a humped distribution in relation to temperature; as temperature moves away from the optimum, performance decreases until a critical thermal minimum or maximum is reached (Lafferty & Mordecai, 2016). Different species exhibit differently shaped thermal response curves depending on whether they are tolerant to a broad range of temperatures, and their method of maintaining an optimum body temperature (Huey & Hertz, 1984; Huey et al., 2003). Depending on their thermal

response curve and their capacity to adapt and migrate, hosts and parasites will respond to higher temperatures in various ways; indeed, parasites and their hosts may respond differently. This could be through shifting their distribution, adapting to increased temperatures or if these are not possible, local extinction (Lafferty & Mordecai, 2016). Consequently, if the distribution of a parasite shifts into a new area, warming will increase prevalence, however, in areas where the habitat is no longer suitable, prevalence will fall. In the current study, only parasite prevalence has been investigated and other metrics, such as infection intensity, have not. The effect of warming on infection intensity consequently remains an important consideration, as infection intensity affects the outcome of infection, for example, higher infection intensity can cause more harm to host fitness and survival (Råberg, 2014; Hidalgo et al., 2022). Further analyses on the effects of warming are needed to understand this relationship.

Interestingly, the temperature dataset demonstrated that when the temperature difference is higher it is less likely that studies will observe a higher prevalence in areas with a higher temperature. When considering the optimal temperature for parasite prevalence, where the temperature of a field site or experimental treatment falls on this temperature curve may affect the likelihood of observing an effect of temperature on prevalence in any given study. For example, measuring prevalence at two temperatures that are higher than the optimal temperature will show a decrease in prevalence with temperature. This indicates that the choice of field site locations and the experimental design of temperature treatment groups is important when investigating the effect of temperature on parasite prevalence. In particular, choosing field sites or experimental treatment groups with a broad range of temperatures will help to encompass a larger proportion of this temperature curve. If known, the shape of the thermal response curve should inform this decision, as the shape of this curve can substantially vary between species as discussed previously (Huey & Hertz, 1984; Huey et al., 2003). Furthermore, future studies should focus on using direct measures of temperature when identifying field sites and designing experiments, as I found that many studies relied on average measures (for example, the monthly mean temperature or mean annual temperature), whereas finer-scale levels of temperature have been shown to affect parasite transmission (eg. malaria, Blanford et al., 2013).

Similar to the effects of temperature, the effects of precipitation on prevalence varied widely. I found some evidence that at larger differences in precipitation between field sites, the higher the chance of observing increased prevalence at higher levels of precipitation. However, this effect was marginal and disappeared when three extreme datapoints were removed, indicating that the precipitation difference between field sites is less influential on results than the temperature difference between

sites discussed above. Again, it is useful to be aware of this relationship when choosing field sites, as the field sites chosen could influence the likelihood of observing an effect of precipitation on prevalence. However, I caution that the precipitation dataset consisted of six studies, limiting the conclusions that can be drawn from it. These conclusions are also limited because all but one study, measured prevalence in mammals and all studies were field surveys, with no control over extraneous variables. Understandably, in many cases laboratory experiments are unrealistic as precipitation is extremely difficult to mimic and manipulate in a laboratory environment. Therefore, field surveys or modelling are the only viable methods to investigate precipitation in many systems. Nonetheless, it is clear that further studies are needed in this area.

The combined temperature and precipitation dataset showed the same wide-ranging effects of these climate variables on prevalence. Unlike the datasets discussed above, no effects of temperature or precipitation difference were identified. This could be explained by the difference in host and parasite taxa included in each dataset; the combined dataset consisted of mostly bird and mammal hosts infected with protozoa whereas in the temperature dataset, which demonstrated the strongest effect, half of the effect sizes were for arthropod hosts infected with a relatively broad range of parasite taxa. Neither the temperature dataset, nor the combined dataset, consisted of a balanced sample of host and parasite taxa. Consequently, I cannot conclude whether the lower likelihood of observing higher prevalence at higher temperatures, when the temperature difference between field sites or experimental groups is larger, is universal, or applies to certain taxonomic groups. This is also the case for the trend observed in the precipitation dataset; I observed a greater likelihood of observing higher prevalence at higher levels of precipitation when the precipitation difference is larger. Nevertheless, this temperature and precipitation difference effect, is important to be aware of when designing field studies and experiments that investigate the effect of climate on prevalence, as failing to encompass a broad section of the thermal response curve of the host or parasite could influence the chance of observing an effect of climate on prevalence.

Whether a parasite was an ectoparasite or endoparasite did not affect the relationship between precipitation on prevalence. One may expect that endoparasites are more buffered from the effects of precipitation compared to endoparasites, as they live within the host. However, endoparasites may also have high levels of environmental exposure. Endoparasites that are transmitted indirectly via surfaces or airborne droplets can be affected by precipitation. For example, precipitation can affect ambient humidity which is linked to the likelihood of airborne coronavirus transmission (Dabisch et al., 2021; Das et al., 2022). It would be interesting to test whether endoparasites with higher

environmental exposure, such as those with free-living stages, are more susceptible to changes in climate compared to those without environmental exposure. Testing this could involve exposing closely related endoparasite species, one with and without a free-living stage, to a range of environmental variables prior to infection and measuring prevalence in hosts. Identifying whether there are key features of parasites, for example of their lifecycle, that make them more sensitive to climate could help to engineer targeted responses in areas that have climate conditions suitable for outbreaks.

The prevalence of parasites with single host lifecycles and either free-living stages or vector-borne lifecycles was not more sensitive to changes in temperature than that of parasites with single host lifecycles without free-living stages. One explanation for this result is that parasites with free-living stages and vector-borne lifecycles may be robust to changes in temperature, due to past selection from large fluctuations in temperature (Nollen et al., 1979; Paaijmans et al., 2013). Alternatively, there may be an interaction between parasite lifecycle and whether the host is a homeotherm or poikilotherm. For example, parasites with single host lifecycles, without free-living stages that inhabit homeothermic hosts may be less affected by temperature changes, compared to those inhabiting poikilothermic hosts. This is because the internal temperature of the homeotherm will remain constant, despite changing environmental temperature, whereas the internal temperature of a poikilothermic host will be more variable. Unfortunately, I could not separate the effects of parasite lifestyle from those of host type due to a limited taxonomic distribution of studies in my dataset. 23 of 26 hosts in the vector-borne lifecycle category were arthropods and all the hosts in the single host lifecycle with free-living stages group were molluscs, arthropods, and amphibians, which are all poikilotherms. Future studies, that investigate a wider variety of host taxonomic groups, would enable analysis of this interaction.

In conclusion, I found that there are no overall effects of temperature and precipitation on parasite prevalence in terrestrial animals, but instead these climate variables have extremely wide-ranging effects on prevalence. This provides quantitative evidence against the 'warmer sicker world' hypothesis and confirms the current view that the effects of global warming on parasite prevalence are highly complex. The fact that the difference in temperature and, to a lesser degree, precipitation between field sites and experimental treatments may affect the likelihood of observing trends in prevalence has implications for both the interpretation of results from previous studies, and the design of future field studies and experiments. Studies that fail to incorporate a large range of the thermal response curve of the host and parasite may make false conclusions about the effect of

climate on prevalence. Furthermore, contrary to popular opinion, the effect of temperature and precipitation on prevalence did not vary between ectoparasites and endoparasites or features of the parasite lifecycle, such as whether it was vector-borne or had free-living stages. This could be a product of the lower representation of some host or parasite taxa in the datasets, however, it could indicate that other factors, such as host habitat, are more important in altering the relationship between climate and prevalence. In addition, the experimental approach used to measure prevalence did not affect the relationship between temperature and precipitation, and prevalence. This is particularly fortunate for testing the impact of precipitation, which is very difficult to test in the laboratory.

In addition to understanding important general trends, I have identified key areas for future research. There is a significant knowledge gap around the effect of humidity on parasite prevalence in terrestrial animals; I found only eight studies that measured the effect of humidity on prevalence. Furthermore, whilst there is substantial research on the effect of climate on arthropod vector-borne parasites, particularly of protozoan parasites of humans, remarkably little is known about either parasite prevalence in reptiles or ectoparasite prevalence more broadly; only two studies measured prevalence in reptiles and one only fifth of effect sizes measured ectoparasite prevalence. Focussing future research in these areas will help to confirm whether the general trends identified here hold true or whether certain types of host-parasite interactions are more or less sensitive to changes in climate, with obvious implications for conservation.

Chapter 6
Transmission of a bumblebee parasite is robust despite parasite exposure to extreme temperatures

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Author contributions:
HSWG: Conceptualisation, experimental design, data acquisition, data analysis, manuscript drafting, manuscript editing.
MFJB: experimental design, manuscript editing.

Abstract

All organisms are exposed to fluctuating environmental conditions, such as temperature. How individuals respond to temperature affects their interactions with one another. Changes to the interaction between parasites and their hosts can have a large effect on disease dynamics. The gut parasite, *Crithidia bombi,* is highly prevalent in the bumblebee, *Bombus terrestris,* and is an established epidemiological model. The parasite is transmitted between bumblebees via flowers, exposing it to a range of environmental temperatures prior to infection. I investigated whether incubation duration and temperature exposure, prior to infection, affects parasite infectivity. Prior to inoculation in *B. terrestris*, *C. bombi* was incubated at 10, 20, 30, 40 or 50°C for either 10 or 60 minutes. These times were chosen to reflect the time that the parasite remains infective for when outside the host and the rate of floral visitation in bumblebees. Prevalence and infection intensity were measured in bees one week later. Incubation duration and the interaction between incubation temperature and duration affected the prevalence of *C. bombi* at 50°C, resulting in no infections after 60 minutes. Below 50°C, *C. bombi* prevalence was not affected by incubation temperature or duration. Extreme temperatures induced morphological changes in *C. bombi* cells, however, infection intensity was not affected by incubation duration or temperature. These results highlight that this parasite is robust to a wide range of temperatures. The parasite was not infective after being exposed to 50°C for 60 minutes, such temperatures likely exceed the flight abilities of bumblebees and thus, the potential for transmission. This study shows the importance of understanding the effects of environmental conditions on both hosts and parasites, which is needed to predict transmission under different environmental conditions.

6.1 Introduction

To survive, all organisms must respond to changing environments. One variable environmental condition is temperature, which fluctuates substantially over a wide range of scales. For example, temperature can vary daily, seasonally, and across the year. In addition, mean temperatures are predicted to increase due to climate change in the coming decades (Wuebbles et al., 2017), posing a further challenge to organisms. All living organisms are sensitive to changes in temperature, and how individuals respond to these changes may affect their interactions with other species (eg. Doney et al., 2011; Boukal et al., 2019). For host-parasite interactions, temperature can have large impacts on disease dynamics (eg. Menti et al., 2000; Frid & Myers, 2002). Consequently, understanding how temperature affects these interactions is necessary for predicting disease dynamics across seasons, in different climates and with climate change.

Temperature can affect disease dynamics directly through altering host susceptibility to infection (eg. Adamo & Lovett, 2011; Murdock et al., 2012). Less obviously, but arguably more importantly, temperature can also affect host susceptibility indirectly. Higher temperatures may result in increased host growth and in turn, host density, leading to higher rates of transmission (Burdon & Chilvers, 1982). Parasites can be exposed to varying temperatures both inside and outside a host, and these temperatures can affect their survival rate, development rate (eg. Leathwick, 2013; Kalinda et al., 2017) and infectivity (O'Connor et al., 2006). Within a host, parasites that infect poikilotherms, such as arthropods, are exposed to wider fluctuations in temperature compared to parasites of homeothermic hosts. Such fluctuations have major impacts on many vector-borne diseases, such as those transmitted by mosquitoes (eg. Liu-Helmersson et al., 2014). In bumblebees and honeybees, temperature variation has been shown to correlate with parasite prevalence and infection intensity (McMullan & Brown, 2005; Chen et al., 2012; Retschnig et al., 2017; Manlik et al., 2023). For example, *N. cerenae* infection in honeybee workers has been shown to correlate negatively with temperature (Chen et al., 2012; Retschnig et al., 2017), but this relationship can change depending on other factors, such as seasonality and host genotype (Manlik et al., 2023). Similar relationships exist outside of invertebrate hosts, for example, amphibian chytrid fungus generally exhibits higher prevalence and infection intensity at lower temperatures (Raffel et al., 2015; Sonn et al., 2017; Ellison et al., 2020).
When outside the host, parasites may be exposed to highly variable environmental conditions. Therefore, parasites with free-living stages and indirect transmission modes are particularly vulnerable to environmental temperature changes due to their period in the external environment.

For example, the survival and infectivity of free-living nematode larvae of sheep are affected by environmental temperature, with the optimum temperature varying between species (O'Connor et al., 2006; Morgan & van Dijk, 2012). Temperature has also been shown to affect the survival and viability of viruses in air-borne droplets (Prussin et al., 2018; Chen, 2020), faeces (Moe & Shirley, 1982) and in water sources (Nasser & Oman, 1999). The direction of temperature effects on viral survival and infectivity can vary between viruses and with other environmental conditions, such as humidity (Moe & Shirley, 1982; Prussin et al., 2018; Chen, 2020).

Many pollinator parasites, including *Vairimorpha apis, V. bombi,* and *Crithidia bombi* are transmitted indirectly via the shared use of flowers (Durrer & Schmid-Hempel, 1994; Graystock et al., 2015; Adler et al., 2018; Figueroa et al., 2019; Pinilla-Gallego et al., 2022). These parasites are deposited across the whole flower, for example, on the petals and in the nectar by workers who may defecate when they forage (Durrer & Schmid-Hempel, 1994; Figueroa et al., 2019; Pinilla-Gallego et al., 2022). The subsequent location of the parasite on the flower will determine its exposure to the ambient temperature. Parasite cells on the petals will exhibit higher temperature variation compared to those in the nectar due to both exposure to direct sunlight and the higher specific heat capacity of nectar, a liquid, compared to the petals, a solid. As one would expect, parasite survival can be higher when deposited inside the floral structure, in the corolla, compared to on more exposed structures, such as the bract (Figueroa et al., 2019). Furthermore, the duration of time the parasite is exposed to the environment depends on the visitation rate of the flower by pollinators. Visitation rate varies between flowers and correlates with the rate of nectar replenishment (Stout et al., 1998). In some cases, the period between flower visits may be up to an hour (Stout & Goulson, 2001).

Crithidia bombi is a prevalent gut parasite of bumblebees and is a well-established epidemiological model (Schmid-Hempel et al., 2019). It is transmitted faecal-orally between colonies via flowers (Durrer & Schmid-Hempel, 1994; Adler et al., 2018; Figueroa et al., 2019; Pinilla-Gallego et al., 2022) and within colonies through contact with infected individuals and with contaminated nest material (Otterstatter & Thomson, 2007; Sah et al., 2021). It has a wide distribution, spanning Europe (eg. Shykoff & Schmid-Hempel, 1991; Rutrecht & Brown, 2008a; Votavová et al., 2022), Australasia (Felden et al., 2022), North and South America (Gillespie, 2010; Cordes et al., 2012; Schmid-Hempel et al., 2014; Gallot-Lavallée et al., 2016; Fernández et al., 2020). *Crithidia bombi* exhibits seasonal variability in prevalence, which peaks in June and July in the northern hemisphere (Popp et al., 2012; Parsche & Lattorff, 2018; Graystock et al., 2020). It is not known whether temperature differences across the seasons contributes to these changes. *Crithidia bombi* growth has been measured *in vitro* and peaks

at 33.7-34.4°C. At temperatures above 37.9°C growth is inhibited (Palmer-Young et al., 2021; Palmer-Young, Raffel, et al., 2018). Interestingly, the temperature of peak growth *in vitro* does not align with the temperature at which hosts exhibit peak infection intensities. Palmer-Young et al. (2019) demonstrated that when the common Eastern bumblebee (*Bombus impatiens*) was incubated at 21°C infection intensities were 81% higher compared to at 37°C. When hosts were incubated at a slightly lower temperature, 29°C, Tobin et al. (2019) found no difference in the prevalence or infection intensity of *C. bombi* compared to those incubated at 21°C.

The effect of temperature on *C. bombi* during environmental exposure, has not been investigated. We do know that the longer the parasite is outside the host the lower the probability of transmission (Schmid-Hempel et al., 1999) and survival (Pinilla-Gallego et al., 2022). Given the changes in *C. bombi* growth *in vitro* at different temperatures (Palmer-Young et al., 2021; Palmer-Young, Raffel, et al., 2018), it is plausible that temperature exposure on flowers affects its transmission between colonies. Furthermore, temperature may affect within colony transmission of the parasite. Although bumblebees try to maintain a constant temperature within the colony, through fanning when it is hot (Weidenmüller et al., 2002), and incubating (O'Donnell & Foster, 2001) and building wax coverings when it is cold (Jones & Oldroyd, 2006), in reality the temperature of the colony will fluctuate with environmental temperature and colony size (Vogt, 1986; Crall et al., 2018). In addition, colony temperature varies between colonies in different climates, particularly at the start of their lifecycle when the colony temperature is similar to the ambient temperature (Hasselrot, 1960).

To test whether temperature exposure affects *C. bombi* infectivity, I exposed *C. bombi* to 10, 20, 30, 40 and 50°C for either 10 or 60 minutes *in vitro* and inoculated *Bombus terrestris audax* hosts. One week later, host prevalence and infection intensity were measured. I expected prevalence and infection intensity to peak at 30°C in alignment with its peak growth *in vitro* (Palmer-Young, Raffel, et al., 2018; Palmer-Young et al., 2021). I also predicted that infection would be impeded by higher temperatures, due to a reduction in growth *in vitro* above 37°C (Palmer-Young, Raffel, et al., 2018; Palmer-Young et al., 2021) and an 81% decrease in infection intensity when bees were incubated at 37°C compared to 21°C (Palmer-Young, Ngor, et al., 2019). Finally, I predicted that prevalence and infection intensity would be higher after *C. bombi* was incubated for 10 compared to 60 minutes (Schmid-Hempel et al., 1999).

6.2 Methods

6.21 Experimental organisms

Eight *Bombus terrestris audax* colonies, with 85-150 workers each, were ordered from Agralan (UK). The colonies were kept at 27.8 (± 0.71) °C, ambient humidity (43.7 (± 4.2)%) and under red light. The colonies were fed with honeybee collected pollen (Agralan, UK) and sterile sugar solution (50% concentration) *ad-libitum.* To ensure colonies were free of infection, 10 individuals per colony were screened for *Crithidia bombi, Apicystis* spp. and *Vairimorpha (Nosema) bombi* by viewing their faeces using a phase contrast microscope (Nikon Eclipse 50i) at X400 magnification (Rutrecht & Brown, 2009). *Crithidia bombi* was obtained from two laboratory stock colonies of *B. terrestris audax* (Agralan, UK). The parasite was originally acquired from post-hibernation spring queens of *B. terrestris audax* caught in Windsor Great Park (Surrey, UK) in March 2021, since when it has been continually cycled through laboratory colonies (Agralan, UK). *Crithidia bombi* has three lifecycle stages. One lifecycle stage is non-motile (amastigote) and two lifecycle stages are motile (choanomastigote and promastigote) (Fig. 1). The prevalence of each lifecycle stage can change over the course of an infection (Logan et al., 2005).

6.22 Experimental design

Treatments

To find out whether temperature exposure prior to inoculation affected *C. bombi* infectivity, *C. bombi* was incubated at 10, 20, 30, 40 and 50°C *in vitro*. These temperatures were chosen after considering the temperatures at which bumblebees forage (Heinrich, 1972; Descamps et al., 2021; Kenna et al., 2021), the evaporation of nectar at higher temperatures (Descamps et al., 2021) and the growth of *C. bombi* at different temperatures *in vitro* (Palmer-Young, Raffel et al., 2018, 2021). *Crithidia bombi* was incubated at these temperatures for 10 and 60 mins, in a fully factorial design. These incubation times were chosen based on the duration of time *C. bombi* remains infective (Schmid-Hempel et al., 1999) and floral visitation rates of bumblebees in the field (Stout et al., 1998; Stout & Goulson, 2001). My pilot experiments also indicated that these temperatures and incubation durations were suitable (results in Appendix 4).

Counting the number of healthy and unhealthy *C. bombi* cells in inoculum prior to inoculation

Pilot experiments (see Appendix 4) identified *C. bombi* cells that appeared less healthy compared to others. Based on pilot observations, criteria for these cells were specified (Table 1, Fig. 1). To assess whether the number of healthy and unhealthy *C. bombi* cells differed between treatments, the number of healthy and unhealthy cells in the inoculum were counted using an improved Neubauer haemocytometer at X400 magnification using a phase contrast microscope.

Table 1 Criteria for identifying healthy compared to unhealthy *C. bombi* cells based on observations from pilot experiments.

Healthy	**Unhealthy**
- Fast swimming choanomastigotes and promastigotes - Larger amastigotes and choanomastigotes (estimated choanomastigote mean length is 6.03 ± 1.09 µm (Schmid-Hempel & Tognazzo, 2010)) - Smoother surface texture of amastigotes and choanomastigotes - Plump amastigotes and choanomastigotes - Cells not burst - Often more lifecycle stages present	- Choanomastigotes and promastigotes that do not swim - Smaller amastigotes and choanomastigotes - Rougher surface texture of amastigotes and choanomastigotes - Shrivelled amastigotes and choanomastigotes - Multiple burst cells - Choanomastigotes have flimsy tails - Amastigotes flatter and darker

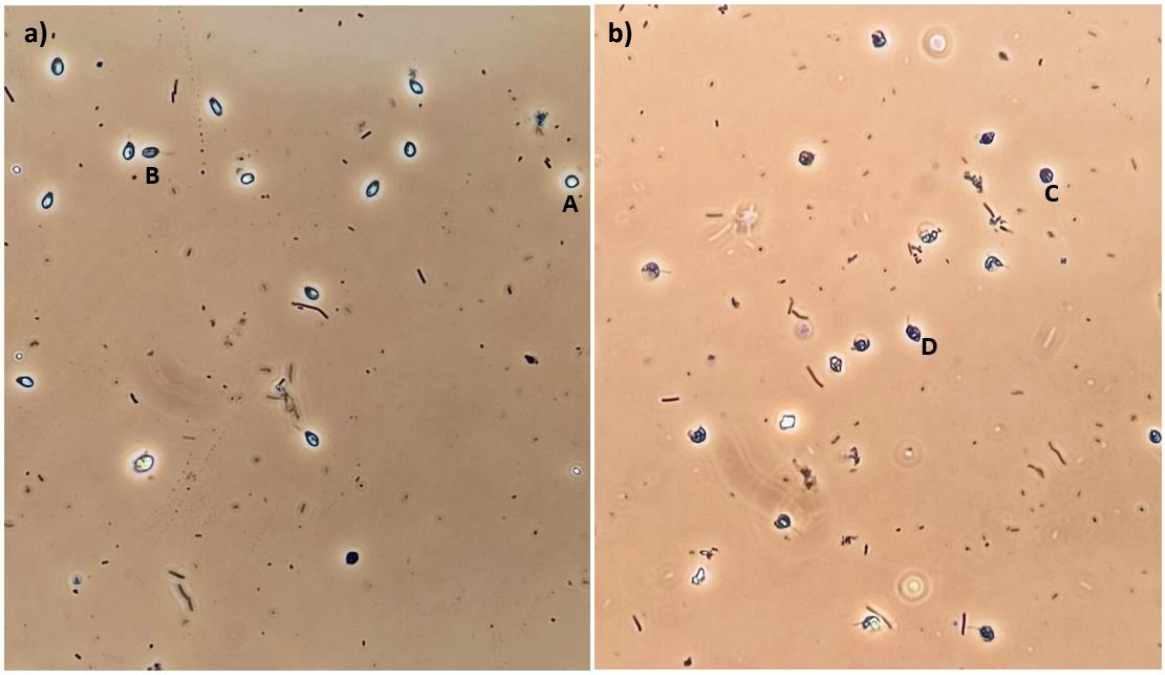


Fig. 1 Photograph of *C. bombi* cells at X400 magnification using a phase contrast microscope. *Crithidia bombi* was suspended in 25% Ringer solution to aid photography. **a)** *Crithidia bombi* following incubation at 30°C for 60 minutes, which exhibits a large proportion of 'healthy' cells (A = healthy amastigote, B = healthy choanomastigote). **b)** *Crithidia bombi* following incubation at 50°C for 60 minutes, which shows a large proportion of 'unhealthy' cells (C = unhealthy amastigote, D = unhealthy choanomastigote).

Inoculation

Individuals workers were inoculated with a dose of 20,000 *C. bombi* cells. This dose was chosen to enable the assessment of prevalence and infection intensity, whilst maintaining field realism (Schmid-Hempel & Schmid-Hempel, 1993). Inoculation dose was also trialled in pilot experiments (see Appendix 4). On each day, two temperatures were tested, one for 10 minutes and one for 60 minutes. To prevent order effects, the order of temperatures was arranged such that a temperature was not tested twice on the same day and the order of temperatures was different for each incubation duration (Table A4.1). On each day, 32 individuals (four per colony) were inoculated per temperature and duration of incubation combination. Therefore, 320 individuals were inoculated in total, 64 each day.

Individuals were removed from their colonies and weighed in pre-weighed vials to the nearest milligram (Scout SKX, Ohaus, Switzerland). Mass was used as a proxy for size since size can affect *C.*

bombi infection intensity (Otterstatter & Thomson, 2006). Mass was used, rather than inter-tegular distance or wing marginal cell length, due to time constraints on inoculation days. I appreciate that body mass may be influenced by sugar consumption, but as all bees had equal exposure to *ad-libitum* food prior to weighing, this seems unlikely to have a meaningful impact on results. Bees were housed individually in nicot cages (Becky's bees, UK), which are cylindrical containers adapted from hair rollers to house bees (Fig. A4.1). Faeces were collected from 20 individuals per *C. bombi* stock colony and purified using a modified triangulation protocol (Cole, 1970; see General methods 2.1). Bees were inoculated following the inoculation protocol in General methods 2.2. *Crithidia bombi* was suspended in sugar solution to mimic the transmission of *C. bombi* in nectar (Durrer & Schmid-Hempel, 1994; Figueroa et al., 2019; but see Cisarovsky & Schmid-Hempel, 2014). The inoculation solution was incubated in a PCR machine (Biometra TProfessional thermocycler, Jena Analytik, 070-951) at the pre-specified temperature for 10 or 60 minutes. Immediately after incubation, the inoculum was given to adult worker bees as is detailed in the inoculation protocol in General methods 2.2.

Housing

Individuals were housed in nicot cages with sterile sugar solution (50% concentration) provided *ad-libitum* via a 5-ml syringe attached to the base of the nicot cage (see Fig. A4.1). As before, the end of syringes was removed to allow access to the sugar. Syringes were replaced every three days to prevent fungal growth.

Measuring infection

One week after inoculation bees were put in specimen tubes and infection prevalence and intensity were measured following the protocol in General methods 2.3. The presence of each lifecycle stage of *C. bombi* (amastigote, choanomastigote and promastigote) and unhealthy cells (Table 1, Fig. 1) were recorded. Prevalence, infection intensity and presence of lifecycle stages were measured once per individual.

6.23 Statistical Analysis

Analyses were performed in RStudio "Prairie Trillium" (RStudio Team, 2022), R version 4.2.0 (R Core Team, 2022). All figures were created using the ggplot() function from the ggplot2 package (Wickham, 2016). For the majority of analyses, temperature and incubation duration were included as categorical variables. When analysing whether the presence of unhealthy cells was affected by the temperature and duration of incubation, temperature was included as a numerical variable because a quadratic

term was required in the model. To test whether the number of unhealthy cells per microlitre of inoculum differed between treatments a generalised linear model with a quasipoisson error distribution was used due to overdispersion. Temperature and incubation duration were included as fixed effects. The interaction between temperature and incubation duration was not included as this led to overdispersion.

To test whether the likelihood of infection varied with parasite incubation temperature and duration, a generalised linear model was constructed with a binomial error distribution and logit link. The full model included temperature, duration of incubation and their interaction as fixed effects. Bee body mass and colony were also included as covariates. For all analyses colony was included as a fixed effect rather than random effect due to the small number of individuals from each colony per treatment (Gelman & Hill, 2006; Arnqvist, 2020). A likelihood ratio Chi-squared test and AIC values were used to compare reduced and full models. Model assumptions were checked graphically and using the DHARMa package (Hartig, 2022).

To investigate whether infection intensity was affected by parasite incubation temperature and duration a generalised linear model with a negative binomial error distribution was constructed using the glm.nb function from the MASS package (Ripley et al., 2002). Only infected bees were included in this analysis. Infection intensity was measured as cells per microlitre rounded to the nearest integer. In the full model, temperature, incubation duration and their interaction were included as fixed effects, in addition to bee mass and colony which were included as covariates. Model fit was assessed as described above.

To test whether the presence of unhealthy cells was affected by the temperature and duration of incubation a generalised linear model with a binomial error distribution and logit link was used. In the full model, temperature, incubation duration and their interaction were included as fixed effects along with colony. Quadratic terms for temperature and the temperature interaction were added after plotting the model results.

To assess whether the presence of each lifecycle stage varied with incubation temperature and duration a separate generalised linear model, with a binomial error distribution and logit link, was used to analyse the presence of each lifecycle stage. The full model included temperature, incubation duration and their interaction and colony as fixed effects.

6.3 Results

A total of 289 bees were successfully inoculated and screened for infection. Some individuals were lost from the sample because they did not drink the entire inoculum (*n* = 12), died before screening (*n* = 11) or did not defecate during screening (*n* = 8).

6.31 Do incubation temperature and duration affect the number of unhealthy cells in one microlitre of inoculum?

Incubation temperature and duration did not affect the number of unhealthy cells in one microlitre of inoculum ($X^2_4 = 39.4$, $p = 0.397$; $X^2_1 = 21.1$, $p = 0.619$; Fig. 2). Figure 2 shows the number of unhealthy cells as a percentage of the total number of counted cells in 1ul. The number of unhealthy cells in one microlitre of inoculum was not analysed as a percentage due to model under dispersion.

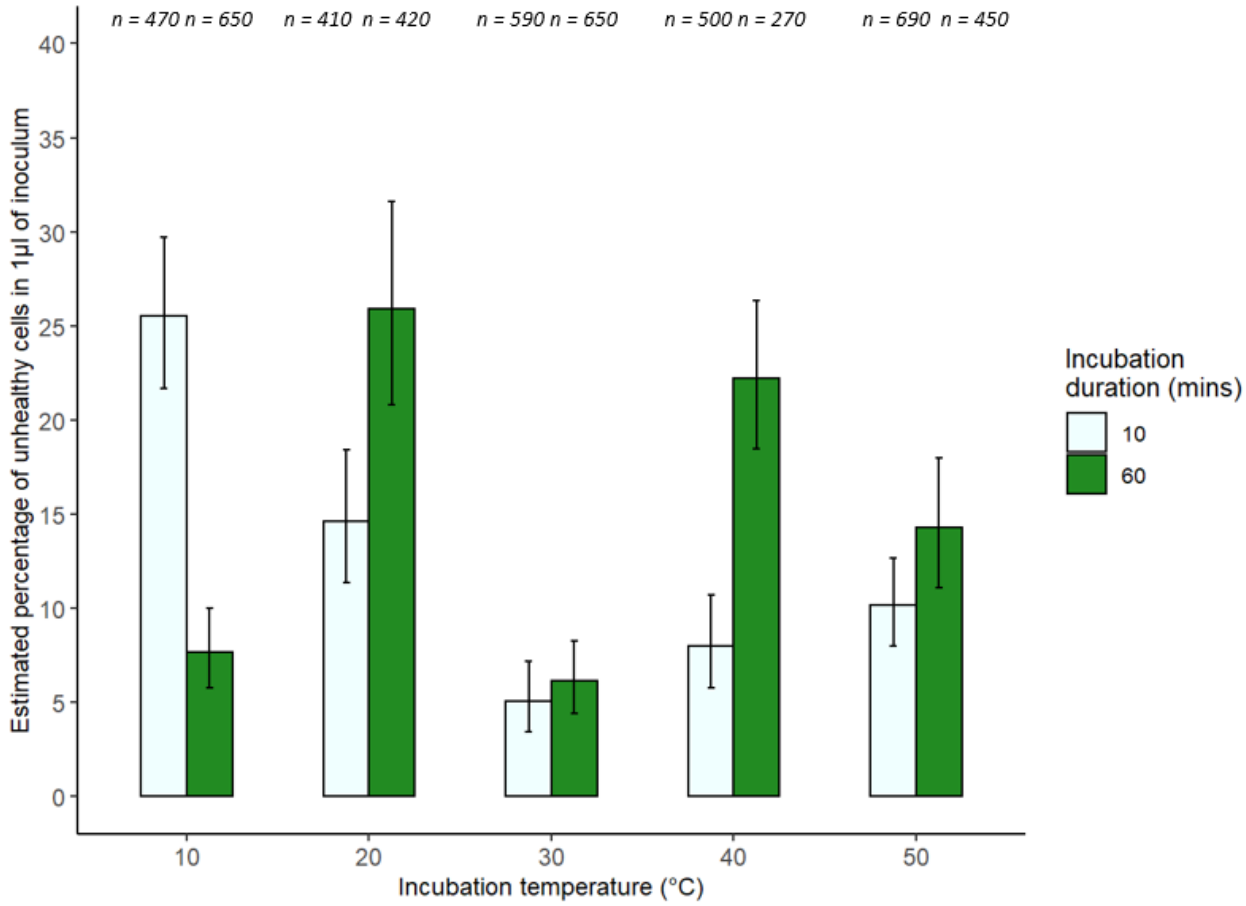


Fig. 2 The estimated percentage of the number of unhealthy *C. bombi* cells in 1µL of inoculum following incubation at five temperatures for 10 (pale green, left) and 60 minutes (dark green, right).

Error bars show 95% binomial confidence intervals and sample sizes of the total number of counted cells are above each bar.

6.32 Do incubation temperature and duration affect the prevalence of infection?

A model that removed colony and included temperature, incubation duration and their interaction, and bee mass had the best fit (see Appendix 4 for full model results). Prevalence of infection was not significantly affected by incubation temperature (b = 0.019, SE = 0.0405, z = 0.472, p = 0.637). However, incubation duration (b = 0.135, SE = 0.0479, z = 2.83, p = 0.005) and the interaction between temperature and incubation duration significantly affected prevalence (b = -0.004, SE = 0.001, z = -3.86, p < 0.001; Fig. 3). Between 20-40°C, longer incubation slightly reduced prevalence, however at 50°C, longer incubation drastically reduced prevalence to 0%. Body mass of individual bees did not significantly affect prevalence (b = 3.5, SE = 2.12, z = 1.65, p = 0.099; Fig. A4.8 and Fig. A4.9).

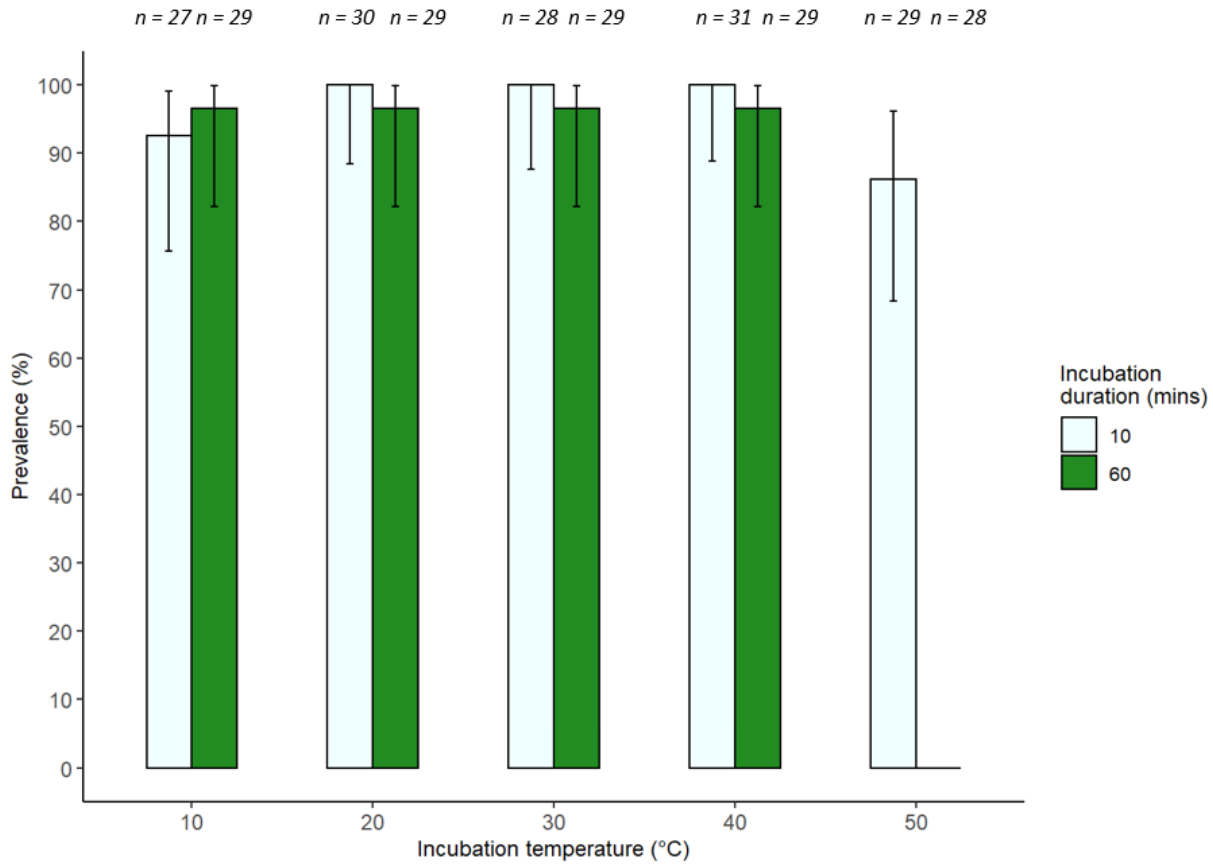


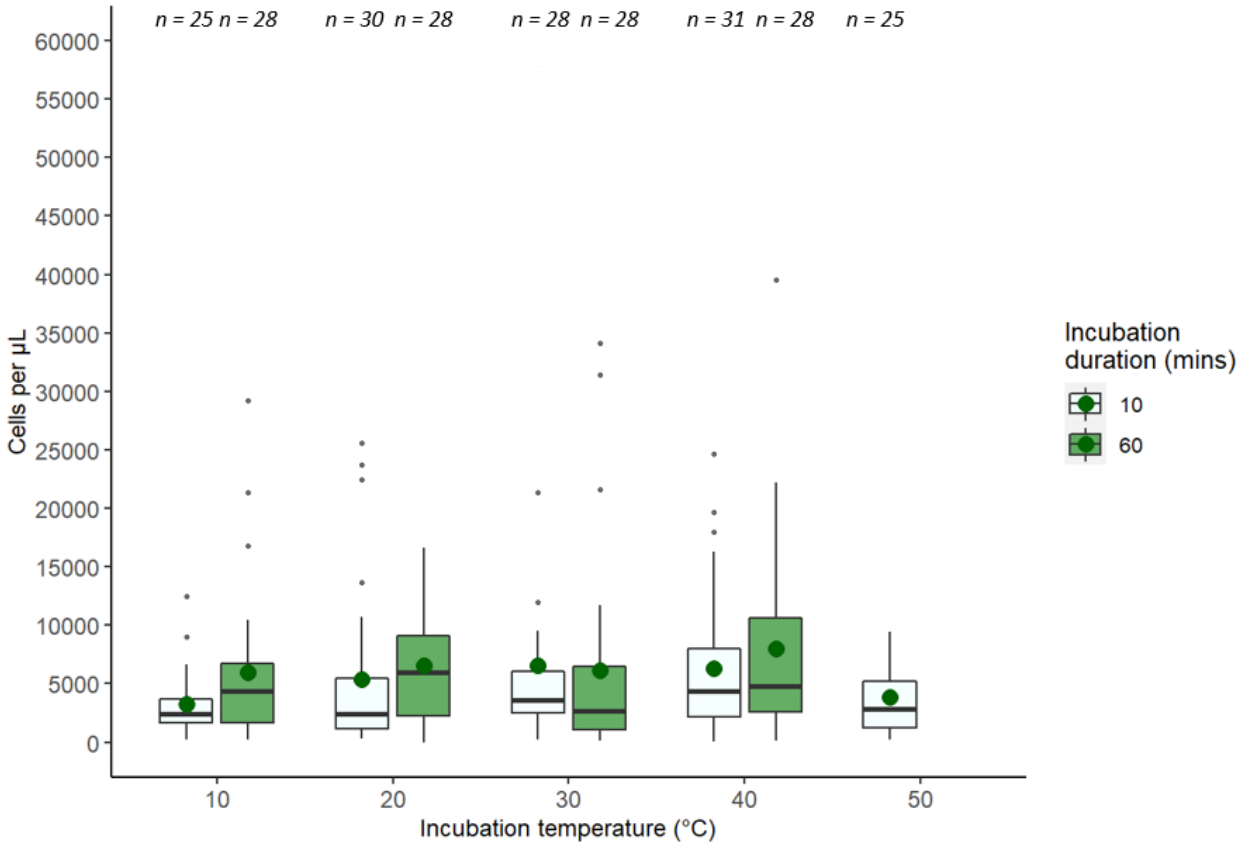
Fig. 3 The prevalence of infection of *C. bombi* in *B. terrestris audax* one week after infection. Prior to inoculation, *C. bombi* was exposed to five temperatures for two time periods. Pale green (left)

indicates exposure to the temperature for 10 minutes and dark green (right) for 60 minutes. Error bars show 95% binomial confidence intervals. Sample sizes given above each bar.

6.33 Do incubation temperature and duration affect the intensity of infection in infected individuals?

A reduced model (including temperature, incubation duration as fixed effects, bee mass and colony as covariates) and excluding the interaction between temperature and incubation duration had the best fit. Temperature and incubation duration did not significantly affect infection intensity ($X^2_4 = 311$, $p = 0.079$; $X^2_1 = 308$, $p = 0.084$; Fig. 4). Figure 4 shows that there was little difference in infection intensity between treatment groups. Furthermore, bee body mass and colony did not affect infection intensity ($X^2_1 = 306$, $p = 0.092$; $X_7 = 308$, $p = 0.063$).

a)



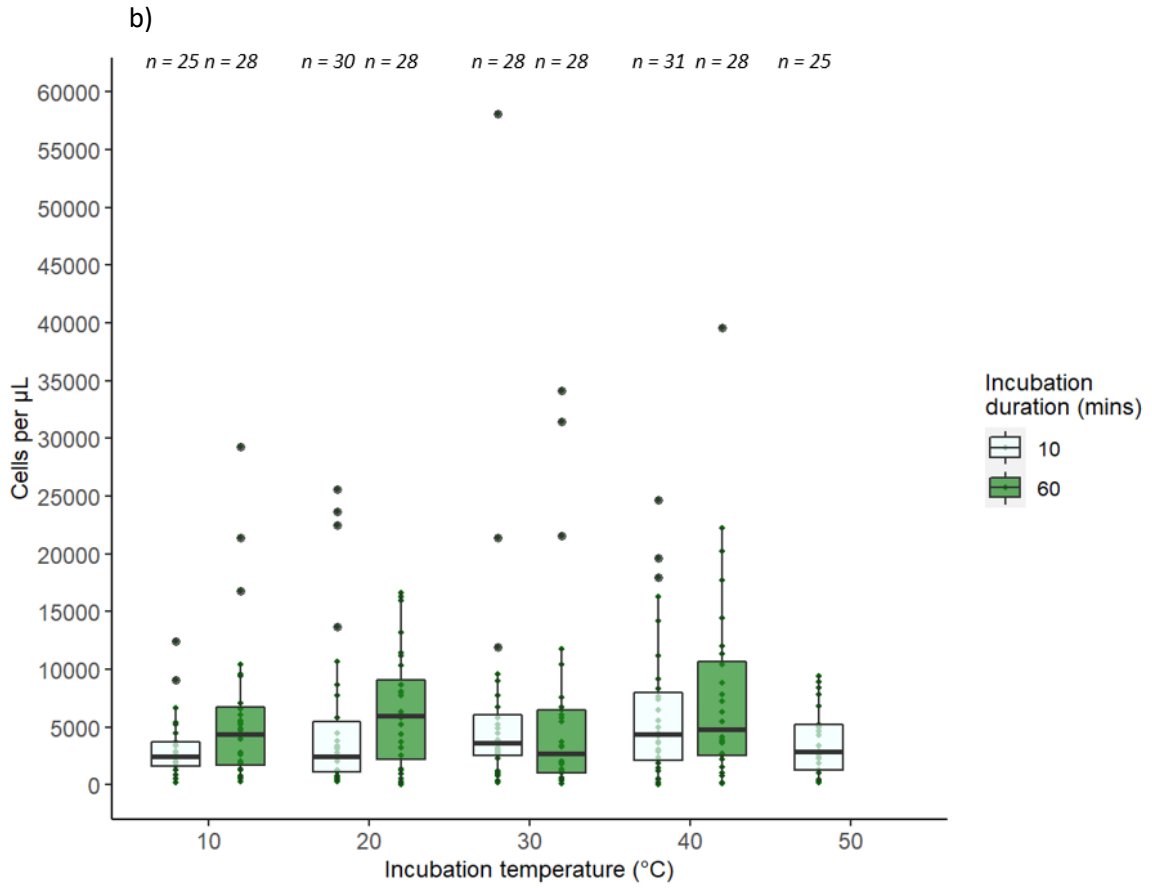


Fig. 4 a) Boxplot showing the infection intensity (cells per µL) of *C. bombi* in *B. terrestris audax* one week after infection. Prior to inoculation, *C. bombi* was exposed to five temperatures for two time periods. Pale green boxplots (left) indicates exposure to the temperature for 10 minutes and dark green boxplots (right) for 60 minutes. Means are indicated by the large, dark green datapoints and sample sizes are above each boxplot. **b)** Same as a) without mean datapoints and including raw data as smaller datapoints.

6.34 Do incubation temperature and duration affect the presence of unhealthy *C. bombi* cells in faeces?

The best model included temperature, the interaction between temperature and incubation duration, the quadratic term for temperature and its interaction, incubation duration and colony. Temperature significantly affected the likelihood of observing unhealthy cells (linear term: $b = 0.22$, $SE = 1.96$, $z = -2.26$, $p = 0.024$; quadratic term: $b = 1.29$, $SE = 1.12$, $z = 2.32$, $p = 0.021$; Fig. 5). After incubation for 10 minutes the prevalence of burst cells was more than two times higher at 10°C compared to 30°C, and three times higher at 50°C compared to 30°C (Fig. 5). A similar pattern was seen after incubation for 60 minutes, with the lowest prevalence of burst cells observed at 30°C. The interaction between

temperature and incubation duration did not affect the prevalence of unhealthy cells (linear term: *b* = 0.239, *SE* =, *z* = -1.16, *p* = 0.248; quadratic term: *b* =1.34 , *SE* = 1.26, *z* = 1.25, *p* = 0.21). In addition, incubation duration and colony did not affect the chance of observing unhealthy cells (incubation duration: $X^2_1 = 1.034$, *p* = 0.309; colony: $X^2_7 = 11.1$, *p* = 0.133)

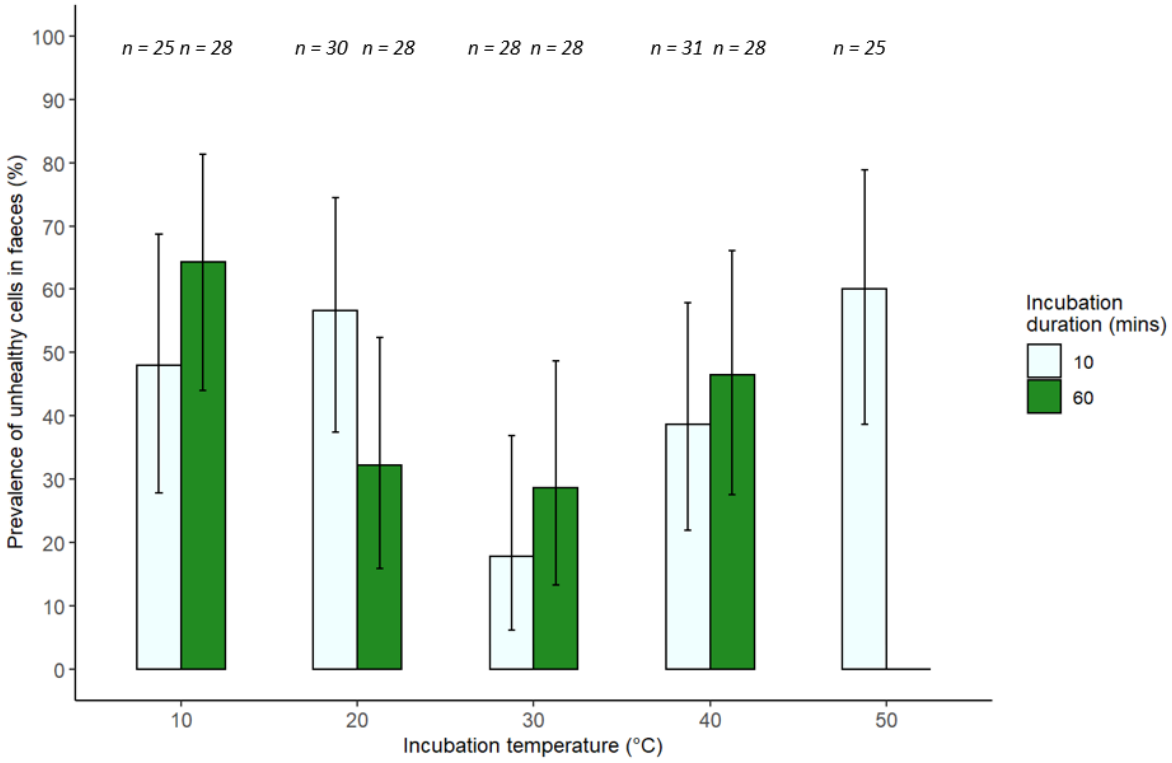


Fig. 5 The prevalence of infected *B. terrestris audax* with unhealthy *C. bombi* cells (based on criteria in Table 1 and Fig. 1) one week after being infected with *C. bombi.* Prior to inoculation, *C. bombi* was exposed to five temperatures for 10 (pale green bars, left) or 60 minutes (dark green bars, right). Error bars show 95% binomial confidence intervals. Sample sizes are above each bar.

6.35 Do incubation temperature and duration affect the likelihood of observing the three *C. bombi* lifecycle stages?

Amastigotes were present in 98% of infected individuals and choanomastigotes in 97.21% of infected individuals. Due to the very high prevalence of these lifecycle stages, the results were not further analysed. Promastigote lifecycle stages were present in 17.93% of infected individuals. While the best model included temperature and incubation duration, neither temperature nor incubation duration significantly affected the presence of promastigotes ($X^2_4 = 230$, *p* = 0.203; $X^2_1 = 229$, *p* = 0.3)

6.4 Discussion

Overall, I found that below 50°C, *C. bombi* prevalence was not affected by incubation temperature or duration, indicating that the parasite remains infectious at a wide range of temperatures. The interaction between incubation temperature and duration affected the prevalence of *C. bombi* at 50°C, resulting in no infections after 60 minutes. Furthermore, the intensity of infection was not affected by incubation duration or temperature. Despite being able to successfully infect and replicate once inside the host, unhealthy, burst *C. bombi* cells were more than twice as prevalent at 10°C compared to 30°C after incubation for ten and 60 minutes and more than three times as prevalent at 50°C compared to 30°C when incubated for 10 minutes. This suggests that temperature may cause physiological changes in the parasite, with the optimum temperature for *C. bombi* approximately 30°C. These changes appear to occur after infection, because the incubation temperature and duration did not affect the presence of unhealthy cells in the inoculum.

The effect of incubation duration on prevalence of infection indicates that the duration of time *C. bombi* spends outside the host affects its ability to infect the next host. Between 20-40°C, incubating the inoculum for 60 minutes resulted in a ~4% decrease in prevalence at a given temperature, compared to incubating the inoculum for 10 minutes. This is a relatively small decrease when prevalence in worker populations in the field is high, for example 77.7% (Popp et al., 2012). However, towards the end of the summer prevalence tends to fall, for example, to 3.9% in August (Parsche & Lattorff, 2018), and at this time a 4% decrease would have a much larger effect on overall prevalence. In contrast to my results, Schmid-Hempel et al. (1999) found a ~16% reduction in prevalence when *C. bombi* was left on a slide for 45 minutes prior to inoculation, compared to if used to infect hosts immediately. In addition, *C. bombi* left on a flower for three hours exhibited reduced motility (Figueroa et al., 2019; Pinilla-Gallego et al., 2022), which may affect its infectivity. I incubated *C. bombi* in solution which suggests that reduced infectivity may not just be a product of it drying out in the environment, but rather other changes that occur when the parasite is outside the host. The larger effect seen by Schmid-Hempel et al. (1999) indicates that in the field *C. bombi* may lose infectivity at a faster rate when deposited on petals compared to in nectar.

When considering the transmission of *C. bombi* in the field, the location of *C. bombi* on the floral structure needs to be considered. Studies have demonstrated that bees defecate in the middle of the flower into the nectar (Figueroa et al., 2019) and that *C. bombi* is transmitted via nectar (Durrer &

Schmid-Hempel, 1994). Sugar in the nectar may exert physiological stress on *C. bombi* as, Cisarovsky & Schmid-Hempel (2014) found that *C. bombi* growth is inhibited at higher concentrations of sugar. This may have implications for *C. bombi* transmission in the field, because the concentration of sugar in plant nectar varies between species (Pamminger et al., 2019). Consequently, *C. bombi* deposited in nectar in some plants with higher nectar sugar concentration may be under greater physiological stress. The likelihood of defecation by bumblebees on different parts of the flower can depend on the floral structure (Figueroa et al., 2019), indicating that the species and trait composition of plants in a landscape could influence the prevalence of *C. bombi.* Furthermore, when considering the duration of time that *C. bombi* is exposed to the environment, the rate of visitation by bumblebee foragers needs to be considered. The visitation rate of different plants varies with multiple floral design features, such as flower colour (eg. Stanton, 1987) and the rate of nectar production (eg. Mitchell, 1994). Therefore, plants with shorter visitation rates may be more likely to transmit *C. bombi,* particularly, for *C. bombi* cells shed on the surface of floral structures rather than in the nectar.

The response of *C. bombi* prevalence to temperature exposure appears not to be linear but a threshold response. The threshold for infectivity was surpassed at 50°C after 60 minutes, as no bees were successfully infected after this treatment. Lack of infectivity at higher temperatures could be due to the denaturation of enzymes and proteins (Copeland, 2000). The fact that infection intensity and the presence of the three lifecycle stages were not significantly affected by temperature shows that once established in a host *C. bombi* was able to successfully replicate, irrespective of its previous exposure. Temperature can affect the separate stages of parasite transmission to a host differently. Increased temperatures, for example, have been shown to increase the ability of the trematode, *Ribeiroia ondatrae,* to penetrate host skin, but reduce the ability of trematode larvae, called cercariae, to encyst after skin penetration (Paull et al., 2012). Furthermore, the increase in the proportion of unhealthy *C. bombi* cells at 50°C compared to 30°C indicates that morphological changes occurred following temperature exposure, but these changes were not visible immediately, because the presence of unhealthy cells in the inoculum did not vary between treatments. When exposed to higher temperatures another trypanosome, *Leishmania* spp, exhibits morphological changes to the cell structure and the parasite loses its ability to multiply (Zilberstein & Shapira, 1994). In *Leishmania* spp. these changes are reversible, and the parasite regains its ability to multiply when the temperature falls. This appears not to be true for *C. bombi* as exposure to 50°C was temporary. The lack of infectivity after prolonged exposure to 50°C suggests that *C. bombi* transmission may be curtailed in climates and seasons with very high temperatures. Interspecific and intercolony transmission may be lower in hotter areas of *C. bombi's* range, such as in Spain (Jabal-Uriel et al., 2017), South America (Schmid-

Hempel et al., 2014; Gallot-Lavallée et al., 2016) and parts of North America (Cordes et al., 2012). Furthermore, due to climate change, temperatures above 40°C are becoming increasingly common and more prolonged (Coumou et al., 2013). For example, in the summer of 2022 temperatures surpassed 40°C in the United Kingdom (Kendon, 2022). Consequently, *C. bombi* is expected to be exposed to temperatures above 40°C more frequently than previously.

However, before drawing conclusions on the effects of temperature on transmission, the impacts of temperature on the host need to be understood. For example, the infectivity of *C. bombi* may decrease at higher temperatures, but the susceptibility of hosts may also change at high temperatures. Whilst I did not examine this, previous work has shown varying effects of temperature on host susceptibility to *C. bombi* (Palmer-Young, Ngor, et al., 2019; Tobin et al., 2019). Tobin et al. (2019) concluded that incubating hosts at 21-29°C had no effect on prevalence or infection intensity. When Palmer-Young et al. (2019) tested a wider range of temperatures, infection intensity of *C. bombi* declined by 81% when hosts were incubated at 37°C compared to 21°C. These studies were conducted in the lab, in a non-stressful environment with food *ad-libitum,* whilst, in the field, food may be less available. Nutritionally stressed bees exhibit higher mortality when infected with *C. bombi* (Brown et al., 2000) and therefore, the effects of temperature on host susceptibility may be different in the field. However, a more important consideration for understanding whether the response of the parasite to high temperatures is relevant for transmission is host behaviour. At temperatures above 24°C, bumblebee flight ability decreases (Kenna et al., 2021) and at extremely high temperatures, such as above 40°C, bumblebees may reach their thermal maximum and enter heat stupor (Martinet et al., 2021). If bumblebees are unable to fly at temperatures above 40°C, transmission will not be affected by reduced *C. bombi* infectivity because the bumblebees will not encounter the parasite. However, thermal tolerance varies between species, with some Mediterranean sub-species, such as *B. xanthopus,* able to withstand extended periods of time at these temperatures (Oyen et al., 2016; Martinet et al., 2021). It is unclear whether these species can fly over 40°C or whether they are just more resistant to heat stupor (Martinet et al., 2021). If they can fly, extreme temperatures may reduce pathogen transmission to heat-tolerant species.

In contrast, at lower temperatures, *C. bombi* infection ability was not impeded, despite a higher proportion of cells looking unhealthy at 10°C compared to 30°C. The fact that *C. bombi* infectivity was approximately constant between 10-40°C suggests that temperature does not play a major role in the seasonal dynamics of *C. bombi* prevalence, which peaks in early summer in the northern hemisphere (Popp et al., 2012; Parsche & Lattorff, 2018). Based on my results *C. bombi* is likely equally as infective

to emerging spring queens at 10°C as it is to foraging workers in the peak of summer. Rather, population demographic changes as the summer progresses likely play a major role in seasonal peaks in prevalence (Popp et al., 2012; Parsche & Lattorff, 2018). However, seasonal fluctuations in prevalence have only been studied in limited parts of *C. bombi's* range, so it would be interesting to test whether seasonal patterns in prevalence are the same across hotter regions, where temperatures surpass 40°C.

Finally, here I only looked at the effect of temperature on infectivity, whereas, when outside the host, *C. bombi* may be exposed to varying levels of humidity, UV radiation and potentially precipitation. These factors may also affect infectivity in isolation or may interact to alter infectivity. Climatic factors other than temperature, have been shown to affect the prevalence of some bumblebee pathogens including *Vairmorpha bombi* (Manlik et al., 2023; McNeil et al., 2020), *C. bombi* and *C. expoeki* (Ivers et al., 2022). Specifically, the prevalence of *V. bombi* can be affected by temperature, humidity, precipitation and cloud cover (Manlik et al., 2023). In addition, some have found a positive correlation between *V. bombi* prevalence and spring precipitation (McNeil et al., 2020). It is likely that UV radiation affects *C. bombi* survival, because *C. bombi* survives for shorter time periods on sunny compared to shaded flowers (Figueroa et al., 2019). Sunlight-level UV radiation has been shown to increase mortality of nematode larvae (Dijk et al., 2009), emphasising the importance of considering all aspects of climate variability. Exposure to different environmental variables whilst *C. bombi* is on flowers compared to when on nest material may mean *C. bombi* is more transmissible in one environment compared to the other. For example, if UV radiation reduces *C. bombi* survival, transmission on flowers may be lower than transmission from contaminated nest material within the colony at the same temperature.

In conclusion, my study showed that the parasite, *C. bombi*, was less infective the longer it spent outside the host under experimental conditions. This may suggest that the likelihood of transmission in the wild is higher on flowers with shorter visitation times. In addition, the infectivity of the parasite was not affected by exposure to temperatures between 10°C and 40°C. *Crithidia bombi* was no longer infective after exposure to 50°C for 60 minutes. However, it is unlikely that many bumblebee species would fly, and therefore encounter, the parasite at this temperature. Infection intensity was not affected by temperature, however, extreme temperatures appear to induce morphological changes to *C. bombi* cells in the faeces of infected individuals. I investigated the effect of one climate variable on parasite infectivity, when in reality multiple climate factors will vary in the environment. Assessing the effect of these on hosts and parasites in isolation, and when interacting, will further our

understanding of the epidemiology of host-parasite interactions across different climates and help to predict the effects of climate change in the future.

Data Availability

DOI for data: 10.5281/zenodo.7760010

Chapter 7
Methods matter: how to quantify *Crithidia bombi* infection intensity in bumblebees

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Author contributions:
HSWG: Conceptualisation, experimental design, data analysis, manuscript drafting, manuscript editing.
AS: Data acquisition.
MFJB: Manuscript editing.

Abstract

The bumblebee gut parasite, *Crithidia bombi*, is widespread and prevalent in the field. Its interaction with *Bombus terrestris* and *B. impatiens* is a well-established epidemiological model. It is spread faecal-orally between colonies via the shared use of flowers when foraging. Accurately measuring the level of infection in bumblebees is important for assessing its distribution in the field, but also when conducting epidemiological experiments. Studies generally use one of two methods for measuring infection. One approach measures infection in faeces whereas the other method measures infection in guts. I tested whether the method of measuring infection affected the estimation of infection. Bumblebees were inoculated with a standardised inoculum and infection was measured one week later using either the faecal or gut method. I found that when the gut method was used to measure infection intensity, estimates were approximately double that from the faecal method. These results have implications for the interpretation of previous study results and for the planning of future studies. Given the importance of bumblebees as pollinators, the impact of *C. bombi* on bumblebee health, and its use as an epidemiological model, I call on researchers to move towards consistent quantification of infections to enable future comparisons and meta-analyses of studies.

7.1 Introduction

Crithidia bombi is a widespread and prevalent gut parasite of bumblebees (eg. Shykoff & Schmid-Hempel, 1991; Rutrecht & Brown, 2008a; Gillespie, 2010; Popp et al., 2012) and is transmitted faecal-orally between colonies via the shared use of flowers by foragers (Durrer & Schmid-Hempel, 1994; Graystock et al., 2015; Adler et al., 2018; Figueroa et al., 2019; Pinilla-Gallego et al., 2022). The interaction between *Bombus terrestris* (and in North America *B. impatiens*) and *C. bombi* is easily manipulated and maintained in the laboratory, making it a well-established epidemiological model (Schmid-Hempel et al., 2019). Consequently, accurate measurement of the prevalence and intensity of *C. bombi* infections in bumblebees is important both for assessing infection levels in wild populations and when testing and investigating epidemiological questions.

There are two commonly used methods to measure the prevalence and intensity of infection; hereafter referred to as the faecal (eg. Schmid-Hempel & Schmid-Hempel, 1993; Brown et al., 2000; Vaughan et al., 2022) and gut sampling method (eg. Biller et al., 2015; Anthony et al., 2015; LoCascio et al., 2019). To measure infection using the faecal sampling method, a faecal sample is taken and viewed under the microscope immediately, and *C. bombi* cells are counted. To measure infection using the gut sampling method, the individual is frozen and at a later date their mid- and hindguts are removed, finely ground in 25% Ringer solution, left to settle and the supernatant is sampled and viewed under the microscope to identify *C. bombi* cells. Previously, the faecal sampling method has been compared to a similar, but not identical, gut sampling method, hereafter referred to as gut sampling method 2 (Otterstatter & Thomson, 2006). In this study, both methods were used to produce two measures of infection intensity for individual bees, but without controlling for inoculation dose. This showed that the two measures of infection intensity were positively correlated, with faecal counts giving higher intensity estimates than gut counts. This is surprising, as one might expect that the gut sampling method 2 is more sensitive to detecting infections as *C. bombi* replicates in the gut prior to shedding cells in the faeces (Logan et al., 2005) and continues to do so throughout the infection.

Whether the two methods described above (faecal and gut sampling method) yield similar results has implications for the interpretation of the results of previous studies. If one method is more sensitive at detecting infection, prevalence estimates will be higher, because the method will be more likely to detect low intensity infections. Estimates of infection intensity will also be higher, because a larger number of cells will be counted. In addition, knowledge of the comparability of the two methods can

inform the design of future studies, for example if low infection intensity is expected the more sensitive method may be suitable. Here, I investigated whether the two methods produce similar estimates of infection by inoculating bumblebees with a standardised *C. bombi* dose and measuring the prevalence and infection intensity in bees one week later using one of two methods. I hypothesised that the faecal sampling method would be a more sensitive measure of infection, leading to higher estimates of prevalence and infection intensity. This is because Otterstatter & Thomson (2006) found higher estimates of infection intensity when the faecal compared to the gut method 2 was used, as described above.

7.2 Methods

7.21 Experimental organisms

Three *Bombus terrestris audax* colonies, of 85-100 workers each, were ordered from Agralan (UK). Colonies were housed under red light at approximately 25°C and ambient humidity. They were fed sterile sugar solution (50% concentration) and honeybee collected pollen (Agralan, UK) *ad-libitum*. *Crithidia bombi* was obtained from two laboratory colonies of *Bombus terrestris audax* (Agralan, UK) that were infected and maintained for *C. bombi* stock (see General methods 2.1).

7.22 Experimental design

Treatments

To investigate whether the measurement method affected the estimate of prevalence and infection intensity bees were infected with a standardised dose of *C. bombi.* Their infection intensity was measured one week later via either the faecal sampling or gut sampling method (for comparison of papers using each method see Table A5.1). Inoculations occurred in three blocks over three days. On each day, five individuals from each of the three colonies were inoculated and their infection intensity measured via each method, resulting in 45 individuals in each method treatment group.

Inoculation

Bees were inoculated with a dose of 20,000 cells to test whether measurement method affected both prevalence and infection intensity. Previous experiments trialled a range of inoculation doses (Otterstatter & Thomson, 2006; Ruiz-González & Brown, 2006b) and this dose resulted in a sufficient number of individuals getting infected to allow investigation of infection intensity, whilst ensuring

enough individuals did not get infected to allow prevalence to be investigated. In addition, this dose is field-realistic (Schmid-Hempel & Schmid-Hempel, 1993).

On the day of inoculation, bees were removed from their colonies and weighed in pre-weighed vials to the nearest milligram (Scout SKX, Ohaus, Switzerland). Infection intensity can covary with size and therefore, mass was used as a proxy for size (Otterstatter & Thomson, 2006). Bees were housed in nicot cages (Becky's bees, UK), which are cylindrical containers adapted from hair rollers to house bees (see Fig. A5.1) and inoculated following the protocol in General methods 2.1 and 2.2. Bees remained housed in nicot cages for one week. 2ml syringes were replaced with 5ml syringes containing sterile sugar solution (50% concentration) and these were replaced every three days to prevent fungal growth.

Measuring infection

One week after inoculation prevalence and infection intensity were measured. Infection was measured in bees in the faecal sampling treatment group following the protocol in General methods 2.3. Bees in the gut dissection treatment group were frozen at -80°C, to be dissected at a later date using a modified General methods 2.4 protocol as follows. On the day of dissection, the crop, mid- and hindguts were removed from the bee. The mid- and hindguts were put in a 2ml Eppendorf with 300µL of 25% Ringer solution (Ohaus, Thermo Scientific, UK). The guts were ground vigorously with a pestle for 30 seconds to release the *C. bombi* from the guts. The mixture was vortexed for 10 seconds and left to stand for three hours to allow gut debris to sink to the bottom and a supernatant to form. Three hours was chosen because previous studies have used this settling time (eg. Anthony et al., 2015; LoCascio, Pasquale, et al., 2019) and this was feasible in an experimental day, given the number of bees being screened for infection. After three hours, 10µL of the supernatant was viewed on an improved Neubauer haemocytometer under a phase contrast microscope at X400 magnification and the prevalence and infection intensity were recorded.

7.23 Statistical analyses

Analyses were performed in RStudio "Prairie Trillium" (RStudio Team, 2022), R version 4.2.0 (R Core Team, 2022). All figures were created using the ggplot() function from the ggplot2 package (Wickham, 2016). The effect of measurement method on prevalence of infection could not be tested since all individuals were infected. To test whether the measurement method affected the estimate of infection intensity a general linear model with a negative binomial error distribution and a log link was used due to overdispersion. The function "glm.nb" was used from the MASS package (Venables &

Ripley, 2002) with cells per microlitre as the response variable. The full model included method as a fixed factor and bee mass, colony and experimental block as covariates. I did not test a sufficient number of colonies to include colony as a random effect in a mixed effects model (Gelman & Hill, 2006; Arnqvist, 2020). Overdispersion was checked using the performance package (Lüdecke et al., 2021) and residuals were checked with the DHARMa package (Hartig, 2022).

7.3 Results

Infection intensity was measured in 42 individuals using the faecal sampling method and 43 using the gut dissection method. Five individuals were lost from the experiment; three from the faecal and two from the gut sampling group, as they did not drink the inoculum. The best model included method and colony as fixed factors. Bee mass and experimental block did not significantly affect infection intensity (X^2_1 = 92.969, p = 0.256; X^2_1 = 93.473, p = 0.18) and were removed from the final model as they did not improve model fit. In the reduced model, method significantly affected infection intensity (X^2_1 = 17.993, p = < 0.001; Fig. 1). When the gut dissection method was used to measure infection intensity the mean infection intensity was approximately double (9,415 [±1.11] cells/µL), that obtained via the faecal sampling method (4,915 [±1.11] cells/µL). Colony also significantly affected infection intensity (X^2_2 = 9.386, p = 0.009; Fig. 2). Colony 1 had significantly higher infection intensity across both methods than Colony 2 (Colony 1: 9,228 [± 1.14] cells/µL, Colony 2: 5,377 [± 1.14] cells/µL; p = 0.009). Infection intensity was not significantly different in Colony 2 compared to Colony 3 (Colony 2: 5,377 [± 1.14] cells/µL, Colony 3: 6,311[± 1.14] cells/µL; p = 0.660) or Colony 1 compared to Colony 3 (p = 0.105).

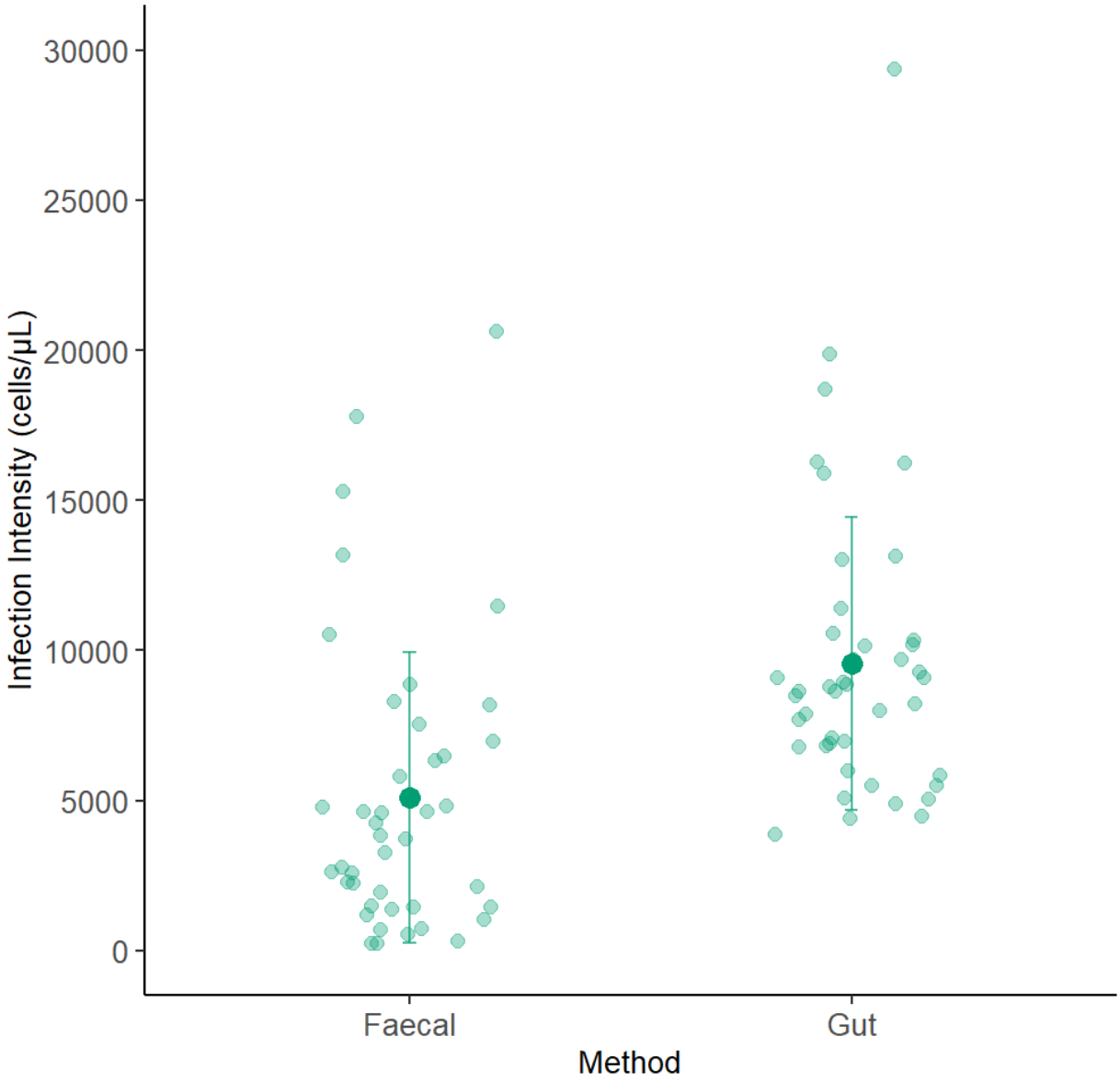

Fig. 1 The infection intensity of *C. bombi* one week after bees were given a standardised dose. Infection intensity was measured using two methods (faecal or gut sampling method). The large, darker datapoints show the mean infection intensity and the bars the standard deviations. Light datapoints show the raw data.

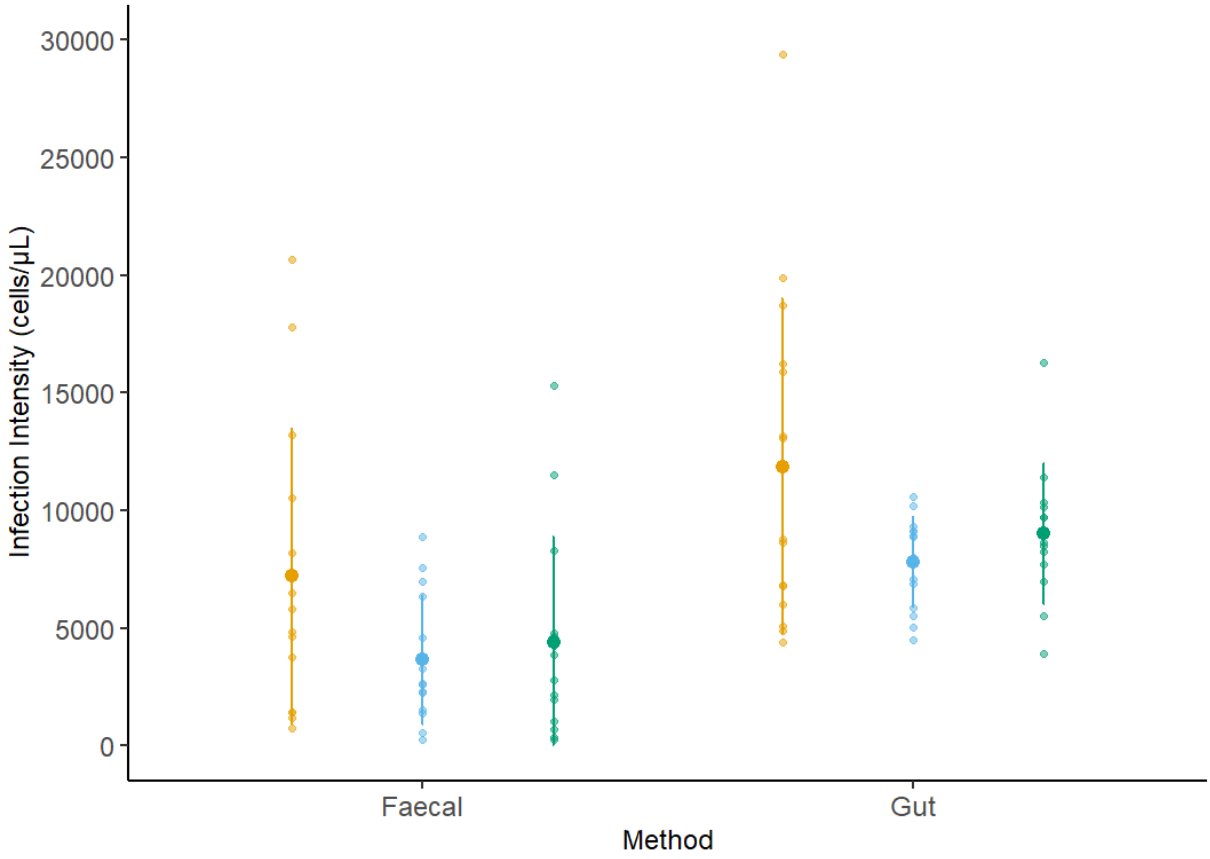

Fig. 2 The infection intensity of *C. bombi* in *B. terrestris* one week after a standardised inoculation dose. Infection intensity was measured using either a faecal or gut sampling method. Colour indicates bee colony (orange: Colony 1, blue: Colony 2 and green: Colony 3). Smaller datapoints are the raw datapoints and mean infection intensity is shown by the larger datapoints. Bars indicate standard deviations.

7.4 Discussion

Here, I found that the method used to measure the infection intensity of *C. bombi* in *B. terrestris* affects the estimate of infection intensity. Specifically, the gut sampling method generated measures of infection intensity that were almost double those from the faecal sampling method. Infection intensity was also affected by the colony bees originated from, with one colony exhibiting higher infection intensities irrespective of the methods used to measure infection intensity.

The higher estimates of infection intensity found when using the gut as opposed to the faecal sampling method demonstrate that these measures are not equivalent or comparable. However, the direction of difference was not as predicted from the previous study of Otterstatter and Thomson (2006), who found higher estimates of infection intensity in faecal compared to gut samples. Interestingly, their faecal estimates were almost double that from the gut, which is a similar magnitude of difference to my results. The difference between the two studies is likely driven by the fact that Otterstatter & Thomson (2006) took both measurements from the same individual, whereas I used individuals for either faecal or gut sampling, but not both. By definition, when both approaches are used on the same individual, gut sampling must always be conducted after faecal sampling. In the Otterstatter & Thomson (2006), it is likely that the number of *C. bombi* cells were lower in the gut because the individual had recently defecated and therefore, cleared a large number of cells from the gut. I used an independent groups design and did not conduct both methods on the same individual. Consequently, I have removed the possibility of this order effect affecting a comparison of the methods.

Although the effect of method on estimates of prevalence could not be tested, the higher infection intensity estimates obtained using the gut sampling method suggests that this method is more sensitive and thus, more likely to identify infection accurately in animals with low infection intensity. This is an important consideration when choosing which method to use when designing a study. If one is expecting low infection intensity the gut sampling method may be more suitable. The gut sampling method may also be able to detect newly established infections earlier when the parasite has just started replicating in the gut, and cell concentration in the faeces is very low, making it a better measure of prevalence. The choice of method should also be determined by the hypotheses being tested, since one could argue that these methods measure slightly different infection responses. The faecal sampling method measures the infectivity of the individual, whereas the gut sampling method measures the parasite load in the gut. Whilst these values are positively correlated within individuals (Otterstatter & Thomson, 2006), my results have emphasised that they are not comparable. Finally, time availability during and after the experiment also needs to be considered when choosing a method. The faecal sampling method is more time-sensitive and will impose a limit on the number of samples that can be collected within one day (approximately 15-18 per hour in my experience), whereas after the samples are frozen the gut sampling method is less time-sensitive. This is because dissection can be done at any time, however, more time is required per sample to obtain an estimate. Approximately 12-15 minutes per sample is required to dissect and measure infection using the gut method, compared to approximately four minutes for the faecal method (H.W.G, pers. obvs).

In addition to informing the designs of future studies, my results are valuable when interpreting the results of previous studies, as higher infection intensities will be expected if the gut sampling method was used to measure infection. My results will aid the interpretation of previous study results in treatment groups where antimicrobials were not applied, when studies specify the timepoint that infection was measured in samples and the inoculation dose is given. However, I note that dose is unlikely to affect infection intensity, as Schmid-Hempel et al. (2019) showed that infection intensity does not vary in bees given inoculation doses above 1,250 cells. Within gut sample methods, settling time also varies between studies (Table A5.1). One may expect that a longer settling duration improves visibility in the sample, as more gut debris sinks to the bottom. However, results from Otterstatter & Thomson (2006) indicate that the duration of settling time does not affect estimates of infection intensity. Given these caveats, it is clear from looking at previous studies that have used the gut method that infection intensity estimates vary (see Table A5.2). For example, Giacomini et al. (2018) estimated mean infection intensity as 5,500 cells/µL 7 days post-inoculation following a 6,000 cell dose and LoCascio, Pasquale, et al. (2019) measured 1,750 cells/µL 7 days post-inoculation following a 6,000 cell dose. In contrast, studies using the faecal method have estimated infection intensity as 4,000 cells/µL 6 days post-inoculation after a 10,000 cell (Schmid-Hempel et al., 1999) and 27,600 cells/µL 7 days post-inoculation after a 25,000 cell dose (Logan et al., 2005). If these estimates are compared, the gut-method estimate is not approximately double the faecal-method estimate, as my results would predict. This could be because studies generally use one method of measuring infection and therefore, direct comparison within one study is not possible. Estimates from various studies may not be directly comparable because different hypotheses are being tested, for example, bees may be fed different pollen diets that can affect infection intensity (eg. Giacomini et al., 2018; Fowler et al., 2022). Furthermore, often laboratory studies that use the gut sampling method use *B. impatiens* as a host (eg. Anthony et al., 2015; Giacomini et al., 2018; Aguirre et al., 2020; Fowler et al., 2022), whereas, studies that use the faecal sampling method use *B. terrestris* as a host (eg. Schmid-Hempel et al., 1999; Logan et al., 2005; Yourth & Schmid-Hempel, 2006; Folly et al., 2020) (see Table A5.2). This confounding use of sampling method and host species makes cross-species comparison of infection estimates challenging. When infection estimates are approximately compared between *B. terrestris* and *B. impatiens* (see Table A5.2), susceptibility appears not to differ between the species, however, it is not possible to attribute differences in infection estimates to species due to the confounding use of sampling method and host species.

In addition, studies use different methods to count *C. bombi* cells, which may also affect infection estimates. For example, some studies count 'live' or 'actively moving' *C. bombi* cells (LoCascio,

Pasquale, et al., 2019; Aguirre et al., 2020). The criteria for which cells are being counted are not explicitly specified in these studies, but 'live' or 'actively moving' likely refers to only two of the cell types of this parasite (choanomastigote and promastigote, both of which have flagella and swim actively). Given that amastigotes are common and abundant across the timeline of infections (Logan et al., 2005), such criteria likely leads to lower intensity estimates when compared to studies that count all of the cell types. Indeed, estimates in these studies are relatively low, for example, LoCascio, Pasquale, et al. (2019) measured 1,750 cells/µL 7 days post-inoculation following a 6,000 cell dose (as detailed above) and Aguirre et al. (2020) estimated infection intensities of 55.5 – 750 cells/µL 7 days post-inoculation with a 6,000 cell dose.

Another significant predictor of infection intensity was colony. On average, one colony consistently exhibited higher infection intensities irrespective of methods. This is a well-established effect in the system, as the host-parasite interaction exhibits genotype-genotype specificity, with some *C. bombi* strains more likely to infect certain colony genotypes (Baer & Schmid-Hempel, 2003; Cisarovsky et al., 2012). In addition, host colonies exhibit a range of immune gene expression, which may affect their susceptibility to infection (Schlüns et al., 2010; Brunner et al., 2013). Furthermore, susceptibility varies between colonies due to differences in the gut microbiome. Similar to host genotype, the host microbiome can have a large effect on infection intensity (Koch & Schmid-Hempel, 2012; Mockler et al., 2018). These two factors are linked as genotype can influence which microbiota establish in the gut (Koch & Schmid-Hempel, 2012). Consequently, some colonies are more susceptible to *C. bombi* infection, as seen in my experiment. Ecologically, this means that some colonies are likely to take the role of super spreaders in driving the annual parasite epidemic.

In conclusion, using the gut sampling method to measure infection intensity, following a standardised inoculation dose, produced cell counts that were almost double that from the faecal sampling method. This has implications for the design of future studies, as the gut sampling method may be more sensitive to low levels of infection. However, the time available during an experiment and the focal response both need to be considered, as the faecal and gut methods measure different infection responses. My results emphasise that an understanding of how results from different methodologies vary can be valuable in the interpretation of results from previous studies. In conclusion, given the importance of bumblebees as pollinators, the impact of *C. bombi* on bumblebee health, and its use as an epidemiological model, I call on researchers to move towards consistent quantification of infections to enable future comparisons and meta-analyses of studies.

Data availability

Data is available at https://doi.org/10.5281/zenodo.10118532.

Chapter 8
Discussion

8.1 Overview

Investigating the intricate relationship between a host and parasite is important for multiple reasons, including measuring the effects of infection on the host, predicting the dynamics of transmission in host populations, and understanding how the relationship may evolve over time. My thesis aimed to further understand the interaction between *B. terrestris* and *C. bombi* to shed light on the mechanisms of *C. bombi* transmission in wild bee populations, whilst also testing hypotheses applicable to parasite transmission in social populations generally. My research has focussed on two main themes, firstly I investigate the effects of host population demography on parasite transmission. I chose to focus on this as little is known about within colony transmission in this model system, particularly how this may vary as the colony progresses through its lifecycle. This system provides a rare opportunity to compare parasite transmission in host populations that naturally transition through contrasting demographics in a relatively short time period. Furthermore, the pandemic highlighted the variation in Covid-19 transmission in populations across the world. Evidently, this was a result of more than differences in our population demography, however, variation in our population structure played a significant role in shaping transmission.

Secondly, my research focussed on the effects of climate on parasite transmission. Understanding the impacts of climate on transmission is becoming increasingly important as our environment becomes more uncertain with climate change. *Crithidia bombi* goes through a period of environmental exposure and consequently, investigating the effects of climate variation on *C. bombi* is necessary for understanding its transmission in the field. In addition to investigating the effects of climate on transmission in this system, I wanted to find out whether there are any broad, overall trends in the effects of climate on parasite prevalence. There is often speculation in the media about increasing disease with climate change, however, to my knowledge there has been no quantitative evidence synthesis on the effects of climate on parasite prevalence in terrestrial animals. Both these research themes emphasise the importance of considering parasite transmission in a wider context, rather than investigating host-parasite interactions in isolation. In this discussion I will summarise my main findings, their wider implications and limitations, and suggest areas for future research.

8.2 Summary of findings

In Chapter 3 and 4 I investigate the effects of population structure and demography on parasite transmission in social populations. Chapter 3 focusses on infection susceptibility at the level of the individual, specifically whether this varies with age. I found that bumblebee age significantly affected susceptibility to *C. bombi* infection, with 0 day old bees harbouring infection intensities that were more than double that of 7 day old bees and quadruple that of 21 day old bees. The expression of antimicrobial peptides (AMP) involved in *C. bombi* defence, defensin and abaecin, did not significantly vary with age, rather the gut microbiome provides the most plausible explanation for the effect of age on *C. bombi* susceptibility. Furthermore, infection intensity did not significantly vary between colonies, whereas AMP expression did significantly vary between colonies.

In contrast to Chapter 3, in Chapter 4 I test whether population demography affects parasite transmission at the colony-level. Bumblebee colonies infected at the start of their ergonomic growth phase exhibited higher prevalence and more prolonged *C. bombi* transmission compared to colonies infected in the middle and the end of their ergonomic growth phase. Overall, prevalence was lowest and persisted for the shortest time period in colonies infected at the end of their lifecycle. However, the estimated R_0 and prevalence after seven days did not vary with the timing of infection. Surprisingly, infection did not cause a persistent epidemic in any colonies, with 14 out of 16 colonies clearing the infection by the end of their lifecycle. As a result, prevalence in males produced at the end of the lifecycle was extremely low and did not differ between colonies infected at different ergonomic growth stages. Colony growth was measured throughout the experiment and treatment 1 colonies exhibited slower growth compared to treatment 2 and 3 colonies. It is unclear whether this was a result of the earlier infection or experimental disruption of treatment 1 colonies.

In Chapters 5 and 6, I investigate the impacts of climate on parasite transmission. In Chapter 5, I examine the effects of climate more generally, conducting a meta-analysis on the effects of climate on parasite prevalence in terrestrial animals. I found that both temperature and precipitation did not have an overall effect on parasite prevalence, rather they had a large variety of effects on parasite prevalence. Interestingly, the temperature difference between two field sites or experimental treatments affected the likelihood of temperature having an effect on parasite prevalence. This indicates that experimental design is important when testing the effects of temperature, and perhaps climate in general, on parasite prevalence. In Chapter 6 I look at the effects climate on transmission

of *C. bombi* specifically, since *C. bombi* is exposed to varying temperatures whilst on flowers in the field. I tested whether temperature exposure affects the infectivity of *C. bombi* to *B. terrestris* by incubating the parasite at a range of temperatures for either 10 or 60 minutes prior to inoculation in *B. terrestris.* Below 50°C, *C. bombi* prevalence was not affected by incubation temperature or duration, but when exposed to 50°C for 60 minutes the parasite was no longer infective. Extreme temperatures induced morphological changes in *C. bombi* cells, however, infection intensity was not affected by incubation duration or temperature. Finally, the colony of origin did not affect infection prevalence or intensity. Together, these results indicate that *C. bombi* transmission is robust to a wide range of temperatures.

In the final empirical chapter, Chapter 7, I compare two commonly used methods of measuring *C. bombi* infection in bumblebees to find out whether they yield similar estimates of infection intensity. One week after inoculation with a standardised dose of *C. bombi*, infection intensity estimates obtained using the gut sampling method were more than double that from the faecal sampling method. In addition, colony of origin significantly affected infection intensity.

8.3 Key themes and implications

8.31 The *C. bombi* – *B. terrestris* model system

Within colony transmission

Despite the significant body of research on this system, little is known about within colony transmission of *C. bombi.* The dynamics of within colony transmission are important to understand as within colony transmission, from foraging workers to new gynes, is one way that *C. bombi* is transmitted to the following generation (Ulrich & Schmid-Hempel, 2015). My results from Chapter 4 show that a single infected worker is not sufficient to cause a persistent epidemic and extremely unlikely to result in transmission to the next generation of sexuals. Albeit, I did not test transmission to new gynes, however, the low level or absence of infection at the end of the colony lifecycle indicates that this is unlikely. This result was the most surprising of my thesis, because of the low number of *C. bombi* cells required to cause an infection (78 cells (Schmid-Hempel et al., 1999)) and the high number of cells released from an infected individual in a single defecation (up to 60,000 cells/µL of faeces (Logan et al., 2005)). Consequently, just one infected individual will release significantly more cells than is needed to cause an infection and given the lack of organisation in a bumblebee colony, *C.*

bombi cells will be spread throughout the colony. This result is also ironic because throughout my research I have been terrified of accidently contaminating and consequently, infecting uninfected colonies, due to the high infectivity of *C. bombi*.

When looking at results from previous studies, Otterstatter & Thomson (2007) found that colonies founded by an infected queen had variable *C. bombi* prevalence 40 days after the emergence of the first worker, with 1/4 workers infected in some colonies and 5/5 workers infected in others. Since this experiment was terminated after 40 days, it is not known whether the epidemic would have persisted for the remainder of the lifecycle or died out as I observed. Repeating this Otterstatter & Thomson (2007) experiment but measuring prevalence for the remainder of the lifecycle, as I did, would elucidate whether founding by an infected queen is sufficient for a prolonged epidemic and subsequent transmission to the following generation. Perhaps, *C. bombi* transmission also dies out in colonies founded by an infected queen when no further infections occur.

The reasons for the difference in prevalence observed between colonies infected at different ergonomic growth stages and thus, of colonies with contrasting demographics, is unclear. Given my results from Chapter 3, the difference in the age structure of colonies could explain some of the variation, however, it is unlikely to provide a full explanation. The difference in susceptibility of workers with age may explain some of the variation because the average age of workers in colonies infected towards the end of the ergonomic growth stage (treatment 3) was higher compared to those infected at the start (treatment 1) and middle (treatment 2) of this phase. In Chapter 3, I found that 21 day individuals had infection intensities four times higher than 0 day old individuals (callows), whereas, 7 day old individuals had double the infection intensities of callows. However, the infection intensity did not significantly differ between 7 and 21 days directly. Consequently, it is possible that as a result of the older average age in treatment 3 colonies, the mean infection intensity of infected individuals may have been lower, leading to fewer *C. bombi* cells shed in faeces and lower transmission. However, age structure of the colony cannot provide a full explanation for my results. This is because the infection intensity in 21 day individuals (9,732 cells/µl, 95% C.I. = [5,130, 18,462]) and thus, the number of *C. bombi* cells shed in their faeces, was more than sufficient to cause an infection in other individuals, as detailed above.

The higher susceptibility of colonies in treatment 1 and thus, smaller colonies may have implications for the susceptibility of different bumblebee species to *C. bombi.* Across bumblebee species, there is large variation in colony size (Cueva Del Castillo et al., 2015; Falk & Lewington, 2019). For example in

the UK, *B. terrestris* colonies can have large colonies of up to 500 individuals, whereas, the Heath bumblebee, *B. jonellus,* has colonies of about 50 workers (Falk & Lewington, 2019). My data on colony growth in Chapter 4, show that colonies with approximately 50 workers (4-6 weeks after inoculation in treatment 1) had high rates of *C. bombi* transmission, whereas as colonies got larger *C. bombi* transmission decreased. This may have implications for *C. bombi* infections across different species because there is a high degree of species mixing on flowers and therefore, some species, perhaps those with smaller colonies, might drive the high prevalence of *C. bombi* often observed in June and July in the UK (Popp et al., 2012; Parsche & Lattorff, 2018). Furthermore, *B. terrestris* is a common species of bumblebee in the UK, whereas some of the species with smaller colonies, such as *B. humilis* (40-50 workers)*, B. muscorum* (40-120 workers) and *B. sylvarum* (up to 50 workers) (Falk & Lewington, 2019) are significantly rarer (Goulson et al., 2005; Darvill et al., 2006; Williams et al., 2007). If smaller colonies are more susceptible to *C. bombi,* some rarer species with smaller colonies may be more susceptible to *C. bombi* compared to more common species, such as *B. terrestris* and *B. lucorum,* which have larger colonies. Assuming the fitness costs of *C. bombi* (Shykoff & Schmid-Hempel, 1991; Brown, Schmid-Hempel et al., 2003; Baron et al., 2017) to all bumblebee species are similar, *C. bombi* may have larger effects on population growth of rarer compared common species, as a result of the fitness cost for queens.

Although I aimed to mimic between colony transmission via nectar, my results from Chapter 6 can also be applied to within colony transmission of *C. bombi* via nectar pots, and to a lesser extent via contaminated surfaces generally. The temperature of bumblebee colonies is regulated, through building wax coverings for insulation when it is cold (Jones & Oldroyd, 2006) and fanning when it is hot (Weidenmüller et al., 2002). Despite regulation, colony temperature can be highly variable and depends on colony size, in addition to the environmental temperature (Vogt, 1986; Crall et al., 2018). My results indicate that within colony transmission is likely largely unaffected by colony temperature, particularly, because bumblebee colonies are underground and therefore, less likely to reach extremely high temperatures such as 50°C, which may occur on floral structures.

Between colony transmission

When transmitted between colonies, *C. bombi* can spend up to an hour on flowers (Stout & Goulson, 2001) and therefore, *C. bombi* must be robust to substantial variation in environmental conditions. During the time that the parasite is on the flower, it may be exposed to a wide variety of temperatures, UV radiation, wind and rain, all of which may affect the ability of *C. bombi* to infect and replicate in the next host. My results from Chapter 6 indicate that *C. bombi* is robust to a wide variety of

temperatures; even the 40°C temperatures seen in the extreme heatwave in the UK in summer 2022 (Kendon, 2022). Despite the ability of *C. bombi* to remain infective across a wide range of temperatures, it appears to be affected by extreme temperatures to some degree, as the prevalence of 'unhealthy' and burst cells was highest at extremely low and high temperatures (Fig. 3, Chapter 6). At 30°C, the prevalence of unhealthy cells was lowest, which corroborates with the results from *in vitro* studies that found the optimum temperature for *C. bombi* growth and replication is 33.7-34.4°C (Palmer-Young, Raffel, et al., 2018; Palmer-Young et al., 2021). It is possible that exposure to extreme temperatures has an effect on *C. bombi* fitness that I did not measure. It would be interesting to repeat the experiment in Chapter 6, with the addition of a serial passage experiment in which the *C. bombi* from infected hosts are used to infect subsequent hosts. Perhaps, using inoculum comprised of a higher proportion of unhealthy cells is less infective to the following hosts.

The meta-analysis in Chapter 5 emphasises that temperature is not the only climate variable that can affect parasite transmission. Although some other climate variables can be challenging to directly manipulate in a laboratory experiment, measuring the effects of temperature on *C. bombi* transmission in isolation is not field-realistic. For example, wind may play an interesting role on the survival and viability of *C. bombi* whilst on flowers, as higher wind speeds are likely to increase the rate of evaporation of contaminated faeces. Schmid-Hempel et al. (1999) found that inoculating bumblebees with contaminated faeces that had dried out for 45 minutes led to approximately a 20% reduction in prevalence in the next host. I also found that *C. bombi* was less infective after being incubated for 60 compared to 10 minutes (Fig. 6, Chapter 6), however, I incubated *C. bombi* in solution and therefore, desiccation was unlikely. In contrast, on flowers faeces are likely to desiccate at a faster rate which may exacerbate the loss of infectivity. The effect of climate on *C. bombi* will vary depending on where the contaminated faeces is deposited on the floral structure. The location of *C. bombi* on the flower can determine its survival (Figueroa et al., 2019; Pinilla-Gallego et al., 2022). This may be because of higher exposure to environmental variation on certain structures, such as on the corolla lip or outer petals, compared to when inside the flower. In contrast, when *C. bombi* is deposited in nectar it will be exposed to different environmental stressors. For example, although it will be less prone to desiccation (unless the nectar evaporates which is possible at high temperatures (Descamps et al., 2021)), it will be exposed to a variety of sugar concentrations, which can affect *C. bombi* survival (Cisarovsky & Schmid-Hempel, 2014). Consequently, when testing the effects of environmental exposure on parasite fitness, it is important to test environmental stressors in combination as they may interact. In addition, one should be aware that environmental stressors can vary substantially

even in environments very close together, as is seen in the environment of nectar compared flower surfaces.

At a larger scale, climate is also likely to exert contrasting pressures on *C. bombi* across different landscapes, for example in cities compared to forests. *Crithidia bombi* prevalence varies between landscapes, with some studies finding a higher prevalence in urban compared to rural landscapes (Goulson et al., 2012; Theodorou et al., 2016; Ivers et al., 2022), whilst Gratton et al. (2023) observed a higher prevalence in forests compared to urban valleys. One climate factor that may vary between landscapes is UV radiation. In forests, *C. bombi* may be exposed to lower UV whilst on flowers as a result of more shade. *Crithidia bombi* survival is higher on shaded compared to exposed flowers (Figueroa et al., 2019) and therefore, UV radiation may affect between colony transmission of *C. bombi* in different landscapes. Furthermore, temperatures in forests compared to urban landscapes are likely to be lower due to the cooling effect of trees (eg. Ellison et al., 2017) and the reduced dissipation of heat in urban areas (Gago et al., 2013; Wang et al., 2021). The difference in climate between landscapes is likely to increase as temperatures rise due to climate change and extreme temperatures become more likely in urban areas (Pörtner et al., 2022). This may enhance the difference in prevalence across contrasting landscapes.

However, it is likely that the difference in prevalence across landscapes is also a result of host population demography. In Chapter 4, I tested the effects of within colony demography on *C. bombi* transmission, but it is important to also consider the demography of whole *Bombus* spp. populations. *Crithidia bombi* prevalence is higher in areas of higher colony density (Parsche & Lattorff, 2018), however, high colony density does not necessarily mean higher population density as colony size must also be accounted for. Colony density is usually measured using microsatellite markers (eg. Parsche & Lattorff, 2018), rather than directly finding colonies. Bumblebee colonies are hard to find (see Goulson (2013) for a comedic method of finding colonies) and even when found it is not ethical to excavate colonies and conduct a colony count. Consequently, estimating population density is extremely difficult. If population density differences between landscapes explain some of the variation in *C. bombi* prevalence, one would expect the visitation rates of flowers to differ between landscapes. Observational experiments that count the number of visits to individual flowers between landscapes could shed light on this. Previous studies have counted bumblebee visits to whole areas in different landscapes (eg. Ahrné et al., 2009), but as far as I am aware not of individual flowers.

Colony differences

In this system it is well-established that there are genotype-genotype host-parasite interactions (Mallon et al., 2003; Barribeau & Schmid-Hempel, 2013; Barribeau et al., 2014). I observed this throughout my research, for example, colonies varied in their expression of defensin and abaecin (Fig. 3, Chapter 3) and in their susceptibility to infection when comparing two methods of measuring infection (Fig. 2, Chapter 7). Furthermore, the pilot experiments in Chapter 6 (see Appendix 4) in which the same inoculation dose was infective to individuals from some colonies but not others, emphasise the differences in infection susceptibility between colonies. However, colony differences were not observed in all experiments. For example, colony did not affect infection intensity in Chapter 3 or infection prevalence or intensity in Chapter 6, despite using bees from five and eight colonies respectively. The lack of a colony effect observed in some areas of my research could be because I only used laboratory colonies, that may have lower genetic variation compared to wild colonies. Furthermore, throughout my PhD I cycled the *C. bombi* used in experiments through two new *B. terrestris audax* colonies every 6-8 weeks. Infected spring queens were collected in spring 2021 and 2022 from Windsor Great Park (Surrey, UK), to create new stock colonies. Collecting *C. bombi* from one location may have limited the number of strains in my sample. This, combined with using laboratory colonies may have reduced my ability to observe colony effects.

8.32 General epidemiology

The environment and parasite transmission

When considering the effects of the environment on parasite transmission, one may expect that parasites with more complex lifecycles, such as vector-borne or those with intermediate hosts, to be more vulnerable to changes in the environment, due to higher levels of environmental exposure and numbers of transmission stages in a single lifecycle. However, hosts with a single host may also undergo periods of time in the environment, which may be very long, such as for some tapeworm eggs (Jansen et al., 2021) or relatively short, for example for Covid-19 (L.-D. Chen, 2020). Regardless of the length of exposure, the period in the environment is likely to have a large effect on parasite survival, infectivity and subsequently, fitness. Interestingly, my meta-analysis in Chapter 5, found the effects of temperature on prevalence were not affected by parasite lifecycle (Fig. 6c, Chapter 5) and the effects of temperature and precipitation were not affected by whether the parasite had free-living stages (Fig. 9a, Chapter 5). This is surprising, firstly because of the large representation of studies on vector-borne parasites and secondly, because vector-borne diseases have been identified as highly sensitive to climate and likely to be affected by climate change (Rocklöv & Dubrow, 2020; Pörtner et al., 2022).

For example, the Asian Tiger mosquito (*Aedes albopictus*) which vectors chikungunya, dengue fever and yellow fever, was previously found in South-East Asia and can now be found in Europe, partly due to increased climate suitability (Del Lesto et al., 2022; Eritja, 2022). The fact that I focussed on animal hosts in general rather than solely human hosts could explain this result. In addition, I only focussed on prevalence, rather than other infection outcomes, such as infection intensity, which may also vary with climate. Furthermore, the lack of studies on some taxon, such as ectoparasites, and on reptiles means that the results may not be applicable to all host and parasite taxon.

The main finding from Chapter 5, which suggests that there is no overall effect of temperature and precipitation on parasite prevalence in terrestrial animals (Chapter 5) provides quantitative evidence against the 'warmer sicker world' hypothesis (Harvell et al., 2002). In contrast, there is large variation in the effects of temperature and precipitation on parasite prevalence. Climate change may mean the prevalence of some parasites with significant human health implications, such as *Plasmodium* spp. or dengue virus increase in some locations. However, the prevalence of other parasites may fall as altered climates provide less suitable conditions for parasite transmission. Perhaps a controversial opinion, but some of the media attention on the effects of climate change on increased disease incidence could partly be a result of a shift in the distribution of some vector-borne diseases from lower-middle income countries (LMIC) to countries in Europe and North America. For example, local outbreaks of malaria now occur in Greece (Trájer, 2022) and West Nile virus has emerged in the United States (Petersen, 2019; Ronca et al., 2021). If transmission of these parasites becomes more frequent and widespread this will evidently have concerning implications, however, I believe it is worth remembering that some of these diseases have been endemic, and in some cases neglected, (for example, dengue virus and chikungunya virus) in many LMIC for decades.

Population structure and demography

The contrasting levels of *C. bombi* prevalence and duration of transmission between treatments in Chapter 4, highlight the significant role that population demography can have on parasite transmission through a host population. The colonies across the three treatments varied in their population size, age demography and sex ratios. It is unclear which of these factors contributed to the different transmission rates observed, however, the sex ratios are unlikely to have played a large role due to the equal susceptibility of workers and males to infection (Ruiz-González & Brown, 2006b). On the other hand, age demography (Chapter 3) and population size may have played a role, but further work is needed to investigate these results (see below). When designing the colony transmission experiment in Chapter 4, I considered each colony representative of an individual population, but

colonies could also be considered as separate social groups or 'households' within one larger population and therefore, represent population demography at a smaller scale. The importance of investigating population demography at a range of scales is shown by the Covid-19 pandemic, when the demography of households (such as whether extended families cohabited), cities and whole countries were important predictors of transmission (Dowd et al., 2020; Nepomuceno et al., 2020). For example, the age demography of populations may be similar, however, in some populations there may be more intergenerational mixing whereas in others similar age groups may be more likely to cohabit. Consequently, I wanted to emphasise that my findings on the effects of colony demography on parasite transmission could be applied to understanding the effects of host population demography on parasite transmission at a range of scales.

Estimating R_0

The high variation in the estimated R_0 between colonies within treatment groups in Chapter 4 (Fig. 4, Chapter 4) indicates that other factors in addition to colony demography affected R_0. Differences in colony susceptibility to *C. bombi* discussed above, could have contributed to this variation. In addition, some of this variation may have arisen because of the challenges of measuring R_0 in this host-parasite system due to the high variation in the time between hosts ingesting *C. bombi* cells and shedding cells in their faeces (Logan et al., 2005). In other host systems, there is also large variation in the time between exposure to the parasite and become infectious (the incubation period) (Ohm et al., 2018; De Giorgio, 2020; Kamiya et al., 2020). For malaria, the incubation rate in mosquitoes varies with temperature. Temperature affects the rate of development of some parasites (eg. O'Connor et al., 2006; Paull et al., 2012) and it is not known whether temperature affects the rate of *C. bombi* development and replication. If temperature does affect the rate of development or replication of *C. bombi,* I would expect temperature to affect R_0 because changes to *C. bombi* development rate may alter the incubation rate. Comparing estimates of R_0 between colonies (at the same ergonomic growth phase) maintained at different temperatures would indicate whether temperature affects R_0 of *C. bombi.* This would have implications for the rate of within colony transmission in different climates and in the future with climate change. The impact of temperature on within colony transmission is discussed in further detail below. Overall, as a result of the difficulties in accurately measuring R_0, variation between populations and the possible effects of the environment, one should be cautious when applying R_0 estimates measured in one population to other population, particularly in high-risk contexts such as was seen in the Covid-19 pandemic.

Methodology

Comparing the two methods for measuring infection intensity in Chapter 7, emphasised the importance of carefully considering the method used to measure parasite infection. Understanding the infection outcome being measured by a particular method is necessary for both choosing an appropriate method when designing an experiment and accurately interpreting the results of an experiment. The importance of experimental design is also shown by the results of the meta-analysis, which suggest the positive effect of temperature on prevalence decreases as the difference in temperature between two field sites or experimental treatments increases (Fig. 5, Chapter 5). This indicates that when investigating the effect of climate on prevalence, thorough research of the host and parasite ecology and thermal range is necessary to ensure the full distribution of the thermal response curve is sampled and false conclusions on the effect of climate on prevalence are not made. I appreciate that knowledge of the ecology and thermal range of hosts and their parasites may not always be known, but perhaps this should be a priority research area prior to conducting wider research on parasite prevalence. In particular, this should be a priority because accurately determining the effect of climate on parasite prevalence will become increasingly important due to climate change.

In addition, by conducting a meta-analysis I saw the importance of transparency when reporting study results, for example, including sample sizes of all treatment groups and including the raw data rather than only summarised data. Being transparent and providing this necessary information is needed for accurate interpretation of study results and assessment of validity, whilst enabling the inclusion of the study in meta-analyses. Furthermore, differences in methods used to measure infection outcomes of host-parasite interactions can make it difficult to synthesise results together in a meta-analysis (see discussion, Chapter 7). For example, when measuring *C. bombi* infections some studies count all cells (Schmid-Hempel et al., 1999), some count 'alive' cells (eg. Aguirre et al., 2020) and some count 'actively swimming' cells (eg. LoCascio et al., 2019) and therefore, it can be hard to compare these results. Perhaps, open communication between lab groups studying the same host-parasite interaction, would help to encourage more consistent methods of measuring infection, particularly, for established epidemiological models. As the body of published literature gets larger and we gain more knowledge it is important to be able to use the data and synthesise it together to draw generalisable conclusions. A large evidence base will be vital for understanding and responding to the complex challenges we currently face, including climate change and the risk of pandemics.

8.4 Future directions for research

In addition to the suggestions made above, investigating the following areas will help to fill the gaps in our knowledge within the *C. bombi* – *Bombus* spp. system and epidemiology more widely. Within the *C. bombi* – *Bombus* spp. model system it would be interesting to test the effect of temperature on within colony transmission. The effect of temperature on *C. bombi* and *Bombus* spp. has been tested separately (*C. bombi:* Chapter 6, *Bombus* spp.: Palmer-Young, Raffel et al., 2018; Tobin et al., 2019), but the effect of temperature has not been tested on both *C. bombi* and *Bombus* spp. simultaneously at the colony level. Despite the results from Chapter 6 suggesting that *C. bombi* is robust to a wide range of temperatures, exposing both *C. bombi* and colonies to extreme temperatures at the same time may reveal other impacts of temperature on transmission. For example, the energy expended by bumblebees to regulate colony temperature may impact their susceptibility to infection, due to trade-offs with other processes such as the immune system. This could result in increased within colony transmission. When considering my results on within colony transmission from Chapter 4 and the fact that smaller colonies have a reduced ability to regulate colony temperature, extreme temperatures may lead to higher rates of *C. bombi* transmission in earlier stage colonies and in species with smaller colonies. Firstly, testing the rate of transmission in colonies maintained at a range of temperatures and secondly, repeating this with colonies at different ergonomic growth phases would indicate whether this is the case. Furthermore, it is unclear whether the slower growth rate of colonies infected earlier in their lifecycle was a result of *C. bombi* or experimental manipulation or disruption. When repeating the experiment in Chapter 4 it would be valuable to add a control treatment group in which 10 workers were removed from the colony and put back twice per week to show whether reduced colony growth was due to colony disruption or *C. bombi* itself. In addition, video tracking experiments, such as those conducted by Otterstatter & Thomson (2007) and Sah et al. (2021), could be used to reconstruct the networks of colonies at different phases in their ergonomic growth phase and may reveal why *C. bombi* transmission was higher in colonies earlier in their lifecycle compared to later (Chapter 4). The network structure may change and evolve as the colony demography changes through their lifecycle.

More generally, I recommend that studies focus on the effects of humidity on parasite prevalence as the meta-analysis (Chapter 5), emphasised a large gap in this area. Furthermore, the majority of studies tested the effects of climate on endoparasites, rather than ectoparasites. Ectoparasites are highly exposed to the environment and consequently, climate could have a large effect on their

fitness. Finally, the parasites of reptiles and amphibians are rarely investigated, with the majority of research conducted on bird and mammal hosts and apart from research on chytrid fungus, parasites of amphibians are underrepresented in the literature.

8.5 Conclusions

My PhD improved our knowledge of the transmission of a widespread and prevalent bumblebee parasite, *C. bombi,* which will aid our understanding of bumblebee health in the field. My research focussed on the effects of host demography and climate on parasite transmission, with the findings having applications for parasite transmission in a range of social species. Research conducted on model systems have their limitations and I appreciate that the experiments conducted in this thesis were conducted in a lab under controlled conditions. Nonetheless, the epidemiological principles of this system can be applied to many other social species and the hypotheses tested can provide a starting point to build upon epidemiological evidence at a larger scale. As the effects of climate change and anthropogenic change continue to occur, the factors shaping host-parasite interactions will become increasingly complex and unpredictable. My research has shown that considering the wider environment and context of parasite transmission, in addition to carefully designing experimental methodology, is necessary for understanding the dynamics of host-parasite interactions in our current and future changing environment.

Appendix 1:
Chapter 3
Newly emerged bumblebees are highly susceptible to gut parasite infection

Hannah S Wolmuth-Gordon, Kazumi Nakabayashi & Mark JF Brown

Fig. A1.1 Callow workers were identified by their greyish legs, white stripes, ruffled fur, sluggish and clumsy behaviour, curved wings, little wing movement and low levels of aggression or resistance when handled.

Table A1.1 Sample for measuring infection intensity divided by age treatment and colony of origin.

	Age (days)			
Colony	**0**	**7**	**14**	**21**
1	4	8	6	5
2	2	3	3	5
3	6	6	5	1
4	7	7	6	2
5	8	6	7	6
Total	27	30	27	19

Table A1.2. Samples collected and lost during the measuring infection experiment.

Treatment	Total collected from colonies	Did not drink inoculum	Did not defecate	Unforeseen circumstances	Died before screening	Final sample
0	30	0	0	0	3	27
7	32	0	0	0	2	30
14	42	1	0	0	13	27
21	42	1	1	15	6	19

Table A1.3. Sample for measuring abaecin and defensin gene expression by age treatment and colony of origin

	Age (days)			
Colony	**0**	**7**	**14**	**21**
1	1	2	3	3
2	1	3	1	2
3	1	2	2	2
4	1	1	2	1
5	1	3	3	2
Total	5	11	11	10

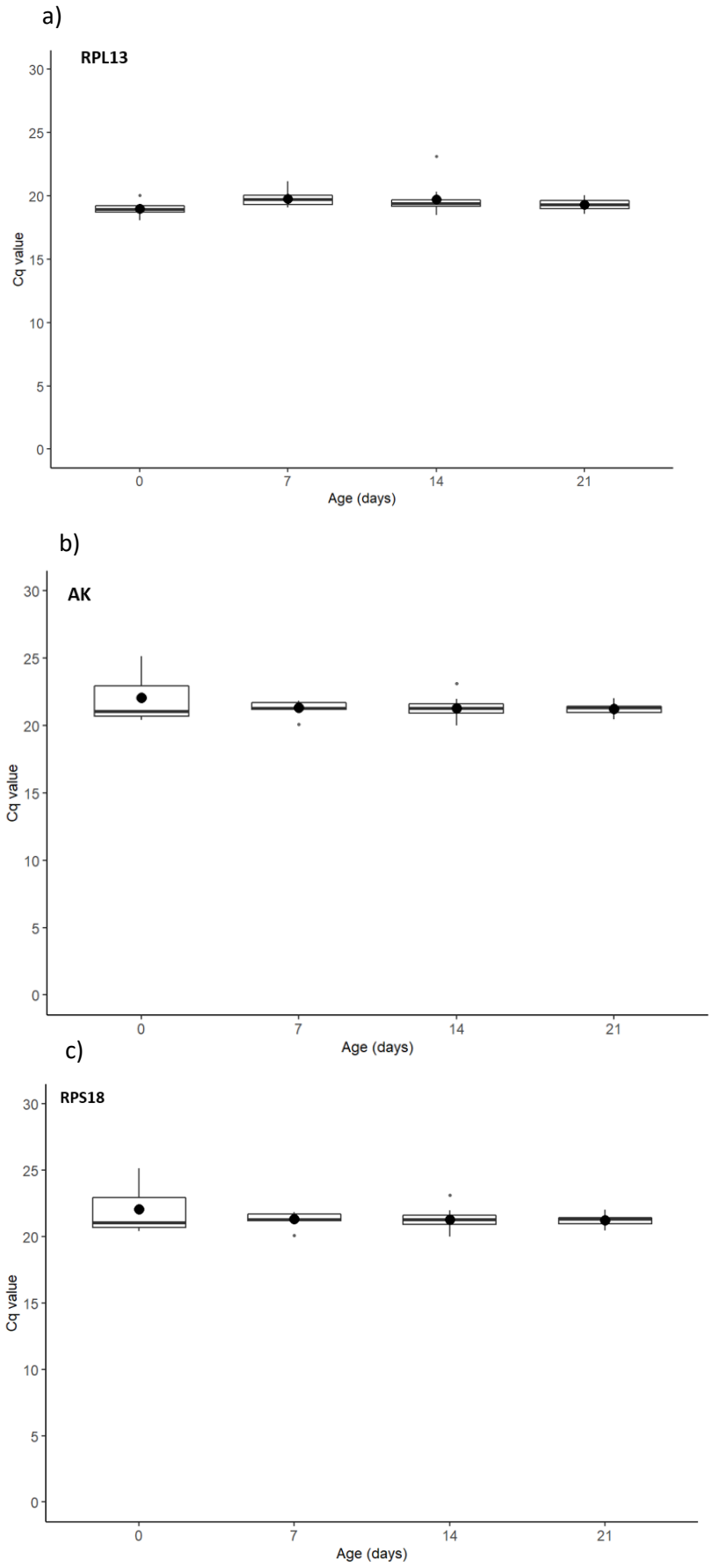

Fig. A1.2 Reference gene expression of **a)** RPL13, **b)** AK and **c)** RPS18 across age treatment groups.

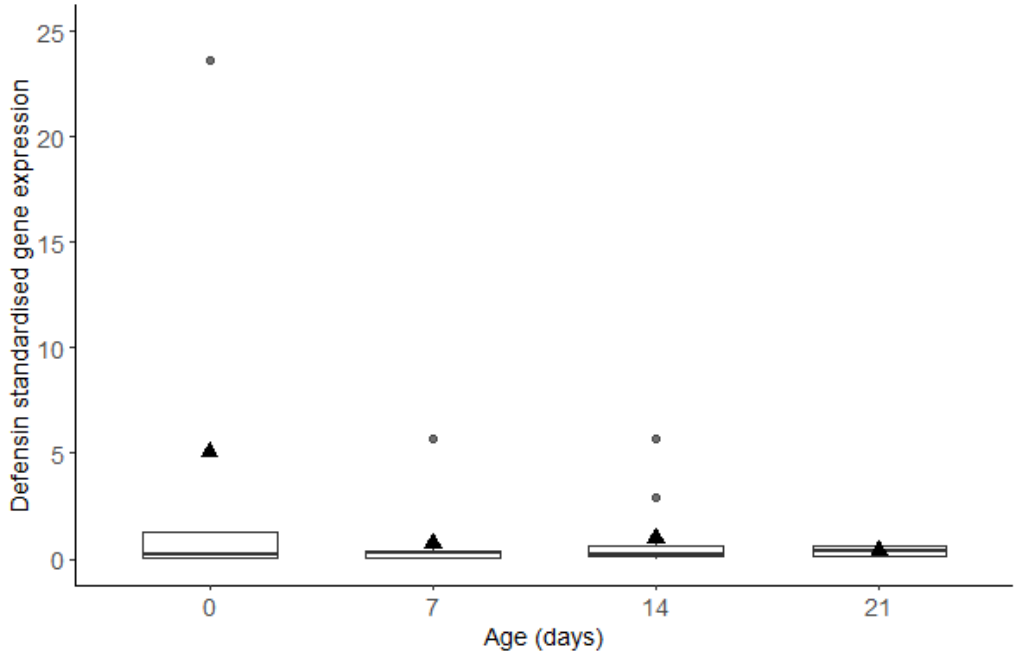

Fig. A1.3 Boxplot showing standardised defensin gene expression of bees of four different ages, 18 hours after inoculation with *C. bombi.* Defensin gene expression is standardised against the expression of RPL13 and AK reference genes. Triangle shows the mean and circular datapoints show outliers. This figure shows the outlier in the 0 day treatment group that was excluded from analysis.

Appendix 2:
Chapter 4
Timing is critical: the timing of exposure determines a parasite's spread in a social host

Hannah S Wolmuth-Gordon & Mark JF Brown

Table A2.1. The inoculation process for each colony, colonies with an asterisk were not carried through the whole experiment.

Colony	Treatment	Inoculation process
1*	1	Both individuals drank but died before screening.
		Colony reinoculated, both individuals drank and individual was infected.
2*	1	Both individuals drank but died before screening.
		Colony reinoculated, both individuals drank and individual was infected.
3	1	Both individuals drank and the individual was infected.
4	1	Both individuals drank and the individual was infected.
5*	1	Two individuals inoculated and both put back into colony by mistake, discarded from experiment
6	1	Both individuals drank and the individual was infected.
7	1	Both individuals drank and the individual was infected.
8	2	Both individuals drank and the individual was infected.
9	2	Both individuals drank and the individual was infected.
10	2	Both individuals drank and the individual was infected.
11	2	Both individuals drank and the individual was infected.
12	2	Both individuals drank, but individual was not infected.
		Colony reinoculated and one individual drank. This individual was infected.
13	3	Both individuals drank and the individual was infected.
14	3	One individual drank and was infected.
15	3	Neither individual drank, colony reinoculated, both individuals drank and the individual was infected.
16	3	One individual dranj and was infected.
17	2	One individual drank, but individual was not infected.
		Colony reinoculated and one individual drank. This individual was infected.
18	3	One individual drank, but individual was not infected.
		Colony reinoculated and one individual drank. This individual was infected.
19	3	One individual drank, but individual was not infected.
		Colony reinoculated and both individuals drank. This individual was infected.
20	3	One individual drank and was infected.

Table A2.2 Number of infected individuals when R_0 was estimated seven days after inoculation of the colony.

Colony	Treatment	Number infected	Sample size	*C. bombi* prevalence (%)	Colony size
1	1	3	20	15	21
2	1	1	19	5.26	23
3	1	3	21	14.29	22
4	1	1	7	14.29	9
6	1	11	21	52.38	35
7	1	10	14	71.43	14
8	2	5	20	25	57
9	2	7	21	33.33	42
10	2	3	21	14.29	43
11	2	9	20	45	58
12	2	3	20	15	48
13	3	3	18	16.67	79
14	3	2	20	10	78
15	3	3	20	15	88
16	3	2	20	10	99
17	2	3	20	15	60
18	3	1	20	16.67	79
19	3	1	20	5	149
20	3	2	20	10	76

Appendix 3:
Chapter 5
Large variation in the effect of climate on parasite prevalence in terrestrial animals: a meta-analysis

Hannah S Wolmuth-Gordon, Julia Koricheva & Mark JF Brown

Searches

Web of science, core collection

Search details

Number of results: 10,429

16/02/21, 3pm
English language
Article and early access
Royal Holloway University of London
1970-2021
Searched title, abstract, key words (topic)
Advanced search

Search string

TS=(parasit* or cestod* or helminth* or nematod* or tapeworm* or virus* or viral or acanthocephala* or copepod* or bacteria* or monogenea* or protozoa* or trematod* or digenea* or ectoparasit* or endoparasit* or pathogen* or flea* or tick* or mite* or leech* or nematomorph* or fungus or fungi or chytrid* or microsporidia* or trypanosome* or fungal)
AND
TS=("host susceptib*" or "host vulnerab*" or “disease vulnerab*” or “disease susceptib*” or “host* resistan*” or “disease resistan*” or prevalence or “disease level” or “disease abundan*” or “disease outcome*” or “disease intensit*” or “level of disease” or “degree of disease” or “pathogen intensit*” or “parasite intensity*" or “pathogen level*” or “level of pathogen*” or “pathogen abundan*” or “abundance of pathogen*” or “pathogen load” or “disease load” or “disease burden” or “parasite burden” or “pathogen burden”)
AND
TS=("climate change" or "global warming" or "climat* varia*" or temperature* or precipitation or rain or rainfall or "water availability" or "water defic*" or "moisture defic*" or heat or thermal or humid* or cold* or chill* or altitud* or “elevation gradient” or latitud* or warm* or “ephemeral water bod*” or “temporary water bod*”)

Scopus

Search details

Number of results: 1,980

17/02/21, 9,30am
Searched title abstract key words

English language
Royal Holloway University of London
Articles only
Advanced search
Source type: journal
Subject area: Agriculture and Biological sciences Biochemistry, genetics and molecular biology earth and planetary sciences immunology and microbiology pharmacology, toxicology and pharmaceutics
All years

Search string

TITLE-ABS-KEY (parasit* or cestod* or helminth* or nematod* or tapeworm* or virus* or viral or acanthocephala* or copepod* or bacteria* or monogenea* or protozoa* or trematod* or digenea* or ectoparasit* or endoparasit* or pathogen* or flea* or tick* or mite* or leech* or nematomorph* or fungus or fungi or chytrid* or microsporidia* or trypanosome* or fungal
) AND TITLE-ABS-KEY ("host susceptib*" or "host vulnerab*" or “disease vulnerab*” or “disease susceptib*” or “host* resistan*” or “disease resistan*” or prevalence or “disease level” or “disease abundan*” or “disease outcome*” or “disease intensit*” or “level of disease” or “degree of disease” or “pathogen intensit*” or “parasite intensity*" or “pathogen level*” or “level of pathogen*” or “pathogen abundan*” or “abundance of pathogen*” or “pathogen load” or “disease load” or “disease burden” or “parasite burden” or “pathogen burden”) AND TITLE-ABS-KEY (
"climate change" or "global warming" or "climat* varia*" or temperature* or precipitation or rain or rainfall or "water availability" or "water defic*" or "moisture defic*" or heat or thermal or humid* or cold* or chill* or altitud* or “elevation gradient” or latitud* or warm* or “ephemeral water bod*” or “temporary water bod*”)

PubMed

Search details

Number of results: 5,461

17/02/21, 9.46am
Searched titles and abstracts
All years-1911-2021
Journal articles only
English language
Royal Holloway university of London

Search string

parasit* or cestod* or helminth* or nematod* or tapeworm* or virus* or viral
or acanthocephala* or copepod* or bacteria* or monogenea* or protozoa* or trematod* or digenea* or ectoparasit* or endoparasit* or pathogen* or flea* or tick* or mite* or leech* or nematomorph* or fungus or fungi or chytrid* or microsporidia* or trypanosome* or fungal
AND
"host susceptib*" or "host vulnerab*" or “disease vulnerab*” or “disease susceptib*” or “host* resistan*” or “disease resistan*” or prevalence or “disease level” or “disease abundan*” or “disease

outcome*” or “disease intensit*” or “level of disease” or “degree of disease” or “pathogen intensit*” or “parasite intensity*" or “pathogen level*” or “level of pathogen*” or “pathogen abundan*” or “abundance of pathogen*” or “pathogen load” or “disease load” or “disease burden” or “parasite burden” or “pathogen burden”
AND
"climate change" or "global warming" or "climat* varia*" or temperature* or precipitation or rain or rainfall or "water availability" or "water defic*" or "moisture defic*" or heat or thermal or humid* or cold* or chill* or altitud* or “elevation gradient” or latitud* or warm* or “ephemeral water bod*” or “temporary water bod*”

Ethos (thesis repository)

Search details

Number of results: 44
17/02/2021
General search

Search string

parasite prevalence temperature

Base (grey literature search engine)

Search details

Number of results 9
17/02/2021
All years
Selected theses, masters theses, doctoral and post doctoral theses
Searched entire document
Advanced search
Language English

Search string

parasite temperature
Repeated with terms: parasite climate change

OpenGrey

Search details

Number of results: 2
18/2/21. 11.35am
English

Search string

Searched: parasite climate

Google scholar

Search details

Number of results: 46,600, only imported first 200
17/02/20, 3.15pm
Title search only

Search string

TI(parasite OR bacteria OR helminth OR fungi OR virus AND prevalence OR "host susceptibility" OR "host resistance" AND rain OR precipitation OR temperature OR "climate change")

Description of which moderators could be analysed due to missing data

In the temperature dataset, all studies measured parasite infection in endoparasites and therefore, the effect of a parasite being an endoparasite or ectoparasite could not be analysed. Effect sizes with multi-host lifecycles were excluded from this moderator analysis as there were fewer than four effect sizes with multi-host lifecycles. To analyse the effects of parasite taxonomy, one study measuring prevalence in a mite was excluded because a sample size of one was not sufficient to generalise the findings. Likewise, when analysing the effect of experimental approach, two studies that used field experimental approaches were excluded from this subgroup analysis. When analysing the precipitation dataset, the effect of parasite lifecycle could not be investigated because only two effect sizes measured the prevalence of parasites without free-living stages, which is below the threshold of four effect sizes per category. Host trophic level had a sufficient sample size to analyse, and within this moderator only the effect of herbivores compared to omnivores could be compared as they each had above four effect sizes. One study that had a carnivore host was excluded from analysis. Furthermore, all studies were field surveys and therefore, methodological approach could not be analysed as there were no studies that used a laboratory or field experimental approach. Whether a host or vector could not be analysed because all studies measured prevalence in hosts. Finally, in the combined temperature and precipitation dataset all studies measured the prevalence of endoparasites and 43 out of 48 effect sizes had vector-borne lifecycles and therefore, differences between endoparasites and ectoparasites and the effect of parasite lifecycle could not be tested. 40 out of 48 effect sizes investigated the prevalence of protozoans, making the sample sizes of other parasite taxa too small to analyse. The methodological approach also could not be analysed because all studies were field studies. Whether a host or vector could not be analysed because all studies measured prevalence in hosts.

The effect of temperature on parasite prevalence

Table A3.1. Three-level random effects model results for all permutations from the temperature dataset.

Permutation	Transformed pooled odds ratio	95% CI		*p*	τ^2		Percentage of total variation explained by:	
		Lower	Upper		Level 2	Level 3	Within study heterogeneity	Between study heterogeneity
2	0.679	0.392	1.175	0.163	0.86	0.908	45.31	43.03
3	0.679	0.392	1.175	0.163	0.86	0.908	45.31	43.03
4	0.972	0.573	1.65	0.915	0.875	0.7	38.61	48.27
5	0.972	0.573	1.648	0.915	0.87	0.7	38.61	48.27
6	0.843	0.529	1.44	0.529	0.74	1.079	52.62	36.08
7	0.885	0.523	1.497	0.644	0.54	1.41	64.72	24.82
8	0.803	0.449	1.435	0.452	1.06	0.891	40.76	48.53
9	0.967	0.557	1.68	0.904	0.871	1.03	48.25	40.96
10	1.002	0.59	1.703	0.993	0.704	1.06	52.39	34.76

Table A3.2 Results from subgroup analysis of the temperature dataset permutation 1. *n* shows the number of effect sizes.

	Odds Ratio (transformed)	95% CI lower	95% CI upper	*p*	$p_{subgroup}$
Methodological approach					0.224
Field survey (*n* = 35)	1.473	0.709	3.06	0.294	
Laboratory experiment (*n* = 27)	0.758	0.124	4.642	0.224	
Host trophic level					0.997
Carnivore (*n* = 32)	1.036	0.481	2.234	0.925	
Herbivore (*n* = 16)	1.098	0.120	10.07	0.937	
Omnivore (*n* = 14)	1.044	0.02	10.92	0.994	
Parasite lifecycle and free-living stages					0.27
Single host, without free-living stages (*n* =11)	1.09	0.36	3.29	0.879	
Single host, with free-living stages	1.64	0.124	21.73	0.58	

(*n* =20)					
Vector-borne without free-living stages (*n* =26)	0.606	0.0519	7.07	0.388	
Parasite taxonomy					0.457
Bacteria (*n* = 8)	0.51	0.122	2.139	0.351	
Fungi (*n* = 4)	2.529	0.0645	99.16	0.157	
Helminth (*n* = 15)	2.359	0.065	83.03	0.155	
Protozoa (*n* = 17)	1.012	0.04	25.51	0.449	
Virus (*n* = 17)	0.695	0.027	17.796	0.734	
Host taxonomy					0.163
Amphibian (*n* = 9)	3.15	0.751	11.673	0.084	
Arthropod (*n* = 32)	0.660	0.041	10.688	0.038*	
Mammal (*n* = *6*)	0.851	0.023	16.885	0.108	
Mollusc (*n* = 9)	1.9	0.048	75.331	0.670	
Whether a host or vector was infected					0.091
Host (*n* = 35)	1.59	0.777	3.26	0.2	
Vector (*n* = 27)	0.4	0.137	1.6	0.091	

The effect of precipitation on parasite prevalence

Table A3.3 Three-level random effects model results of all the permutations of the precipitation datasets. Significant *p*-values are shown in bold.

Permutation	**Transformed pooled odds ratio**	**95% CI**		***p***	**τ^2**		**Percentage of total variation explained by:**	
		Lower	**Upper**		**Level 2**	**Level 3**	**Within study heterogeneity**	**Between study heterogeneity**
2	1.833	0.549	6.127	0.31	1.21	0.214	13.095	73.81
3	1.346	0.558	3.246	0.493	0.363	1.00	63.305	22.92
4	1.346	0.558	3.246	0.493	0.363	1.00	63.31	22.919
5	1.678	0.579	4.85	0.326	0.81	0.495	32.58	53.48
6	1.76	0.62	5.01	0.274	0.783	0.463	31.49	53.27
7	1.831	0.647	5.19	0.242	0.82	0.351	25.87	60.18
8	1.98	0.903	4.32	0.085	0.377	0.399	40.63	38.43
9	1.7	1.001	2.87	**0.047**	0.033	0.763	77.76	3.37
10	1.66	0.64	4.35	0.234	0.601	0.58	41.3	43.76

Table A3.4 Results from moderator analysis of the precipitation dataset using a random effects three-level model and data from permutation 1. *n* shows the number of effect sizes.

	Odds Ratio (transformed)	95% CI lower	95% CI upper	*p*	$p_{subgroup}$
Host trophic level					0.749
Herbivore (*n* = 12)	1.86	0.6	5.73	0.264	
Omnivore (*n* = 12)	1.539	0.149	15.916	0.749	
Endoparasite or ectoparasite					0.515
Endoparasite (*n* = 10)	2.256	0.0216	257.47	0.968	
Ectoparasite (*n* = 15)	1.04	0.122	8.88	0.515	

Combined effects of temperature and precipitation on parasite prevalence

Table A3.5 Results of three-level random effects models from all permutations of the combined temperature and precipitation dataset.

Permutation	Transformed pooled odds ratio	95% CI		*p*	τ^2		Percentage of total variation explained by:	
		Lower	Upper		Level 2	Level 3	Within study heterogeneity	Between study heterogeneity
2	0.713	0.402	1.26	0.241	0.386	2.11	79.85	14.6
3	0.766	0.421	1.36	0.344	0.689	1.45	63.19	29.95
4	0.845	0.495	1.44	0.528	0.19	0.098	85.88	7.78
5	0.9	0.513	1.577	0.707	0.239	2.285	85.1	8.89
6	0.986	0.545	1.783	0.961	0.529	1.77	72.02	21.49
7	1.08	0.634	1.84	0.769	0.327	1.734	78.41	14.77
8	0.794	0.394	1.6	0.512	1.12	1.83	59.12	36.15
9	0.92	0.562	1.51	0.936	0.149	1.79	85.72	7.15
10	1.069	0.612	1.87	0.812	0.377	1.91	78.24	15.48

Table A3.6 Results from subgroup analysis using a three-level random effects model and the combined temperature and precipitation dataset. *n* shows the number of effect sizes.

	Odds Ratio (transformed)	**95% CI lower**	**95% CI upper**	***p***	$p_{subgroup}$
Parasite has free-living stages					0.204
Yes (*n* = 7)	0.518	0.0631	1.457	0.204	
No (*n* = 44)	1.34	0.724	2.49	0.343	
Host taxonomic group					0.561
Mammal (*n* = 18)	1.304	1.89	9.00	0.896	
Bird (*n* = 31)	1.377	0.459	4.132	0.561	
Host trophic level					0.994
Carnivore (*n* = 6)	1.14	0.217	5.998	0.874	
Herbivore (*n* = 6)	1.242	0.021	72.595	0.943	
Omnivore (*n* = 39)	1.126	0.037	34.636	0.988	

All studies included in the meta-analysis

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Appendix 4:
Chapter 6
Transmission of a bumblebee parasite is robust despite parasite exposure to extreme temperatures

Hannah S Wolmuth-Gordon & Mark JF Brown

Table A4.1 Blocking design for the order of incubation duration and temperatures tested on each day to prevent order effects.

Incubation duration (mins)	Temperature (° C)				
	Day 1	**2**	**3**	**4**	**5**
10	20	30	10	50	40
60	30	40	50	20	10

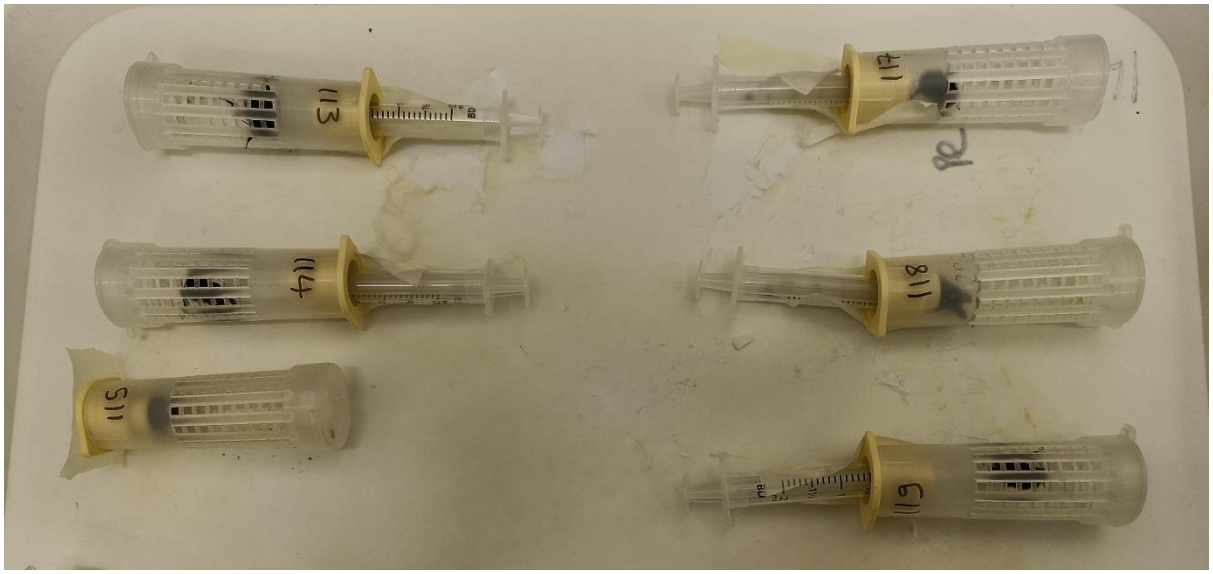

Fig. A4.1 *Bombus terrestris audax* were housed in nicot cages (Becky's bees, UK) throughout the experiment. Here, bees are being inoculated using 2ml syringes attached to the base of the nicot cage with masking tape. After inoculation, 2ml syringes were replaced with 5ml syringes containing sterile sugar solution (50% concentration) provided *ad-libitum*. Syringes were replaced every three days to prevent fungal growth.

Pilot experiment 1

A pilot experiment was conducted to determine the inoculation dose, and temperature and incubation duration ranges. The same protocol was followed as outlined in the methods section. Two *Bombus terrestris audax* colonies, with 85-150 workers were ordered from Agralan (UK). *Crithidia bombi* (please see Materials and Methods in the Main text for details of origin) was exposed to 10°C and 40°C for 10 and 60 minutes. 32 individuals (16 per colony) were used in each temperature, incubation duration combination. An inoculation dose of 15,000 cells was trialled to maximise the chance of infection whilst, also enabling the effect of treatment on prevalence to be investigated.

Results

Table A4.2 Number of bees lost from the sample and the reason why.

Reason bees were lost from sample	Frequency
Failed to drink inoculum	4
Died before screening	3
Failed to defecate during screening	1

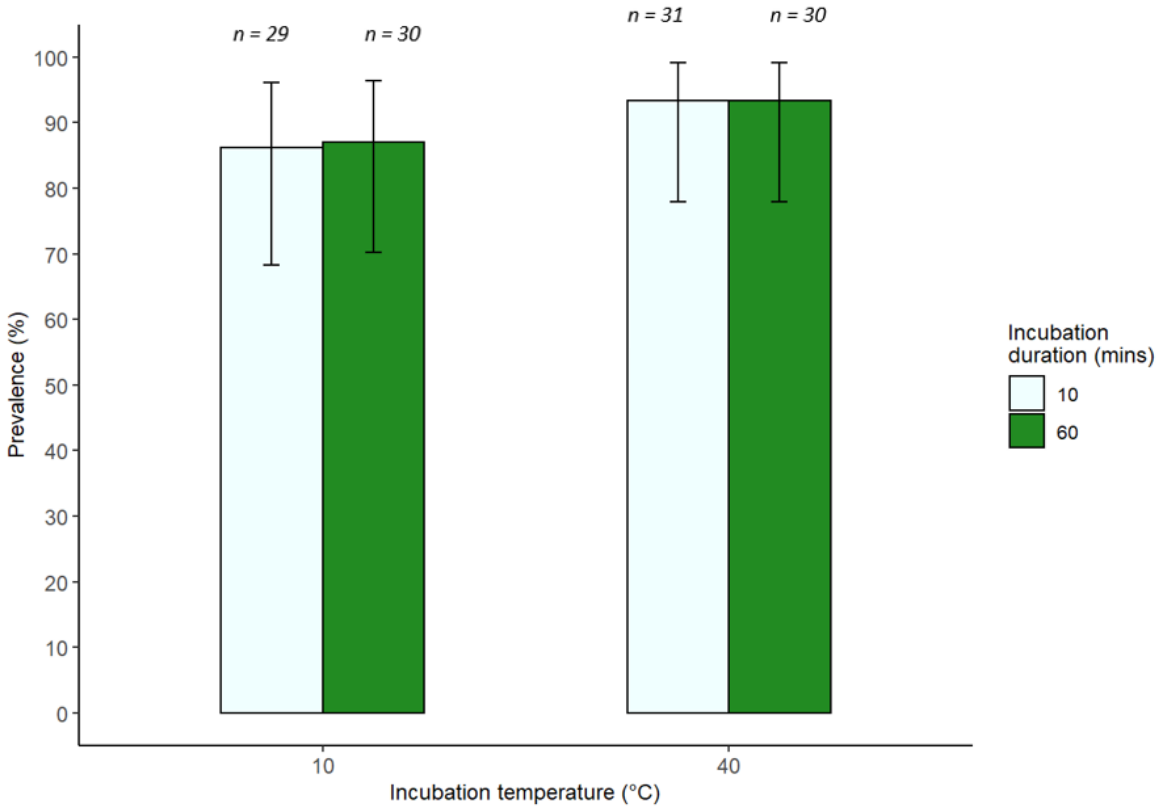

Fig. A4.2 Prevalence of *C. bombi* in bees one week after pilot inoculation of 15,000 cells. *Crithidia bombi* was exposed to two temperatures for 10 (pale green, left) and 60 minutes (dark green, right) prior to inoculation. Error bars are 95% binomial confidence intervals.

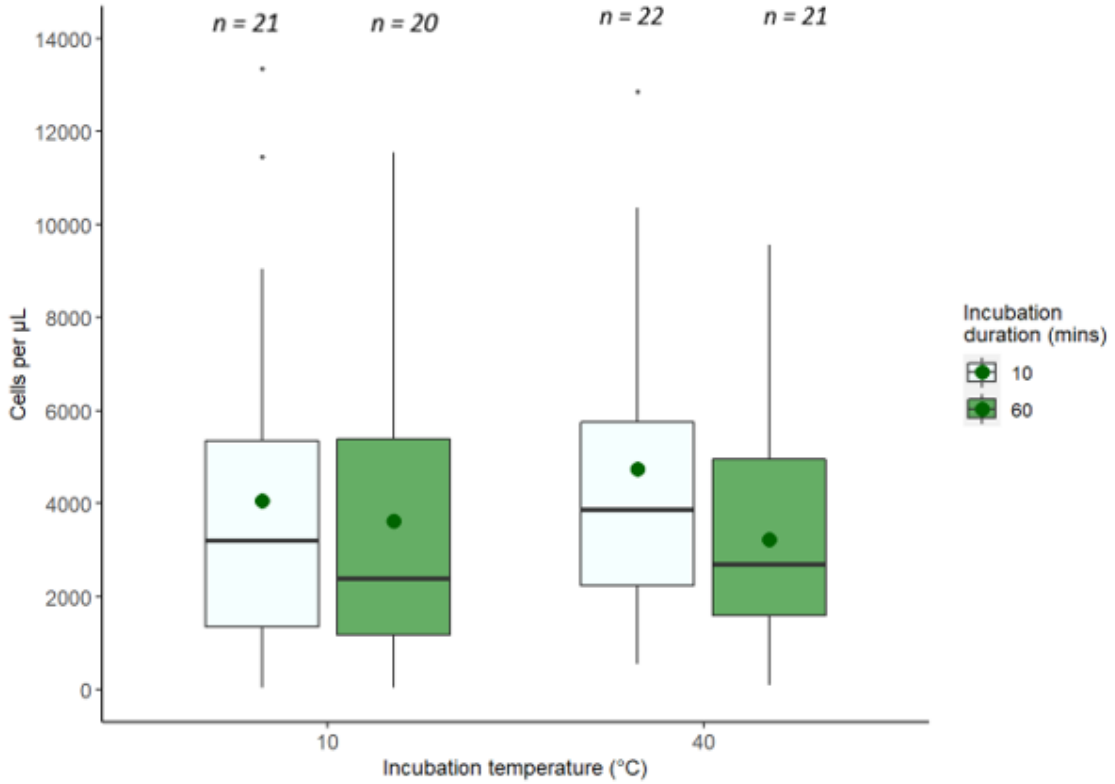

Fig. A4.3 Boxplot showing the intensity of infection of *C. bombi* in bees one week after a pilot inoculation of 15,000 cells. *Crithidia bombi* was exposed to two temperatures for 10 (pale green, left) and 60 minutes (dark green, right) prior to inoculation. Means are indicated by the large, dark green datapoints and sample sizes are given above.

Pilot experiment 2

The prevalence was higher than expected in pilot experiment 1. A lower inoculation dose of 8,000 cells was trialled to lower the rate of infection and enable us to test the effect of temperature and incubation duration of prevalence. Furthermore, a higher maximum temperature of 50°C was tested. The inoculum was incubated at these temperatures for 10 minutes. 60 minutes was not tested at 50°C because it was assumed that the rate of infection would be lower than for 10 minutes at 50°C. 16 bees were tested in each treatment group, 8 from each colony.

Results

Table A4.3 Number of bees lost from the sample and the reason why.

Reason bees were lost from sample	Frequency
Failed to drink inoculum	2
Died before screening	1
Failed to defecate during screening	1

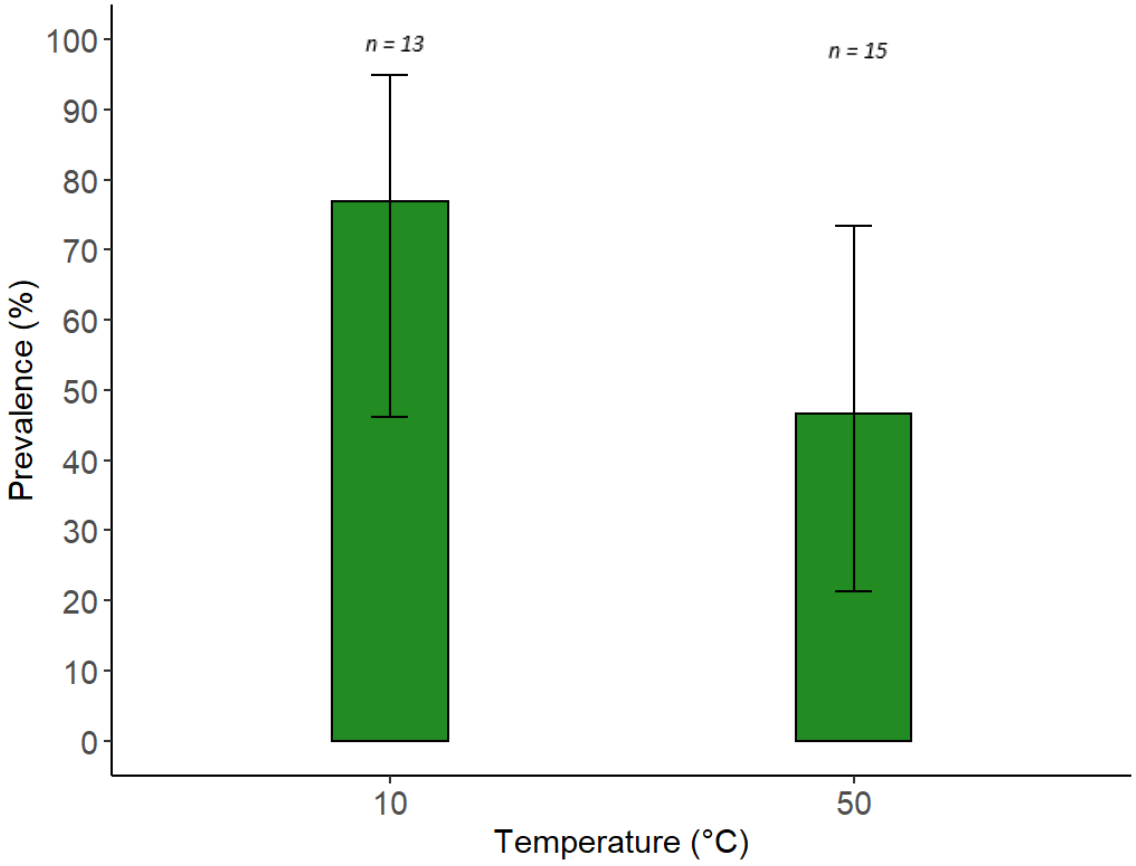

Fig. A4.4 Prevalence of *C. bombi* in bees one week after pilot inoculation of 8,000 cells. *Crithidia bombi* was exposed to two temperatures for 10 minutes prior to inoculation. Error bars are 95% binomial confidence intervals. Sample sizes given above.

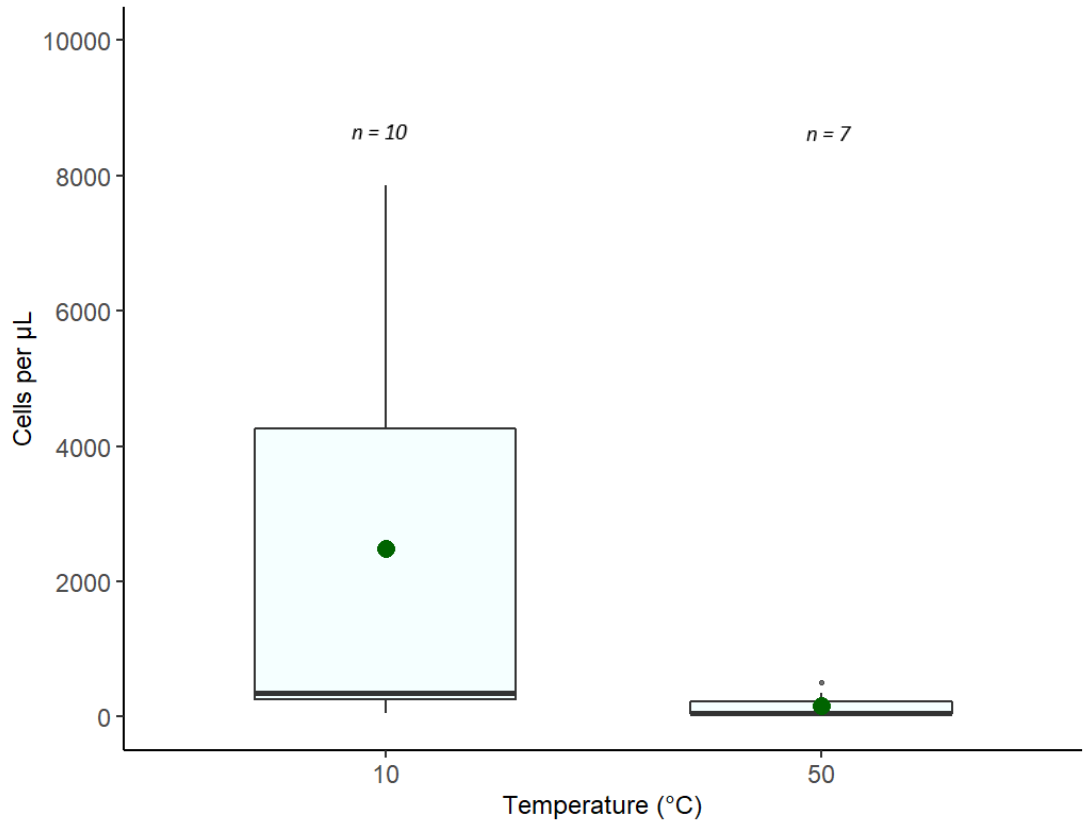

Fig. A4.5 Boxplot showing the infection intensity of *C. bombi* in bees one week after a pilot inoculation of 8,000 cells. *Crithidia bombi* was exposed to two temperatures for 10 minutes prior to inoculation. Means are indicated by the large, dark green datapoints and the sample sizes are given above.

Experiment 1. Inoculation dose of 8,000 cells

This experiment was conducted using a dose of 8,000 cells, based on results from the pilot experiments. After observing the prevalence, I decided to increase the inoculation dose to 20,000 as the prevalence was not as expected given previous data and the pilot results.

Table A4.4 Number of bees lost from the sample and the reason why.

Reason bees were lost from sample	Count
Failed to drink inoculum	20
Died	4
Failed to defecate	5

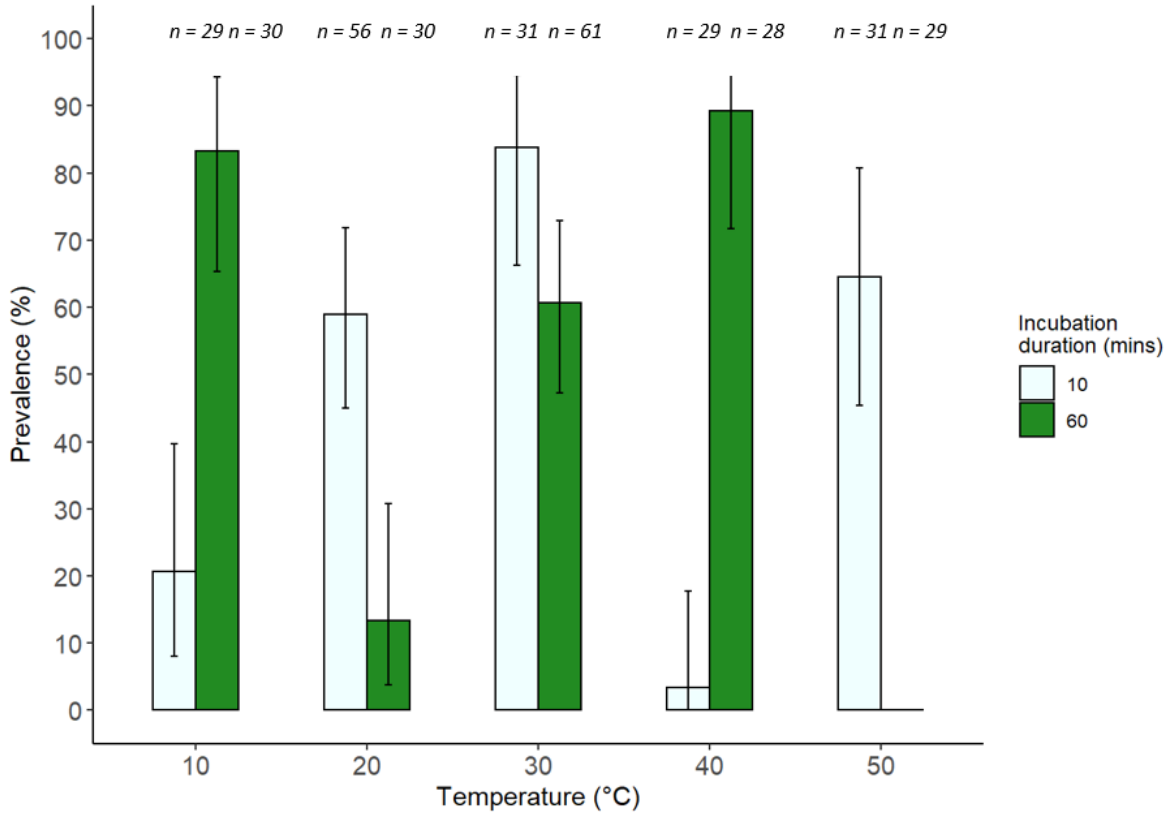

Fig. A4.6 The prevalence of infection of *C. bombi* in individuals one week after infection with 8,000 cells. Prior to inoculation, *C. bombi* was exposed to five temperatures for two time periods. Pale green (left) indicates exposure to the temperature for 10 minutes and dark green (right) for 60 minutes. Error bars show binomial 95% confidence intervals. Sample sizes are above the bars.

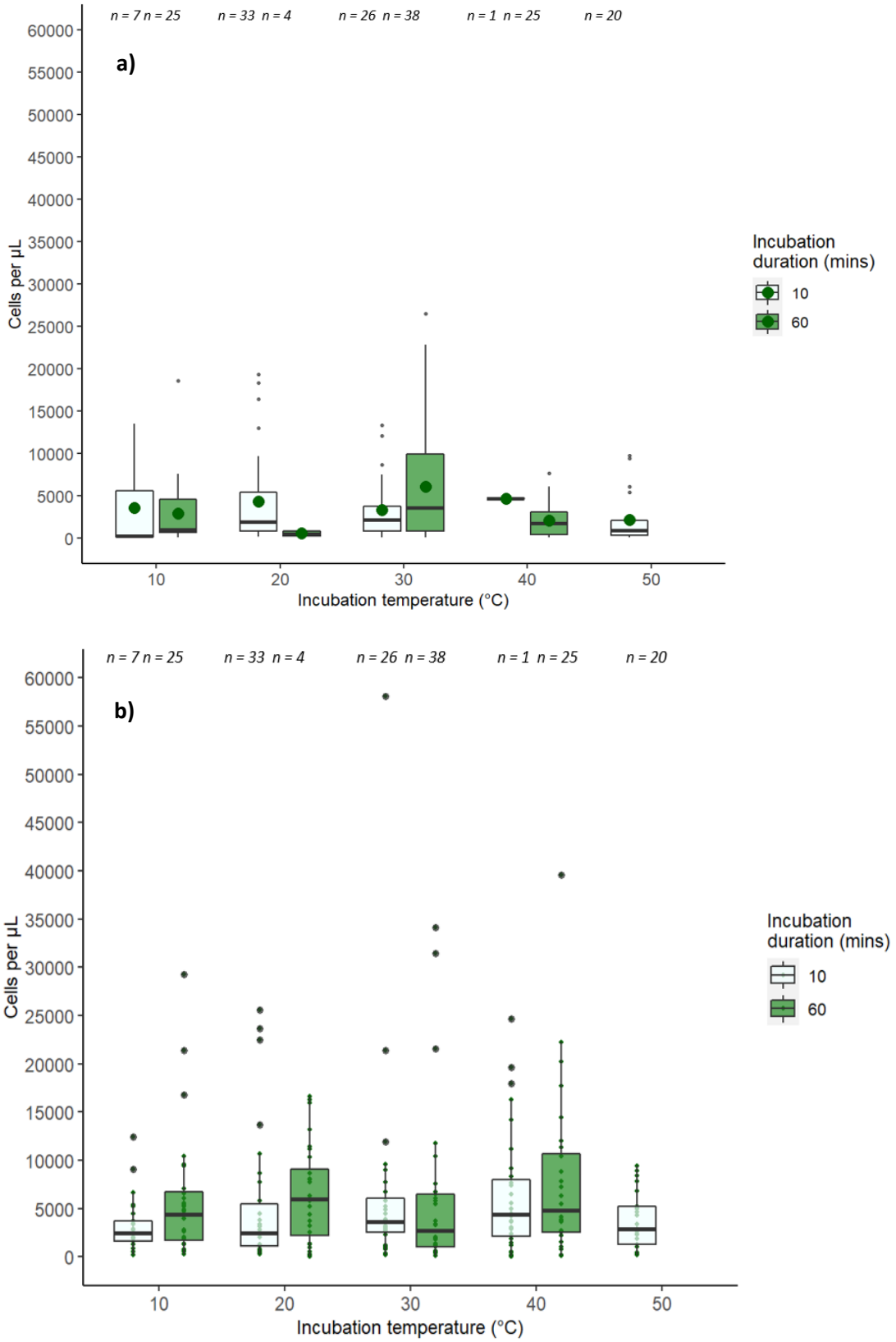

Fig. A4.7 a) Boxplot showing the infection intensity (cells per µL) of *C. bombi* in individuals one week after infection with a dose of 8,000 cells. Prior to inoculation *C. bombi* was exposed to five

temperatures for two time periods. Pale green (left) indicates exposure to the temperature for 10 minutes and dark green (right) for 60 minutes. Means are indicated by the large, dark green datapoints and sample sizes are given above. **b)** Same as a) without mean datapoints and including raw data as smaller datapoints.

Experiment 2. Inoculation with 20,000 (experiment in manuscript)

Do incubation temperature and duration affect the prevalence of infection?

In the full model (including temperature, incubation duration and their interaction, bee mass and colony), prevalence of infection was significantly affected by incubation temperature (X^2_4 = 132, p < 0.001), incubation duration (X^2_1 = 89.6, p < 0.001) and their interaction (X^2_4 = 52.953, p < 0.001; Fig. 3). Body mass of individual bees and colony did not significantly affect prevalence (X^2_1 = 89.0, p = 0.424; X_7 = 76.3, p = 0.0808; Fig. A4.8 and Fig. A4.9). This model was not presented in the main analyses because colony was insignificant and resulted in a worse fitting model.

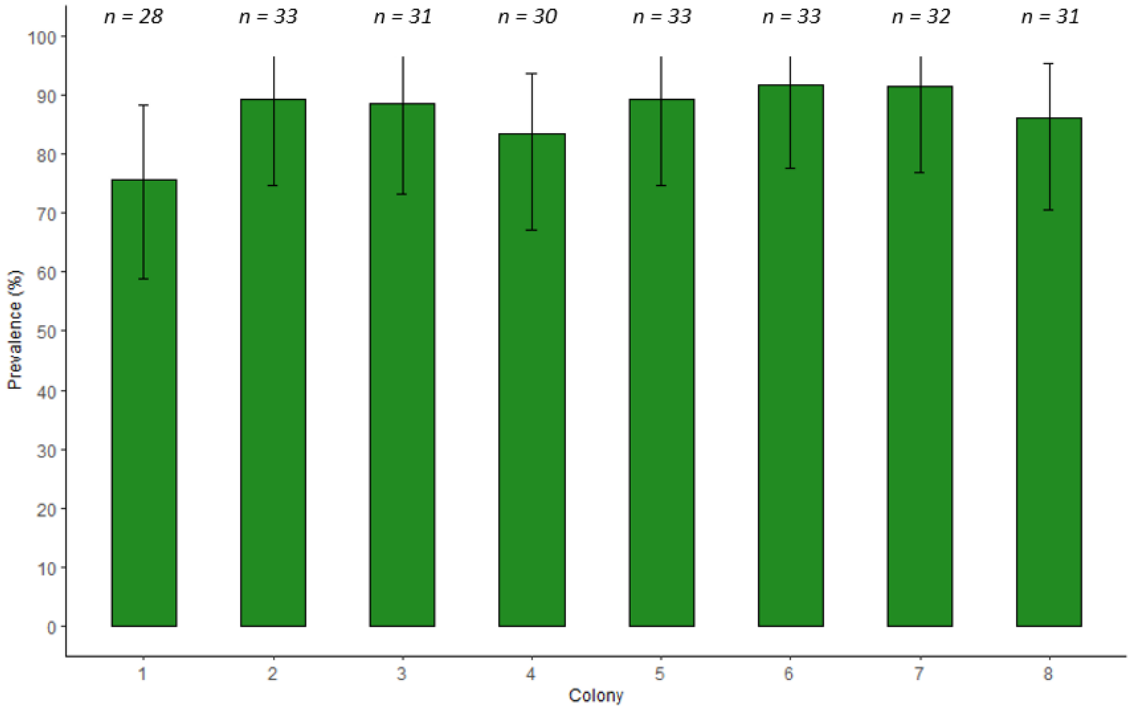

Fig. A4.8 Prevalence of *C. bombi* in individuals from each colony after inoculation with 20,000 cells. Error bars show 95% confidence intervals. Samples sizes are given above bars.

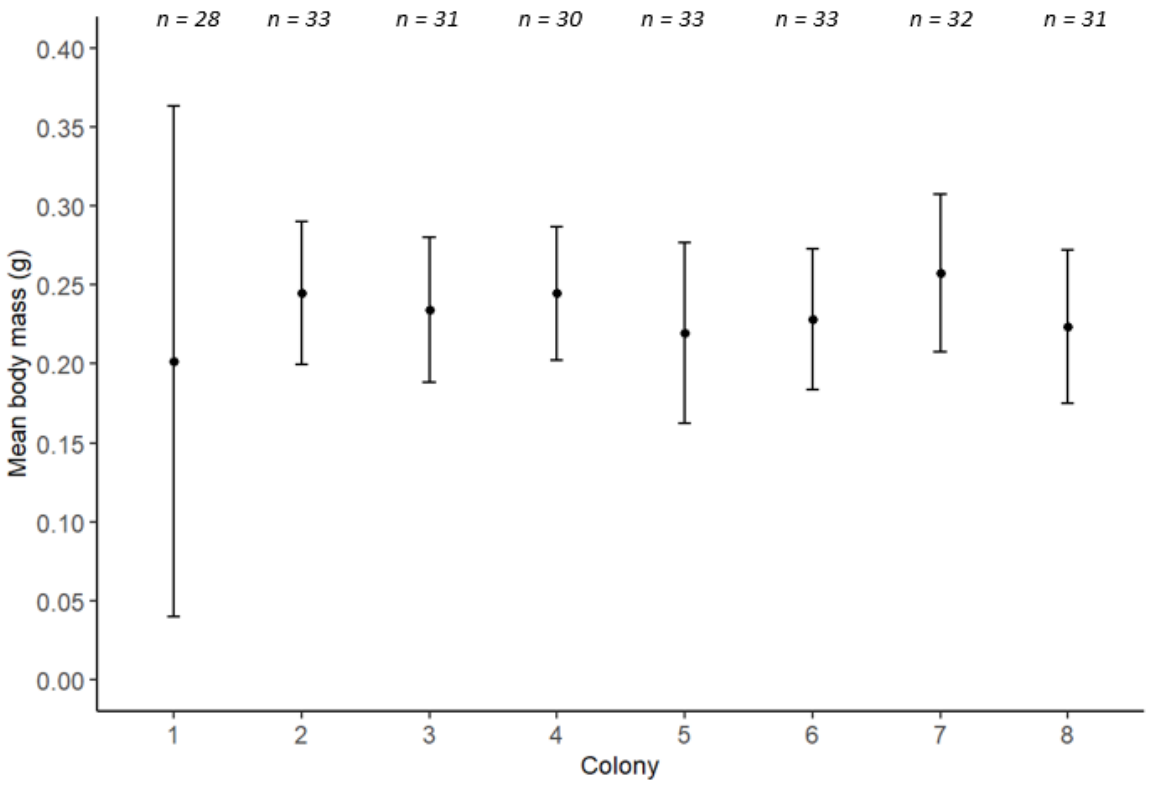

Fig. A4.9 Mean body mass of bees from each colony before inoculation with *C. bombi.* Error bars show upper and lower standard deviations and sample sizes are given above the points.

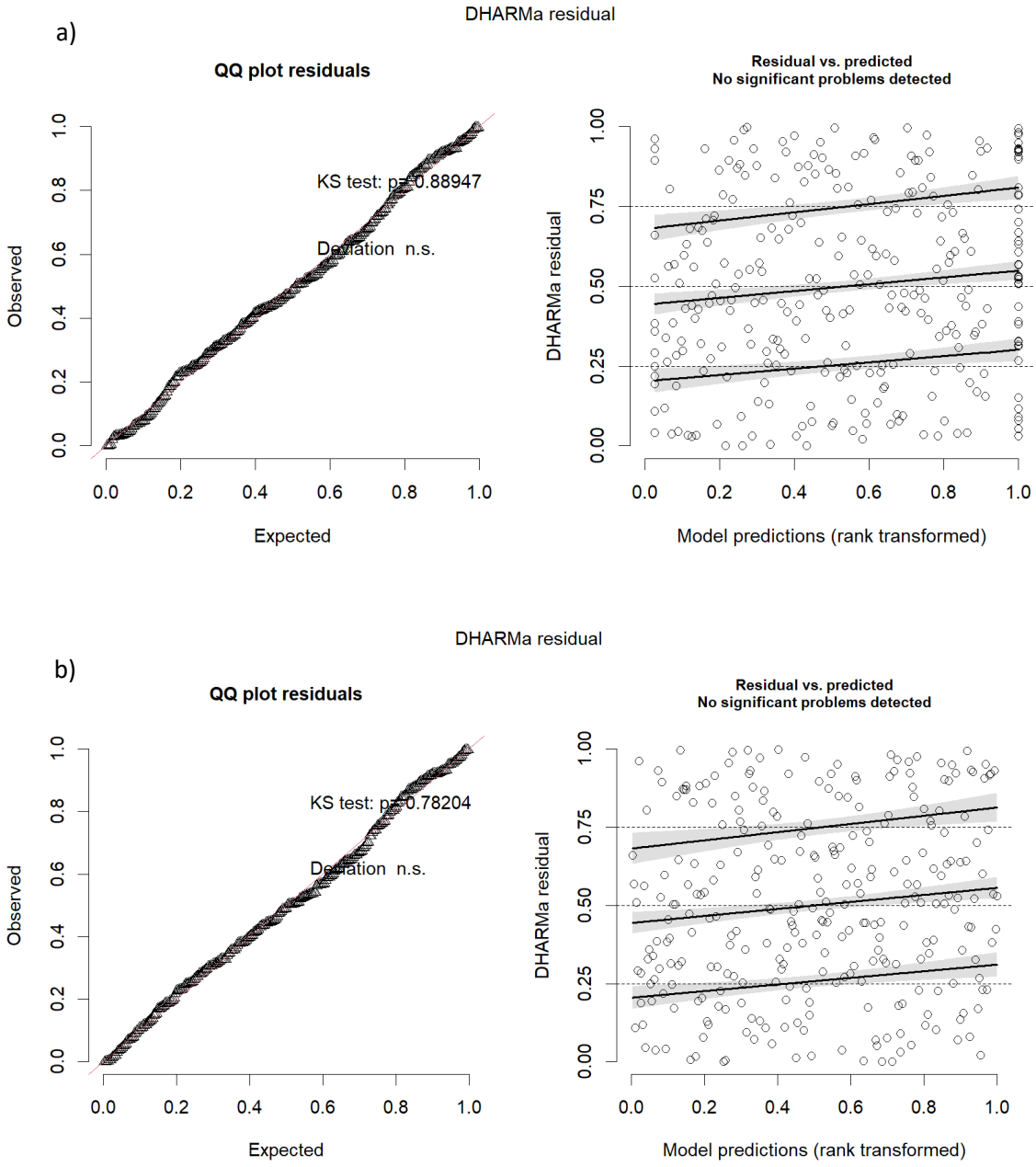

Fig. A4.10 Residual plots for checking assumptions of (a) the full model and (b) the reduced model without colony, using the package "DHARMa" (Hartig, 2022)

Appendix 5:
Chapter 7
Methods matter: how to quantify *Crithidia bombi* infection intensity in bumblebees

Hannah S Wolmuth-Gordon, Anisah Sharmin & Mark JF Brown

Table A5.1 Comparison of gut methods used in previous studies.

Paper	Settling time (hrs)	Gut dissected	Grinding guts	Other notes
Anthony, W. E., Palmer-Young, E. C., Leonard, A. S., Irwin, R. E., & Adler, L. S. (2015). Testing dose-dependent effects of the nectar alkaloid anabasine on trypanosome parasite loads in adult bumblebees. *PLoS ONE*, **10**, e0142496. https://doi.org/10.1371/journal.pone.0142496	3-8	Mid and hindguts	Ground guts in 300ul distilled water	
Biller, O. M., Adler, L. S., Irwin, R. E., McAllister, C., & Palmer-Young, E. C. (2015). Possible synergistic effects of thymol and nicotine against *Crithidia bombi* parasitism in bumblebees. *PLoS ONE*, **10**, e0144668. https://doi.org/10.1371/journal.pone.0144668	4	Gut tracts	Ground guts in 300ul distilled water	
Palmer-Young, E. C., Hogeboom, A., Kaye, A. J., Donnelly, D., Andicoechea, J., Connon, S. J., Weston, I., Skyrm, K., Irwin, R. E., & Adler, L. S. (2017). Context-dependent medicinal effects of anabasine and infection-dependent toxicity in bumblebees. *PLoS ONE*, **12,** e0183729. https://doi.org/10.1371/journal.pone.0183729	5	Intestinal tracts	Ground guts 300ul distilled water	
Giacomini, J. J., Leslie, J., Tarpy, D. R., Palmer-Young, E. C., Irwin, R. E., & Adler, L. S. (2018). Medicinal value of sunflower pollen against bee pathogens. *Scientific Reports*. https://doi.org/10.1038/s41598-018-32681-y	4-5	Digestive tracts excluding honey crop	Ground guts in 300ul 25% strength Ringer solution	

LoCascio, G. M., Pasquale, R., Amponsah, E., Irwin, R. E., & Adler, L. S. (2019). Effect of timing and exposure of sunflower pollen on a common gut pathogen of bumblebees. *Ecological Entomology*. **44**, 702-710**.** https://doi.org/10.1111/een.12751	3-4	Guts (not specified)	Ground guts with 300ul 25% Ringer solution	Counted 'live and actively moving *C. bombi'*
LoCascio, G. M., Aguirre, L., Irwin, R. E., & Adler, L. S. (2019). Pollen from multiple sunflower cultivars and species reduces a common bumblebee gut pathogen. *Royal Society Open Science*, **6**, 190279. https://doi.org/10.1098/rsos.190279	4-5	Mid and hindguts	Ground guts in 300ul of 25% Ringer solution	
Aguirre, L. A., Davis, J. K., Stevenson, P. C., & Adler, L. S. (2020). Herbivory and time since flowering shape floral rewards and pollinator-pathogen interactions. *Journal of Chemical Ecology*, **46**, 978–986. https://doi.org/10.1007/s10886-020-01213-2	4	Hindguts	Ground hindguts in Ringer solution	Counted 'live' *C. bombi* cells

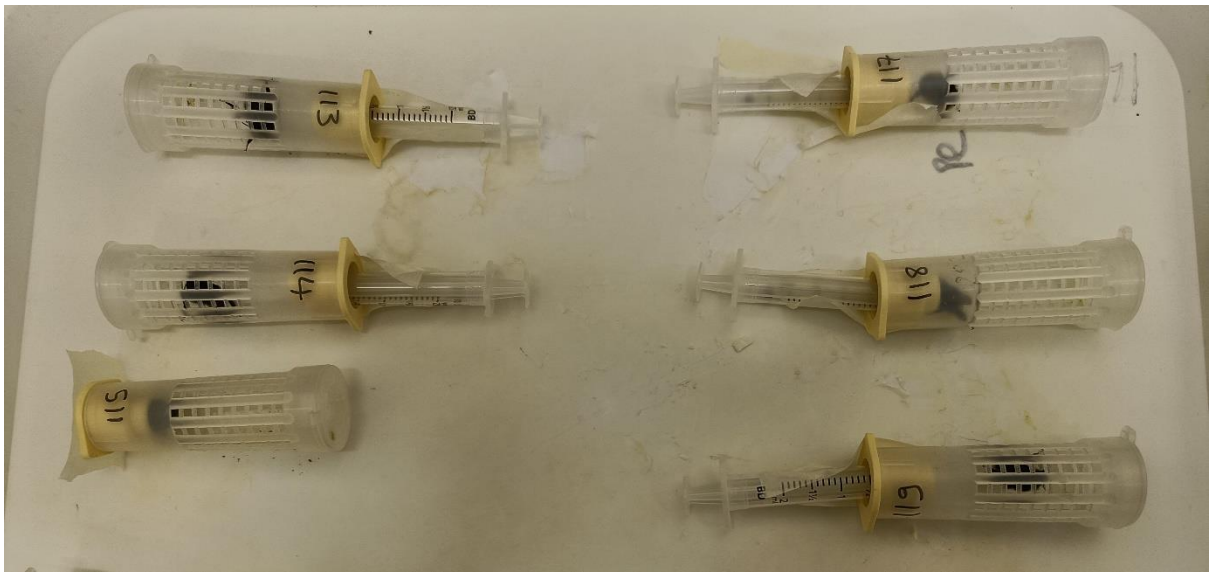

Fig. A5.1 *Bombus terrestris audax* were housed in nicot cages (Becky's bees, UK) throughout the experiment. Syringes were taped to the base of the nicot cage and used to inoculate and feed the bees.

Table A5.2 Comparison infection prevalence and intensity estimates from studies that use the gut and faecal sampling method. When empirical data was not available, Webplot digitizer was used to extract data from figures.

Sampling method	Study	Host species	Dose (cells)	Days post-infection	Infection intensity (cells/µL)	Infection prevalence (%)	Notes
Gut	Aguirre LA, Davis JK, Stevenson PC, Adler LS. (2020) Herbivory and time since flowering shape floral rewards and pollinator-pathogen interactions. *Journal of Chemical Ecology*, **46**, 978–986. doi:10.1007/s10886-020-01213-2	*B. impatiens*	6,000	7	555		Counted 'live' cells Estimate taken from 'within 1 month undamaged' control treatment.
	Fowler AE, Giacomini JJ, Connon SJ, Irwin RE, Adler LS. (2022) Sunflower pollen reduces a gut pathogen in the model bee species, *Bombus impatiens*, but has weaker effects in three wild congeners. *Proceedings for the Royal Society B Biological Sciences*, **289**, 20211909. doi:10.1098/rspb.2021.1909	*B. impatiens*	6,000	35	wild colony 2016: 14,450 commercial colony 2016: 2,250 wild colony 2020: 2,600	100 88	Estimate taken from buckwheat control treatment.
	Giacomini JJ, Leslie J, Tarpy DR, Palmer-Young EC, Irwin RE, Adler LS (2018) Medicinal value of sunflower pollen against bee pathogens. *Scientific Reports*, **8**, 14394. doi:10.1038/s41598-018-32681-y	*B. impatiens*	6,000	7	Buckwheat pollen treatment: 5,500 Rapeseed pollen treatment: 2,000	93	Estimate taken from buckwheat and rapeseed control treatments.

	Anthony WE, Palmer-Young EC, Leonard AS, Irwin RE, Adler LS. (2015) Testing dose-dependent effects of the nectar alkaloid anabasine on trypanosome parasite loads in adult bumblebees. *PLoS One*, 10, e0142496. doi:10.1371/journal.pone.0142496	*B. impatiens*	6,000	Upon death	885		70	Estimate taken from 0ppm anabasine treatment.
Faecal	Schmid-Hempel P, Puhr K, Krüger N, Reber C, Schmid-Hempel R. (1999) Dynamic and genetic consequences of variation in horizontal transmission for a microparasitic infection. *Evolution*, **53,** 426-434. doi:10.1111/j.1558-5646.1999.tb03778.x	*B. terrestris*	10,000		6	4,000		Estimate taken from 'early, immediate' treatment group
	Yourth CP, Schmid-Hempel P. (2006) Serial passage of the parasite *Crithidia bombi* within a colony of its host, *Bombus terrestris*, reduces success in unrelated hosts. *Proceedings for the Royal Society B Biological Sciences*, **273,** 655-659. doi:10.1098/rspb.2005.3371	*B. terrestris* and *B. lucorum*	10,000		7		71	Estimate taken from single passage between unrelated colonies.

Logan A, Ruiz-González MX, Brown MJF. (2005) The impact of host starvation on parasite development and population dynamics in an intestinal trypanosome parasite of bumblebees. *Parasitology*, **130,** 637-642. doi:10.1017/S0031182005007304	*B. terrestris*	25,000	7	27,600		Estimate taken from pollen treatment group.
Folly AJ, Barton-Navarro M, Brown MJF. (2020) Exposure to nectar-realistic sugar concentrations negatively impacts the ability of the trypanosome parasite (*Crithidia bombi*) to infect its bumblebee host. *Ecological Entomology*, **45,** 1495-1498. doi:10.1111/een.12901	*B. terrestris*	10,000	7	Sugar conc. treatments: 10%: 5,922 20%: 7,608 40%: 12,835 60%: 4,350	69 64 55 71	Estimates taken from four sugar concentrations.

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Adler, L. S., Michaud, K. M., Ellner, S. P., McArt, S. H., Stevenson, P. C., & Irwin, R. E. (2018). Disease where you dine: plant species and floral traits associated with pathogen transmission in bumblebees. *Ecology*, **99**, 2535–2545. https://doi.org/10.1002/ecy.2503

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