The role of parasites in the invasion ecology of *Harmonia axyridis*

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SUMMARY ABSTRACT

The success of an invasive alien species is often attributed to the ecological advantage gained from natural enemy release. Numerous factors have been suggested as contributing to the success of *Harmonia axyridis* as an invasive alien species, including enemy release. This thesis studied the interactions of several parasites with *H. axyridis*, investigating parasite transmission, growth and virulence as well as host immune responses, thereby shedding light on the potential role of enemy release in the invasion biology of this ladybird.

Benefits gained by invasive alien species from enemy release diminish if parasites of native species shift hosts to exploit the novel invader. The fungal ectoparasite *Hesperomyces virescens* began infecting *H. axyridis* shortly after it invaded the UK, probably as a result of a host shift from *Adalia bipunctata*. This study found a rapid increase in *H. virescens* prevalence over three years in London *H. axyridis* populations. Laboratory study showed *H. virescens* transmission and growth to be more efficient on *A. bipunctata* than the novel host. In addition, reciprocal interspecific transfers of *H. virescens* strains isolated from *A. bipunctata* and *H. axyridis* revealed that the infection characteristics of the fungi from these two hosts differed, suggesting strains may have diverged after the initial shift from *A. bipunctata* to better exploit the host from which they were derived.

Laboulbenialian fungi were previously thought to have negligible impacts on host fitness. A detailed examination of *H. virescens* infecting *H. axyridis* found distinct virulence, with infections resulting in a 50% reduction in host lifespan. In addition, chronic *H. virescens* infection in males caused acceleration in the age-associated decline in body condition while for females, infection triggered fecundity senescence and a faster age-related decline in fertility. While their role in accelerating ageing is debated, the results presented here provide evidence that infectious diseases can drive the ageing process in this insect species.

In nature, multiple parasites affecting a single host are common. The effect of co-infection on the virulence caused by two fungal infections was characterised using *H. axyridis* and *A. bipunctata* hosts. The ability of two ladybird species to defend against an acute fungal

parasite, while infected with the relatively avirulent *H. virescens* was found to be sex-specific. While for females, the presence of co-infection did not alter the virulence seen in singly infected females, a higher mortality rate existed for co-infected males compared with those infected singly. Previously, *H. virescens* has been considered to be avirulent, however, this study provides evidence that this chronic fungal parasite may be important when considering the mortality associated with co-infections in the field.

The invasive success of *H. axyridis* has, in part, been attributed to a more vigorous immune ability compared with other competitor species. Previously, field studies have shown that the prevalence of the parasitoid wasp *Dinocampus coccinellae* in *H. axyridis* is considerably lower than in the UK primary host of this wasp, *Coccinella septempunctata*. The extent to which the prevalence asymmetry in the field is driven by differences in host encapsulation response was tested by first comparing the encapsulation ability of *C. septempunctata* and *H. axyridis* directed against an artificial implant. Following this, the encapsulation response of *D. coccinellae* parasitized individuals was assessed and compared between the two host species. While encapsulation ability did not differ between the host species, and *D. coccinellae* did not affect the immune response of *H. axyridis,* wasp parasitism did alter the encapsulation ability of *C. septempunctata,* although it was inconsistent across sexes and populations.

Overall, this thesis furthers our understanding of the fungal parasite *H. virescens* and its association with the notorious invader *H. axyridis.* The research presented here also demonstrates the use of *H. axyridis* as a model system in areas other than invasion ecology and furthermore, contributes to understanding the role of infectious disease in the rate of ageing. Finally, sex-specific effects were found across the chapters of this thesis, demonstrating the use of *H. axyridis* in the study of sex-specific effects of infections.

DECLARATION OF AUTHORSHIP

I, Katharine Mary Berry, declare that this thesis has been composed by myself and that it embodies the results of my own research. Where appropriate, I have acknowledged the nature and extent of work carried out in collaboration with others.

Signed …….

Date …….

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Furth, Fortune and Fill the Fetters

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General introduction

In 1949, Haldane speculatively examined the idea that infectious diseases play an important role in influencing evolution, and discussed how disease can be an advantage or disadvantage for species when in competition with others. One question he asked was "*how important is disease as a killing agent in nature?*" (Haldane 1949). Indeed, this question is also worth careful deliberation when we consider the role of parasites in biological invasions, where the mortality induced by parasites in one species may benefit another, aiding or adversely affecting the establishment of an invasive alien species. The resistance and immune ability of invasive alien hosts to novel infectious agents can, therefore, influence the overall dynamics of biological invasions, where a highly resistant invader can remain unpressured by parasites in comparison with native species.

1.1 Invasive alien species (IAS)

Invasive Alien Species (IAS) are often thought to be one of the major global threats to biodiversity (Verneau *et al.* 2011; Roy *et al.* 2014) especially when considered with other drivers of ecosystem change (Pyšek & Richardson 2010; Vilà *et al.* 2011; Grarock *et al.* 2014). IAS can be defined as a non-native species that, when established, have a negative impact on biodiversity, human health or the economy (ISAC 2006). While the introduction can be deliberate or accidental, humans are often responsible in mediating IAS transportation, which can occur via multiple different pathways (Drake & Lodge 2004; Tatem *et al.* 2006; Hulme *et al.* 2008; Pergl *et al.* 2017; Saul *et al.* 2017). IAS cause billions of dollars worth of damage each year, annually costing £1.7 billion to the British economy, and $120 billion to the USA (Torchin & Mitchell 2004; Pimentel *et al.* 2005; Williams *et al.* 2010).

The reason some species become invasive, while others do not, varies from species to species. It must inherently be driven by differences in competitive ability, which may be influenced by, amongst other factors, body size, reproductive strategy, and adaptability to new climates: although the ability to generalise the role of these traits is debated (Dukes & Mooney 1999; Mooney & Cleland 2001; Mills *et al.* 2004; Torchin & Mitchell 2004). In addition,

interactions with infectious disease agents may also be important when determining the invasive ability of a species (Keane & Crawley 2002). Horizon scanning to detect species with the highest invasion risk recently resulted in a list of 30 species of potential concern to Britain, the majority of which are likely to be introduced as stowaways (Roy *et al.* 2014). Biosecurity has also increasingly been utilised as a preventative action against IAS and emerging disease, although its usefulness relies on widespread implementation (Dunn & Hatcher 2015).

Biological invasions are responsible for documented dramatic reductions in native species. The arrival of the European green crab (*Carcinus maenas;* Linnaeus; Brachyura: Portunidae) to the estuaries of Nova Scotia has decimated local eelgrass beds (*Zostera marina*; Linnaeus; Alismatales: Zosteraceae), an important habitat for migrating Canadian geese (*Branta canadensis;* Linnaeus; Anseriformes: Anatidae) (Garbary *et al.* 2014). The zebra mussel, *Dreissena polymorpha* (Pallas) (Veneroida: Dreissenidae), reduces the zooplankton community and causes the decline of other mussel populations (Aldridge *et al.* 2004; Sousa *et al.* 2011). The bumblebee *Bombus terrestris* (Linnaeus) (Hymenoptera: Apidae) caused the widespread disappearance of the native *Bombus dahlbomii* (Guérin-Méneville) (Hymenoptera: Apidae), since its introduction into South America in 1998 (Schmid-hempel *et al.* 2014). Japanese Knotweed (*Fallopia japonica;* Houtt; Caryophyllales: Polygonaceae) impacts plant species composition in areas where it has invaded, significantly reducing species diversity by 80% in comparison to non-invaded plots (Hejda *et al.* 2009). The presence of the invasive *Alliaria petiolata* (Cavara & Grand; Brassicales: Brassicaceae) in North American forests alters the belowground associations between native tree seedling and mycorrhizal fungi, suppressing their growth (Stinson *et al.* 2006). Multiple studies have linked the arrival of an IAS with the decline or extinction of native species (Aldridge *et al.* 2004; Kelly *et al.* 2006; Clavero *et al.* 2009; Sousa *et al.* 2011), however, specifically testing the direct role of IAS in native species declines can prove to be difficult (Gurevitch & Padilla 2004). The arrival of an IAS in some cases is undoubtedly responsible for the near extinction of native species, for example the arrival of the brown tree snake (*Boiga irregularis;* Merrem; Reptilia: Colubridae) to the island of Guam just

after world war II (Wiles *et al.* 2003; Rodda & Savidge 2007). However the interactions between IAS and native communities are more often likely to be complex, involving both direct and indirect interactions between species (Pearson & Callaway 2003; Byers *et al.* 2010; Weidenhamer & Callaway 2010; Preston *et al.* 2012).

Direct effects of IAS on native communities, such as predation and competition, while undisputedly important, are only part of the whole picture. Indirectly, IAS may alter the interaction between species already present in a community through apparent competition or indirect mutualism (White *et al.* 2006). Apparent competition can occur in the presence of a natural enemy (predator, parasite or parasitoid) shared between the IAS and a native species (Holt 1977). The invasion of the California Channel Islands by the pig, *Sus scrofa* (Linnaeus) (Artiodactyla: Suidae), increased the predatory population of the golden eagle, *Aquila chrysaetos* (Linnaeus) (Accipitriformes: Accipitridae), which in turn led to increased pressure, and subsequent decline, of the native fox population, *Urocyon littoralis* (Baird) (Carnivora: Canidae), which shared the eagle as a predator (Roemer *et al.* 2002). Alternatively, if the presence of an IAS reduces the pressure of a natural enemy on a native population, for example by acting as a sink in which parasites are unable to fully develop (Hoogendoorn & Heimpel 2002), then the presence of an invader can act indirectly as a mutualism (Rodriguez 2006; White *et al.* 2006).

1.2 The role of parasites in invasions

Parasites are important mediators of biological invasions and, indeed, are often themselves considered IAS as emerging diseases (Torchin *et al.* 2002; Prenter *et al.* 2004; Dunn 2009; Hatcher *et al.* 2012; Dunn & Hatcher 2015; Roy *et al.* 2016b). The world's worst IAS list as compiled by the IUCN contains multiple parasites and pathogens, including the causative agents of Dutch elm disease (*Ophiostoma ulmi*) and rinderpest (*Morbillivirus*) (Hatcher *et al.* 2012). Emerging disease and the introduction of disease vectors can have a large impact on the health of both humans and livestock (Hatcher *et al.* 2012). The arrival of the Asian tiger

mosquito (*Aedes albopictus;* Skuse; Diptera: Culicidae) in the USA has been associated with many human infections including the Dengue fever virus, West Nile virus and Japanese Encephalitis virus (Enserink 2008). In addition, parasites play an important role in ecosystem function and community structure (Hudson *et al.* 2006) and are often considered to be important in dictating the success of IAS, for example through enemy release (Keane & Crawley 2002), parasite spillover or spillback (Kelly *et al.* 2009; Britton 2013).

1.2.1 Enemy Release Hypothesis (ERH)

IAS are often considered to have an ecological advantage in comparison to native species due to a lack of co-evolved natural enemies, and a reduced number of natural enemies able to utilise the IAS in the invaded range, more generally known as the enemy release hypothesis (ERH) (Keane & Crawley 2002; Daehler 2003; Roy *et al.* 2011a; Strauss *et al.* 2012; Dunn & Hatcher 2015). Indeed, Torchin *et al.* (2003) report that over twice as many parasites are found in native populations of IAS than in the invasive alien populations. Although the term natural enemies covers predators as well as parasites, from here on, I refer to only parasites when mentioning natural enemies. Under the term parasite, I refer to organisms that require a host in order to complete their life cycle and which reduce the fitness of that host; within this definition I include endo- and ectoparasites, as well as micro- and macroparasites. Furthermore, one distinct subset of parasitic organism is parasitoids: parasites that live on or in their host and which obiligately kills the host to complete their lifecycle.

The benefit from enemy release can manifest as regulatory or compensatory release, or a combination of the two (Colautti *et al.* 2004). Regulatory release occurs immediately, when natural enemies in the native range of the IAS strongly negatively affect host life history traits, such that the absence of co-evolved natural enemies upon invasion results in fast population growth. In comparison, compensatory release can refer to an evolutionary response, where the absence of infection by co-evolved parasites selects to remove genotypes exhibiting costly high levels of defence from the population, or an adaptive shift where resources can be reallocated

from immune defence to other traits, enabling faster population growth in well-defended IAS (Colautti *et al.* 2004).

The major assumption behind the ERH is that natural enemies have the ability to significantly impact host populations (Roy *et al.* 2011a), and if natural enemies were entirely excluded from the invaded range then native species would competitively exclude the IAS (Keane & Crawley 2002). There are multiple mechanisms behind the lower diversity and prevalence of natural enemies which affect IAS compared with native species. The founder effect resulting from establishment by relatively few individuals reduces the probability that parasites will be introduced alongside their hosts (Colautti *et al.* 2004; Phillips *et al.* 2010). Where natural enemies are present infecting founder individuals, parasites whose transmission is principally governed by density dependent factors, and those with complex lifecycles may be unable to successfully establish (Torchin *et al.* 2002, 2003; Torchin & Mitchell 2004). However, some natural enemies are successful in establishing alongside IAS hosts; vertically transmitted parasites, and those with low host virulence (defined as the fitness loss to the host caused by infection) are more likely to persist with an IAS at the time of invasion (Prenter *et al.* 2004).

Multiple reviews and meta-analyses have found definitive proof to be lacking that ERH occurs universally in all biological invasions (Colautti *et al.* 2004; Hawkes 2007; Heger & Jeschke 2014). While at a biogeographical scale, all IAS will lose natural enemies during their invasions; community studies provide little general evidence supporting the ERH (Colautti *et al.* 2004). The study of multiple plant species has, however, found fewer natural enemies infecting invasive alien plants in their invaded range, supporting enemy release in this system (Mitchell & Power 2003). The effect of enemy release varies depending on the type of enemy and the time since invasion (Cornell & Hawkins 1992; Hawkes 2007). Indeed, as time passes after an invasion event, IAS may be exploited by new natural enemies in their invaded range, potentially removing any ecological advantage gained at the time of invasion (Mitchell *et al.* 2010). However, even IAS established for over 400 years still had only 40% of the total parasite

diversity reported in their native range (Torchin & Mitchell 2004; Mitchell *et al.* 2010). This trend does not however appear to be universal, for example Poulin & Mouillot (2003) found that the diversity of helminths accumulated over time on introduced salmonid fishes in their invaded range was greater than the diversity found in their native range.

1.2.2 The importance of parasite host shifts in mediating the success of biological invasions

While the number of natural enemies able to utilise IAS in the invaded range does not commonly reach the same level as in the IAS' native range (Torchin & Mitchell 2004; Mitchell *et al.* 2010), some natural enemies are able to perform host shifts to utilise a novel invading species that provides a relatively unexploited ecological resource (Poulin & Mouillot 2003a; Pettersen *et al.* 2016). Generally, parasites with low host specificity can utilise a novel host more easily than parasites with high specificity (Cornell & Hawkins 1992; Bertheau *et al.* 2010). The phylogenetic relatedness, and similarity of ecological niche between the IAS and native host species that harbour candidate infections can also influence the likelihood of a shift (Poulin & Mouillot 2003b; Bertheau *et al.* 2010).

In disease ecology, the terms 'spillover' and 'spillback' have been applied when considering the emergence of infectious disease (Daszek *et al.* 2000). The 'spillover' of a disease from an infected reservoir population to sympatric, but as yet uninfected, population can occur, for example, between populations of domestic animals and wildlife, posing a distinct threat to endangered species (Daszek *et al.* 2000). In Africa, an outbreak of canine distemper among domestic dogs resulted in the decline of African wild dogs in the same region due to the spillover of this disease to the wild population (Alexander & Appel, 1994). Considering biological invasions, the invasion of IAS with their natural enemies in-tow can result in parasites shifting to utilise hosts in the invaded range, increasing the success of the invasion. Disease can mediate invasions by introducing a parasite with low virulence in an IAS to a susceptible native host, which experiences high virulence when infected (Lymbery *et al.* 2014; Dunn & Hatcher 2015; Blackburn & Ewen 2016). In the UK, an important factor in the

decimation of the native red squirrel, *Sciurus vulgaris* (Linnaeus) (Rodentia: Sciuridae), after the arrival of the American Grey Squirrel, *S. carolinensis* (Gmelin) (Rodentia: Sciuridae), was the transmission of a *Parapoxvirus* into the native population (Tompkins *et al.* 2003). While spillover of disease from IAS to native populations can strongly influence the invasion success of an IAS, 'spillback' can also occur. In invasive populations, a parasite shift from native species to the IAS population can amplify the size of the natural enemy population in the native species, aiding invasion success of the IAS (Kelly *et al.* 2009). The high density of zebra mussels (*D. polymorphia*) in Lake Naroch in Belarus has amplified the prevalence of the trematode, *Echinoparyphium recurvatum* (Echinostomida: Echinostomatidae), resulting in higher parasite prevalence in local aquatic bird populations (Mastitsky & Veres 2010). Spillback as a driver of biological invasion requires that a parasite makes a host shift to an IAS, that this infection is of lower virulence in the IAS than in native species, and that transmission back to native species is frequent enough that it elevates the prevalence of infection in the native species.

In this thesis, the role of parasites in the invasion biology of the invasive alien ladybird *Harmonia axyridis* (Pallas) (Coleoptera: Coccinellidae) was assessed, studying two parasite species with low host specificity. The association of *H. axyridis* with the sexually transmitted fungal parasite *Hesperomyces virescens* (Thaxter) (Ascomycota: Laboulbeniaceae) and the generalist braconid wasp parasitoid, *Dinocampus coccinellae* (Shrank) (Hymenoptera: Braconidae), was examined. Differences in the encapsulation ability of *H. axyridis* and a UK ladybird species were tested, as well as the ability of *D. coccinellae* to modulate the encapsulation ability of both species. In addition, the effect that *H. virescens* has on the lifespan and senescence of *H. axyridis* was assessed, as well as the infection characteristics of this parasite on the invader and the native *A. bipunctata*.

1.3 Insect Immunity

Generally, the immune system is comprised of both cellular and humoral immunity in order to defend against multiple different microorganisms (fungi, viruses and bacteria) and larger parasites. Although much simpler than those of higher vertebrates, insect immune systems also consist of humoral and cellular defences, including epithelial immunity, a diverse range of parasite receptors and multiple signalling pathways (Elrod-Erickson *et al.* 2000; Ribeiro & Brehelin 2006; Lemaitre & Hoffmann 2007; Cronin *et al.* 2009; Davies & Dow 2009). When considering insect immunity, the best-characterised immune system is that of *Drosophila melanogaster* (Meigen) (Diptera: Drosophillidae). The defence against invading organisms starts with the cuticle, which as well as acting as a physical barrier, is also involved in immune function, with epithelial producing antimicrobial peptides (Tzou *et al.* 2000; Leclerc & Reichhart 2004). If an invading microorganism or parasite passes through the epithelium, the humoral and cellular immune defences are activated to attack the foreign organism (Leclerc & Reichhart 2004; Stanley 2012; Salazar-Jaramillo *et al.* 2014).

The production of antimicrobial peptides and other effector molecules is the role of the humoral immune response (Hedengren *et al.* 1999; Elrod-Erickson *et al.* 2000). Antimicrobial peptides are produced in the fat body, immune cells and epithelia as defensive agents against fungi, gram-positive, and gram-negative bacteria (Bulet *et al.* 1999; Hoffmann 2003; Leclerc & Reichhart 2004). The production of antimicrobial peptides involves multiple signalling pathways, including the IMD and Toll pathways (Engström 1999; Leclerc & Reichhart 2004).

While less is known about the cellular immune response (Leclerc & Reichhart 2004), it plays a central role in allowing hosts to phagocytise or encapsulate an invading organism (Salazar-Jaramillo *et al.* 2014). In *Drosophila,* several cell types defend the host against invading parasites and microorganisms. Plasmatocytes, lamellocytes and crystal cells are all forms of hemocyte cell found in *Drosophila* (Strand 2008). Plasmatocytes play a central role in phagocytosis, while lamellocytes and crystal cells are involved in the encapsulation and

melanisation of parasitoid eggs and other macro-parasites (Fauvarque & Williams 2011; Salazar-Jaramillo *et al.* 2014). Once multiple layers of lamellocytes have assembled around an invading parasite, crystal cells rupture to release prophenyloxidase (proPO), which is converted to phenyloxidase (PO), catalysing the production of melanin and a range of reactive oxygen species, ultimately resulting in parasite death (Rizki & Rizki 1990; Schmidt *et al.* 2001; Cerenius *et al.* 2008; Fauvarque & Williams 2011). PO also plays a role in repairing the cuticle of the host, when haemocytes are responsible for creating a melanised scab where damage occurs (Jiravanichpaisal *et al.* 2006; Wood *et al.* 2006; Strand 2008).

1.3.1 Ladybird defence

While *D. melanogaster* may have the best-characterised immune response of all invertebrates, it is not a perfect model for all insects, including beetles. In addition to the antimicrobial peptides found in other insects, two beetle specific antimicrobial peptides are produced to act against gram-negative bacteria (Iwanaga & Lee 2005). The cellular immune response of coleopterans is also more complex than in flies, containing a wider variety of haemocyte types (Giulianini *et al.* 2003; Manachini *et al.* 2011; Firlej *et al.* 2012). Although different haemocytes perform similar roles in most insect taxa, the naming of the cells differs among species, with those in *Drosophila* being different to those found in many other insects (Strand 2008). Therefore, the cells described in *Drosophila* in the previous section perform a similar function to those in many coleopterans (Strand 2008). In the invasive *H. axyridis* five types of hemocytes are found: plasmatocytes, granular hemocytes I and II, oenocytoids and spherule haemocytes, which have different roles in ladybird defence (Firlej *et al.* 2012). In *D. melanogaster,* phagocytosis is the role of the plasmatocyte cells, however in *H. axyridis* there are three hemocytes akin to the plasmatocytes in *D. melanogaster,* plasmatocytes and granular hemocytes I and II (Strand 2008, Firlej 2012). These three cells perform slightly different functions and, while granular hemocyte I cells phagocytise bacteria, plasmatocytes and granular hemocyte II cells provide defence against larger parasites, including the development of a

capsule around an invading egg, much like the lamellocytes found in *D. melanogaster* (Fauvarque & Williams 2011; Firlej 2012).

While the immunity of ladybirds has not been well studied, more generally their defence includes both behavioural reactions and some more specific anti-predator defence adaptations, including physical and chemical processes. The majority of ladybirds are well defended chemically by species-specific toxic alkaloids, physically by spines in their larval stages, hard elytra as adults and aposematic colouration in both the adult and larval stages (Bezzerides *et al.* 2007; Ware & Majerus 2008). Reflex bleeding (secretion of haemolymph droplets from the leg joints) releases the species-specific alkaloids, making the ladybirds foul tasting and creating a strong smell to deter potential predators (Daloze *et al.* 1995; Sloggett *et al.* 2011).

1.3.2 Harmonia axyridis defences

Harmonia axyridis is very well defended against multiple natural enemies, which is thought to have played a role in its success as an IAS. As well as large larval spines, all *H. axyridis* life stages contain the alkaloid harmonine in their haemolymph (Laurent *et al.* 2002; Ware & Majerus 2008; Sloggett *et al.* 2011). This particularly strong alkaloid found in *H. axyridis* is lethal to other ladybird species following predation, and a strong antimicrobial agent (Rieder *et al.* 2008; Sloggett *et al.* 2011; Rohrich *et al.* 2012). Intraguild predation is a common phenomenon within aphidophagous communities and particularly well-documented between different species of ladybird (Gardiner *et al.* 2011; Thomas *et al.* 2013; Katsanis *et al.* 2013). The association of *H. axyridis* with a microsporidian parasite has been hypothesised to act as an additional 'biological' defence mechanism for this invader, resulting in the death of other species of ladybird predating the eggs, larvae and adults of this IAS (Vilcinskas *et al.* 2013b). However, while it has been demonstrated that the consumption of *H. axyridis* by native species causes mortality, the precise role, if any, that this infection may have played in the invasion biology of *H. axyridis* is debated (Sloggett 2013).

1.4 Sexually transmitted infections

Sexually transmitted infections (STIs) are found throughout the animal kingdom, including in both vertebrates and invertebrates (Lockhart *et al.* 1996; Knell & Webberley 2004). STIs often have more chronic long-term virulence effects than other non-STIs (Knell & Webberley 2004). Indeed, the virulence associated with STIs is often lower than that of horizontally transmitted infectious disease, due to selection on the parasite to maintain the host in suitable mating condition (Knell 1999). Nevertheless, fertility reduction, sterility and parasitic castration are common symptoms of STIs (Knell & Webberley 2004). While it has been suggested this fertility virulence is adaptive to the parasite as it results in increased host mating rates to assist infection spread (Apari *et al.* 2014), there is little evidence to support this (Knell & Webberley 2004; Lafferty & Kuris 2009). Indeed, these effects of STIs on host reproduction may be a product of directly targeting the reproductive system, or a result of nutritional drain, for example mounting an immune response (Hurd 2001; Lafferty & Kuris 2009).

While the majority of mammal STIs are bacteria or viruses, in insects, 62% of reported STIs are multicellular (fungi, helminths or mites), the bulk of which have been reported from coleopterans (Knell & Webberley 2004). The prevalence of STIs in insect host populations is often high, exceeding 70% in some cases (Knell & Webberley 2004). Unlike many horizontally transmitted infections, the transmission of STIs is not generally thought to be density dependent, rather their transmission is governed by host mating frequency (Thrall *et al.* 1995; Kokko *et al.* 2002). Ryder *et al.* (2005), however, demonstrated that host encounter rate increases in a density dependent manner, leading to a higher mating frequency, in the ladybird host *Adalia bipunctata* (Linnaeus) (Coleoptera: Coccinellidae) of the sexually transmitted mite *Coccipolipus hippodamiae* (McDaniel and Morrill) (Acari: Podapolipidae).

1.5 Ladybird diversity

Coccinellids are a subgroup of the insect order Coleoptera, consisting of over 4500 species globally, 47 of which are found in the UK (Roy *et al.* 2012b). Although there are five UK species that not predatory, feeding on mildew or plants, the vast majority of the ladybird species in the UK are carnivorous, feeding on aphids, scale insects or mites (Majerus 1994; Roy *et al.* 2013). Many of these predatory species specialise in terms of habitat (for example the conifer specialising eyed ladybird, *Anatis ocellata* (Linnaeus) (Coleoptera: Coccinellidae), and the reedbed specialising water ladybird, *Anisosticta novemdecimpunctata* (Linnaeus) (Coleoptera: Coccinellidae) (Roy *et al.* 2012b). However there are some species that are considered generalists in habitat and food preference, these include *A. bipunctata*, *C. septempunctata*, and the well-established IAS *H. axyridis*. The lifecycle of all ladybirds follows the same development pattern from egg to larvae to pupae and finally to an adult ladybird. The majority of ladybirds oviposit their eggs in clutches, some in groups as small as 2, while others will lay up to 100 eggs in a clutch (Roy *et al.* 2012).

1.6 Ladybird natural enemies

Multiple different natural enemies attack ladybirds, including predators (spiders and birds), parasites (mites, nematodes, fungi, and bacteria) and parasitoids (Hymenoptera and Diptera) (Riddick *et al.* 2009; Roy *et al.* 2013). In addition to ladybird-specific parasites, many, such as the entomopathogen *Beauvaria bassiana* (Balsamo) (Ascomycota: Hypocreales), infect a wide range of hosts, including multiple ladybird species (Pathan *et al.* 2007). While many natural enemies have a worldwide distribution (Roy *et al.* 2011b), some have a more limited distribution due to their transmission mode (Ryder *et al.* 2005). For example, STIs rely on the overlap of adult generations, constraining parasites like *C. hippodamiae* to geographic areas with climatic conditions allowing their *A. bipunctata* hosts to be multivoltine (Ryder *et al.* 2007). Vertically transmitted pathogens which infect adult ladybirds can manipulate ladybird reproduction (Majerus & Hurst 1997). Multiple bacterial agents have been recognised as male-

killing parasites from over 10 separate ladybird species, causing the death of male host embryos (Weinert *et al.* 2007; Roy *et al.* 2013). In Britain, no horizontally transmitted parasites are known to infect ladybird eggs, however, all other life stages are infected by natural enemies (Roy *et al.* 2013).

The majority of the parasitoids that utilise ladybirds are from the Hymenoptera and Diptera orders (Ceryngier & Hodek 1996). Two Phorid species from the genus *Phalacrotophora* (Diptera: Phoridae) commonly parasitize the ladybird pre-pupa that forms shortly before pupation (Hurst *et al.* 1998). Several *Phalacrotophora* are able to parasitize a single pupa, although the total number depends upon the size of the host ladybird (Roy *et al.* 2013). In contrast, the Tachinid fly *Medina separata* (Meigen) (Diptera: Techninidae) only oviposits a single egg per adult ladybird host (Roy *et al.* 2013).

Although multiple species of hymenopteran are parasitoids of ladybirds, arguably the best studied is the braconid *D. coccinellae.* The solitary koinobiont parasitoid *D. coccinellae* has been reported utilising over 50 different ladybird species globally, including the IAS *H. axyridis* (Koyama & Majerus 2008). While *D. coccinellae* primarily parasitizes adult ladybirds, it has also been reported to successfully parasitize final stage larvae (Berkvens *et al.* 2010). Once *D. coccinellae* has approached and laid an egg into the adult ladybird, the egg hatches within the host's body tissues and develops through three instars. In the final fourth-instar, the larvae emerges from the abdomen of the ladybird and spins a silk cocoon between the legs of the ladybird, fastening it to the substrate, where the larvae develops from into an adult *D. coccinellae* wasp (Roy *et al.* 2012). Preferentially, *D. coccinellae* oviposits in female rather than male hosts, a behaviour that is probably driven by the increase in developmental success that occurs with size, combined with the larger size of female ladybirds (Davis *et al.* 2006). The invasion of the IAS *H. axyridis* has sparked multiple studies investigating the ability of parasites to utilise this species as a host. Despite *D. coccinellae* parasitising *H. axyridis* both in the native and invaded range of this species, the eclosion rate is still considerably lower in the invader (Koyama & Majerus 2008; Comont *et al.* 2013). Experimental tests have shown no preference

in oviposition of *D. coccinellae* between *H. axyridis* and a UK primary host species *C. septempunctata* (Koyama & Majerus 2008). Therefore, the presence of *H. axyridis* in the UK could influence the interaction between *D. coccinellae* and *C. septempunctata*, due to the reduced ability of this wasp to fully develop in the invader (Hoogendoorn & Heimpel 2002). However, despite the lower eclosion rate of *D. coccinellae* from the IAS, Comont *et al.* (2013) found no difference in the parasitism rate of *C. septempunctata* between populations where *H. axyridis* was present and absent, refuting the idea this species acts as an egg sink.

1.6.1 Laboulbeniales (Ascomycota: Laboulbeniales)

Of the reported fungal STIs of insects, the majority come from the order Laboulbeniales (Knell & Webberley 2004). The Laboulbeniales are obligate ectoparasitic fungi of arthropods containing approximately 2000 described species (Weir & Hammond 1997; Ceryngier & Twardowska 2013). Unlike many fungal species, the Laboulbeniales do not produce mycelia, instead forming a thallus (plural: thalli) on the cuticle of the host (Weir & Beakes 1995). Once an ascospore comes in contact with the cuticle of a suitable host, they elongate, attach and penetrate the host cuticle with small rhizoids, 3μm in diameter (Weir & Beakes 1996). These fungi possess both male and female reproductive structures, which are present on a single mature thallus, either on the same, or separate appendages (Shanor 1955; Weir & Beakes 1996). After fertilisation and maturation, multiple mature ascospores will be contained within the perithecium of the thallus (Weir & Beakes 1996). Many of the Laboulbenialian fungi are sexually transmitted, and upon the contact of thalli with a new host, the mature spores are transferred onto the mating partner's cuticle (Weir & Hammond 1997).

The Laboulbeniales typically have a high degree of host specificity (Weir & Beakes 1995; Weir & Hammond 1997). Some species have also been reported to be highly location-specific on their hosts, for example fungi in the *Chitonomyces* genus (Shanor 1955; Weir & Blackwell 2005). Indeed, molecular analysis has found two separate species with distinct position specificity to be present on the ladybird *Coleomegilla maculata* (De Greer)

(Coleoptera: Coccinellidae) in South America (Goldmann *et al.* 2013). However, generally the majority of the Laboulbeniales are restricted to infecting a single species or a group of closely related species (De Kesel 1996).

Although rhizoids have been reported penetrating the host cuticle, the Laboulbeniales have generally been thought to cause little to no damage to their hosts with minimal fitness consequences (Weir & Beakes 1995). Although longevity effects have been reported in earwigs (Strandberg & Tucker 1974), a recent study reviewed the current laboulbeniales literature and found that fitness costs were unknown for over half of the fungus-host associations detailed (Tragust *et al.* 2016). Interestingly, long-term infection by the Laboulbenialian fungi *Laboulbenia formicarum* (Thaxter) (Ascomycota: Laboulbeniales) triggers increased grooming and immune gene expression in the garden ant *Lasius neglectus* (Van Loon, Boomsma & Andrásfalv) (Hymenoptera: Formicidae), resulting in a higher survival probability after exposure to an entomopathogenic fungus (Konrad *et al.* 2015). The authors, however, did not look at any effects other than survival and gene expression.

1.6.2 Hesperomyces virescens

Four members of the *Hesperomyces* genus have been reported to infect coccinellids, however *H. virescens* is by far the most common, while the other three species (*H. chilomenis, H. hyperaspidis* and *H. coccinelloides*) are only known from a few records or single type specimens (Ceryngier & Twardowska 2013). Like all members of the Laboulbeniales order, the lifecycle of *H. virescens* is dependent on contact between two host individuals. Primarily, *H. virescens* is sexually transmitted, however in coccinellid overwintering aggregations where individuals are in close contact with one another, social transmission is also possible (Riddick & Schaefer 2005). Upon contact with a suitable host, mature ascospores are released from the tip of the thallus, which then attach to the cuticle of the new host, where they elongate and develop to form a new thallus (Weir & Beakes 1996). *Hesperomyces virescens* is self fertile, with both male and female appendages present on each thallus to allow the maturation of ascospores

(Weir & Beakes 1996). *Hesperomyces virescens* has been reported to infect 13 species of coccinellid, including the UK native *A. bipunctata* and the IAS *H. axyridis* (Riddick *et al.* 2009). While population declines of *Chilocorus bipustulatus* (Linnaeus) (Coleoptera: Coccinellidae) in Israel have previously been linked to the effect of *H. virescens*, these declines have since been shown to more likely be linked to the availability of prey items rather than fungal virulence (Kamburov *et al.* 1967; Applebaum *et al.* 1971).

Hesperomyces virescens has been found infecting *H. axyridis* in several countries around the world (Figure 1.1), including Ohio, USA in 2002, the Netherlands in 2008, Belgium in 2007, South Africa in 2013, and in the UK in 2011 (Garcés & Williams 2004; De Kesel 2011; Haelewaters *et al.* 2014, 2016a; Raak-van den Berg *et al.* 2014). All of these associations in the invaded range of *H. axyridis* were recorded several years after the first reported record of *H. axyridis*. The delayed establishment of the association between *H. axyridis* and *H. virescens* strongly indicates that it is the result of host shifts from native coccinellids. Although *H. virescens* is mainly transmitted during sexual contact, transmission is also possible during mixed-species over-wintering aggregations (Riddick & Schaefer 2005). Interestingly, it is possible to see infection by the Laboulbeniales on museum specimens, and by this method, it has been found that *H. virescens* is present on *H. axyridis* in its native range (Haelewaters *et al.* 2014). The prevalence of *H. virescens* is often high in *H. axyridis* ladybird populations, for example, in Kentucky *H. axyridis* populations, where there have been reports of a prevalence of over 80% (Harwood *et al.* 2006).

Figure 1.1: The distribution of *H. virescens* parasitizing *H. axyridis* in a) the USA and b) Europe. Areas highlighted in black show countries, or states in the USA, where *H. virescens* has been reported. (Map taken from Haelewaters *et al.* 2016b).

1.7 Harmonia axyridis

The harlequin ladybird (*H. axyridis*) is a notorious IAS, and is described by Roy *et al.* (2006) as 'the most invasive ladybird on Earth'. This invasive ladybird has caused drastic declines of native ladybird species in areas where it has become established (Brown *et al.* 2011a). Native to Asia, this species was introduced into North America in 1916 as a biological control agent against crop pests (Gordon 1985), and has now has become fully established in the Americas, South Africa and Europe (Brown *et al.* 2011b; Figure 1.2). Despite multiple releases through the 1900s, *H. axyridis* didn't establish in North America as a 'feral' species until the 1980s (Chapin & Brou 1991). By this time *H. axyridis* was already being introduced in Europe, and it only took 10 years until there were widespread reports of establishment (Pell *et al.* 2008). In 2003, *H. axyridis* was recorded in the South-East of England, despite never being introduced into the UK (Brown 2010). Since then *H. axyridis* has rapidly increased in numbers; its range increased at a rate of over 100 km per year for two years after the initial invasion of the UK to cover much of England and Wales (Brown *et al.* 2008).

There are multiple reasons considered to be important to the global invasive success of *H. axyridis*, including its morphological, physical, behavioural and chemical traits (Soares *et al.* 2008). Comont *et al.* (2012) argue that ladybird species geographic range sizes are better explained by their resource-use traits than their life-history traits; for example, exploitation of a wide array of prey items was a more important predictor of range extent than body size. Indeed, the generalist nature of the diet and habitat of *H. axyridis* has been suggested many times as one of the explanations for the success of this species (Majerus *et al.* 2006; Alhmedi *et al.* 2010; Clercq & Bale 2011; Evans *et al.* 2011).

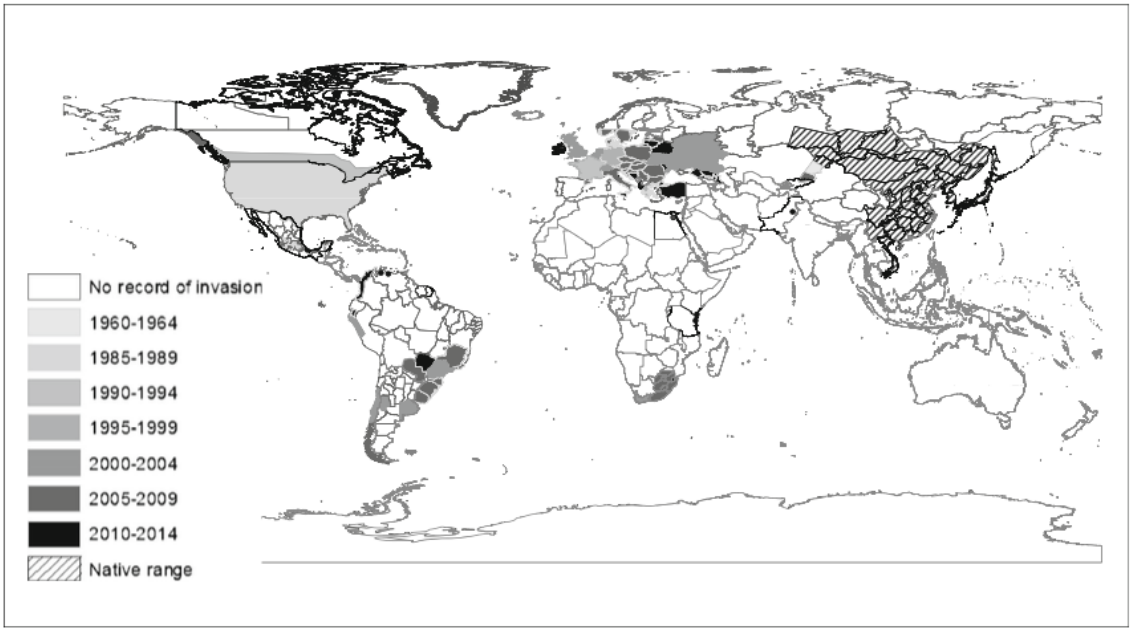

Figure 1.2: Global distribution of *H. axyridis* (Taken from Roy *et al.* 2016)

The voracious appetite and generalist nature of *H. axyridis* make this species a very effective biological control agent. Unfortunately, however, these traits also make this species an effective intra-guild predator, putting other aphid predators and their ecosystem services, including other coccinellids, at risk of competition and predation (Koch 2003; Majerus *et al.* 2006; Harmon *et al.* 2007; Pell *et al.* 2008; Gardiner *et al.* 2011; Brown *et al.* 2011a, 2015; Roy *et al.* 2012a). Indeed, Ware & Majerus (2008) found the larvae of multiple UK native ladybird species to be predated by *H. axyridis* larvae in laboratory experiments. The predation of other ladybirds has also been documented in the field, with the gut contents of field-collected *H. axyridis* found to contain the DNA of both *A. bipunctata* and *A. decempunctata* (Linnaeus) (Coleoptera: Coccinellidae) (Thomas *et al.* 2013). The generalist nature of *H. axyridis* and its role as an intraguild predator have led to the decline of native ladybird species that were once considered locally common, for example *A. bipunctata* in the UK (Brown *et al.* 2011a). The decline of native ladybirds has not only occurred in the UK, it has also been recorded in North America, South America and mainland Europe (Martins *et al.* 2009; Roy *et al.* 2012a, 2016a; Grez *et al.* 2016).

1.8 Aims and objectives

IAS can have a large impact on native communities in their invaded range. The release from natural enemies is often considered as an important determinant of the successful establishment of IAS. However, multiple studies have shown that the ERH does not apply universally to IAS, and is likely to interact with a number of other mechanisms to influence host population size. The immune ability of IAS, in addition to the ability of native parasites to perform a host shift to exploit them, will impact the dynamics of an invasion. Given the dramatic effect IAS can have on native populations, it is vital to consider the role parasites may play in their long-term population dynamics. The recent establishment of an association between the IAS *H. axyridis* and the fungal parasite *H. virescens* in the UK provides an opportunity to investigate the role this natural enemy might play in reducing any benefit from enemy release in this species. Therefore, the overall aim of this thesis is to address the role of this fungal parasite in the invasion biology of *H. axyridis*. Furthermore, this thesis sought to identify whether the activity of the *H. axyridis* encapsulation immune response explains its low susceptibility to a global natural enemy, the parasitoid wasp *D. coccinellae*. This thesis therefore aims to:

(i) test whether the sexual transmission rate and thalli development of *H. virescens* differs in two host species: the UK native *A. bipunctata* and the invasive *H. axyridis* (chapter 2)

(ii) assess whether variation in *H. virescens* infection biology between *H. axyridis* and *A. bipunctata* results from differences in the hosts, or in differences in the fungal strains naturally associated with them, by performing interspecific transfers of *H. virescens* (chapter 2)

(iii) test whether *H. virescens* infection influences lifespan and the rate of ageing in the invasive host *H. axyridis,* by measuring the virulence resulting from this chronic sexually transmitted infection (chapter 3)

(iv) investigate whether infection with *H. virescens* influences the susceptibility of the host to a second infection, the generalist fungal parasite *B. bassiana*, studying both the native species *A. bipunctata,* and invasive *H. axyridis* (chapter 4)

(v) examine whether differences in the developmental success of *D. coccinellae* in two species, *C. septempunctata* and *H. axyridis,* can be explained by differences in the immune response of these ladybirds. In addition, to test whether *D. coccinellae* is able to suppress immune responses in either host (chapter 5).

Chapter 2:

Breakdown of enemy release as a fungal parasite switches hosts to infect an invasive alien ladybird

2.1 Abstract

During biological invasions, alien species can benefit from enemy release if parasites native to the new range are unable to infect the novel species efficiently. Host shifts by parasites can subsequently be important in determining the future dynamics of biological invasions. This study investigated the exploitation of the invasive alien ladybird *Harmonia axyridis* by the fungal parasite *Hesperomyces virescens*. A host shift by this parasite, from the UK native ladybird *Adalia bipunctata* to *H. axyridis*, probably occurred in the years after *H. axyridis* invaded the UK. We show a dramatic increase in the prevalence of *H. virescens* in invasive alien *H. axyridis* populations in London since 2013. In the laboratory, we first compared the efficiency of sexual transmission and growth of *H. virescens* on *H. axyridis* with that on *A. bipunctata.* Then we conducted artificial infections of both host species using fungal isolates from conspecific and heterospecific hosts, testing whether variation in infection characteristics between the host species were predominantly driven by host differences or differences in the fungal strains they carried. The sexual transmission rate of *H. virescens* was lower on the invasive alien species; furthermore fungal burdens were lower and fungal development slower. Artificial transmission of *H. virescens* found a higher rate of intraspecific, compared with interspecific, transmission. While the ability to determine the driver of this effect was limited, a combination of host and fungal traits are likely to be involved in driving this effect, and that strains infecting *H. axyridis* may have diverged from those infecting *A. bipunctata* to better infect the invasive alien host. Our results demonstrate that *H. virescens* has a poorer ability to exploit the invasive alien *H. axyridis* than *A. bipunctata* after a recent shift, yet despite this the field prevalence of this infection on *H. axyridis* has increased substantially. The ecological advantage gained by the invader from enemy release may be breaking down as *H. virescens* begin to better exploit the novel invasive alien host.

2.2 Introduction

During biological invasions invasive alien species are exposed to novel native parasites in the invaded range. Invasive alien species are often considered to be at an ecological advantage upon invasion due to enemy release: the lack of co-evolved natural enemies associated with an invader at the point of invasion (Keane & Crawley 2002; but see Colautti *et al.* 2004; Heger & Jeschke 2014). Any benefit from enemy release is likely to decline over time as parasites adapt to exploit an invasive species (Mitchell *et al.* 2010; Stricker *et al.* 2016), although the accumulation of parasites rarely, if ever, reaches the same level as that of native species in the invaded area (Torchin & Mitchell 2004; Mitchell *et al.* 2010).

Host switches by parasites are common, and novel hosts can experience high levels of virulence from infection (Longdon *et al.* 2015). Indeed, emerging diseases that switch hosts, many of which are highly virulent and infectious, often result in devastating consequences for human health, for example the Ebola outbreak between 2013 – 2016 (Urbanowicz *et al.* 2016). The ability of native parasites to perform a host shift, and the level of virulence experienced by a novel host, will depend on multiple factors. The capacity of a parasite to develop on, and transmit from, a novel host plays a key role in the success of a host shift, phenomena that can be strongly influenced by the relatedness of the current and novel host species (Longdon *et al.* 2014, 2015). To successfully exploit a host, parasites must be able to physically gain access (e.g. the invasion of host tissues), withstand or evade immune defences, and successfully draw nutrients to develop, mature and transmit to a new host (Hellgren *et al.* 2009). These factors can act as barriers to a parasite, preventing immediate establishment in a novel host. Therefore, in order for a host shift to occur, a parasite has to either adapt to utilise a novel host, or have pre-existing adaptations allowing the immediate exploitation of a host (Agosta *et al.* 2010). Here, we investigate the exploitation of an invasive alien species following a recent host shift by a fungal parasite, and test for fungal adaptation with a cross-species transmission experiment.

Ladybirds and their parasites have been intensively studied in the UK for many years, including during the initial arrival of *Harmonia axyridis* in 2003 and its subsequent population growth and expansion (for example Roy *et al.* 2008, 2011, Brown *et al.* 2008, 2011; Comont *et al.* 2013). The distribution of ladybird species both pre- and post- the invasion of *H. axyridis* has also been recorded by the long running UK Ladybird Survey (and the Cambridge Ladybird Survey before this). While parasites have been reported in association with *H. axyridis* globally, multiple investigations have noted that infections often result in low virulence for this species (Roy *et al.* 2008; Comont *et al.* 2013; Haelewaters *et al.* 2016b). In addition, a lower infection prevalence is often reported for *H. axyridis* in comparison with other ladybird species utilised by the same parasites (Comont *et al.* 2013; Haelewaters *et al.* 2016b). The highly effective physical, chemical and immune defences of *H. axyridis*, including antimicrobial peptides and alkaloids, may contribute to the low virulence and field prevalence of parasites in this invasive alien species (Rohrich *et al.* 2012).

The sexually transmitted Laboulbenialian fungus, *Hesperomyces virescens* is known to have low host specificity, infecting multiple coccinellid species, including the UK native *A. bipunctata* (Riddick & Schaefer 2005; Haelewaters *et al.* 2016a). Unlike most fungi, these biotrophic fungi develop thalli on the cuticle of the host, containing both male and female reproductive organs (Weir & Beakes 1995; Gorczak *et al.* 2016). Transmission of *H. virescens* relies on direct contact between individuals as ascospores are unable to survive without a host for any period of time (Haelewaters *et al.* 2016a). Upon contact with a new host, mature ascospores are released from a mature thallus, and attach to the host cuticle with a haustoral foot, before continuing development into a new thallus allowing onward transmission (Weir & Beakes 1996; Weir & Hammond 1997).

Hesperomyces virescens has previously been studied in association with the UK native ladybird *Adalia bipunctata* (Welch *et al.* 2001) and, while it is now also found in UK populations of *H. axyridis,* this association was only reported in 2011, 8 years after *H. axyridis* invaded in 2003 (Haelewaters *et al.* 2014). Indeed, a delay between the establishment of *H.*

axyridis and initial reports of *H. virescens* infection seem to be common, for example this delay was 5 years in Belgium (Adriaens *et al.* 2003; De Kesel 2011) and 11 years in the USA, where *H. virescens* is now almost exclusively associated with *H. axyridis* (Chapin & Brou 1991; Garcés & Williams 2004; Ceryngier & Twardowska 2013). The replicated delay in the establishment of *H. virescens* on *H. axyridis* indicates that these observations probably represent multiple independent host shifts of strains that previously infected native insect species. These novel associations may have occurred as a result of contact between *H. axyridis* and native host species in overwintering aggregations (Nalepa & Weir 2007).

While it is possible the *H. axyridis* individuals that invaded the UK already carried *H. virescens*, but that this infection went undetected for 8 years, a number of arguments suggest this is unlikely. The majority of biological invasions are initiated by a small number of individuals (Torchin & Mitchell 2004) which reduces the likelihood they bring natural enemies with them (Keane & Crawley 2002). The global invasion of *H. axyridis* resulted from repeated intentional introductions of this species as a biological control agent in both the USA and mainland Europe (Gordon 1985; Koch 2003); it seems unlikely that biological control laboratory stocks would be released into the field contaminated with *H. virescens* infections. The invasion of *H. axyridis* into the UK was not the result of a purposeful biological control release. Therefore the initial invasion of *H. axyridis* in 2003 is likely to have consisted of only a few individuals invading from mainland Europe, where *H. virescens* had not at that time been observed infecting *H. axyridis* (Haelewaters *et al.* 2014). The invasion of *H. axyridis* in the UK rapidly spread to include a range covering much of Southern England (Brown 2010), yet early UK observations of *H. virescens* infection on *H. axyridis* were all located in similar areas to the only recorded location (London) where *H. virescens* is known to infect *A. bipunctata* (Welch *et al.* 2001; Haelewaters *et al.* 2014).

This study aimed to investigate the novel exploitation of *H. axyridis* by the fungal parasite *H. virescens*. After observing a dramatic epidemic of *H. virescens* on *H. axyridis* in the field, this investigation compared the ability of the fungus to transmit and develop on this novel

host with that on the ancestral host, *A. bipunctata*. We hypothesised that *H. virescens* would not be able to utilise *H. axyridis* to the same extent as *A. bipunctata*, predicting lower transmission and fungal growth on the invader. A fully reciprocal experimental design then tested whether interspecific differences in the transmission and development of *H. virescens* resulted from host or fungal traits.

2.3 Materials and Methods

Experimental material

To investigate the temporal changes in *H. virescens* prevalence on *H. axyridis*, ladybirds were collected from four sites in London (Hyde Park, Green Park, Regent's Park and St. James' Park) in each of the summers of 2013, 2014 and 2016. All collections were conducted between the months of July and September (2013, July 24th to 26th; 2014, September, between 1st and 10th; and 2016, July 5th and 6th). In each collection year, observations of the intensity of elytral pigment allowed ladybird age to be assessed (Roy *et al.* 2013). Ladybirds were scored for infection in the laboratory using a dissection microscope.

In order to compare the transmission and development ability of *H. virescens* infections between the invasive alien *H. axyridis* and the ancestral host, *A. bipunctata*, field infected 'donor' and uninfected 'recipient' ladybirds were collected from multiple locations in the summer of 2016 (Table 2.1). *Hesperomyces virescens* is found globally and has been reported to infect 13 different coccinellid species (Riddick *et al.* 2009). Although this fungal parasite is mainly sexually transmitted, it can also be transmitted through close contact between individuals, allowing potential host shifts between coccinellid species (Riddick & Schaefer 2005). Therefore, one aim of this study was to test whether fungal strains were better adapted to infect the host species they were isolated from. While we hypothesise that a host shift of *H. virescens* from *A. bipunctata* individuals to *H. axyridis* in the UK resulted in the current parasite presence in these populations, field collection found no *H. virescens* infected *A. bipunctata*

donor individuals in the London populations sampled. In addition, in samples collected from Stockholm (Table 2.1), no *H. virescens* infected *H. axyridis* were found, therefore a comparison of recipient ladybirds from this geographic location infected with a donor fungus sourced from this area for each donor species was not possible. Therefore, to avoid the possibility that geographic variation in host and parasite characteristics could confound this analysis, due to potential local adaptation of the parasite to infect local host genotypes, fungal donors and recipient ladybirds for both ladybird species were collected from separate geographical locations (Table 2.1). Each recipient species was sourced from two (*H. axyridis*) or three (*A. bipunctata*) separate populations. Each individual field-infected donor ladybird represented an independent *H. virescens* fungal strain in the experimental design, which used 40 fungal strains in total (20 from each donor ladybird species).

Table 2.1: A summary of collection locations for the two ladybird species used in this study. Donor ladybirds refer to field infected fungal sources, while recipient ladybirds are uninfected ladybirds that were exposed to *H. virescens* in the laboratory over the course of the experiment.

Location Collected	Species Collected	Donor/Recipient
Strandvägen & Humlegården, Stockholm, Sweden	*Adalia bipunctata*	Donor
Green, Hyde, St. James' & Regents Park London, UK	*Harmonia axyridis*	Donor
City park, Zvolen, Slovakia	*Harmonia axyridis*	Recipient
Kongens Have, Copenhagen, Denmark	*Harmonia axyridis*	Recipient
Green, Hyde, St. James' & Regents Park, London, UK	*Adalia bipunctata*	Recipient
Forthside Way, Stirling, UK	*Adalia bipunctata*	Recipient
Princes Street Gardens, Edinburgh, UK	*Adalia bipunctata*	Recipient

Field collected ladybirds were checked for *H. virescens* infection in the laboratory using a dissection microscope, then separated by sex and divided into two age categories, based upon the level of pigment in their elytra (Roy *et al.* 2013). Dark elytral pigmentation early in the season (May to August) is indicative that ladybirds eclosed in the previous year and have overwintered, while light pigmentation is observed on individuals that eclosed in the summer of collection (Roy *et al.* 2013). However, later in the season (September), darker pigmented

individuals may also include ladybirds that eclosed from a first summer generation. Only ladybirds with light pigmentation, indicating that they had eclosed in the summer of study, were used in infection experiments. The uninfected status of recipient ladybirds collected from populations that carried *H. virescens* was confirmed by holding them in the laboratory for approximately one month and using a microscope to verify latent *H. virescens* infections were not present.

Experimental protocol

This study was conducted across four experimental blocks (A - D, first to last) over 23 days (minimum 4 days, maximum 11 days between blocks). Of the 40 fungal strains, 20 (10 from each donor species) were used in infection trials in the first pair of blocks, A and B, and the remainder used in C and D. Within each block, the transmission success, infection burden (total thalli produced) and the developmental stage (immature or mature thalli) of *H. virescens* fungal strains were compared between the two ladybird species. Successful transmission was defined as the presence of *H. virescens* infection on recipients by the end of the experiment, excluding any individuals that died before the end of a 6-week period.

Two routes of transmission were examined in this study: firstly, artificial transmission, using fungal isolates from conspecific and heterospecific hosts, and secondly, the sexual transmission of conspecific isolates. For each fungal strain used in each block two ladybirds of each species were exposed to artificial transmission, and two conspecific ladybirds to sexual transmission. To perform comparable interspecific and intraspecific artificial transmission of the infection, each donor individual was manually rubbed onto two *H. axyridis* and two *A. bipunctata* individuals per block, in a random order. Each fungal strain was artificially transmitted onto approximately equal numbers of each sex per recipient species. Immediately following this, each donor ladybird was placed into a separate 9cm Petri dish to assess sexual transmission rates; they were allowed to mate with two conspecific ladybirds of the opposite sex

for four days. Subsequently, infection characteristics were assessed on the recipients. This process of artificial and sexual transmission was repeated in the second pair of blocks with an independent set of fungal donors. The total number of recipient ladybirds exposed to artificial transmission was 320 (160 of each recipient species), and 160 were exposed to sexual transmission (80 of each ladybird species).

Throughout the experiment, all ladybirds were kept individually in 9cm Petri dishes at a constant temperature of 20°C with an 18:6 hr L:D cycle and 60% relative humidity. Ladybirds were moved into new Petri dishes once per week and fed *ad lib* three times per week with artificial diet (Roy *et al.* 2013) and 'Entofood' (*Ephestia kuehniella* and *Artemia* spp. eggs) (Koppert Biological Systems). Beginning 9 or 10 days after exposure, ladybirds were checked weekly until 6 weeks post-exposure; each individual was scored for infection, thalli burden, and thalli developmental stage.

Statistical analysis

All analyses used R, version 3.3.2 (R Core Development Team 2016). All models were sequentially simplified using AIC comparisons; terms were removed if their inclusion did not improve AIC by 2 points. The P-values for all models were calculated using likelihood ratio tests, comparing models with and without the term of interest. The 'CAR' package (Fox & Weisberg 2011) was used to test for variance inflation in all models.

Field prevalence of H. virescens

A binomial generalised linear model was used to assess the increase in *H. virescens* field-prevalence by including year collected (2013, 2014 and 2016) as a covariate and using infection status as a response variable.

Fungal donor ladybirds

A generalized linear model with a Poisson distribution was used to compare the mean thalli burdens on the field collected donor individuals for the two ladybird species. The sex and species of the fungal donor, as well as their interaction, were included as fixed effects in this model.

Transmission success of H. virescens

The transmission success of *H. virescens* in the sexual and artificial transmission treatments was scored as a binomial variable, describing whether recipient individuals were infected or not at the end of the experiment. Individuals that died before the end of the experiment were excluded. Each individual was represented once in this analysis. Binomial mixed effects models had extremely poor fit and poor estimation of the random effects of *H. virescens* fungal strain and recipient population. Therefore a chi-squared test was used to investigate the difference in transmission success of *H. virescens* on the two host species. These chi-squared tests produced qualitatively similar results to the poorly fitting generalised linear mixed effects models.

Ladybird mortality

The number of individuals that died over the duration of the experiment, in both the sexual and artificial transmission treatments, was also used as a binomial variable in analyses testing determinants of mortality. As with the transmission success analysis, a poor fit in binomial mixed effects models and poor estimation of the random effects meant a chi-squared comparison was used to investigate the difference in the number of deaths that occurred in both recipient ladybird species. The results gained from chi-squared tests were qualitatively similar to the generalised linear mixed effects models.

Infection characteristics: sexual transmission

Analyses of the data for the characteristics of both sexual and artificial *H. virescens* infection included only individuals that became infected during the experiment, and examined the total fungal burden, thalli maturation and total mature thalli (to examine onward transmission potential). For each of these infection characteristics, multiple observations were conducted on each individual. Due to the distribution of the data, it was not possible to test for temporal autocorrelation with a correlation continuous-time autoregressive structure using the R package 'nlme' (Pinheiro *et al.* 2017). Therefore, repeated measures were accounted for in all models using a random effect for ladybird individual in mixed effects models, using the R package 'lme4' (Bates *et al.* 2014) and the optimizer "bobyqa", with a maximum iteration limit of 100000. In addition, all models included a random effect of fungal strain. Although host population was tested as a random effect in each model, its poor estimation meant it was not included in models. Unless otherwise stated, the base set of terms included in all models was time (in weeks), thalli burden of the fungal donor, recipient species, recipient sex, experimental block and recipient lifespan. Two-way interactions between the covariate 'time' and the following terms were included in models: fungal donor burden, recipient sex and recipient species. In addition, to assess potential differences between the infectivity of the donor species, an interaction between recipient species and the thalli burden of the fungal donor was also included. Furthermore, models assessed the effect of interactions between block and recipient species, and block and recipient sex to test consistency of effects across blocks.

Total fungal burden

The fungal burden of *H. virescens* infections was considered as the total number of thalli, immature and mature, counted on recipient ladybirds each week throughout the experiment. The base set of terms and random effects described above were included in

negative binomial generalised linear mixed effects models to assess the rate of increase in the total fungal burden between the two ladybird species.

Thalli maturation

The factors influencing the probability of mature thalli developing on a recipient ladybird during the experiment were assessed using binomial generalized linear mixed effects models, and the base set of terms described above, with the exception of time, as only a single observation was included for each individual. A random effect of fungal strain, but not ladybird individual (as there were no repeated measures), was included in this analysis.

In addition, the degree of maturation of the whole thalli burden on infected individuals was assessed by categorising thalli loads as a binary variable, indicating whether more than 50% of the thalli were mature at the time of each observation. A binomial generalized linear mixed effects model was used with the same set of base terms and random effects as described above. Only data from experimental batches A, B, and D were included in this analysis, as in batch C, no *H. axyridis* individuals matured over 50% of their thalli.

Onward transmission potential

The total number of mature thalli on recipients was used as a metric to assess onward transmission potential. Due to zero-inflation in the data, the analysis excluded data collected before week 2 of the experiment, where no mature thalli were recorded. In order to improve model fit, the response variable of mature thalli burden was scaled, dividing each value by the standard deviation of the total sample, before adding one. A square root transformation of the scaled response variable was used to improve the fit of the data to a normal distribution. The transformed response variable was included with the full base set of terms and random effects, as detailed above, in generalised linear mixed effects models with Gamma distributions using log link functions.

Infection characteristics: artificial transmission

The intraspecific and interspecific transmission of *H. virescens* was assessed using artificial exposure to fungal strains. The low number of individuals that became infected limited the analysis of this data set. All models containing artificial transmission data were analysed with the R package 'lme4' (Bates *et al.* 2014).

Total fungal burden

To investigate differences in the performance of fungal strains derived from the two ladybird species, the data for fungal burdens on each of the recipient species was considered separately. Due to the limited number of individuals that became infected, we considered only the fungal burden at 6 weeks after exposure to *H. virescens,* excluding the rest of the data from the analysis. To account for over-dispersion, negative binomial generalised linear mixed effects models containing a random effect for fungal strain were used to investigate the impact of the donor species and fungal burden of the donor, as well as their interaction, on the total number of thalli produced on each recipient species.

Onward transmission potential

The onward transmission potential of *H. virescens* on each recipient species was assessed by investigating the total number of mature thalli on each host at 6 weeks post-exposure to *H. virescens.* To test the impact of the thalli burden of the fungal donor and the species of the fungal donor, these terms were included in generalised linear mixed effects models with Poisson distributions, along with their interaction. In addition, a random effect of fungal strain was included in these models.

2.4 Results

Dramatic increase in H. virescens *field-prevalence between 2013 and 2016*

A dramatic increase occurred in the field-prevalence of *H. virescens* infecting *H. axyridis* London populations between the years 2013 and 2016, jumping from below 10% to above 70% in the three years sampled ($X^2_{[2]} = 481.14$, p = <0.001; Figure 2.1). The infection prevalence of *H. virescens* is likely to be higher in older individuals compared with newly eclosed ladybirds. Therefore larger numbers of ladybirds with darker pigmentation could drive the change in *H. virescens* prevalence over the sample years, however the proportion of older ladybirds at the time of collection was approximately similar between the years (2013, 40.6%; 2014, 38.6%; and 2016, 50.7%).

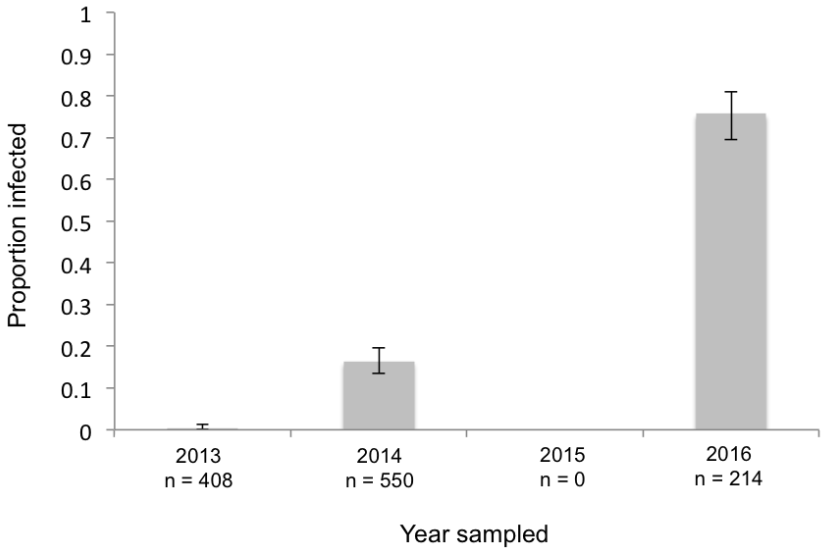

Figure 2.1: The field prevalence of *H. virescens* increased across the years sampled, from 2013 to 2016. Sample sizes shown on x-axis, no samples were taken in 2015. Error bars show 95% confidence intervals.

Higher fungal burden on field-infected A. bipunctata *than* H. axyridis *donors*

Forty fungal donor ladybirds, 20 *H. axyridis* and 20 *A. bipunctata* (Table 2.1), were used in laboratory transmission experiments. The mean thalli burden on these field-caught donors was considerably lower on *H. axyridis* than *A. bipunctata* (*H. axyridis*: 93.91, 95% CI 68.27 to 129.18; *A. bipunctata*: 219.67, 95% CI 175.78 to 274.51; $X^2_{[1]} = 984.46$, p = <0.001;

Figure 2.2). In addition, the thalli number was higher on male than female *A. bipunctata* donors, however for *H. axyridis* donors this trend was reversed, such that females had higher burdens than males; nevertheless, however this was not significant (donor sex by donor species interaction: $X^2_{[1]} = 102.88$, p = 0.066; Figure 2.2).

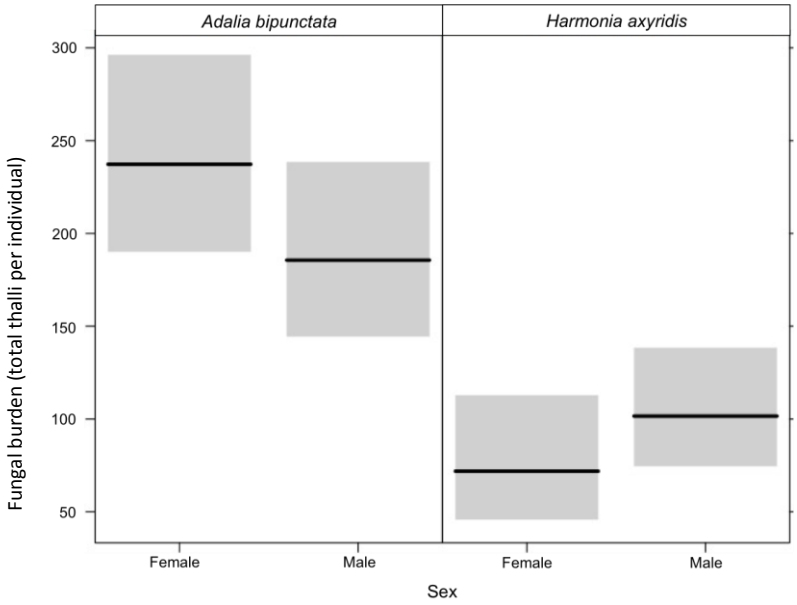

Figure 2.2: The fungal burden (total number of thalli per individual) was higher on *A. bipunctata* than *H. axyridis* field-caught donors. Female *A. bipunctata* had a higher burden than males, while the reverse was seen for *H. axyridis* donors. Grey bars show 95% confidence intervals.

Sexual Transmission

Higher sexual transmission rate of H. virescens *on the ancestral host,* A. bipunctata*, than the invasive host species*

Each recipient ladybird (61 *A. bipunctata* & 73 *H. axyridis*) in the sexual transmission treatment was exposed to a *H. virescens* strain from one of twenty conspecific fungal donors. Averaging across both host species, transmission took place on 85.07% (n = 134) of occasions. However, the transmission rate was 15.36% higher for *A. bipunctata* recipients compared with *H. axyridis* recipients ($X^2_{[1]} = 6.310$, p = 0.007; Figure 2.3).

Over the course of the 6-week experiment, 15.72% (± 2.89 SEM) of recipients died in the sexual transmission treatment. Averaging over both infected and uninfected individuals, the

death rate for *A. bipunctata* (22.78% ± 4.72 SEM) was higher than that for *H. axyridis* (8.75% ± 3.16; $X^2_{[1]} = 5.951$, p = 0.008). For both species, more *uninfected* than infected individuals died (*A. bipunctata*: $X^2_{[1]} = 11.213$, p = <0.001; *H. axyridis*: $X^2_{[1]} = 7.288$, p = 0.004).

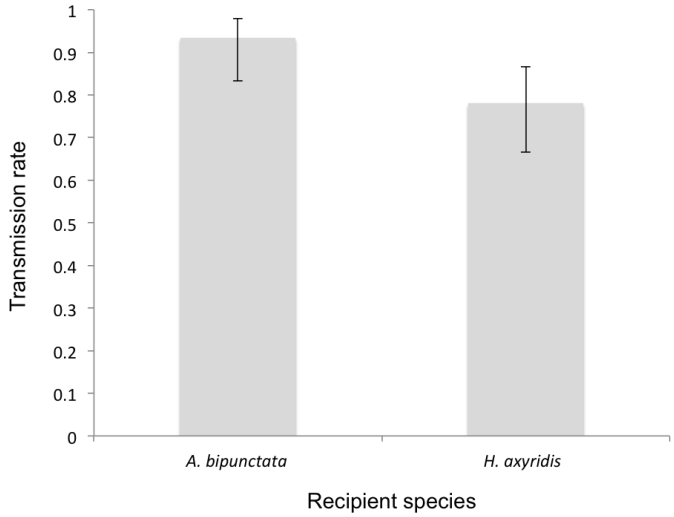

Figure 2.3: The rate of intraspecific transmission following sexual contact for both ladybird species. The rate of transmission was higher for *A. bipunctata* (n = 61) compared to *H. axyridis* (n = 73), (error bars = 95% binomial CIs).

Fungal burdens did not differ between host species for sexually transmitted infections

Excluding those individuals that did not become infected during sexual exposures, the total thalli burden on recipient ladybirds increased during the 6-week experiment ($X^2_{[1]} = 82.150$, p = <0.001). No difference was seen between the rate of increase in infection burden for *H. axyridis* and *A. bipunctata* (time by recipient species interaction: $X^2_{[1]} = 0.048$, p = 0.826; Figure 2.4). By 3 weeks post exposure, although generalized linear models indicated *A. bipunctata* hosts developed higher mean thalli numbers (19.10, 95% CI 9.23 to 39.52), in comparison with *H. axyridis* hosts (8.42, 95% CI 2.90 to 24.49), this difference was not significant ($X^2_{[1]} = 1.032$, p = 0.310; Figure 2.4). Variation in the total thalli burden between fungal strains accounted for 20.67% of random effect variation in this analysis.

The infections established in the four different experimental blocks varied. There was a general increase in thalli burdens that developed on recipient ladybirds across blocks A to D, such that hosts infected in block D had over twice as many thalli as those in block A ($X^2_{[3]} =$

12.009, p = 0.007; Figure 2.5). In line with our prediction that *H. virescens* can better exploit the ancestral host species, *A. bipunctata* developed larger thalli burdens than *H. axyridis* hosts in three of the four blocks, controlling for other covariates, however this between-block variation was not significant (recipient species by block interaction: $X^2_{[3]} = 6.366$, p = 0.095; Figure 2.5).

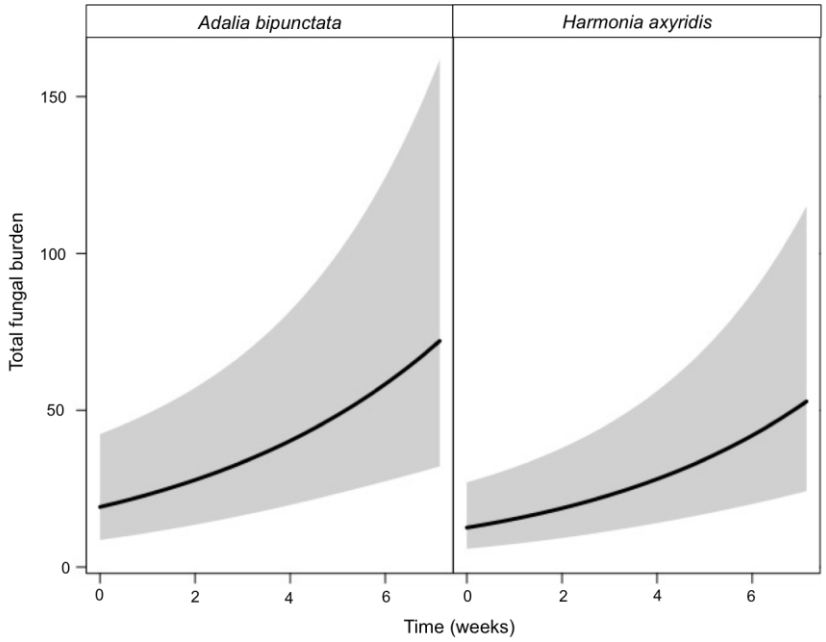

Figure 2.4: The total number of *H. virescens* thalli growing on recipient ladybirds following sexual exposure did not differ between ladybird species during the course of the experiment. *A. bipunctata:* n = 68, and *H. axyridis:* n = 59. Error bars = 95% CIs.

Pooling across both recipient species, there was no difference between males and females in the mean burden at 1 week post infection, the first point of thalli detection ($X^2_{[1]} = 0.669$, p = 0.413), nor at 3 weeks post infection ($X^2_{[1]} = 1.055$, p = 0.304). However, across the full six week experimental period the growth speed of the infection varied between the sexes: the rate of increase in thalli numbers was faster on males than females (time by host sex interaction: $X^2_{[1]} = 8.162$, p = 0.004). Donor ladybirds with higher thalli burdens caused higher recipient ladybird thalli numbers by the 3-week experimental midpoint ($X^2_{[1]} = 7.625$, p = 0.006), an effect that did not differ between the two host species (recipient species by donor burden interaction: $X^2_{[1]} = 0.024$, p = 0.878). In addition, sexually transmitted infections from

donors with lower burdens had a faster rate of thalli increase than infections derived from donors with higher burdens (time by fungal donor burden interaction: $X^2_{[1]} = 8.735$, $p = 0.003$).

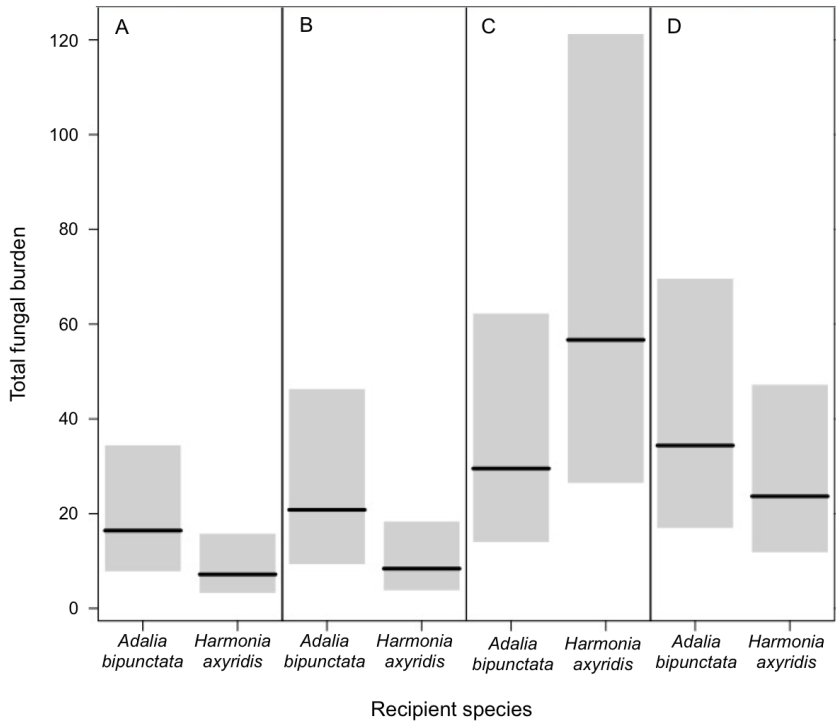

Figure 2.5: The total number of thalli on ladybirds after sexual exposure to an infected conspecific ladybird increased across blocks A to D. All blocks, except C, were consistent in showing a lower number of thalli on *H. axyridis* hosts compared with the ancestral host, *A. bipunctata*. The number of thalli is estimated at the experimental midpoint (3 weeks post infection) for each block from a model containing data for the full 6 weeks of the experiment. Error bars = 95% CIs.

In order to control for a potential effect of selective disappearance of ladybirds on thalli loads in these analyses, which might have resulted from non-random host death during the experiment, we fitted ladybird lifespan as a covariate in models. The majority of ladybirds in the sexual transmission treatment that became infected lived beyond the end of the experiment (89.76%, n = 127). However, the burden of thalli that developed on infected ladybirds was associated with their eventual lifespan: longer lived ladybirds had a greater thalli burden than shorter lived individuals at the midpoint of the experiment ($X^2_{[1]} = 24.941$, $p = <0.001$).

Fungal infections mature faster on the ancestral host species

Thalli grow through an immature phase, before maturing to carry spores capable of transmission. We hypothesized that *H. virescens* would be better able to exploit and mature on

the ancestral host species, *A. bipunctata,* compared with *H. axyridis.* In agreement with this, a higher proportion of *A. bipunctata* individuals (76.47%, n = 68) developed mature thalli than *H. axyridis* individuals (66.10%, n = 59) during the study ($X^2_{[1]} = 9.464$, p = 0.002). The proportion of ladybirds that developed mature thalli was not dependent on host sex ($X^2_{[1]} = 0.038$, p = 0.845), nor on the thalli burden of the fungal donor ($X^2_{[1]} = 0.022$, p = 0.883). Furthermore, 45.86% of the random effect variance in this analysis was accounted for by fungal strain. A main effect of experimental block indicated that recipients in block C had a lower probability of thalli maturation than those in other blocks ($X^2_{[3]} = 23.750$, p = <0.001). The probability of producing mature thalli was associated with the eventual lifespan of the recipient: individuals with a longer lifespan had a higher probability of producing mature thalli by week 3 of the experiment ($X^2_{[1]} = 15.484$, p = <0.001).

In addition to testing the probability of mature thalli development on infected individuals, we also assessed the rate of thalli development within individual burdens by measuring whether over 50% of the total thalli burden of the host matured. In accordance with our prediction, by the 3-week experimental midpoint, the proportion of individuals on which the majority of thalli had matured was higher for *A. bipunctata* individuals in comparison with *H. axyridis* individuals ($X^2_{[1]} = 19.674$, p = <0.001; Figure 2.6). Indeed, in block C where fungal maturation was low, no *H. axyridis* individuals matured over 50% of their thalli in block C. Despite this, the synchrony with which the thalli matured on each infected host was marginally faster on *H. axyridis,* although this effect was not significant (time by recipient species interaction: $X^2_{[1]} = 3.274$, p = 0.070; Figure 2.6).

Across the two recipient species, male ladybirds were more likely to have the majority of their thalli fully developed by 3-weeks post-exposure to *H. virescens,* in comparison with female individuals ($X^2_{[1]} = 5.139$, p = 0.023); however, synchrony of maturation was no different between the sexes (time by sex interaction: $X^2_{[1]} = 1.357$, p = 0.244). This sex difference in the proportion of individuals with mature thalli loads was consistent across the experimental blocks A, B and D (block by host sex interaction: $X^2_{[2]} = 1.378$, p = 0.502). In

block C, no *H. axyridis* of either sex matured over 50% of their thalli; however, considering the remaining 3 experimental blocks, the probability over 50% of thalli were mature on individuals by 3-weeks post-fungal exposure was generally greater in later experimental blocks ($X^2_{[2]}$ = 21.172, p = <0.001).

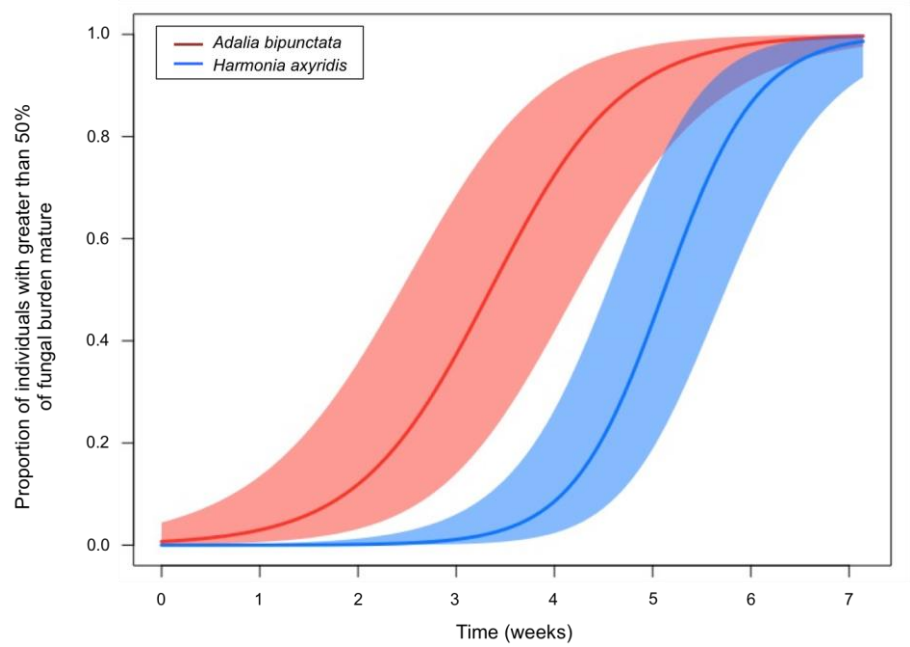

Figure 2.6: A comparison of *H. virescens* maturation on *A. bipunctata* and *H. axyridis* over the 6-weeks post-infection. The y-axis shows the proportion of host individuals for which greater than 50% of the thalli burden were mature. Red line = *A. bipunctata*, blue line = *H. axyridis.* Shaded areas are 95% CIs.

The thalli burden of the fungal donor did not affect the proportion of recipients that had the majority of their thalli mature ($X^2_{[1]}$ = 0.801, p = 0.371), and this non-significant trend did not differ between the two host species (host species by donor burden interaction: $X^2_{[1]}$ = 0.291, p = 0.590). Individuals were more likely to have a higher proportion of mature thalli at 3-weeks post–exposure if they subsequently had a longer lifespan ($X^2_{[1]}$ = 34.131, p = <0.001). In addition, the random effect of fungal strain accounted for 15.20% of the random effect variance in this analysis.

Earlier transmission potential on the ancestral host species

We assessed species differences in the onward transmission potential of recipient ladybirds by comparing the total number of mature thalli on the two host species, beginning from week two of the experiment. In accordance with our prediction that *H. virescens* would be less able to exploit the novel invasive alien host than the ancestral host, recipient *A. bipunctata* ladybirds had a very considerably higher mean burden of mature thalli (31.19 ± 6.60 SEM) than *H. axyridis* (0.17 ± 0.10 SEM) at 3-weeks post sexual exposure to *H. virescens* ($X^2_{[1]} = 5.637$, p = 0.018; Figure 2.7). Indeed, considering only recipients that developed mature thalli by the end of the study (*A. bipunctata*: 86.7%; *H.* axyridis: 67.8%), only 2% of the 40 *H. axyridis* individuals had done so by week 3, compared to 86.4% of the 59 *A. bipunctata*. After establishing this early difference mature burden difference, the rate of increase in transmission potential was considerably faster for infected *A. bipunctata* than for *H. axyridis* individuals (time by recipient species interaction: $X^2_{[1]} = 14.084$, p = <0.001).

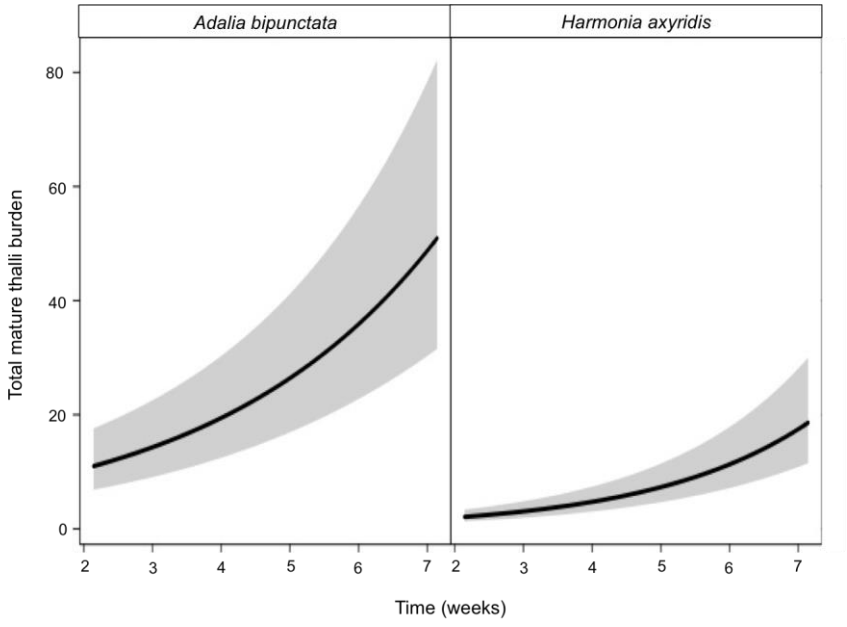

Figure 2.7: The total onward transmission potential as measured by the mature thalli burden was higher on *A. bipunctata* than on *H. axyridis* individuals as was the rate at which transmission potential increased. The x-axis shows the time in weeks post-infection, beginning from 2 weeks, when the first mature thalli appeared.

At 3-weeks post exposure to *H. virescens*, the transmission potential was no different between the two sexes ($X^2_{[1]} = 2.345$, p = 0.126), nor was there any detectable sex difference in

the rate of increase in mature thalli across the experiment (time by sex interaction: $X^2_{[1]} = 2.107$, $p = 0.147$). While higher thalli burdens on fungal donor ladybirds resulted in higher mature thalli burdens, this was not significant ($X^2_{[1]} = 2.898$, $p = 0.089$), and the effect was no different between recipient species (donor burden by recipient species interaction: $X^2_{[1]} = 0.448$, $p = 0.503$). In addition, the thalli burden of the donor ladybird did not affect the rate of increase in mature thalli numbers on the recipient (time by donor burden interaction: $X^2_{[1]} = 0.686$, $p = 0.408$).

The transmission potential of hosts increased across the duration of the experiment ($X^2_{[1]} = 161.830$, $p = <0.001$); this was consistent between the blocks of the experiment (block by time interaction: $X^2_{[3]} = 4.42$, $p = 0.237$). Between-block variation did not differ across recipient species (block by recipient species interaction: $X^2_{[3]} = 0.251$, $p = 0.969$), nor the two sexes (block by recipient sex interaction: $X^2_{[3]} = 1.306$, $p = 0.728$). However, a higher number of mature thalli were more likely to be present on individuals with a longer lifespan ($X^2_{[1]} = 11.479$, $p = <0.001$). The variation in transmission potential associated with the random effect of fungal strain accounted for only 3.12% of the residual variance after fitting the fixed effects.

Artificial transmission

For infections derived from sexual transmission, as described above, the fungal parasite *H. virescens* was not able to exploit the invasive alien species *H. axyridis* to the same extent as the ancestral host, *A. bipunctata.* This difference in fungal growth and development could result from differences between characteristics of the two ladybird host species, or from differences in the fungal isolates that grow on each species in the field. In order to test this, a fully reciprocal experiment exposed 160 individuals of each recipient species to either a fungal strain from their own host species, or from a heterospecific donor (40 *H. virescens* strains split equally between the two fungal donor species) by manually rubbing a donor ladybird onto uninfected recipients of both species. The transmission rate resulting from artificial exposure to *H. virescens* was low

in comparison to that from sexual exposure (Figure 2.2; Figure 2.8). Of the 320 artificially exposed ladybirds, only 8.13% of *A. bipunctata* (n = 160) and 9.38% of *H. axyridis* (n = 160) individuals became infected with *H. virescens.*

Intraspecific transmission more effective than interspecific transmission for both host species

The key aim of this reciprocal artificial transmission part of the study was to determine whether host species or fungal strain differences were responsible for the variation in host exploitation by *H. virescens* in the sexually transmitted section of the study. Averaging across the interspecific and intraspecific infection routes, the infectivity of *H. virescens* did not differ between the two recipient species ($X^2_{[1]} = 0.162$, p = 0.688). However, for both species more infections resulted from intraspecific fungal exposure than from interspecific exposure ($X^2_{[1]} = 10.602$, p = 0.001; Figure 2.8). The degree of this difference between the rate of transmission for the interspecific and the intraspecific infection routes was greater for *A. bipunctata* than *H. axyridis*, however this effect was not significant (recipient species by infection type interaction: $X^2_{[1]} = 3.064$, p = 0.080; Figure 2.8). This strong effect of infection origin in *A. bipunctata* was exemplified by the fact that only a single infection developed on *A. bipunctata* following interspecific artificial exposure to *H. virescens* from *H. axyridis* (n = 80), considerably fewer than the 12 infections resulting from intraspecific fungal transmission (n = 80) ($X^2_{[1]} = 11.795$, p = 0.001). While this trend was the same in *H. axyridis* recipients, the infectivity difference between inter- and intraspecific exposures was not significant ($X^2_{[1]} = 1.871$, p = 0.171; Figure 2.8).

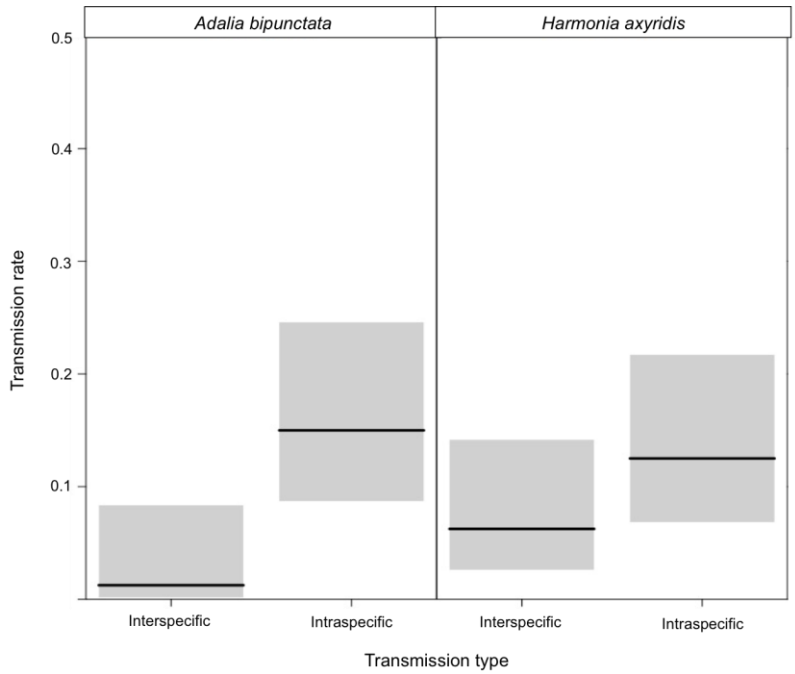

Figure 2.8: The transmission rate of *H. virescens* following either inter- or intra-specific exposure to fungal strains for the two recipient species *A. bipunctata* and *H. axyridis*. The transmission rate was higher for intraspecific exposure for both recipient species (Error bars = 95% confidence intervals).

The low transmission success in this artificial exposure part of the study meant many of the subsequent statistical tests had low power. However, the total fungal burden and the onward transmission potential (the number of mature thalli) were considered for individuals that became infected by the end of the study. Artificially exposed *H. axyridis* hosts infected with fungal strains from *A. bipunctata* had slightly higher mean burdens (14.8 ± 7.32 SEM) and slightly higher total mature thalli numbers (7.0 ± 4.47 SEM) than *H.* axyridis hosts infected from *H. axyridis* donor ladybirds (total thalli: 12.3 ± 5.03 SEM; mature thalli: 5.2 ± 2.56 SEM; Figure 2.9). However, these differences were not significant (total thalli: $X^2_{[1]} = 0.247$, $p = 0.619$; mature thalli burden: $X^2_{[1]} = 1.009$, $p = 0.315$; Figure 2.9) and mature thalli began to appear on *H. axyridis* hosts at week 2 regardless of infection source.

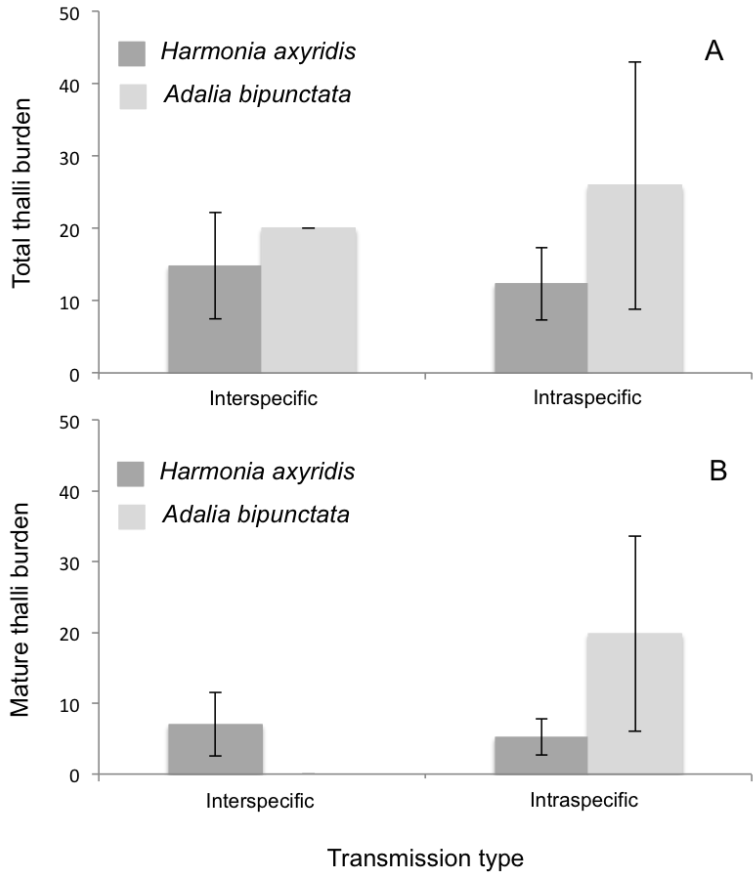

Figure 2.9: The fungal burden of artificially infected *H. axyridis* and *A. bipunctata* hosts at the end of the 6-week experiment. A: total thalli burden on host ladybirds; B: onward transmission potential, assessed as the total number of mature thalli on ladybird hosts. Note that only a single individual is represented in the *A. bipunctata* interspecific transmission category, none of whose thalli became mature by the end of the 6-weeks. (Error bars = SEM).

By 6 weeks post exposure, only a single *A. bipunctata* individual was infected with a *H. axyridis* derived fungal strain. This individual carried 20 thalli, which fell within the burden range for the twelve *A. bipunctata* that carried *A. bipunctata* derived infections (range = 1 – 212 thalli) and was close to the mean burden for these intraspecifically infected individuals (25.9 ± 17.12 SEM; Figure 2.9). Considering the onward transmission potential of infected ladybirds, this single *A. bipunctata* individual infected with *H. axyridis* derived fungus did not develop any mature thalli by the end of the 6-week experiment, whereas for infections resulting from intraspecific transmission, a mean of 19.8 (± 4.47 SEM) mature thalli were observed on infected *A. bipunctata* (Figure 2.9).

2.5 Discussion

The ecological advantage gained by biological invaders resulting from enemy release can be influenced if native parasites are able to perform a host shift and utilise invasive alien species (Keane & Crawley 2002). In this study, we investigated the exploitation of an invasive alien species by a fungal parasite following a recent host shift from an ancestral host species in the UK. Despite a dramatic epidemic of *H. virescens* in field populations of *H. axyridis*, the fungal parasite displayed a lowered ability to exploit *H. axyridis* in comparison with an ancestral host species, *A. bipunctata.* In addition to a lower transmission rate, the growth and development of sexually transmitted *H. virescens* infections on *H. axyridis* hosts was slower and results in a lower onward transmission potential than infections on *A. bipunctata* hosts. While the immune response of *H. axyridis* to this fungal parasite is unknown, this invader had a higher resistance to numerous parasites in comparison to other ladybird species (see Haelewaters *et al.* 2016b for a review). To test whether the difference in the infectivity of *H. virescens* was due to traits of the host species or the fungal strains infecting them, we undertook a cross-infection study. This was inconclusive, however it demonstrated a higher rate of intraspecific, than interspecific transmission for fungal strains isolated from both *H. axyridis* and *A. bipunctata*, suggesting that fungal isolates are adapted to infect the species they were isolated from in the field.

Sexually transmitted infections often reach a high prevalence in insects (Knell & Webberley 2004). The results of this study demonstrate a dramatic increase in the prevalence of the sexually transmitted parasite *H. virescens* in London populations of *H. axyridis*. In 2016 it reached a level over 20% higher than that reported infecting *A. bipunctata* in the same area by Welch *et al* in 2001, prior to the *H. axyridis* invasion. Although sites were sampled once per year, the first and final samples occurred in July, the month of the lowest reported prevalence in the 2001 study by Welch *et al*. We therefore argue that the substantial increase in *H. virescens* prevalence over three years in the London *H. axyridis* population is a real phenomenon and unlikely to be explained by variation in the time of sampling combined with seasonal

prevalence variation. In addition, the proportion of ladybirds having overwintered from the previous generation in each sample (as determined by elytral pigment differences) was approximately equal. As ladybirds were collected in the September of 2014, compared with July in 2013 and 2016, it is possible the darker pigmented individuals in this year included individuals eclosing early in the season, rather than only ladybirds from the previous generation. However, as 2014 was the middle year of our three samples, it seems likely the prevalence increase seen in this study is not an artefact of older individuals, which are more likely to be infected, being more common in later samples. In line with our findings, Belgian populations of *H. axyridis* have also suffered a wave of *H. virescens* infection, rising to a prevalence of over 96% since the first observation in 2007 (De Kesel 2011). *Hesperomyces virescens* has a low host specificity (Riddick & Schaefer 2005; Haelewaters *et al.* 2016a) and has been found naturally infecting multiple populations of both *A. bipunctata* (Welch *et al.* 2001; Webberley *et al.* 2006) and *H. axyridis* (Garcés & Williams 2004; Riddick & Schaefer 2005; Nalepa & Weir 2007; De Kesel 2011; Haelewaters *et al.* 2014, 2016a; Gorczak *et al.* 2016). Despite this, our laboratory-based results examining the growth and development of *H. virescens* on a novel invasive alien host, *H. axyridis,* show that this fungal parasite is unable to exploit the novel host to the same extent as the ancestral host, *A. bipunctata.*

Our data show that the ability of *H. virescens* to sweep through London populations of *H. axyridis* is not driven by high intrinsic susceptibility of *H. axyridis*. It therefore seems likely that epidemiological factors causing high transmission in this species may be responsible. The reproductive biology of hosts can have a strong impact on the prevalence of sexually transmitted infections, which rely on the overlap of adult generations for successful inter-annual transmission (Knell & Webberley 2004). The incidence of generation overlap in ladybirds is higher in those species that are able to reproduce multiple times per year. In southern areas of the UK *H. axyridis* is commonly bivoltine, and, although in warmer years *A. bipunctata* also displays this dual reproductive cycle, it is less consistent than that seen in *H. axyridis* (Roy *et al.* 2013). The rate of effective inter-year transmission of *H. virescens* infection in ladybird

populations is therefore likely to be higher in *H. axyridis* in the south of the UK than *A. bipunctata.* As a result, it is plausible that the range of *H. virescens* in the UK could expand following the host shift by *H. virescens* to infect *H. axyridis*, due to intergenerational mating occurring over a wider geographic area of the UK.

The reduced ability of *H. virescens* to exploit *H. axyridis* observed in this study could be driven by either host or fungal differences; however, determining the main driver of this effect was hampered by low transmission rates in the artificial, cross-infection part of the experiment. If the observed effects had been driven by fungal differences, fungal transmission and growth would be expected to have been most efficient for *A. bipunctata* derived fungal strains, regardless of the recipient species. Alternatively, higher transmission and growth on *A. bipunctata* than *H. axyridis* recipients, regardless of fungal strain, would suggest that host differences drove the observed effects. Our results, however, appear to fall between these two alternatives, indicating that intraspecific *H. virescens* transmission was more effective than interspecific transmission for both recipient species. In addition, despite small sample sizes, following artificial transmission thalli burdens were no different for either of the two recipient species between *H. axyridis* and *A. bipunctata* derived fungal strains. While the mechanics of the artificial transmission method may have had lower efficiency between, than within, species, it is speculatively more likely the effect is derived from a combination of host and fungal differences; suggesting that fungal strains are best adapted to transmit between members of the host species they were isolated from. This effect may have occurred as a result of genetic change in *H. virescens* strains following the initial host shift from *A. bipunctata* to *H. axyridis*, however, as this fungus is known to infect multiple ladybird species (Riddick & Schaefer 2005; Haelewaters *et al.* 2016a), it is perhaps more likely that some fungal strains had a pre-existing ability to exploit *H. axyridis.*

While the field prevalence of *H. virescens* has increased in multiple *H. axyridis* populations, including the London population monitored in this study (Haelewaters *et al.* 2016b), our results demonstrate *H. virescens* is better able to exploit *A. bipunctata* than *H.*

axyridis when transmitted sexually. It is possible the fungal burden of donor ladybirds used in this experiment may have influenced the resulting transmission and growth of *H. virescens* infections on recipients: importantly, higher fungal burdens were present on *A. bipunctata* donors compared with *H. axyridis* donors. However, it is unlikely the effects seen in this study were driven by higher donor burdens in *A. bipunctata*, in comparison with *H. axyridis,* as across all infection characteristic analyses, with the exception of the total thalli burden on the recipient, the fungal burden on the donor ladybird was accounted for. While the total thalli burden of recipient ladybirds was influenced by donor burden (higher donor burdens resulted in higher recipient burdens), this effect was present even while controlling for different donor species in the analysis. In addition, although the total thalli burden on ladybird recipients was influenced by the fungal burden of the donor ladybird, the effect was consistent across both host species.

Although this study was not designed to specifically detect the sex-specific effects of infection, our results are consistent with previous studies showing males are more susceptible to infection than females (Zuk 2009; Cordoba-Aguilar & Munguia-Steyer 2013; Stephenson *et al.* 2016). *Hesperomyces virescens* infections grew more quickly on male recipients of both species, resulting in an earlier appearance of mature thalli and a higher transmission potential at 3-weeks post exposure to *H. virescens*, although no difference in the initial thalli burden was seen between the two sexes. The higher thalli burden on male hosts is likely to be a result of autoinfection from pre-existing mature thalli, which appear sooner on males. The lack of difference in the initial thalli burden between the sexes, yet the presence of faster maturation and higher fungal burdens on males suggests males may have a lower immune capability to resist infection. Sex-biased parasite infections are common in multiple animal host species (Cordoba-Aguilar & Munguia-Steyer 2013; Stephenson *et al.* 2016). Multiple studies have examined the reasons behind this phenomenon, including the role of hormones, behaviour, and life history differences between the sexes (Zuk 2009; Bacelar *et al.* 2011; Schmid-Hempel 2011). Although the precise mechanism behind sex-biased infections is debated, it has been suggested that males allocate fewer resources to immune defence than females (Bacelar *et al.*

2011; Schmid-Hempel 2011). In *Heliothis virescens* moths, where females are the sexual signallers, variation in immune defence investment occurs, such that bacterially challenged female *He. virescens* have higher immune gene expression than males (immune deployment), which in contrast, maintain higher baseline immunity (immune maintenance) than females (Barthel *et al.* 2015). Barthel *et al.* (2015) also demonstrated that female reproductive success was reduced following immune challenge in infected females but not in males, demonstrating a trade off between immune and reproductive investment in *He. virescens.* The parasite used in the current study is able to penetrate the cuticle, gaining nutritional value from the host (Weir & Beakes 1996) and, while the sex-specific effect observed here may be driven by immune differences, it is also possible the effect could be driven by variation between males and females in the nutrition that the fungus can draw from the host.

In addition to the impact of ladybird sex, experimental block was also found to have a strong impact on the infection characteristics throughout the sexual transmission section of the study. The higher thalli burdens seen in later experimental blocks could result from a variety of reasons. All ladybirds in all blocks were exposed to infection by *H. virescens* within one month of each other. It is therefore possible that the donors used in later blocks gave a larger dose to recipients, as infections were incubated in these donors for longer, or that recipients may have been more susceptible to infection at this later time point in the investigation. An alternative explanation, an increase in susceptibility resulting from the senescence of immune ability, seems improbable over the short delay between the blocks, given the short proportion of ladybird lifespan this covers (Roy *et al.* 2013).

The enemy release hypothesis proposes that the benefit gained from the impaired ability of native parasites to infect invasive alien species results in an ecological advantage for the invader (Keane & Crawley 2002; Roy *et al.* 2011a). This study finds evidence to support this, demonstrating a reduced ability of *H. virescens* to exploit the invasive alien *H. axyridis* in sexual transmission experiments when compared with an ancestral host species, *A. bipunctata.* However, our results also observe a dramatic epidemic of *H. virescens* in field populations of *H.*

axyridis. In addition, we provide some evidence that *H. virescens* fungal strains on *H. axyridis* in the field have diverged from those infecting *A. bipunctata,* exhibiting a higher ability to infect the invader. It therefore seems likely that we are observing the breakdown of enemy release as the fungal parasite switches to infect the novel invasive alien host.

Chapter 3:

Chronic sexually transmitted infection causes accelerated senescence in the ladybird beetle *Harmonia axyridis*

3.1 Abstract

Human lifespan has increased dramatically since pre-industrial times, driven largely by changes in the prevalence of infectious disease, particularly in children. However, the role of infectious disease in accelerating ageing in adults remains unclear. Chronic inflammatory conditions may develop as a result of acute childhood infections, or as chronic adult infections, and have been proposed to accelerate ageing; however experimental tests are rare. We experimentally investigated whether a chronic sexually transmitted infection influences the rate of senescence for a range of host fitness traits in a model insect species, *Harmonia axyridis*. We show strongly elevated late life mortality in infected hosts. In addition, infected males display a dramatic acceleration in the normal rate of ageing-associated loss of body condition. Infection caused fecundity senescence in females, a phenomenon not seen in uninfected beetles. Furthermore, female fertility senesced more quickly in response to high infection burdens than in uninfected ladybirds. Our results demonstrate chronic infection can strongly accelerate the senescence of multiple life history traits, providing compelling evidence for the role of infectious disease in driving the ageing process and shortening lifespan.

3.2 Introduction

Variation in lifespan can be driven by differences in the rate at which organisms age or by differences in the rate of age-independent mortality (Stearns 1992; Rose 1994; Johnson *et al.* 1999). The definitions of ageing and senescence can be tricky to separate, however, ageing occurs in chronological terms, but does not necessarily refer to the reduction in fitness as individuals may increase in fitness when they are younger (Gems 2015). By contrast, senescence refers to a decline in fitness over time, although individuals can senesce at different rates over the same time period, and thus senescence does not necessarily closely relate to chronological time (Gems 2015). The dramatic increase in the average human lifespan during the last century is generally attributed to a reduction in mortality caused by infectious disease, particularly in children (Wilmoth 2000; World Health Organisation 2002). Thus, historical increases in human lifespan are frequently proposed to result from a reduction in baseline mortality rates, rather than a reduction in the rate at which people age (Fries 1980; Johnson *et al.* 1999; Finch & Crimmins 2004). Nevertheless, the extent to which infectious diseases directly influence the rate of ageing in animal populations is debated (Fries 1980; Franceschi *et al.* 2000; Gavazzi & Krause 2002; Finch & Crimmins 2004; Kirkwood 2005; Pawelec *et al.* 2005; Hayward *et al.* 2016). The cohort morbidity hypothesis proposes that acute infections suffered in childhood can cause chronic inflammation in adult life, which in turn affects adult lifespan (Finch & Crimmins 2004); however, the study of some human populations suggest this to be unlikely (Hayward *et al.* 2016). Alternatively, chronic inflammatory infections affecting the adult population have also been proposed to alter lifespan and accelerate ageing (Danesh *et al.* 1997; Appay *et al.* 2007; Beirne *et al.* 2014). In this study we experimentally investigate the impact of a chronic fungal infection on senescence in a model insect species.

Negative impacts of infection on host lifespan may result both from infection pathology and from the impacts of immune system stimulation. Chronic viral infections of humans such as HIV (Human Immunodeficiency Virus) and CMV (Cytomegalovirus) can result in immunological shifts akin to those seen in uninfected elderly humans, for example a

lower rate of T-cell renewal and telomere shortening (Spencer *et al.* 2002; Fletcher *et al.* 2005; Desai & Landay 2010; Kaplan *et al.* 2011; Pawelec *et al.* 2014). The early onset of age-related diseases, such as cardiovascular disease, can also result from chronic infection in humans (Danesh *et al.* 1997; Ngeh *et al.* 2002; Currier *et al.* 2008; Desai & Landay 2010; Deeks 2013). Evidence of accelerated ageing in response to chronic infection has also been found in non-human vertebrates, for example the reduction of telomere length in birds infected by avian malaria (Merino *et al.* 2000; Asghar *et al.* 2015), and badgers infected with *Mycobacterium bovis* (Beirne *et al.* 2014). Infection with *M. bovis* has also been associated with long-term fitness effects in buffalo (Pollock & Neill 2002; Jolles *et al.* 2005; Joly & Messier 2005). When we consider invertebrates, while there are no examples of long-term studies directly examining the effects of chronic infection on senescence, there are studies investigating the effect of immune activation on ageing insects. The chronic stimulation of the *D. melanogaster* immune response, for example, reduces lifespan and increases inflammation in the insect (Libert *et al.* 2006). Indeed, immune activation in the early-life of the model beetle *Tenebrio molitor* damages the vital Malpighian tubules, resulting in a greater impact of infection in later life (Khan *et al.* 2017). In invertebrates, it is therefore considered that immune activation and stimulation can have late-life effects. This study investigates this further by examining a long-term association with a fungal parasite, considering both long-term immune stimulation and several host fitness traits over the adult lifespan of an invertebrate.

The level of virulence caused by parasites may be influenced by host condition, at least in part because host immune responses are often resource limited (Norris & Evans 2000; Povey *et al.* 2009). Immune responses can be less effective and parasite burdens higher in hosts feeding on sub-optimal diets (Wallace *et al.* 1995; Strain & Stear 2001; Cunningham-Rundles *et al.* 2005; Cotter *et al.* 2011). In extreme cases, mounting immune responses in the absence of adequate nutrition can result in early host death (Moret & Schmid-Hempel 2000). Dietary protein plays an important role in maintaining elements of immune defence and protein consumption has been shown to increase the survival of infected individuals

(Lochmiller *et al.* 1993; Lee *et al.* 2006, 2008; Povey *et al.* 2014). Diet also has a direct impact on lifespan, with dietary restriction classically resulting in lifespan extension (Masoro 2000, 2002; Lee *et al.* 2015).

In this study, the ladybird *Harmonia axyridis* was used as a model organism to study the effects of a chronic fungal parasite. *Harmonia axyridis* was originally introduced as a biological control agent to North America and Europe and has since become notorious as an invasive alien species almost worldwide (Roy *et al.* 2016a). The spread of *H. axyridis* has resulted in the decline of multiple other species, principally due to its ability to out-compete native species and its abilities as an intraguild predator; although it is also thought *H. axyridis* also benefits from enemy release (Brown *et al.* 2015; Roy *et al.* 2016a).

The sexually transmitted fungal parasite *Hesperomyces virescens* has been found associated with *H. axyridis* in its native range (Haelewaters *et al.* 2014), and throughout much of its invaded range (Cottrell & Riddick 2012; Haelewaters *et al.* 2016b), where the field-prevalence can reach up to 82% (Harwood *et al.* 2006). Like other members of the order Laboulbeniales, *H. virescens* produces finger-like thalli on the host cuticle, which mature to contain ascospores that are discharged upon contact with a new host (Weir & Beaks 1996). Unlike other fungal pathogens, there are no penetrating mycelia beyond the haustorial foot attaching the thallus to the cuticle surface (Shanor 1955); however there is some evidence of rhizoidal haustorial appendages which penetrate the cuticle (Weir & Beakes 1996). It has generally been assumed *H. virescens* has no virulent effect on its host, however, some studies have suggested negative longevity effects associated with this group of pathogens, for example in earwigs (Strandberg & Tucker 1974). Kamburov *et al.* (1967) observed a lifespan reduction in infected *Chilocorus bipustulatus* ladybirds, attributing this to a virulence effect of *H. virescens*; however Applebaum *et al.* (1971) demonstrated these lifespan effects were, in fact, due to other factors.

This study aimed to test whether the chronic infection *H. virescens* influences senescence in the ladybird *H. axyridis*. Infection caused a substantial elevation in late-life

mortality. To test whether this may have occurred because of an alteration in the ageing rate of infected individuals, multiple life history traits were examined for senescence over the duration of adult lifespan. In addition, we tested whether these effects were resource mediated, by comparing the impact of this infection on the host under two different diet regimes.

3.3 Materials and Methods

Experimental material

Adult *H. axyridis* were collected in the first two weeks of September 2014 from the areas of Regents Park, St James's Park and Green Park in London, UK. Adults were collected using a beating tray under trees and by visually searching nettle and bramble patches. Ladybirds were brought back to the laboratory where they were sexed and checked for infection with *H. virescens.* Uninfected adult ladybirds were bred in the laboratory in pairs under a controlled temperature, humidity and light regime (20°C, 60% RH, 18:6 hr L:D). Once eggs hatched, ladybird larvae were kept individually in 9cm Petri dishes until they had pupated; resulting adults were pooled into dishes, divided by sex and family line. All ladybirds were reared on a combination of *ad lib Ephestia kuehniella* eggs (Entofood, Koppert Biological Systems) and artificial diet (Roy *et al.* 2013). Adult ladybirds were then artificially overwintered in the fridge for 3 months, during which time they were provided with artificial diet.

Seventeen *H. virescens* strains were sourced from infected *H. axyridis* adults collected from the field as described above. Fungal strains were maintained on *H. axyridis* ladybirds not involved in this study by mating an infected ladybird with an uninfected individual of the opposite sex. All 17 *H. virescens* strains were used to provide a source of infection in this study and were represented in each diet regime and on each ladybird sex.

Experimental protocol

To generate comparable infected and uninfected ladybirds, two virgin recipient ladybirds of the same sex and family line were randomly allowed to mate with either a *H. virescens* infected or uninfected *H. axyridis* ladybird donor of the opposite sex. After 24 hours, the two recipient ladybirds were removed from their mating partner, and randomly allocated into either a low or high nutrient diet treatment. The high nutrient diet consisted of regular artificial diet: including yeast, liver, sugar, maple syrup, multivitamins, agar and water (Roy *et al.* 2013); while the low nutrient diet included only sugar, water and agar. For the duration of the study, ladybirds in both diet treatments were also given *E. kuehniella* eggs *ad lib* and kept individually in a 9cm Petri dish. Fresh food and a clean dish were provided daily. Uninfected *H. axyridis* individuals, also bred from the same London field collections, were provided on a weekly basis for 24 hours to each ladybird in the study, allowing for sperm replenishment in females and for males to maintain sexual activity. Mating never occurred between siblings. Of the 136 ladybirds in this experiment, a total of 92 ladybirds were exposed to *H. virescens.* Individuals exposed to *H. virescens* that did not become infected (15.22%) were excluded from all further analyses.

All ladybirds were monitored daily for mortality. At the beginning of the study, the pronotum width of each ladybird was measured using a microscope eyepiece graticule. Each week ladybirds were weighed, using a balance reading to 1mg, and checked for the presence of mature *H. virescens* thalli, which were then counted if present. Observations continued until the death of each ladybird. Dishes containing female *H. axyridis* were monitored daily for eggs; eggs were counted and their hatch rate determined. The daily monitoring of eggs continued for the first 84 of the experiment.

Statistics

All statistical analyses were conducted using R version 3.3.2 (R Core Development Team 2016). For all models, with the exception of those for fecundity, P-values were

calculated using likelihood ratio tests to compare models with and without the term of interest, and tested for variance inflation using the 'CAR' package in R (Fox & Weisberg 2011). The optimal models for lifespan, body condition and fertility analyses were selected based on AIC, terms were removed if their inclusion did not improve AIC by 2 points. Due to multiple measures on the same individuals during their lifespan, temporal autocorrelation was tested for in the condition and fertility models using a continuous-time autoregressive (of order 1) correlation structure in a linear mixed effects model, with the optimizer 'optim', using R package 'nlme' (Pinheiro *et al.* 2017). In all models the base set of explanatory variables tested were (unless otherwise stated) ladybird sex, infection status at death, pronotum width, mature thalli burden and time since the start of the experiment (time). All two-way interactions between these variables were also included. Three way interactions between diet, mature thalli burden and time; diet, time and infection status at death; diet, mature thalli burden and sex; and diet, infection status at death and sex were also included in the full model. In all models, with the exception of those for lifespan, random effects were included for ladybird individual and family line. Models for ladybird condition and fertility contained a nested random effects structure, allowing a random intercept and slope for each individual within family line.

Lifespan

Ladybird lifespan variation was analysed using a parametric survival regression model with the R package 'Survival' (Therneau & Grambsch 2000). Explanatory variables were: infection status at death, ladybird sex, pronotum width and diet. A three-way interaction was also included between infection status at death, sex and diet treatment, as well as all possible two-way interactions. To investigate the trends driving the significance in the three-way interaction, separate models were included for each sex, using the same model structure as above (excluding the variable of sex).

Condition

Ladybird weight (in milligrams) was measured weekly and used to assess body condition by including ladybird pronotum width as a covariate in the models. The presence of temporal autocorrelation required this analysis to be conducted with a linear mixed effects model using the R package 'nlme' (Pinheiro *et al.* 2017). Models used the fixed and random effects structure described above.

Fecundity

Female fecundity was assessed daily. Many individuals did not oviposit every day, causing zero-inflation in the data. Therefore, we ran Zero-Altered Poisson (ZAP) models using the R package 'MCMCglmm' (Hadfield 2010) to separately model the impact of infection on the probability of oviposition and on the clutch size. Mean trends in clutch size and laying probability increased for the first 24 days of the experiment, before falling thereafter; to assess senescence we analysed fecundity trends from days 24 to 84. Non-informative priors were used and prior sensitivity analysis undertaken. Models were run for 1010000 iterations with a burn-in of 10000 and sampled every 250 iterations. Fixed effects and interactions were as detailed above, with the exclusion of sex, as only females were studied. One random effect used an unstructured covariance matrix, testing for variance between family lines in oviposition probability and variance in clutch size assessed by the binomial and Poisson parts of the model respectively, as well as the covariance between these terms. A second random effect accounted for repeated measures on each individual; using an unstructured matrix to assess between-individual variance in both early fecundity and the age-dependent decline in fecundity for both the binomial and Poisson parts of the model.

Fertility

The hatch rate of all egg clutches was assessed daily from 24 to 84 days, thereby ignoring a phase of increasing fecundity during the early part of the experiment. Temporal

autocorrelation was tested for as above, using an arc sine square root transformed response variable of the daily proportion of eggs hatched, but was found to be absent. Therefore, the analysis of ladybird fertility was carried out using a generalised linear mixed effects model, using R package 'lme4' (Bates *et al.* 2014) with a two vector binomial response variable of hatched and unhatched eggs, using the optimizer 'bobyqa'. The model was run for 100000 iterations. The explanatory terms included in the model include the base set of variables and their interactions, with the exception of ladybird sex, as only females were included in this analysis.

3.4 Results

Infection reduces lifespan in infected ladybirds

We tested the impact of *H. virescens* infection on the lifespan of male and female *H. axyridis* under two different diet regimes: 122 adult ladybirds were split approximately equally between treatment combinations (Figure 3.1). After a 3 month simulated overwintering period, subsequent lifespan ranged from 61 to 392 days (mean = 166.87 ± 6.53 SEM). Survival analysis demonstrated that there was a very strong negative impact of infection on lifespan ($X^2_{[1]} = 116.175$, $p = <0.001$; Figure 3.1). Median lifespan of uninfected beetles was 250 days, whereas this was reduced by 50.4% to 124 days for those infected with *H. virescens*.

The impact of infection on host lifespan was sex-specific, and this sexual dimorphism in virulence was further influenced by diet (sex by infection status at death by diet interaction: $X^2_{[1]} = 5.586$, $p = 0.018$; Figure 3.1). This effect was driven by several complex trends. The reduction in lifespan caused by infection was greatest for male ladybirds (sex by infection status at death interaction: $X^2_{[1]} = 5.110$, $p = 0.024$). When considering only the male ladybirds, there was a diet-specific impact of infection: the lifespan of male ladybirds given the high diet treatment was impacted more strongly by the fungal infection than those in the low diet treatment, although not significantly so (infection status at death by diet interaction:

$X^2_{[1]} = 3.418$, p = 0.064). However, for female ladybirds, while infection strongly reduced lifespan ($X^2_{[1]} = 39.404$, p = <0.001), the reduction did not differ between the two diet treatments (infection status at death by diet interaction: $X^2_{[1]} = 2.258$, p = 0.133), nor was there any difference in lifespan between the high and low diets ($X^2_{[1]} = 0.340$, p = 0.560) when averaging across both infection statuses.

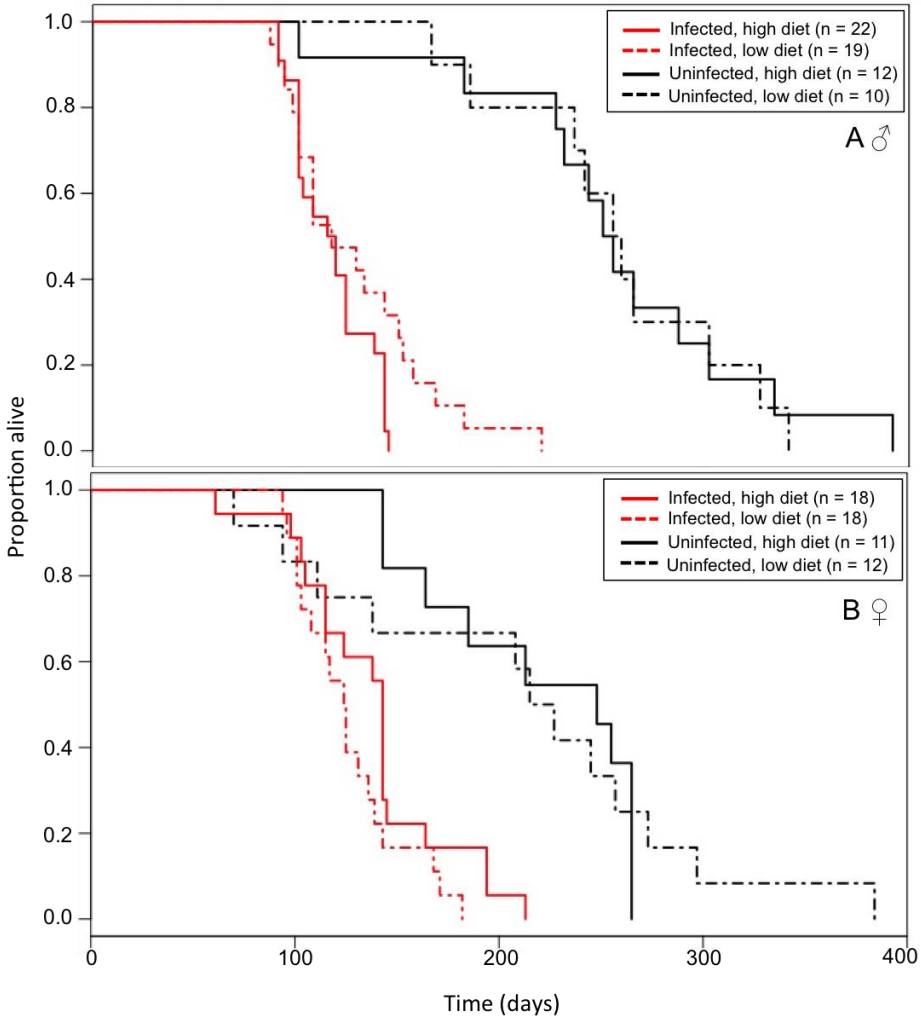

Figure 3.1: Lifespan was significantly reduced for *H. axyridis* ladybirds infected with *H. virescens*. Survival curves are for male (A) and female (B) ladybirds, and have been split by infection status (red line = infected, black line = uninfected) and diet treatment (solid line = high diet, dashed line = low diet). The legend displays the number of individuals per treatment. A greater impact of infection occurred for male ladybirds on the high diet treatment compared with those in the low diet treatment, this was, however, not seen for females.

Infection accelerates the lifetime loss of body condition

The impact of *H. virescens* infection on the rate at which ladybirds lost body weight as they aged was assessed using weekly weight measurements. The initial weight of male ladybirds (23.66mg ± 0.76 SE) was less than that of females (30.81mg ± 0.72 SEM) ($X^2_{[1]} =$

108.588, p = <0.001). Body condition senesced markedly in uninfected beetles ($X^2_{[1]}$ = 10.593, p = 0.001). Across the whole data set, this senescence was strongly accelerated in response to infection by the fungal parasite (time by infection status at death interaction: $X^2_{[1]}$ = 6.381, p = 0.012), however this effect was sex-specific (time by sex by infection status at death interaction: $X^2_{[1]}$ = 6.391, p = 0.012; Figure 3.2). While for male ladybirds, the rate of body condition senescence was faster in the presence of infection (time by infection status at death interaction: $X^2_{[1]}$ = 19.112, p = <0.001), when female ladybirds were analysed alone, no such effect occurred (time by infection status at death interaction: $X^2_{[1]}$ = 0.210, p = 0.647), despite an overall decline in uninfected female body condition over the duration of the experiment ($X^2_{[1]}$ = 5.383, p = 0.020; Figure 3.2).

The degree to which infection accelerated senescence of body condition in males was not altered by the diet treatment received (time by diet by infection status at death interaction: $X^2_{[1]}$ = 3.058, p = 0.080), although males senesced more slowly in the low diet treatment across both *H. virescens* infection categories (time by diet interaction: $X^2_{[1]}$ = 6.789, p = 0.009). For females, however, the rate of age-related decline in body condition did not vary between the two diet treatments (time by diet interaction: $X^2_{[1]}$ = 1.557, p = 0.212).

The negative effect of infection on body weight senescence in males occurred despite the increase in weight resulting from increasing mature thalli burdens as individuals aged, which was controlled for in the analysis ($X^2_{[1]}$ = 40.127, p = <0.001). The increasing burden of mature thalli on ladybirds influenced the weight of ladybird hosts differently between diet treatments, and further, the direction of the trend was opposite in males and females (diet by sex by thalli burden interaction: $X^2_{[1]}$ = 6.920, p = 0.009). While for males, the increase in body weight resulting from higher thalli burdens was steeper on the high diet treatment (burden by diet treatment: $X^2_{[1]}$ = 4.908, p = 0.027), for females, the opposite occurred: females on the low diet had a steeper increase in weight resulting from higher burdens compared with those on the high diet (burden by diet treatment: $X^2_{[1]}$ = 9.445, p = 0.002).

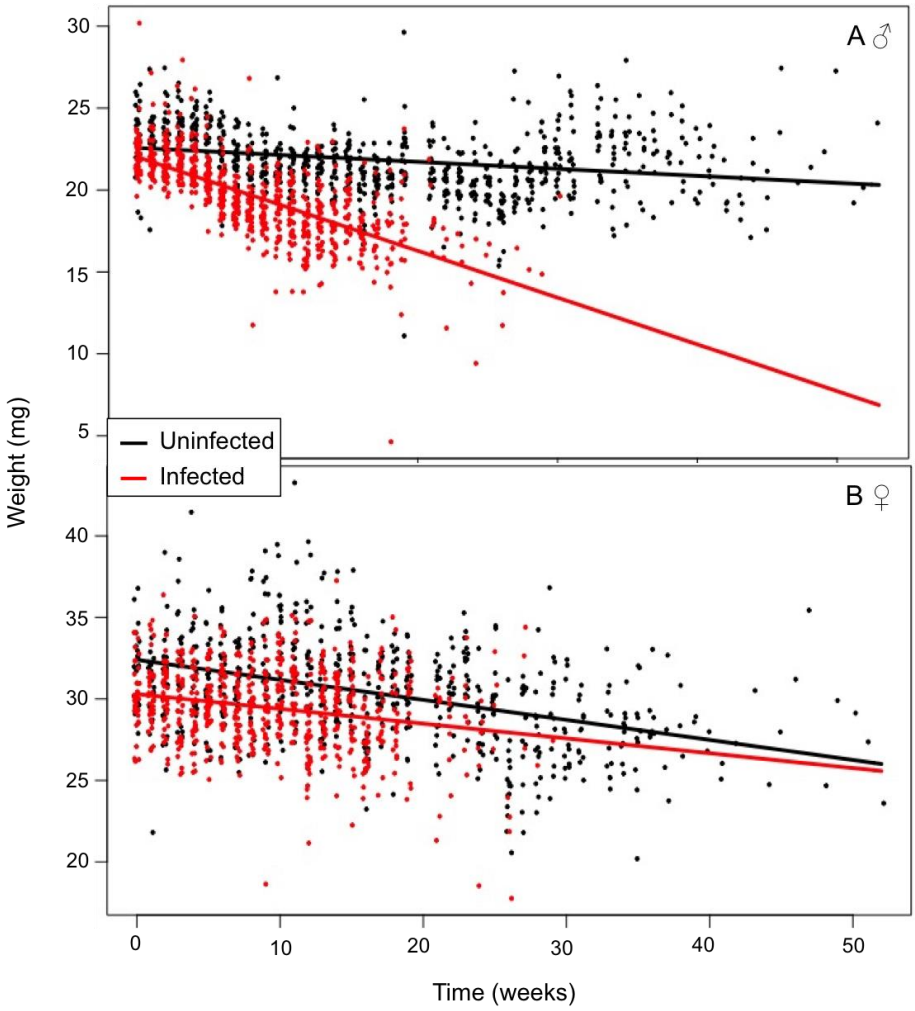

Figure 3.2: The body condition of all ladybirds senesced over time. A sex-specific effect of infection was present in the decline of ladybird body condition. Males (A) displayed accelerated senescence in response to infection with *H. virescens*. Females (B) showed no change in the rate of body condition senescence in response to fungal infection. *Hesperomyces virescens* infected beetle observations in red and uninfected observations in black. The data points on this plot are not all independent as each ladybird individual was weighed multiple times across the duration of the study.

All analyses included a variable controlling for ladybird size (pronotum width), which allowed the comparison of body condition in individuals of different overall size. Initial body weight was strongly positively associated with ladybird size pooling data across both sexes ($X^2_{[1]} = 48.034$, $p = <0.001$). However, the rate of body weight senescence was only influenced by body size in females (size by time interaction: $X^2_{[1]} = 6.877$, $p = 0.009$): smaller females senesced more quickly than larger female ladybirds, a trend not seen in males (size by time interaction: $X^2_{[1]} = 0.040$, $p = 0.842$). After fitting the fixed effects, variation in body condition associated with the random effect of family line accounted for 9.34% of the residual variance.

Infection causes fecundity senescence

We studied the fecundity of adult female *H. axyridis* in response to infection with *H. virescens*. Adult beetles were monitored daily for 84 days and the number of eggs produced were counted. To investigate the senescence in female fecundity, the data included in this analysis start at day 24, the point at which fecundity began to decline. At 55 days after the start of egg observations (the midpoint of the data included in this analysis) the mean clutch size for females, excluding 0s, was 19.05 (± 1.62 SE), while that of infected females was 16.39 (± 2.06 SE). A zero-altered Poisson model separately modelled the impact of factors on the probability of oviposition and on clutch size. None of the variables considered in this study influenced the oviposition probability of females; however, the age-dependent change in the size of egg clutches was strongly influenced by the mature thalli burden of *H. virescens* on female ladybirds (Figure 3.3, **Error! Reference source not found.**). While there was no significant evidence of clutch size senescence in uninfected ladybirds, or those with low thalli burdens (pMCMC = 0.388), larger infection burdens caused an age-dependent decline in the size of egg clutches, which became progressively steeper with increasing thalli burdens (**Error! Reference source not found.**; Figure 3.3). Beyond this impact of increasing thalli burden, there was no significant difference between the uninfected and infected individuals in age-dependent fecundity change, either when considering oviposition probability (day by infection status at death interaction: pMCMC = 0.636) or clutch size (day by infection status at death interaction: pMCMC = 0.621).

Female fecundity was not influenced by diet treatment in either the binomial (pMCMC = 0.352) or Poisson sections of the model (pMCMC = 0.555), nor did diet influence age-dependent changes in oviposition probability (day by diet interaction: pMCMC = 0.858) or clutch size (time by diet interaction: pMCMC = 0.872).

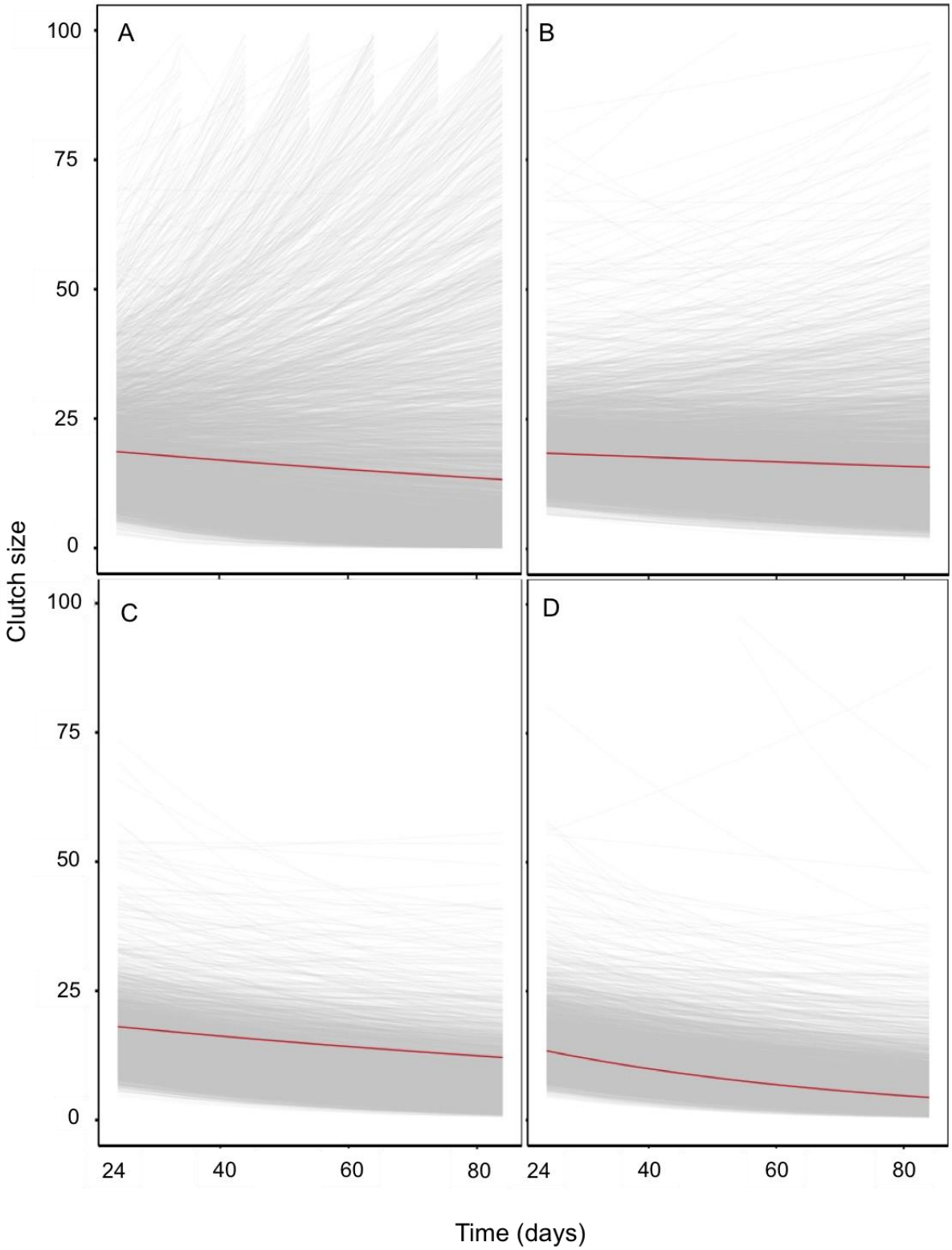

Figure 3.3: Egg clutch size senesced in females with higher mature *H. virescens* thalli burdens. This figure was plotted from a MCMCglmm model containing a categorical burden variable. A = 0 thalli, B = 1 to 50 thalli, C = 51 to 100 thalli, D = 101 to 455 thalli. Each grey line on the plots shows a single estimate of the age-dependent slope of fecundity change from the models posterior distribution for each level of the categorical burden variable; red lines depict mean estimates. Due to the scale on the y-axis, some grey lines (model estimates) have been truncated.

Table 3.1: Egg clutch size senesced in females with high infection burdens of *H. virescens* thalli. Diet treatment had no effect on female fecundity, or the age-dependent change in oviposition probability of clutch size. The parameter estimate table displays the estimates from the best-fit MCMCglmm ZAP model. The binomial section of the table shows estimates for the zero-altered binomial part of the model that assesses oviposition probability, while the Poisson section describes the effects on clutch size.

Poisson

	Mean	***Lower 95% CI***	***Upper 95% CI***	***Effective Sample***	***pMCMC***
(intercept)	2.796	2.343	3.235	3786.8	**<0.001**
Time (days)	-0.018	-0.036	0.019	315.1	0.388
Thalli burden	0.0034	-0.0005	0.00743	4000.00	0.096
Time (Days):thalli burden	-0.00007	-0.0001	-0.00002	4000.00	**0.006**

Binomial

	Mean	***Lower 95% CI***	***Upper 95% CI***	***Effective Sample***	***pMCMC***
(intercept)	-3.729	-4.454	-2.997	4000.00	**<0.001**
Time (days)	0.0023	-0.0371	0.0424	825.9	0.893
Thalli burden	-0.0005	-0.01552	0.00482	4000.00	0.314
Time (Days):thalli burden	0.00008	0.000036	0.00019	4000.00	0.161

Infection accelerates fertility senescence

The daily hatch rate of egg clutches produced by females was monitored for 84 days, however, to study the senescence of this trait, data were analysed from day 24, the point at which fecundity began to senesce. Fertility variation between family lines accounted for 14.00% of the total random effect variance in the analysis. The fertility of uninfected females declined over the duration of the experiment, although not significantly so ($X^2_{[1]} = 3.410$, p = 0.065). This pattern of senescence in female fertility was affected by the burden of thalli on hosts (time by burden interaction: $X^2_{[1]} = 4.418$, p = 0.036): the age-dependent decline in egg hatch rate occurred more strongly in ladybirds as infection burdens increased (Figure 3.4).

Female fertility was also influenced by the diet treatment received ($X^2_{[1]} = 29.279$, p = <0.001), with females on the low diet treatment having a lower hatch rate than females receiving the high quality diet treatment. Pooling across infection categories, the mean initial hatch rate for ladybirds on the high nutrient diet was 0.91 (95% CI 0.82 to 0.95), while on the low diet treatment it was 0.54 (95% CI 0.38 to 0.69). There was, however, no difference in

the overall rate of fertility senescence between the two diets (time by diet interaction: $X^2_{[1]}$= 1.863, p = 0.172).

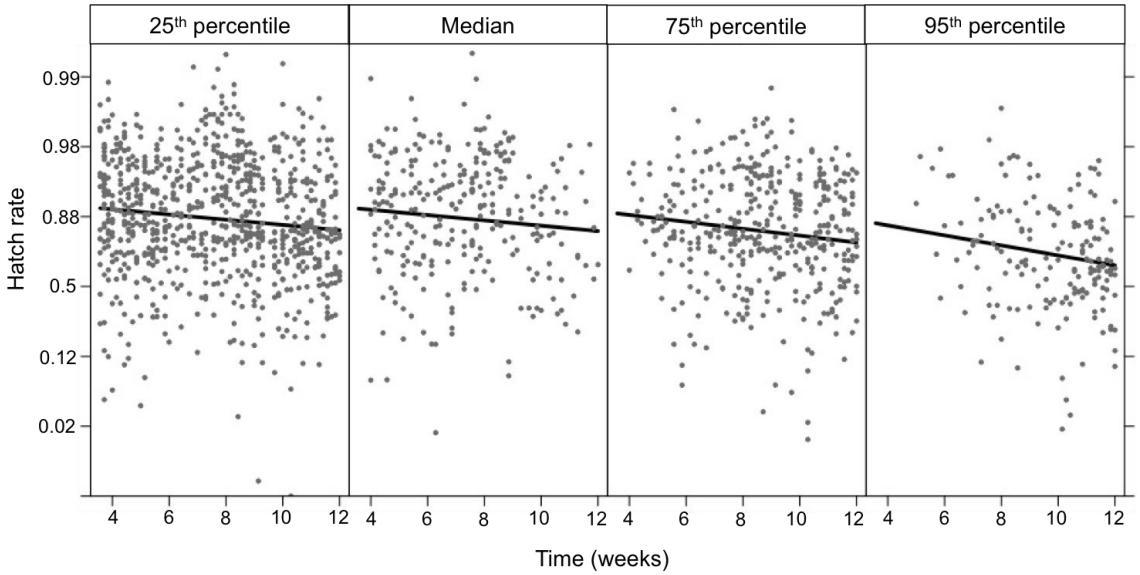

Figure 3.4: The hatch rate of eggs senesced for all females. The rate of age-dependent senescence was accelerated in response to high mature thalli burdens of *H. virescens* on hosts. Thalli burdens in the 95th percentile resulted in the strongest decline in hatch rate over the experiment. The panels in the figure show the age-dependent hatch rate decline for thalli burdens in the 25th, median, 75th and 95th percentiles. Y-axis is back transformed from a logit scale, data plotted in the un-transformed space.

Ladybird size differences were associated with variation in ladybird fertility. Female fertility declined more rapidly with age in smaller ladybirds (size by time interaction: $X^2_{[1]}$= 8.599, p = 0.003). The fertility of larger ladybirds was more strongly affected by high thalli burdens than that of smaller females (size by burden interaction: $X^2_{[1]}$= 6.919, p = 0.009).

3.5 Discussion

Parasite infection commonly results in early host death, however the role infection plays in senescence itself is debated (Fries 1980; Franceschi *et al.* 2000; Gavazzi & Krause 2002; Finch & Crimmins 2004; Kirkwood 2005; Pawelec *et al.* 2014; Hayward *et al.* 2016). The impact infectious disease has on ageing has been proposed to arise from chronic inflammation, either resulting from acute infection suffered in childhood, or long-term adult infections (Finch & Crimmins 2004; Hayward *et al.* 2016). This study tests the latter hypothesis, experimentally investigating a chronic fungal infection in a model insect species. Our results provide clear evidence that, at least in this insect host species, the presence of a

chronic infection accelerates the senescence of multiple host life-history traits; suggesting a potential general role of infection in driving the ageing process.

Although in this study the presence of infection reduced host lifespan by 50%, almost no mortality occurred until 100 days post infection. The delayed nature of virulence in this chronic infection suggests that in insect systems the virulence associated with chronic infections may be underestimated. The accelerated senescence and early late-life mortality associated with the sexually transmitted fungus observed in this study displays parallels with some chronic STIs of humans, such as Hepatitis C Virus and HIV (Paradis *et al.* 2001; Appay & Rowland-Jones 2002; Papagno *et al.* 2004; Desai & Landay 2010). As we have shown for *H. virescens*, STIs often cause sterility in their hosts, both in human (Thompson & Washington 1983; Ochsendorf 2008; Apari *et al.* 2014) and animal infections (Lockhart *et al.* 1996; Knell & Webberley 2004). In mammals, STIs tend to more commonly affect fertility rather than mortality (Lockhart *et al.* 1996), while invertebrate STIs can result in increased mortality for hosts (Abbot & Dill 2001; Knell & Webberley 2004; Ryder *et al.* 2007). However, invertebrate STIs commonly display rather more acute effects than those observed here, for example the complete sterility caused by the mite *Coccipolipus hippodamiae* on *A. bipunctata* in as little as three weeks (Webberley *et al.* 2004). The infection-accelerated fertility senescence in the females studied here could result from an inability of old infected females to make fertile eggs, alternatively it could be due to mating reluctance in infected females or the males they were paired to. However, the lack of complete sterility indicates females did not run out of sperm, and that another direct or indirect mechanism is involved in the fertility decline.

While this study did not specifically aim to investigate sex-specific effects of infection, male *H. axyridis* were more negatively affected by *H. virescens* infection than female beetles. While no effect of *H. virescens* infection was seen on the age-related decline in body condition in females, for male *H. axyridis,* the presence of the fungus resulted in an acceleration of this body condition decline. In addition, the lifespan of both male and female beetles was considerably shorter in the presence of *H. virescens* infection, however the

reduction was greater in males. Males of multiple taxa have been shown to have a reduced immune ability in comparison to females (Zuk 2009; Stephenson *et al.* 2016). Indeed, the trade-off between reproduction and immune defence within individuals has been demonstrated in some invertebrates, for example the increase in immune defences at the point of sexual maturation in females, but its decrease in males (Barthel *et al,* 2015; Adamo *et al.* 2001, Ahtiainen *et al.* 2005). In this study, we are able to compare the response to *H. virescens* between the sexes only for lifespan and body condition, as the remaining traits that were examined were done so only in females. It would be interesting for future studies to consider other traits that could be compared in both sexes to further examine these sex-specific effects of infection.

Our study cannot conclusively discriminate whether the infection-mediated reduction in the lifespan of adult beetles is caused by a general acceleration in the senescence of the host's physiological systems, or whether it results from a delayed but direct effect of parasite virulence. The thalli of *H. virescens* develop on the host's cuticle, with minimal growth within the body cavity (Weir & Beakes 1996), which suggests that infection induced mortality is less likely to occur as a direct result of parasite virulence, as the host is not physiologically overwhelmed by fungal growth. The observed acceleration of senescence in unrelated traits resulting from infection (male body condition and female reproductive traits) across this study could also be indicative of a more general effect in multiple physiological systems of the host, ultimately resulting in a reduction of host lifespan.

Whether the cause of early death is a direct effect of infection, or an indirect effect due to early senescence, these reductions in host fitness could be due to one of two mechanisms. The parasite may cause pathology by directly damaging the host, for example the release of toxins into host cells (Cornelis & van Gijsegem 2000) or the drawing of resources directly from the host. Alternatively, infection by the parasite may invoke fitness costs indirectly, due to the costs of mounting immune defences (Brown *et al.* 2000; Hurd 2001; Sabri *et al.* 2011). Chronic stimulation of the immune response can be costly (Borkow *et al.* 2000; Hayes *et al.* 2004; van Baarle *et al.* 2005; Johansen *et al.* 2006; Desai & Landay

2010). Humans infected with HIV often suffer earlier from age-related disease, resulting from long-term immune stimulation (Appay & Rowland-Jones 2002; Desai & Landay 2010; Deeks 2013), while in invertebrates, chronic activation of the immune response in *Drosophila melanogaster* results in inflammation and premature death (Libert *et al.* 2006). In addition, early life immune activation in *T. molitor,* causes damage of Malpighian tubules, leading to a greater risk of infection in later life (Khan *et al.* 2017). While we are unable to specifically determine whether the virulence effects of *H. virescens* are the result of infection pathology or chronic immune stimulation, it has previously been demonstrated that the fitness effects induced by an acute parasite in *D. melanogaster* resulted from the immune activation rather than directly by infection pathology (Bashir-Tanoli & Tinsley 2014). Indeed, although the host immune response to *H. virescens* has not been characterised, immune genes in ants infected with a Laboulbenialian parasite were upregulated in response to infection (Konrad *et al.* 2015).. In addition, the chronic stimulation of the *D. melanogaster* immune response had a similar effect on lifespan as we see in this study, resulting from the creation of a chronic inflammatory condition (Libert *et al.* 2006). Together, these previous studies perhaps indicate that the virulence effects seen in our study are likely to be driven by immune stimulation, rather than directly by the pathology of *H. virescens.* In addition, while *H. virescens* probably draws directly on host resources to some extent, in this study, the senescence in female body condition did not change with *H. virescens* infection. Furthermore, the age-related decline of body condition was not accentuated by the low diet treatment in either host sex. In the event *H. virescens* pathology resulted from the direct removal of resources, virulent effects would have been expected to be amplified for all individuals receiving the low quality diet that lacked the protein provided by the liver found in the high quality diet, however this was not the case.

Dietary protein is important for both immune defences and when considering senescence (Cotter *et al.* 2011; Piper *et al.* 2011). It is, however, worth considering that all ladybirds in this study, regardless of diet treatment, received *E. kuehniella* eggs *ad lib* in addition to artificial diet to maintain egg production in females, making it possible our low

quality diet may not have substantially limited resources enough to cause an effect. However, while the diet manipulation did not influence the magnitude of *H. virescens* virulence, female fertility appeared to be resource limited, and was dramatically reduced for individuals receiving the low quality diet. If resources were diverted away from host reproduction, this could be driven by the parasite to facilitate parasite growth, or alternatively it might be a host strategy to reallocate resources to immune defence (Hurd 2001; although see Bashir-Tanoli & Tinsley 2014). However, for male hosts, while a small effect of diet was seen on male lifespan, a greater impact of infection occurred for males on the high diet treatment, which is the reverse of what might be expected. It could be argued that thalli growth on the head and mouthparts of infected hosts could directly interfere with resource acquisition, creating the virulent effect we observed in our infected ladybirds; however, the lack of acceleration in female body condition senescence implies this cannot be the case. It would, however, be interesting for future studies to investigate this in more detail.

The ecological and evolutionary relevance of ageing investigations on animals is frequently questioned because researchers commonly study individuals at ages rarely seen outside the laboratory. However, ladybirds such as *H. axyridis* commonly live for around one year, including a period of winter diapause. This timescale is similar to that in our study, where ladybirds initially diapaused for 3 months, and the subsequent median experimental lifespan was either approximately 9 or 4.5 months, depending on *H. virescens* infection. Furthermore, because, like many temperate invertebrates, ladybirds usually overwinter before reproducing, the late-life senescence effects driven by infection that we observed will have strong relevance to the fitness of ladybirds in the field.

The virulence *H. axyridis* experiences from *H. virescens* infection could have significant impacts on individuals in infected wild populations. The field-prevalence of *H. virescens* can reach over 80% in some populations (Harwood *et al.* 2006), and we have shown a prevalence in a UK *H. axyridis* population of over 70% (chapter 2). While *H. virescens* does not transmit or develop as well on *H. axyridis* compared to *A. bipunctata*, a UK ancestral host of *H. virescens* (chapter 2), it is clear from this study that this chronic fungal STI has

substantial virulence on the invader. The timing of the virulent effects in the wild could be crucial in determining the impact *H. virescens* could have on *H. axyridis* populations. In this experiment, we artificially diapaused all individuals before exposing them to infection, however, in the field, individuals may become infected after they eclose towards the end of the reproductive season, resulting in a heavy thalli burden going into an overwintering period. Indeed, Nalepa & Weir (2007) reported a higher infection prevalence in populations towards the end of the overwintering period, which could result in increased mortality for individuals emerging in the spring. While this fungal infection does achieve some non-sexual transmission, resulting from host contact during overwintering aggregations (Nalepa & Weir 2007), as with any insect STI, sexual transmission requires mating between the adult and offspring generations (Knell & Webberley 2004). The early mortality of infected individuals that we demonstrate here may reduce the opportunity for contact between overwintered adults and the new season cohort, potentially limiting infection prevalence. Ladybirds in this study were kept in stable laboratory conditions and did not suffer from the presence of competition, repeated reinfection or other parasites, therefore field populations affected by multiple stressors could suffer elevated virulence compared with the results seen here.

An association between *H. virescens* and multiple other ladybird species has been reported worldwide (Riddick & Schaefer 2005; Haelewaters *et al.* 2016b): across Europe and the USA this infection has spread in many populations of *H. axyridis* (Haelewaters *et al.* 2016b). It is likely these infections stem from a host shift, which has potentially occurred when beetles are in close proximity to each other in overwintering aggregations (Nalepa & Weir 2007). While we have studied the virulence effects of *H. virescens* on an invasive alien species, it would be interesting to further investigate these effects on other ladybird host species, especially in pre- and post- invasion populations.

The role infectious disease plays in altering host senescence is debated. Lifelong inflammation following an acute childhood infection and chronic stimulation of the immune response later in life have both been proposed to shorten lifespan by accelerating ageing (Finch & Crimmins 2004; Pawelec *et al.* 2005). Our results provide compelling evidence for

the role of long-term chronic infections in driving the ageing process. The extent to which these findings can be generalised to other infections and host species deserves further study. Nevertheless, our finding may shed light on the processes underlying the substantial lifespan extension that has occurred in human populations over the last century associated with declining burdens of infectious disease.

Chapter 4:

Sex-specific effects of co-infection in ladybirds

4.1 Abstract

Single host-parasite associations are not generally found in nature; instead, the presence of multiple parasite species within a single host is far more common. Co-infection with multiple pathogens has the potential to alter the virulence of the infectious agents, exacerbating or reducing the negative effects caused by each infection. This study investigated the ability of two ladybird species to defend against the lethal entomopathogen *Beauveria bassiana* while already infected with a relatively avirulent chronic fungal parasite, *Hesperomyces virescens*. Our results demonstrate a sex-specific effect of co-infection in both *Adalia bipunctata* and *Harmonia axyridis*. While the virulence of *B. bassiana* was unaltered by the presence of co-infection in females, *B. bassiana* virulence in male hosts was magnified in the presence of the second parasite. Furthermore, our analysis demonstrates that this male-specific effect of co-infection was principally driven by the presence of *H. virescens* infection, rather than the higher *H. virescens* infection burden that was present on males of both species. It has previously been assumed that *H. virescens* is avirulent to its hosts, however this study demonstrates that this chronic parasite may be an important determinant of host fitness in the field for hosts co-infected by other pathogens.

4.2 Introduction

In wild populations simultaneous infection of hosts with more than one parasite or parasite is commonplace. Co-infection can exacerbate the virulence of individual infections, increasing the likelihood and severity of symptoms, both in human (Zarski *et al.* 1998; Graham *et al.* 2001; Effros *et al.* 2008) and animal host-parasite systems (Opriessnig *et al.* 2004; Van Loock *et al.* 2006). Alternatively, interactions between co-infecting infectious agents and the immune responses they generate can reduce infection virulence, or even protect a host from the effects of other pathogens (Bilenko *et al.* 2004; Konrad *et al.* 2015); knowledge of this meant that humans have historically attempted to use co-infection to their advantage (Snounou & Pérignon 2013; Faure 2014)

Co-infecting pathogens may interact for many reasons. The presence of multiple pathogens within a single host can result in direct or indirect competition between them for host resources (Smith & Holt 1996; Graham 2008; Mideo 2009; Fenton & Perkins 2010). The specific nature of many immune responses can result in inadequate responses of a host against secondary pathogens (Bradley & Jackson 2008; Sears *et al.* 2011); non-specific downregulation of host immunity by one parasite may benefit another by leaving a host unable to challenge the establishment of a new parasite (Pritchard *et al.* 1984; Behnke *et al.* 1992; Hayes *et al.* 2004). In mammalian systems, the trade-off between the activation of T-helper type 1 and 2 cells can reduce the effectiveness of host defences against secondary pathogens, for example the role of hookworms in increasing susceptibility to malaria (Ezenwa *et al.* 2010; Nacher 2011; Potian *et al.* 2011). However, the effect helminths have in the occurrence of malaria is species-dependent, with some helminths appearing to protect hosts against this disease (Hartgers & Yazdanbakhsh 2006; van Riet *et al.* 2007; Nacher 2011). The presence of microbes that ameliorate parasite virulence may be common place in invertebrates, where heritable vertically transmitted bacterial endosymbionts can protect hosts from other invading parasites (Mateos *et al.* 2006; Hamilton & Perlman 2013).

The additional upregulation of host immune defences resulting from simultaneous infection by multiple pathogens can have negative effects on host fitness. In humans, co-

infection by HIV and hepatitis C virus chronically activates the T-cells of the immune response, resulting in increased incidence of liver disease (Gonzalez *et al.* 2009). In addition, chronic stimulation of the immune response by a parasite can make hosts more susceptible to other infections (Borkow *et al.* 2000; Hayes *et al.* 2004). Understanding the impact of co-infection is therefore vital when considering parasite dynamics in host populations, especially where parasite or host control is required (Fenton 2008; Nacher 2011).

The virulence associated with laboulbenialian fungi on their arthropod hosts is often thought to be negligible (Whisler 1968; Weir & Beakes 1995, although see Kamburov *et al.* 1967; Strandberg & Tucker 1974). However, we have demonstrated that *Hesperomyces virescens* accelerates the rate of ageing in the invasive alien ladybird *Harmonia axyridis* (chapter 3). Other studies demonstrate that the fitness consequences of laboulbenialian infections are likely to be complex: in ants, infection elicits an immune response in the host and can protect the host against secondary infection by a lethal entomopathogen (Konrad *et al.* 2015). In this study we investigate the impact of host co-infection by a chronic laboulbenialian parasite and *Beauveria bassiana*, an acutely virulent entomopathogen that infects multiple coccinellid species, including *H. axyridis* and *Adalia bipiunctata* (Cottrell & Shapiro-Ilan 2003; Roy *et al.* 2008).

Although both *H. virescens* and *B. bassiana* have virulent effects on *H. axyridis,* this ladybird species shows a high level of resistance against these fungal pathogens when singly infected (Roy *et al.* 2008; chapter 2 & 3). The immune capabilities of *H. axyridis* are often cited as superior to those of other ladybird species (Gross *et al.* 2010; Schmidtberg *et al.* 2013; Vilcinskas *et al.* 2013a). A large number of antimicrobial peptides and proteins have been identified within *H. axyridis,* many of which have been demonstrated to act strongly against entomopathogenic fungi (Vilcinskas *et al.* 2013a). It is possible these aid the resistance of *H. axyridis* to individual infections by both *B. bassiana* and *H. virescens*. To date, however, a clear understanding of how *H. axyridis* is impacted by co-infection has not been achieved (Haelewaters *et al.* 2016b).

This study aims to investigate whether infection with a chronic, relatively avirulent parasite influences a hosts' ability to defend against secondary infection by an acute, highly virulent fungal parasite. This was tested in two ladybird species, *A. bipunctata* and *H. axyridis,* for two reasons. Firstly, *H. axyridis* has been demonstrated to be more resistant to the acute entomopathogen, *B. bassiana,* than other ladybird species (Roy *et al.* 2008), and therefore it might be predicted to suffer less from any negative effects of co-infection. Secondly, the chronic parasite *H. virescens* is better able to exploit *A. bipunctata* than *H. axyridis* as a host (chapter 2); therefore the effects of co-infection may be more apparent in *A. bipunctata*.

4.3 Methods

Experimental material

The ability of ladybirds infected with *H. virescens* to defend against an acute fungal infection was tested using two host species, *H. axyridis* and *A. bipunctata*. *Hesperomyces virescens* infected and uninfected individuals were exposed to the generalist fungal parasite, *B. bassiana,* and then monitored daily for survival. The ladybirds used in this experiment were collected in the summer of 2016, and experienced the experimental procedure described in chapter 2 which investigated the exploitation of *H. axyridis* and *A. bipunctata* by *H. virescens*. Briefly, *H. virescens*-infected *H. axyridis* and *A. bipunctata* were collected from sites in London and Stockholm respectively (for detailed locations, see table 1 in chapter 2). These field-infected donors were used to sexually transmit *H. virescens* to uninfected conspecific recipient ladybirds across four experimental blocks. In addition, within each block, artificial interspecific and intraspecific transmissions (by manual contact) were also conducted with each donor species. Control (unexposed) individuals were also set up in the last two experimental blocks (blocks C and D of chapter 2) and kept in the same conditions as the exposed ladybirds. Not all *H. virescens* exposures resulted in a successful infection establishing by six weeks post-exposure; ladybird individuals in the present study therefore fell into three *H. virescens* infection classes: successfully infected, exposed but not infected,

or true control individuals. In the current study, all these ladybirds were exposed to either *B. bassiana* or a control treatment on the same day; some *H. virescens* infections had developed further than others by this time because the experimental blocks in chapter 2 were staggered over 23 days.

Throughout these experiments, ladybirds were kept individually in 9cm Petri dishes in a controlled temperature facility (20°C, 18:6 hr L:D cycle, and 60% relative humidity). Ladybirds were fed artificial diet (Roy *et al.* 2013) and 'Entofood' (*Ephestia kuehniella* and *Artemia* spp. eggs) (Koppert Biological Systems) three times per week; Petri dishes were changed once per week.

Experimental protocol

For both ladybird species, individuals of the three *H. virescens* infection categories (*H. virescens* infected, failed *H. virescens* infection and controls) were divided into two treatments: exposure to *B. bassiana* or control (0.03% solution of Tween 20 in distilled water) (Table 4.1). *Beauveria bassiana* spores (Botanigard 22WP-1, strain GHA) were suspended in 0.03% Tween 20 solution at a concentration of 7.73×10^8 spores ml^{-1} for *H. axyridis* and 7.73×10^7 spores ml^{-1} for *A. bipunctata* individuals; these concentrations were the approximate LD50 for each species (which had been determined during previous trials). Each ladybird was placed in an Eppendorph tube containing 1ml of *B. bassiana* spores suspended in Tween 20, or 1ml Tween 20 alone, which was inverted 5 times; then ladybirds were placed onto a damp piece of filter paper in a 9cm Petri dish for 12 hours. Following this, the ladybird was transferred into a clean Petri dish and fed normally. Individuals were monitored on a daily basis for survival up to 16 days post exposure. Upon death, each ladybird was placed into a clean Petri dish with damp filter paper, allowing the growth of *B. bassiana* to confirm the cause of death. Control individuals that died were also treated in this manner to verify no *B. bassiana* infection was present. All *B. bassiana*-exposed ladybirds that died over the course of the experiment presented the phenotype consistent with this infection, with white spores evident between the elytra and between the plates of the cuticle.

Table 4.1: The number of individuals of each host species included in each treatment (*B. bassiana* or Tween) and infection category (*H. virescens* infected, failed or uninfected).

	Beauveria bassiana		**Tween 20 (control)**	
	H. axyridis	***A. bipunctata***	***H. axyridis***	***A. bipunctata***
***H. virescens* infected**	32	39	21	20
***H. virescens* failed**	118	81	54	39
Uninfected	26	25	10	10

Statistical analysis

All analyses were conducted using R, version 3.3.2 (R Core Development Team 2016). The experiment generated 'time to event' survival data, requiring the censoring of data for those individuals that survived beyond the end point of the experiment at day 17. The R package 'survival' (Therneau & Grambsch 2000) was used for all analyses; parametric survival models with Weibull errors were implemented. A comparison of two models, one containing 3 levels of *H. virescens* infection category (infected, failed infection and control), and the other 2 levels (infected and uninfected) found the latter to have the lowest AIC (AIC: 1331.849 and 1326.928 respectively). Therefore, the two uninfected *H. virescens* treatment categories were pooled.

The base set of covariates and factors included in models (unless otherwise specified) was a 2-level term for *H. virescens* infection category, *B. bassiana* infection status, ladybird species, ladybird sex, and experimental block (see chapter 2). In addition, to investigate whether co-infection generally influenced the mortality caused by the two fungal pathogens, a two-way interaction between *H. virescens* infection status and *B. bassiana* infection status was included in the model. To specifically test whether these effects of co-infection differed between the sexes and between the two ladybird species, we fitted three-way interactions between ladybird sex, *B. bassiana* treatment and *H. virescens* infection status, as well as ladybird species, *B. bassiana* treatment and *H. virescens* infection status.

The low number of deaths in the Tween control treatment (Figure 4.1) caused models containing data for both *B. bassiana* infection categories to produce large standard errors on parameter estimates. Therefore, the impact of *H. virescens* infection was considered for *B. bassiana* and Tween control individuals separately in models containing the base set of terms

outlined above (with the exceptions that the *B. bassiana* treatment factor was removed and an interaction between ladybird sex, species and *H. virescens* infection status was added). In addition, to test whether the effect of co-infection occurred across all blocks, an interaction between block and *H. virescens* infection category was also included in *B. bassiana* infection models.

To investigate whether the magnitude of co-infection effects was influenced by *H. virescens* burden, we considered only co-infected individuals in models containing the same base set of terms detailed above, with the exception of *H. virescens* infection status and *B. bassiana* treatment. For all models containing *H. virescens* burden, the variable of total thalli was mean-centred at the point of each division of the dataset.

Model simplification was conduced by comparing the AIC of models, removing terms that did not improve the model by two points. P-values were generated by comparing models with and without the term of interest using a likelihood ratio test.

4.4 Results

Co-infection reduces male survival

Hesperomyces virescens infected and uninfected ladybirds (261 *H. axyridis* and 214 *A. bipunctata*) were exposed to either *B. bassiana* spores or a control of Tween, and monitored for survival for 16 days. *Beauveria bassiana* was highly virulent: of the 154 control individuals, 95.5% (n = 154) survived to the end of the experimental period, whereas only 46% (n = 321) of individuals in the *B. bassiana* treatment survived ($X^2_{[1]} = 126.834$, p = <0.001). The low number of Tween control individuals that died over the duration of the experiment meant two separate models were used to investigate the impact of *H. virescens* infection on *B. bassiana* infected and Tween control ladybirds. Considering only individuals infected with *B. bassiana,* a sex-specific virulent effect occurred when ladybirds were additionally infected with *H. virescens* (ladybird sex by *H. virescens* infection interaction: $X^2_{[1]} = 10.720$, p = 0.001; Figure 4.1). The proportion of male ladybirds surviving to day 16

post-exposure fell from 0.55 (± 0.05 SEM) when singly infected with *B. bassiana* to 0.21 (± 0.02 SEM) in the presence of *H. virescens* ($X^2_{[1]} = 20.152$, p = <0.001). This effect of co-infection was not present in females, for which the survival probability of ladybirds infected solely with *B. bassiana* was 0.58 (± 0.04 SEM), compared with 0.61 (± 0.07 SEM) in the presence of both fungal infections ($X^2_{[1]} = 0.020$, p = 0.889). This sex-specific co-infection effect was no different in the two ladybird species (ladybird sex by *H. virescens* infection by ladybird species interaction: $X^2_{[1]} = 0.037$, p = 0.847).

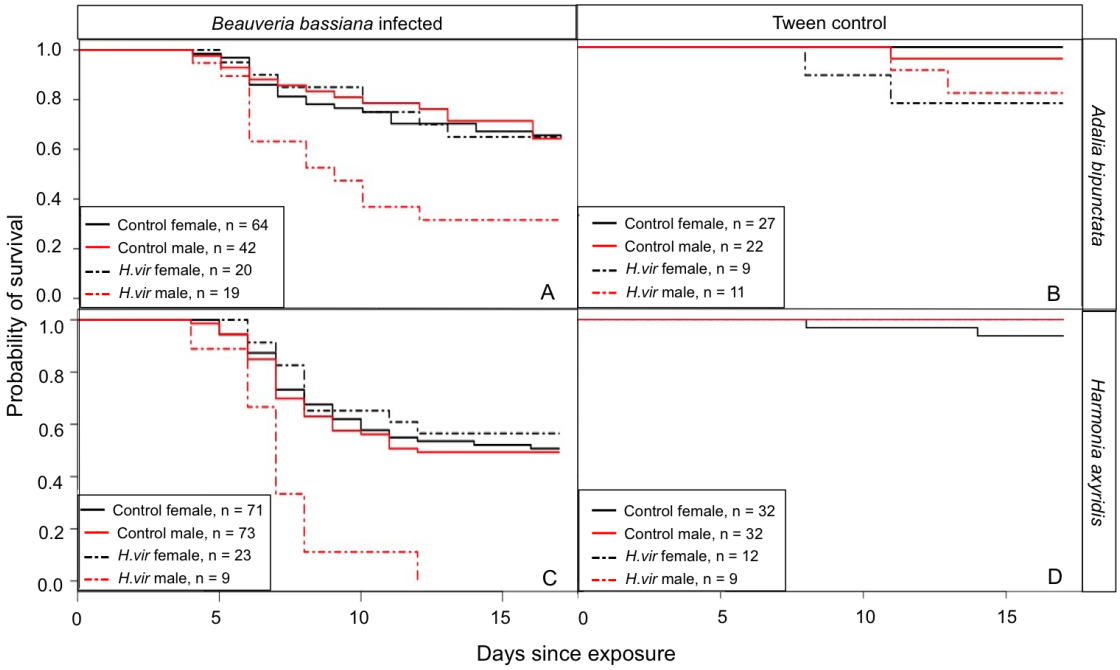

Figure 4.1: The survival probability of both *A. bipunctata* (A and B) and *H. axyridis* (C and D) decreased when infected with *B. bassiana* (A and C) in comparison with those exposed to a control of Tween (B and D). Black lines show female ladybirds and red depicts males. Solid lines show *H. virescens* uninfected individuals, and dashed lines represent *H. virescens* infected individuals. In plot D, the control males obscure the dashed lines for *H. virescens* infected individuals, as both these treatment groups had 100% survival. The survival of males declined faster than females when co-infected with *H. virescens* and *B. bassiana.*

Considering only Tween control individuals and pooling across both host species, while proportional survival of *H. virescens* infected ladybirds was 0.90 (± 0.05 SEM), compared to 0.97 (± 0.02 SEM) for those that did not carry this infection, the difference was not significant ($X^2_{[1]} = 2.992$, p = 0.084). In addition, no sex-specific effect of *H. virescens* infection was observed for Tween control individuals (ladybird sex by *H. virescens* infection interaction: $X^2_{[1]} = 2.212$, p = 0.137). The mortality associated with *H. virescens* infection differed between the two host species in the Tween control treatment: a greater proportion of

A. bipunctata individuals infected with *H. virescens* (survival proportion of 0.80 ± 0.09 SEM) had died by the end of the 16 day experimental period compared with the 100% survival of *H. virescens* infected Tween treatment *H. axyridis* individuals (ladybird species by *H. virescens* infection interaction: $X^2_{[1]} = 4.379$, p = 0.036; Figure 4.1).

Sex-specific effects of co-infection were not driven by differences in H. virescens *burden between males and females*

The mean thalli burden of *H. virescens* infected males in this study was 61.9% higher than that of females at the time they were exposed to *B. bassiana.* Therefore, to investigate whether the sex-specific effect of co-infection was either driven, or further influenced by *H. virescens* thalli burden, the data for co-infected ladybirds were considered separately. In these individuals that carried both infections, higher thalli burdens were indeed associated with shortened lifespans ($X^2_{[1]} = 3.990$, p = 0.046). However, co-infected males had substantially shorter lifespans than females regardless of their infection burden ($X^2_{[1]} = 19.470$, p = <0.001; Figure 4.2) demonstrating that they were intrinsically less able to survive co-infection. Furthermore, the negative effect of burden was not different between the two sexes (ladybird sex by *H. virescens* burden interaction: $X^2_{[1]} = 1.351$, p = 0.245). In a model containing both ladybird species, the negative impact of higher thalli burdens on lifespan did not differ between the host species (ladybird species by *H. virescens* burden interaction: $X^2_{[1]} = 0.861$, p = 0.353).

To investigate whether high *H. virescens* thalli burdens influenced mortality in ladybirds that did not carry *B. bassiana*, individuals infected with *H. virescens* in the Tween control treatment were considered separately. The mortality of these Tween treatment ladybirds singly infected with *H. virescens* was not influenced by the burden of thalli ($X^2_{[1]} = 0.156$, p = 0.692), nor did this differ between the two sexes (ladybird sex by *H. virescens* burden interaction: $X^2_{[1]} = 2.825$, p = 0.093).

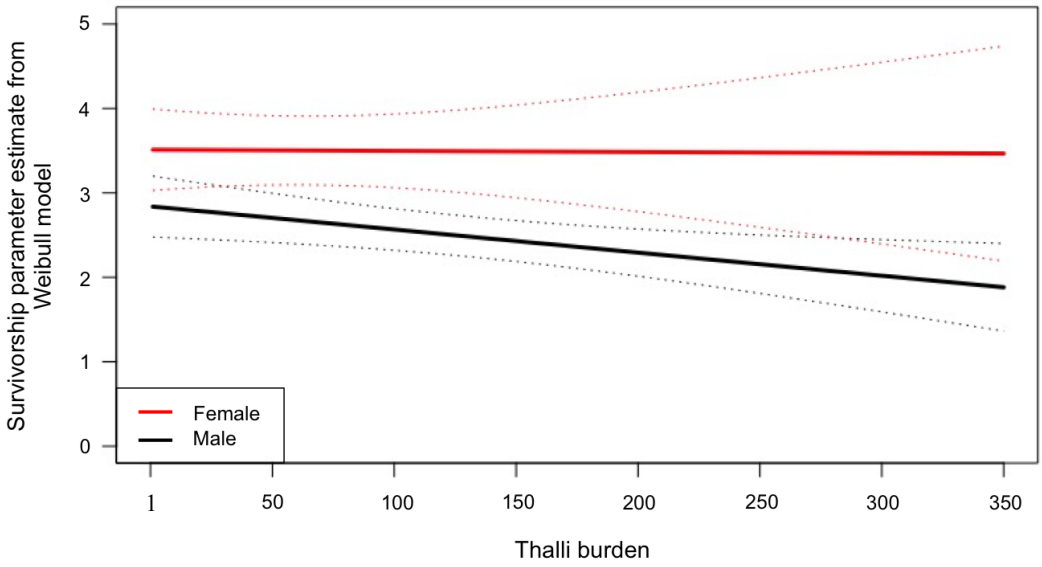

Figure 4.2: The survival of ladybirds co-infected with *H. virescens* and *B. bassiana* decreased with higher thalli burdens of *H. virescens,* but this difference was not sex-specific (red = females, black = males). This figure is plotted from the parameter estimates of the parametric survival model and contains an interaction between thalli burden and ladybird sex. The mean thalli burden on male ladybirds was 61.9% higher than on females. Dotted lines = SEM.

Block effects

The ladybirds used in this experiment were derived from chapter 2 in which ladybirds were exposed to *H. virescens* in four blocks, and therefore the variable 'block' was included in all models. After 16 days, the variation in proportional survival between all blocks ranged from 0.57 (± 0.05 SEM) in block C to 0.77 (± 0.04 SEM) in block D ($X^2_{[4]} = 15.304$, p = 0.004). Considering only those ladybirds exposed to *B. bassiana,* the impact of *H. virescens* infection was not different between the experimental blocks (block by *H. virescens* infection interaction: $X^2_{[4]} = 5.207$, p = 0.267).

4.5 Discussion

Co-infection can alter the virulence that a parasite causes individually. In this study, we investigated the ability of two ladybird species, *H. axyridis* and *A. bipunctata,* to defend against acute *B. bassiana* infection while infected with the chronic fungal parasite *H. virescens.* We found a sex-specific effect of co-infection. Infection mortality was strongly magnified in co-infected males of both host species, compared to males singly infected with

either fungus. However, this co-infection-driven exacerbation of virulence was not present in females for either host species.

Very little is known about the immune response of ladybirds to *H. virescens* infection, however, a related laboulbenialian fungus upregulated immune gene expression in infected ants and was protective against a lethal entomopathogen, *Metarhizium brunneum* (Konrad *et al.* 2015). It might therefore have been hypothesised that in the current study, where ladybirds were co-infected with a laboulbenialian parasite and the lethal *B. bassiana,* some protective effect might occur from *H. virescens* infection. However, the opposite trend was observed. While it is not possible to definitely conclude whether this co-infection mortality principally resulted from exacerbation of either *H. virescens* or *B. bassiana* virulence*,* it seems most likely that *H. virescens* infection amplifies the mortality caused by *B. bassiana*, as the former parasite causes negligible mortality over the timescales studied here (chapter 3). The insect immune response contains multiple different mechanisms to defend against pathogens (Lavine & Strand 2002), and thus the response mounted against *H. virescens* infection is likely to be different from that produced against *B. bassiana*. This difference is likely in part to be driven by the fact that the pathogens in this study infect different locations on the host. While *H. virescens* establishment occurs on the hosts' cuticle, which may induce epithelial immune responses, *B. bassiana* also stimulates immune responses beyond the cuticle after it has invaded the hosts' body cavity. Infection with a parasite can leave hosts unable to defend against a secondary infection if the two pathogens stimulate competing immune responses (Jolles *et al.* 2008; Dunn *et al.* 2012). Alternatively, a chronic infection causing the long-term stimulation of parasite defences could exhaust the hosts' ability to defend against a secondary parasite (Borkow *et al.* 2000; Hayes *et al.* 2004), and as hosts had been infected with *H. virescens* for 6 to 8 weeks at the start of this study*,* it is possible immune stimulation may have caused the observed co-infection effect. Another mechanism that may drive this result is the active non-specific suppression of host defences by one of the pathogens (Hayes *et al.* 2004). Infection with *H. virescens* might reduce the hosts' immune response, resulting in an impaired ability to defend against secondary

pathogens. However, another laboulbenialian parasite has been shown to upregulate immune genes in ants (Konrad *et al.* 2015), suggesting that these ladybird hosts may mount an immune response to *H. virescens* infection. While we only considered survival of the ladybirds in this study, a number of possibilities exist when considering the immune function of individuals, as discussed above.

While many studies show that co-infection exacerbates parasite virulence (Zarski *et al.* 1998; Abu-Raddad *et al.* 2006; McCullers 2006), our study shows no such effect for female ladybirds of either species. One mechanism that could drive the sex-biased result seen in this study is an asymmetry in immune investment between the two sexes. Many studies have demonstrated that males of multiple taxa have a lower immune ability than females (Zuk 2009; Cordoba-Aguilar & Munguia-Steyer 2013; Stephenson *et al.* 2016). Indeed, we have previously demonstrated that the reduction in late-life survival following chronic *H. virescens* infection only occurs in males (chapter 3). In this study, however, the results demonstrate that male survival does not differ from that of females in the short term when exposed singly to either fungal parasite. While this result differs from that in chapter 3, the maximum time that hosts had carried the *H. virescens* infection in this study was only half of the 100 days where infection induced mortality was observed in chapter 3. The thalli produced by *H. virescens* attach to the surface of the hosts cuticle with a haustorium, and small rhizoid structures penetrate into the host (Weir & Beakes 1996). While it is plausible large thalli burdens could drain host resources to the extent that the host is unable to mount a defence against a secondary infection, this would predict that there would be higher mortality rates in *A. bipunctata*, which develops higher fungal burdens than *H. axyridis*; this species difference was not observed here. Male survival was, however, considerably reduced in the presence of co-infection, which may be driven by an insufficient level of resources available to mount an effective immune response against a secondary infection (Sandland & Minchella 2003; Martin *et al.* 2007; Ezenwa & Jolles 2011). An alternative explanation is that the sex-specific co-infection effect could derive from the faster growth, and therefore larger burdens, of *H. virescens* on males compared to females (chapter 2). We tested this by investigating the

impact of fungal burden on mortality in co-infected hosts. Our results demonstrate that while higher thalli burdens did reduce survival in both males and females, controlling for burden differences, males suffered higher co-infection mortality costs.

While immune effects may be responsible for the low survival of co-infected males in this study, another possibility could be the physical effects of a combined infection. Indeed, the lethal effect of *B. bassiana* has been demonstrated to be strongly linked to the site of the host where infection occurred (Ishii et al. 2017). The thalli produced by *H. virescens* are present on the ladybird cuticle, however their position varies between the sexes due to the parts of the cuticle in contact during mating: thalli are generally located on the elytra on infected females and the ventral surface and legs on males (Riddick 2006). While *B. bassiana* is a systemic parasite, infection is initiated when spores germinate on and then penetrate the ladybird cuticle. The attachment of *H. virescens* thalli creates additional holes in the surface of the cuticle (Weir & Beakes 1996), which may weaken the barrier of the cuticle. In addition, the presence of thalli on the surface of the ladybird, especially in tight clusters, could prevent efficient cuticle cleaning by the ladybird, making it easier for *B. bassiana* spores to penetrate the host. While the mechanical effects described here are not obviously sex-specific, the location of *H. virescens* thalli on the ladybird might lead to more *B. bassiana* spores penetrating males. In addition, the access to the haemocoel required by *B. bassiana* hyphae might possibly be more easily achieved if the parasite enters the host through the ventral surface of the abdomen rather than the elytra.

Previous studies have shown *H. axyridis* to be more resistant to multiple parasites and pathogens, including *B. bassiana,* than native ladybird species (Roy *et al.* 2008; Haelewaters *et al.* 2016b). Indeed, *H. virescens* grows more effectively on *A. bipunctata* than *H. axyridis* hosts (chapter 2). However, the result of this growth asymmetry meant that in this study the burden of *H. virescens* thalli infecting *H. axyridis* was three times lower than that infecting *A. bipunctata*. While it might be anticipated that this would mean the effects of co-infection would be stronger in *A. bipunctata* than *H. axyridis,* our results show that the effect of co-infection is no different between the males of both species, despite no co-infected *H. axyridis*

males surviving to the end of the study. An alternative explanation for the lack of difference in the effect of co-infection between the species is that is the dosage of *B. bassiana* spores that each species received differed. This study aimed to infect both *H. axyridis* and *A. bipunctata* with an LD50 dose of *B. bassiana* spores. To achieve this, ten times the dose of *B. bassiana* spores for *H. axyridis* was required than *A. bipunctata* (Roy *et al.* 2008b; unpublished data). Our attempt to normalise the mortality between the two species could have prevented *H. axyridis* resisting the effects of co-infection, thereby causing the same virulent effects observed in *A. bipunctata.* However, these effects of co-infection were only present in male ladybirds, therefore it is possible that *B. bassiana* dose was not the main driver in the species effects seen here.

This study tested the ability of two ladybird species to defend against a generalist fungal parasite, while infected with another, more chronic parasite. The strains of *H. virescens* used here were experimentally transmitted from field caught ladybirds, the majority generated from conspecific interactions (chapter 2); however, the *B. bassiana* strain was not isolated from field populations, and was a general pesticide strain. Therefore, it is possible field-caught isolates of *B. bassiana* may cause different virulence for both ladybird species tested here. In the field, *Coccinella septempunctata* is more commonly infected with *B. bassiana* than either *A. bipunctata* or *H. axyridis* (as discussed in Roy *et al.* 2008b). However, although field reports of *B. bassiana* infections in *H. axyridis* are rare, the field prevalence of *H. virescens* has increased markedly in some UK *H. axyridis* populations (chapter 2), therefore there may be a strong likelihood that these two pathogens will infect a single individual in the field. Yet, the field association between *H. virescens* and *A. bipunctata* has existed for considerably longer than that with *H. axyridis* (Welch *et al.* 2001; Haelewaters *et al.* 2014). While the same sex-biased virulence of co-infection was seen in *A. bipunctata* in this study, mortality associated with *B. bassiana* is rarely reported for this species (Roy *et al.* 2008b). Future studies should investigate these possibilities further, with the addition of using field-derived *B. bassiana* strains.

The results of this study have demonstrated a sex-specific effect of co-infection in hosts infected with an acute and a chronic fungal infection in two ladybird species. Previously it has been assumed that the chronic parasite *H. virescens* is avirulent, however, in chapter 3 we show it accelerates the rate of ageing in infected *H. axyridis* ladybirds. In this study we have demonstrated another important effect of infection with *H. virescens,* showing that this chronic parasite may enhance the virulence of co-infecting pathogens, demonstrating the potential for *H. virescens* to have significant fitness consequences for hosts in the field.

Chapter 5:

Differences in the field prevalence of the parasitoid *Dinocampus coccinellae* between an invasive alien and native ladybird species are not driven by host encapsulation ability

5.1 Abstract

Natural enemies can influence the competitive interactions between native and invasive alien species in multiple ways, including enemy release, apparent competition or because they exploit interspecific differences in immune ability. In order to develop in a potential host, a parasite must overcome immune defences, which in insects, includes the encapsulation ability of the cellular immune response. While the formation of a melanised capsule around macroparasites can prevent parasite development, parasitoids are often able to suppress this response. In the UK, the parasitoid *Dinocampus coccinellae* parasitizes both the invasive alien *Harmonia axyridis* and the native *Coccinella septempunctata*, however its prevalence is far lower in the invader. This study investigated the impact of this parasitoid wasp on the encapsulation ability of these two ladybird species. First, we compared whether the encapsulation response of the UK native *C. septempunctata* differed from that of the invasive alien *H. axyridis*. Secondly, we tested the ability of the braconid wasp, *D. coccinellae*, to downregulate the encapsulation response of both ladybird host species, before finally comparing whether the impact of *D. coccinellae* on the host encapsulation response varied between *C. septempunctata* and *H. axyridis*. While it might be predicted that *D. coccinellae* would downregulate the encapsulation response of the primary host, *C. septempunctata*, our results show encapsulation was upregulated in Scottish samples of this species following parasitism. In contrast, in ladybirds sampled from England *D. coccinellae* oviposition generally resulted in the suppression of the hosts' encapsulation ability; however, this effect did not differ between the two host ladybird species. Both ladybird species displayed similar encapsulation abilities when parasitized with *D. coccinellae*, which suggests that the low field prevalence of *D. coccinellae* in *H. axyridis* is not due to this immune response. Alternative aspects of the immune system, or perhaps the internal environment of the invasive alien host, are potential explanations for the differences between the two host species in the field prevalence of this parasitoid that could be tested in the future.

5.2 Introduction

Competitive interactions between native and invasive alien species can be influenced in multiple ways, one of which is the influence of natural enemies (Price *et al.* 1986; Prenter *et al.* 2004). The presence of enemy release, shared natural enemies (leading to apparent competition) and asymmetries in immune defences between native and invasive alien species can all impact the dynamics of the interactions (Prenter *et al.* 2004; Lee & Klasing 2004; Dunn 2009; Dunn *et al.* 2012). The ability of a parasite to infect and develop within a host relies on overcoming or avoiding host immune defences. In insects, the innate immune response contains multiple mechanisms to protect the host from invading parasites. While phagocytosis and the production of antimicrobial peptides are triggered by smaller pathogens, such as bacteria, larger macroparasites frequently induce cellular encapsulation (Schmidt *et al.* 2001; Jiravanichpaisal *et al.* 2006; Sparks *et al.* 2008; Strand 2008). Once the presence of a macroparasite is detected by the host immune system, circulating haemocytes adhere to its surface forming a multilayer capsule (Salt 1970; Cerenius *et al.* 2008). Melanin, produced after the inactive enzyme prophenyloxidase is converted to phenyloxidase, is then added to the capsule, which ultimately results in the death of the parasite (Schmidt *et al.* 2001; Cerenius *et al.* 2008).

Parasitoids, however, have developed a variety of mechanisms to avoid encapsulation by insect immune defences. While some parasitoids completely evade host immune defences, by embedding developing eggs into host tissue or covering their eggs in encapsulation-preventing proteins, others are able to actively suppress the immune response (Asgari *et al.* 1998; Kinuthia *et al.* 1999; Moreau *et al.* 2003; Prevost *et al.* 2005). At the time of oviposition, some parasitoids inject viruses or virus-like particles that result in impaired function or destruction of host immune cells (Chiu & Govind 2002), interrupt the melanisation pathway (Labrosse *et al.* 2003), and prevent the encapsulation response (Edson *et al.* 1981; Kadash *et al.* 2003). Viruses and virus-like particles are often present as the result of a evolutionarily long-term association with the parasitoid, to the extent that their genomic material may be encoded in the parasitoid genome (Whitfield 2002; Drezen *et al.* 2003). Braconid wasps are one group of

parasitoids which characteristically use polydnaviruses to attack the haemocytes involved in the host's immune defence, rendering hosts unable to form a capsule around the invading egg (Beckage 1998). This study investigated the encapsulation ability of two host ladybird species in response to an artificial implant and furthermore, it tests whether a braconid parasitoid is able to downregulate the encapsulation ability of the two hosts.

The braconid parasitoid *Dinocampus coccinellae* is found globally associated with over 50 ladybird species in the subfamily coccinellidae, including the invasive alien *Harmonia axyridis* in both its native and invaded ranges (Ceryngier & Hodek 1996; Koyama & Majerus 2008). The adult female *D. coccinellae* oviposits into the body cavity of a ladybird host, where it hatches and develops through four larval instars (Dheilly *et al.* 2015). Once in the final instar, the *D. coccinellae* larva emerges from the ladybird and spins a silk cocoon between the legs of the host; the host then remains alive on top of the developing wasp until an adult emerges (Dheilly *et al.* 2015). Like many species of braconid wasp, *D. coccinellae* produces teratocyte cells that absorb nutrients from the infected host and aid the growth and development of the parasitoid larva (Strand 2014). Although it has been suggested that teratocyte cells are involved in the avoidance of host immunity, a greater number of studies provide evidence supporting their role in the disruption of host growth and metamorphosis (Beckage & Gelman 2004; Strand 2014). In *H. axyridis* parasitized by *D. coccinellae,* Firlej *et al* (2007) found fewer wasp teratocytes and altered cell growth patterns when compared with parasitism of native host ladybird species, supporting the notion that *H. axyridis* as a suboptimal host for this parasitoid. While no polydnavirus has been isolated from *D. coccinellae*, a paralysis virus has been identified from the head of parasitized coccinellid beetles, which may be transmitted to the host during oviposition (Dheilly *et al.* 2015). In addition to the paralysis virus, it is possible other viruses or virus-like particles could be injected during oviposition to assist in the suppression of the host immune response.

In the UK, the primary host of *D. coccinellae* is the coccinellid *Coccinella septempunctata,* however, this parasitoid is also found at a much lower prevalence in the

invasive alien *Harmonia axyridis* (Koyama & Majerus 2008; Comont *et al.* 2013). Indeed, a lower field prevalence of *D. coccinellae* infecting *H. axyridis* is also found in this ladybird's native Japan, in comparison with a subspecies of *C. septempunctata, C. septempunctata bucki* (Koyama & Majerus 2008b; Maeta 1969 as cited in Koyama *et al.* 2013). The arrival of *H. axyridis* in the UK in 2003 resulted in the establishment of this invader across much of England, overlapping with populations of the ubiquitous *C. septempunctata* (Brown 2010; Roy *et al.* 2012b). The low prevalence of natural enemies associated with *H. axyridis* is often thought to result from enemy release (Keane & Crawley 2002; Roy *et al.* 2011a), although the immune ability of the invader is also cited as stronger than that of other ladybirds (Rohrich *et al.* 2012; Vilcinskas *et al.* 2013a). While laboratory based studies have previously shown no oviposition preference of *D. coccinellae* between *C. septempunctata* and *H. axyridis,* a lower eclosion rate still exists in the invasive alien host when compared with the primary host (Koyama & Majerus 2008; Berkvens *et al.* 2010). It has therefore been suggested that invasive alien *H. axyridis* populations act as a *D. coccinellae* egg sink: parasitoids oviposit into both *H. axyridis* and *C. septempunctata,* however the wasp rarely successfully ecloses from *H. axyridis* and thus the wasp population might reduce where both host species co-exist (Hoogendoorn & Heimpel 2002; Berkvens *et al.* 2010). The mechanism driving the low eclosion rate of *D. coccinellae* infecting *H. axyridis* is currently unknown, but explanations include the immune capacity of the host, or the inability of the parasitoid to downregulate the hosts' defences. While it has been demonstrated that *H. axyridis* is able to encapsulate *D. coccinellae* eggs, the ability to do so was variable (Firlej *et al.* 2012).

This study aimed to investigate whether the low field prevalence of *D. coccinellae* in *H. axyridis* is due to the encapsulation capability of the host or the ability of the parasitoid to downregulate the encapsulation response. To test this, we first examined the encapsulation response of *H. axyridis* and *C. septempunctata* to an artificial implant. We then investigated whether *D. coccinellae* is able to downregulate the encapsulation ability of either host species. Finally, we tested whether the impact of *D. coccinellae* on the host encapsulation response

varied between *C. septempunctata* and *H. axyridis*, by comparing the encapsulation ability of each host species in response to *D. coccinellae* oviposition. We predicted that *D. coccinellae* would be able to downregulate the encapsulation response of *C. septempunctata,* but would be less effective in doing so in *H. axyridis*. This result would help explain the patterns of field prevalence of *D. coccinellae* seen for each species in both the native and invasive range of *H. axyridis*.

5.3 Materials and Methods

Study material

In order to investigate how the encapsulation response of *C. septempunctata* was influenced by *D. coccinellae* oviposition, *C. septempunctata* ladybirds (n = 72) were collected from the centre of Stirling, Scotland, in the summer of 2014 (here on known as the Scottish population). Ladybirds were kept in the laboratory, details below, in single-sex groups in Petri dishes for two to four weeks and monitored for the appearance of field-derived infections of *D. coccinellae.*

To compare the encapsulation ability of the primary and invasive alien hosts of *D. coccinellae, H. axyridis* (n = 237) and *C. septempunctata* (n = 121) adults and pupa were collected from populations in South East England in the summers of 2014 and 2015 (Table 5.1). As above, ladybirds were kept in the laboratory and monitored for field infections of *D. coccinellae.*

Dinocampus coccinellae wasps that eclosed from field collected ladybirds were transferred into plastic fly vials (7.5cm by 2.2cm) and fed a 10% honey solution. All ladybirds and wasps were kept in a controlled environment facility at 20°C, 16:8 hr L:D cycle and 60% relative humidity for the duration of the experiment. To diagnose *D. coccinellae* field infection, ladybirds were kept in single sex, 9cm Petri dishes containing 5 to 10 individuals. Subsequently, during the experiment, ladybirds were kept in individual Petri dishes. At all times, ladybirds

were fed with 'Entofood' (*Ephestia kuehniella* and *Artemia* spp. eggs, Koppert Biological Systems) and artificial diet (Roy *et al.* 2013). Ladybird sex, weight and pronotum width were determined at the start of the experimental period. Ladybird condition was calculated as weight (mg) divided by pronotum width (mm).

Table 5.1: Sample locations and sizes of *C. septempunctata* and *H. axyridis* collected in England in 2014 and 2015.

	2014		**2015**	
	C. septempunctata	***H. axyridis***	***C. septempunctata***	***H. axyridis***
Richmond, Battersea & Bushy Parks, London	21	116	0	0
Cholsey, Crowmarsh Gifford & Wallingford, Oxfordshire	3	29	89	79
Pangbourne, Berkshire	0	0	8	13

Experimental procedure

Ladybirds were divided into two treatment categories: parasitized or un-parasitized with *D. coccinellae*. In each of the encapsulation assays, only *D. coccinellae* individuals from the same English (n = 14) or Scottish (n = 10) populations as the hosts were used. Ladybirds in the *D. coccinellae* parasitism treatment were placed singly in a 9cm Petri dish with a single *D. coccinellae* wasp and observed until an oviposition event had taken place. Ladybirds in the un-parasitized treatment were similarly placed in a Petri dish but without the presence of *D. coccinellae*. The encapsulation ability of invertebrates can be assessed by the measurement of melanised cells surrounding an artificial implant (Lackie, 1979). In order to assess the encapsulation ability of ladybirds, a nylon implant was inserted into the abdomen of each ladybird 24 hours after *D. coccinellae* oviposition (or control treatment): nylon fishing wire implants were 0.15mm diameter and varied in length from 0.75 to 1.75mm. To insert the implants, ladybirds were anaesthetised with CO_2, their elytra separated and the implant pushed through the abdominal cuticle, ensuring the implant was entirely within the haemocoel and no part was in contact with the insect cuticle. After a further 48 hours, ladybirds were dissected, the implants removed and mounted onto a microscope slide under a coverslip using Eukitt mounting medium. If any un-emerged parasitoid larvae resulting from field-parasitism prior to

the experiment were observed at the time of implant removal, ladybirds were removed from the analysis.

To prevent the mounted implants from fading, slides were stored in the dark in a fridge prior to processing (approximately 1 week). Each slide was photographed 2 to 3 times against a white background under a microscope to generate a mean score for the average encapsulation across the whole implant and the darkest encapsulation point on the implant (Leica MZ125 microscope with an Olympus SP-500UZ camera). To ensure consistency across all photographs, the same white background was used for all implants. In addition, the laboratory was blacked out with blackout blinds to ensure the only illumination in the photographs came from the microscope light source. All images were analysed using the computer software Image J (Schneider *et al.* 2012). For each photograph, the implant, including any surrounding encapsulation, was outlined using the polygon section tool and the measure function was used to assess the mean encapsulation and darkest point (darkest pixel in the selected area).

Statistical analysis

All analyses were done with the software 'R' (R Core Development Team, 2016), version 3.3.2. In order to test whether the encapsulation ability of ladybirds was affected by the presence of *D. coccinellae,* the response variables of mean and darkest encapsulation (measured by image J) were transformed (see below), so that higher scores equated to greater encapsulation. The explanatory power of the models was assessed by AIC comparisons and models simplified by removing terms that did not improve model fit by two AIC points. P-values were calculated for all models using likelihood ratio tests, comparing models with and without the term of interest.

To test the ability of ladybirds to encapsulate an artificial implant, the mean encapsulation value was used as a response variable. In order to improve the fit of the data to model assumptions, mean encapsulation values were multiplied by 100 (allowing the variability

of the values to be maintained in the transformed data) and square root transformed. To investigate the maximum encapsulation ability of ladybird hosts in response to a *D. coccinellae* egg, the darkest point on the implant was also used as a response variable (maximum encapsulation), which was logit transformed after dividing by the highest maximum value of encapsulation to improve the fit of the data to a normal distribution.

All models tested the influence of a base set of factors and covariates on the encapsulation ability of *H. axyridis* and *C. septempunctata* ladybirds. The base set of terms was: ladybird sex, ladybird species, wasp treatment, ladybird body condition (weight divided by pronotum width) and implant length. The covariates of body condition and implant length were mean centred to allow model intercept comparisons for an average ladybird with an average implant length. Interactions were also tested between wasp treatment and implant length, and wasp treatment and body condition in all models. To test whether *D. coccinellae* affected the encapsulation ability of the ladybird species differently, and further, whether this was also influenced by ladybird sex, a three-way interaction between ladybird species, ladybird sex and wasp treatment (including all component two-way interactions) was included in all models.

Scottish C. septempunctata

The effect of *D. coccinellae* parasitism on Scottish *C. septempunctata* encapsulation ability was tested using the mean and maximum encapsulation values as response variables. The transformed mean encapsulation response variable was included in a generalised linear model with a Gamma distribution using a log link function and the explanatory variables detailed above, with the exception of ladybird species. The transformed response variable of maximum encapsulation was also tested with these variables, but using a linear model with a Gaussian distribution.

Wasp origin

In 2015, 50% (n = 14) of *D. coccinellae* used to parasitize ladybirds in this study originated from *H. axyridis* and the remainder from *C. septempunctata*. Using only ladybirds in the parasitized treatment, we tested whether wasp origin affected the encapsulation ability of either host species with generalised linear models using a Gamma distribution and log link function. The factors tested in these models were ladybird sex, ladybird species and wasp origin, with the covariates of implant length and ladybird body condition. In addition, to test whether variation in encapsulation ability between the ladybird species was dependent on the wasp origin, these factors were included together in an interaction. We also tested for a sex-specific effect for wasp origin by including host sex and wasp origin as an interaction. Ladybird species and ladybird sex were also included in an interaction in this model.

English C. septempunctata *and* H. axyridis

The impact of *D. coccinellae* on the mean and maximum encapsulation ability of English *H. axyridis* and *C. septempunctata* was tested using generalised linear models with a Gamma distribution and a log link function. In addition to the explanatory variables detailed above, a factor for sampling year was included as a main effect and in interactions with all other main effects, testing for any variation between the two sample years. To more closely examine the three-way interaction between ladybird species, ladybird sex and wasp treatment, models were fitted for the data from *H. axyridis* and *C. septempunctata* separately for both the response variables (mean and maximum encapsulation scores).

Host population

To investigate whether *C. septempunctata* populations differed in their encapsulation response to *D. coccinellae* parasitism, the mean and maximum encapsulation ability of ladybirds sampled in England and Scotland were compared using a generalised linear model

with a Gamma distribution and a log link function. The influence of host population was also tested separately for *H. axyridis* ladybirds in 2014 (London and Oxfordshire) and in 2015 (Berkshire and Oxfordshire) in generalised linear models with log link functions and Gamma distributions. All population models included the base set of terms detailed above, with the exception of species, and the inclusion of host population a factorial variable. In addition, to test the influence of population and ladybird sex on the ability of *D. coccinellae* to influence the encapsulation response, wasp treatment was included in an interaction with population and host sex. All possible combinations of sex, population and treatment were also included in the models.

5.4 Results

Scottish C. septempunctata *upregulate encapsulation ability in response to* D. coccinellae *parasitism*

We first tested whether *D. coccinellae* parasitism influenced the subsequent mean and maximum encapsulation responses in *C. septempunctata* collected from Scotland, outside the invaded range of *H. axyridis.* These two measures of encapsulation were correlated, but not perfectly so (Pearson's correlation r = 0.671), therefore analyses were conducted on both immune metrics separately. The mean encapsulation score for *C. septempunctata* ranged from 1 to 64, with an overall mean of 13.06 (± 1.29 SEM), while the mean maximum encapsulation was 82.24 (± 4.56 SEM, range: 3 - 135). Both encapsulation scores were higher in parasitized beetles. The mean encapsulation response of un-parasitised *C. septempunctata* was 11.26 (95% CI 8.36 - 16.75, n = 32), compared to 14.44 (95% CI 10.44 - 19.98, n = 40) for parasitized individuals, a difference that was not significant ($X^2_{[1]} = 0.272$, p = 0.135). A significant effect of wasp treatment was, however, present for the maximum encapsulation of individuals ($X^2_{[1]} = 11.848$, p = 0.004), such that *C. septempunctata* parasitized by *D. coccinellae* had an 11.8% higher maximum encapsulation response than un-parasitized individuals (Figure 5.1).

While male *C. septempunctata* had a lower mean encapsulation than females in both treatments, this sex difference was only significantly different for ladybirds in the un-parasitized treatment (males = 7.28, 95% CI 4.26 - 12.42; females = 11.26, 95% CI 8.36 - 16.75; $X^2_{[1]}$ = 0.775, p = 0.012). The strength of the effect of parasitism on mean encapsulation did not vary between male and female hosts (treatment by ladybird sex interaction: $X^2_{[1]}$= 0.002, p = 0.902). Indeed, the small increase in the mean encapsulation response after wasp parasitism was not significant when the data for females ($X^2_{[1]}$ = 0.086, p = 0.389), or males ($X^2_{[1]}$= 0.243, p = 0.514, Figure 5.1) were tested separately. However, considering the maximum encapsulation ability of *C. septempunctata,* a sex-specific effect of parasitism saw males increase their maximum encapsulation by 42.75% ($X^2_{[1]}$= 11.483, p = <0.001), an effect not seen in females ($X^2_{[1]}$= 2.301, p = 0.252, Figure 5.1) or when the data for both sexes were considered in the same model (treatment by ladybird sex interaction: $X^2_{[1]}$= 2.412, p = 0.444).

For Scottish ladybirds, body condition was an important factor in determining the mean encapsulation ability of both male and female *C. septempunctata* ($X^2_{[1]}$ = 1.021, p = 0.004). In females, averaging over both parasitism treatments, a 35.16% decrease in mean encapsulation occurred between the 25th and 75th quartile of ladybird body condition, indicating females in a poor condition had a greater encapsulation responses; in females this influence of body condition on the mean encapsulation ability was not affected by parasitism (treatment by body condition interaction: $X^2_{[1]}$= 0.036, p = 0.576). For males, however, a parasitism-specific effect of body condition was present in *C. septempunctata* (treatment by body condition interaction: $X^2_{[1]}$ = 0.654, p = 0.021): parasitized male ladybirds had lower encapsulation scores when in better condition, in contrast with un-parasitized males, where ladybirds in better condition displayed a higher mean encapsulation ability (Figure 5.2).

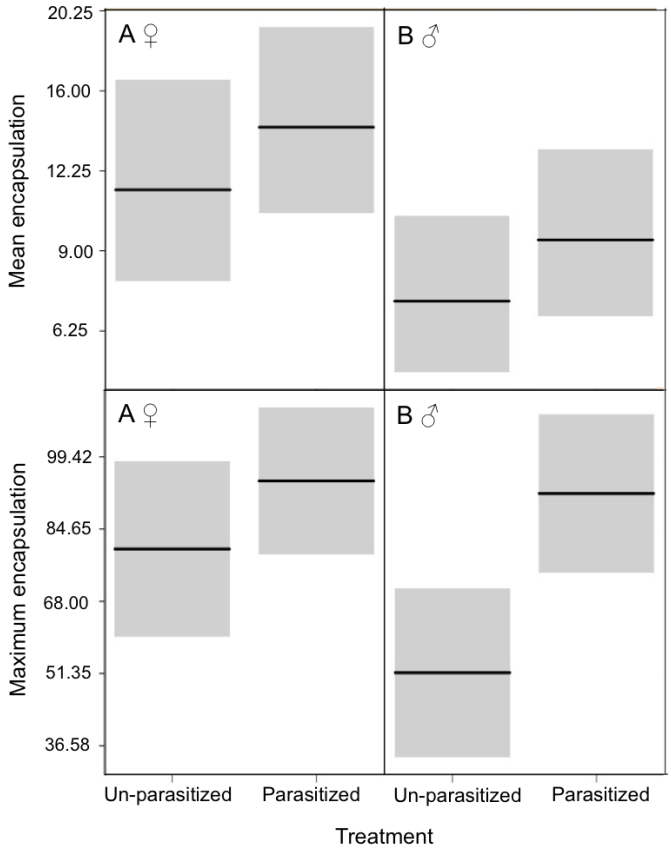

Figure 5.1: The mean and maximum encapsulation response of *C. septempunctata* sampled from Scotland was upregulated in the presence of *D. coccinellae* parasitism (A = females, B = males). The y-axes of mean and maximum encapsulation have been back-transformed to raw values (see methods), however results predicted from the statistical models are plotted in the transformed space. Grey bars are 95% CIs.

Considering the maximum encapsulation response of *C. septempunctata* ladybirds, ladybird body condition, again, did not influence the ability of females to encapsulate the implant ($X^2_{[1]} = 4.660$, $p = 0.114$), however, the maximum encapsulation response was 33.80% lower for males in the 75th, compared to the 25th, quartile of ladybird body condition ($X^2_{[1]} = 11.533$, $p = <0.001$). The encapsulation ability of *C. septempunctata* individuals did not vary according to the length of implant used (mean: $X^2_{[1]} = 0.006$, $p = 0.828$; maximum: $X^2_{[1]} = 2.314$, $p = 0.195$).

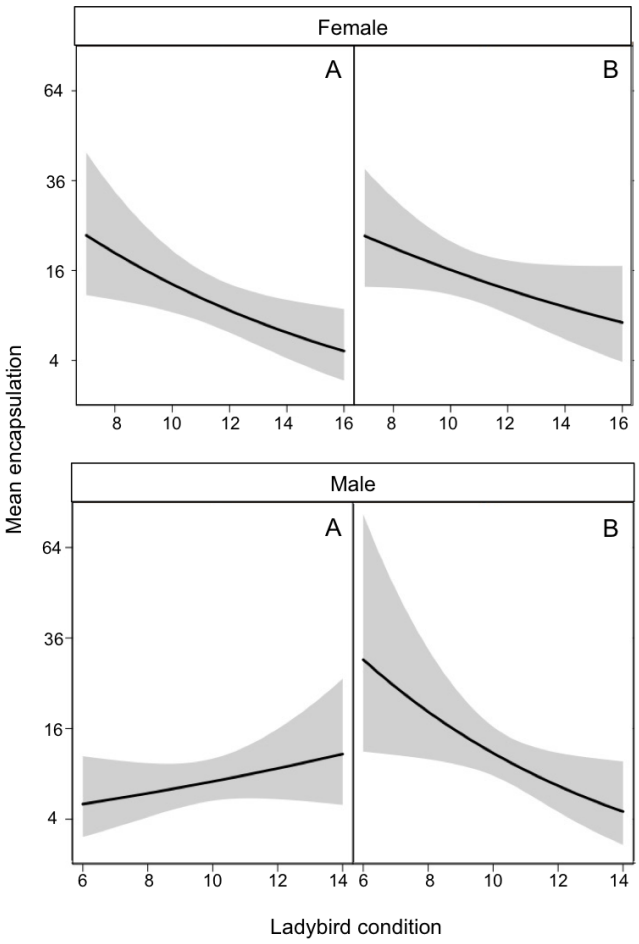

Figure 5.2: The mean encapsulation response of Scottish *C. septempunctata* males and females varied according to ladybird body condition. All but un-parasitized male ladybirds had a higher encapsulation response in poorer condition, however un-parasitized males showed the reverse (A = un-parasitized (females: n = 15; males: n = 17), B = parasitized (females: n = 21; males: n = 19)). The y-axes of mean encapsulation has been back-transformed to raw values (see methods), however results predicted from the statistical models are plotted in the transformed space. Shared areas are 95% CIs.

The impact of D. coccinellae *on host immune responses does not consistently differ between H. axyridis and C. septempunctata*

To test whether the impact of *D. coccinellae* on the host encapsulation response varied between the two ladybird species, we compared the mean and maximum encapsulation ability of *H. axyridis* and *C. septempunctata* from English ladybird populations (Pearson's correlation for these two encapsulation metrics: r = 0.674). The mean encapsulation values ranged from 0 to 76 (11.76 ± 0.49 SEM) and the maximum from 0 to 162 (69.16 ± 2.47 SEM). The encapsulation ability of ladybirds was not affected by the host origin of *D. coccinellae* (mean:

$X^2_{[1]} = 0.005$, p = 0.795; maximum: $X^2_{[1]} = 0.134$, p = 0.425), therefore *D. coccinellae* originating from both *H. axyridis* and *C. septempunctata* were used throughout the study.

The two ladybird species, *H. axyridis* and *C. septempunctata* collected from English populations, did not differ in their un-parasitized encapsulation ability (mean: $X^2_{[1]} = 0.065$, p = 0.473; maximum: $X^2_{[1]} = <0.001$, p = 0.913). We initially predicted *D. coccinellae* parasitism would reduce the encapsulation response of *C. septempunctata,* but not *H. axyridis.* However, in this dataset, parasitism did not cause a species-specific change either for the mean (treatment by species interaction: $X^2_{[1]} = 0.090$, p = 0.393) or maximum (treatment by species interaction: $X^2_{[1]} = <0.001$, p = 0.992) encapsulation responses, despite a general encapsulation decrease following *D. coccinellae* parasitism, with the exception of *C. septempunctata* females (Figure 5.3).

Considering the two ladybird species separately, *D. coccinellae* parasitism did not affect the mean ($X^2_{[1]} = 0.060$, p = 0.395) or maximum ($X^2_{[1]} = 0.033$, p = 0.649) encapsulation ability of *C. septempunctata*. However, while female *C. septempunctata* displayed a 26.43% upregulation in mean encapsulation response when parasitized by *D. coccinellae*, the opposite was seen for male *C. septempunctata* for which there was a decrease of 46.78% (mean encapsulation: treatment by sex interaction: $X^2_{[1]} = 0.678$, p = 0.003; maximum encapsulation: treatment by sex interaction: $X^2_{[1]} = 1.000$, p = 0.011, Figure 5.3).

The encapsulation ability of *H. axyridis* was not affected by *D. coccinellae* parasitism when measured as either mean ($X^2_{[1]} = 0.076$, p = 0.476) or maximum ($X^2_{[1]} = 0.021$, p = 0.541) encapsulation. However, while *H. axyridis* demonstrated a slight downward trend in encapsulation ability in both sexes in response to *D. coccinellae* parasitism, this trend did not differ between the sexes: males and females downregulated their immune response to the same extent following the *D. coccinellae* treatment (mean encapsulation: treatment by sex interaction: $X^2_{[1]} = 0.099$, p = 0.416; maximum encapsulation: treatment by sex interaction: $X^2_{[1]} = <0.001$, p = 0.913, Figure 5.3).

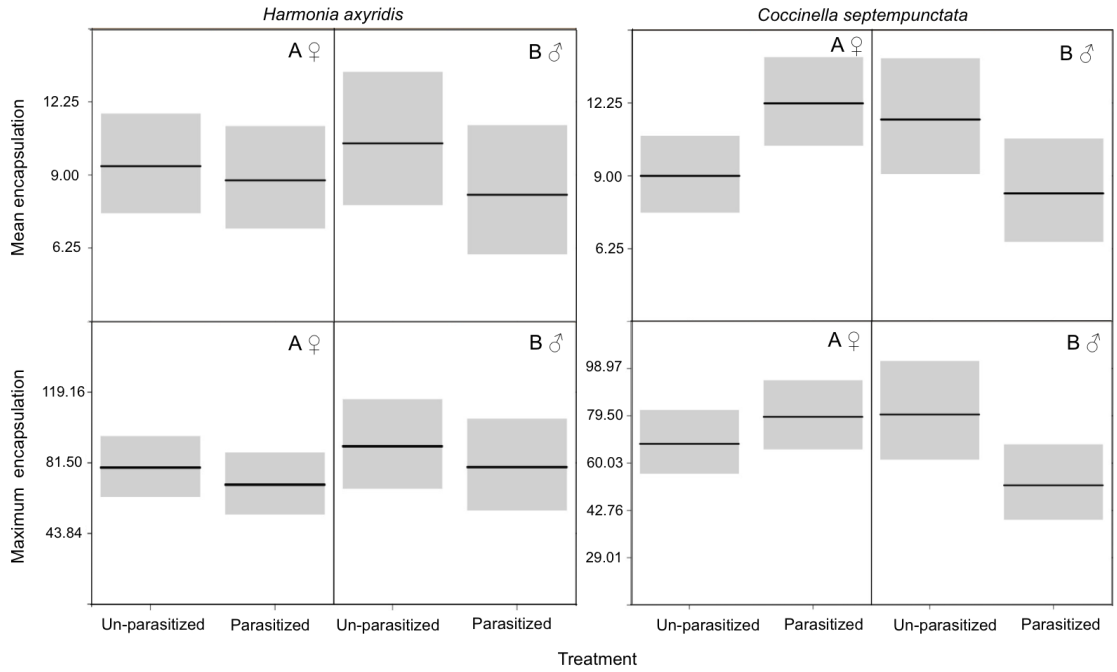

Figure 5.3: The mean and maximum encapsulation ability of *H. axyridis* and *C. septempunctata* individuals sampled from English populations (A = females, B = males). Parasitism by *D. coccinellae* generally resulted in a downregulation of the immune response, with the exception of *C. septempunctata* females; this was the opposite trend to the upregulation seen in the Scottish population (see Fig 5.1). The y-axes of mean and maximum encapsulation have been back-transformed to raw values (see methods), however results predicted from the statistical models are plotted in the transformed space. Grey bars are 95% CIs.

Samples of *H. axyridis* and *C. septempunctata* were collected in two years, 2014 and 2015; therefore year was included as a factor in all models containing this data. Un-parasitized *H. axyridis* ladybirds had a higher mean encapsulation response in 2015 (12.72 ± 0.78 SEM) compared to 2014 (11.37 ± 0.90 SEM), but this was marginally not significant ($X^2_{[1]} = 0.539$, p = 0.057). In addition, the maximum encapsulation ability of un-parasitized *H. axyridis* was no different between the two sample years ($X^2_{[1]} = 0.011$, p = 0.654). The impact of parasitism did not differ between years for the mean (year by treatment interaction: $X^2_{[1]} = 0.040$, p = 0.573) or maximum (year by treatment interaction $X^2_{[1]} = 0.015$, p = 0.574) encapsulation ability when both *H. axyridis* and *C. septempunctata* were considered in the same model.

In contrast to the Scottish *C. septempunctata* population, ladybird body condition did not significantly affect the mean encapsulation ability of un-parasitized *C. septempunctata* ($X^2_{[1]} = 0.233$, p = 0.078), or *H. axyridis* ladybirds ($X^2_{[1]} = 0.007$, p = 0.825) sampled in England. Ladybird body condition did, however, influence the maximum encapsulation response of un-

parasitized *C. septempunctata* ladybirds: a 22.80% lower encapsulation response was seen in *C. septempunctata* ladybirds in better condition compared to those in poor condition when comparing the between the 25th and 75th quartiles of ladybird condition ($X^2_{[1]}$ = 1.583, p = 0.001). The body condition of *H. axyridis* did not influence the maximum encapsulation ability of this species ($X^2_{[1]}$ = 0.020, p = 0.552).

The length of the nylon implant inserted into host ladybirds ranged from 0.75 to 1.75mm. Higher maximum encapsulation scores were seen on larger implants when both ladybirds species were considered together in a single model ($X^2_{[1]}$ = 0.193, p = 0.045). While implant length did not affect the mean encapsulation ability of *H. axyridis* ($X^2_{[1]}$ = 0.189, p = 0.529), the maximum encapsulation ability of un-parasitized *H. axyridis* was influenced by implant length ($X^2_{[1]}$ = 0.231, p = 0.041): longer implants produced a higher maximum encapsulation score than shorter implants, however, the same effect was not seen for *C. septempunctata* ladybirds ($X^2_{[1]}$ = 0.062, p = 0.649).

The immune capability of *C. septempunctata* males appeared to be inconsistent across the Scottish (Figure 5.1) and English (Figure 5.3) populations. Therefore, the Scottish and English ladybirds were compared to test the degree to which *D. coccinellae* differently affected the ladybirds' encapsulation ability. The mean and maximum encapsulation ability of *C. septempunctata* were higher in the Scottish than the English population, pooling across both treatment categories (mean: $X^2_{[1]}$ = 0.930, p = <0.001; maximum: $X^2_{[1]}$ = 0.980, p = <0.001). Despite the fact that an opposite trend was seen in the encapsulation ability of parasitized males between English and Scottish populations, the contrast was not statistically significant for the mean encapsulation ability of *C. septempunctata* (sex by treatment by population interaction: $X^2_{[1]}$ = 0.009, p = 0.100). However, the contrasting effect of parasitism on the maximum encapsulation ability of *C. septempunctata* males between the two populations was found to be significant (sex by treatment by population interaction: $X^2_{[1]}$ = 0.206, p = 0.038). While the encapsulation trend resulting from parasitism for female *C. septempunctata* did not differ between the two populations for the mean (population by treatment interaction: $X^2_{[1]}$ = <0.001,

p = 0.987), or maximum (population by treatment interaction: $X^2_{[1]} = 0.010$, p = 0.678) abilities, when considering only male individuals, the effect of parasitism differed between the Scottish and English populations for both the mean (population by treatment interaction: $X^2_{[1]} = 0.012$, p = 0.022) and maximum (population by treatment interaction: $X^2_{[1]} = 0.818$, p = 0.002) encapsulation responses.

Considering only *H. axyridis* individuals, a comparison of encapsulation ability between different English populations found no difference in the mean or maximum encapsulation ability of individuals sampled in either 2014 (mean: $X^2_{[1]} = 0.006$, p = 0.858; maximum: $X^2_{[1]} = 0.003$, p = 0.940), or 2015 (mean: $X^2_{[1]} = 0.021$, p = 0.625; maximum: $X^2_{[1]} = <0.001$, p = 0.970).

5.5 Discussion

Interactions with natural enemies can influence the dynamics between native and invasive alien species. The braconid wasp *D. coccinellae* is a generalist coccinellid parasitoid, which has a lower prevalence in *H. axyridis* than *C. septempunctata* populations, both in the UK and Japan (Koyama & Majerus 2008b; Maeta 1969 as cited in Koyama *et al.* 2013). While multiple mechanisms could drive the observed difference in field prevalence, this study focused on the encapsulation immune response of two ladybird species, comparing the encapsulation ability of *H. axyridis* and *C. septempunctata* and the effect *D. coccinellae* oviposition has on this response. The results of this study show that the change in encapsulation ability of ladybirds in response to *D. coccinellae* parasitism differed between two sample locations for male *C. septempunctata* hosts between Scotland and South East England. While *C. septempunctata* collected from Scotland, as well as females from England, upregulated their encapsulation response following *D. coccinellae* oviposition, a downward trend existed for male *C. septempunctata* and also for both sexes of *H. axyridis* collected from England. Our results demonstrate variation in the effect of *D. coccinellae* parasitism on encapsulation responses, which could indicate geographic variation in host-parasite adaptation in this system.

In order to successfully complete development, parasitoids are often able to suppress or evade host immune defences, including downregulating the encapsulation response generated by the host (Edson *et al.* 1981; Rizki & Rizki 1994; Schmidt *et al.* 2001). Indeed, multiple species of braconid wasp use viruses or virus-like particles to defend against the host immune system by preventing melanisation or killing cells used in the encapsulation response (Beckage 2008; Asgari 2012). While the presence of immunosuppressive viral infections has not yet been reported in *D. coccinellae,* our results show that in both ladybird species collected from South East England, with the exception of female *C. septempunctata,* encapsulation is reduced in the presence of *D. coccinellae* parasitism. This is consistent with our hypothesis that the generalist braconid *D. coccinellae* is able to impair the immune defences of its host. We predicted that differences in the efficacy of the encapsulation response between *C. septempunctata* and *H. axyridis* might explain the interspecific difference in the field prevalence of *D. coccinellae*. However, we found no difference between the host species in this immune metric. We conclude that the low prevalence of *D. coccinellae* seen in field *H. axyridis* populations is not driven by elevated encapsulation ability in this IAS, nor is there any evidence that *D. coccinellae* lacks an ability to downregulate the encapsulation response in this host species. Multiple studies have shown that *H. axyridis* has a high resistance to many different parasites (see Haelewaters *et al.* 2016 for a review). It is often thought that this is driven by enemy release, or the lack of co-evolved enemies in the invaded range, however, in this case, *D. coccinellae* is found in the native range of *H. axyridis* (Koyama & Majerus 2008). The immune capabilities of *H. axyridis* are thought to be considerably stronger than in other ladybird species (Rohrich *et al.* 2012; Schmidtberg *et al.* 2013; Vilcinskas *et al.* 2013a). Indeed, the defensive alkaloids have properties to kill even human pathogens (Rohrich *et al.* 2012). However, in this case, the encapsulation ability does not appear to be higher in *H. axyridis* when compared to the primary host *C. septempunctata*.

In contrast to the low field prevalence of *D. coccinellae* in *H. axyridis*, in Scottish populations of *C. septempunctata*, parasitoid prevalence has been reported at over 60%

(Geoghegan *et al.* 1997). In this study we observed an increase in encapsulation ability in Scottish *C. septempunctata* when hosts were parasitized by *D. coccinellae.* The high encapsulation ability in response to parasitism we have observed in Scottish *C. septempunctata* populations is surprising given that these populations suffer very high prevalence of *D. coccinellae* (Geoghegan *et al.* 1997). One possible interpretation is that although encapsulation is upregulated following parasitism, it is not effective in defending against the developing wasp and therefore does not affect the prevalence of this parasitoid in the field. Indeed, while a greater increase in encapsulation was observed in response to parasitism in the mean encapsulation metric, only the maximum encapsulation ability was significantly upregulated in response to *D. coccinellae* oviposition. The parasitoid may, instead of suppressing the immune system, passively avoid the defences of *C. septempunctata* (Asgari & Schmidt 1994; Eslin & Prévost 2000; Nappi 2010). While the results collected from English sampled populations, with the exception of *C. septempunctata* females, are consistent with *D. coccinellae* suppressing the immune response*,* the result from the Scottish populations suggests that downregulation of the encapsulation response does not occur ubiquitously for all *D. coccinellae* parasitism. The sex-specific reduction in encapsulation ability of English *C. septempunctata* ladybirds may indicate geographic variation in *D. coccinellae* or the host ladybird species; however, our study was not designed to test this.

This study observed the encapsulation of nylon implants by ladybirds three days after parasitism by *D. coccinellae*, before the time that the parasitoid wasp egg hatches (Firlej *et al.* 2012), and during the time viruses, virus-like particles, teratocytes and non-viral proteins typically affect host immune function (Stoltz *et al.* 1988; Lavine & Beckage 1995; Le *et al.* 2003; Asgari 2012). Therefore, while we did not test what the factor influencing host immune function might be, wasp-derived factors typically impact host immunity during the time frame of our encapsulation assay. Eggs oviposited by *D. coccinellae* develop teratocyte cells from the serosa membrane once inside the parasitized host (Dahlman 1991). Teratocytes play an important role in the nutrition of developing parasitoid larvae (de Buron & Beckage 1997;

Strand 2014), and in *H. axyridis,* Firlej *et al* (2007) found fewer of these nutritional cells in parasitized *H. axyridis* in comparison to *Coleomegilla maculata,* a suitable host for *D. coccinellae* in Canada. The same authors also found differing growth patterns of teratocytes in *H. axyridis,* which may result from unsuitability of the internal environment of *H. axyridis*. In addition, an observation of ladybird behaviour in response to *D. coccinellae* approach and attack found a greater behavioural defence ability in *H. axyridis* compared to *C. maculata* (Firlej *et al.* 2010). Although the host's encapsulation ability and its downregulation by *D. coccinellae* could be hypothesised to play a role in the lower eclosion ability of this parasitoid, in this study we have shown this is not the case. Behavioural traits and internal suitability, rather than encapsulation ability, could therefore play a role in explaining the low prevalence of *D. coccinellae* in field populations of *H. axyridis*.

The body condition of *C. septempunctata* hosts determined the extent of the encapsulation response in both the Scottish and English populations sampled. While this study did not explicitly aim to test this, ladybird hosts in poorer condition displayed a higher encapsulation response than those in better condition. It is generally considered that individuals in better condition possess resources to mount better immune responses (Lawniczak *et al.* 2007; Povey *et al.* 2014). However, a trade-off between encapsulation and antibacterial immune responses to infection have also been demonstrated (Cotter *et al.* 2004; Rantala & Roff 2005), as have trade-offs between immune function and food intake (Adamo *et al.* 2007, 2010). The higher encapsulation ability of ladybirds in poor condition may therefore mean they are resolving these trade-offs differently to individuals in better condition.

This study investigated the encapsulation response of two ladybird species, *H. axyridis* and *C. septempunctata*, comparing the encapsulation ability of each in response to parasitism by *D. coccinellae*. While no difference in encapsulation ability was seen between the two ladybird species, generally for populations sampled in England, there was an indication that the presence of parasitism resulted in a reduction of the hosts' encapsulation response. In contrast, Scottish populations of *C. septempunctata* displayed an increase in encapsulation ability in the presence

of *D. coccinellae* parasitism. The opposite trends observed in these two populations could indicate the presence of geographically differentiated *D. coccinellae* strains, however further study is needed to determine this. The invasive species *H. axyridis* has a lower field prevalence of *D. coccinellae* than *C. septempunctata,* and our results indicate this is not due to asymmetries in the encapsulation ability of the hosts in the presence of this parasitoid. However, we do not rule out that the disparity could be due to other aspects of immune defence, for example the chemical alkaloids seen in *H. axyridis*, which are known to be potent defences against other parasites, and may affect teratocyte cell development.

Chapter 6:

General Discussion

This PhD thesis aimed to address the role of parasites in the invasion biology of *Harmonia axyridis*. This research presents the first detailed examination of the transmission ability and virulence effects of the fungal parasite *Hesperomyces virescens*. A comparison of *H. virescens* infection characteristics found a poorer ability of the parasite to exploit *H. axyridis* in comparison with the ancestral host species, despite high field prevalence in this invasive alien ladybird. Indeed, assessing the role *H. virescens* plays in the rate of ageing, this thesis provides evidence that this fungal parasite accelerates the ageing process in *H. axyridis*. In addition, the research presented here shows *H. virescens* infection increases the virulence of an acute fungal parasite in male hosts of two ladybird species. While *H. axyridis* has been previously noted to have high resistance to multiple parasites, this thesis finds no strong evidence to suggest that *H. axyridis* has a stronger encapsulation response than a UK native ladybird species when parasitized by generalist parasitoid wasp. Primarily, *H. axyridis* has been a model species in invasion ecology; however, the research presented here demonstrates this ladybird is also a useful system for other studies, such as in the study of ageing and the sex-biased effects of infection.

6.1 A model system beyond invasion ecology?

As a highly successful invader, *H. axyridis* is frequently used as a model system in invasion ecology (Roy & Wajnberg 2008; Roy *et al.* 2016a). However, in this thesis, *H. axyridis* has instead been used as a model to investigate the role of infection in accelerating the rate of ageing. Infection with the fungal STI, *H. virescens,* resulted in an acceleration of the age-related decline in body condition for male *H. axyridis,* as well as causing fecundity senescence and accelerating fertility decline in females. There are few studies on chronic infections in invertebrates, however in humans and other vertebrates chronic infections have been examined in detail: e.g. malaria in great reed warblers, *Acrocephalus arundinaceus*; hepatitis B virus in humans (Shepard *et al.* 2006); and Simian Immunodeficiency Virus (SIV) in monkeys (McClure *et al.* 1989). Yet, is it really possible to compare the effects resulting from infection seen in this thesis to those found in human and vertebrate chronic infections? In humans, acute childhood infection and chronic adult infections have both been suggested

as drivers of accelerated ageing in response to infectious disease (Finch & Crimmins 2004; Hayward *et al.* 2016). However, in humans, the ability to thoroughly test these hypotheses is limited.

The innate immune system, responsible for the inflammation response, is conserved in both vertebrates and invertebrates (Hoffmann 2003) and age-related shifts in immune function have been documented in the model invertebrates *Caenorhabditis elegans* and *Drosophila melanogaster* (Müller *et al.* 2013). Indeed, chronic immune stimulation in *D. melanogaster* has been shown to result in long-term immune up-regulation and a reduction of lifespan (Libert *et al.* 2006). The methods used in this thesis to study the influence of infectious disease on the rate of ageing in *H. axyridis* have allowed the examination of the entire adult lifespan of the infected ladybirds, something that is limited in many human studies. The results presented here provide compelling evidence that chronic infection plays a direct role in the acceleration of ageing in this model system, and causes virulence that seems to be comparable to those seen in human infections. However, without an understanding of the underlying processes behind the observed effects in ladybirds, it is difficult to determine how closely these effects may mirror those seen in vertebrates.

In addition to the observation that *H. virescens* infection produces analogous virulence effects to those seen in vertebrate chronic infections, some effects are also similar to those observed more generally in STIs. In invertebrates, as well as humans, STIs can result in sterility or castration, for example the nematode *Mehdinema alii* infecting the cricket *Gryllodes sigillatus;* and *Neisseria gonorrhoeae* causing pelvic inflammatory disease in humans (Lockhart *et al.* 1996; Knell & Webberley 2004; Luong & Kaya 2005; Lafferty & Kuris 2009; Apari *et al.* 2014). While full sterility was not observed in *H. axyridis* in laboratory conditions, a reduction in the number of eggs produced by females, and the fertility of those eggs, is consistent with other STIs of invertebrates and humans. The laboratory conditions used in this thesis maintained ladybirds individually, restricting the possibility of re-infection or competition for resources; therefore, under field conditions it is plausible the virulence effects might be amplified. However, the ladybirds maintained in the laboratory

were fed artificial diet, rather than a more field-realistic diet of aphids. Despite this, no effect of diet treatment (high and low quality artificial diet) was apparent in chapter 3, suggesting that host nutrition is perhaps not a principal factor affecting the virulence of *H. virescens.*

Considering the similarities of the effects between human and invertebrate chronic infection, it seems that the characterisation of ladybird immune responses to chronic infection, as well as understanding age-related changes in ladybird immune function, would be a logical starting point for future research. While human chronic infections are undeniably important and certainly warrant continued study, future research into the underlying mechanisms of ageing by performing experimental studies on shorter lived, invertebrate organisms with comparable, yet simpler immune systems, could prove invaluable to the understanding of the ageing process.

6.2 To what extent are these parasites causing immune responses?

This thesis has studied the growth of *H. virescens* on ladybirds, identifying that the growth rate varies between species, and sexes, and that virulence effects also vary between males and females. While some sections of this thesis have speculated that this variation reflects differences in host immunity, this has not been verified. The virulence effects of *H. virescens* were greater for male than female *H. axyridis* in this thesis. Males of multiple species have a lower ability to defend against infection than females (Zuk 2009; Stephenson *et al.* 2016), and in chapter 2, the fungal burden of *H. virescens* was considerably higher on males than on females. As resource limitation can reduce immunocompetence (Moret & Schmid-Hempel 2000; Hanssen *et al.* 2004; Cotter *et al.* 2011) a dietary manipulation was included in chapter 3, however, the low quality diet did not amplify the virulence caused by *H. virescens.* It is, however, possible the dietary manipulation used was not sufficient to limit host resources enough to show an effect. It is unclear whether infected ladybirds are physiologically passive to *H. virescens* growth, or whether a strong immune response is generated against the fungal haustorium. While the immune response of ladybirds to *H. virescens* has not been tested, a related Laboulbenialian fungus has been demonstrated to

upregulate immune genes in ant hosts (Konrad *et al.* 2015). This response does not demonstrate that the immune system is able to control a Laboulbenialian infection, only that an immune response is triggered.

In addition to the sex-specific effects of *H. virescens* infection, the results of chapter 2 demonstrate this parasite transmits and develops more effectively on an ancestral host, *A. bipunctata*, than on *H. axyridis*. Several studies have identified the strength of *H. axyridis'* toxic alkaloids, including harmonine, and the antimicrobial peptides generated by the host's humoral defences (Sloggett *et al.* 2011; Fischer *et al.* 2012; Rohrich *et al.* 2012; Schmidtberg *et al.* 2013). While one explanation of the lower ability of *H. virescens* to exploit the IAS in comparison with *A. bipunctata* could be a higher immune ability of *H. axyridis,* the results of chapter 2 do not support this hypothesis. Artificial intra- and interspecific transmission indicated that some species-specific adaptation has occurred in the parasite, with each set of fungal strains transmitting at a higher rate to the host species from which they were derived. Additional support that the defence ability of *H. axyridis* against infection does not drive the species-specific results of chapter 2 can be found in chapter 4, where *H. axyridis* lacked superiority over *A. bipunctata* in dealing with co-infection, although as discussed in chapter 4, it is possible this was driven by a higher dose of the secondary fungal parasite *Beauveria bassiana.* In addition, the results presented in chapter 5 do not demonstrate the encapsulation ability of *H. axyridis* to be any greater than that of *Coccinella septempunctata.*

The ability of *H. virescens* to exploit *A. bipunctata* more effectively than *H. axyridis* observed in chapter 2 therefore must result from a mechanism other than superior immunity in the invasive alien species. The artificial infections in chapter 2 were designed to detect whether host or fungal parasite differences drove the different transmission and growth rates in the host ladybird species. The results appear inconclusive, showing that the actual situation is neither directly related to the suitability of a particular host species to all fungal strains, nor of particular fungal strains to both host species. In reality, chapter 2 suggests a level of species-specific adaptation has occurred, showing intraspecific transmission rates to be the highest for both species. The enemy release hypothesis is usually focused on genetic

compatibility of host and parasite species in the invaded range. However, the increase in the field prevalence of *H. virescens* in populations of the invader indirectly suggests that ecological factors, such as the reproductive biology of *H. axyridis*, as well as species-specific adaptation, may drive high transmission rate in this species. In light of these findings, a thorough investigation of the immune response generated in response to *H. virescens* infection would considerably add to the understanding of the effects observed in this thesis.

6.3 How has the arrival of *H. axyridis* changed the parasite dynamics in UK ladybirds?

One of the observations made in this thesis was the dramatic increase in *H. virescens* prevalence in invasive alien London populations of *H. axyridis* (chapter 2). In chapter 4, infection with *H. virescens* was demonstrated to exacerbate the virulent effect of the fungal parasite *B. bassiana* in males of two ladybird species. In addition, *H. virescens* infection has also been demonstrated to cause virulence in *H. axyridis* (chapter 3), although these effects did not occur until approximately 100 days after the initial exposure to infection, and infected ladybirds are still able to reproduce. Indeed, a field prevalence of above 70% in the *H. axyridis* population observed in chapter 2 demonstrates this fungal parasite persists in field populations of this invader, and at a higher level than the maximum reported UK prevalence in *A. bipunctata* of just below 50% (Welch *et al.* 2001).

The initial host shift of *H. virescens* from *A. bipunctata* to *H. axyridis* is likely to have occurred during mixed species overwintering aggregations where these two ladybird species co-occur (Nalepa & Weir 2007). Indeed, the *H. virescens* field infections of UK *H. axyridis* are found in the same area as those reported in *A. bipunctata.* Therefore, the high field prevalence of this fungal parasite in the *H. axyridis* populations sampled in chapter 2 make it plausible that spillback from the IAS to *A. bipunctata* could occur (Figure 6.1). Furthermore, *H. axyridis* has high population densities in areas of South East England, and its habitat overlaps considerably with *A. bipunctata* (Roy *et al.* 2012b; a). While interspecific transmission is considerably less efficient than intraspecific transmission (chapter 2; Figure 6.1), the combination of a large number of individuals and the high prevalence of *H. virescens*

within some of these populations could increase the likelihood of infectious contact between these two ladybird species, which may cause spillback to be relatively common. However, during the fieldwork of this PhD, I have not seen a single *H. virescens* infected *A. bipunctata* from the sampling sites in London (approximately 150 observed under a microscope, plus unknown others inspected in the field). Chapter 2 of this thesis suggests that a level of species-specific adaption by *H. virescens* has occurred since the initial host shift, which could limit the interspecific transmission of this fungus in London ladybird populations. Furthermore, the arrival of *H. axyridis* into the UK has resulted in the decline of many native ladybird species, including *A. bipunctata* (Roy *et al.* 2012a). Previously, the prevalence of *H. virescens* in *A. bipunctata* was found to be highest in the centre of London, and lower towards the edge of the city (Welch *et al.* 2001), where the likelihood of more than one generation per year, and therefore adult inter-generational cohort overlap in the early summer, is more consistent. It is plausible that the displacement of *A. bipunctata* by *H. axyridis* in the centre of London restricts the number of generations *A. bipunctata* is able to complete annually, limiting adult overlap and therefore restricting the intraspecific transmission of *H. virescens* in this native species. In addition, the sexual contact rate of *A. bipunctata* is linked to host density (Ryder *et al.* 2005), therefore the decline of this native ladybird species, as a result of *H. axyridis* invasion (Brown *et al.* 2011a), may influence the field prevalence of *H. virescens* in *A. bipunctata.*

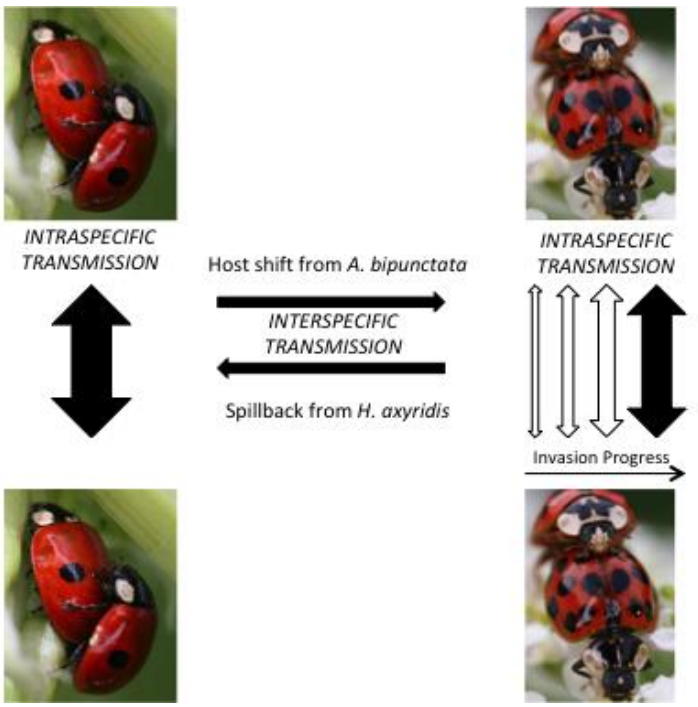

Figure 6.1: Conceptual diagram depicting the initial host shift of *H. virescens* from *A. bipunctata* to *H. axyridis*. Following a host shift, within species transmission continues in both species, although at a lower rate per contact in *H. axyridis* than in *A. bipunctata,* due to a poor fungal growth of *H. virescens* on the IAS (chapter 2). While interspecific transmission was responsible for the initial host shift, the results of chapter 2 demonstrate this transmission route to be far less efficient than intraspecific transmission. Intraspecific transmission is likely to increase, and the infection become more prevalent, as an invasion progresses over time as the fungus adapts to utilise the invader. The high field prevalence of *H. virescens* in *H. axyridis* populations (chapter 2) suggests that the fungus could spillback from *H. axyridis* to *A. bipunctata* at a higher rate than the initial host shift occurred, however, chapter 2 also suggests spillback from *H. axyridis* to *A. bipunctata* should be inefficient due to evidence of some host-specific adaptation, and the lower *H. virescens* burden on *H. axyridis* in comparison with *A. bipunctata.* (photos: Mike Majerus).

If the maintenance of a fungal STI is indicative of the reproductive biology of *H. axyridis* being permissive of such infections, then it is possible other STIs not currently in the UK may be able to establish as emerging diseases. Although primarily associated with *A. bipunctata,* the sexually transmitted mite, *Coccipolipus hippodamiae,* has been found naturally occurring in some European populations of *H. axyridis,* although as yet not in the UK (Rhule *et al.* 2010; Raak-van den Berg *et al.* 2014). *Coccipolipus hippodamiae* has never been able to establish in UK *A. bipunctata* populations, likely due to the inconsistent overlap of generations in this ladybird species (Webberley *et al.* 2006; Pastok *et al.* 2016). In contrast to *A. bipunctata*, *H. axyridis* is more commonly bivoltine in the South of England (Roy *et al.* 2013), and the resulting overlap of adult generations required for the transmission of STIs between cohorts raises the possibility of a *C. hippodamiae* epidemic occurring in UK populations of this IAS. An association between this parasitic mite and *H. axyridis* in the UK

could result in two outcomes. Firstly, *C. hippodamiae* could act as a naturally occurring biological control agent for *H. axyridis* due to the fact that it causes female host sterility, thereby reducing the dominance of this invasive ladybird species. Secondly, while the mite species may be able to infect *H. axyridis*, spillback could occur such that mites are introduced into the native *A. bipunctata* population already under pressure from *H. axyridis.* The former of these two outcomes could reduce the ecological advantage provided by enemy release in the invader, allowing the recovery of UK native ladybirds, which have declined in the presence of *H. axyridis.* In addition, the decline of *A. bipunctata* resulting from the invasion of *H. axyridis* may have reduced the sexual contact rate in this species, therefore restricting the ability of STIs to spread in the UK population of this ladybird. However, in the event of *C. hippodamiae* appearing in UK *H. axyridis* populations*,* continued low-level spillover from infected *H. axyridis* may enable *C. hippodamiae* infections to be sustained in *A. bipunctata* populations, causing further declines of this UK native ladybird.

6.4 Sex–biased effects of infection

In all of the chapters of this thesis sex-specific effects were observed. While chronic *H. virescens* infection of *H. axyridis* significantly reduced the lifespan of all individuals, the reduction was greater for male ladybirds (chapter 3). In addition, the acceleration in body condition senescence was greater for male than female *H. axyridis* when infected with *H. virescens.* In chapter 2, *H. virescens* developed more effectively on males of both *H. axyridis* and *A. bipunctata* than it did on females, while in chapter 4, co-infection between two fungal pathogens significantly reduces the lifespan of UK native and invasive alien male ladybirds, yet not females. The fact that the negative impacts of infection observed in this thesis are focussed on males could be due to the ability of females to either restrict parasite infection and growth, for example via superior immune ability (resistance), or could indicate that females are better able to reduce the negative fitness consequences associated with infection (tolerance). While chapters 2, 3, and 4 in this thesis could suggest male ladybirds may have a greater immune ability than females, the results of chapter 5, which measured the encapsulation response of *H. axyridis* and *C. septempunctata,* appear inconsistent between

host populations and species. The encapsulation response of *H. axyridis* did not differ between the sexes in *Dinocampus coccinellae* parasitized or un-parasitized individuals, however male *C. septempunctata* varied in their response to parasitism across populations (chapter 5). While in Scottish populations, male *C. septempunctata* upregulated their encapsulation to a greater extent than females, English *C. septempunctata* males had a downregulated encapsulation response in the presence of *D. coccinellae* parasitism.

Across all chapters of this thesis there is consistent evidence of the sex-biased effects of infection. With the exception of the Scottish population in chapter 5, male ladybirds were consistently more negatively impacted by infection; observed as lifespan effects, the ability of parasitoids to downregulate the encapsulation response, and the faster growth and development of a fungal parasite on males. Male-biased parasitism exists across multiple taxa (Zuk 1990, 2009), however the mechanism behind the observed bias has been debated (Rolff 2002). The sex difference in immune function is one mechanism given consideration for male-biased parasitism (Restif & Amos 2010; Schmid-Hempel 2011). Immune defences are important when considering the fitness of an individual, and certain sex-specific trade-offs exist within the sexes for various life history traits (Stoehr & Kokko 2006; Zuk 2009). The trade off between immune and reproductive investment has been shown to exist in males and females, resulting in an increase in female immune investment at reproductive age in *G. texensis,* but a reduction in the immune investment of males at the time sexual signalling begins (Adamo *et al.* 2001). Similarly, a reduction in immune investment in male wolf spiders (*Hygrolycosa rubrofasciata)* signalling to females also demonstrates the trade off between reproduction and immunity (Ahtiainen *et al.* 2005). Bacelar *et al.* (2011) predict that male-biased parasitism exists more so for males with shorter lifespans or who suffer greater resource competition than females. In addition, sexual selection has also been suggested to result in male-biased parasitism, with polygamous males allocating more resources away from immune defences into reproductive effort than monogamous species (Zuk 1990, 2009). Ladybirds are a polygamous group of insects, and while the immune defences of this group

are not fully characterised, the sex-biased effects of infection seen in this thesis are consistent with those seen in other taxa.

6.5 Conclusions

This thesis provides the first detailed examination of the fungal parasite *H. virescens* in association with two ladybird species found in the UK. While the research surrounding *H. axyridis* has commonly focused on invasion biology, this thesis has demonstrated the use of this species as a model insect in the study of ageing; demonstrating that a chronic infectious disease can accelerate the rate of ageing in an invertebrate, a concept that has been debated in humans. In addition, this thesis provides some evidence to support a level of species-specific adaptation of *H. virescens* in *H. axyridis* and *A. bipunctata*. The infection of *H. axyridis* by *H. virescens* is likely the result of a host shift by the parasite to exploit the IAS. While this fungal parasite was previously considered to be avirulent, the work presented here indicates not only that this parasite is virulent on an IAS, but also that infection with this parasite can exacerbate the effect of secondary infection. Although *H. virescens* is unlikely to significantly reduce the invasive population of *H. axyridis*, its high prevalence may result in sustained spillback of this parasite into native ladybird populations.

Chapter 7:

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