# Investigating the Biology of Host Shifts of Heritable Microbes

Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of Doctor in Philosophy

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# Abstract

Arthropods are commonly infected with chronic bacterial infections that are passed from mother to offspring, typically transovarially. Obligate mutualists provide arthropod hosts with nutrients, facultative mutualists protect hosts against natural enemies, and reproductive parasites alter the host's offspring sex ratio. These phenotypes ensure future transmission events of the endosymbiont. Heritable microbe frequencies vary across host populations. Some species, for example *Wolbachia*, are predicted to be in over 50% of all arthropod species and do not appear to have any focussed taxonomic affiliations whereas *Cardinium* is present in 7% of species, with spiders and mites a pronounced incidence hotspot. The incidence of heritable microbes in nature is in part a product of their capacity to host shift. The success of the host shift depends on host compatibility (ability to receive and transmit a novel endosymbiont without the bacteria causing excess pathology or eliciting the host's immune response). Compatibility evolves independently of the novel endosymbiont and a key determinant of the bacteria's success is the phylogenetic distance of the novel host from the ancestral host. However, the extent to which host compatibility is an evolvable trait is unknown. In this thesis, I address key factors affecting host compatibility to novel endosymbiont infection. I assess the speed of evolution of host compatibility in the *melanogaster* subgroup of drosophilids, to novel endosymbiont, *Spiroplasma poulsonii* and find closely related species do differ in compatibility, as predicted by the phylogenetic clade model of compatibility. I serendipitously observed a phenotype switch in the bacterium in laboratory culture and compile preliminary evidence to form a better representation of the endosymbiont's behaviour. Within this system, I also investigate the role of gut microbiota in determining host compatibility and find that gut microbiota and *Spiroplasma* do not interact to influence host life history traits. The thesis then examines interactions of a very different heritable microbe, *Arsenophonus nasoniae*. *A. nasoniae* relies on mixed modes of transmission to spread throughout host populations and is highly infectious. I observe that *A. nasoniae* presents an immune challenge to both adult female and diapausing larvae of its native host, *Nasonia vitripennis*. The bacterium presents fitness costs to novel host, *Nasonia girault*i, reducing fecundity and upregulating immune genes. Further, I present a novel mechanism of symbiont control through oxidative stress and iron sequestration. These findings have important implications for host shift biology of heritable microbes and highlight the importance of including life history traits in evaluations of host-symbiont dynamics.

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# Chapter 1: General introduction

## 1.1 Symbiosis

Symbiosis encompasses a range of long term and intimate associations between unlike organisms, with the larger of these commonly referred to as the host, and the smaller the symbiont. Symbiotic interactions vary along a continuum, but we most often discuss the extremes, which are parasitism, where the host's fitness is compromised by the presence of the symbiont and mutual dependence, where host fitness is compromised in the absence of the symbiont (de Bary, 1879). The most common type of symbiosis is facultative and ecologically contingent mutualism, whereby the symbiont shifts from beneficial to costly depending on the host environment (damage to benefit ratio) (Werren *et al*, 2008).

Symbiotic interactions can occur between organisms of distinct biological kingdoms, and requires at least two individuals from disparate species. One party typically lives on or in close proximity to the other. Microbes tend to live inside their host and are often restricted to symbiosis-dedicated organs (Braendle *et al*, 2003). A symbiont may only be required during a particular developmental stage of its host, or it may exert an impact throughout the host's lifetime. Reciprocally, the symbiont may need to be of a particular age, or in the case of microbes, titre before it can exert an impact on its host.

The relationship between symbiont and host may be transient and end within a host's lifetime, as can be the case for environmentally acquired microbiota. Other relationships may be ancient, where the strict congruence between host and symbiont phylogenies implies the symbiont is transmitted from one generation to the next over many millions of years. Over evolutionary time, symbioses have led to increased biological and ecological complexity as well as enabling species diversification – for instance living on different dietary niches or in different environments. New symbioses are commonly formed via transmission of a symbiont from one host species to another but the host and symbiont factors that determine whether an organism will be a suitable host or not are often unclear.

## 1.2 Heritable symbiosis in insects

### *1.2.1 Overview of bacterial endosymbiosis*

Symbionts that live within the host's body are referred to as endosymbionts. Pertri first noted insect-microbe endosymbioses in 1909 following the discovery of a bacterium associated with the Italian olive fly (Petri, 1909). The bacterium was later characterised as the uncultivable endosymbiont, *Candidatus* Erwinia dacicola (Capuzzo *et al*, 2005). Associations between insects and microbes were soon discovered to be widespread amongst the insect orders Hemiptera, Blattaria and Coloeptera (Buchner, 1965). Today, insect-microbe symbioses are accepted as being widespread in nature (Moran and Baumann, 2000; Duron *et al* 2008). A recent study by Weinert *et al*, 2015, estimates that approximately 52% of arthropod species harbour the intracellular endosymbiont *Wolbachia*.

Where the host depends upon their symbiont for normal growth and reproduction and vice versa, the symbionts are called primary or obligate endosymbionts (Douglas, 1998). Primary endosymbionts are often associated with hosts that live on nutritionally restricted diets such as phloem sap or vertebrate blood and supplement their diet with otherwise unavailable amino acids or vitamins (Moran and Telang, 1998). Primary endosymbionts reciprocally require the association and cannot be cultured *in vitro*. Secondary or facultative endosymbionts are not required by their host for survival but the bacteria may nevertheless require a host to survive. These microbes may co-occur with primary endosymbionts in bacteriocytes or reside elsewhere in the host. Both primary and secondary endosymbiotic bacteria influence their hosts life history and incidence in a given environment.

Most endosymbionts are maternally inherited through trans-ovarial transfer of the bacteria from the mother to the developing eggs or embryos (Buchner, 1965). There are also peroral routes of maternal transmission whereby bacteria injected into the offspring's host are ingested by the developing offspring (Werren *et al*, 1986; Skinner, 1985).

### *1.2.2 Phenotypes*

Where vertical transmission is imperfect, purely heritable microbes are maintained by 'drive' phenotypes that ensure their persistence in the host. Beneficial phenotypes include protection against viruses or predators (Oliver *et al*, 2003; Hedges *et al*, 2008; Teixeira *et al*, 2008; Hamilton and Perlman, 2013), and metabolic provisioning. Parasitic phenotypes include alteration of the host's offspring sex ratio (Hurst *et al*, 1993; McCutcheon *et al*, 2009). Some endosymbionts can combine beneficial and parasitic phenotypes, such as the *Spiroplasma* symbiont of *Drosophila melanogaster*, which kills male offspring, but protects female hosts from wasp attack (Xie *et al*, 2014).

Buchner, a pivotal scientist in developing our understanding of the diversity and importance of endosymbiosis, first observed that bacteria associated with sap-feeding aphids (later named *Buchnera aphidicola*) were restricted to specialised host cells called mycetocytes. Later they were found to be maternally transmitted (Buchner, 1965; Baumann *et al*, 1995). *Buchnera* supplements the host's diet, which lacks key amino acids required for growth and development, and thus the host is able to exploit unusually restricted nutritional resources. It also limits the global distributions of the aphid due to extensive genome reduction in the symbiont, which makes it sensitive to high temperatures, negatively affecting host fecundity and performance (Dunbar *et al*, 2007).

Facultative endosymbionts with protective phenotypes allow their arthropod hosts to persist in environments where natural enemies are present, thereby allowing ecological release and niche exploitation (Jones *et al*, 2011). For example, when the pea aphid is infected with the facultative bacterial symbiont *Hamiltonella defensa* and its associated phage, parasitic wasp larvae are unable to develop inside the aphid host (Oliver *et al*, 2005). Strains of symbionts in the *Spiroplasma and Wolbachia* genera also confer protection against various parasitic elements and thus increase their host's fitness in their environment (Jaenike *et al*, 2010a; Jaenike *et al*, 2010b; Xie *et al*, 2014).

For a maternally transmitted symbiont. a male host represents a 'dead end' and thus symbionts have evolved methods to manipulate host reproduction to favour the production of female hosts. These include: feminization, where male embryos are converted to female embryos; parthenogenesis, where females produce offspring without requiring fertilization; cytoplasmic compatibility (CI), where reproduction between a male infected with a specific symbiont strain and an uninfected female is incompatible and finally male-killing, where selective destruction of male embryos or larvae presents a fitness advantage to females of the same brood (Engelstadter and Hurst, 2009). Reproductive parasitism has evolved de novo numerous times in different genera of endosymbiont, including *Wolbachia*, *Spiroplasma*, *Cardinium*, *Arsenophonus* and *Rickettsia* (Duron *et al*, 2008).

## 1.3 Host shift biology of heritable endosymbionts

A host-shift occurs when either a heritable element (such as a mobile element, reproductive parasite, microbe or beneficial symbiont), or a non-heritable infecting microbe, jumps from a permissive host species in which it routinely circulates to a naïve host species. The

frequency of movement is variable: some symbionts move commonly, some rarely. Some move to only closely related host species and could be considered specialist, others can make large jumps across phylogenetically distinct clades and are said to have a 'broad host range' (BHR). Like plasmids the bacterial endosymbiont, *Wolbachia*, has a BHR and can transfer between members of different arthropod orders (Heinemann and Sprague, 1989; Weinert *et al* 2015). In contrast, 'specialists' such as the cicada endosymbiont, *Hodgkinia cicadicola,* replicate autonomously within their hosts and typically undergo extensive genome reduction (Gil *et al*, 2002). A host shift event may result in a dead end, with no onward transmission. Alternatively, a symbiont may transmit within the new host species, resulting in an epidemic or fixation within the new population.

There are three processes that determine whether a symbiont can jump from its ancestral host species to a novel host species. First, there must be exposure and infection of an individual of the novel host, which depends on the behaviour and ecology of both species. Second, compatibility is required such that the parasite or symbiont can proliferate in the novel host and establish infection without excess pathology, and with opportunity for onward transmission. Third, the host must be one in which the novel symbionts achieves the basic reproductive number, $R_0$, > 1. If the $R_0$ is < 1, each existing infection causes less than one novel infection and thus there is little transmission potential amongst the novel host species (Dietz, 1993; Woolhouse, 2002). $R_0$ is partly determined by compatibility but may also be influenced by symbiont density (e.g. peroral transmission routes (Nadal-Jimenez *et al*, 2019) or other host features such as natural enemy pressure (e.g. for a protective symbiont (Xie *et al*, 2015)).

Many symbionts combine both vertical and horizontal transfer to persist in host populations (Itoh *et al*, 2014). Some endosymbionts may persist purely via their ability to infect. For certain symbionts, horizontal transfer may be of equal if not greater importance at ensuring trans-generational transmission. For example, *Arsenophonus nasoniae,* an extracellular male-killing bacterial endosymbiont of the parasitic wasp *Nasonia vitripennis*, depends on horizontal transfer to spread and reach fixation in the host population (Parratt *et al*, 2016). Infected parasitoids will often coparasitize fly pupal hosts with uninfected conspecifics, a phenomenon called superparasitism, which creates transmission opportunity. Individuals among different parasitoid wasp species may also infectiously transmit *A. nasoniae* amongst themselves, in a process called multiparasitism. *A. nasoniae* has also shown mobility in the filth fly wasp community, likely mediated by multiparasitism events (Duron *et al*, 2010).

Obligate intracellular symbionts require trans-generational transmission to remain associated with the host population. For these symbionts, vertical transmission is the dominant method of symbiont acquisition and maintenance. For a heritable endosymbiont to become established in a novel host population, it must first transmit horizontally and then infect the female germline or ovipositor where it can be passed on to the offspring (Herren *et al*, 2013). Jaenike *et al*, 2007 observed that ectoparasitic mites of *Drosophila* can transfer *Spiroplasma* from an infected individual to an uninfected host. Furthermore, they showed that post transinfection, *Spiroplasma* can vertically transmit to the novel host's offspring. According to Herren and Lemaitre, 2011, spiroplasmas that are unable to access the female reproductive organs cannot be transmitted to the host's offspring.

Once a symbiont has entered host cells and caused infection, it must proliferate and avoid inducing a hyper-immune response. If the novel host does not have the same genetic machinery as the ancestral host then *de novo* mutations in the symbiont directly following a host-shift may be of great importance to its maintenance. These mutations may alter the efficiency of binding to host cell receptors and in turn alter the symbiont's success in entering host cells. Mutations and deletions of genes may reduce symbiont virulence thereby reducing the cost of infection in the novel host (Shigenobu *et al*, 2000; Dale *et al*, 2002). The number of mutations required, the mutational order and the mutational target size will differ between symbiont and their host's genotypes (Longdon *et al*, 2014). Parasites with high mutation rates may establish and adapt to their novel host more quickly than parasites with low mutation rates. On the other hand, if the symbiont has a high rate of deleterious or even lethal mutations, then a high mutation rate is likely to be disadvantageous (Carrasco *et al*, 2007). Post host-shift evolution could also impose fitness costs on the ancestral host and alter the symbionts compatibility to the ancestral host (Ferris *et al*, 2007). Duffy *et al*, 2006 show that mutations in an RNA virus P3 gene, which are important for host attachment, are antagonistically pleiotropic. In other words, the mutations that aid parasite establishment in the novel host present a fitness cost in the ancestral host.

A host shift may result in species diversification of either the symbiont or the host, or both, via adaptation to the novel host or cospeciation. Where speciation of parasites occurs independently of host speciation, it is often a result of a host shift, whereby the parasite becomes isolated from its ancestral lineage following lateral transfer (de Vienne *et al*, 2013). McTaggart *et al* 2016, show that pathogenic rust fungi diversified in a short amount of time and more importantly, they did not evolve at the same tempo as their current plant hosts. The divergence ages for rust fungi mirror the host ages of ancestral plant species. This

phylogenetic incongruence suggests that frequent host shifts were the main driver of speciation in rust fungi (McTaggart *et al*, 2016).

## 1.4 Emerging questions in host shift biology

### *1.4.1 Speed of evolution of compatibility amongst closely related species*

Horizontal transmission events are rare but they are of ecological importance (Werren *et al*, 1995; Charleston and Roberston, 2002). A good predictor of host compatibility is the genetic distance of the novel host from the ancestral host whereby the success of parasite infection decreases with increasing phylogenetic distance from the host-of-origin (Engelstadter and Hurst *et al*, 2006). Increasing genetic distance is likely associated with decreasing similarity in cellular environments, immune response and cell surface receptors between the host species. Tinsley and Majerus, 2007, observed that compatibility of a male-killing *Spiroplasma* of the coccinellid beetle, *Adalia bipunctata* decreases with increasing phylogenetic distance from the ancestral host and the ability of the bacterium to male-kill reduces with increasingly distant inter-specific transfers. Thus, phylogeny may constrain male-killer host-shifts. Gilbert and Webb, 2007 show that the proportion of plant species that developed disease following inoculation with a fungal pathogen decreases with phylogenetic distance from the natural host. Moreover, phylogeny is sufficient in describing spillover and cross-species transmission events of rabies virus in bats (Streicker *et al*, 2010; Faria *et al,* 2013). The studies outlined above suggest that host compatibility is simple product of general divergence between species, a trait where one can predict easily which novel species are likely to be infected based on their phylogenetic proximity to the ancestral host.

Geographical overlap has less of an influence on the incidence of cross-species transmission events. For example, Perlman and Jaenike, 2003 show that the potential host range of parasitic nematodes in drosophilids is greater than the observed host range. In this case, genetic distance is still a better predictor of successful host-shifts versus sympatry. For primates, who share similar niches, behaviours and geographical overlap, high contact rates may lead to host-shifts. However, Faria *et al*, 2013, show that geographical overlap is only a modest predictor of host-shift events between primate species.

Closely related host species do not always share the same components of the immune response or internal machinery due to mutational loss and gain of functions. This process leads to a more nuanced view of host compatibility, where variation in susceptibility and

compatibility to novel infections can arise over small phylogenetic distances, affecting symbiont incidence within host clades. This is referred to as host phylogeny or the phylogenetic clade effect (Longdon *et al*, 2011). This theory implies that host compatibility is an evolutionarily labile trait whereby closely related hosts may respond differently to novel symbiont infection, regardless of their phylogenetic distance from the ancestral host.

Further, symbiont phylogeny may affect symbiont incidence. For example, a novel symbiont may be able to bypass the host's immune recognition system if the novel host is already infected with a closely related bacterium. Longdon *et al*, 2011 show that viral titre in novel *Drosophila* hosts decreases with increasing phylogenetic distance from the ancestral host. However, they also show that viral replication and persistence is determined equally by phylogenetic distance and host phylogeny. A similar case may be true for bacterial endosymbionts.

### *1.4.2 Heritable vs microbiota compatibility links*

In most animals, the largest and most diverse community of microbes resides in the gut lumen. Gut microbes are ecologically contingent mutualists and range in phenotype from beneficial to pathogenic, depending on environmental factors such as diet and microbiome composition. In *Drosophila*, gut microbiota are important for normal development and can alter host nutritional allocation by interacting with the insulin pathway (Shin *et al*, 2011; Storelli *et al*, 2011). Gut microbes also alter host nutrition by producing dietary supplements or consuming ingested nutrients (Bäckhed *et al*, 2004; Newell and Douglas, 2014).

The *Drosophila* microbiome provides an ideal model for investigating the effects of gut microbiota on host nutrient allocation and physiology. The composition of the host microbiome is shaped by diet (Chandler *et al*, 2011). Typically *Drosophila* species feed and lay their eggs on fermenting or decaying fruit and other plant material, although it is unclear whether the microbes they ingest pass through the gut transiently, or whether they take up permanent residency (Pais *et al*, 2018). In laboratory culture, the microbiome is made up of less than 30 microbial species, of which two species of *Acetobacter* and two species of *Lactobacillus* dominate (Chandler *et al*, 2011; Wong *et al*, 2011). Upon removal of the gut microbiota via egg dechorionation and growth in axenic medium, larval development time of *D. melanogaster* increases (Newell *et al*, 2014; Wong *et al*, 2014). Thus, microbes present on the surface of the egg chorion are important for larval development of the fruit fly (Ridley *et al*, 2012).

Axenic (germ-free) flies have altered nutrient allocation patterns, with elevated glucose and triglyceride concentrations. Newell and Douglas, 2014, show that normal glucose levels can be restored in axenic flies upon the re-introduction of any of the 4 dominant gut bacterial species. However, to attain normal lipid concentrations and larval development time, *Acetobacter* and *Lactobacillus* must both be present. By altering the composition of fly microbiota, we can measure its impact on host nutrient allocation and in turn the effects of nutrient allocation on symbiont-host interactions.

Like Acetobacters, an endosymbiont of fruit flies, *Spiroplasma*, utilizes host lipid to proliferate. When lipid is limited, *Spiroplasma* proliferation is reduced or even inhibited (Herren *et al*, 2014). An insect's bacterial endosymbionts should be characterized within the context of the host's microbiota. Conflict for host resources may create a mismatch between the microbiota and endosymbiont populations and could ultimately determine whether a host is compatible for novel endosymbiont infection.

Compatibility driven by the gut microbiota has been evidenced in *Anopheles gambiae* mosquitoes. This species is naturally unable to vertically transmit transinfected *Wolbachia*. Whilst some strains of the bacteria are able to access the germ line, they are not passed to the offspring. In another *Anopheles* species, *Wolbachia* somatic transinfection causes blood-meal induced death. Following antibiotic treatment, Hughes *et al*, 2014 observed perfect maternal transmission of *Wolbachia* in both *Anopheles* species and reduced titres of one particular bacterial species, *Asaia*, in the gut of the mosquito. Thus, components of the gut microbiota can inhibit vertical transmission of maternally inherited endosymbionts and may in part explain why some insect species are infected in nature whilst other are not.

### *1.4.3 Drosophila/Spiroplasma/parasitoid system*

To address the emerging questions in host shift biology of heritable microbes, I use the *Drosophila/Spiroplasma/*parasitoid system to measure the rate of evolution of host compatibility. Nakayama *et al*, 2015 observed that *Spiroplasma* native to *Drosophila hydei* performed poorly in novel host *Drosophila melanogaster*. Thus, there is opportunity to investigate the compatibility status of other species in the *melanogaster* subgroup, as they are equivalent in their genetic distance to the endosymbiont's ancestral host. Furthermore, *Spiroplasma* is easily transferable between *Drosophila* species, via microinjection and can be identified via PCR analysis.

*1.4.3.1 Drosophila melanogaster species subgroup*

Species in the *Drosophila melanogaster* subgroup originate from tropical sub-Saharan Africa. *Drosophila melanogaster*, the common fruit fly, first moved out of Africa to Europe and Asia between 10,000 to 15,000 years ago (David and Capy, 1988). More recently it has spread as a human commensal to the Americas and Australia in association with human travel and colonization. Today *D. melanogaster* has a global distribution and resides on every continent except Antarctica. The species primarily feeds on rotting fruits and plant material (Markow, 2015). Its short generation time and ease of laboratory culture has led to its extensive study over the last 100 years and provided much insight into key biological processes.

There are nine species in the *melanogaster* subgroup, of the Sophophora subgenus. *Drosophila simulans*, the closest relative of *D. melanogaster* is also a cosmopolitan species and is thought to have diverged from *melanogaster* around 3 million years ago (Mya). Despite its cosmopolitan status, *D. simulans* is not found worldwide (David *et al*, 2007). *D. sechellia* is endemic to the Seychelles archipelago and is highly specialized to toxic *Morinda citrifolia* fruit. It is a close relative of *D. simulans*, which co-occurs on the same islands in the Seychelles archipelago. It is thought to have separated from *simulans* around 500,000 years ago (Garrigan *et al*, 2012). Anatomically, these 3 species are very similar but they can be differentiated based on the male genitalia (Kliman *et al*, 2000). *D. yakuba* is prevalent in sub-Saharan Africa, where it is associated with human activity.

*1.4.3.2 Spiroplasma poulsonii*

*Drosophila* species harbour two maternally transmitted bacterial endosymbionts, *Wolbachia* and *Spiroplasma* (Mateos *et al*, 2006). *Wolbachia* is the best studied endosymbiont and different strains have wide ranging effects on the behaviour and life history of their hosts (Werren *et al*, 2008). Less is known about the significance of *Spiroplasma* infections. Spiroplasmas belong to the Gram-positive division, and are helical and actively motile bacteria of the *Mollicutes* class (Sakaguchi and Poulson, 1961). Unlike *Wolbachia* which resides in the cytoplasm of host cells, *Spiroplasma* occupies a largely extracellular niche in *Drosophila*, being present in numbers in the haemolymph (Sakaguchi and Poulson, 1961). The spiroplasmas that infect *Drosophila* species originate from a clade of plant pathogenic strains, *Spiroplasma citri*, *S. kunkelii* and *S. phoeniceum* (Bové, 1997; Haselkorn *et al*, 2009). All 3 plant pathogens are restricted to the phloem sieve tubes and are infectiously transmitted between plants by phloem feeding leafhoppers (Bové *et al*, 2003). Some

spiroplasmas are entomopathogens. For example, *S. melliferum* and *S. apis* induce mortality in the honey bee whilst other spiroplasmas are closely associated with insect guts and are thought to be commensals (Wedincamp *et al*, 1996).

Maternal transmission has arisen several times in the genus *Spiroplasma*. Like *Wolbachia*, *Spiroplasma* can alter the host's offspring sex ratio to increase the number of females and thus increase the number of infected individuals in a population (Hurst *et al*, 1999a; Hurst *et al*, 1999b). *Spiroplasma* has been reported in 16 *Drosophila* species including *D. melanogaster* and *D. simulans*, out of 200 species tested (Haselkorn *et al*, 2009). However, only a single *D. simulans* individual out of 236, tested positive for the infection (Watts *et al*, 2009).

The *Spiroplasma* Melanogaster Sex Ratio Organism (MSRO) strain was originally described as the cause of reproductive parasitism through male-killing, but has more recently been observed to protect *Drosophila melanogaster* against parasitoid wasp attacks (Xie *et al*, 2014). In this case, the survival advantage to the *Drosophila* host is small but significant and provides additional drive to maintain the symbiont in host populations. The strength of *Spiroplasma*'s protective phenotype differs with the parasitoid wasp strain used. In our lab, we have observed variation in virulence between two *Leptopilina heterotoma* strains, Lh14 and France. The Lh14 strain, provided by Mariana Mateos in Texas, US is a highly virulent strain. *Spiroplasma* provides little survival advantage to the *Drosophila* host, in concordance with Xie *et al*, 2014. However, there is *c.* 80% larva-adult fly survival in MSRO-infected *D. melanogaster* post exposure to the France strain (described in Chapter 2 of thesis). Thus wasps vary in virulence, affecting the ability of *Spiroplasma* to rescue parasitized flies.

A different *Spiroplasma* strain, HY1, also protects its natural host, *D. hydei*, against attack from *L. heterotoma* (Xie *et al*, 2010). HY1 confers *c.* 50% larva-adult survival against the Lh14 strain and does not cause reproductive parasitism (Kageyama *et al*, 2006; Xie *et al*, 2010). In North America, a different *Spiroplasma* appears to be spreading throughout the woodland mycophagous fly species, *Drosophila neotestacae.* Jaenike *et al*, 2010a showed that *Spiroplasma* protects *D. neotestacea* against the sterilizing effects of the parasitic nematode, *Howardula aoronymphium*. Defensive symbiont phenotypes drive the rapid spread of the symbiont through natural *Drosophila* populations.

*1.4.3.3 Leptopilina heterotoma*

Drosopholids are commonly parasitized by other insect species. The larval parasitoid wasp *Leptopilina hetertoma*, of the Eucoilidae family, is a generalist and lays its eggs in first- and

second-instar larvae of several *Drosophila* species, including *D. melanogaster* and *D. simulans*. Wasp eggs hatch around 40-43 hours post oviposition, depending on the sex of the wasp (Eijsackers and Bakker, 1971). First-instar wasp larvae possess mandibles which they use to extricate themselves from the egg, remove supernumerary eggs or larvae and consume host hemolymph (Pexton and Mayhew, 2004). The developing wasp larva continues to feed on the developing host, finally killing the host in its pupal stage before metamorphosis. Third instar wasp larvae exit the body of the fly pupa and continue to develop in the gap between the pupa and puparium. A single adult wasp emerges from a single fly puparium.

Flies defend themselves against wasp attack via induction of the immune transcriptional response or egg encapsulation (Nappi and Vass, 1993; Russo *et al*, 1996; Schlenke *et al*, 2007). Encapsulation results in wasp larval death due to the inability of the larva to move and feed (Salt, 1970). However, wasps can overcome and even suppress their host's innate immune response of egg encapsulation using virus-like particles (VLPs) (Whitfield and Asgari, 2003; Lee *et al*, 2009). Upon injection into the host hemolymph, the VLPs enter immune cells and lyse them, thus preventing melanisation (Rizki and Rizki, 1994).

## 1.5 Immunity roles in heritable symbiont/host combinations, both natural and novel

### *1.5.1 Insect Immunity Overview*

Innate immunity is the first line defence against invading parasites and infectious microorganisms. It constitutes physical barriers, such as chitinous exoskeleton and barrier epithelia (Ferrandon *et al*, 1998; Tingvall *et al*, 2001) and a set of dedicated immune genes that are highly conserved across phyla (Engstrom *et al*, 1993; Hoffmann *et al*, 1999). Insects are highly resistant to pathogenic microorganisms (Hultmark, 1993). The immune response discriminates between self and non-self and keeps chronic beneficial microbial infections 'in check' whilst eliminating pathogens. Responses are commonly characterised into cellular and humoral components. The cellular response is comprised of differentiated populations of hemocytes which carry out surveillance and phagocytosis, whereby invading pathogens are engulfed and encapsulated (Lanot *et al*, 2001). The humoral component of innate immunity consists of antimicrobial peptides (AMPs), which are made in the insect fat body (the insect equivalent of the mammalian liver), and lysozymes. AMPs are secreted into the hemolymph

where they have proteolytic function (Steiner *et al*, 1981; Bulet *et al*, 1999). The insect immune system relies on rapid, systemic and nonspecific recognition of invading microorganisms. It does not require the development of long-term immunity.

The Imd and Toll transcriptional signalling pathways are launched against Gram-negative and Gram-positive bacteria, fungi or yeast, respectively and detect patterns of molecular structures on invading cell surfaces (Lemaitre *et al*, 1995; Lemaitre *et al*, 1996). Once the microbial motifs have been recognized by host pattern recognition proteins, genes encoding signal and effector molecules are transcribed. The Imd and Toll pathways differ in the effectors they induce. The Janus Kinase (JAK)/signal transducers and activators of transcription (STAT) signalling cascades, which are implicated in mammalian innate immunity, also appear to play a role in insect immunity (reviewed in Agaisse and Perrimon, 2004). The Toll pathway does not interact directly with cell surface ligands of invading microorganisms. Instead, recognition proteins (e.g. PGRP) identify the structure patterns (peptidoglycans) on Gram-positive bacteria (Levashina *et al*, 1999). Subsequent serpin-controlled proteolytic cleavage of the polypeptide, *Spaetzle*, triggers the activation of NF-κB Rel proteins *DIF* and *Dorsal* and the transcription of AMPs (Ip *et al*, 1993; Dushay *et al*, 1996). The Imd pathway is activated by a transactivator Rel protein, *Relish,* which like *Spaetzle*, needs to be cleaved to become active (Stoven *et al*, 2000). Once active, transcription of AMPs such as *Diptericin* occurs (Lemaitre *et al*, 1995).

Other important components of the humeral response are hemolymph coagulation and deposition of melanin at sites of cuticular injury and infection (Ashida and Brey, 1995; Muta and Iwanaga, 1996). A key enzyme that mediates melanin synthesis is phenoloxidase (PO), which aids with wound healing and immobilization of large parasites such as parasitoid wasp eggs (Rizki and Rizki, 1990; Russo *et al*, 1996; Binggeli *et al*, 2014). Once immobilized, parasites or pathogens can be targeted by effector molecules for elimination. High levels of reactive oxygen species (ROS) and nitric oxide (NO) are also important elements of the humeral immune response.

Having been extensively studied in genetics laboratories over the past 100 years and having no adaptive immunity, *Drosophila melanogaster* is the workhorse for insect innate immunological studies. Homologs of *Drosophila* genes have been identified in mammalian systems. Most notable are the Toll-like receptors (TLRs), which activate NF-κB in response to microbial stimulation (Aderem and Ulevitch, 2000; Akira *et al*, 2001). Characterisation of defence pathways in other, phylogenetically distinct insects such as the honey bee *Apis*

*mellifera*, *Bombus* spp., the pea aphid *Acyrthosiphon pisum* and *Anopheles* mosquitoes have subsequently provided a greater insight to the insect innate immune response (Dimopoulos *et al*, 2002; Evans *et al*, 2006; Gerardo *et al,* 2010; Riddell *et al*, 2011). Recent immunological studies of the order Hymenoptera have revealed a depauperate collection of immune genes in social species relative to solitary insects (Barribeau *et al*, 2015). The lack of an immune gene repertoire is thought to predate the evolution of sociality and may explain in part why bees have developed strong hygienic behaviours.

Hymenoptera are only second to Diptera in the number of sequenced genomes. Transcriptomic studies on the immune catalogue of the genus *Nasonia* revealed Hymenoptera- and *Nasonia*-specific immune genes (Tian *et al*, 2010). Comparative analysis of transcripts from RNA-seq platforms of immune-challenged (post septic injury) and uninfected wasps provides an unbiased method of identifying and characterizing genes that are regulated as part of the immune response. This does not always require homology-based annotations from other, better characterized insects.

Fast evolution in the immune recognition gene family is observed, both in terms of the copy number and variation of the elements, and the sequence of the genes. Analysing patterns of positive selection across characterized and candidate immune genes in the *melanogaster* subgroup of drosophilids revealed some immune genes are highly dynamic and that certain pathogens may drive adaptive evolution amongst closely related species (Sackton *et al*, 2007).

### *1.5.2 Insect immune response to bacterial endosymbionts*

Despite having an effective innate immune systems that eliminates invading pathogens, insects are commonly associated with chronic and sometimes obligate bacterial endosymbionts (Buchner, 1965). *Wolbachia* is an intracellular Gram-negative bacteria present in around 50% of arthropods, making it one of the most ubiquitous bacteria (Weinert *et al*, 2015). The second most common chronic bacterial infection found in insect species is *Spiroplasma*. Both are maternally-transmitted endosymbionts that display a range of phenotypes in their different hosts (Ota *et al*, 1979; Rousset *et al*, 1992; Anbutsu and Fukatsu, 2003). Previous studies have focused on immune interactions with invaders of pathogenic origin due to their detrimental effect on the host. However, little is known about the molecular mechanisms of host tolerance to resident symbionts versus resistance to closely related pathogens or parasites.

### *1.5.3 Endosymbiont resistance*

Symbionts that manipulate host reproduction via male-killing, cytoplasmic incompatibility (CI), feminization or parthenogenesis increase the number of infected females in the host population (Werren *et al*, 2008). In cases where the host and symbiont have coevolved, elimination of the symbiont via induction of the innate immune system should be selected against. Instead of curtailing symbiont abundance to counter the negative effects of male-killing, hosts can develop strong selection for suppressor loci (Hornett *et al*, 2006). The drive to suppress male-killing can result in the rapid spread of suppressor alleles through the host population (Charlat *et al*, 2007), increasing the number of males and reducing the frequency of the symbiont (Hayashi *et al*, 2018). Interestingly, in the case of *Hypolimnas bolina*, the rapid spread of a suppressor allele is not associated with a reduction in *Wolbachia* frequency. This is because when male-killing is suppressed a second phenotype, CI, is induced. This suggests that the two phenotypes are functionally linked and that *Wolbachia* has multiple ways of maintaining itself in the host population (Hornett *et al*, 2008).

In the case of *Wolbachia*-induced CI, infected females that mate with either infected or uninfected males produce viable offspring. Uninfected females that that mate with infected males undergo high embryo mortality. Thus females infected with the symbiont are selected for in the host population (Hurst and Werren, 2001). Despite the arms race between symbiont and host counter-defence through rapid suppressor evolution, the insect immune system, which is present to eliminate parasitic or pathogenic microorganisms, has been tailored to accommodate the bacteria.

Given the high prevalence of *Wolbachia* in arthropods and its propensity to successfully host shift, one might expect *Wolbachia* to have functional mechanisms to suppress or evade the insect innate immune system. In *D. simulans*, naturally occurring *Wolbachia* neither induces nor suppresses the expression of key AMPs associated with Gram-negative bacteria, including cecropin and diptericin. *Wolbachia*-infected flies challenged with *Escherichia coli* however, express high levels of these genes (Bourtzis *et al*, 2000). The same lack of AMP induction was found in *Aedes albopictus* naturally infected with *Wolbachia*.

In stark contrast, the virulent strain of *Wolbachia*, *w*MelPop or 'Popcorn' overreplicates in its native host, *D. melanogaster*, causing degeneration of host tissue and shortened life-span (Min and Benzer, 1997). *w*MelPop displays the same phenotype in transinfected *Aedes aegypti* mosquitos, and also causes CI (McMeniman *et al*, 2009). It is hypothesised that the life-shortening phenotype in *A. aegypti* is a result of constitutive immune gene expression,

which has a high fitness cost and one expects to observe following septic injury (Kambris *et al*, 2009). Despite the induction of a plethora of immune genes including cecropins, defensins, lysozymes and pathogen surveillance proteins (PGRPs), *w*MelPop is faithfully transmitted from mother to offspring in *A. aegypti* suggesting that this strain of *Wolbachia* is resistant or tolerant to host immunity (Kambris *et al*, 2009; McMeniman *et al*, 2009).

In contrast to Bourtzis *et al*, 2000, the *Wolbachia* surface protein (WSP) has been shown to elicit a strong immune response in cell lines of naturally uninfected *Anopheles gambiae* (Pinto *et al*, 2012). The same WSP elicits only a mild immune response in cells of naturally infected *Aedes albopictus*. However, the WSP used in this study was of nematode origin and may differ from *A. albopictus'* native *Wolbachia* strain. It is also possible that *A. albopictus* will show varying degrees of tolerance to different *Wolbachia* strains.

*Spiroplasma*, a bacterial endosymbiont of *Drosophila* spp., does not activate the *Drosophila* Toll or Imd immune pathways (Hurst *et al*, 2003; Herren and Lemaitre, 2011; Hutchence *et al*, 2011). Hurst *el al*'s, 2003, Northern blot analysis of seven key AMP genes in wild type (WT) and *Spiroplasma* infected flies revealed little or no expression of any of the AMP-encoding genes. Hutchence *et al*, 2011 show that *Spiroplasma* male-killing strains MSRO and NSRO induce proteolysis and peptidoglycan catabolism; however, this does not correspond with downstream production of AMP. The *Spiroplasma* of *Drosophila hydei*, HY1, does not induce expression of any immune related genes in native or novel hosts (*D. melanogaster*). These studies are complemented by Herren and Lemaitre's, 2011 investigation of *Spaetzle* and *Relish* null mutants, which do not alter *Spiroplasma* titre. They also performed RT qPCR on *Drosomycin* and *Diptericin*, genes that encode AMP in the Toll and Imd pathways, respectively. Expression levels of the two genes did not differ between flies harbouring *Spiroplasma* and WT flies. Overall, there is comprehensive evidence to suggest that *Spiroplasma* evades or resists the host immune system. The mechanisms by which *Spiroplasma* achieves this are unknown, although the bacteria do lack a cell wall (Tully *et al*, 1987; Tully *et al*, 2009). Thus *Spiroplasma* may avoid recognition by the host's peptidoglycan recognition proteins (PGRPs).

Established endosymbionts undergo the phenomenon of genome reduction whereby redundant genes are lost (Andersson and Andersson, 1999; Shigenobu *et al*, 2000). Often these symbionts lack genes that are required by their free-living counterparts, and only retain genes that fulfil the most essential functions (Oakeson *et al*, 2014; Manzano-Marin and Latorre, 2014). *Buchnera aphidicola*, the obligate mutualist of the pea aphid has lost the

genes for MAMPs (microbe-associated molecular patterns e.g. peptidoglycan (PGN)) synthesis (Shigenobu *et al*, 2000) and therefore cannot be detected by the pea aphid's immune receptors. On the host side, the pea aphid appears to lack genes critical for the recognition, signalling and killing of microbes (Gerardo *et al*, 2010). Furthermore, AMP activity was not detected following infection with either Gram-negative *Escherichia coli* or Gram-positive *Micrococcus luteus* (Laughton *et al*, 2011). Taken together, loss of genes that affect immune protection in the host or symbiont, or both, due to their co-obligate dependence, may facilitate host survival.

### *1.5.4 Endosymbiont tolerance*

Another method of restricting endosymbiont proliferation and virulence is to spatially constrict bacteria to bacteriocyte cells. Clusters of bacteriocytes bound by an epithelial layer form an organ called the bacteriome (Douglas, 1989). The bacteriome reduces direct contact between the bacteria and the host's immune system. The cereal weevil, *Sitophilus oryzae*, is associated with the Gram-negative intracellular mutualist, *Sodalis pierantonius* (Heddi *et al*, 1998). *S. pierantonius* has only recently been acquired by its host (30,000 years ago) and thus retains many genes associated with self-regulation and PGN motif synthesis. It has however lost genes required for the synthesis of several amino acids and vitamins (Clayton *et al*, 2012; Oakeson *et al*, 2014).

Anselme *et al*, 2006, found that the peptidoglycan recognition protein gene (PGRP) that recognises PGN motifs on the cell walls of the invading bacteria, wPGRP, was significantly upregulated in the bacteriome of the weevil, *Sitophilus zeamais*. In addition, wPGRP expression correlated with symbiont virulence. wPGRP is an ortholog of the *Drosophila* PGRP-LB protein, an amidase that specifically degrades Gram-negative bacteria PGN. In *Drosophila*, PGRP-LB is expressed in the midgut and is associated with downregulation of the Imd pathway (Zaidman-Rémy *et al*, 2006). It prevents flies from activating the immune system in response to beneficial gut bacteria. Thus the seclusion of the endosymbiont and localised downregulation of the host immune pathways likely mediates host-symbiont interactions.

In a subsequent study, Anselme *et al*, 2008 observed that weevils challenged with *S. pierantonius* in the hemolymph (outside the bacteriome) mount a systemic immune response, resulting in the induction of AMP-encoding genes. To prevent bacterial invasion into the hemolymph an AMP, coleoptericin A (*colA*), is constitutively overexpressed in the weevil bacteriome. This suggests that outside the symbiont-restricted bacteriome, *S.*

*pierantonius* is recognized as a microbial intruder. More recently, Maire *et al*, 2018 showed that proteins *imd* and *Relish*, of the Imd pathway, are required for colA induction in *S. zeamais* and a closely related weevil species, *Sitophilus oryzae*. Here, endsymbiont regulation is under the control of the host's Imd pathway.

Interaction with the host immune system is also important in the tse tse fly- Sodalis symbiosis. The tsetse fly harbours the facultative bacterium, *Sodalis glossinidius,* which is the sister taxa to SOPE, a lineage of *Sodalis* from the grain weevil, *Sitophilus oryzae.* Weiss *et al*, 2008 show that native *Sodalis* infection does not affect fly survival. However, flies challenged with the closely related human enteric pathogen, *E. coli* K12 display compromised immunity. Flies infected with an *E. coli* OmpA mutant strain had increased survival and recombinant *Sodalis* expressing *E. coli* outer membrane protein A (OmpA) became pathogenic. These data suggest that bacterial outer membrane proteins are important in host infection outcomes. When flies ingested the same number of live *E. coli* K12 cells as they were injected with, they were able to clear the infection. This suggests that the tsetse fly has compartmentalised immune mechanisms.

OmpA consists of 8 membrane-traversing β-barrels joined by 4 loop structures (L1-L4). Weiss *et al*, 2008 show that L1 in several symbionts including *Sodalis* has amino acid insertions and substitutions that differ to pathogenic bacteria. Thus bacterium-specific polymorphisms in OmpA are likely determinants of infection phenotype.

Expression of *pgrp-lb* was upregulated in Tsetse flies infected with pathogenic *E. coli* (Weiss *et al*, 2008). *Drosophila* PGRP-LB is a negative regulator the immune system (Zaidman-Rémy *et al*, 2006). RNAi-knockdown of Tsetse *pgrp-lb* resulted in survival of *E. coli* K12-infected flies. Thus, when induced, the immune system eliminates bacterial infection. Tsetse flies infected with avirulent *E. coli* (either *E. coli* OmpA mutant strain or *E. coli* expressing *Sodalis* OmpA) expressed AMPs from both the Toll and Imd immune pathways and were able to clear infection. *Sodalis* on the other hand is able to persist in its host despite induction of immune gene expression. *Sodalis* and other symbionts could be resistant to the induced AMPs, or in some cases they may be able to enter and hide in host hemocytes using a type three secretion system (TTSS).

*Arsenophonus nasoniae*, is a maternally inherited reproductive parasite of *Nasonia* wasps (Huger *et al*, 1985). For an endosymbiont *A. nasoniae* has an unusually large genome size (3.5 Mb) and retains many genes associated with virulence and metabolism (Darby *et al*, 2010). It is understood that these genes are retained so that the bacteria can survive and proliferate

in the wasp's fly pupal host. The bacterium is ingested by early instar wasp larvae and reinvades via the larval gut wall. *A. nasoniae* can also be infectiously transmitted when an uninfected conspecific shares a pupal host with an infected wasp (Skinner, 1985; Duron *et al*, 2010). With its large genome and ability to self-replicate *A. nasoniae* can be cultured on cell-free media (Huger *et al*, 1985).

The genome of *A. nasoniae* contains at least two complete TTSS, one of which appears to be functional and closely related to the TTSS of virulent *Yersinia* and a *Pseudomonas* species (Darby *et al*, 2010). It also contains a full control mechanism for TTSS gene expression. TTSS are present in Gram-negative bacteria and facilitate bacterial invasion via the injection of proteins into eukaryotic cells (Mecsas and Strauss, 1996). They are often associated with pathogenicity (Hensel *et al*, 1998). The second TTSS found in *A. nasoniae* is homologous to the TTSS found on *Salmonella spp*. pathogenicity island 1 (SPI-1) (Darby *et al*, 2010), which is responsible for invasion across the gut epithelia (Hueck, 1998). *A. nasoniae* also encodes 12 ORFs (open reading frames) with sequence similarity to TTSS effectors. Three of these ORFs are found within the TTSS operon itself and show sequence similarity to effector molecules that alter host cell signalling and immune pathways.

*A. nasoniae* possesses a pathogenicity island of particular note. This site contains ORFs with multiple leucine rich repeat (LRRs) (Darby *et al*, 2010). Proteins that contain LRRs bind ligand and may be important for host/symbiont interactions (bacterial pathogenicity and host immunity) (Kobe and Deisenhofer, 1995; Spaink, 2002). One such gene encodes a cytotoxin which is essential for pathogenicity in *Yersinia pestis* (Hines *et al*, 2001). Other ORFs in the *A. nasoniae* genome show sequence similarity to toxin genes, in particular to *Aip56* (apoptosis inducing protein 56) found on a plasmid in *Photobacterium damselae* ssp. *Piscicida* (Darby *et al*, 2010). Functional studies of the protein show that it can kill neutrophils and is necessary for bacterial virulence (do Vale *et al.* 2007). An ORF with sequence similarity to the putative toxin gene, *cnf1* (cytotoxic necrotizing factor 1), has also been identified. The gene carries domains for eukaryotic cell adhesion and transfer of the C terminal. The C terminal, once translocated into a eukaryotic cell, could activate host Rho GTPases. Other genes for example, Insecticidal toxin complex (Tc) genes, which have been previously described in Gram-negative entomopathogens, are undergoing pseudogenization (Darby *et al*, 2010).

Outer membrane proteins are an important factor in determining the response of the host to bacterial endosymbionts. As mentioned previously, the sequence of the external loops of *OmpA* in *Sodalis glossinidius* determines whether the Tsetse fly host induces an immune

response. *A. nasoniae*'s *OmpA*-like ORF contains an insertion in the first loop (L1). Whilst an insertion is characteristic of symbionts and not of closely related pathogenic bacteria (Weiss *et al*, 2008), this particular motif is unique and positioned in a functionally important part of the protein (Darby *et al*, 2010). This motif may influence whether insect hosts detect *A. nasoniae* as a beneficial symbiont or an invading pathogen.

*Cardinium*, a genus of bacteria belonging to the Bacteroidetes, has been identified in *Culicoides spp*., mites, ticks and spiders and it is thought to infect around 7% of all arthropods (Zchori-Fein and Perlman, 2004; Nakamura *et al*, 2009). In the whitefly *Bemisia tabaci, Cardinium* cBtQ1 is a facultative bacterial endosymbiont (Gottlieb *et al*, 2008; Santos-Garcia *et al*, 2014). Its genome reveals cBtQ1-specific genes, many of which are associated with toxicity and pathogenicity (Santos-Garcia *et al*, 2014). Of note, the chromosome has 4 duplicated copies of rtxBDE and a single copy of tolC, which are ABC and transmembrane transporters of the type 1 secretion system (TSS1), respectively. TSS1s allow secretion of proteins across from the cytoplasm into the extracellular media (Delepelaire, 2004). The rtxBDE genes are related to the RTX toxin transport system of *Vibrio*. Moreover, a 17 kb segment of chromosome containing the rtxBDE and tolC genes has been duplicated. Other toxin-related genes such as *CHV_p018* and *CHV_p021*, the latter of which is associated with bacterial insecticidal toxins and intercellular signalling proteins, can be found on the symbionts multicopy plasmid (Santos-Garcia *et al*, 2014). Although there is good evidence to suggest that *Cardinium* cBtQ1 has the ability to elicit an immune response, interactions between the symbiont and its host innate immune system have not been characterized.

To date, studies on host immunity have focused on Gram-negative alpha- and gamma-proteobacterial pathogens, and *Wolbachia* and *Spiroplasma* (wall-less microbes derived from the gram positive relatives) endosymbionts. Few studies have investigated host tolerance and resistance to bacterial endosymbionts. It is commonly understood that insects possess only the innate branch of immune system however, insects arguably feature elements of adaptive immunity. All of the insect systems described above possess established and long lasting responses to bacterial endosymbionts, which are likely to have been parasitic or pathogenic upon initial infection. In some cases, immune genes are induced in the presence of endosymbionts however, the bacteria appear to have some level of resistance or tolerance, ability to persist and be transmitted to the host's offspring.

### *1.5.5 The Arsenophonus/Nasonia system*

To investigate immunity as a possible determinant of host compatibility to novel endosymbiont infection, I utilize the *Arsenophonus/Nasonia* system.

*1.5.5.1 Arsenophonus*

*Arsenophonus* is a diverse genus of Gram-negative bacteria belonging to Enterobacteriaceae, of the gammaproteobacteria. *Arsenophonus* is commonly an inherited symbiont, infecting around 5% of arthropods. Infections have been described in a range of orders such as Hymenoptera, Diptera, Hemiptera, Coleoptera and Araneae and infection prevalence has been shown to be very high or fixed in the different host species (Duron *et al*, 2008). Unlike *Cardinium*, *Arsenophonus* does not show a bias in the taxa it infects, demonstrating a typical BHR. The *Arsenophonus* of *Nasonia* wasps has a large genome and can grow in cell-free media (Huger *et al*, 1985). It is the only known reproductive parasite of this genus and there is some evidence to suggest that it supplements its host's diet with B vitamins (Santos-Garcia *et al*, 2018). *Arsenophonus* species that are required by their host have undergone more extensive genome reduction and can only be grown in cell culture. In the UK, *Arsenophonus* has been associated with poor health in *Apis mellifera* colonies (Budge *et al*, 2016) and in the US the bacteria is associated with colonies suffering from Colony Collapse Disorder (CCD) (Cornman *et al*, 2012).

*1.5.5.2 Arsenophonus nasoniae*

As the origin of the name of the bacteria suggests ('Arsen' meaning male and 'phonus' meaning slayer), *Arsenophonus nasoniae*, is indeed a son-killer and kills around *c.*80% of haploid male offspring in a *Nasonia* brood. It was first described by Skinner, 1985 as the causal agent of maternally inherited female-biased offspring sex ratios in the parasitoid wasp, *Nasonia vitripennis*. Skinner, 1985 also demonstrated that the bacteria could be infectiously transmitted from an infected female to an uninfected conspecific, via superparasitism (a phenomenon common in nature where wasps coparasitize fly pupal hosts).

*A. nasoniae* has a large genome (3.5 Mb), although it is smaller than two of its most closely related species, *Proteus mirabilis* (3.96 Mb, Pearson *et al,* 2008) and *Photorhabdus luminescens* (5.40 Mb, Duchaud *et al,* 2003). However, it is much larger than the genomes of other facultative secondary endosymbionts such as *Spiroplasma poulsonii* (1.82 Mb, Paredes *et al*, 2015) and *Cardinium* cBtQ1 (1.065 Mb, Santos-Garcia, 2014). It shows evidence of becoming increasingly AT rich, following a pattern typical of vertically transmitted

endosymbionts, despite its unique transmission biology. There is also evidence of gene pseudogenization (Darby *et al*, 2010).

Around 5% of *Nasonia vitripennis* females are naturally infected with the bacterium, which increases the number of females in the population by preventing the development of haploid male embryos. Specifically, the maternal centrosomes do not form correctly, and development is arrested (Ferree *et al*, 2008). The male-killing phenotype has arisen de novo multiple times in the evolutionary history of bacterial endosymbiosis. Like other maternally inherited bacteria, *Arsenophonus* cannot pass from father to son and thus the male-killing phenotype benefits the bacterium by increasing the proportion of infected females in the host population. A potential benefit of male killing for the host is less clear, although it does reduce resource competition amongst related females within the fly host pupa. Also, male-killing has been shown to reduce deleterious effects associated with inbreeding (Hurst and Majerus, 1993).

The transmission biology of *A. nasoniae* is unusual and unique amongst maternally inherited bacteria, which typically transfer bacteria intracellularly via oocytes. In contrast, *A. nasoniae* is injected into the wasp's fly pupal host along with venom and wasp eggs. Bacterial cells are ingested by developing wasp larvae, which infect the larvae to different degrees depending on the amount of bacteria ingested (Nadal-Jimenez *et al*, 2019). *A. nasoniae* then travels across the gut epithelia to colonize the female ovipositor. This method of peroral transmission means that the bacterium is frequently horizontally transmitted to uninfected conspecifics or individuals of a different species developing in the same fly host (Duron *et al*, 2010)

*A. nasoniae* has been detected in wild-caught *Nasonia longicornis*, a closely related and sympatric species of *N. vitripennis* (Balas *et al*, 1996). Both *N. longicornis* and *Nasonia giraulti* can horizontally acquire *A. nasoniae* in laboratory multiparasitism conditions (where two species simultaneously parasitize the same host), where the bacteria also causes male-killing (Duron *et al*, 2010). *Nasonia* species often share the same filth fly hosts (Taylor *et al*, 2011), creating an opportunity for horizontal transmission.

#### *1.5.5.3 Nasonia vitripennis*

Species of the hymenopteran genus *Nasonia* have haplodiploid sex-determination. In other words, fertilized diploid eggs develop into females and unfertilized haploid eggs develop into

males (Whiting, 1967). There a 4 recorded species in the *Nasonia* genus that parasitize the pupal stage of filth- and blowflies. *N. vitripennis* is a gregarious parasitoid, laying multiple eggs within a host pupa. It has a global distribution and is associated with human populations. *N. giraulti* and *N. oneida* are found in the Northeast of North America and *N. longicornis* is predominantly found in the Northwest (Darling and Werren, 1990; Raychoudhury *et al,* 2010). *N. vitripennis* diverged from the other 3 species around 1 MYA and the other 3 are separated by 300,000 – 400,000 years.

The wasps can be easily cultured under laboratory conditions and have a generation time of 14 days at 25°C. Larvae emerge around 24 hours post egg deposition and proceed to feed on the fly host hemolymph, killing the fly in the process (Whiting, 1967). After 4 larval instars they pupate and eclose inside the host pupa before emerging as adult wasps. *N. giraulti* tend to mate before emerging from the fly pupal host whereas *N. vitripennis* typically mate after emerging. The 4 species can produce fertile viable hybrid offspring, but only after each of the species has been cured of their otherwise incompatible *Wolbachia* infections (Breeuwer and Werren, 1990). All *Nasonia* species can cope with a wide range of climatic conditions (Darling and Werren, 1990).

In temperate regions, *Nasonia* species respond to fluctuations in seasonal light and temperatures by entering a facultative diapause stage, which is a physiological state of dormancy (Saunders, 1965a; Saunders, 1965b). By synchronising their life cycle with the seasons, the wasps reproduce and develop during favourable conditions. Diapause is mediated by neuronal-hormonal cues, predominantly in response to light:dark cycles (photoperiod), but temperature, moisture and food availability can also influence the induction of diapause (Saunders, 2002). Diapause is induced in adult females following exposure to a specific photoperiod. During diapause the wasps have arrested development at the fourth larval instar, and metabolism is greatly reduced. As photoperiod changes gradually with latitude *Nasonia* species have evolved distinct diapause responses (Paolucci *et al*, 2013). It is possible for *N. vitripennis* to lose its *Wolbachia* infection during this period of metabolic dormancy. However, how diapause affects *A. nasoniae* proliferation and maintenance during diapause and vice versa, is unknown.

## 1.5 Project overview

In this thesis, I address important questions concerning host shift biology of heritable microbes, and the biology of these interactons more generally.

First, I discuss potential host factors that determine compatibility to novel endosymbiont infections, following host shift events. In chapter 2, I evaluate the speed of evolution of compatibility to novel *Spiroplasma* endosymbiont infection among species in the *Drosophila melanogaster* subgroup. Following on from chapter 2, I discuss in the high lability of endosymbiont phenotypes and propose mechanisms that make *Spiroplasma* a successful, yet rapidly evolving endosymbiont.

In chapter 4, I examine the role of diet and the *Drosophila* gut microbiota in determining host compatibility to native *Spiroplasma* infection, and conversely, the role of *Spiroplasma* in altering host metabolites.

I also asses the role of insect innate immunity in determining host compatibility to novel endosymbiont infection, using the *Nasonia/Arsenophonus* model system. In chapter 5, I investigate the whole organismal transcriptomic response of *Nasonia vitripennis* to its native *Arsenophonus nasoniae* infection and also the response of *N. vitripennis*'s sibling species, *Nasonia giraulti,* to novel *A. nasoniae* infection. In chapter 6, I investigate *A. nasoniae* interaction with its host during diapsue and measure the impact of *A. nasoniae* on diapause induction and its interaction with the host during diapause.

# Chapter 2

## Rapid evolution of compatibility to novel heritable microbes in the *melanogaster* subgroup of drosophilids.


### Abstract

Heritable symbionts represent important components of host biology, both as antagonistic reproductive parasites, and beneficial protective partners. An important component of heritable microbes' biology is their ability to establish in new host species, a process equivalent to a host shift for an infectiously transmitted parasite or pathogen. The ability to host shift will, for instance, partly determine the fraction of species that carry particular heritable symbiont infections. For a host-shift to occur, the symbiont must be compatible with the host: it must not cause excess pathology, must have good vertical transmission, and a drive phenotype that enables spread. In this chapter, I investigate the evolutionary lability of compatibility to heritable microbes, a key determinant of the incidence of symbiotic infections. Variation in compatibility for bacterial *Spiroplasma* endosymbionts from *Drosophila hydei* in host flies from the *melanogaster* subgroup of *Drosophila* is observed, which indicates compatibility evolves. Interactions with the 'foreign' protective symbiont from *D. hydei* varied from pathological with low vertical transmission, to being asymptomatic with high vertical transmission. This variation occurred between closely related species pairs, such as *D. simulans* (high compatibility) and *D. melanogaster* (high pathology). The close relatedness of this pair of host species emphasises the rapidity with which host-symbiont compatibility can evolve, which explains in part the observation that only a fraction of species carry protective symbionts. However, the protective phenotype is not always conveyed upon lateral transfer, even if the host is seemingly compatible. Moving forward, the variation between sibling species pairs observed above provides an opportunity to identify the mechanisms behind variable compatibility between closely related host species, which will drive hypotheses as to the evolutionary drivers of compatibility variation.

## 2.1 Introduction

Bacterial endosymbiont infections are widespread in nature and exert various effects on insect host reproduction, provide defence against natural enemies and aid in nutrient allocation (Breeuwer *et al*, 1992; Heddi *et al*, 1991; Hurst *et al*, 1996; Hamilton and Perlman, 2013). Symbioses can be obligate or facultative, and mutually beneficial as well as costly and parasitic (Buchner, 1965). Facultative and obligate endosymbionts confer important properties that allow their host to colonize new ecological niches and can strongly influence the host's life history (Dunbar *et al*, 2007). Endosymbionts are typically maternally inherited (Cosmides and Tooby, 1980) but they can also be transmitted horizontally.

Symbionts vary in their patterns of infection across hosts. Some symbionts are found in many species, whereas others are present in just a small fraction. Some are found in a taxonomically widespread set of hosts (broad host range) whereas others are found in particular groups of host species. For example, over 50% of all arthropods are predicted to be infected with the intracellular bacteria, *Wolbachia*, which is considered to have a broad host range (Weinert *et al*, 2015). *Cardinium* on the other hand is present in only 13% of arthropods and is highly associated with arachnid groups (Duron *et al*, 2008; Martin and Goodacre, 2009; Nakamura *et al*, 2009; Weinert *et al*, 2015). It is thus considered more specialist. It is unclear why symbionts are present in certain host species and not others. The observed range and diversity of symbionts may be due to a) the evolution of reproductive isolation in their host and co-cladogenesis of host and symbiont, which causes an increase in the number of infected species (Bruckner and Bordenstein, 2012) or b) host-shift events whereby the symbiont jumps from its native host to a novel, previously uninfected host (Woolhouse and Gowtage-Sequeria, 2005).

A host shift may result in a dead end where an individual of a novel host species is exposed but there is no onward transmission of the microbe. Alternatively, the symbiont may transmit onward to individuals in the new host species, creating an epidemic. Whether spread into the novel host occurs is partly determined by compatibility (the ability to cause infection and persist in a novel host), but may also be influenced by host density (for infectiously transmitting agents) or other host features such as natural enemy pressure (for a protective symbiont) (Xie *et al*, 2015).

Compatibility to receive novel symbionts depends at least in part on host relatedness (Gilbert and Webb, 2007; Streicker *et al*, 2010). For example, pathogenic viruses are more likely to infect species that are closely related to their ancestral host, such that the success of

infection decreases with increasing phylogenetic distance from the ancestral host (Longdon *et al*, 2011). Alternatively, closely related host species may share similar levels of susceptibility regardless of their genetic distance from the ancestral host. This phylogenetic effect may create variable incidence of symbiont infection between different host clades, with some host clades that are distantly related to the symbiont's natural host being compatible with the symbiont (Longdon *et al*, 2014).

For a heritable endosymbiont to become established in a novel host population, it must first transmit horizontally into it and then infect the female germline (Herren *et al*, 2013). The mechanisms underlying lateral transmission of bacterial symbionts are not well understood. Jaenike *et al*, 2007 observed that ectoparasitic mites of *Drosophila* could transfer *Spiroplasma* from one host species to an uninfected host. Furthermore, they showed that the *Spiroplasma* could vertically transmit to the novel host's offspring. Vertical transmission immediately following a symbiont host-shift event is typically poor and there are fitness costs incurred by the symbiont (Clancy and Hoffmann, 1997; Russell and Moran, 2005; Kageyama *et al*, 2006; Tinsley and Majerus, 2007; Nakayama *et al*, 2015), but once established in the germline cells, specifically the oocytes, the symbiont can be transmitted to the host's offspring. Spiroplasmas that are unable to access the female reproductive organs via the host's yolk proteins cannot be transmitted to the host's offspring (Herren and Lemaitre, 2011). Symbionts in novel hosts may also show maladaptive virulence phenotypes, which may attenuate over time (McGraw *et al*, 2002; Carrington *et al*, 2010; Nakayama *et al*, 2015) via genetic changes in the symbiont and host adaptation.

The *Drosophila* endosymbiont, *Spiroplasma,* belongs to the gram-positive division, and is a helical and actively motile bacterium of the class Mollicutes (Williamson *et al*, 1999). Unlike *Wolbachia*, which resides in the cytoplasm of host cells, *Spiroplasma* occupies a largely extracellular niche in *Drosophila*, in the haemolymph (Sakaguchi and Poulson, 1961). The relationship between host and *Spiroplasma* can vary from beneficial defensive mutualism to reproductive parasitism (Jaenike *et al*, 2010a; Xie *et al*, 2010; Xie *et al* 2014). In *Drosophila hydei*, *Spiroplasma* protects against the generalist parasitoid wasp, *Leptopilina heterotoma*, without manipulating host reproduction (Xie *et al*, 2010). *Spiroplasma* is recorded in 17 species of *Drosophila* from across the phylogeny. Thus the symbiont can undergo regular host shift events (Watts *et al*, 2009; Jaenike *et al*, 2010b).

Two phylogenetically distant hosts, *Drosophila melanogaster* and *Drosophila nebulosa* are infected with distinct yet closely related spiroplasmas. In both cases, they cause male killing.

The *Spiroplasma* native to *D. nebulosa*, nebulosa sex ratio organism (NSRO), has lower transmission efficiency in *D. melanogaster* compared to *D. melanogaster*'s native *Spiroplasma*, MSRO (Hutchence *et al*, 2011). Native and introduced infections did not, however, differ in their ability to male-kill (Hutchence *et al*, 2011).

Here I investigate the rate of evolution of host compatibility in four *Drosophila* species in the *melanogaster* subclade. Previous work indicated that the *Spiroplasma* strain from *D. hydei*, HY1, misfits *D. melanogaster* due to poor vertical transmission and virulence in the novel host (Kageyama *et al*, 2006; Nakayama *et al*, 2015). *D. melanogaster* and *D. hydei* are highly diverged from one another and last shared a common ancestor 40-62 million years ago (MYA) (Ranz *et al*, 2001). In contrast *D. melanogaster* and *Drosophila simulans* share a more recent common ancestor, about 2-3 MYA (Li *et al*, 1999). To date, no one has explored the capacity of other species in the *melanogaster* subgroup to propagate HY1 infection. I artificially transferred HY1 from its native host, *D. hydei*, to the novel hosts, *D. melanogaster, D. simulans, Drosophila sechellia* and *Drosophila yakuba* and measured transmission efficiency and fitness costs. I also examined whether the artificially transferred *Spiroplasma* retains its protective phenotype in *D. melanogaster* and *D. simulans* upon exposure to the common parasitoid wasp, *L. heterotoma*.

## 2.2 Materials and Methods

*2.2.1 Symbiont and host*

*Spiroplasma* infected *D. hydei* (*Spiroplasma* strain TEN 104-106 haplotype 1, HY1, Mateos *et al,* 2006) was used in this study. HY1 falls as an out-group of *Spiroplasma poulsonii* and shows no evidence of reproductive manipulation in the *D. melanogaster* (Hutchence, 2011). Two recipient strains of *D. melanogaster* were used, one was *Wolbachia* (*wMelCS*)-infected strain of *D. melanogaster,* Canton S (CS), derived from Montenegro *et al*, 2005 and the other a *Wolbachia* uninfected *D. melanogaster* strain, Oregon-R. The laboratory populations of *Wolbachia* infected (*wRi*) and uninfected *D. simulans* (F15 and F7, respectively) were derived from iso-female lines supplied by the Centre for Environmental Stress and Adaptation Research, La Trobe University, Australia. These strains were provided by Prof Nina Wedell. The *D. sechellia* line was acquired from a Drosophila Stock Centre in the US and provided by Dr Chloe Heys. I used *D. sechellia* line 21, carrying the *w*Sh strain of *Wolbachia*. I used a *Wolbachia* uninfected strain of *D. yabuka,* provided by Dr Ben Longdon.

*D. hydei*, *D. melanogaster* and *D. simulans* strains were maintained on a cornmeal-based medium (ASG) consisting of yeast, sugar, maize and nipagin, supplemented with live yeast granules, at 25°C under a 12 h:12 h light:dark cycle with overlapping generations. *D. yabuka* and *D. sechellia* were maintained on malt medium (yeast, malt, maize, nipagin and propionic acid) supplemented with live yeast granules, at 25°C under a 12 h:12 h light:dark cycle with overlapping generations.

*2.2.2 Artificial lateral transfer of Spiroplasma*

Microinjections were carried out as described by Nakayama *et al*, 2015. In brief, hemolymph was extracted from the thorax of *Spiroplasma* infected *D. hydei* and mixed with sterile PBS. Virgin female *Drosophila* recipients were artificially injected in the abdomen with 0.1-0.2µl of PBS-hemolymph, using a hydraulic positive-pressure microinjection apparatus (Model IM-6, Narushige Ltd, Tokyo, Japan).

Injected females were aged for 14 days to let the *Spiroplasma* infection establish in the new host. Each female was then placed with 2 males of the same *Drosophila* strain- to ensure mating success- in a vial containing 15 ml *Drosophila* medium.

Adult injected flies were allowed 4 days to oviposit, after which time the males were discarded and the females frozen at -80°C. All injected females were screened for *Spiroplasma* using a PCR assay. Offspring from unsuccessfully infected females were used as

negative controls. The DNA extraction and PCR assays were carried out as described by Nakayama *et al*, 2015. PCR assays were conducted as follows. Each female was macerated in a 50 µl 5% Chelex (Chelex 100 Resin, Bio-Rad Laboratories, Hercules, CA, USA) solution and 1 µl proteinase K, and incubated at 37 °C overnight (Walsh *et al*, 1991). Samples were then heated at 95°C for 10min to denature the proteinase K. PCR amplifications were performed using *Spiroplasma*-specific primers SpoulF (5'-GCTTAACTCCAGTTCGCC-3') and SpoulR (5'-CCTGTCTCAATGTTAACCTC-3') as in Montenegro *et al*, 2005. PCR cycling conditions were an initial denature of 1 min 30 s at 94°C, followed by 35 cycles of 15 s at 93 °C, 1min annealing at 47 °C and 1min at 72 °C. The injected females were designated as the parental generation.

*2.2.3 Measurement of fitness*

Virgin female offspring from the successfully injected females and an uninfected control were aged for 10 days and then placed individually with two males in small plastic vials (2cm height x 2cm diameter) containing grape jelly agar. Grape jelly aids the collection of first instar larvae. Flies were permitted to mate and oviposit for 24 h at 25°C, and then tipped onto fresh agar once a day for further 2 days. Larvae from the first day were discarded. On day 4, the males were discarded, and the adult female *Drosophila* were removed and screened for *Spiroplamsa* via PCR assay. Larvae from F1 females that tested negative for *Spiroplasma* were discarded. First instar larvae from infected females were picked into vials of fly food at a constant density of 20 larvae per vial. Eclosed virgin females of the F2 generation were collected and aged for 10 days.

To measure the cost of *Spiroplasma* infection in a novel host, I measured the number of adult offspring produced by the F2 generation of infected parental females. Each F2 female was placed with two males in a vial of fly medium to mate. They were moved into fresh vials every day for five days to allow continuous oviposition. On the sixth day adult flies were removed and the females were screened for *Spiroplasma*. Vials that had been occupied by uninfected females (other than the uninfected controls) were kept and noted as flies that had lost infection between the F1 and F2 generations. The eclosed offspring from days 2 to 5 were counted. This provided an index of the direct influence of *Spiroplasma* on host fitness.

To ensure that the fitness measures were robust I performed the fitness assay of infected females alongside an uninfected control. The variation in fecundity observed between the different fitness assays of the same *Drosophila* strains could be due to temperature and

humidity changes in the laboratory associated with changes in seasons (Hoffmann, 2010), which were beyond our control. PCR assays included (a) a positive control re-extracted alongside the test individuals (*D. melanogaster* carrying Melanogaster Sex Ratio Organism: MSRO) and (b) repeat PCR assays of negative specimens. Quality control for PCR assays included screening all individuals for the mitochondrially encoded cytochrome c oxidase I (MT-CO1). This allowed us to eliminate any false positives.

*2.2.4 Vertical transmission in the absence of ageing and selection*

The F1 generation of 16 successfully transinfected *D. simulans W-* females were divided into 6 replicate populations. The 6 replicate populations were permitted to reproduce and oviposit on days 0-5 post-eclosion. Vertical transmission from one generation to the next was quantified via conventional PCR analysis with QC PCR as described above. Only females were analysed.

*2.2.5 Wasp maintenance*

I used an inbred laboratory strain of *Leptopilina heterotoma,* originally collected from Sainte Foy-lès-Lyon and la Voulte, France. The strain was donated by Dr Fabrice Vavre and harboured *Wolbachia*. Wasps were maintained on *D. melanogaster* Oregon-R with standard ASG food, at 25°C under a 12 h:12 h light:dark cycle. The wasps take approximately 21 days to complete one life cycle under these conditions.

*2.2.6 Fly and wasp survival post parasitoid attack*

The *D. simulans* protection assay was carried out on F3 generation larvae, following artificial infection of adult females with HY1 (see above). For *D. melanogaster,* F2 generation larvae were exposed to the wasps, following artificial infection with HY1.

I set up 6 treatments per assay: transinfected larvae with and without wasps (S+ Lh+, S+ Lh-), uninfected controls with and without wasps (S- Lh+, S- Lh-) and a naturally infected *D. melanogaster* with and without wasps (MSRO Lh+, MSRO Lh-). For each treatment I established 10 replicate vials, each containing 20 first and second instar *Drosophila* larvae. The larvae were picked onto the surface of standard ASG food from grape agar (as described above). Five mated female *L. heterotoma* adult wasps were immediately added to each wasp treatment vial and left to attack larvae for 3 days at 25°C under a 12 h:12 h light:dark cycle. The wasps had previously been matured to at least 5 days of age at 25°C, with males. The number of pupae, eclosed adult flies and later the number of eclosed wasps were counted

for each vial. This meant that fly and wasp fitness could be assessed in terms of the number of flies and wasps surviving the fly pupal stage.

*2.2.7 Statistical analyses*

All statistics were performed using RStudio Software for Statistical Computing, version 3.5.0 (R Core Team, 2018). All data was tested for normality.

*Fecundity*: The number of offspring produced by transinfected and uninfected control females was analysed using general linear models. Where there were selection replicates, they were nested within the infection status in the analyses. Where there was only one infected replicate, a paired t-test was used.

*Infection prevalence*: Due to separation of data, I implemented the brglm package which estimates binomial-response GLMs on iteratively updated pseudo-data. Bias reduction provides finite estimates of the model paramenters, wherease maximum likelihood estimates are infinite. Only successfully infected females were used at the start, but infection decreased in the F1 generation and continued to decrease in the F2 and F3 generations (see Fig.1.3). Nakayama *et al*, 2015 showed a drastic decline in HY1 *Spiroplasma* infection immediately following transinfection into *D. melanogaster*. Here I am interested in whether *Spiroplasma* infection can be maintained in *D. simulans* in the absence of directional selection for transmission efficiency of *Spiroplasma*.

*Protection assays*: A generalized linear model with binomial errors was used to test the effect of *Spiroplasma* on fly pupae-to-adult survival, in the presence and absence of wasps. For fly fitness, I used a two-vector response variable (number of adult flies that emerged/pupae that did not give rise to adult flies). To do this I used the function 'cbind', which combines two columns into a vector in this order: number of successes, number of failures. For wasp fitness, I used a two-vector response (number of emerging adult wasps/number of fly pupae that did not give rise to a wasp). The independent variables were *Spiroplasma* infection status and wasp attack (for fly fitness data only).

## 2.3 Results

*2.3.1 Direct cost of Spiroplasma on host fitness*

I compared the response to novel *Spiroplasma* infection in species of the melanogaster sub group, *Drosophila melanogaster*, *Drosophila simulans*, *Drosophila sechellia* and *Drosophila yakuba*.

*D. melanogaster W*+ females infected with *Spiroplasma* produced significantly fewer offspring relative to the uninfected control (Fig. 2.1A, t value = 6.217, $P < 0.001$). There was no significant effect of replicate (replicate 2, t value = 0.079, $P = 0.937$; replicate 3, t value = 0.504, $P = 0.616$; replicate 4, t value = -0.547, $P = 0.586$). *Spiroplasma*-infected *W*- females also produced fewer offspring relative to an uninfected control (t = -3.61, $P < 0.001$). *Wolbachia* does not appear to influence the suitability of *D. melanogaster* to novel *Spiroplasma* infection. In total, 43/69 10-day old *D. melanogaster W*+ F2 females were infected with *Spiroplasma* (62.3%, CI 49.8% - 73.7%). The infection prevalence of 75 *D. melanogaster W*- F2 females was 62.7% (CI 50.7% - 73.6%).

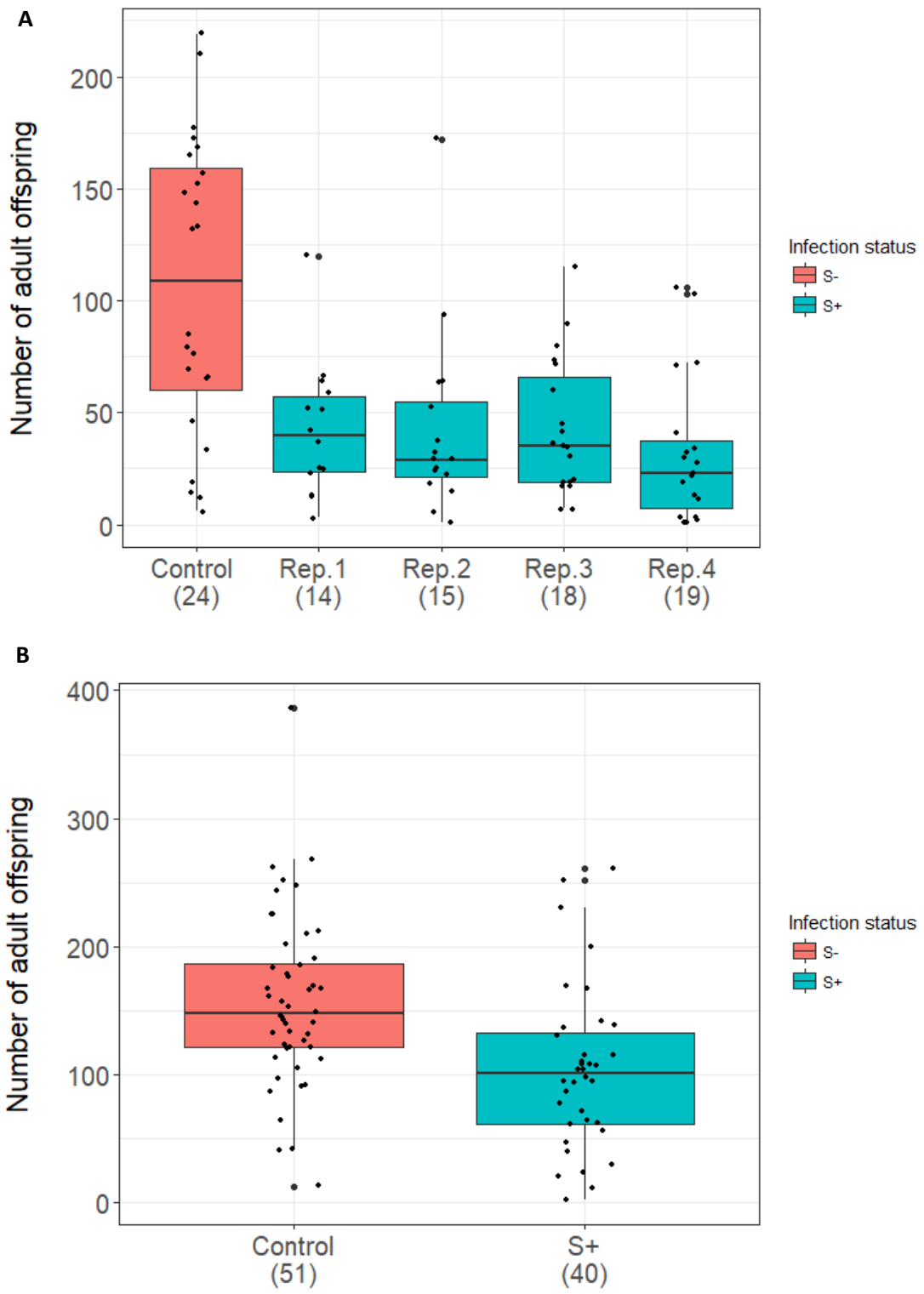



**Figure 2.1.** Direct cost of *Spiroplasma* on host fecundity. The average number of offspring produced over 4 days by the F2 generation of *D. melanogaster* females following artificial lateral transfer of *Spiroplasma* and an uninfected control. **A** *Wolbachia*-infected flies (4 selected infected lines) and **B** *Wolbachia*-uninfected flies (1 selected line). The box plots display the upper and lower quartiles, the median and the range. Points represent each measurement obtained. Values in paratheses show sample size.

In contrast, I found no difference in the number of offspring produced by *Spiroplasma*-infected *D. simulans W*+ and uninfected control individuals (Fig.2.2A, t = -0.486, *P* = 0.628). There was no effect of replicate (replicate 2, t value = -1.25, *P* = 0.219; replicate 3, t value = 0.848, *P* = 0.400). The same was true for *D. simulans W*- (Fig. 2.2B, t = -0.835, *P* = 0.406). Again, there was no effect of replicate (replicate 2, t value = -0.666, *P* = 0.508; replicate 3, t value = -1.567, *P* = 0.121). Fifty-six out of 60 F2 generation *D. simulans W*+ females were infected with *Spiroplasma* (93.3%, CI 83.8% - 98.2%). Vertical transmission efficiency from the F1 to F2 generation in *D. simulans W*- females was 56.0% (N = 75, CI 44.1% - 67.5%).

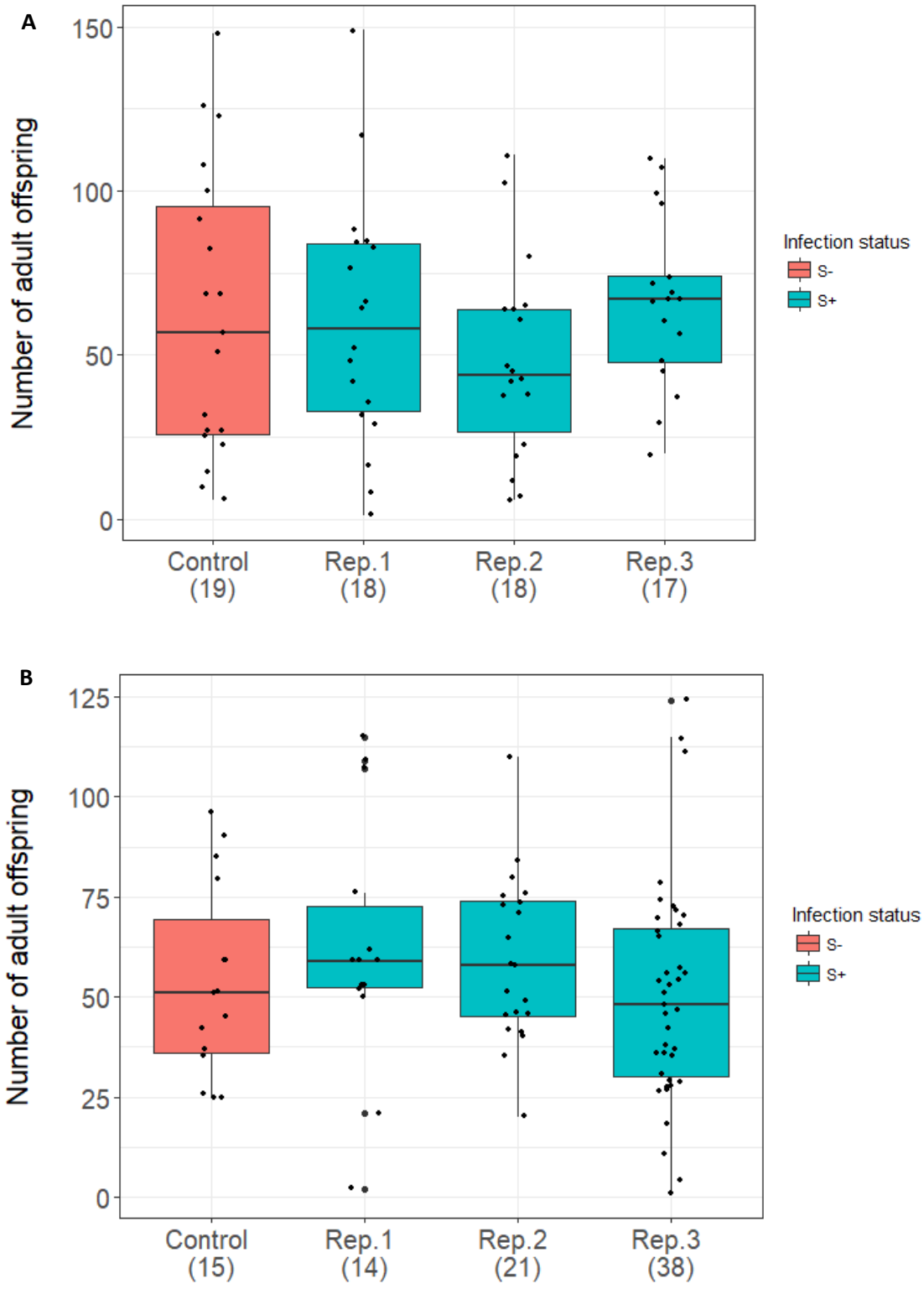



**Figure 2.2.** The average number of offspring produced over 4 days by *D. simulans* following artificial lateral transfer of *Spiroplasma*, and an uninfected control. **A** *Wolbachia*-infected flies (3 selected infected lines) and **B** *Wolbachia*-uninfected flies (3 selected line). The box plots display the upper and lower quartiles, the median and the range. Points represent each measurement obtained. Values in paratheses show sample size.

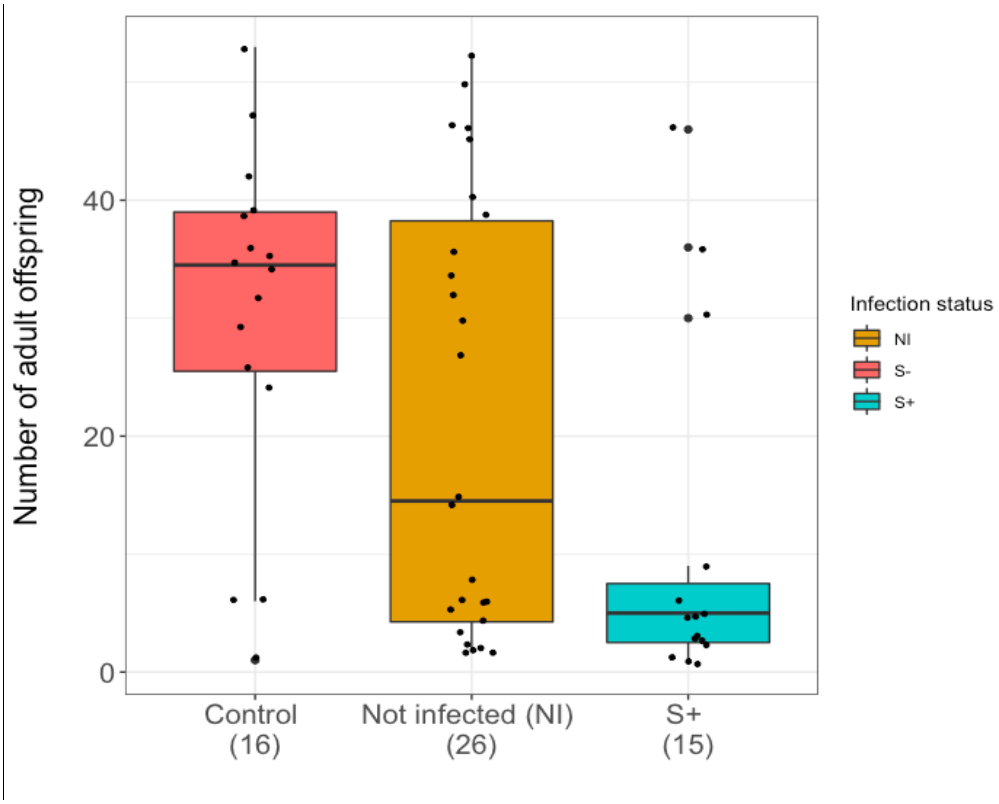



**Figure 2.3.** Mean number of offspring produced over 4 days by *D. sechellia W+* following artificial lateral transfer of *Spiroplasma*, and an uninfected control. Females that lost *Spiroplasma* infection between the F1 and F2 generations are called 'Not infected (NI)'. The box plots display the upper and lower quartiles, the median and the range. Points represent each measurement obtained. Values in paratheses show sample size.

F2 generation *D. sechellia W+* females infected with *Spiroplasma* produced significantly fewer offspring than uninfected control females (Fig.2.3. t value = -3.34, $P < 0.001$). Vertical transmission efficiency between the F1 and F2 generations was relatively poor, with 36.6% of the F2 maintaining infection (N = 41, CI 22.1% - 53.1%). This gave rise to a group of 'not-infected' (NI) F2 females, which descended from F1 infected mothers, but tested negative for *Spiroplasma*. The NI group did not produce significantly fewer offspring than the uninfected control (t value = -1.70, $P = 0.08$).

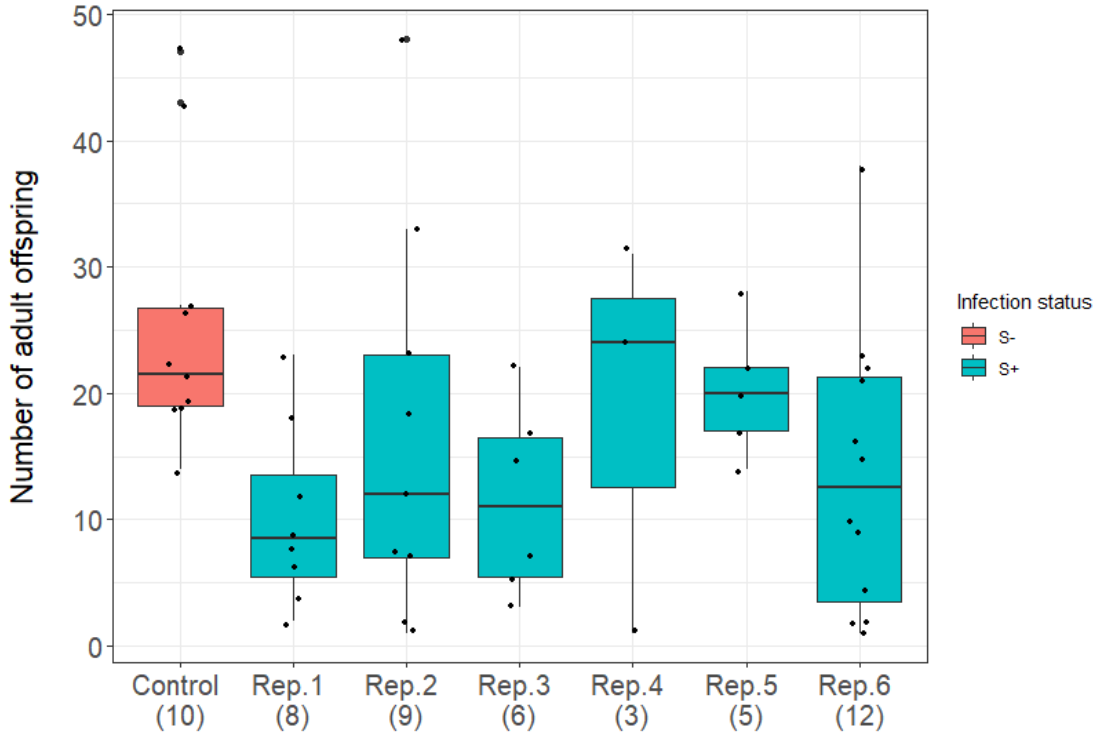



**Figure 2.4.** The average number of offspring produced over 4 days by *D. yakuba W-* following artificial lateral transfer of *Spiroplasma*, and an uninfected control. The box plots display the upper and lower quartiles, the median and the range. Points represent each measurement obtained and values in paratheses show sample size.

Four out of 6 *Spiroplasma*-transinfected *D. yakuba W-* lines produced significantly fewer offspring than uninfected controls. Statistically, replicates 5 and 6 did not produce fewer offspring relative to the uninfected control line, but these were characterized by low replication and power (Fig.2.4. replicate 1, t value = -2.63, $P < 0.05$; replicate 2, t value = -2.05, $P < 0.05$; replicate 3, t value = -2.52, $P < 0.05$; replicate 4, t value = -2.39, $P < 0.05$; replicate 5, t value = -1.92, $P = 0.06$; replicated 6, t value = -1.27, $P = 0.21$). Transmission of *Spiroplasma* between the F2 and F3 generations was exceptionally high, with infection present in 81/83 females (97.6%, CI 91.6% - 99.7%).

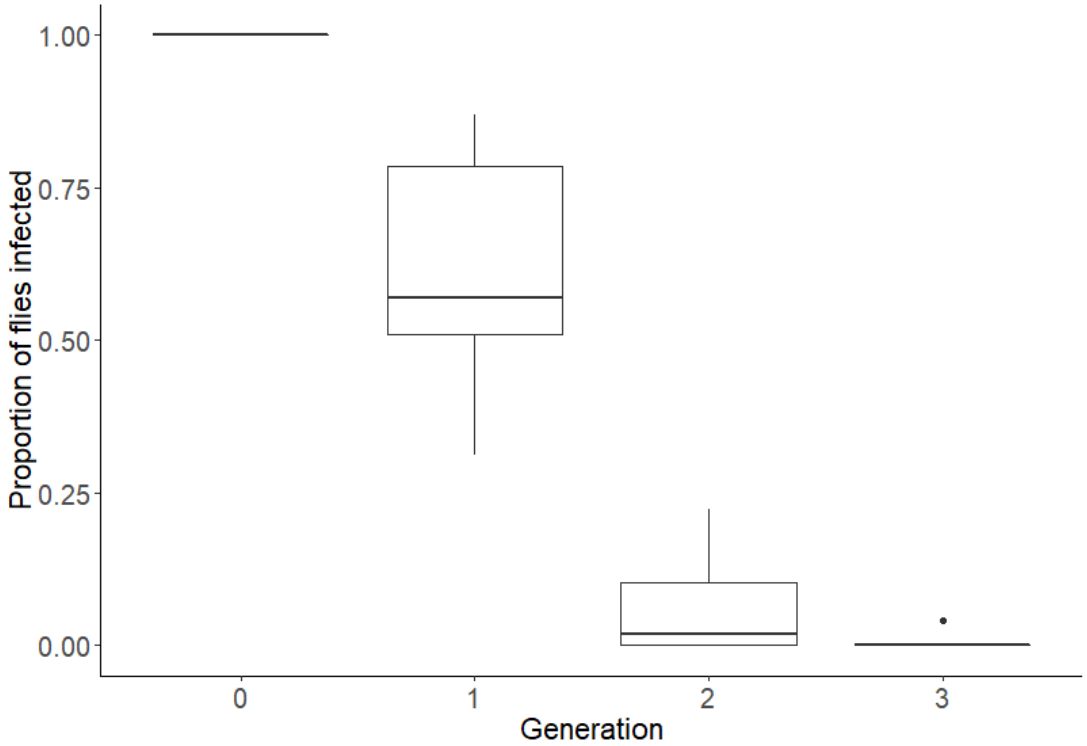



**Figure 2.5.** *Spiroplasma* infection prevalence over 3 generations following lateral transfer to *Drosophila simulans W-*. The box plots display the upper and lower quartiles, the median and the range. Points represent each measurement obtained.

### *2.3.2 Vertical transmission efficiency in the absence of directional selection*

Figure 2.5 shows the changes in infection prevalence of *Spiroplasma* following lateral transfer into *D. simulans W-*. The flies were not selected based on *Spiroplasma* infection at each generation and instead left to reproduce and oviposit on days 0-5 post eclosion. There was a rapid decline in the proportion of flies infected in each generation, from generation 0 (the injected mothers) to the F3 females (generation 1, z value = -2.184, $P < 0.05$; generation 2, z value = -4.408, $P < 0.01$; generation 3, z value = -5.041, $P < 0.01$). In the absence of selection, *Spiroplasma* infection in juvenile female *D. simulans* is lost by the F3 generation (Fig.2.5).

### *2.3.3 HY1 protection in transinfected Drosophila species*

I compared the survival of *Spiroplasma* transinfected- and *Spiroplasma*-free *D. simulans* and *D. melanogaster* in the presence and absence of the parasitoid wasp, *L. heterotoma* (Fig.2.6A; Fig.2.7A). *D. melanogaster* naturally infected with *Spiroplasma* strain MSRO, that is protective, was used as a positive control (data not shown). I included results of transinfected flies whose parents had their infection status confirmed by PCR analysis.

In the absence of wasps, fly pupa-to-adult survival did not differ between *Spiroplasma*-transinfected and *Spiroplasma*-free *D. simulans*. Flies from both treatments had a mean survival of *c.* 100%. The proportion of *Spiroplasma*-transinfected and *Spiroplasma*-free *D. simulans* surviving attack of *L. heterotoma* was low (*c.* 14.3% and *c.* 7.10%, respectively). There was a minor positive effect of *Spiroplasma* on the pupae-to-adult survival of *D. simulans* in the presence of *L. heterotoma* but this difference was not significant (nonsignificant; $P = 0.402$). Overall, *Spiroplasma* did not enhance the survival of *D. simulans* in the presence of parasitoid wasps. I also compared pupae-adult survival of wasps in the presence and absence of *Spiroplasma* infection (Fig.2.6B). *Spiroplasma*-transinfected flies gave rise to fewer wasps than *Spiroplasma*-free flies (*c.* 53.7% and *c.* 78.2%, respectively). In other words, wasp survival was significantly lower in the *Spiroplasma*-transinfected treatment ($t = 3.56$, $P < 0.05$), suggesting that HY1 has a negative effect on wasp development.

In the presence of wasps, the pupae-adult survival of transinfected *D. melanogaster* was significantly higher than *Spiroplasma*-free flies ($z = 3.35$, $P < 0.05$). Thus HY1 protects *D. melanogaster* against wasp-induced mortality (Fig.2.7A), albeit not to the same extent as MSRO (data not shown). As expected, *D. melanogaster* transinfected with HY1 gave rise to fewer wasps than uninfected flies ($t = 3.60$, $P < 0.05$). Pupae-adult wasp survival in infected *D. melanogaster* was *c.* 20%, compared to *c.* 54.1% in uninfected flies (Fig.2.7B).

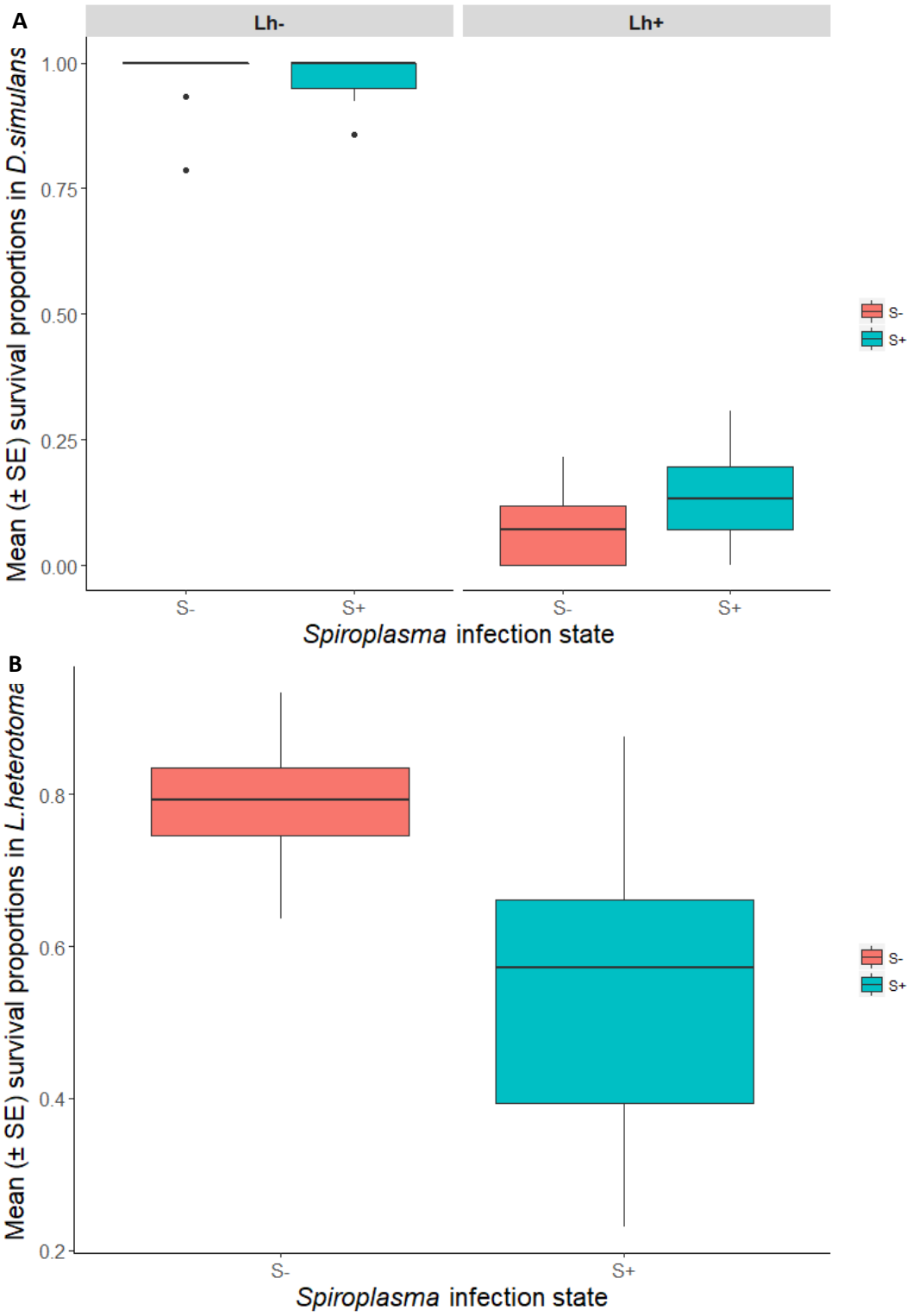



**Figure 2.6.** Impact of *Spiroplasma* on wasp parasitisation outcome following lateral transfer to *Drosophila simulans* W-. **A** Fly pupa-adult survival in the presence and absence of *L. heterotoma*. **B** Average wasp survival in the presence/absence of *Spiroplasma*. Blue box = *Spiroplasma*-transinfected (S+) *D. simulans*; peach box = *Spiroplasma* uninfected (S-) *D. simulans*. The box plots display the upper and lower quartiles, the median and the range.

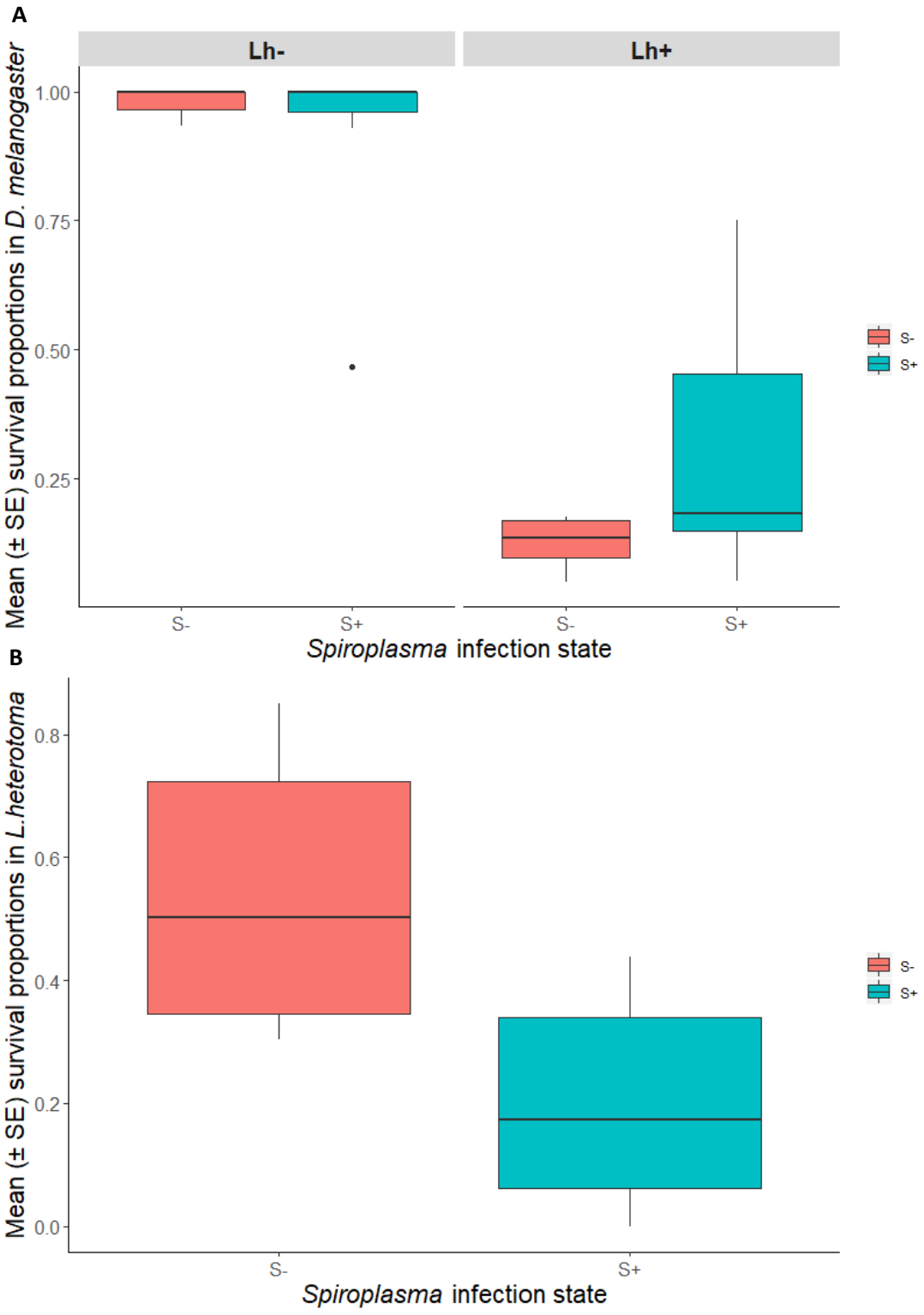



**Figure 2.7.** Impact of *Spiroplasma* on wasp parasitization outcome following lateral transfer to *Drosophila melanogaster* W+. **A** Fly pupae-adult survival in the presence and absence of *L. heterotoma*. **B** Average wasp survival in the presence/absence of *Spiroplasma*. Blue box = *Spiroplasma*-transinfected (S+) *D. simulans*; peach box = *Spiroplasma* uninfected (S-) *D. simulans*. The box plots display the upper and lower quartiles, the median and the range.

## 2.4 Discussion

The ability of a symbiont to infect and spread in a new host species without causing it harm determines the success of the symbiont in nature. Our current model of host compatability holds that the success of a novel infection decreases with increasing distance from the native host. An alternative theory developed for pathogens, the phylogenetic clade effect, suggests that patches of susceptible hosts may be scattered across host phylogeny independently of their distance to the native host, as compatibility evolves (Longdon *et al*, 2014).

In this chapter, I examine the tempo of evolution of host compatibility to novel *Spiroplasma* infection in the *D. melanogaster* subgroup. All species in the *D. melanogaster* subgroup are equally distantly related from *Spiroplasma*'s natural host, *D. hydei*. Similar to Kageyama *et al*, 2006 and Nakayama *et al*, 2015, I found that *Spiroplasma* imposed a direct cost to host fitness and had poor vertical transmission in *D. melanogaster*, and this observation was paralleled in *D. sechellia* and *D. yakuba*. However, *Spiroplasma*-transinfected *D. simulans* did not exhibit pathology relative to the uninfected controls, although vertical transmission was variable in the generations post lateral transfer. The presence of the secondary endosymbiont, *Wolbachia*, did not appear to alter *Spiroplasma*'s phenotype in the novel hosts. However, to test conclusively whether *Wolbachia* affects *Spiroplasma*'s pathological phenotype, one could cure the *D. melanogaster W+* CS strain of *Wolbachia* and then measure the impact of *Spiroplasma* infection alongside *W+* infected female replicates. Due to the differences in genetic backgrounds of the *W+* and *W-* strains used and the fact that the experiments were carried out at different times, it is not possible to conclude that *Wolbachia* does not affect the offspring reduction caused by *Spiroplasma*.

Importantly, species in the *D. simulans* complex diverged only recently. *D. sechellia* and *D. simulans* for example, are estimated to have diverged from one another 413,000 years ago and they can produce fertile female hybrids. Nevertheless, these host species differ markedly in their response to novel *Spiroplasma* infection. *D. simulans* is the only species tested where *Spiroplasma* does not exhibit a pathological phenotype. However, in the presence of early reproduction, and in the absence of selection for infection at each generation *Spiroplasma* rapidly disappeared. Despite the absence of obvious pathology, *Spiroplasma* was not maintained in the novel host population and thus *Spiroplasma* would be very unlikely to successfully invade. Inefficient vertical transmission indicates a misfit between symbiont and host (Clancy and Hoffmann, 1997; Hutchence *et al*, 2011; Nakayama *et al*, 2015), although this misfit does not necessarily affect *Spiroplasma* titre (Kageyama *et al*, 2006). As suggested

by Nakayama *et al*, 2015, the poor transmission efficiency is likely due to inefficient movement of *Spiroplasma* into the germ line due to a divergence of yolk proteins among the different species (Herren *et al*, 2013).

*Drosophila hydei*, the native host of HY1, is in the *repleta* species group and has a different life history to recipient hosts in the *D. melanogaster* subgroup. At 24ºC *D. hydei* takes 14 days to develop from egg to adult and females do not reach sexual maturity until 3 days after eclosion and the males 9 days (Markow, 1985). In contrast, species in *the D. melanogaster* subgroup develop from egg to adult in around 8.5 days (Ashburner *et al*, 2005). Thus the genetic distance and subsequent differences in life history between *D. hydei* and species in the *D. melanogaster* subgroup, could create a mismatch between the symbiont and novel host.

I hypothesize that the pathological agent of *Spiroplasma* is ineffective in *D. simulans* due to a loss or alteration of a host target molecule, or the development of resistance in the host, which has occurred since the two species diverged from one another. I was not able to further investigate the causative agent of pathology in these 3 species due to a change that occurred in *Spiroplasma* whilst in *D. hydei* stock. This phenotypic switch of *Spiroplasma* is described in chapter 3.

I investigated whether the protective phenotype of *Spiroplasma* could transfer to novel hosts, and whether this element of compatibility has also diverged. *Spiroplasma* did not increase fly survival in *D. simulans* following parasitoid wasp attack but it did however, have a negative effect on wasp survival. In contrast, *Spiroplasma* transinfected into *D. melanogaster* conferred protection, where infected hosts showed increased fly survival in the presence of parasitoids. These data suggest that host background is important in predicting compatibility to novel symbiont infection. The CantonS strain of *D. melanogaster* used in this assay can also carry its own *Spiroplasma* native strain, MSRO, which is present in wild populations of *D. melanogaster* at frequencies ranging from 1.1 to 17% (Montenegro *et al*, 2005). Genetic variation and functional differences among host species could lead to varying levels of compatibility potential. 'Preadaptation' could be a pre-requisite for successful host-shifts and could be an important general limit on symbiont host-shifts. One caveat is that these assays were carried out two years after the virulence phenotype observations, such that the strain may have diverged in protection property during laboratory passage.

The control females used in this experiment received the same injection as the infected females, they were not randomly allocated control females. Arguably, it would have been more appropriate to use females injected with PBS as controls as it is not evident whether the female controls used in my experiments eliminated *Spiroplasma* through an unknown mechanism or whether *Spiroplasma* was for some reason unable to infect these females. However, the results from experiments in this chapter are highly comparable to Nakayama *et al*, 2015. The controls used are indeed uninfected and show no pathology compared to the infected female replicates.

In this chapter I show that *Spiroplasma* has a direct physiological cost to all but one of the *Drosophila* hosts tested in the *Drosophila melanogaster* subgroup, which are all equivalent in their relatedness to the natural host. This supports the phylogenetic clade theory that susceptible hosts are scattered across *Drosophila* phylogeny independently of their distance to ancestral hosts. In addition, it demonstrates that host competence to receive *Spiroplasma* is an evolutionarily labile trait, varying between sibling species. Where *Spiroplasma* did not exhibit a pathological phenotype following lateral transfer, the symbiont did not express its protective phenotype and it was not maintained in the host population. In another instance where *Spiroplasma* had previously shown pathology, the protective phenotype was expressed. Overall, compatibility of closely related hosts to novel *Spiroplasma* infection is an evolutionarily labile trait, in terms of vertical transmission, pathology and protective phenotype.

# Chapter 3

## Heritable *Spiroplasma* rapidly change phenotype.


### Abstract

Endosymbionts typically have a small population size, reproduce asexually, may lack repair mechanisms and undergo a bottleneck event at each host generation. Thus, they can have faster mutational rates and accelerated rates of molecular evolution compared to their free-living bacterial counterparts. Here, I serendipitously observe a phenotype switch in the bacterial endosymbiont of *Drosophila* species, *Spiroplasma*. During simple maintenance of *Spiroplasma* strain, HY1, of *Drosophila hydei*, the bacteria evolved increased compatibility (reduced pathology) to novel hosts. Thus, not only is compatibility to receive a novel endosymbiont an evolvable trait but also endosymbionts can be highly labile in terms of their phenotype and interactions with novel hosts.

## 3.1 Introduction

Arthropods are commonly associated with chronic bacterial infections which are maintained in host populations via faithful cytoplasmic inheritance (Buchner, 1965). Host compatibility, the ability to receive and transmit a novel symbiont without activating the immune response or exhibiting pathology, typically reduces with phylogenetic distance from the ancestral host (Longdon *et al*, 2011), such that distantly related species make less compatible hosts. In nature, symbionts undergo host-shifts at different rates; for example, *Wolbachia* is present in around 50% of arthropod species and has thus undergone many host shift events (Weinert *et al*, 2015) and *Cardinium* only 7% (Zchori-fein and Perlman, 2004). The widespread movement of these microbes indicates new associations form commonly, but there may be factors other than sympatry that determine whether a symbiont can invade a novel host species. For instance, heritable symbionts rely on host reproduction to pass to the next generation and thus their prevalence (and ability to colonise a novel host species) may be affected by any fitness costs they impose on the host.

Previously, I observed that the ability of a host to receive and transmit a novel endosymbiont following a host shift is an evolvable trait that can differ between closely related species, including sibling species. Kageyama *et al*, 2006 and Nakayama *et al*, 2015 suggest, along with data I present in chapter 2, that *Spiroplasma* from *Drosophila hydei* (HY1) reduces the fitness of the novel host, *Drosophila melanogaster*. I have also shown that HY1 is effectively lethal in *Drosophila sechellia* and that it does not elicit a pathological phenotype in *Drosophila simulans*.

Following on from chapter 2, I was motivated to repeat the high pathology observed in *D. sechellia* to determine the mechanistic cause of the pathological phenotype. Ribosome inactiving proteins (RIPs), encoded in *Spiroplasma*'s genome (Masson *et al*, 2018), mediate protection against parasitic nematodes and wasps in *Drosophila melanogaster* and *Drosophila neotestacea* (Hamilton *et al*, 2015; Ballinger and Perlman, 2017). RIP activity causes the depurination of wasp 28S rRNA, soon after the wasp egg hatches in the *Drosophila* larvae. Although fly ribosomes show little evidence of targeted depurination from *Spiroplasma*, one could hypothesize that *Spiroplasma* recognizes novel hosts as foreign and thus the observed pathology in novel *Drosophila* hosts is related to the depurination of their 28S rRNA by RIPs (Ballinger and Perlman, 2017).

Up until now there has been strong repeatability of the pathogenic phenotype of HY1 in the novel host, *D. melanogaster* (Kageyama *et al*, 2006; Hutchence *et al*, 2011; Nakayama *et al*,

2015; Chapter 2). However, here I observe an unexpected lack of repeatability of this assay. Instead of causing pathology in novel host, *D. sechellia, Spiroplasma* strain, HY1, imposed no fitness cost to adult females. To confirm that the change had indeed occurred in *Spiroplasma* and not the host, I repeated the transinfections and subsequent fecundity assays in *D. melanogaster* and again in *D. sechellia*. I observed that *Spiroplasma* has an altered phenotype relative to that described in chapter 2 and by Kageyama *et al*, 2006, Hutchence *et al*, 2011 and Nakayama *et al*, 2015.

## 3.2 Materials and Methods

*3.2.1 Symbiont and hosts*

*Drosophila hydei* wild-type stocks harbouring *Spiroplasma* (*Spiroplasma* strain TEN 104-106 haplotype 1, HY1, Mateos *et al,* 2006) were used. This stock was originally established in Mexico in 2004, from a single HY1-infected female. HY1 falls as an out-group of *Spiroplasma poulsonii* and shows no evidence of reproductive manipulation in the *Drosophila* strains that were used as novel fly hosts (Hutchence *at al*, 2011). Other *Spiroplasma* lineages used in this chapter include Ancestral and M14. Both strains are derived from the Mexican isoline but have been maintained independently of one another in the laboratory for many years.

A *Wolbachia* (*wMel*) infected strain of *D. melanogaster,* Canton S (CS), derived from Montenegro *et al*, 2005, was used. *D. hydei* and *D. melanogaster* stocks were maintained on a cornmeal-based medium (ASG) consisting of agar, yeast, sugar, maize and nipagin, supplemented with live yeast granules, at 25°C under a 12 h:12 h light:dark cycle with overlapping generations. The *D. sechellia* line was acquired from a Drosophila Stock Centre in the US and given to me by Dr Chloe Heys. I used *D. sechellia* line 21, carrying the *w*Sh strain of *Wolbachia*. *D. sechellia* was maintained on malt medium (agar, yeast, malt, maize, nipagin and proprionic acid) supplemented with live yeast granules, at 25°C under a 12 h:12 h light:dark cycle with overlapping generations.

*3.2.2 Timeline of HY1 strains*

The *Spiroplasma* HY1 strain (Mateos *et al,* 2006) has been split into independent lineages in the laboratory many times over in the past decade. I will refer to the historical *Spiroplasma* strain used by Hutchence *et al*, 2011 and Nakayama *et al*, 2015 as Ancestral. In May 2014, fellow PhD student, Dr Chris Corbin, injected the hemolymph of *D. hydei* infected with the Ancestral *Spiroplasma* strain into tetracyclin-treated (and thus *Spiroplasma* uninfected) *D. hydei*. The resulting infected lineage is referred to as M14 (Fig.3.1).

The transinfection and protection assays described in chapter 2 were conducted from December 2015 to May 2017. The *Spiroplasma* used in these assays derives from the Ancestral stock, but it has been maintained separately since October 2015. I therefore refer to the lineage used in the experiments for this chapter as HY1-2015. Repeat assays using HY1-2015 were conducted from August 2017 whereupon *Spiroplasma* showed an altered phenotype. I refer to *Spiroplasma* with the novel phenotype as HY1- 2017.

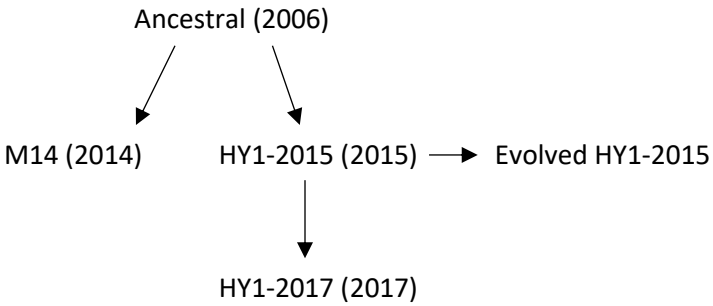



**Figure 3.1** Schematic of laboratory *Spiroplasma* lineages. The bacterial strain used in this chapter, HY1-2015, has been maintained separately from the Ancestral strain since 2015.

### *3.2.3 Artificial lateral transfer of Spiroplasma*

Microinjections were carried out as described by Nakayama *et al*, 2015. Hemolymph was extracted from the thorax of *Spiroplamsa* positive *D. hydei* and mixed with sterile PBS. Virgin female *Drosophila* were artificially injected in the abdomen with 0.1-0.2µl of PBS-hemolymph, using a hydraulic positive-pressure microinjection apparatus (Model IM-6, Narushige Ltd, Tokyo, Japan).

Injected females were aged for 14 days to let the *Spiroplasma* infection establish in its new host. Each female was then placed with 2 males of the same *Drosophila* strain in a vial containing 15 ml *Drosophila* medium. Adult flies were allowed 4 days to oviposit, after which the males were discarded and the females frozen at -80°C. All injected females were screened for *Spiroplasma* using a PCR assay. Offspring from unsuccessfully infected females (females that did not take up *Spiroplasma* upon injection) were used as negative controls. The DNA extraction and PCR assays used are described in chapter 2. The injected females were designated as the parental generation.

### *3.2.4 Maintenance of Spiroplasma in the novel hosts, D. melanogaster* and *D. sechellia*

Virgin female offspring from the successfully infected parental females and an uninfected control were aged for 10 days and then placed with two males in small mating cages (2cm height x 2cm diameter) containing grape juice medium (concentrated grape juice, agar, water and nipagin), as described in chapter 2. Larvae were transferred onto ASG fly medium in standard 75 mm vials, at a constant density of 20 larvae per vial. Eclosed virgin females of the F2 generation were collected and aged for 10 days.

*3.2.5 Measurement of fitness*

To measure the fitness costs of *Spiroplasma* infection in novel hosts, I measured the number of adult offspring produced 2 generations after transinfection, comparing the fecundity of infected and uninfected females. Details of the methodology of the fecundity assay can be found in chapter 2. The eclosed offspring from each day were counted and sexed.

An earlier assay where *D. melanogaster W+* was transinfected with HY1 (described in chapter 2), was revisited in this study. Following the fitness assay, F2 females from each parental line were collected (X, Y and Z), aged for 10 days and then placed individually in vials of *Drosophila* medium with 2 CS males for one day, allowed to mate, and then allowed to oviposit for a further 4 days. After, the adult flies were removed and the females screened for *Spiroplasma*. Offspring from uninfected individuals were discarded. Virgin female offspring from infected vials were collected and mixed with other females from the same parental line. Four females were randomly selected per line to be mothers of the next generation. This enabled selection of both cost of infection and vertical transmission efficiency. These females were aged for 10 days and then crossed and screened, as described above. This procedure was continued for 20 generations. *Spiroplasma* of the F20 generation is herein referred to as 'evolved HY1-2015'. A fitness assay was carried out using 'evolved HY1-2015' alongside *Spiroplasma* of the HY1-2017 strain. To ensure that the fitness measurements were comparable between the newly transinfected females and the F20 selected lines, I transferred the 'evolved' *Spiroplasma* from the selected lines into virgin CS females and then measured the fitness of the F2 generation.

To ensure that the fitness measures were robust, I performed the fitness assay of infected females alongside an uninfected control. The variation in fecundity observed between the different fitness assays of the same *Drosophila* strain could be due to temperature and humidity changes associated with changes in seasons (Hoffmann, 2010), which were beyond our control. PCR assays included (a) a positive control re-extracted alongside the test individuals (*D. melanogaster* carrying Melanogaster Sex Ratio Organism: MSRO) and (b) repeat PCR assays of negative specimens. Quality control for PCR assays included screening all individuals for the mitochondrially encoded cytochrome c oxidase I gene (CO1).

*3.2.6 Statistical analyses*

The number of offspring produced by transinfected and uninfected control females was analysed using general linear models. Where there were selection replicates, they were

nested within the infection status in the analyses. All statistics was performed using RStudio Software for Statistical Computing, version 3.5.0 (R Core Team, 2018).

## 3.3 Results

*3.3.1 Loss of direct cost of Spiroplasma on host fitness in D. sechellia*

I compared the response to novel HY1-2017 infection in two *Drosophila* species in the *melanogaster* subgroup, *D. melanogaster* and *D. sechellia,* following a change in HY1 phenotype. This phenotype switch occurred 3 months after the previous transinfection assay in *D. sechellia* which was reported in chapter 2.

*Spiroplasma* strain, HY1-2017, showed no evidence of causing pathology in the novel host *D. sechellia* (Fig.3.2). HY1-2017 infected F2 females produced a comparable number of offspring to the uninfected controls (S+, $t$ = -0.202, $P$ = 0.840) as did the uninfected F2 females that were descended from infected F1 mothers (NI, $t$ = -0.768, P = 0.444). This observation contrasts to the almost lethal effects of HY1-2015 in *D. sechellia*, reported in chapter 2.

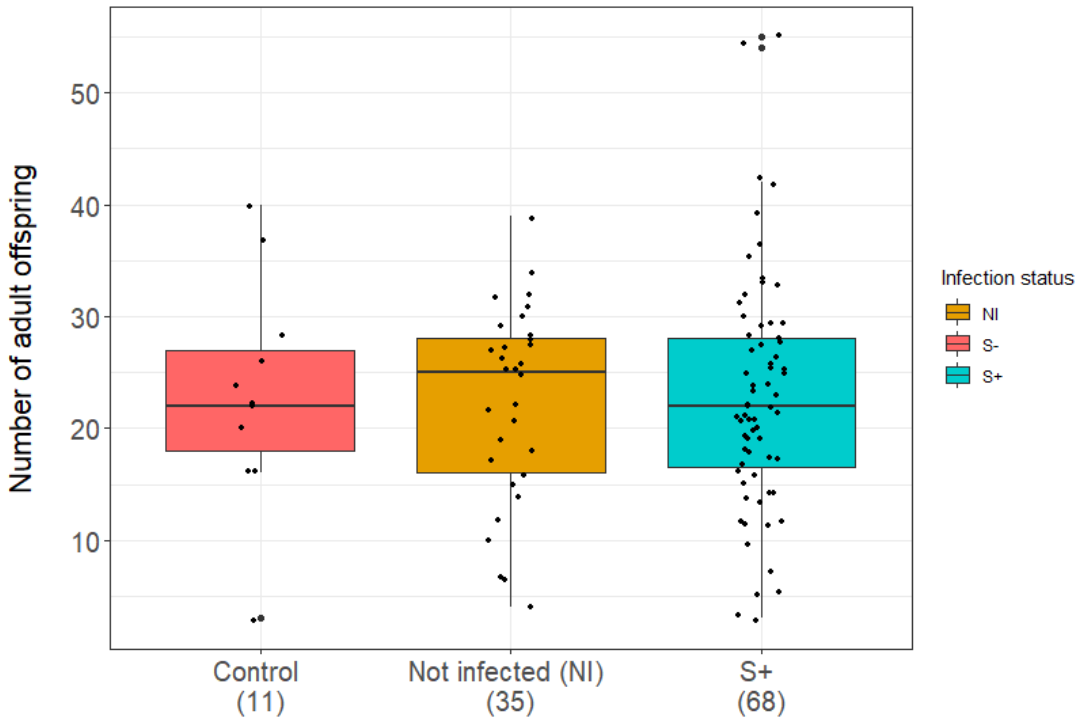



**Figure 3.2** Direct cost of *Spiroplasma* on host fecundity following lateral transfer from *D. hydei* to *D. sechellia*. The average number of offspring produced over 4 days by F2 *D. sechellia* females infected with *Spiroplasma*, females that have lost infection and an uninfected control. Females that lost *Spiroplasma* infection between the F1 and F2 generations are called 'Not infected (NI)'. The box plots display the upper and lower quartiles, the median and the range. Points represent each measurement obtained and values in paratheses show sample size.

In a second experiment, I transinfected *D. sechellia* with additional laboratory strains of *Spiroplasma,* that had been maintained independently of one another in stock culture. The number of offspring produced by females infected with HY1-2015, as well as the number of offspring produced by females infected with Ancestral and M14 strains, was comparable to the number of females produced by uninfected controls (Ancestral, $t = -0.348$, $P > 0.05$; S+, $t = 0.531$, $P > 0.05$; M14, $t = -1.182$, $P > 0.05$) (Fig.3.3). Thus, the phenotype switch appears to have affected all *Spiroplasma* stocks.

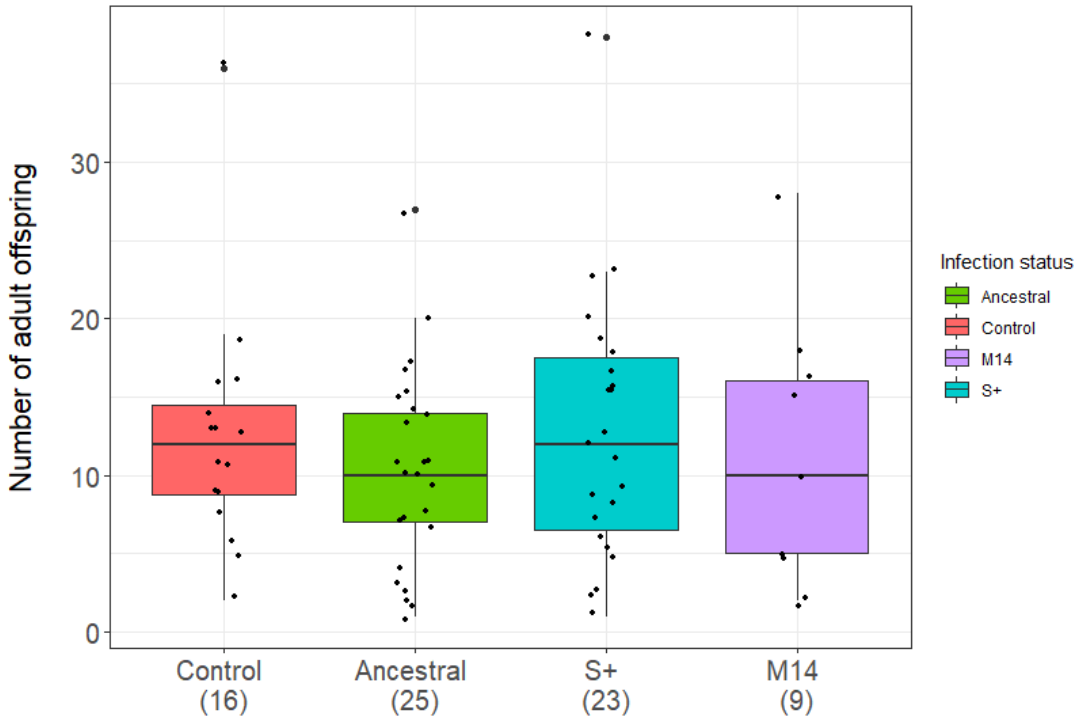



**Figure 3.3** The average number of offspring produced over 4 days by *D. sechellia* following artificial lateral transfer of different *Spiroplasma* strains, and an uninfected control. The box plot displays the upper and lower quartiles, the median and the range. Points represent each measurement obtained. Values in paratheses show sample size.

### *3.3.2 Loss of direct cost of infection in D. melanogaster*

In chapter 2, I reported that following lateral transfer, HY1-2015 causes pathology in *D. melanogaster W+*. The results support the findings of Kageyama *et al*, 2006 and Nakayama *et al*, 2015. However, in a subsequent repeat assay, *D. melanogaster* females infected with the native HY1-2017 strain did not display pathology (Fig. 3.4). F2 generation females infected with the HY1-2017 strain from *D. hydei* stock culture produced fewer offspring than the uninfected controls, but the difference was not statistically significant (S+, $t = -1.753$, $P >$

0.05). Two of the evolved HY1-2015 lines, X and Y, that were passaged through *D. melanogaster* for 20 generations, produced significantly fewer offspring compared to the uninfected controls (X, $t$ = -3.099, $P$ = 0.002; Y, $t$ = -2.667, $P$ = 0.009). Although evolved HY1-2015 line, Z, produced fewer offspring than the uninfected control, it was not significantly different (Z, $t$ = -1.810, $P$ > 0.05). Line Z appears to have attenuated relative to the fitness costs the same selection line imposed after only 2 passages (see chapter 2). Thus the phenotype switch appears to have affected the stock cultures only and not the X, Y and Z lines which have been maintained independently of the stocks over the preceding year.

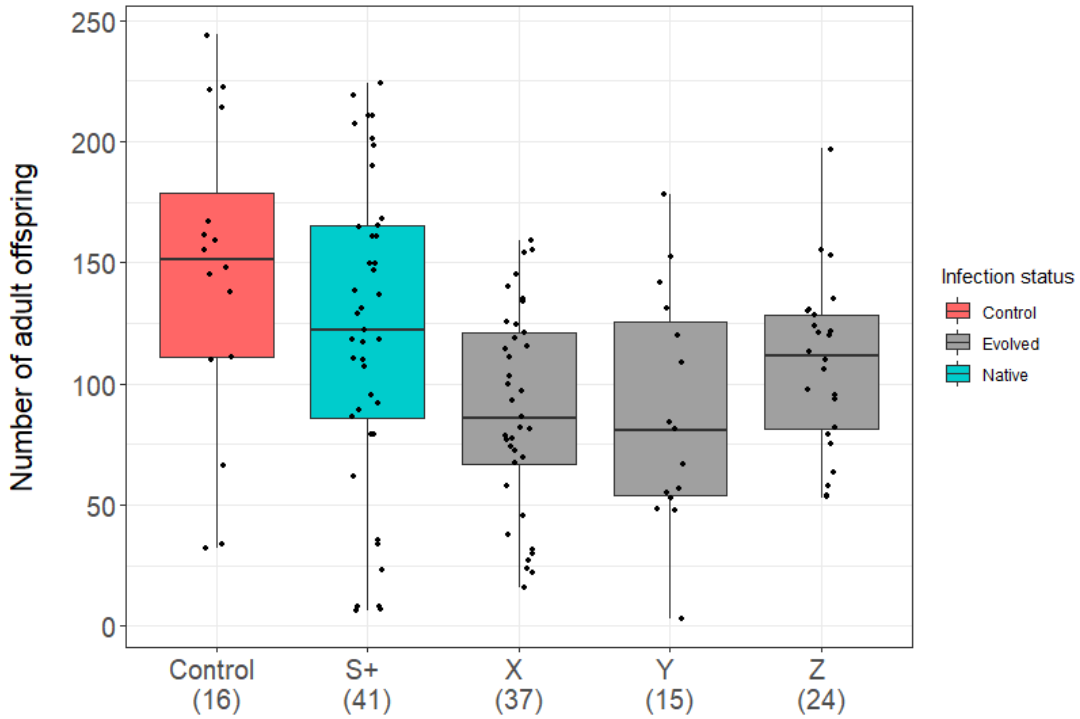



**Figure 3.4** Direct cost of native and evolved strains *Spiroplasma* on host fecundity, following lateral transfer to *D. melanogaster W+*. The average number of offspring produced over 4 days by F2 *D. melanogaster* females infected with *Spiroplasma* and an uninfected control. The box plot displays the upper and lower quartiles, the median and the range. Points represent each measurement obtained. Values in paratheses show

## 3.4 Discussion

Bacteria differ in their evolutionary history and mutational rate. Endosymbionts for example, have a small population size (typically smaller than their free-living counterparts), reproduce asexually and undergo little or no recombination between host lineages (Moran, 1996). They may also lack systems for DNA repair. Thus, the relative rates of substitution are 1.7- to 2.7-fold higher for endosymbionts than their free-living counterparts, equivalent to hypermutator strains that have lost mismatch repair systems. In particular, the symbiont of the pea aphid, *Buchnera aphidocola,* displays accelerated evolution in 16S rRNA sequences and an accumulation of A + T rich codon families in amino acids. Furthermore, *Buchnera* has a lower ratio of synonymous to nonsynonymous substitutions compared to ratios for the same genes in free-living *Escherichia coli* and *Salmonella typhimurium*. This is due to elevated rates of nonsynonymous substitutions. Thus the increased rate of DNA sequence evolution in *Buchnera* compared to other symbionts is a result of the fixation of mildly deleterious mutations and codon bias toward [A + T].

Endosymbionts also undergo a bottleneck each generation during inoculation of the host's progeny. Due to their population structure, endosymbionts accumulate mutations that are deleterious at the level of polypeptide function (Moran, 1996). These changes could result in a shift in the bacteria's phenotype.

Previous studies suggest that *Spiroplasma poulsonii* causes pathology and/or has poor vertical transmission efficiency following transinfection into *D. melanogaster* (Counce and Poulson, 1966; Kageyama *et al*, 2006; Nakayama *et al*, 2015). In chapter 2, I observed that *Spiroplasma* has a direct physiological cost among closely related hosts, *D. melanogaster, D. sechellia* and *D. yakuba,* but not in *D. simulans*. These novel host species are all equivalent in their relatedness to the natural host. Together, these results suggest that transinfected HY1-2011 and HY1-2015 do not perform well in terms of vertical transmission and virulence in the majority of novel hosts in the *D. melanogaster* subgroup, compared to its native host (Xie *et al*, 2010) and *D. simulans* (see Chapter 2). Moreover, compatibility to receive novel *Spiroplasma* infection in the *D. melanogaster* subgroup is an evolutionarily labile trait.

In this study, I reassessed the evolution of host compatibility to novel *Spiroplasma* infection in the *D. melanogaster* subgroup 2 years after the first experiment. I focused on two *Drosophila* species, *D. melanogaster* and *D. sechellia*, where *Spiroplasma* had previously shown the greatest effects (see chapter 2). Contrary to previous findings, I observed that HY1-2017 from *D. hydei* did not cause pathology in either of these novel host species. I also

checked additional lineages of HY1 *S. poulsonii*, Ancestral and M14 in *D. sechellia* that have been maintained independently in the laboratory. Overall, I tested 4 independently maintained lineages of HY1 in *D. sechellia* and *D. melanogaster* ('evolved' HY1-2015, HY1-2017, Ancestral and M14). The virulent phenotype was unexpectedly and repeatedly undetectable. The fitness measurements were conducted in multiple host species and thus the change in *Spiroplasma* phenotype can be attributed to evolution of *Spiroplasma*, and not the hosts.

Recently, Harumoto and Lemaitre, 2018 identified a *Spiroplasma* MSRO mutant laboratory strain with reduced male-killing capacity (MSRO-SE), where almost half of the male progeny survive to adulthood. Typically, MSRO infected flies have an average egg hatch rate of 42% and produces broods that are 98.56% female (Montenegro *et al*, 2005). Similar to the reduced *Spiroplasma* virulence reported in this chapter, the reduced male killing was not associated with host genetic background. A comparison between the MSRO-SE and MSRO genomes revealed deletion of a protein with ankyrin repeats and the OTU (ovarian tumour) deubiquitinase domain, called Spaid (*S. poulsonii* androcidin). Expression of Spaid was sufficient to cause male killing. This study reveals that other *Spiroplasma* strains, not just HY1 can rapidly change phenotype (Harumoto and Lemaitre, 2018). Indeed, previous reports of trait lability were recorded in *Spiroplasma* strain NSRO in the 1980s, characterised as the strain NSRO-A (Yamada *et al,* 1982) that lacked male-killing ability.

The likelihood that all four independently maintained HY1 lineages changed at or around the same moment in time could be considered low. A change in conditions or a drop in temperature in the 25°C CT room could be responsible for independent evolutionary changes across the different lineages. Another possible explanation could be the spread of a virus through the *D. hydei* stocks which results in repression or alteration of *Spiroplasma*'s phenotype.

Since Harumoto and Lemaitre's publication in 2018, further reports of rapid phenotypic change in *Spiroplasma* have been reported. For example, *Spiroplasma* grown in culture can lose the capacity to re-establish symbiosis with insect hosts. In addition, *S. poulsonii* shows rapid spontaneous evolution of antibiotic resistance *in vitro* when challenged with kanamycin, even with relatively small population sizes and slow generation times (F. Masson, pers. Comm.).

Following the phenotype change in the Liverpool HY1 stock, HY1 has been sequenced and compared to the genomes of an additional Liverpool strain, M14 and *Spiroplasma* from the

Texan stock of *D. hydei* (TX). Preliminary findings suggest that HY1 has a very high substitution rate at the nucleotide level. Based on estimates from M14 and TX, HY1 evolves 1000x faster than *Wolbachia* (Richardson *et al*, 2012) and 30x faster than *Buchnera* (Moran *et al*, 2009), (M. Gerth, pers. comm.). This rapid rate of evolution could be caused by a lack of DNA repair mechanisms, but also by *Spiroplasma*'s small population size, lack of recombination and bottlenecks, similar to Buchnera (Moran, 1996).

Moreover, there have been larger structural changes such as duplications and deletions of over 1000bp among the different *Spiroplasma* strains. Specifically, there were 14 structural changes in TX and 6 in M14 strains (M. Gerth, pers. comm.). Similar to *Spiroplasma melliferum* (Lo *et al*, 2013), HY1 has undergone rapid structural rearrangement despite having an average nucleotide sequence similarity of *c*. 99% to TX. Thus genome rearrangements may be a typical feature of *Spiroplasma.* HY1 contains a copy of the male killing gene, Spaid; however it lacks the signal peptide and thus the male killing phenotype is not observed in *D. hydei*. It also contains an ETX (epsilon toxin) domain which is typically found in pathogenic *Spiroplasma*.

Overall, the data presented indicate that *Spiroplasma* is a highly labile symbiont in terms of the phenotype of interaction with the host. Previous work had established changes from virulence to avirulent during laboratory passages in a novel host species (Nakayama *et al,* 2015). In this case, simple maintenance in the native host led to evolution of increased compatibility (lower virulence) in novel hosts. The rapid evolution towards benign impacts of *Spiroplasma* infection on the fitness of novel hosts occurred between experiments, in the native host, *D. hydei*. There is now a growing wealth of phenotypic and genomic evidence to suggest that laboratory strains of *Spiroplasma* change quickly, particularly at loci that are important for generating phenotypes. Thus we are able to start building a range of phenotype evolution that reflects the rapid molecular evolution of *S. poulsonii*.

Caution should be exercised over assuming that identified strains of *Spiroplasma* are identicall. Due to *Spiroplasma*'s high lability, stocks are likely to vary over time within and between laboratories. Thus, a reference collection should be kept and frozen, ideally in the form of an isofemale line, which can be used for comparison of independently maintained stock cultures.

# Chapter 4

## Gut microbes do not strongly influence *Spiroplasma*/*Drosophila* phenotypes.


### Abstract

There are two major groups of arthropod symbionts that are studied, those that are transmitted transoviarially and those that are transmitted on the egg chorion and subsequently reside in the gut. Animal physiology is a consequence of the dynamic interplay between diet and gut microbes. The physiological impact of endosymbionts and gut microbes have traditionally been considered in isolation of each other, despite evidence that both endosymbionts and gut microbes can present as metabolic drains to the host. In this chapter, I exploit the *Drosophila melanogaster* model system to examine the interaction between diet, gut microbiota and heritable *Spiroplasma* bacteria. I observed that nutrient availability affects larval developmental time, particularly in axenic *Drosophila*. *Spiroplasma* further delays development of axenic *Drosophila,* but only on a low yeast diet. I did not observe a reduction in *Drosophila* hemolymph lipid in response to diet or *Spiroplasma* infection. *Spiroplasma* titre is stable and buffered against changes in the gut microbial community and there is no correlation between lipid availability and *Spiroplasma*. Overall, all microbial species tested were functionally equivalent in regulating host nutrition and there were no strong interactions between diet, microbiota and symbiont infection. In future research, heritable microbes should be considered in conjunction with gut microbiota.

## 4.1 Introduction

Arthropod- bacterial symbioses are common in nature, and diverse in form and function (Duron *et al*, 2008; Weinert *et al*, 2015). This diversity includes variation in the impact of the symbiont on the fitness of the host individual, which can vary from required, to beneficial (but not required), to costly and parasitic (Ewald, 1987). Diversity also includes the route by which the host-microbe combination is formed. This may be through trans-ovarial inheritance via the egg or embryo (O'Neill *et al*, 1992), acquisition from the mother on the surface of the egg (Wong *et al*, 2011), mechanical transmission into the arthropod's host during oviposition (Werren *et al*, 1986) and finally acquisition following environmental exposure. These routes are reflected in symbionts that inhabit the exterior surface, the gut lumen, colonize gut epithelia, or live inside the main body of the insect, commonly inside cells. The microbes involved in these symbioses are also broadly derived from across the eubacterial tree of life. Many symbiotic microbes are members of proteobacteria, but a range of gram positive microbes and Mollicutes are also observed living symbiotically within insects (Ota *et al*, 1979; Moran and Telang, 1998).

There have been two major groups of symbioses studied in recent years. First, there are those that are heritable, passing from a female to her offspring, commonly inside eggs or embryos (Ota *et al*, 1979; Rousset *et al*, 1992; Werran *et al*, 1995; Sacchi *et al*, 2008). Heritable microbes can contribute to host physiology through amino acid or vitamin synthesis (Douglas, 1998; Moran *et al*, 2003 Sabree *et al*, 2009; Santos-Garcia *et al*, 2018). They may also represent a metabolic drain on their host by utilizing host metabolites (Herren *et al*, 2014). Second, there are gut microbes. Gut-associated microbes have various impacts on host physiology, development, longevity, behaviour and immunity (Ryu *et al*, 2008; Storelli *et al*, 2011; Shin *et al*, 2011; Dantoft *et al*, 2016; Wong *et al*, 2017). Typically, insects reared in axenic (germ-free) conditions perform worse than microbe-colonized individuals on a range of life history and physiological parameters. This is most obvious in species with coadapted microbiomes, such as termites and bees, but is also observed more widely, such as in species such as *Drosophila* (Brune and Ohkuma, 2010; Wong *et al*, 2014; Kwong and Moran, 2016).

Bacterial gut microbes and endosymbionts have traditionally been considered in isolation of each other. However, a recent study in mosquitos indicated that gut microbes were a primary determinant of host compatibility (the ability to spread in novel host species) to symbiont infection. Hughes *et al*, 2014 demonstrated that the native gut microbiota in *Anopheles*

*gambiae* prevented the vertical transmission of horizontally transferred *Wolbachia*. When the gut microbiome was removed, specifically the bacterium *Asaia* via antibiotic treatment, *Wolbachia* was transmitted perfectly from mother to offspring. In certain cases, the native host microbiome may act as a biological barrier to novel symbiont infection. Reciprocally, symbionts may affect the composition of the gut microbiome. In *Drosophila melanogaster*, the presence of *Wolbachia* has been shown to reduce *Acetobacter* abundance, although this phenomenon is host genotype dependent (Simhadri *et al*, 2017). Furthermore, the symbiotic bacterium, *Enterococcus mundtii*, is essential in maintaining the gut microbiota of *Galleria mellonella* during metamorphosis (Johnston and Rolff, 2015). In the absence of the symbiont the adult gut microbiota is dominated by pathogenic bacteria, which cause host mortality. Thus it is important to consider gut microbes and endosymbionts together when investigating the impact of endosymbionts on host development, physiology and behaviour and vice versa.

Here, I exploit the *Drosophila melanogaster* system to examine the interaction between gut microbiota and heritable *Spiroplasma* bacteria. Gut symbionts affect *Drosophila* ontogeny, nutrient allocation and reproduction (Newell and Douglas, 2014; Wong *et al*, 2014, Elgart *et al*, 2016; Morimoto *et al,* 2017). Elimination of the gut microbiota via egg dechorionation, extends larval development time of *D. melanogaster* (Ridley *et al*, 2012). Axenic flies also display an altered metabolism for example, glucose and lipid concentrations are higher and body mass is greater than conventional flies (with unmanipulated microbiota). Acetobacters in particular utilize *Drosophila* metabolites and are essential in maintaining 'standard' fly lipid concentrations and weight (Newell and Douglas, 2014). In addition, *Spiroplasma* heritable symbionts have also been observed to alter lipid metabolism. Reciprocally, *Spiroplasma* titre is sensitive to nutrition, with proliferation limited by access to lipid (Herren *et al,* 2014).

Evidence that both gut and *Spiroplasma* symbionts affect host lipid indicates that aspects of host physiology, such as available lipid, may be codetermined by gut microbiota status and *Spiroplasma* presence/absence. I test for the response of life history parameters, and physiological variables (carbohydrate, protein, and lipid) to gut microbiota and *Spiroplasma* presence across standard and low nutrient diets in a factorial design. Additionally, I investigate whether *Spiroplasma* titre is affected by gut microbiome composition and diet.

## 4.2 Material and Methods

I compared the following metrics in *Drosophila*; development rate, nutritional indices and *Spiroplasma* titre across six different gut microbiota conditions (axenic, *A.pomorum*-only, *A.tropicalis*-only, *L.brevis*-only, *L.plantarum*-only and 4-species) in the presence/absence of heritable *Spiroplasma* bacteria, under standard and low nutrient availability.

*4.2.1 Cultivation of bacteria and insects*

Experiments compared gut microbiota and physiological measures between *Wolbachia* (*wMel*) infected *D.melanogaster* Canton S (CS) strain and an isogenic strain carrying Melanogaster Sex Ratio Organism (MSRO) as derived from Montenegro *et al*, 2005. These strains were maintained at 25°C under a 12 h:12 h light:dark cycle on a standard diet (SY) comprising 100 g $l^{-1}$ glucose (Sigma, St Louis, MO, USA), 100 g $l^{-1}$ inactive yeast (MP Biomedicals, Santa Ana, CA, USA) and 12 g $l^{-1}$ agar (MP Biomedicals) and preservatives (0.04% phosphoric acid, 0.42% propionic acid; Sigma). A low yeast (LY) diet comprised of 25 g $l^{-1}$ yeast and 100 g $l^{-1}$ glucose (2.5:10). Preservatives (nipagin, propionic acid) were added to autoclaved food once cooled to 60 °C.

Four bacteria, originally derived from *D. melanogaster* guts, were used: *Acetobacter pomorum* DmelCS_004*, Acetorbacter tropicalis* DmelCS_006*, Lactobacillus brevis* DmelCS_003 and *Lactobacillus plantarum* DmelCS_001 (Newell *et al*, 2014). The bacteria were cultured in modified MRS (mMRS), containing the following (all from Sigma unless otherwise noted): 1.25% vegetable peptone (Becton Dickinson), 0.75% yeast extract, 2% glucose, 0.5% sodium acetate, 0.2% dipotassium hydrogen phosphate, 0.2% triammonium citrate, 0.02% magnesium sulfate heptahydrate, 0.005% manganese sulfate tetrahydrate, 1.2% agar (Apex) at 30°C under aerobic conditions (*Acetobacter*) or with reduced oxygen in sealed containers that were filled with $CO_2$ gas prior to sealing (*Lactobacillus*).

Axenic *Drosophila* were generated by the method of Newell and Douglas, 2014. Briefly, freshly laid eggs (<18 h old) were collected from grape juice agar plates and washed 3 times in 0.6% sodium hypochlorite solution followed by 3 washes in sterile water and then transferred aseptically to sterile food, with approximately 30 CS eggs for *Spiroplasma*-negative treatments and 60 MSRO eggs for *Spiroplasma*-positive treatments (double the quantity of *Spiroplasma*-positive eggs were transferred to compensate for the male-killing phenotype in the MSRO strain). Three vials were set up on the first day and another three were set up the following day so that there were six vials in total per treatment. Three

additional vials of axenic CS flies were created to supply axenic males as required later in the experiment. To generate gnotobiotic *Drosophila* (i.e. containing one or more defined microbes), a bacterial suspension comprising 5 x $10^6$ cells in 50 µl and prepared from an overnight culture by the procedure of Newell and Douglas, 2014, was added to each corresponding vial. Where *Drosophila* were colonized with the four bacterial strains, the inoculating suspension included 1.25 x $10^5$ cells of each bacterial strain.

Axenic and gnotobiotic insects were maintained under the same conditions as the routine fly stocks at 25°C under a 12 h:12 h light:dark cycle. After eclosion, the vials containing MSRO *Drosophila* (which were exclusively female) were supplemented with two axenic males, to ensure that MSRO females had the opportunity to mate.

*4.2.2 Development time assays*

The time of development to pupation and to adulthood was scored. Observations were made three times daily: at 0, 6, and 11.5 hours post dawn (h.p.d.) for pupation and at 2, 7, and 11 h.p.d. for eclosion to adulthood, until all individuals had eclosed.

*4.2.3 Nutritional indices*

At 10 days post-eclosion, two pools of three mated females from each of the 6 vials from each treatment were collected on ice and weighed to the nearest µg using a Mettler Toledo (MX5) microbalance, and then homogenized in 125 µl of TET buffer (10 mM Tris pH 8, 1 mM EDTA, 0.1% Triton X-100) with 100 µl lysis matrix D (MP Biomedicals) with shaking for 30 s in a FastPrep-24 instrument with the default settings (MP Biomedicals). The homogenates were centrifuged for 3 min at 15,000 x g to pellet debris. Two 50 µl samples of supernatant were heated at 72°C for 30 mins to inactivate endogenous enzymes. Both these samples and a further 10 µl sample were flash frozen and stored at -80°C prior to assay. Protein content was analyzed using the Bio-Rad DC kit according to manufacturer's instructions. Triglyceride (TAG) was measured using the Free Glycerol Detection Kit in combination with Triglyceride Reagent, following manufacturer's instructions (Sigma). Glucose content was measured by the Glucose Oxidase (GO) method as described previously (Newell and Douglas, 2014). Another two groups of three flies from each vial from each treatment were collected for CFU determination to verify microbiome infection status. Finally, 2 pools of 3 flies were collected for DNA extraction and *Spiroplasma* titre analysis (MSRO flies only).

*4.2.4 Abundance of bacteria in experimental flies*

For assays of *Acetobacter* and *Lactobacillus,* two groups of three female flies were taken from the same vials used for nutritional indices and homogenized in 200 µl PBS, as described above. All samples were brought to 1 ml with PBS, and the number of colony forming units (CFUs) was scored by spiral plating (on a WASP-2 instrument, Microbiology International) on mMRS plates. For samples from flies colonized with both *Acetobacter* and *Lactobacillus*, samples were plated twice: once on medium supplemented with 10 µg ampicillin $ml^{-1}$ to exclude *Lactobacilus* growth, and the other incubated in $CO_2$ atmosphere (as above) to suppress *Acetobacter* growth. All plates were incubated at 30°C for 2-3 days, when CFUs were scored using Protocol 3 colony counter (Microbiology International). Species of *Acetobacter* and *Lactobacillus* were differentiated by morphological criteria, as described by Newell and Douglas, 2014.

*4.2.5 DNA extraction and qPCR*

Three *Drosophila* were homogenized per tube (as described above), with 678 µl Cell Lysis Buffer (108 mM Tris-HCl, pH 8.0; 1.5 M NaCl; 21.6 mM EDTA) and 20 µl 20 mg proteinase K $ml^{-1}$. Each sample was incubated at 56 °C for 2 h. Then 5 µl RNaseA were added and samples were incubated at 37 °C overnight. DNA was extracted with 750 µl phenol:chloroform:isoamyl alcohol (25:24:1) as described by Adair *et al*, 2018. In brief, samples were centrifuged at 8000×g for 15 min at 4 °C and 500 µl aqueous phase was transferred to a new tube. To precipitate DNA, 37.5 µl 3M sodium acetate and 500 µl isopropanol were added to all samples and then incubated at −20°C overnight. Following centrifugation at 8000×g for 15 min at 4 °C, the pellets were washed in 750 µl cold 75% ethanol, air-dried, resuspended in 50 µl sterile endonuclease-free water and stored at −20 °C.

For quantification of *Spiroplasma* titre, a modified version of the comparative (Ct) method of quantitative PCR (qPCR) assay by Anbutsu and Fukatsu (2003) was carried out using a Fast Real-Time PCR system (Applied Biosystems). Each 10 µl of qPCR reagent included 5 µl PowerUp SYBR Green Master Mix (Applied Biosystems), 2.5 µl nuclease-free $H_2O$, 2 µl DNA template and 0.25 µl of 20 µM primers. The *Spiroplasma dnaA* gene primers were DnaA109F 5′-TTAAGAGCAGTTTCAAAATCGGG-3′ and DnaA246R 5′-TGAAAAAAACAAACAAATTGTTATTACTTC-3′ (Anbutsu and Fukatsu, 2003). The *Drosophila RPS17* gene primers were Dmel.rps17F 5′-CACTCCCAGGTGCGTGGTAT-3′ and Dmel.rps17R 5′-GGAGACGGCCGGGACGTAGT-3′ (Osborne *et al*, 2009). Reactions were carried out using the following programme: one cycle of 95°C for 10 min, followed by 40 cycles of 95°C 5 s. 56°C 5

s, 72°C 30 s and finally one cycle of 72°C for 10 min. Delta Ct was calculated by subtracting the RPS17 Ct value from the dnaA Ct value for the same sample.

### *4.2.6 Statistical analyses*

Data for development time were analysed in R Software for Statistical Computing, version 1.0.153, using the Survival, coxme, and multcomp (Therneau, 2015a; Therneau, 2015b; Hothorn *et al*, 2008) packages. The development data were formatted as survival objects for each treatment using the Surv function. Kaplan-Meier plots were generated from survival objects using plot- (Survfit(survival object)). A Cox mixed-effects model was applied to survival objects using experiment as a random effect. Pairwise Tukey's tests were made with the general linear hypothesis test (glht) function, correcting *P* values for multiple comparisons by the single-step method (default procedure in multcomp). This approach allowed me to account for any 'block' variation among the two experiments which were set up on two consecutive days. Mass, nutrient content and *Spiroplasma* titre of the flies were analysed with linear models using the multcomp package. All data, apart from titre, were fitted to a three-way interaction of *Spiroplasma* infection, diet and microbiota treatment using experiment as a main effect. Log2 transformed *Spiroplasma* titre was fitted to a two-way -diet by microbe- interaction. The CFU values were log transformed to achieve normal distributions and then analysed with a linear model with microbiota treatment as the only main effect.

## 4.3 Results

*4.3.1 Drosophila development time*

I compared the development time of axenic *Drosophila* (lacking gut microbes) and *Drosophila* colonized with the four test bacteria (*A. pomorum*, *A. tropicalis*, *L. brevis* and *L. plantarum*), either in mono-association or in combination (4-species), in the presence or absence of symbiont, *Spiroplasma*, and reared on either the standard diet (SY) or low-yeast diet (LY).

*Drosophila* displayed slower development on low-yeast diets (Table 4.1, Cox: Z=20.91, $P$ <0.05). CS 4-species individuals reared on a yeast-rich diet eclosed 20 hours before CS 4-species flies reared on a LY diet. Furthermore, axenic flies take significantly longer to develop than flies colonised with all 4 bacteria species (Cox: Z=-23.93, $P$ <0.05) supporting the findings in Ridley *et al*, 2012 and Ridley *et al*, 2013. *Spiroplasma* delayed *Drosophila* development in axenic flies by 5 hours, but only on a LY diet (Cox: Z=3.85, $P$ <0.05). On a yeast-rich diet, the presence of *Spiroplasma* increased larval development time of axenic flies by 5 hours (Fig. 4.1D). *Spiroplasma* also delayed the development of flies monoassociated with either *A. pomorum* or *L. brevis,* on a LY diet (Fig. 4.1C, Cox: Z=2.52, $P$ <0.05, Cox: Z=2.91, $P$ <0.05). Whilst gut microbes influence eclosion time in *Drosophila* reared on a low yeast diet, they had little effect on the development time of *Drosophila* reared on a standard yeast diet. Similar results were observed for time to pupation (supplementary material, Table 1 and Fig. 4.1).

| | Cox statistic for time to: | | | |
|---|---|---|---|---|
| | Eclosion: | | | |
| Treatments | Estimate | SE | *z* value | *P* value |
| DietNormal | 1.961 | 0.094 | 20.91 | <0.001 |
| *A. pomorum* | -0.187 | 0.098 | -1.89 | 0.059 |
| *A. tropicalis* | 0.050 | 0.098 | 0.51 | 0.61 |
| Axenic | -2.106 | 0.088 | -23.93 | <0.001 |
| *L. brevis* | -0.549 | 0.099 | -5.53 | <0.001 |
| *L. plantarum* | -1.007 | 0.097 | -10.33 | <0.001 |
| MSRO | 0.129 | 0.107 | 1.21 | 0.23 |
| DietNormal-*A. pomorum* | 0.054 | 0.138 | 0.39 | 0.7 |
| DietNormal-*A. tropicalis* | 0.048 | 0.131 | 0.36 | 0.72 |
| DietNormal-axenic | 1.532 | 0.116 | 13.20 | <0.001 |
| DietNormal-*L. brevis* | 0.109 | 0.132 | 0.82 | 0.41 |
| DietNormal-*L. plantarum* | 0.728 | 0.131 | 5.57 | <0.001 |
| DietNormal:MSRO | -0.224 | 0.166 | -1.34 | 0.18 |
| *A. pomorum*:MSRO | -0.164 | 0.152 | -1.08 | 0.28 |
| *A. tropicalis*:MSRO | -0.166 | 0.154 | -1.08 | 0.28 |
| axenic:MSRO | -0.355 | 0.130 | -2.73 | 0.0064 |
| *L. brevis*:MSRO | -0.434 | 0.154 | -2.81 | 0.0049 |
| *L. plantarum*:MSRO | 0.016 | 0.154 | 0.11 | 0.92 |
| DietNormal: *A. pomorum*:MSRO | 0.577 | 0.229 | 2.52 | 0.012 |
| DietNormal: *A. tropicalis*:MSRO | 0.343 | 0.227 | 1.51 | 0.13 |
| DietNormal:axenic:MSRO | 0.709 | 0.198 | 3.58 | <0.001 |
| DietNormal: *L. brevis*:MSRO | 0.660 | 0.227 | 2.91 | 0.0036 |
| DietNormal: *L. plantarum*:MSRO | 0.252 | 0.231 | 1.09 | 0.28 |

**Table 4.1.** Multiple comparisons of Cox mixed-effects survival models. The cox model makes a global comparison of eclosion observations, using experiment replicate as a random effect, which accounts for "block" variation among the two experiments. Diet, microbe treatment and Drosophila strain are the fixed effects. Pairwise comparisons of the Cox models were made by Tukey's HSD test implemented in the multcomp package for R.

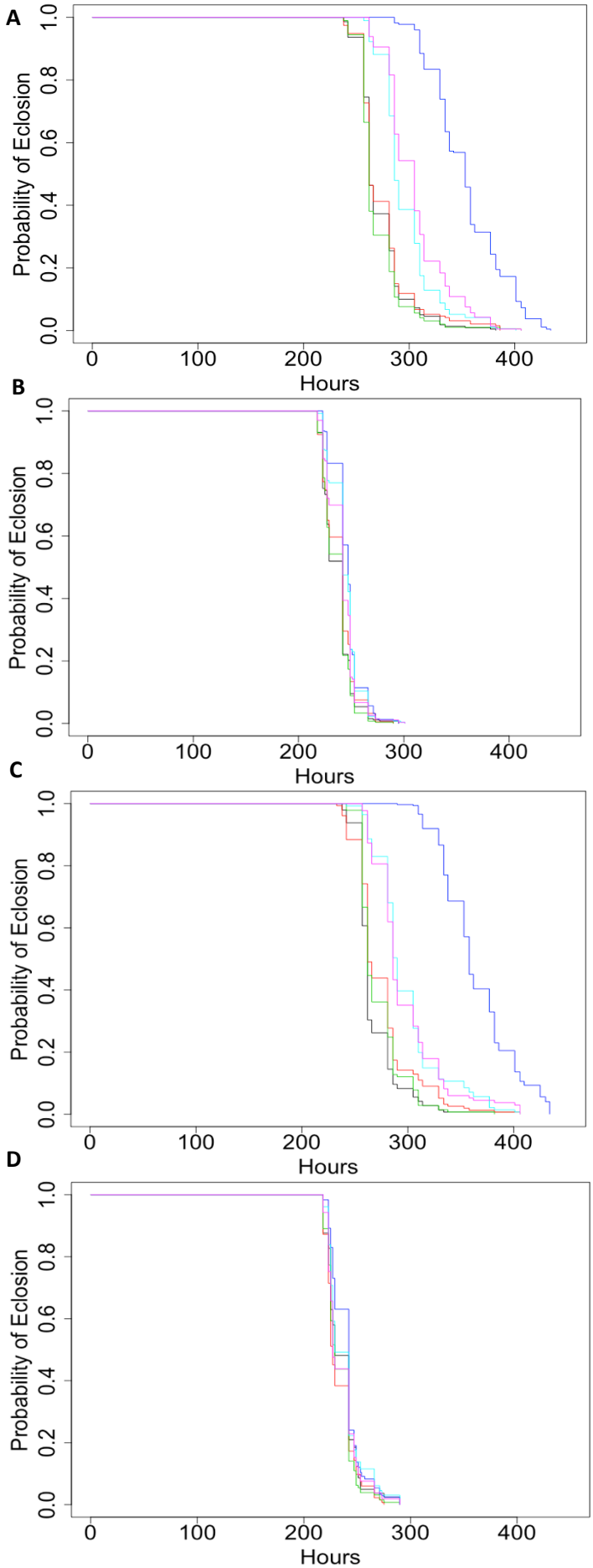



**Figure 4.1.** Development time of *Drosophila*. Median time to completion of eclosion shown as hours post egg-deposition for 4-species (black), *A. pomorum* (red), *A. tropicalis* (green), *L. brevis* (cyan), *L. plantarum* (magenta) and axenic (blue) flies. **A** (CS flies on a LY diet), **B** (CS flies on an SY diet), **C** (MSRO on a LY diet) and **D** (MSRO on an SY diet).

*4.3.2 Quantification of bacterial species abundance*

The numbers of bacteria in 10-day old flies were quantified in all microbiota treatments to confirm that the bacterial inoculations were successful and that the axenic *Drosophila* had few, if no bacteria. I was unable to quantify bacterial abundance in *Spiroplasma*-infected flies reared on the standard diet due to a lack of flies surviving till day 10 post eclosion. I compared the colony forming units (CFUs) observed in single-species microbiota and microbe-free treatment groups to the 4-species microbiota group. Axenic flies had significantly fewer bacteria compared to the 4-species treatment group (GLM, t value = -5.004, $P < 0.05$) (supplementary materials Fig. S.4.2), however it is clear there was some bacterial contamination during the 10 days after the flies had eclosed. Herein, axenic *Drosophila* are referred to as microbe-depleted individuals.

*4.3.3 Effects of Spiroplasma, diet and gut microbe composition on the nutritional indices of Drosophila*

To measure the effects of *Spiroplasma*, diet and microbiota composition on host nutrient allocation in microbe-depleted *Drosophila* and the single- and 4-species treatments, I compared adult weight, glucose, protein and Triglyceride (TAG) content in the presence and absence of *Spiroplasma* and on low and standard yeast diets. There was no significant effect of *Spiroplasma* on adult body mass (Fig. 4.2). The 4-species treatment had higher fresh weight relative to the other microbiota treatments in CS and MSRO flies reared on the low yeast diet, and MSRO flies reared on the standard diet. Fresh weight in CS flies reared on the standard diet showed a more varied response to microbiota treatment. Fresh weight was significantly higher in the microbe-depleted and *Acetobacter* treatment groups relative to the 4-species and *Lactobacillus* treatment groups (Fig. 4.2B). Subsequently, the values for protein, glucose and TAG were standardized to the fly weight.

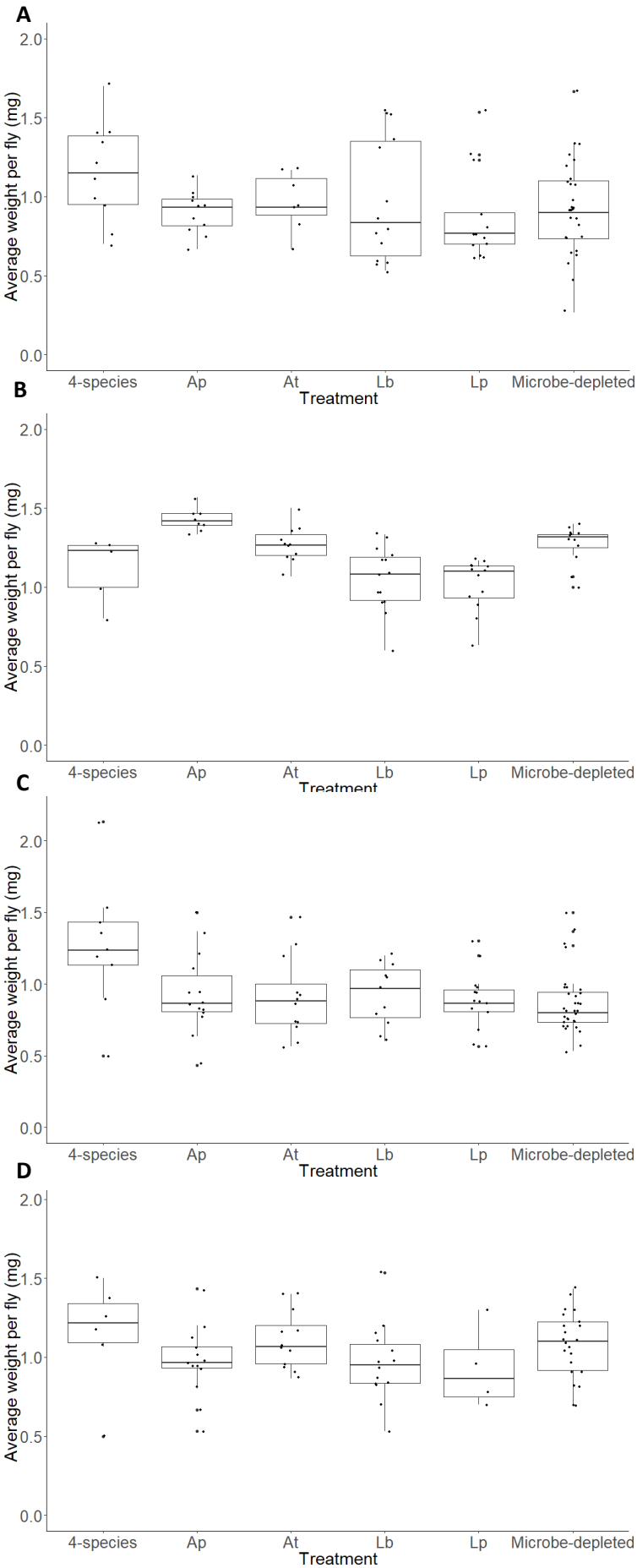



**Figure 4.2.** Effects of single- and 4-species microbiota and microbe-depleted treatments on adult *Drosophila* fresh fly weight. Fresh weight of female flies is reported as means ± standard errors from 2 independent experiments, for *Spiroplasma* infected and uninfected flies reared on a low yeast or a standard diet. **A** (CS flies on a low yeast diet), **B** (CS flies on a standard diet), **C** (MSRO on a low yeast diet) and **D** (MSRO on a standard diet).

There were no significant effects of *Spiroplasma* infection and diet on TAG content (GLM, t value = 0.454, P > 0.05 and t value = -0.445, P > 0.05 respectively). TAG levels were lower in the 4-species microbiota treatment relative to all other microbiota treatments however, only the *Lactobacillus* and *A. pomorum* treatments groups had significantly higher TAG (Fig. 4.3).

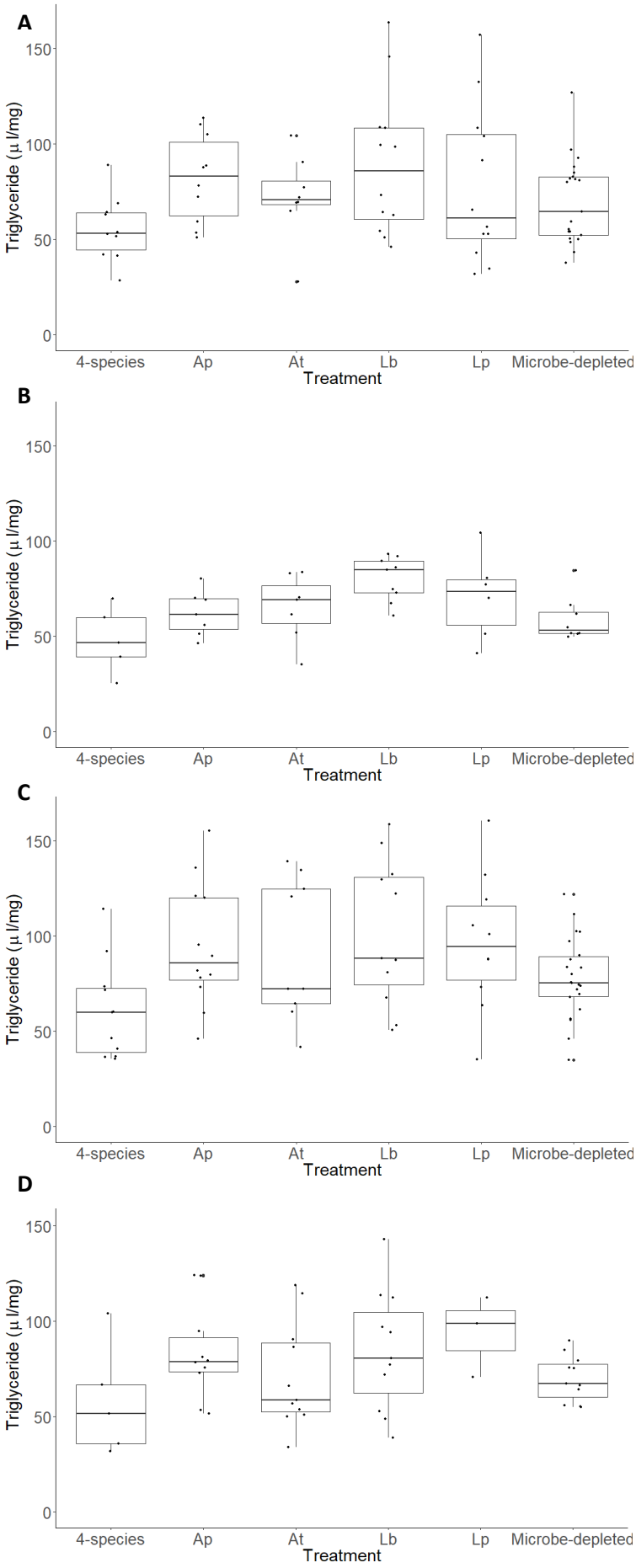



**Figure 4.3.** Effects of single- and 4-species microbiota and microbe-depleted treatments on *Drosophila* triglyceride (TAG) content. TAG content of female flies is reported as means ± standard errors from 2 independent experiments, for *Spiroplasma* infected and uninfected flies reared on a low yeast or a standard diet. TAG content was standardised to fresh weight. **A** (CS flies on a low yeast diet), **B** (CS flies on a standard diet), **C** (MSRO on a low yeast diet) and **D** (MSRO on a standard diet).

There were no significant effects of diet and *Spiroplasma* infection alone on *Drosophila* protein content (GLM, t value = 0.769, $P > 0.05$ and t value = -0.069, $P > 0.05$ respectively, Fig. 4.4). The effect of *Spiroplasma* on host protein content depended on both diet and gut microbiota. *Spioplasma*-infected, microbe-depleted *Drosophila* had significantly lower protein content relative to the 4-species treatment group, but only on the standard diet (Fig. 4.4D) (GLM, t value = -2.125, $P < 0.05$).

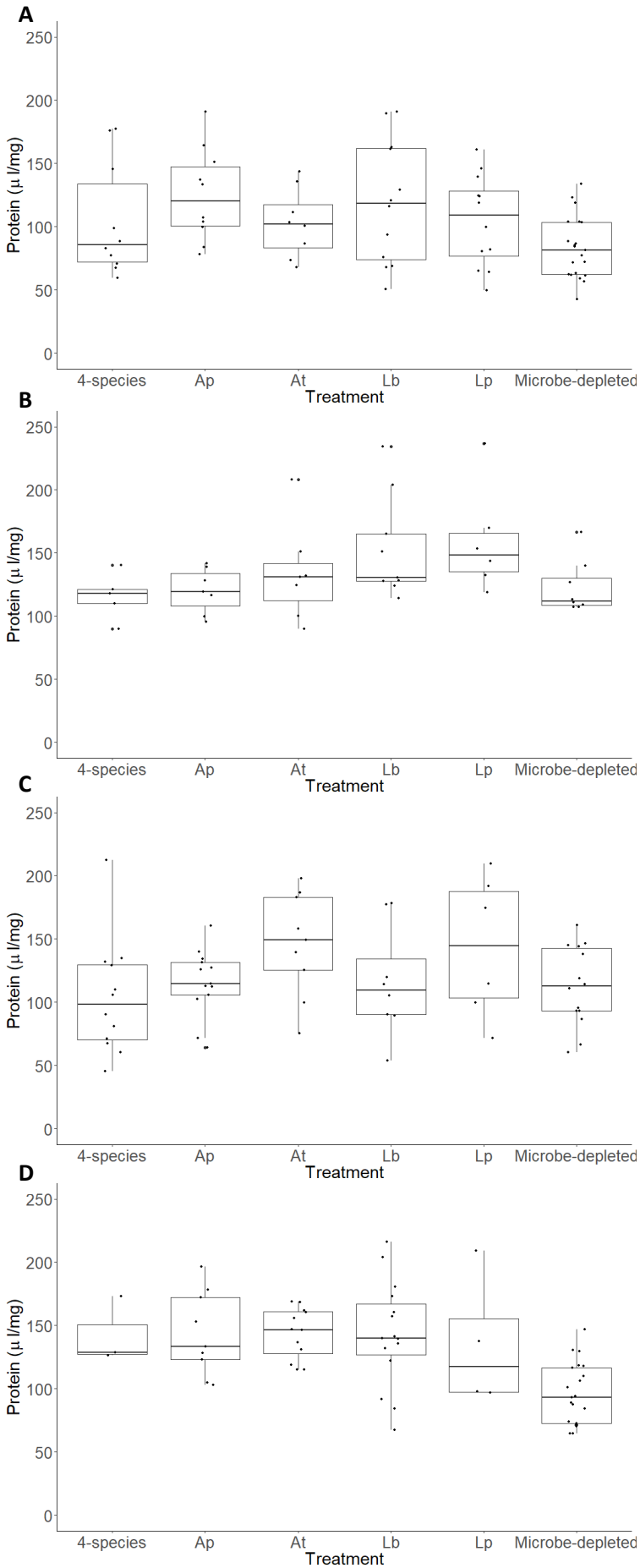



**Figure 4.4.** Effects of single- and 4-species microbiota and microbe-depleted treatments on *Drosophila* protein content. Protein content of female flies from 2 independent experiments, for *Spiroplasma* infected and uninfected flies reared on a low yeast or a standard diet. The box plots display the upper and lower quartiles, the median and the range. Points represent each measurement obtained. Protein content was normalised to adult fresh weight. **A** (CS flies on a low yeast diet), **B** (CS flies on a standard diet), **C** (MSRO on a low yeast diet) and **D** (MSRO on a standard diet).

Individually, diet and *Spiroplasma* infection did not affect *Drosophila* glucose content (GLM, t value = 1.117, $P > 0.05$ and t value = 0.968, $P > 0.05$ respectively). The *A. pomorum* treatment group in *Spiroplasma* infected flies reared on the standard diet had significantly higher glucose content relative to the 4-species treatment group (Fig. 4.5D).

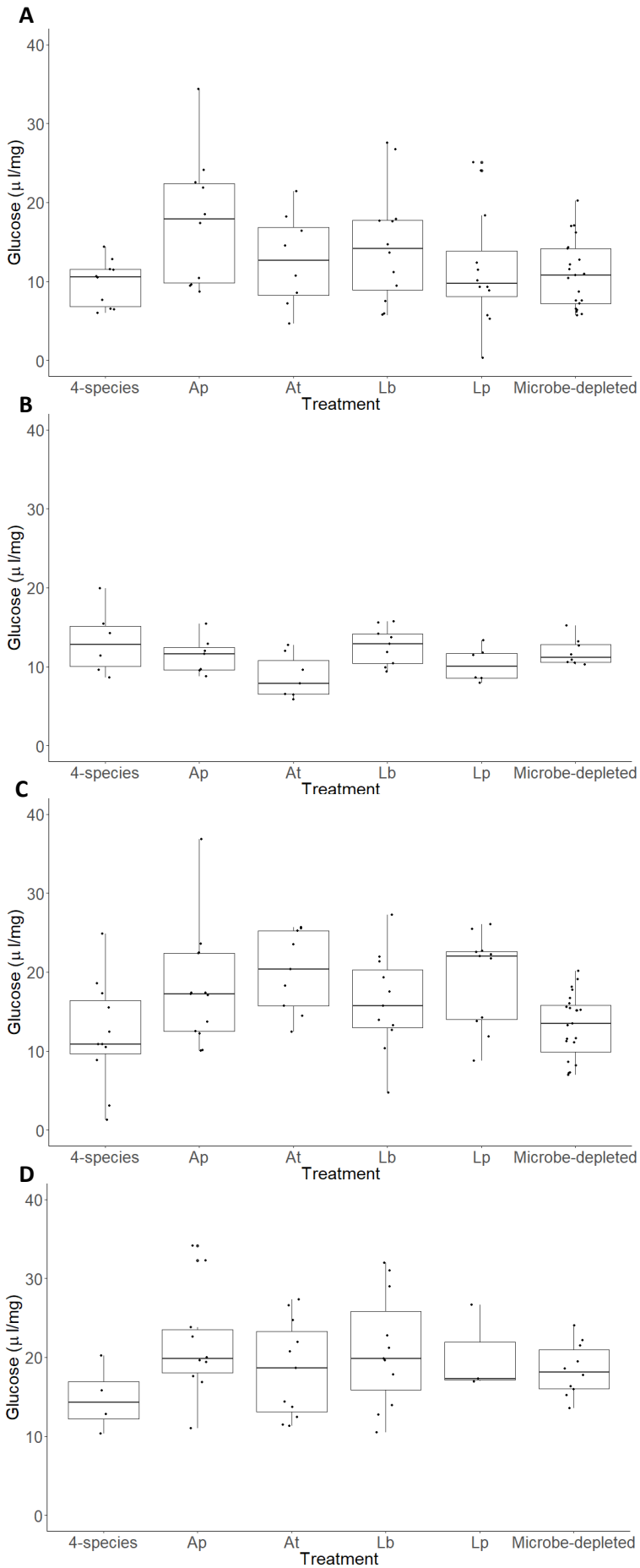



**Figure 4.5.** Effects of single- and 4-species microbiota and microbe-depleted treatments on *Drosophila* glucose content. Glucose content of female flies from 2 independent experiments, for *Spiroplasma* infected and uninfected flies reared on a low yeast or a standard diet. The box plots display the upper and lower quartiles, the median and the range. Points represent each measurement obtained. Glucose content was normalised to adult fresh weight. **A** (CS flies on a low yeast diet), **B** (CS flies on a standard yeast diet), **C** (MSRO on a low yeast diet) and **D** (MSRO on a standard yeast diet).

*3.3.4 Effect of gut microbes and diet on Spiroplasma proliferation*

To measure the effects of diet and microbe composition on *Spiroplasma* proliferation, I compared *Spiroplasma* titres of MSRO flies with single- and 4-species microbiota to the microbe-depleted flies, on the standard and low yeast diets. Under nutrient limitation, *Spiroplasma* titre is significantly higher relative to when nutrients are not limiting (GLM, t(67) value = -2.45, $P < 0.05$) (Fig. 4.6). There are no significant interactions between microbiota treatments and diet, despite microbe- and diet-mediated alterations to host nutrient allocation.

*Spiroplasma* titre is not associated with TAG concentration on either the low or standard yeast diets (Fig. 4.7) (r = 0.17, $P > 0.05$ and r = -0.05, $P > 0.05$ respectively). This suggests that *Spiroplasma* titre is maintained at stable titres regardless of alterations to gut microbe composition.

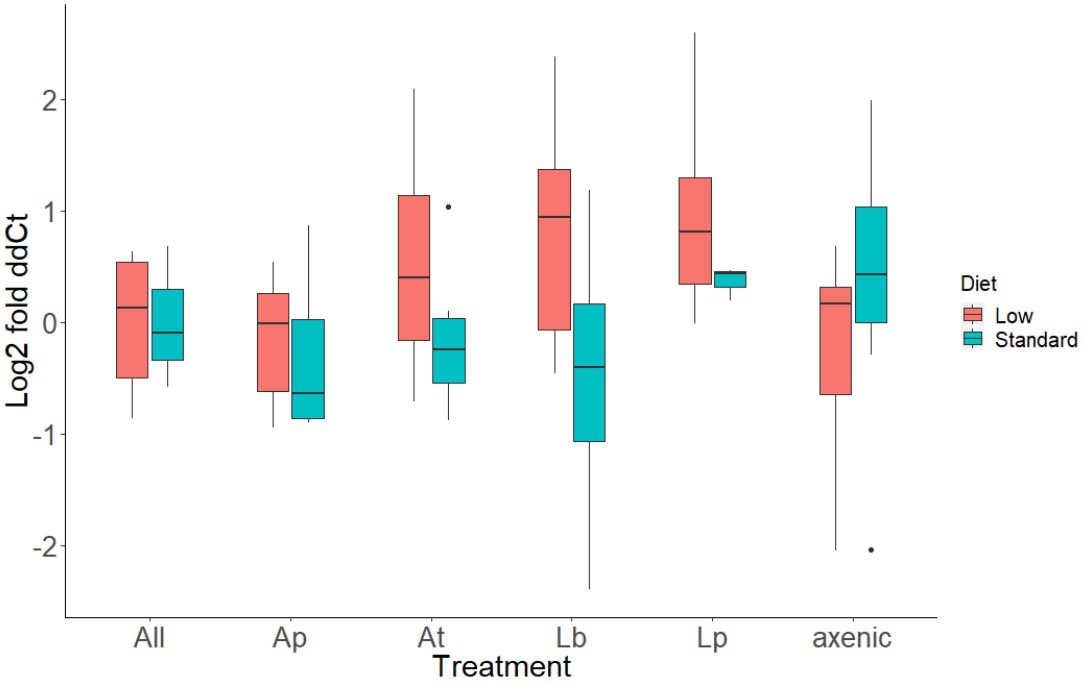



**Figure 4.6.** The implications of host nutritional depravation and gut microbe composition on *Spiroplasma* proliferation. Quantification of *Spiroplasma* titres by qPCR on a low yeast diet (peach) and standard diet (turquoise). Values for each treatment have at least 2 samples of 3 flies.

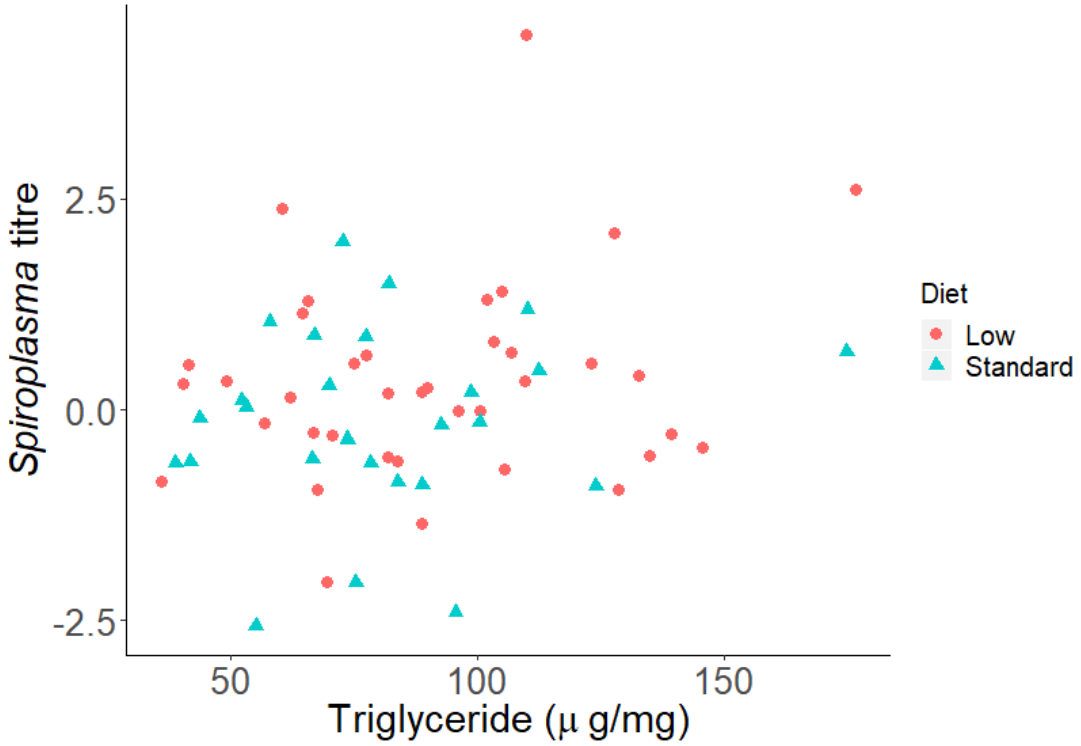



**Figure 4.7.** *Spiroplasma* proliferation is unaffected by TAG concentration. Pearson correlation analysis of *Spiroplasma* titre and TAG concentration: each titre value corresponds with a value for TAG concentration. Flies came from the same experimental vials.

## 4.4 Discussion

Animal physiology is likely to be a consequence of the dynamic interplay between diet and microbes; this is most obviously the case for gut microbes that may either use food or modify it in a way to be accessible. Recent work has highlighted the additional potential role of heritable microbes on host physiological indicators. Where most historical research has focused on the combined effects of diet and gut microbiota (Ridley *et al*, 2012; Wong *et al*, 2014; Newell and Douglas, 2014) and the individual and combined effects of diet and the heritable symbiont *Spiroplasma* on host physiology (Herren *et al*, 2014; Paredes *et al*, 2016), I investigated the interplay between the tripartite factors in the model organism, *D. melanogaster*.

Previous work established that *Drosophila* and their microbiota do not compete for shared resources (Wong *et al*, 2014). I hypothesised that gut microbiota, specifically Acetobacters, compete with *Spiroplasma* for hemolymph lipid and thus limit host performance on a standard diet. I did not observe the previously reported microbe-mediated alterations to host nutrient levels, nor did I observe the predicted interactions between the Acetobacters, *Spiroplasma* and lipid. Furthermore, *Spiroplasma* and diet together did not affect protein or lipid availability across the different microbe treatments. I did observe a reduction in protein concentration in a three-way interaction between *Spiroplasma,* standard diet and the microbe-depleted treatment. These alterations came without evidence for the gut microbial community driving changes in *Spiroplasma* titre, although *Spiroplasma* titre is lower on a yeast rich diet.

These findings differ from previous research, which has focused on gut microbiota and endosymbionts independently of one another. With respect to heritable microbes, Paredes *et al*, 2016, observed that *Spiroplasma* depletes host hemolymph lipid and Herren *et al*, 2014, observed that *Spiroplasma* proliferation is limited by host lipid availability. I did not observe a reduction in hemolymph lipid in response to *Spiroplasma* infection. Examining the gut microbiota, Wong *et al*, 2014, observed that protein content was significantly reduced in female 5-day old axenic *Drosophila*. I observed the same phenomenon in microbe-depleted 10-day old females relative to the single- and 4-species treatments, but only in the presence of *Spiroplasma* and on a standard diet. This suggests that the gut microbiota does not affect the demand for dietary protein when yeast is

limiting. It is unclear why the presence of *Spiroplasma* is associated with a depletion of protein.

The data leads to two questions. First, why can I not recapitulate past studies on the single effects of *Spiroplasma* and microbiota? Failure to recapitulate previous experiments may be explicable by methodological differences. For instance, *Spiroplasma* MSRO kills male *Drosophila* and the experiments of Paredes *et al*, 2016 and Herren *et al* 2014 are performed in virgin flies. Mating and reproduction provides a strong basis for altering host physiology, as reproduction is costly, and the act of oviposition itself involves 'excretion' of *Spiroplasma* through eggs. Considering mated females produce a far greater number of eggs relative to unmated *Drosophila* (Herren *et al*, 2014), the rate of *Spiroplasma* shedding is likely to be greater in this study compared to previous studies on virgin flies. Here, I show that *Spiroplasma* proliferation is lower under standard nutritive conditions, and this is likely a result of greater egg production compared to flies reared on a low yeast diet. In addition, the metabolomics assays were carried out on older flies (10 days old vs 5 days old), after a longer period of reproduction, and with more laboratory manipulation, which may explain the higher CFUs and different results.

In conclusion, I did not observe strong interactions between diet, microbiota and symbiont infection. By utilizing *Drosophila* naturally infected with *Spiroplasma*, with microbiota-depleted and single- and 4-species microbiota treatments, I have shown that all microbial species tested are functionally equivalent in regulating host nutrition. In contrast to my predictions, *Spiroplasma* does not interact with gut microbiota to impact host traits such as development time and nutrient allocation. I also show that *Spiroplasma* titre is stable and buffered against changes in the gut microbial community. However, there is a clear interaction term for protein content which suggests that *Spiroplasma* interacts with the host when gut microbes are absent and nutrients are not limiting. This is important when considering the effects of microbes on host physiology. In future research, heritable microbes should be considered in conjunction with gut microbiota.

## 4.5 Supplementary Materials

| | Cox statistic for time to pupation: | | | |
|---|---|---|---|---|
| Treatments | Estimate | SE | *z* value | *P* value |
| DietNormal | 2.092 | 0.095 | 22.13 | <0.001 |
| *A.pomorum* | 0.135 | 0.099 | 1.37 | 0.17 |
| *A.tropicalis* | 0.107 | 0.098 | 1.09 | 0.28 |
| axenic | -2.531 | 0.091 | -27.84 | <0.001 |
| *L.brevis* | -0.722 | 0.098 | -7.34 | <0.001 |
| *L.plantarum* | -1.088 | 0.097 | -11.26 | <0.001 |
| MSRO | 0.178 | 0.107 | 1.66 | 0.097 |
| DietNormal-*A.pomorum* | -0.401 | 0.137 | -2.92 | <0.001 |
| DietNormal-*A.tropicalis* | -0.103 | 0.131 | -0.78 | 0.43 |
| DietNormal-axenic | 1.744 | 0.118 | 14.78 | <0.001 |
| DietNormal-*L.brevis* | 0.219 | 0.131 | 1.66 | 0.096 |
| DietNormal-*L.plantarum* | 0.726 | 0.130 | 5.60 | <0.001 |
| DietNormal:MSRO | -0.948 | 0.177 | -5.35 | <0.001 |
| *A.pomorum*:MSRO | -0.531 | 0.154 | -3.45 | <0.001 |
| *A.tropicalis*:MSRO | -0.097 | 0.155 | -0.63 | 0.53 |
| axenic:MSRO | -0.560 | 0.131 | -4.43 | <0.001 |
| *L.brevis*:MSRO | -0.490 | 0.153 | -3.20 | <0.001 |
| *L.plantarum*:MSRO | 0.028 | 0.154 | 0.18 | 0.85 |
| DietNormal: *A.pomorum*:MSRO | 1.089 | 0.237 | 4.58 | <0.001 |
| DietNormal: *A.tropicalis*:MSRO | 0.400 | 0.235 | 1.44 | 0.15 |
| DietNormal:axenic:MSRO | 1.223 | 0.208 | 5.87 | <0.001 |
| DietNormal: *L.brevis*:MSRO | 0.934 | 0.236 | 3.96 | <0.001 |
| DietNormal: *L.plantarum*:MSRO | 0.208 | 0.246 | 0.85 | 0.4 |

**Table S.4.1.** Multiple comparisons of Cox mixed-effects survival models. The cox model makes a global comparison of pupation observations, using experiment replicate as a random effect, which accounts for "block" variation among the two experiments. Diet, microbe treatment and Drosophila strain are the fixed effects. Pairwise comparisons of the Cox models were made by Tukey's HSD test implemented in the multcomp package for R.

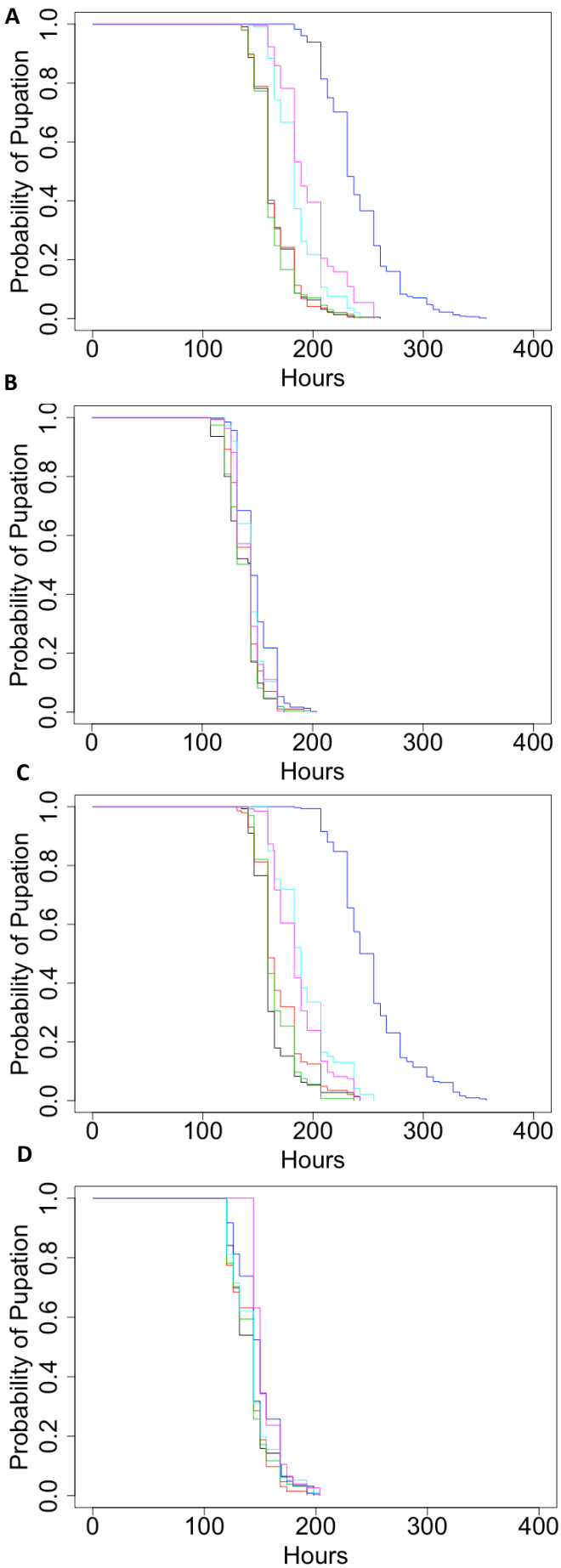



**Figure S.4.1.** Development time of *Drosophila*. Median time to completion of pupation, shown as hours post egg-deposition (p.e.d) for 4-species (black), *A.pomorum* (red), *A.tropicalis* (green), *L.brevis* (cyan), *L.plantarum* (magenta) and axenic (blue) flies. **A** (CS flies on a low yeast diet), **B** (CS flies on a standard yeast diet), **C** (MSRO on a low yeast diet) and **D** (MSRO on a standard yeast diet).

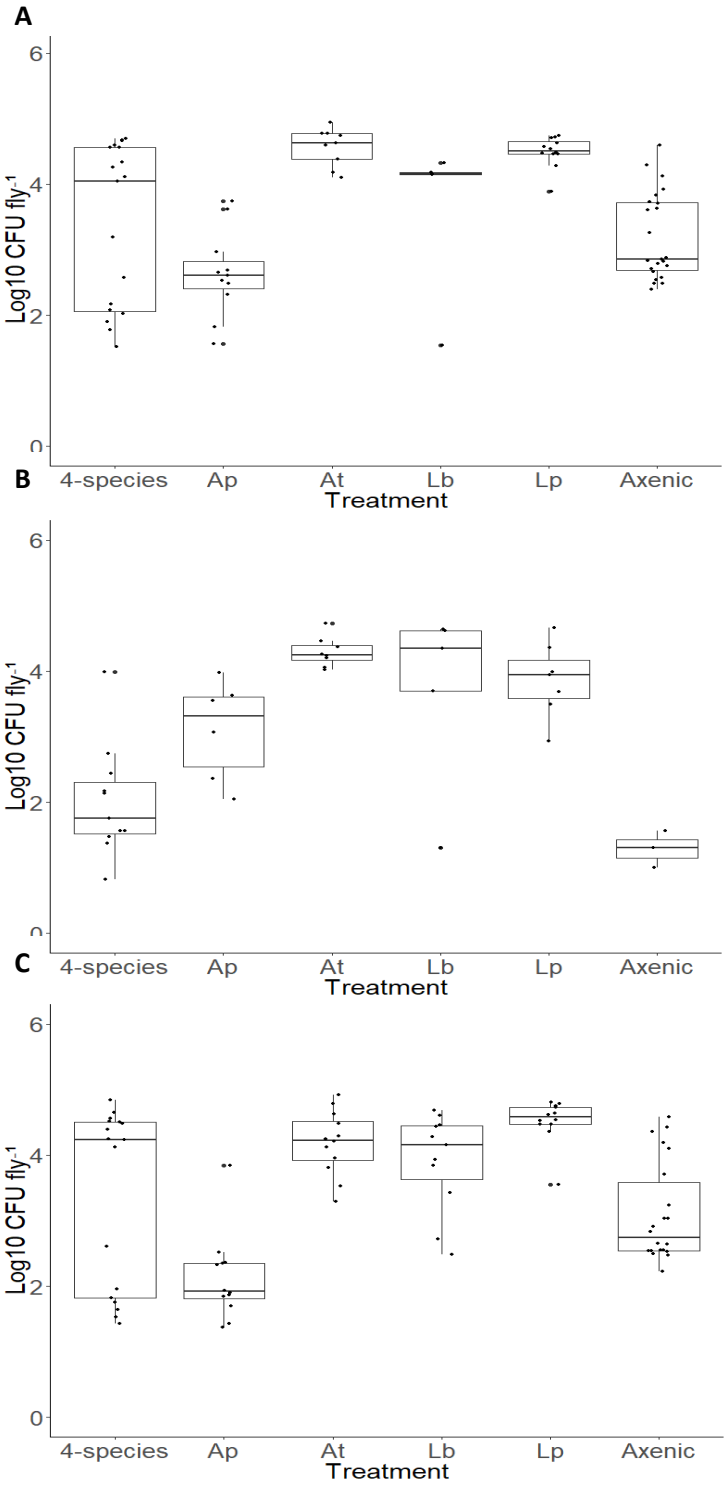



**Figure S.4.2.** Bacterial species abundance in axenic, single- and 4-species *Drosophila* microbiota. CFU counts from whole flies are shown for each bacterial treatment. Each box delineates the first and third quartiles and the median. The whiskers show the range. **A** (CS flies on a low yeast diet), **B** (CS flies on a standard yeast diet), **C** (MSRO on a low yeast diet).

# Chapter 5

## Transcriptional response of *Nasonia* to infection with *Arsenophonus nasoniae* in natural and host shift associations


## Abstract

*Arsenonophonus nasoniae* is a facultative endosymbiont of *Nasonia* parasitoid wasps that manipulates host reproduction to produce a female biased offspring sex ratio. To date, transcriptomic or microarray studies suggest that native endosymbionts do not induce the host immune response but they can induce small-scale organismal responses. Immune responses tend to be greater upon transinfection, but this has only been determined for *Wolbachia* and *Spiroplasma*. To understand better the biological interactions between *Nasonia* hosts and *A. nasoniae*, which retains many characteristics of a pathogen, I used RNAseq to generate the transcriptome of adult female wasps with and without *A. nasoniae*. I observed greater differential gene expression and extensive upregulation of genes in *Nasonia vitripennis* in response to its native *A. nasoniae* infection, compared to transinfected *Nasonia giraulti*. *A. nasoniae* thus has a widespread effect on its native host, inducing immune genes and oxidase reduction and heme-binding pathways. I consolidated the RNAseq results for key immune genes using RT qPCR. I outline several potential means through which *A. nasoniae* infection might induce a genome-wide responsein its host. *A. nasoniae* and *N. vitripennis* form a unique symbiosis whereby coexistence has led to an immunoreaction, which is not observed upon transinfection.

## 5.1 Introduction

Endosymbiosis is where two disparate species for example, an arthropod and an intracellular prokaryote, form a close association, with one (the symbiont) living and replicating within the body of the other (the host) (Buchner, 1965). It can lead to rapid adaptive evolution of certain host traits, give rise to ecological release, and also promote speciation. The nature of symbioses vary along a parasitism-mutualism continuum and the benefit or cost of symbiont carriage may depend on the host's environment. Approximately 60% of arthropod species are estimated to harbour vertically inherited bacterial endosymbionts (Weinert *et al*, 2015). These bacteria can confer ecologically contingent benefits such as defence against natural predators, nutrient provisioning and improvement of metabolism and reproduction (Douglas *et al*, 2001; Jaenike et al, 2010a; Xie et al, 2010; Weiss *et al*, 2011; Pais *et al*, 2008). These mutualistic phenotypes drive symbionts into host populations, improving their adaptive traits and invasive power.

Some symbionts are very common in host communities. For example, *Wolbachia* is thought to infect 50% of all arthropods (Weinert *et al*, 2015) and *Buchnera* is present in nearly all species of aphid, including the pea aphid, *Acyrthosiphon pisum* (Buchner, 1965). Symbiosis can also be associated with costs such as reduced life span, reproductive abnormalities, immune activation and even host death (Werren *et al*, 2008; Anbutsu and Fukatsu, 2011). Often the bacteria are essential for host survival, although in some cases they also pose a significant immunological challenge (Login *et al*, 2011).

Certain endosymbionts completely avoid the host immune system. For example, the second most common endosymbiont of *Drosophila* spp., *Spiroplasma,* resides in the hemoplymph and does not have a cell wall. Thus it is not recognized by the host (Hutchence *et al*, 2011; Herren and Lemaitre, 2011). Furthermore, *Wolbachia* is able to enter host cells, within which it is hidden from the host's immune response (Bourtzis *et al*, 2000; Teixeira, 2012; Chrostek *et al*, 2014). *Buchnera* on the other hand is localised to bacteriocytes and has lost the genetic machinery to synthesise conserved microbe-associated molecular patterns (MAMPs) (Shigenobu *et al*, 2000). Thus, like *Spiroplasma, Buchnera* is not be detected by the host's immune system.

Coevolution between host and symbiont results in genome erosion and loss of redundant and harmful genes such as those involved in biosynthesis of metabolic intermediates and virulence (McCutcheon and Moran, 2011). Extensive genome reduction of symbionts leads to the inability to grow outside the host, in cell free culture (Baumann *et al*, 1995). Less is

known of the genomic changes that occur on the host side of the partnership. The pea aphid has lost genes underlying the immune-deficiency (IMD) signalling pathway, which may have facilitated the development of symbiosis with *Buchnera* (Gerardo *et al*, 2010). The subsequent trade-off of this partnership is an elevated risk of pathogen attack (Nakabachi *et al*, 2003), which may have led to the presence of diverse protective symbionts.

Genome-wide analysis of the interaction between *Wolbachia* and its native host, *Drosophila melanogaster*, suggests that antibacterial immune genes are not differentially expressed between *Wolbachia* infected and uninfected flies (Wong *et al*, 2011). In contrast, Caragata *et al*, 2017 observed an upregulation of 159 contigs and downregulation of 98 contigs in *Aedes fluviatilis,* in response to native *Wolbachia* infection. None of the differentially expressed genes were associated with the Toll and IMD signalling pathways; however, bacterial recognition genes and genes associated with *Plasmodium* infection, oxidative stress, lipid metabolism, DNA/RNA processing and membrane transport were upregulated in response to *Wolbachia*.

A similar number of genes were differentially expressed in *w*Mel transinfected *Aedes aegypti,* including immune genes. However, the same immune genes were not upregulated in *w*Mel's native host, *D. melanogaster* (Rancès *et al*, 2012). A larger number of genes were differentially expressed in *w*MelPop transinfected *A. aegypti*, which likely reflects the pathogenic nature of this *Wolbachia* strain (Kambris *et al*, 2009; Rancès *et al*, 2012).

In *Tetranychus urticae*, CI- inducing *Wolbachia* induces an upregulation of oxidative reduction pathways and a few immune related genes; however, there is no evidence of *Wolbachia* mediated immune priming in this species (Zhang *et al*, 2014). Li *et al*, 2018, observed that *Wolbachia* did not induce an upregulation of immune or reproductive proteins in the parasitoid wasp *Nasonia vitripennis*. *Wolbachia* was however, associated with differential expression of catalytic, metabolic and binding proteins.

Thus, *Wolbachia* does not appear to cause widespread upregulation of immune genes in native hosts and where there is differential expression it is typically observed between oxidase reduction activity genes in infected and uninfected individuals. Induction of immune genes and antimicrobial pathways is more common following lateral transfer of *Wolbachia* from its native host to novel hosts.

Insects that harbour endosymbionts are under selective pressure to reduce the costs associated with symbiont carriage. To accommodate symbionts, hosts may modify or actively suppress their immune system to avoid launching a costly hyper-immune attack, which could

kill the bacteria and end the symbiotic partnership (Koga *et al,* 2007; Wang *et al*, 2009; Ratzka *et al*, 2013). Hosts may also keep symbionts 'in check' by restricting symbiont proliferation and confining them to specialised host cells called bacteriocytes, which prevent the bacteria from circulating in the hemolymph and coming into contact with immune recognition proteins (Buchner, 1965; Douglas, 1989; Moran and Telang, 1998). The cereal weevil *Sitophilus spp*. produces the AMP coleoptercin A (colA) in bacteriocyte cells to prevent its endosymbiont, *Sodalis pierantonius,* from leaking or escaping. This response is mediated by an IMD-like pathway, which is highly conserved and utilized for pathogen removal. In this system, the pathway mediates endosymbiont control in this system (Maire *et al*, 2018). Nonetheless, the host must retain the ability to recognize pathogenic bacterial invaders and launch successful immune responses against them. This is complicated by the fact that many symbionts and bacterial pathogens have evolutionarily conserved MAMPs (e.g. peptidoglycans) (Maire *et al*, 2018).

Although there are conserved core immune pathways across insect orders (Lemaitre *et al*, 1995; Zou *et al*, 2007), there is evidence to suggest that protein-coding genes involved in pathogen recognition and removal are under rapid selection (Sackton *et al*, 2007; Waterhouse *et al*, 2007). Certain insect species such as the jewel wasp, *Nasonia vitripennis,* have taxonomically-restricted immune genes (Sackton *et al*, 2013). *N. vitripennis* has developed a complex antimicrobial arsenal through gene duplication and exon reshuffling (Tian *et al*, 2010). A possible, but unexplored, cause for the rapid evolution of taxonomically-restricted proteins is the presence of its endosymbiont.

*Nasonia vitripennis*, is infected with *Arsenophonus nasoniae*, a vertically transmitted Gram negative bacteria in the Enterobacteriacae family of the class Gammaproteobacteria. *A. nasoniae* is present in around 4% of female *N. vitripennis* and manipulates host reproduction. The bacteria kills haploid (unfertilized) males which can result in the mortality of c. 80% males in a single brood to create a female biased offspring sex ratio (Skinner, 1985). *A. nasoniae* is injected by adult females into the fly pupal host along with eggs and venom/calyx fluid. Bacteria are then ingested by developing wasp larvae (Huger *et al*, 1985; Werren *et al*, 1986; Nadal-Jimenez *et al*, 2019). The bacteria cross the gut barrier in the developing wasps, and colonize the ovipositor tubes during host pupation. In developing larvae, infection is concentrated around the mouth and digestive tract but some larvae become systemically infected (Nadal-Jimenez *et al*, 2019).

Whilst *A. nasoniae* has undergone significant genome minimization, it retains genes involved in replication and survival, so that it can survive in the intermediary fly host and cross the wasp gut barrier (Darby *et al*, 2010). The microbe also retains functional Type III secretion systems (TTSSs), akin to pathogenic *Yersinia* and *Salmonella* sp., which are required for virulence in these species (Wilkes *et al*, 2010). *A. nasoniae* possesses the machinery that allow it to acquire chelated and ferrous iron from the host. In *E. coli* this machinery is thought to be a 'virulence determinant' (Payne and Finkelstein, 1978; Wilkes *et al*, 2010). There are multiple ORFs and islands that have sequence similarity to Type III secreted effectors, which typically alter the host innate immune response. There are also toxin genes including *Aip56* (apoptosis inducing protein 56) which can kill neutrophils (do Vale *et al*, 2007) and *cnf1* (cytotoxic necrotizing factor 1) which can activate Rho GTPases. Further to this, the genome contains a variety of genes predicted to encode cell surface glycoproteins and capsular polysaccharide synthesis. These features would likely make the microbe immunogenic.

*Arsenopohonus nasoniae* is unusual among endosymbionts in that it can transmit infectiously, via super- and multi-parasitism (where conspecifics or females from different species share the same fly pupal host) (Huger *et al*, 1985), and it retains many characteristics of a pathogen (Wilkes *et al*, 2010). The combination of transmission modes characteristic of *A. nasoniae* indicates this endosymbiont may deviate from the classic model for a maternally inherited microbe, in terms of its pathogenicity and whether it induces any wholescale defence or immune pathways in the host.

Mechanisms of symbiont control other than immunity have often been overlooked. Insects may respond to chronic endosymbiont infection in a defensive manner that does not solely depend on the Toll and IMD immune pathways. Of the investigations into immunological expression that have been conducted, they tend to be restricted to established model insects such as *Drosophila* and mosquito species. The recent reannotation of the *N. vitripennis* genome (Rago *et al*, 2016) provides an opportunity to compare the transcriptomes of symbiont-infected and uninfected wasps. Using RNAseq, I investigated the *A. nasoniae*-induced transcriptome of native host *N. vitripennis* and novel host, *Nasonia girault*i. Upregulation of candidate immune genes was confirmed by quantitative reverse transcription polymerase chain reaction.

## 5.2 Materials and Methods

*5.2.1 Approach*

Two analyses are presented. The first is an RNAseq analysis of *Nasonia* host gene expression in the presence and absence of *Arsenophonus nasoniae* in the natural host *N. vitripennis* and in the novel host and sibling species, *N. giraulti*. The second is a validation of immune expression in *N. vitripennis* using qRT PCR.

*5.2.2 Nasonia and symbiont strains*

*N. vitripennis* strain AsymC and *N. giraulti* strain RV1xTetra were maintained using house fly pupae as hosts in Drosophila vials. Neither strain harbours *Wolbachia*. Five fly pupae (up to 30 days old) and five mated females were added to each vial. The vials were sealed with cellulose acetate flugs and placed in a 25 °C incubator with 14:10 L:D cycle for 14-15 days until the new wasp generation emerged.

*5.2.3 Arsenophonus nasoniae strain Fin'13*

*N. vitripennis* is naturally infected with *A. nasoniae* (isolated from Turku, Finland in 2013). This symbiont strain was genetically manipulated to carry the GFP expressing plasmid, pOM1-gfp, by fellow lab member, Dr Pol Nadal. The strain emits green light under epifluorescent illumination, allowing easy tracking of symbiont status, tropism and titre. This strain is henceforth termed An-GFP.

*5.2.4 Lateral transfer of A. nasoniae from N. vitripennis to N. giraulti*

Transfer of GFP-tagged *A. nasoniae* to *N. giraulti* was carried out by Dr Pol Nadal. The protocol is as follows: *A. nasoniae* was grown in BHI medium at 30 °C and 250 rpm for 6 days until an $OD_{600}$ = 0.6-0.8 was obtained. An-GFP was pelleted and re-suspended in 10% sterile glycerol and then injected into 10 fresh *Sarcophaga bullata* pupae. Small quantities of the bacteria-glycerol cocktail were injected into each pupa at the junction between pupal segments, using 0.2 mm diameter needles. The injected pupae were left to dry for 5-10 minutes in *Drosophila* vials and then 10 mated female *N. giraulti* were added. Ten days later the fly pupae were opened and observed under a M165 FC Leica stereoscope equipped with epifluoresence to visualise GFP. Non-injected pupae were used as negative controls. Any GFP-negative pupae were discarded and the GFP-positive pupae were placed in Drosophila vials to continue development. Transinfected *N. giraulti* were then maintained as described above.

### *5.2.5 Annotation of Assembly Nvit 2.1*

Putative functional characteristics of *N. vitripennis* transcripts were annotated using Trinotate v3.1.1 (Bryant, 2017) as the OGS2 annotation (Rago *et al*, 2016) was unavailable at the time the analysis was conducted. Transcript similarities to known proteins were detected via a BLASTX search which searches the SwissProt (Boeckmann *et al*, 2005) and UniRef90 protein databases (UniProt Consortium, 2015). Candidate coding regions within transcript sequences were detected with TransDecoder v5.5.0 (https://github.com/TransDecoder/TransDecoder/wiki) and sequence similarities to the protein products were identified using BLASTP. Conserved protein domains were identified based on hidden Markov models (HMM), using HMMER v3.2.1 (Finn *et al*, 2011) and Pfam 32.0 (Xfam Consortium, 2018) databases. Signal peptides predictions were made with signal v5.0 (Armenteros, 2019) and transmembrane predicted regions were predicted with TmHMM v2.0 (Krogh *et al*, 2001). All results were combined in Trinotate, stored in a SQLite database and reported in a tab delimited summary file.

### *5.2.6 Library preparation and sequencing*

Three pools each of adult female *A. nasoniae* infected *N. vitripennis* (n = 50), *A. nasoniae* uninfected *N. vitripennis* (n = 50), *A. nasoniae* infected *N. giraulti* wasps (n = 50), *A. nasoniae* uninfected *N. giraulti* (n = 50) wasps were collected and frozen in liquid nitrogen. All samples were maintained at -80°C until required for RNA extraction. Total RNA was extracted by standard Trizol protocols. Samples were DNAse treated with DNAse I (Thermofisher) to remove any contaminating DNA. Preparation of dual-indexed, strand-specific mRNA libraries using RiboZero rRNA depletion and NEBNext Ultra Directional RNA library preparation kits was conducted by Margaret Hughes from the Centre for Genomic Research (CGR), Liverpool. Library preparation was successful for all 12 samples. The samples were sequenced on one lane of Illumina HiSeq 4000 (150 bp paired-end) by Anita Lucaci, from CGR.

### *5.2.7 Mapping reads to N. vitripennis genome*

Raw transcript reads were filtered for quality and trimmed by CGR. Sequences with poor sequence quality or adapter contamination were removed. The reads were then trimmed for the presence of Illumina adapter sequences using Cutadapt v1.2.1 (Martin, 2011) and then further trimmed using Sickle version 1.2 with a minimum window quality score of 20. Reads shorter than 20 bp after adapter sequence trimming were removed.

Filtered adapter-trimmed reads were subsequently mapped to the *N. vitripennis* transcriptome (Assembly Nvit 2.1: NCBI Accession: GCF_000002325.3) using Salmon (v0.13.1) (Patro *et al*, 2017). A reference transcriptome is available for *N. giraulti* however, it was not deemed to be of good enough quality to use for this analysis. Therefore the *N. giraulti* libraries were also aligned to the *N. vitripennis* transcriptome.

*5.2.8 Differentially expressed genes*

I used the tximport package (Soneson *et al*, 2015) to import and summarize transcript level abundances, estimated counts and transcript lengths in R (R Core Team, 2016), for statistical inference. To identify genes that are regulated by *A. nasoniae* in *N. vitripennis* and *N. giraulti*, I used a pair-wise approach and quasi-likelihood method in edgeR Bioconductor package (Robinson *et al*, 2010). EdgeR performs differential abundance analysis on a simple list-based object called a DGEList. Estimated counts from Salmon were normalised to gene length. A design matrix was created to assign treatment conditions to each sample and parameterize the experimental effects to each sample. From a biological point of view, genes must be expressed to a threshold level before being translated. Thus, genes with low counts were filtered across all libraries. Filtering in EdgeR takes into account library size and experimental design. The non-filtered genes were then normalized by trimmed means of M (TMM) (Robinson and Oshlack, 2010) to eliminate composition bias between the libraries.

I used the kegga function in the limma package v3.28.14 (Ritchie *et al*, 2015) to conduct an analysis on the overrepresentation of Gene Ontology and Kegg Pathway terms. The gene ID system used by kegga for each species is determined by KEGG. I assigned the OrgBb object to *N. vitripennis*, whose annotation is available from AnnotationHub (Morgan, 2019).

*5.2.9 RT qPCR validation of immune gene expressionon individual N. vitripennis wasps*

To confirm the results of the RNAseq analysis, I assessed the expression of 5 candidate immune genes that have previously been shown to increase expression upon exposure to Gram positive and Gram negative bacteria, in *N. vitripennis* (Sackton *et al*, 2013). Antimicrobial peptides, Nahymenoptaecin-2 and Nabaecin-2 (characterised by Tian *et al*, 2010) showed the highest log2 fold expression after immune challenge, followed by Navidefensin1-1. I also investigated an immune-inducible signalling gene, CLIP domain and a catalytic peptidoglycan recognition protein, PGRP-LB. In Sackton *et al*, 2013, the induced PGRP-LB gene is characterised as 'PGRP-LC', based on sequence homology. However, after analysis of interpro domains, it was clear the protein had zinc binding residues typical of

PGRP-LB genes. Thus, I have re-characterised the PGRP-LC gene as PGRP-LB, and a likely negative regulator of immune expression.

As a positive control for immune induction, I challenged individual *A. nasoniae* uninfected female adult wasps with the entomopathogen, ***Serratia*** *marcescens.* Each of the females were given a septic injury by poking a 0.1 mm dissecting pin dipped in bacterial culture of ***Serratia*** *marcescens,* into the abdomen. *S. marcescens* used in this study was provided by Paul Loughnane. Ten pools of *S. marcescens* infected (n = 30), *S. marcescens* uninfected (n = 30) and *S. marcescens* uninfected- *A. nasoniae* infected (n = 30) wasps were frozen in liquid nitrogen 6 hours after experimental manipulation, or collection. All samples were kept at -80 °C until required for RNA extraction.

*5.2.10 Determining immune expression*

Pools of *S. marcescens* infected, *S. marcescens* uninfected and *S. marcescens* uninfected-An-GFP *N. vitripennis* adults females were homogenized in 500 µl of Trizol (Ambion) and incubated at room temperature (RT) for 5 min. Two hundred microliters of chloroform (Sigma) were added and samples were mixed by shaking for 15 sec. The homogenized wasps were then spun in the centrifuge at 12000 g for 15 min at 4 °C. Following centrifugation, the top aqueous phase was removed with care not to disturb the interphase and placed in a fresh, RNAse free Eppendorf tube. Five hundred microliters of isopropanol were added to each tube after which the tubes were incubated for 10 min at RT. The samples were spun again at 12000 g for 10 min at 4 °C and the supernatant was subsequently removed. All samples were washed in 75% EToH and spun at 12000 g for 5 min at 4 °C. The supernatant was removed and discarded and the pellets air-dried for 15-20 min at RT. Fifty microliters of nuclease-free water were added to each tube and samples were frozen at -80 °C until further notice. Prior to freezing, RNA quality was verified using nanodrop and only samples with 260/230 ratios > 1.8 and 260/280 ratios > 1.8 were used for subsequent analysis.

RNA samples were DNAse treated with DNAse I (Thermofisher) to remove any contaminating DNA and then quality assessed again using nanodrop. RNA was transcribed to cDNA using the Transcriptor First Strand cDNA Synthesis Kit (Roche). cDNA was stored at -80 °C until required for qPCR analysis. To measure expression of key immune genes in An-gfp and uninfected females, a modified version of the comparative (Ct) method of quantitative PCR (qPCR) assay by Anbutsu and Fukatsu (2003) was carried out using a Fast Real-Time PCR system (Roche Lightcycler 480 II). Each 10 µl of qPCR reagent included 5 µl PowerUp SYBR

Green Master Mix (Applied Biosystems), 2.5 µl nuclease-free $H_2O$, 2 µl cDNA and 0.25 µl of 20 µM primers.

Primer sets for each gene of interest were designed using either OLIGO6 or Primer3 programs. Combinations of primers were tested against immune-challenged wasps at different temperatures until there was high amplification efficiency and no evidence of primer dimerization. The primers used in this study are presented in Table 5.1.

qRT PCR analyses were carried out using the following programme: one cycle of 95°C for 10 min, followed by 40 cycles of 95°C 5 s. 57°C 5 s, 72°C 30 s and finally one cycle of 72°C for 10 min. Expression of all genes was standardised relative to a housekeeping gene, EF1a (elongation factor α 1), yielding 2^-ΔΔCt. All calculations are in logarithm base 2.

| Gene | Function | Primers |
|---|---|---|
| Ef1a | Internal control (Verhulst, 2011) | 5'- CACTTGATCTACAAATGCGG -3'<br>5'- GAAGTCTCGAATTTCCACAG -3' |
| Navidefensin1-1 | AMP | 5'- CTCCAGCTTTGCCCATACTCA -3'<br>5'- GACGCCGGTGTAAGAC- 3' |
| Nahymenoptaecin-2 | AMP | 5'- CTCGCACTTCTCGCCTTTG -3'<br>5'- GGATTTGGCGGTCGGTTTAT -3' |
| Nabaecin-2 | AMP | 5'- ACGCTTCACCATACAGACCA -3'<br>5'- GTTGATTTCGACTTGGCCGT -3' |
| PGRP-LB | Recognition | 5'- TTGGGATGACATTGGCTACAG -3'<br>5'- GCCAGATTCCACTCCGTATT -3' |
| CLIP domain HMM | Signalling | 5'- TCTCGTGATGACCAGTTGCT -3'<br>5'- GATGGTCAGCTCGTCGTAGA -3' |

**Table 5.1.** List of genes and primers designed with OLIGO6 and Primer3.

## 5.3 Results

*5.3.1 Sequencing and mapping the Nasonia symbiont-regulated transcriptome*

| *Nasonia* species | Sample identifier | Total reads | % mapping success |
|---|---|---|---|
| *N. vitripennis* | Positive1 | 17,930,634 | 65.9 |
| | Positive2 | 18,887,111 | 68.6 |
| | Positive3 | 16,916,769 | 66.1 |
| | Negative1 | 15,499,179 | 64.2 |
| | Negative2 | 16,029,004 | 66.7 |
| | Negative3 | 13,956,847 | 57.0 |
| *N. giraulti* | Positive1 | 15,124,868 | 56.1 |
| | Positive2 | 14,756,438 | 58.4 |
| | Positive3 | 16,160,319 | 56.8 |
| | Negative1 | 14,790,110 | 60.8 |
| | Negative2 | 15,399,938 | 60.1 |
| | Negative3 | 13,916,492 | 55.1 |

**Table 5.2**. Number of reads from each library produced on one lane of Illumina HiSeq 4000.

In total, I recovered 53,734,514 high quality transcript reads from the *N. vitripennis A. nasoniae* infected treatment, 45,485,030 reads from the *N. vitripennis A. nasoniae* uninfected treatment, 46,041,625 from the *N. giraulti A. nasoniae* infected treatment and 44,106,540 from the *N. giraulti A. nasoniae* uninfected treatment. Transcript read lengths from individual libraries and mapping success to the Nvit2.1 assembly is summarised in Table 5.2.

*5.3.2 Functional annotation of N. vitripennis using Trinotate*

I annotated the assembled genome of *N. vitripennis* using an annotation toolkit, Trinotate (Bryant *et al*, 2017) which concatenates information on predicted coding regions, predicted coding domains, signal peptides and transmembrane domains. I applied Trinotate to the 26,502 transcripts in the *N. vitripennis* assembly. The vast majority of transcripts had detectable homologs in other species.

*5.3.3 Differential gene expression analysis in Nasonia wasps in response to A. nasoniae*

I examined the expression profiles of 6 samples of whole adult female *N. vitripennis* wasps and 6 samples of *N. giraulti* from *A. nasoniae* infected and uninfected treatments. For *N. vitripennis*, samples from each treatment clustered together on a multi-dimensional scaling (MDS) plot, indicating that the leading biological coefficient of variation (BCV) between samples of the same treatment is small. In Fig 5.2A, dimension 1 separates the two treatments by 1-2 units, corresponding to a 2-4-fold change between samples. *N. giraulti* sample Positive3 was removed from the *N. giraulti* differential expression analysis as it did not cluster in either dimension with samples Positive1 and Positive2 (Fig. 5.2B).

Using a negative binomial GLM approach implemented in the R/Bioconductor package 'edgeR' (Robinson *et al*, 2010) I identified 11,371 expressed genes. 2625 genes were at least 2-fold significantly differentially expressed (FDR ≤ 0.05) between *A. nasoniae* infected and uninfected *N. vitripennis* wasps. Of the significantly differentially expressed genes, 1,716 were upregulated and 909 were downregulated (Fig.5.3A). Genes that showed the greatest differential expression between the 2 treatments in *N. vitripennis* correspond to those encoding immune protein serine protease 142 and chitin binding protein. Both genes are significantly upregulated ($P < 0.05$, see Fig. 5.4A and Table 5.3).

*A. nasoniae* infected *N. vitripennis* wasps were enriched for physiologically relevant functional categories based on an analysis of Gene Ontology (GO) terms. Of note, genes associated with oxidoreductase, catalytic, iron binding and heme-binding activities were most enriched. For example, of the 238 genes associated with oxidoreductase activity, 96 were upregulated and only 9 were downregulated ($P < 0.05$) (Fig. 5.3A). Of the 45 genes associated with iron binding activity, 27 were upregulated and 1 was downregulated ($P < 0.05$). Genes associated with chromatin and the nucleus were significantly downregulated ($P < 0.05$).

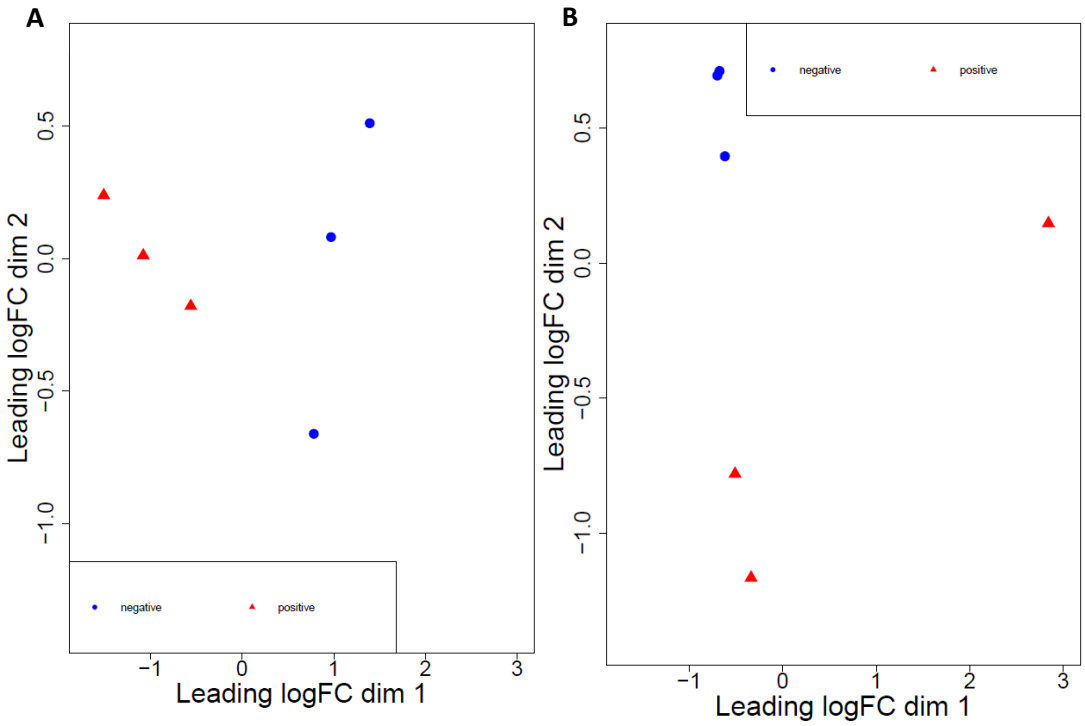



**Fig. 5.2**. MDS plots of logCPM values over dimensions 1 and 2 with samples coloured by *A. nasoniae* treatment groups, of **A,** *N. vitripennis* and **B**, *N. giraulti*. Distances on the plot correspond to leading fold-change, which is the average (root-mean-square) log2-fold-change for the 500 genes most divergent between the 3 samples in each treatment.

There is a very marked difference in the number of genes that are differentially expressed in response to *A. nasoniae* between the 2 *Nasonia* species (Fig.5.3). In *N. giraulti*, I identified 11,036 genes expressed in either An-gfp or uninfected wasps or both. Of these, 202 genes were upregulated in An-gfp wasps, and 134 were downregulated (Fig.5.3B). *Arsenophonus nasoniae* infected *N. giraulti* were enriched for GO terms associated with the nucleosome, protein-DNA complex, DNA packaging complex and chromatin. All were downregulated in the *A. nasoniae* treatment ($P < 0.05$). Genes that clustered into the defence response were the most highly upregulated, with 3 out of 10 genes upregulated and 0 downregulated ($P < 0.05$). Heme-binding proteins were also significantly upregulated, with 5 genes out of 45 upregulated and 0 downregulated. However, compared with that observed in *N. vitripennis*, the response is much smaller. Individual genes showing the greatest differential expression were cytochrome B, molybdenum cofactor synthesis protein cinnamon and a putative ankyrin repeat protein. Together with the gene enrichment analysis, there appears to be an over-representation of genes associated with chromatin structure and respiration (Fig. 5.4B, Table 5.4)

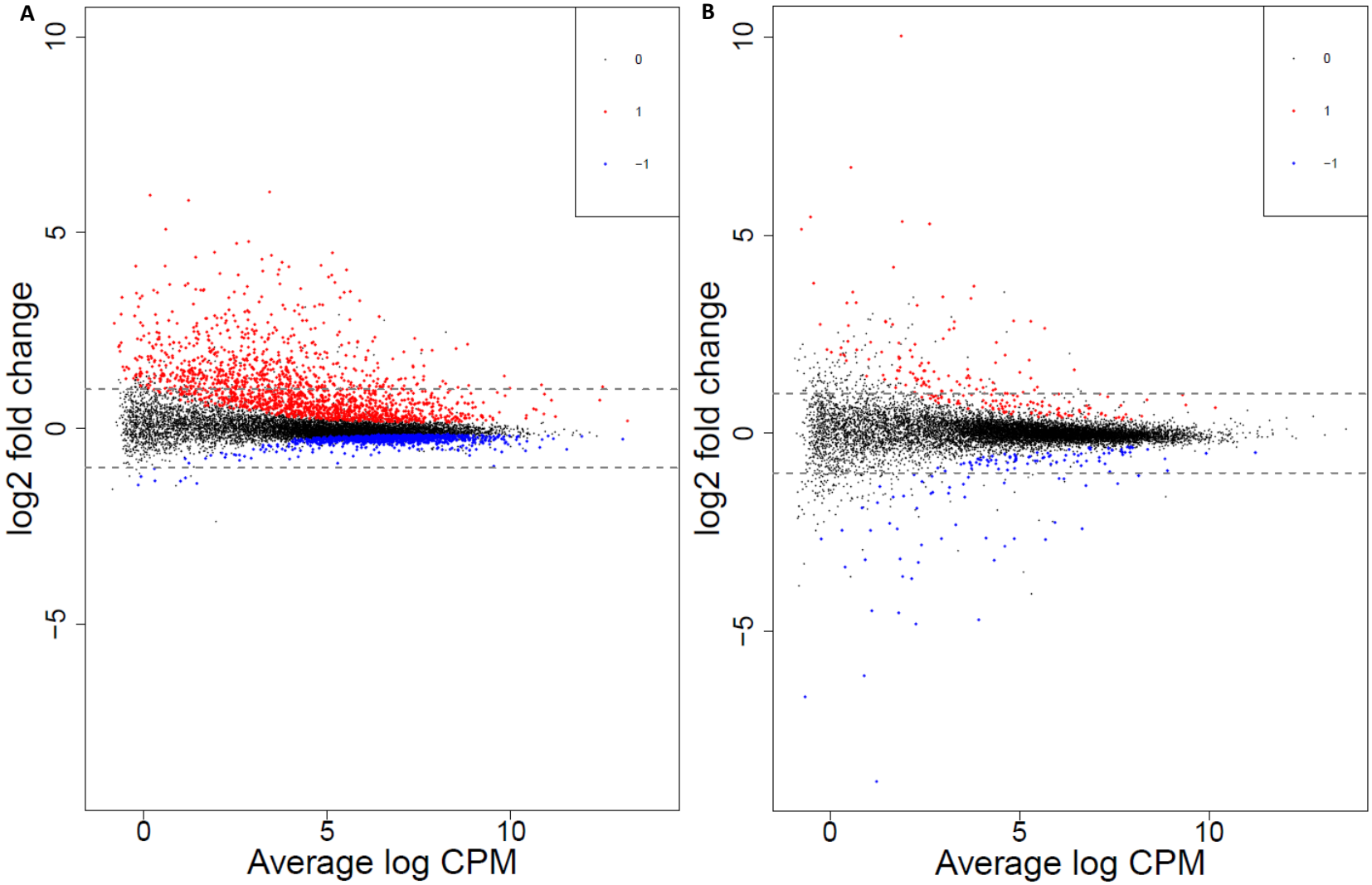



**Figure 5.3**. Mean-difference (MD) plot showing the log-fold change and average abundance of each gene in **A**, native host, *N. vitripennis* and **B**, novel host, *N. giraulti*. Significantly up and down DE genes (at 5% FDR) are highlighted in red and blue, respectively.

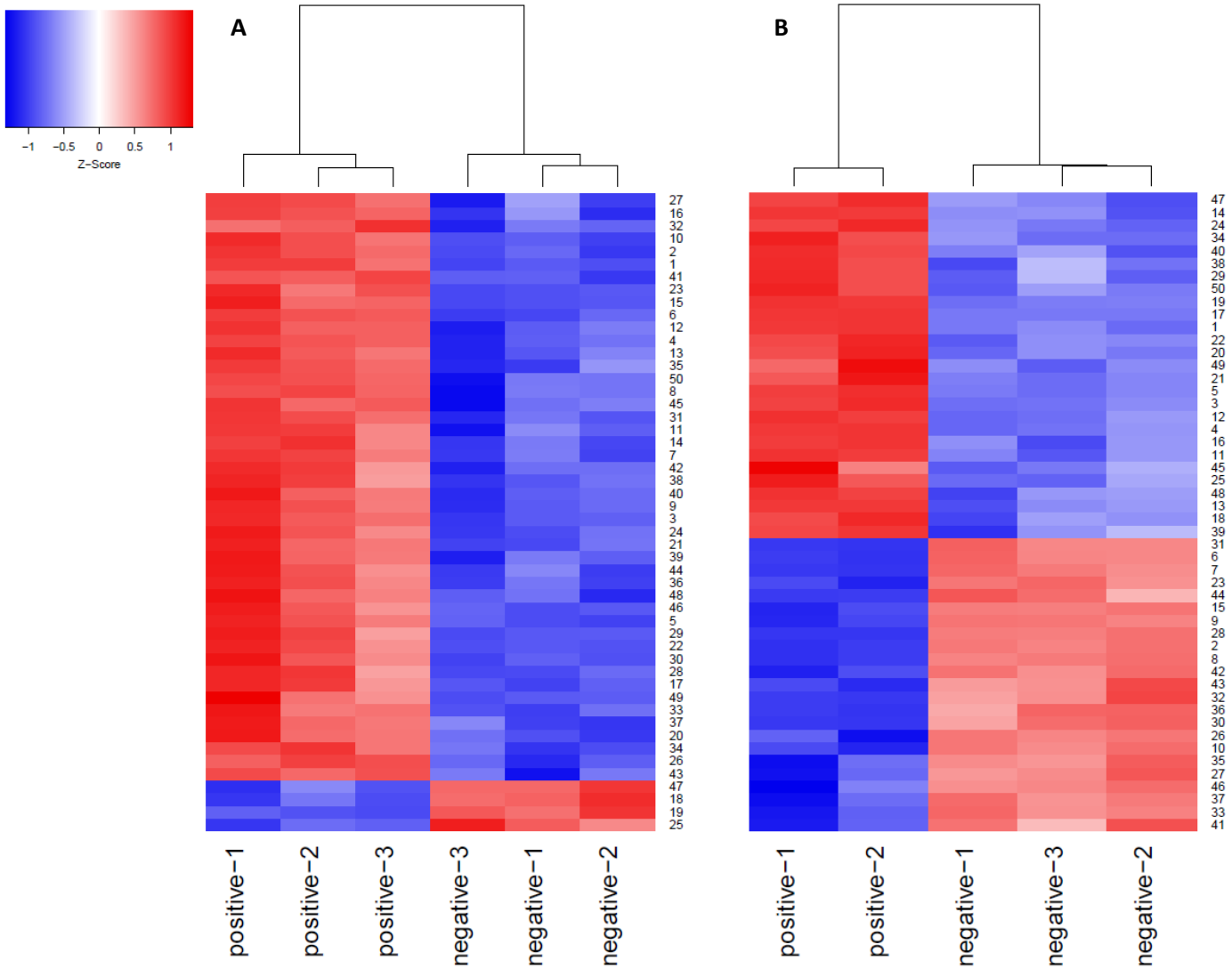



**Figure 5.4**. Heat map across all the samples of **A**, *N. vitripennis* and **B**, *N. girault*i, using the top 50 most DE genes between *A. nasoniae* infected and uninfected treatments.

| No. | Gene ID | Gene Name |
|---|---|---|
| **1** | 100124049 | serine protease 142 |
| **2** | 100119225 | uncharacterized LOC100119225 |
| **3** | 100116525 | prisilkin-39-like |
| **4** | 100119140 | YTH domain-containing protein 1-like |
| **5** | 100678913 | adenylate cyclase, terminal-differentiation specific-like |
| **6** | 100123271 | cytochrome P450 4AB18 |
| **7** | 100123012 | protein shifted-like |
| **8** | 100678580 | uncharacterized LOC100678580 |
| **9** | 100122687 | chymotrypsin inhibitor-like |
| **10** | 100120458 | uncharacterized LOC100120458 |
| **11** | 100379125 | carboxypeptidase N-like protein |
| **12** | 100678004 | uncharacterized LOC100678004 |
| **13** | 100121985 | myrosinase 1-like |
| **14** | 100120307 | indole-3-acetaldehyde oxidase-like |
| **15** | 103315534 | uncharacterized LOC103316634 |
| **16** | 100678078 | uncharacterized LOC100678078 |
| **17** | 100116079 | regucalcin-like |
| **18** | 100120946 | protein lethal(2)essential for life-like |
| **19** | 100678914 | G-protein coupled receptor Mth2-like |
| **20** | 100114921 | dehydrogenase/reductase SDR family member on chromosome X-like |
| **21** | 100121156 | facilitated trehalose transporter Tret1-like |
| **22** | 100216345 | sex comb on midleg |
| **23** | 100121133 | uncharacterized LOC100121133 |
| **24** | 100116702 | heat shock protein 83-like |
| **25** | 100679963 | uncharacterized LOC100679963 |
| **26** | 100114302 | cytochrome P450 6k1 |
| **27** | 100114279 | isocitrate dehydrogenase [NAD] subunit gamma, mitochondrial-like |
| **28** | 100124170 | cytochrome P450 4BW5 |
| **29** | 100118611 | acyl-CoA Delta(11) desaturase-like |
| **30** | 100118928 | dentin sialophosphoprotein-like |
| **31** | 100120476 | uncharacterized LOC100120476 |
| **32** | 100120370 | alpha-tocopherol transfer protein-like |

| | | |
|---|---|---|
| **33** | 100116954 | uncharacterized LOC100116954 |
| **34** | 100116344 | uncharacterized LOC100116344 |
| **35** | 100678573 | toll-like receptor 3 |
| **36** | 100679797 | uncharacterized LOC100679797 |
| **37** | 100115114 | acyl-CoA Delta(11) desaturase |
| **38** | 100124192 | carboxylesterase clade A, member 5 |
| **39** | 100678238 | putative odorant binding protein 41 |
| **40** | 103315672 | uncharacterized LOC103315672 |
| **41** | 100120331 | uncharacterized oxidoreductase YrbE-like |
| **42** | 100680373 | uncharacterized LOC100680373 |
| **43** | 100122119 | 5-phosphohydroxy-L-lysine phospho-lyase |
| **44** | 100122238 | multidrug resistance-associated protein 4-like |
| **45** | 100120743 | SPARC |
| **46** | 103316634 | uncharacterized LOC103315534 |
| **47** | 100118419 | UBX domain-containing protein 6 |
| **48** | 100116008 | uncharacterized LOC100116008 |
| **49** | 100122803 | synaptic vesicle glycoprotein 2B |
| **50** | 107981429 | uncharacterized LOC107981429 |

**Table 5.3**. The top 50 DE genes between *A. nasoniae* infected and uninfected *N. vitripennis*.

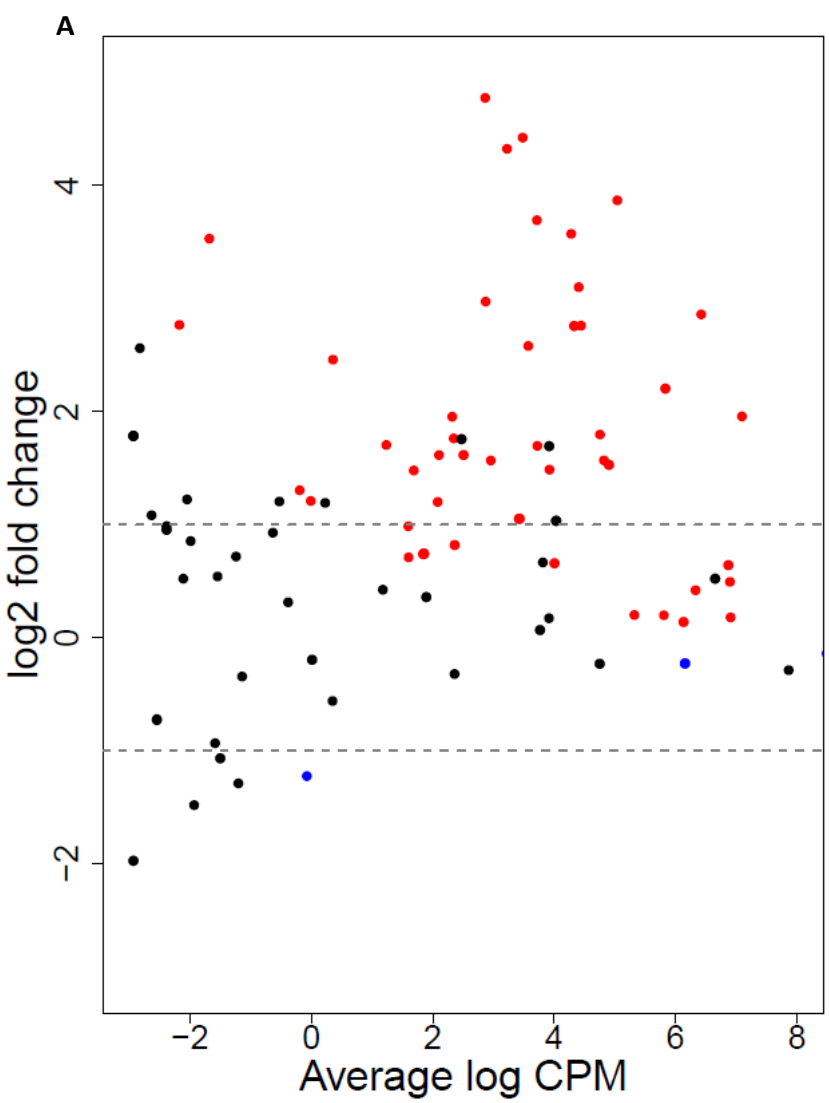
| No. | Gene ID | Gene Name |
|---|---|---|
| **1** | 107981441 | cytochrome b-like |
| **2** | 103317394 | uncharacterized LOC103317394 |
| **3** | 100678248 | uncharacterized LOC100678248 |
| **4** | 100117270 | molybdenum cofactor synthesis protein cinnamon |
| **5** | 103317515 | putative ankyrin repeat protein RF_0381 |
| **6** | 107981424 | uncharacterized LOC107981424 |
| **7** | 100116900 | probable multidrug resistance-associated protein lethal(2)03659 |
| **8** | 100114082 | protein HGH1 homolog |
| **9** | 100679339 | uncharacterized LOC100679339 |
| **10** | 107981423 | uncharacterized LOC107981423 |
| **11** | 107982227 | uncharacterized LOC107982227 |
| **12** | 103318172 | uncharacterized LOC103318172 |
| **13** | 100117364 | DDB1- and CUL4-associated factor 5 |
| **14** | 100113858 | uncharacterized LOC100113858 |
| **15** | 100123528 | histone H2B |
| **16** | 100116112 | uncharacterized LOC100116112 |
| **17** | 107981501 | NADH-ubiquinone oxidoreductase chain 1-like |
| **18** | 100116035 | GATA zinc finger domain-containing protein 14-like |
| **19** | 100678521 | 1,5-anhydro-D-fructose reductase-like |
| **20** | 100118376 | UDP-glucose 4-epimerase-like |
| **21** | 100678896 | uncharacterized LOC100678896 |
| **22** | 100118565 | hexamerin 70b |
| **23** | 103317597 | uncharacterized LOC103317597 |
| **24** | 100122185 | apidermin 3 |
| **25** | 100116041 | dynein heavy chain-like protein PF11_0240 |
| **26** | 107982140 | uncharacterized LOC107982140 |
| **27** | 100117301 | histone H4 |
| **28** | 100679050 | uncharacterized LOC100679050 |
| **29** | 100120370 | cytochrome P450 4BW5 |
| **30** | 100678103 | 4-coumarate--CoA ligase 1-like |
| **31** | 100679213 | uncharacterized LOC100679213 |
| **32** | 100680003 | uncharacterized LOC100680003 |

| | | |
|---|---|---|
| **33** | 103315496 | histone H1B-like |
| **34** | 103316634 | uncharacterized LOC103316634 |
| **35** | 100122816 | histone H3 |
| **36** | 100122143 | cuticular protein RR-1 family member 39 |
| **37** | 100117697 | histone H1C-like |
| **38** | 100120850 | ornithine decarboxylase-like |
| **39** | 100678737 | ornithine decarboxylase-like |
| **40** | 103316059 | uncharacterized LOC103316059 |
| **41** | 100679242 | neprilysin-21 |
| **42** | 107980639 | uncharacterized LOC107980639 |
| **43** | 100121980 | putative ATP-dependent RNA helicase DHX57 |
| **44** | 103315965 | speckle-type POZ protein A-like |
| **45** | 100116955 | farnesol dehydrogenase-like |
| **46** | 100117327 | histone H3 |
| **47** | 100678607 | uncharacterized LOC100678607 |
| **48** | 103316249 | uncharacterized LOC103316249 |
| **49** | 100118531 | hexamerin 83 |
| **50** | 100114526 | beta-ureidopropionase-like |

**Table 5.4**. The top 50 DE genes between *A. nasoniae* infected and uninfected *N. giraulti*.

*5.3.4 Expression of homology-annotated immune genes*

I investigated the expression of homology-annotated *N. vitripennis* immune genes, in *N. vitripennis* and *N. giraulti*. Ninety-one immune genes were expressed in *N. vitripennis*, 88 of which were upregulated and 3 downregulated in the presence of *A. nasoniae* (Fig.5.5A). The 3 downregulated genes encode signalling molecules, CLIP and ras85D. Among the highly expressed immune genes were Nahymenoptaecins, Nasonins and Navidefensins, including Nahymenoptaecin-2 and Navidefensin-1-1. In *N. giraulti*, 50 immune genes were expressed with 9 genes significantly upregulated and 7 downregulated (Fig.5.5B). The upregulated genes include Nasonin-3, Navidefensin-1-2, Nahymenoptaecin-2 and Nabaecin-1.



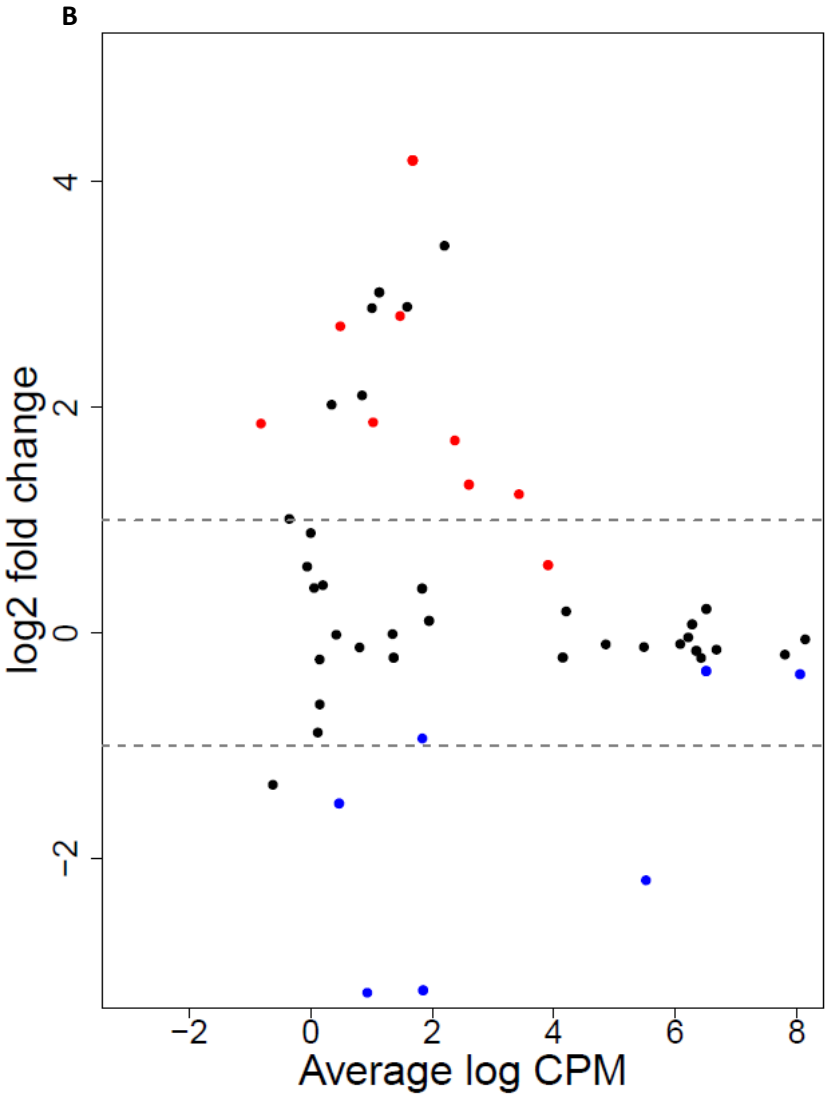




**Figure 5.5**. MD plots showing the log-fold change and average abundance of *N. vitripennis* immune genes in **A**, *N. vitripennis* and **B**, *N. girault*i. Significantly up and down DE genes are highlighted in red and blue, respectively.

*5.3.5 Inducible expression profiles of adult N. vitripennis immune genes*

I used RT quantitative PCR to determine whether 5 candidate immune genes were expressed in individual adult female *N. vitripennis* in response to *A. nasoniae* infection. I measured expression in *A. nasoniae* infected and uninfected wasps. The *A. nasoniae* treatment (A+) was significantly different from the uninfected treatment (A-) (MANOVA, $F_{1,18} = 10.39$, $P < 0.005$). Within the *A. nasoniae* treatment, two AMP genes, Nahymenoptaecin_2 and Navidefensin_1_1 were actively expressed and recognition receptor *peptidoglycan receptor protein LB* (PGRP-LB) was downregulated (Fig. 5.6, Table 5.5). Of the 2 upregulated AMP genes, Nahymenoptaecin-2 was more strongly expressed. Expression of Clip-domain serine protease (CLIP) and AMP, Nabaecin_2, were was not induced by *A. nasoniae* in this experiment.

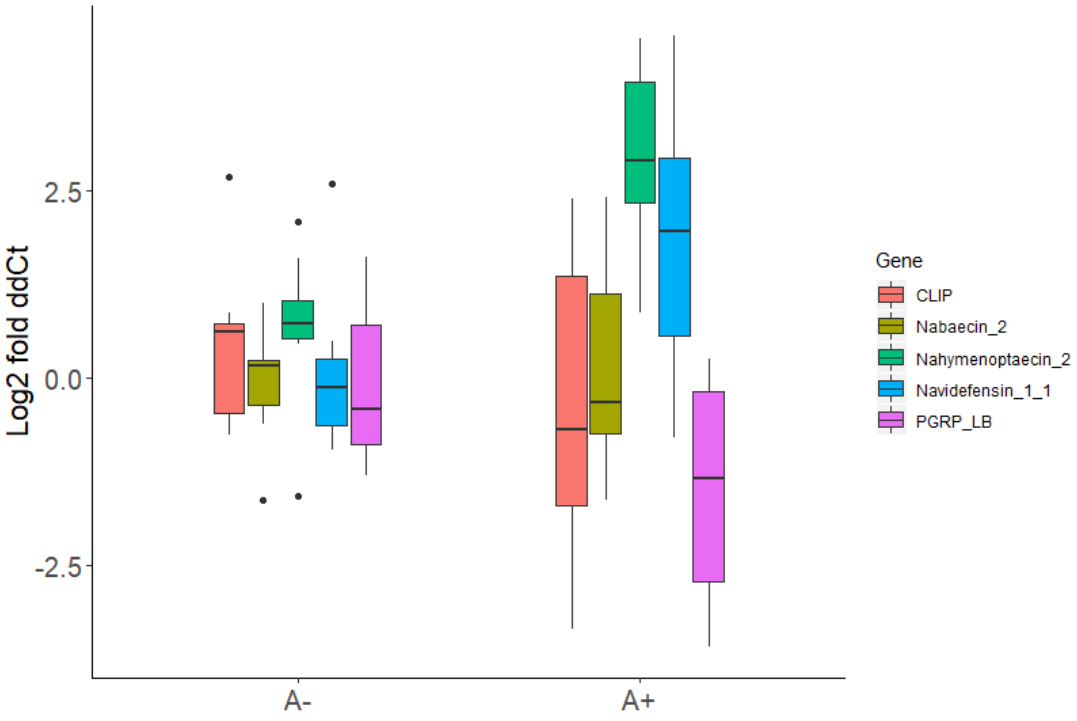



**Figure 5.6.** Log2-fold gene expression relative to an invariant housekeeping gene in adult female *Nasonia vitripennis*, in the presence and absence of native *Arsenophonus nasoniae* infection (A+: *A. nasoniae* infected, A-: *A. nasoniae*- free). The box plots display the upper and lower quartiles, the median and the range.

| Gene | Df | F value | P | |
|---|---|---|---|---|
| **CLIP** | 1 | 0.132 | 0.720 | - |
| **Nabaecin_2** | 1 | 0.117 | 0.736 | - |
| **Nahymenoptaecin_2** | 1 | 11.5 | 0.00326 | ** |
| **Navidefensin_1_1** | 1 | 7.55 | 0.013 | * |
| **PGRP_LB** | 1 | 6.25 | 0.022 | * |
| **Residuals** | 18 | | | |

**Table 5.5.** Univariate ANOVA results for each gene tested in the MANOVA. Transformed expression values ($\delta\delta$Ct) were treated as dependent on the presence of *A. nasoniae* (A-, A+). -, P > 0.1; ~, P < 0.1; *, P < 0.05; **, P < 0.01; ***, P < 0.001

## 5.4 Discussion

The true extent to which the immune system represents an important interface with symbionts is poorly understood. Typically, whether they're reproductive parasites or mutualists, endosymbionts do not elicit an immune response in their native hosts, and in some cases, novel hosts (Hurst *et al*, 2003; Anbutsu and Fukatsu, 2010; Herren and Lemaitre, 2011; Hutchence *et al*, 2011; Chrostek *et al*, 2014; Caragata *et al*, 2017; Li *et al*, 2018). In all described cases of mutualism, either the symbiont or host has evolved mechanisms to reduce the fitness costs imposed by the bacterium. Even the reproductive parasite, *Wolbachia*, does not activate or repress AMP production in *Drosophila simulans* and *Aedes albopictus* (Bourtzis *et al*, 2000). A recent study using the *Sodalis/Sitophilus* system, where the endosymbiont is restricted to a host-dedicated organ, identified a localised immune response targeted at the endosymbiont (Maire *et al*, 2018).

Whilst few studies have investigated how insects utilize their innate immune systems to control intra- and extra- cellular, obligate and facultative endosymbiont proliferation and virulence, less is known of whole organismal responses. *Spiroplasma* for example, is associated with the downregulation of egg production (chorion) genes following transinfection to a novel host (Hutchence *et al*, 2011) and *Wolbachia* induces oxidative stress and genes involved in protein, carbohydrate and lipid metabolism (Caragata *et al*, 2017). In this study I set out to investigate the transcriptomic response of *Nasonia* parasitoid wasps in their natural symbiotic state and following novel endosymbiont transinfection.

From both qPCR and RNAseq analyses, I observed that immune and defence genes are significantly up-regulated in *A. nasoniae* infected wasps. In *N. vitripennis* the gene that showed the greatest differential expression between the two treatments was serine protease 142 (SP142). SP142 has been implicated in the innate immune response of silkworms and is commonly important at the start of immune cascades, during which they cleave and activate proteins that induce Toll and imd pathways (Li *et al*, 2017).

Other than host immunity, the main factor that limits bacterial growth and fitness within hosts is iron availability. Many microbes that live inside hosts typically face a low-iron environment in host tissues (Messenger and Barclay, 1983a; Messenger and Barclay, 1983b). Iron plays an important role in cell composition, metabolism and enzyme activity. Iron is also important in host-symbiont interactions. Deficiency causes growth inhibition, a decrease in RNA and DNA synthesis and altered cell morphology. It is crucial for bacterial survival that cells acquire iron from their environment. Competition can emerge between host-symbiont

partners for iron and sequestration of iron is regarded as a determinant of virulence (Payne and Finkelstein, 1978). Organisms whose virulence has been shown to be enhanced by iron include *E. coli, Pseudomonas aeruginosa, Yersinia pestis* and *Staphylococcus aureus*.

Iron is required for catalytic activity. Most notably, it is utilized by enzymes involved in oxidative metabolism. To acquire essential iron, microbes produce iron-chelating compounds called siderophores which remove ferric iron from the environment and make it available to the microbe for growth. Altered expression of genes involved in oxidative stress and iron metabolism has been observed in response to *Wolbachia* infection (Kremer *et al*, 2009; Caragata *et al*, 2017). For example, *Wolbachia* infection in *Aedes fluviatilis* causes an upregulation of iron-binding proteins (Caragata *et al*, 2017) and *Asobara tabida* wasps that lack *Wolbachia* overproduce ferritin, a protein involved in regulation of iron homeostasis (Kremer *et al*, 2009). Brownlie *et al*, 2009 suggest that *Wolbachia* behaves as a nutritional mutualist, whereby *Wolbachia* infected *D. melanogaster* produce a greater number of eggs relative to uninfected flies, on high iron and low iron diets.

Wilkes *et al*, 2010 observed that the genome of *A. nasoniae* contains ORFs that encode translocation systems for chelated iron. One system is based on one Fe3+ ABC transporter system and the other on the TonB-dependent *FepABCDG* translocation system of *Escherichia coli*, with which it is syntenous. Whether *A. nasoniae* makes siderophores itself is not known and requires further investigation however, the bacteria does possess a fragment of an ORF with polyketide synthase domains whose most likely role is in siderophore production. *A. nasoniae* also possesses an operon which has sequence homology to a *FepABC* transporter from *E. coli*, which transports free ferrous (unchelated) iron (Wilkes *et al*, 2010).

There is strong evidence to suggest that *A. nasoniae* interferes with iron metabolism by actively sequestering it from its host, *N. vitripennis* (Wilkes *et al*, 2010). Iron homeostasis is important for regulation of oxidative stress. Removal of too much iron from the host may result in a stress situation whereby functional homeostasis is upset in the host. Enrichment for gene terms associated with oxidoreductase activity suggests that infected wasps are indeed under oxidative stress. During oxidative stress reactive oxygen species (ROS) are produced, which may act to damage or inhibit the iron sequestration abilities of *A. nasoniae*, albeit with a cost to the individual itself. ROS typically damage lipids, proteins and nucleic acids. Thus, the observed upregulation of genes associated with DNA package complex, DNA binding and chromatin may well be a response to protect vital cellular functioning from oxidative stress damage. I also observed that *N. vitripennis* responds by upregulating the

production of iron-binding compounds. Thus the host directly competes for iron *with A. nasoniae*.

A contrasting explanation could be that iron depletion by *A. nasoniae* causes *N. vitripennis* to induce oxidoreductase and catalytic activities, which require iron. By utilizing and binding iron itself, *N. vitripennis* may find that it is able to control *A. nasoniae* proliferation and virulence. Thus instead of being a negative by-product of *A. nasoniae* infection, over-expression of oxidoreductase genes could form part of the hosts defence response and endosymbiont control mechanism. Another possible mechanism driving the observed changes in iron metabolism could be infection-induced anorexia. The lifespan of *Drosophila* infected with the pathogen, *Salmonella*, can be extended through diet restriction (Ayres and Schneider, 2009). Infection-induced anorexia alters host defences and leads to increased tolerance to infection. Diet restriction in response to *A. nasoniae* infection may affect *Nasonia* immune defences, resulting in altered metabolic processes and oxidative stress. I did not measure and compare feeding rates in *A. nasoniae* infected and uninfected *Nasonia* however, it may in part explain the observed changes in iron metabolism. Infection-induced anorexia in developing *Nasonia* larvae would lead to asynchrony between infected and uninfected wasp stocks and anecdotally, I did not observe this in the laboratory stocks.

The observed wide-scale upregulation of immunity and defence genes and genes responsible for iron homeostasis in *N. vitripennis* may simply be non-adaptive symptoms to illness however, the response to *A. nasoniae* infection differs greatly between the native and novel host species. One might hypothesize that the novel host produces a stronger response to infection compared to the native host. *A. nasoniae* in this study derives from Finland and has no history of exposure to *N. giraulti*, a North American species. However, *A. nasoniae* is able to successfully host-shift from *N. vitripennis* to *N. giraulti* in the laboratory setting and it is likely to occur in *N. giraulti* in the wild (Duron *et al*, 2010). Although it is possible that the host genetic background of *N. giraulti* will have been exposed to the North American strain of *A. nasoniae*, it will not have been exposed to the European strain of *A. nasoniae*. Thus, in this study *A. nasoniae* is a novel infectious agent to *N. giraulti*.

Many more genes in *A. nasoniae*-infected *N. giraulti* were upregulated than downregulated, but these genes did not correspond to the oxidative stress genes observed in *N. vitripennis*. Iron-binding proteins were also over-expressed in *N. giraulti* but there were far fewer relative to *N. vitripennis*. It may be that *A. nasoniae* is less- or un-able to exploit the novel host for iron due to differences in cellular components and receptors. Thus *N. giraulti* may not

become deficient in iron and enter a state of oxidative stress. It may also be that the immune response is more effective against *A. nasoniae* in *N. giraulti* than in *N. vitripennis*. Immune effectors such as antimicrobial peptides are small, rapidly evolving molecules (approx. 100 amino acids in length). Whilst *Nasonia* species are thought to have effectors that do not show sequence homology to AMP in other Hymenoptera (Sackton *et al*, 2013), the diversity of effectors could differ between the individual *Nasonia* species. Furthermore, the lack of available iron for *A. nasoniae* may render the bacteria more susceptible to immune attack.

Broad scale changes in gene expression and phenotypes are often associated with changes in DNA methylation patterns. In *N. vitripennis*, methylated genes typically have higher expression levels and lower variation in expression across developmental stages (Wang *et al*, 2013). The large and widespread upregulation of genes in *A. nasoniae* infected *N. vitripennis* could be associated with altered methylation patterns. Manipulating methylation on a large scale can be achieved in *Nasonia* using the demethylating agent, 5-aza-2'-deoxcytidine (5-aza-dC) (Cook *et al*, 2018). Assessment of methylation patterns across the genome can be accomplished using bisulphite sequencing (Wang *et al*, 2013). To investigate further the gene expression patterns caused by *A. nasoniae*, 5-aza-dC treatment could be applied to *A. nasoniae* uninfected adult female *N. vitripennis,* and differential gene expression re-assessed to see if it corresponds to that of *A. nasoniae* infected females, either using RT qPCR or repeating the RNAseq analysis. An alternative approach would be to carry out a Bisulphite sequencing (BS-Seq) experiment on *A. nasoniae* infected and uninfected adult female *N. vitripennis.*

The data presented in this chapter strongly suggest that the bacterial endosymbiont *A. nasoniae* induces a major systemic response in its natural parasitoid wasp host, *N. vitripennis*. A plethora of host genes, most notably those involved in oxidative stress and iron sequestration are upregulated. Key antimicrobial peptides and signalling molecules are also over-expressed relative to uninfected wasps. This transcriptional response differs greatly from the response of *N. vitripennis*'s sibling species, *N. giraulti*, to novel *A. nasoniae* infection. Alterations to the amount of iron of iron-binding proteins in the host may be a determining factor in the host's susceptibility to bacterial infection. Conversely, the efficiency of iron acquisition by the bacteria may affect its growth and virulence. Moreover there could be wasp species-specific iron requirements. Instead of using predominantly oxidative phosphorylation pathways which require metal ions including iron, *N. giraulti* may utilize or shift to utilizing glycolytic enzymes for metabolism, which do not require iron.

This study redefines what we understand about host-symbiont interactions and immunity. *A. nasoniae* is able to successfully maintain itself within host populations however; this comes at an overwhelming cost to its coevolved host through the upregulation of hundreds of immune, metabolic and enzymatic genes. *A. nasoniae* has a profound effect on various physiological functions of the host and is both parasitic and pathogenic in behaviour. Following a host shift, the endosymbiont elicits an immune response but nothing else of great note.

Here I propose a novel mechanism for controlling endosymbiont proliferation and virulence. *A. nasoniae* activates systemic upregulation of stress and immune responses in *N. vitripennis*. The induction of oxidoreductase and catalytic activities are likely part of an evolutionary arms race between *N. vitripennis* and *A. nasoniae*, whereby *A. nasoniae* has evolved methods of iron sequestration and *N. vitripennis* has adapted by removing iron from the internal environment. The fact that this systemic stress response was not observed in *N. girault*i, provides insight into host shift biology and suggests that there are incompatibilities between host and microbe that prevent *A. nasoniae* from tapping into host iron resources. Taken together, the results suggest that *A. nasoniae* is not only a reproductive parasite but also a pathogen in its native and novel hosts.

# Chapter 6

## Symbiont–host interactions in diapause: a case study in *Nasonia spp*.


### Abstract

Insects in temperate regions enter a life history stage called diapause to survive the harsh winter condtions, whereby development is arrested and metabolism greatly reduced. Despite the importance of diapause in insect biology, there are few accounts of how symbiosis affects, and is affected by, diapause. In this chapter, I outline the relationship between *Arsenophonus nasoniae* and *Nasonia* wasps during diapause in terms of whether the symbiont affects the likelihood of the wasp host entering diapause, and the nature of the symbiosis during diapause in terms of infection behaviour and host immune response. These analyses are completed for the native host *N. vitripennis* and the novel host *N. giraulti*. I observe that *A. nasoniae* had little impact on the tendency of the *N. vitripennis* to enter diapause. There was however evidence to suggest that *A. nasoniae* negatively affected fecundity and thus diapause in *N. giraulti*. *A. nasoniae* was associated with a host immune reaction (nodulation) during diapause, in the native host, with occasional examples of host necrotic death alongside symbiont proliferation. Host Immune gene expression, in terms of AMP induction, was observed in the novel host, *N. girautli*, but not in the native host, *N. vitripennis*.

## 6.1 Introduction

Microorganisms share a long evolutionary history with many insect lineages and can influence insect ecology and evolution (Buchner, 1965). From the host perspective, interactions with these bacteria can be obligate, where they provide a necessary service such as nutrient provisioning, cuticle formation or aiding reproduction (Douglas, 1998). Secondary, or facultative, symbionts are not required by their host, but can be important for protection against natural enemies (Oliver *et al*, 2003; Hamilton and Perlman, 2013; Xie *et al*, 2014). Both obligate and facultative symbionts are vertically transmitted, from mother to offspring, often transovarially.

To maintain themselves within host populations, facultative symbionts can shift along the parasitism-continuum spectrum, depending on the host's environment (Ashby and King, 2017). For example, common symbionts of fruit flies, *Wolbachia* and *Spiroplasma,* can manipulate their host's reproductive biology via cytoplasmic incompatibility, parthenogenesis or male killing (Werren *et al*, 2008; Montenegro *et al*, 2005). *Wolbachia* can also cause feminization in the European woodlouse, *Armadillidium vulgare* (Rigaud and Juchault, 1992). The result of these mechanisms is the same- an increase in the number of females and thus symbiont prevalence in the host population. The prevalence of endosymbionts in a host population depends on the efficiency of maternal transmission and the effect of the symbiont on host fitness. Symbionts rely on host reproduction to pass to the next generation and thus their prevalence may be affected by any fitness costs they impose on the host.

Incongruences between host and symbiont phylogenies indicate that horizontal transfer of symbionts has occurred among different insect species (Russell *et al*, 2003; Sandstrom *et al*, 2001). Facultative endosymbionts must therefore retain some ability to infect and proliferate in naive and even distantly related host species. However, compatibility to novel symbiont infection reduces with phylogenetic distance from the ancestral host (Longdon *et al*, 2011), such that distantly related species make less compatible hosts. The widespread movement of endosymbionts provides opportunity for establishment of new associations, but there may be factors other than sympatry that determine whether a symbiont can invade a novel host species.

Generally, studies of these parameters are made in laboratory conditions, at constant temperature and long photoperiod. However, insects from temperate regions are subject to annual changes in photoperiod and temperature and adjust their phenology accordingly so

that they can be active, grow and reproduce when resources are most abundant (Tauber *et al,* 1986). Most insects undergo some form of reproductive latency or diapause in response to seasonally unfavourable conditions, where development is arrested and metabolism is greatly reduced. Diapause is often instigated at a genetically determined developmental stage such as embryonic, larval or pupal stage.

Despite the importance of diapause for insect population biology, studies of the interaction between symbiont and host during diapause are very rare. In some *Drosophila* populations, CI- inducing *Wolbachia* can reach fixation but elsewhere *Wolbachia* frequency can be moderate. For example, *w*Mel *Wolbachia* frequency is generally high in the tropics of Australia but declines with latitude, possibly due to winter photoperiod and temperatures (Hoffmann *et al*, 1994; Kriesner *et al*, 2016). Kriesner *et al*, 2016, observed that *w*Mel infected *Drosophila* that survived an induced period of dormancy as adults, produced fewer eggs, fewer viable offspring and had a lower proportion of viable ova compared to uninfected females that had been exposed to dormancy-inducing conditions. Thus, *w*Mel infection has a negative fitness cost on female *Drosophila* in temperature regions. In contrast, infection with a male-killing *Rickettsia* in adult female two spot ladybirds, *Adalia bipunctata*, caused a reduction in oviposition and longevity. However; the bacterium did not affect the overwinter survivorship of the beetles (Hurst *et al*, 1994).

Host diapause may also affect the onward transmission of the symbiont. During cold winter months *Nasonia* species enter diapause (Saunders, 1965a; Saunders, 1965b). Following diapause-inducing cues, prolonged artificial refrigeration of *Nasonia vitripennis* can result in a reduction in density and loss of single or double *Wolbachia* infections (Perrot-Minot *et al*, 1996). Loss of either *Wolbachia* strain A or B from a double infection can result in segregation of infected female lines and lead to reproductive isolation.

The success and failure of symbiont host shifts may also be affected by host diapause. Following artificial lateral transfer from their native *Drosophila melanogaster* and *Drosophila simulans* hosts to *Aedes* mosquioes respectively, *w*MelCS and *w*Ri *Wolbachia* strains did not affect egg viability following 8 weeks of egg storage (diapause). However, following 10 weeks of egg storage, both *Wolbachia* strains cause a reduction in egg hatch rate relative to a tetracycline treated control. In contrast, the *w*Pip strain of *Wolbachia* from *Culex quinquefasciatus* caused a rapid and significant reduction in egg viability with an egg hatch rate of less than 20% following 3 weeks of storage, relative to tetracycline treated control

females (Fraser *et al*, 2017). Thus, symbiotic interactions in diapause can determine host-symbiont compatability.

In this chapter, the interaction of *N. vitripennis* and *N. giraulti* with *A. nasoniae* is examined in the context of diapause. Diapause in *Nasonia* is mediated via neuro-hormonal signals (Nelson *et al*, 2010) and also maternally induced in response to daily light:dark cycles (Tauber *et al*, 1986). In response to these signals, a maternal transcript is passed into eggs, which causes the progeny to arrest at the third larval instar. As both diapause signals and bacteria are maternally transmitted, any environmental cues the mother perceives that signal a change in photoperiod may influence bacterial transmission.

*Nasonia giraulti*, the sibling species of *N. vitripennis* can obtain *A. nasoniae* in laboratory multiparasitism events (Duron *et al*, 2010) and the distributions of the two wasp species overlap in eastern North America (Darling and Werren, 1990). Thus, there is opportunity for *A. nasoniae* to transfer from *N. vitripennis* to *N. giraulti* in nature. The costs and benefits of *A. nasoniae* carriage in *N. vitripennis* are not known, although recently it has been suggested that *Arsenophonus* species supplement the diet of whiteflies with B vitamins (Santos-Garcia, 2018). Costs associated with transinfection of *A. nasoniae* are also unknown.

The *Arsenophonus*- *Nasonia* symbiosis is relatively recent and although *A. nasoniae* has undergone genome degradation, it retains active metabolic pathways and genes important for self-regulation (Darby *et al*, 2010). *A. nasoniae* also displays an array of virulence factors and putative genes encoding toxins such as apoptosis-inducing protein (Aip)-like ORFs and type III secretion systems (Darby *et al*, 2010; Wilkes *et al*, 2010; S. Siozios pers. comm.). Thus, this system could provide insight into the early stages of host-symbiont co-evolution. With the information presented thus far, one could hypothesize that *A. nasoniae* activates the innate immune system in diapausing larvae of *N. vitripennis.*

In this chapter, I investigated the effect of *A. nasoniae* on the photoperiodic induction of diapause in its native host, *N. vitripennis* and novel host, *N. giraulti*. I assessed the prevalence and pattern of infection of *A. nasoniae* in diapause larvae and looked for evidence of pathology and immune induction. I also assessed the expression profile of key immune genes in the two wasp species.

## 6.2 Materials and Methods

*6.2.1 Nasonia and symbiont strains*

*N. vitripennis* strain AsymC and *N. giraulti* strain RV1xTetra were maintained using house fly pupae as hosts. Five fly pupae (up to 30 days old) and five mated females were added to each tube. The tubes were sealed with cellulose acetate flugs and placed in a 25 °C incubator with 14:10 L:D cycle for 14-15 days until the new wasp generation emerged.

*6.2.2 Arsenophonus nasoniae strain Fin'13*

*N. vitripennis* is naturally infected with *A. nasoniae* (isolated from Turku, Finland in 2013). This symbiont strain was genetically manipulated to carry the GFP expressing plasmid, pOM1-gfp, by fellow lab member, Dr Pol Nadal. The strain emits green light under epifluorescent illumination, allowing easy tracking of symbiont status, tropism and titre. This strain is henceforth termed An-GFP.

*6.2.3 Lateral transfer of A. nasoniae from N. vitripennis to N. giraulti*

Transfer of An-GFP to *N. giraulti* was carried out by Dr Pol Nadal, as described in chapter 5.

*6.2.4 Ascertaining the impact of A. nasoniae on Photoperiodic induction of diapause*

Prior to their use, wasps were maintained in standard conditions at 25 °C with a 14:10 L:D cycle. Newly emerged females were permitted to mate for 1 day (with siblings). Fifty mated An-GFP *N. vitripennis* females and 50 mated wild type females were removed and individually placed in *Drosophila* vials with 2 fresh (2-3 day old) *S. bullata* pupae. The vials were sealed with cellulose acetate flugs and placed in an incubator at 15 °C with 8:16 L:D cycle. The two host pupae were replaced every other day for 10 days. Following parasitism, hosts were placed at 25 °C with 14:10 L:D for 4 weeks.

*6.2.5 Diapause scoring*

Normally developing *Nasonia* emerge from the host pupae after 14-15 days at 25 °C. Thus it is easy to score diapause by opening host pupae after 4 weeks at 25 °C and scoring the presence of larvae. Diapause was measured as a binary trait such that broods with larvae were scored 'diapause +ve' and broods with only adult wasps were scored 'diapause –ve'. Mixed broods, where larvae and adults were both present, were scored 'diapause +ve'. Mixed broods were very rare and occurred more frequently in *N. giraulti*.

*6.2.6 Visualizing A. nasoniae infection in diapausing N. vitripennis and N. giraulti*

For *N. vitripennis*, day 10 parasitized pupae were counted and assessed for An-GFP infection. All diapause larvae were observed under a M165 FC Leica stereoscope under epifluoresence and the number of infected larvae (green fluorescent) in each brood was recorded. For *N. giraulti*, infection prevalence was assessed in day 22 parasitized pupae, due to the larger threshold of light/dark cycles required to induce diapause in this species.

### *6.2.7 Determining immune expression in diapausing larvae*

Six pools of 30 uninfected and 30 An-GFP diapausing *N. vitripennis* larvae and 2 pools of 30 uninfected and 30 An-GFP diapausing *N. giraulti* larvae were collected, flash frozen in liquid nitrogen and frozen at -80 °C overnight. RNA extractions were carried out the next day using standard Trizol protocols, as described in chapter 5.

To measure expression of genes that have previously been shown to increase expression upon exposure to Gram positive and Gram negative bacteria, in *N. vitripennis,* (Sackton *et al*, 2013) I used a modified version of the comparative (Ct) method of quantitative PCR (qPCR) assay by Anbutsu and Fukatsu (2003), as described in chapter 5. Antimicrobial peptides, Nahymenoptaecin-2 and Nabaecin-2 (characterised by Tian *et al*, (2010)) showed the highest log2 fold expression after immune challenge, followed by Navidefensin1-1. I also investigated an immune-inducible signalling gene, CLIP domain and a catalytic peptidoglycan recognition protein, PGRP-LB. Primer sets for each gene of interest are described in chapter 5.

### *6.2.8 Statistical analysis*

I carried out Fisher's exact tests of independence to analyse differences in diapase induction between An-GFP and uninfected wasps. I carried out a nonparametric one-way multivariate analysis of variance (MANOVA) for qRT PCR gene expression for the independent variables: An-GFP and uninfected. MANOVA accounts for multiple testing and is thus robust to type I error. When MANOVA effects were significant, I explored the univariate individual gene effects.

## 6.3 Results

*6.3.1 Induction of diapause*

Diapause was induced in *N. vitripennis* larvae after adult female wasps from the maternal generation were exposed to a low temperature and 8:16 L:D cycle. Females started producing diapause larvae within 3 days of exposure to the diapause-inducing cues (Fig. 6.1). By day 10, the 31 An-GFP, and 37 uninfected *N. vitripennis* females all produced offspring that were in the physiological state of diapause. An-GFP did not affect the induction of diapause in adult females (100%, *P > 0.05)*.

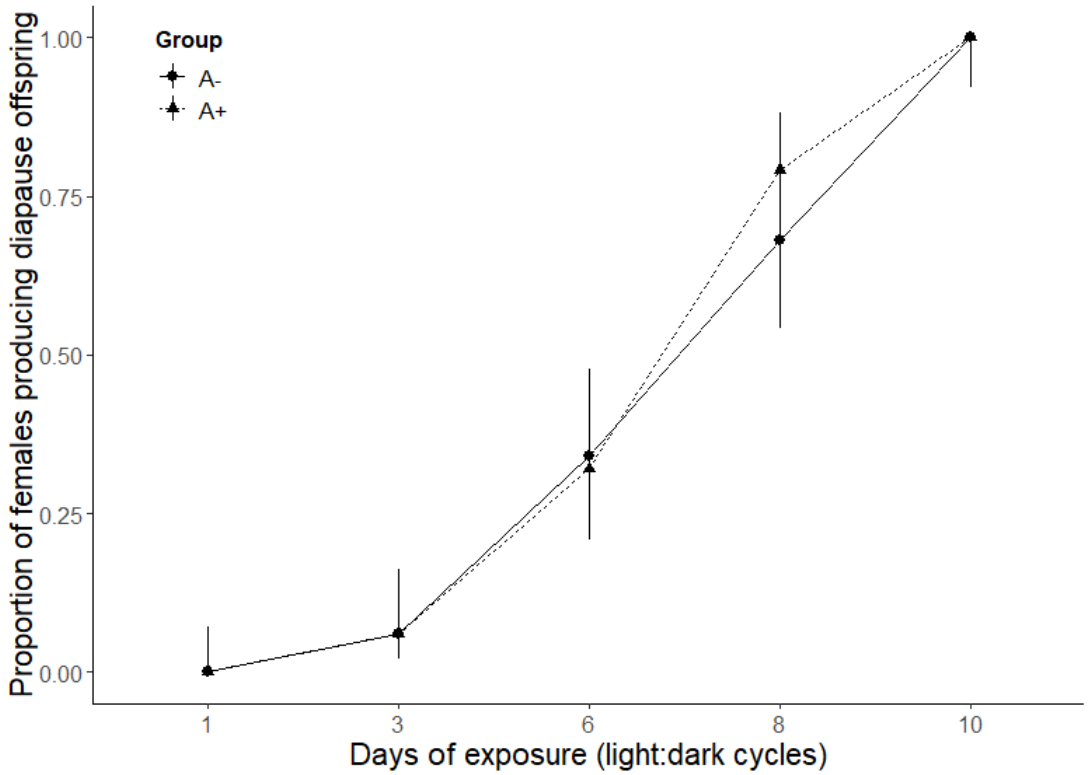



**Figure 6.1.** Diapause response of *N. vitripennis* females infected and uninfected with An-GFP (A+, A-) at 15 °C with 8:16 light:dark cycle. The proportion of diapausing broods is in relation to the total number of broods produced by females. Error bars represent the 95 % binomial confidence interval.

The proportion of diapause offspring produced by *N. vitripennis* and *N. giraulti* differed greatly (Table 6.2). *N. giraulti* produced very few diapause larvae following 10 days of diapause inducing cues, such that an analysis could not be conducted. In a repeat assay, the females were left undisturbed in diapause conditions from day 10 to day 22 and given fresh

hosts on day 22. The proportion of diapause larvae produced by females infected with An-gfp and uninfected females after 22 days of exposure to diapause conditions was assessed. Six out of 27 An-GFP infected females oviposited successfully (22.2%, CI 8.62%-42.3%), and all larvae entered into diapause. All other hosts were unparasitized. Of the 28 uninfected females, 18 oviposited (64.3%, CI 44.1%-81.4%), and these broods all entered diapause. Thus oviposition of An-GFP transinfected *N. giraulti* following diapause inducing cues was lower relative to uninfected wasps ($P < 0.05$).

*6.3.2 Visualisation of An-GFP in N. vitripennis diapause larvae*

Frequency of An-gfp infection in each diapause brood was assessed in *N. vitripennis*. Transmission of An-gfp is lower when females lay singly on a host pupa compared to when wasps are able to superparasitize (Parratt *et al*, 2016). In total, 137/754 diapause larvae (18.1%, CI 15.5-21.1%) showed systemic infections, fluorescing green throughout the body.

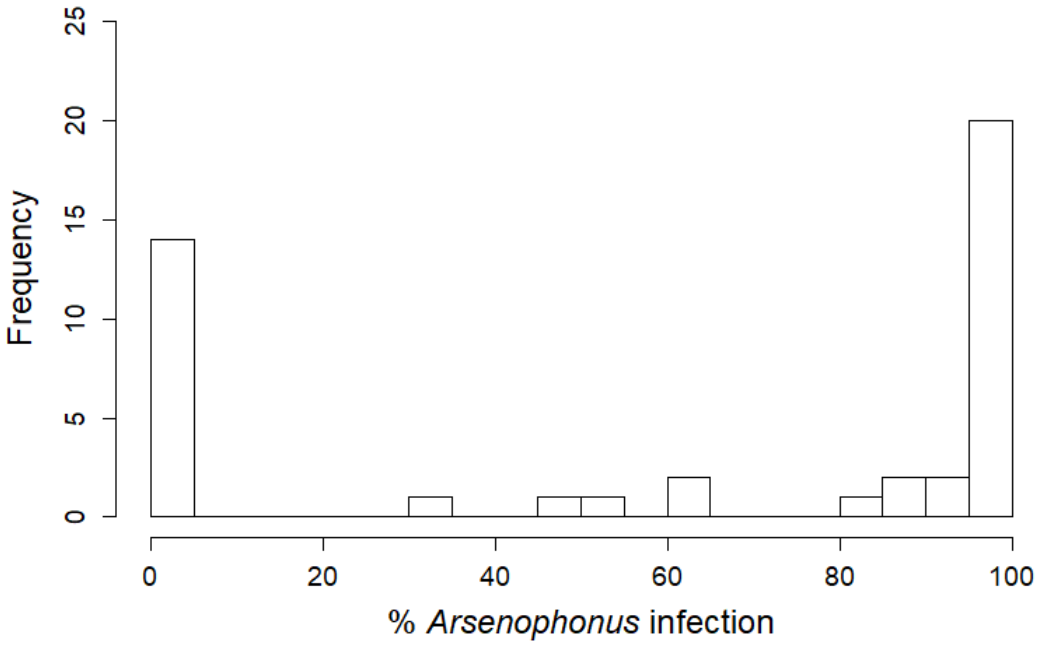



**Figure 6.2.** Frequency of An-gfp infection in diapause larvae. Infected females were each given 2 host pupae every other day for 10 days in diapause inducing conditions. An-gfp infection in each brood was assessed by visualization with GFP under a stereoscope.

| **Nasonia** ***species*** | ***Number of 8:16 L:D cycles*** | ***A-/A+*** | ***Females producing diapause offspring*** | ***Total number of females*** |
|---|---|---|---|---|
| **vitripennis** | 10 | A+ | 31 | 31 |
| | 10 | A- | 37 | 37 |
| **girault**i | 10 | A+ | 0 | 16 |
| | 10 | A- | 5 | 43 |
| | 22 | A+ | 6 | 27 |
| | 22 | A- | 18 | 28 |

**Table 6.1.** Number of diapause offspring produced by An-gfp infected 10-day old *N. vitripennis* and 10- and 22-day old *N. giraulti* and uninfected control females.

Similar to non-diapause larvae (Nadal-Jimenez *et al*, 2019), I found tropisms with foci of infection evident in the mouthparts and gut. 87 % of larvae in infected broods (n=31 broods, n=900 larvae) carried An-GFP infection. Thus I observed broods with mixed infection status, with some larvae harbouring An-GFP and others not. Broods where all larvae were infected displayed a range of infection phenotypes, with tropisms and systemic infections. Few broods were exclusively systemically infected.

Three out of the 31 An-GFP diapause broods examined contained necrotic larvae, which were not observed in any of the 37 control broods where An-gfp was absent. In total, 11 necrotic larvae were observed in 754 infected larvae observed (1.46%, CI 0.73%-2.6%), and these glowed intensely with An-GFP indicating the symbiont remained viable and had grown to higher titre than in viable wasp pupae. The necrotic larvae presented signs of melanized nodules throughout the body (Fig. 6.3: A, B). Further to this, living diapause larvae were also observed to display localized melanized spots in the areas surrounding An-GFP growth (Figure 6.3: C, D), and these melanised spots were not observed in any of the control uninfected *N. vitripennis* larvae.

From these data, we conclude An-GFP interacts with the *N. vitripennis* immune system during diapause, inducing nodule formation, and that symbiont proliferation is usually maintained 'in check' by this. Occasionally, proliferation is observed alongside a widespread nodulation response and necrotic death of the host. The lack of necrotic death in *Nasonia* uninfected

with An-gfp indicates that *A. nasoniae* drives these occasional deaths, which occur when An-GFP is not suppressed by the nodulation response.

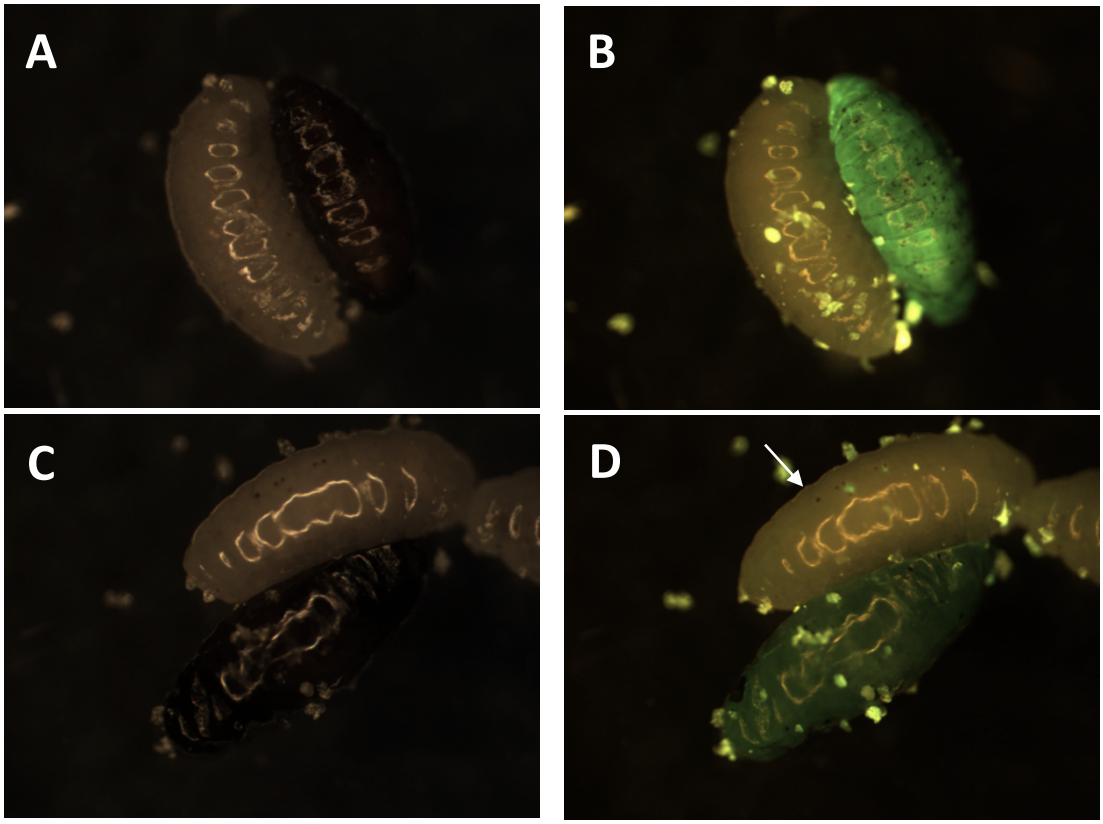



**Figure 6.3.** An-gfp infection in necrotic (black) and live (white) diapausing *Nasonia vitripennis*. Necrotic larvae fluoresce brightly with An-GFP. Necrotic larvae also show melanization and thus activation of the immune system (B). Live larvae also show melanin deposited at the sight of An-GFP infection indicated by the arrow (D).

### *6.3.3 Inducible expression of immune genes in N. vitripennis and N. giraulti diapause larvae*

Up-regulation of immune effectors is a common response to microbial invasion in insects. As a result, insects are highly resistant to microbial pathogens. In contrast, in the majority of symbioses, insects do not exhibit a systemic immune response against their co-evolving endosymbiotic partners. To investigate whether *N. vitripennis* and *N. giraulti* change their expression profiles during diapause in response to An-gfp, I measured expression of 5 genes (3 AMP, 1 signalling and 1 peptidoglycan receptor). Using RT-qPCR I show that 4 of the 5 candidate immune genes are downregulated in *N. vitripennis* diapause larvae infected with An-gfp, relative to uninfected individuals (Fig.6.4, MANOVA, $F_{1,9} = 25.9$, $P < 0.0005$). Nahymenoptaecin_2 was removed from this analysis due to technical error.

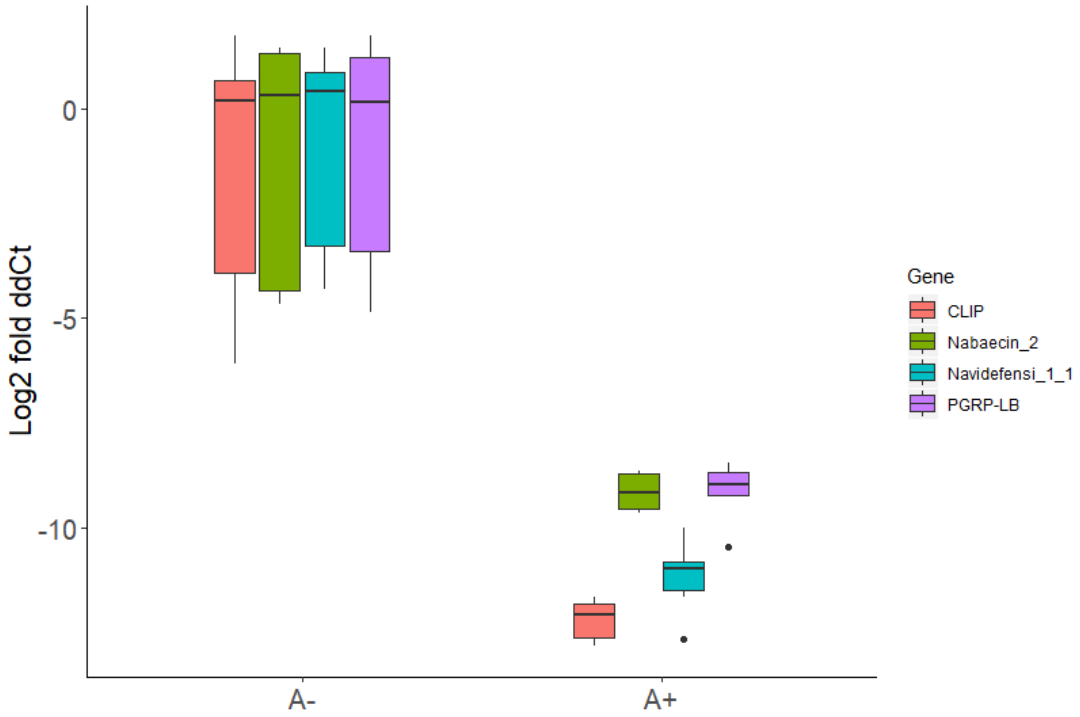



**Figure 6.4.** Log2fold gene expression relative to an invariant housekeeping gene in diapausing *Nasonia vitripennis* larvae, in the presence and absence of An-gfp infection (A+: An-gfp infected, A-: uninfected).

In contrast, expression of all 5 candidate immune genes appears to up-regulated in transinfected *N. giraulti* relative to uninfected control larvae. However, a lack of samples due to low diapause incidence in this species means that these data cannot be statistically analysed. Expression of the immune genes is initiated by An-gfp infection. In particular, CLIP signalling protein and PGRP-LB showed the greatest increase in expression. The differential expression patterns among immune genes represent just a small component of the wasp's immune response to An-gfp and may be part of a larger combinational strategy to control symbiont proliferation and virulence.

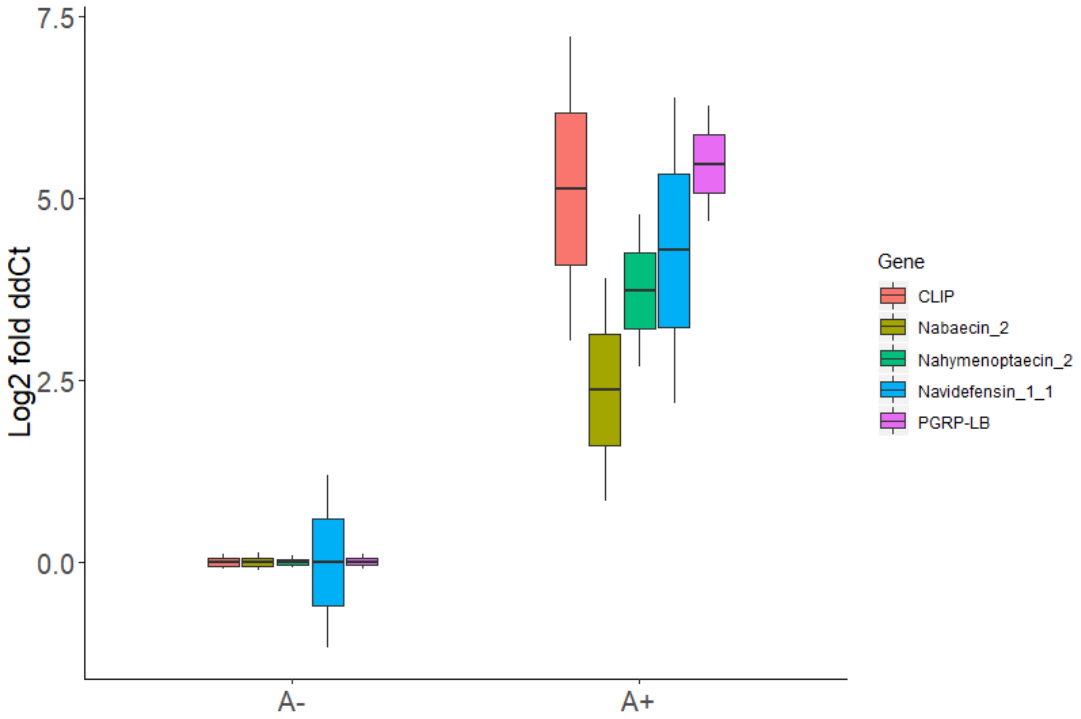



**Figure 6.5.** Log2fold gene expression relative to an invariant housekeeping gene in diapausing *Nasonia giraulti* larvae, in the presence and absence of novel An-gfp infection (A+: An-gfp infected, A-: uninfected).

## 6.4 Discussion

Temperate regions are subject to annual changes in photoperiod and temperature. Organisms living within these regions adjust their phenology accordingly so that they can be active, grow and reproduce when resources are most abundant. For insects that reside in temperate regions, diapause – the process through which insects survive harsh winter conditions – is an important component of insect population biology. However, the majority of studies on host-symbiont interactions neglect to consider interactions within diapause. The few studies that do exist demonstrate that certain endosymbiont strains can impose negative fitness costs to their hosts during and upon exit of diapause (Fraser *et al*, 2017; Kriesner *et al*, 2016).

Similar to Grillenberger *et al*, 2009, I observed interspecific variation for the response to diapause inducing cues. All *N. vitripennis* females that successfully parasitized their fly pupal hosts produced diapause offspring following 10 days of short photoperiod and low temperature, regardless of their *A. nasoniae* infection status. None of the 16 *A. nasoniae* infected *N. giraulti* females produced diapause offspring following 10 days of diapause inducing cues and of the 43 *A. nasoniae* uninfected females, only 5 produced diapause larvae. These data suggest there is intraspecific variation in the response to environmental cues but also, that there is a difference in the occurrence of diapause between the two species.

*A. nasoniae* does not affect the photoperiodic response to diapause cues in *N. vitripennis*. The proportion of *A. nasoniae*-infected and control females producing diapause offspring increased in a similar manner over time. After 10 days of diapause inducing cues, all 31 *A. nasoniae*-infected and 37 control females produced diapause offspring. However, *A. nasoniae* appears to have an adverse effect on *N. giraulti*'s fitness by reducing fecundity in the novel host. *A. nasoniae* infected *N. giraulti* parasitize fewer hosts and produce few or no offspring at day 22 of the diapause induction regime. However, due to the low incidence of diapause and thus small sample sizes, these data have limited power and require further investigation.

I observed that *A. nasoniae* presents an immune challenge to its native host, *N. vitripennis*, which responds by producing melanotic nodules. Melanin production is typically triggered upon wounding or bacterial infection in insects and aids wound healing and the sequestration of invading microbes. *N. vitripennis* keeps *A. nasoniae* infection 'in check' to prevent it from proliferating and becoming pathogenic. In the majority of cases, the host is able to contain *A. nasoniae* successfully to small confined infections but occasionally diapause larvae will die

from sepsis or symbiont over-proliferation. The necrotic phenotype was not observed in *N. girault*i; however, it would be premature to conclude the phenotype is not present, as the incidence of diapause was greatly reduced in this species. Thus, *A. nasoniae* presents as a pathogen during the larval diapause stage of *N. vitripennis* and occasionally has the ability to go 'rogue'. The necrotic phenotype is distinct among host-symbiont interactions.

Many parasitoid wasp species have overlapping host ranges. For example, in North America *N. vitripennis* and *N. girault*i often co-parasitize fly pupa (Grillenberger *et al*, 2009). *A. nasoniae* can transmit between sympatric parasitoid wasps, *N. vitripennis* and *Nasonia longicornis,* via multiparasitism in the wild (Balas *et al*, 1996). Duron *et al*, 2010 estimated that *N. vitripennis* has a 12% chance of transmitting *A. nasoniae* to *N. girault*i in nature, based on multiparasitism rates in the wild and per contact transmission rates in the laboratory. In fact, many wasp and filth fly species found to be harbouring *A. nasoniae* are distantly related from one another. Thus, the symbiont can be identified as a generalist that infects divergent species without causing known excess pathology (Taylor *et al*, 2011). The *N. vitripennis* and symbiont strains used in this study are of European origin. Although *N. girault*i, which is native to North America, may have been exposed to *A. nasoniae* from *N. vitripennis* in North America and thus its genetic background may be compatible with the symbiont, it will not have been exposed to the European strain that infects European *N. vitripennis*. Thus *N. girault*i in this study represents a naïve host species. Although comparative genomics of the North American and European *A. nasoniae* strains have not been carried out, the two strains are likely to be divergent such that the European strain is virulent in *N. girault*i.

Four immune genes that were previously shown to respond to bacterial immune challenge in adult *N. vitripennis* females (Sackton *et al*, 2013), are actively suppressed in *A. nasoniae* infected *N. vitripennis* diapausing larvae. A common feature of symbiosis is the coevolution of the host and symbiont. *N. vitripennis* and *A. nasoniae* have likely been associated for many thousands of years and thus the symbiont may have developed mechanisms that enable it to dampen the host's immune response so that the host does not perceive the symbiont as a bacterial invader and mount an attack. Over time, this may have turned into active suppression of AMP, signalling and receptor genes. Producing an immune response is costly and potentially costlier when metabolism is reduced during diapause. To avoid the costly induction of the immune system during diapause, *A. nasoniae* may have evolved to suppress non melanin-related genes involved in immunity.

As an aside, the internal control gene (EF1a) in uninfected *N. vitripennis* control diapause larvae displayed low expression levels compared to the *A. nasoniae* infected larvae. This may be a biological artefact of diapause but should be treated with light caution. To understand better the response of *N. vitripennis* diapause larvae to *A. nasoniae* infection, numerous temporal repeat assays should be conducted using different housekeeping genes as internal controls.

In *N. giraulti*, the 5 immune genes were not up-regulated relative to the internal control gene in uninfected larvae. However, in the presence of *A. nasoniae* infection, their expression was upregulated. There is clear induction of the immune system upon transinfection and in *N. giraulti*, *A. nasoniae* is recognized as an invading pathological agent. The immune response may become attenuated or even suppressed over time as the host continues to vertically transmit the bacteria to offspring.

In conclusion, I observe for the first time the interaction between *A. nasoniae* and diapause induction in the symbiont's natural host, *N. vitripennis*. I show that while *A. nasoniae* induces the production of melanin at sites of infection in *N. vitripennis*, other immune genes are suppressed. Upon transinfection, *A. nasoniae* is a pathological agent that reduces host fecundity and induces an immune response. Taken together, I observe strong interactions between a bacterial endosymbiont and its diapausing hosts, which do not support the general hypothesis that endosymbionts avoid or are undetected in their coevolved hosts.

# Chapter 7: General Discussion

*7.1 Host-shifts and host compatibility*

Bacteria have evolved diverse relationships with insects (Buchner, 1965). Mutualists increase the fitness of their host in a given environment (Jaenike *et al*, 2010b). In contrast, reproductive parasites provide little known benefit to their host. Whilst symbionts were traditionally treated as either beneficial or parasitic, many symbionts are both reproductive parasites and mutualists (Hedges *et al*, 2008; Teixeira *et al*, 2008; Xie *et al*, 2014). Their fitness effects and efficient transmission ensure their maintenance and spread in host populations. Typically, heritable microbes are vertically transmitted which aligns the interest of the symbiotic partners as the bacteria rely on the female host to be able to reproduce successfully for their own transmission.

Symbiont host shifts have, over evolutionary time, led to an increase in biological and ecological diversity through niche separation enabled by the traits they carry and reproductive isolating barriers they establish, accelerating the creation of novel species. Host shift events have led to endosymbionts becoming common in insects; for example, *Wolbachia* is estimated to be present in around 50% of arthropods (Weinert *et al*, 2015). Nevertheless, little is known about what determines a symbiont's host range. Endosymbionts form a key component of natural communities and an endosymbiont's host range must be determined by the intrinsic suitability of the novel host and its ecological suitability.

In this thesis, I address potential host and symbiont factors that determine host compatibility to novel endosymbiont infection in the genetically tractable *Spiroplasma/Drosophila* and *Arsenophonus/Nasonia* systems.

*7.2 How fast does compatibility evolve amongst closely related species?*

Susceptibility to infection is often determined by phylogeny, such that susceptibility to novel infection decreases with increasing phylogenetic distance (Longdon *et al*, 2014). This relationship has been observed in viral, bacterial and fungal pathogens and symbionts (Charleston and Robertson, 2002; Gilbert and Webb, 2007; de Vienne *et al*, 2009; Streicker *et al*, 2010; Longdon *et al*, 2011). The data presented in this thesis suggest that whilst host phylogeny may be an important indicator of host susceptibility, other host factors may be important in determing endosymbiont compatibility to novel hosts. I observed that host

compatibility to novel endosymbiont infection is an evolutionarily labile trait that is not solely a function of genetic distance. Species in the *Drosophila melanogaster* subgroup, that are equal in their genetic distance from the focal ancestral host of Spiroplasma, *Drosophila hydei,* differ in their ability to support the bacterial endosymbiont *Spiroplasma*. Of great interest is the difference between *D. simulans* and its island endemic, *D. sechellia*, which diverged from one another very recently. Compatibility is high (though insufficient to allow invasion) in *D. simulans*, but very low in *D. sechellia*, where pathology is high. Conversely, a feature that would drive the symbiont in host populations – protection against natural enemies- was higher in *D. melanogaster* than in *D. simulans*.

Whilst the results from the experiments conducted in the first 2 chapters of this thesis do not replicate the phylogenetic distance effect, it does not mean that phylogeny does not influence host suitability in the *Drosophila/Spiroplasma* system. To test whether phylogenetic distance does indeed drive host suitability, one could artificially transfer *Spiroplasma* from *D. hydei* into species most closely related to *D. hydei*, followed by a selection of species of increasing phylogenetic distance. In this thesis, I measure the fitness costs imposed by *Spiroplasma* in one clade of *Drosophila,* where the species tested are allof equal phylogenetic distance from the endosymbiont's ancestral host. Althought the data suggest that phylogony does not adequately explain all the variation observed in host compatibility, it does not necessarily identify clade differences. The difference in host compatibility between *D. simulans* and *D. sechellia* may be unique and not replicated elsewhere in the *melanogaster* species group of Drosopholids.

This distinction presented a promising case for examining the evolution of compatability. However, I observed that *Spiroplasma* evolves too quickly to assess differences amongst host species. Notably, the pathology phenotype was lost in laboratory passage. A slower evolving symbiont, with a lower mutational rate would be more appropriate for future investigatory work in this area. Nevertheless, the evolution of compatibility in the laboratory presents an opportunity for understanding the microbial factors that are important at the host interface.

### *7.3 Determinants of host compatibility, what could they be?*

#### *7.3.1 Infection density*

The different host responses to *Spiroplasma* infection may be due to variation in the nature of infection in each novel host species. Kageyama *et al*, 2006 observed that a non male-killing

*Spiroplasma* native to *D. hydei* reached higher titres in *D. melanogaster,* following artificial transfer. However, infection density was lower than a male-killing *Spiroplasma* strain, NSRO, following horizontal transfer from *D. nebulosa*. Kageyama *et al*, 2006 argued that the mechanism of male-killing could be *Spiroplasma* density, in other words, a density threshold of *Spiroplasma* must be reached in the host before it can cause male-killing. However, recent work by Harumotu and Lemaitre, 2018, indicates that male-killing is induced by expression of the toxin, Spaid, and thus infection density is not responsible for the parasitic phenotype. Furthermore, increased titre in novel hosts does not correspond to an induction of host immunity (Hutchence *et al*, 2011). In fact, induction of the Toll and Imd immune pathways increases *Spiroplasma* titre (Herren and Lemaitre, 2011). If *Spiroplasma* titre is responsible for the observed pathology in novel hosts, it must occur independently of host responses to infection.

Vertical transmission of *Spiroplasma* in novel hosts was variable (chapters 2 and 3). In *W+* and *W- D. simulans*, *Spiroplasma* did not induce pathology. However, transmission of the bacteria to the *W+* strain was extremely high (93.3%) and transmission to the *W-* strain was low (56.0%). High vertical transmission efficiency occurs when bacterial titre is also high thus one could predict that the *D. simulans* transinfected females transmitting at a rate of 93.3% have high *Spiroplasma* titre. Yet in this case, high titre in *D. simulans W+* does not correspond to pathology. Thus, titre is unlikely to be the causative agent of pathology and other, currently unknown, differences in host characteristics are likely to be responsible for the pathological phenotype. Furthermore, *Spiroplasma* titre undergoes a bottleneck each generation, when it is vertically transmitted. Infection density remains low during larval development and then increases during metamorphosis (Herren and Lemaitre, 2011). One could investigate pathology in the larval stage of transinfected individuals, to see if pathology is absent when bacterial titre is low.

### *7.3.2 Gut microbes*

Compatibility to novel symbiont infection occurs in the absence of coadaption with the symbiont. Phylogeny has been shown to be an important determinant of host compatibility (Longdon *et al*, 2011), but the ultimate source of the phylogenetic effect is unknown. Gut microbes and host immunity are posited to play a key role. In *Anopheles* mosquitoes, the gut microbiota presents a barrier to vertical transmission of novel *Wolbachia* infection. In one *Anopheles* species, *Wolbachia* causes blood meal induced mortality, which can be alleviated

upon the removal of one particular bacterium, *Asaia*, residing in the gut (Hughes *et al*, 2014). Past work indicated metabolic interference potentially exists between gut microbes and *Spiroplasma*, mediated through lipid. Thus, the interaction between *Spiroplasma* and gut microbes was tested. However, the data I present in chapter 4 suggest that gut microbes are unlikely to be a determinant of host compatibility to novel *Spiroplasma* infection amongst closely related *Drosophila* species.

Gut microbes may have a larger role in determining compatibility to novel *Arsenophonus nasoniae* infection in *Nasonia* wasps. To successfully transmit to the next generation, *A. nasoniae* is taken up through feeding, and must pass through the gut barrier, to the ovipositor. Thus, *A. nasoniae* will encounter other gut microbes and may affect microbe diversity and number. High titre gut microbes such as *A. nasoniae* may compete with other bacteria in the gut and this competition may have adverse fitness implications for the host. The implications to the host and the direct impacts of *A. nasoniae* on other gut microbes and *vice versa* are unknown. However, the direct competition that likely exists suggests this is a potentially important interaction.

### *7.3.3 Immunity*

As discussed earlier, host compatibility to receive a novel endosymbiont is determined by evolutionary processes that occur in the absence of the endosymbiont. Aside from gut microbes, other host factors such as immune system function may determine whether the host can support the nutritional requirements and facilitate the transmission of the bacteria.

Invading microbes typically encounter an insect's well-defined and effective innate immune system. Endosymbionts commonly represent an exception to this rule. We know that endosymbionts switch hosts. *Wolbachia* for example, is the most common endosymbiont in nature (Weiner *et al,* 2015). However, *Wolbachia* does not activate the immune system in native and novel insect hosts (Bourtzis *et al*, 2000; Chrostek *et al*, 2014). *Spiroplasma*, which does not have a cell wall, also does not trigger the immune response in native and novel *Drosophila* hosts (Hurst *et al*, 2003; Herren and Lemaitre, 2011; Hutchence *et al*, 2011). Thus, the lack of innate immune response suggests that immunity cannot explain the differences observed in host compatibility amongst different insect species, for these endosymbionts. This leaves us to question whether we are approaching host compatibility with too simplistic a view.

Herren *et al*, 2014, suggest that host lipid availability limits *Spiroplasma* proliferation. I could not recapitulate these results. However, the study does suggest that other physiological pathways in the host may influence and seek to control endosymbiont proliferation and virulence.

*7.4 Does A. nasoniae elicit an immune response in native and novel hosts?*

It is commonly believed that endosymbionts, as a rule, do not activate the immune system of their arthropod hosts. In cases where endosymbionts do activate the immune response, it is typically part of a coevolved control mechanism that is localised to specific organs which spatially restrict the bacteria within the host (Maire *et al*, 2018). If endosymbiont carriage is too costly in terms of the immune response, then either the host could perish, which is not in the interest of the microbe, or the host could remove the microbe via the immune response. Although these are all plausible hypotheses, the data in this thesis suggest that they are too simplistic.

*Arsenophonus nasoniae* retains many of the pathogenic characteristics of its free-living counterparts (Wilkes *et al*, 2010) and relies on infectious transmission in addition to vertical transmission, to sustain itself in host populations (Parratt *et al*, 2016). These mixed modes of transmission mean that the bacterium does not have to provide a benefit to its host, as it can rely solely on infectivity and reproductive parasitism to spread and be maintained in host populations. In fact, the genome of *A. nasoniae* resemble that of a pathogen, not a mutualistic endosymbiont. Here, I observe that the *Nasonia* immune system is strongly induced in *A. nasoniae* infected *N. vitripennis* and *N. giraulti*. The response includes activation of a vast array of AMPs, signalling and receptor molecules.

The reaction of native and non-native hosts differed. Interestingly, more immune genes are differentially expressed (largely upregulated) in the native host, *N. vitripennis* compared to the novel host, *N. giraulti*. However, the reliance of transcriptome analysis on the *N. vitripennis* model means that there may be *N. giraulti*-specific effectors, or effectors that were orthologous but divergent in sequence, that have been missed during the analysis process. Despite the induction of immunity, *A. nasoniae* is able to persist in natural populations. It is even able to persist through diapause in the face of a nodulation response. Innate immunity is therefore not a determinant of host compatibility to *A. nasoniae* infection.

In addition to the upregulation of the immune response, pathways involved in oxidoreducatsase and iron- and heme-binding are upregulated in *N. vitripennis*. The *A. nasoniae* genome encodes genes related to iron sequestration (Wilkes *et al*, 2010). Together with the response of *N. vitripennis* to *A. nasoniae* infection, there is evidence to suggest that *A. nasoniae* sequesters iron from *N. vitripennis*. Iron depletion in the host may have negative fitness consequences and thus it is in the host's interest to limit the amount of iron uptake by the bacteria, and this may be an important component of host-mediated control of symbiont titre. The data suggest that *N. vitripennis* achieves this control via the upregulation of oxidoreductase activities and iron-binding. This observation appears to be an adaptive response in *N. vitripennis* as an upregulation of these pathways is not observed following lateral transfer to the novel host, *N. giraulti*.

*A. nasoniae* can be maintained in *N. giraulti* wasps under laboratory conditions. It is possible that differences in cellular machinery and receptors will alter the capacity to undertake a natural host shift into this species, for instance if *A. nasoniae* is not able to access iron in *N. giraulti*. This incompatability may explain why infection is rarely reported in the wild. An alternative hypothesis is that *A. nasoniae* binds iron in *N. giraulti* but the host does not respond with the same adaptive response as *N. vitripennis*. Instead, *A. nasoniae* may display uncontrolled proliferation and cause pathology. *A. nasoniae* titres should be assessed and compared in *N. vitripennis* and *N. giraulti* adult females.

In temperature regions most insect species enter a quiescent period or state of metabolic and developmental arrest during winter, called diapause (Tauber *et al*, 1986). Insects will remain in this state of physiological dormancy until favourable conditions return. Studies typically ignore 'natural' life history traits when investigating host-symbiont interactions, including diapause. Typically, symbiosis is studied in insects that are maintained in laboratory settings, at constant long-day photoperiod and constant temperature. How symbionts, which continue to require nutrients during diapause, affect their insect hosts during this important life stage is not known, and is particularly important as these periods are often stressful for the host.

Importantly, *N. vitripennis* perceives the cue to enter diapause in adult females (Skinner, 1985). Females pass the cue to their offspring, which enter developmental arrest as larvae. Similar to diapause cues, endosymbionts are maternally transmitted and thus environmental factors that affect the information passed to the female's offspring may also influence transmission and maintenance of *A. nasoniae*. During diapause, wasp development and

metabolism are arrested. The ability to control iron sequestration and thus symbiont titre and virulence may be affected. The data here suggest that diapause larvae are less able to regulate symbiont proliferation as necrosis can be observed, although this phenotype is rare.

*N. girault*i is more refractory to entering diapause than *N. vitripennis* (Grillenberger *et al*, 2009). Given *N. girault*i must experience winter in North America, the cause for this inhibition to entering diapause is unknown. Perhaps diapause is more costly in *N. girault*i. Here, I present evidence that *A. nasoniae* is pathogenic in *N. girault*i adults and diapause larvae. Immune genes are upregulated and adult females that receive the diapause cues produce fewer offspring relative to uninfected females.

### *7.5 Conclusions*

The two symbioses I have chosen to work on have endosymbionts with contrasting biology and behaviour. *Spiroplasma* is a rapidly mutating and evolving mutualist. Following lateral transfer, *Spiroplasma* can cause pathology. However, the pathology is not associated with an upregulation of an immune response (Hutchence *et al*, 2011). *Spiroplasma*'s protective phenotype is not transferred upon lateral transfer, although weak protection can be observed in novel hosts whose genetic background has previously been exposed to *Spiroplasma*. *Spiroplasma* titres appear to be incredibly stable despite perturbances in microbiota.

In contrast, *A. nasoniae* molecular evolution occurs at a much slower rate (Crystal Frost, pers. comm.) and infection with this symbiont provides no known benefit to the host. Furthermore *A. nasoniae* induces upregulation of nearly 2000 genes in its native host and 200 in a closely related but novel wasp host. Microbiota may play a role in the success of *A. nasoniae* in novel hosts as the bacteria has to cross the gut barrier and thus will come into contact with other microbes in the gut. It appears that *N. vitripennis* harnesses oxidoreductase activity and iron binding to control *A. nasoniae* proliferation and virulence, and may provide an explanation as to why *A. nasoniae* is able to successfully persist in *N. vitripennis* in the wild. The *A. nasoniae- N. vitripennis* symbiosis is unique in that the endosymbiont displays mixed modes of transmission. These mixed modes allow *A. nasoniae* to remain pathogen-like instead of evolving to attenuate any negative fitness costs it imposes on the host.

### *7.6 Future perspectives*

In this thesis, I investigate two very different bacterial endosymbionts. *Spiroplasma* adapts to novel environments very quickly (Nakayama *et al*, 2015) and is able to spontaneously mutate in stock culture. This rapid mutational rate and evolvability might explain how *Spiroplasma* is able to persist in nature. Whilst steps are being taken to sequence and analyse the *Spiroplasma* genome, assignment of the term 'hyper-mutator' should only be given following confirmation of a selection experiment. Future work should involve transinfecting *Spiroplasma* from a single female *D. hydei*, into *D. melanogaster* and *D. simulans*. Multiple, independent lines should be created following transinfection and after selecting for *Spiroplasma* infection for 20 generations in each line, in each species, *Spiroplasma* should be sequenced. The substitution rate can then be quantified.

Why *Spiroplasma* has an elevated mutation rate is also a question that would be useful to resolve. Other endosymbionts including *Wolbachia* and *Arsenophonus* evolve at a much slower rate than *Spiroplasma*. A plausible hypothesis is that *Spiroplasma* lacks functional DNA repair mechanisms. The DNA repair system could have been lost during the process of genome reduction and thus mutations that occur during replication may not be corrected. This lack of proof reading may lead to rapid loss and gain of functions, which have been anecdotally noted by myself and members of the Lemaitre group, as well as evidenced in past laboratory mutations and changes of phenotype on passage. Thus, whilst analysing the genome of *Spiroplasma*, one should look for presence/absence of functional DNA repair mechanisms.

As is the case with most tripartite systems, the gut microbiota are often neglected. In the case of *A. nasoniae*, where the bacteria are ingested by the host during the larval stage and passed through the gut and through the gut barrier to the ovipositor, the gut microbiota should be taken into account in future studies. A high titre microbe such as *A. nasoniae* may perturb the gut microbiota and cause dysbiosis in the gut, which may in turn impose negative fitness costs in the host. Furthermore, *Nasonia* wasps undergo metamorphosis, and just before metamorphosis, they are able to enter a facultative state of diapause. During metamorphosis the wasps must retain its gut microbes, but also *A. nasoniae* must ensure that it survives too. Thus the interaction between the endosymbiont and gut microbiota during this obligatory anatomical re-organisation should be investigated, particularly now, when we know that *A. nasoniae* is pathogenic.

A knockout Tn5 mutant library now exists for *A. nasoniae*. The transcriptomics work described in this thesis should be consolidated with investigation of responses in the *A.*

*nasoniae* knockouts. In particular, the importance of iron in virulence should be investigated using *A. nasoniae* loss of function mutants lacking siderophores and iron transporters. One might expect that the absence of iron sequestration by *A. nasoniae* might result in the disappearance of the upregulation of oxidoreductase activity. Furthermore, the inability to sequester iron may result in the failure of *A. nasoniae* to infect and persist in the host.

Methylation patterns should be assessed in *A. nasoniae* infected *N. vitripennis* to see if a generalised methylation response to infection is responsible for the widespread changes in gene expression. This can easily be achieve using bisulfite-sequencing of *A. nasoniae* infected adult females, or experimentally analysed via 5-aza-dC treatment and subsequent RT qPCR or RNAseq analysis of uninfected females.

In summary, there remain many unanswered questions in host shift biology. Evidence is presented that host compatibility to symbionts is labile, and more subtle than the caricature of increasing genetic distance relates to lower compatibility. Symbiont compatibility was also observed to evolve as a symbiont trait, without selection, during lab passage. Future work should address more widely the role of gut microbes as a host factor when investigating interactions between hosts and their endosymbionts, particularly for endosymbionts that have to cross the gut barrier. Further, the thesis indicates that the prevailing wisdom that heritable microbes do not interface with the host immune system, and thus host immunity is not important in compatibility, is not always correct; this interface is highly active in the *A. nasoniae* – host interaction. The relationship between an endosymbiont and its host's immune system appears to be complex and there is no one rule that encompasses all systems.

# Chapter 8: References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