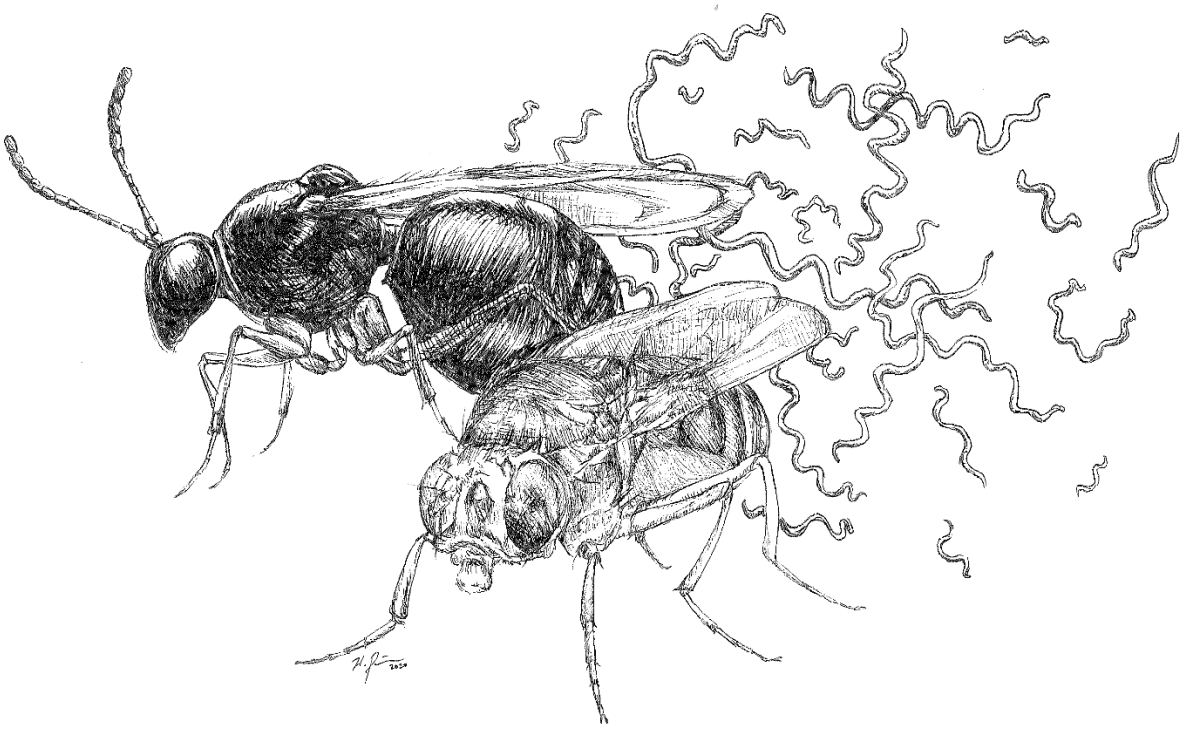
UNIVERSITY OF LIVERPOOL

The evolutionary ecology of a defensive symbiont in a host-parasitoid interaction



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

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- November 2021 -

Abstract

All insects are subject to attack from natural enemies. Classically, the outcome of natural enemy attack was considered to be dependent on factors encoded within the host and parasite genomes. However, it is now widely appreciated that defensive bacterial symbionts residing within the host can dictate the outcome of natural enemy attack in insects. In this thesis, I utilise the *Spiroplasma-Drosophila melanogaster* system to evaluate the factors which determine the outcome of *Spiroplasma*-mediated defence against *Leptopilina* parasitoid wasps. I first examine whether the strength of protection conferred by *Spiroplasma* varies with the strain of *Leptopilina heterotoma* wasp attacking the host. This experiment was conducted in the presence and absence of ethanol, an environmental factor thought to impact the outcome of parasitism. The strength of protection conferred by *Spiroplasma* was observed to strongly depend on the strain of *L. heterotoma*. However, contrary to previous research, environmental ethanol did not substantially aid survival against parasitoid wasps, although it did contribute to a composite measure of protection which included the chance of fly surviving attack and the relative fertility of survivors. I then examine whether the strain of *Spiroplasma* alters the strength of protection conferred against *Leptopilina* wasps. I compared the response of flies carrying two strains of male-killing *Spiroplasma* to challenge by two strains of *L. boulardi* and two strains of *L. heterotoma* wasp. There was no evidence to suggest that the strength of protection conferred was dependent on the strain of *Spiroplasma* in any case. Finally, I consider the impact of the environment, examining the effect of temperature on the protection phenotype. I observed that *Spiroplasma*-mediated protection was weaker at cooler temperatures. However, this effect was only observed when flies were subject to cooler temperatures before, but not during or after wasp attack, suggesting that the thermal history of the fly determines the efficiency of *Spiroplasma*-mediated protection in this system. This effect appeared to be mediated at least partially through temperature effects on host *Spiroplasma* titre. Collectively, these results provide a more general understanding of defensive symbiont evolutionary ecology beyond the well studied aphid systems and demonstrate that the outcome of symbiont-mediated protection is much more complex than the mere presence or absence of the defensive symbiont. The results highlight the importance of host, symbiont and natural enemy genetics as well as the environment when considering the dynamics of a defensive symbiont in natural populations.

Acknowledgements

First and foremost, I would like to thank my supervisor, Greg Hurst. Ever since I first walked into your office as an undergraduate, you have constantly supported me and truly believed in my abilities as a researcher – often more so than myself. My confidence as a person has drastically improved over the past four years and I believe this is mainly due to your constant reassurance and encouragement albeit, in a lot of cases, via snack bribes. Without a doubt, my PhD would not have been as enjoyable and straightforward without you as a supervisor for which I am incredibly grateful.

Thanks to all past and present Hurst lab group members for their helpful discussions about my research and assistance around the lab. Specifically, Jo Griffin and Ewa Chrostek – from trying to understand how the numbers on a pipette work to finally mastering qPCR, you both provided me with invaluable knowledge and guidance that greatly helped my lab work.

Thank you to my fellow micro-adventurers, Louise Reynolds and Olga Pawlowska. Our tea, gossip and art meetings were about as adventurous as we got after that incredibly long walk and slightly uncomfortable camp on Formby beach.

Of course, my PhD journey would not have been as enjoyable without every single PhD student passing through the DEEB offices. You all provided me with constant distraction and offered extremely helpful discussions about ~~science~~ Love Island.

I would also like to thank everyone who I met during my JSPS fellowship in Japan. A personal thank you to Kazuki Miura, Wakako Funatski, Taka Miyatake and Daisuke Kageyama. Your kindness and overwhelming hospitality made my stay in Japan truly unforgettable. Only in Japan could you be eating a raw egg on a hill side one minute, praying to the salamander gods the next, and ending the day with a trip to a shrimp farm. Arigato gozaimashita!

Thank you to my cheerleaders and best friends, Jenny Hill and Nicole Phillips. You have always taken an interest in my 'fly stuff' and have been constantly there to listen and support me throughout the highs and lows – I really am very grateful.

I'd also like to thank my fabulous housemates over the past four years, Jess Humphreys, Lorna Brooks and Issy Makin. Your nursing stories always put life into perspective and our garden prosecco afternoons were always much needed. Not forgetting the one and only, Greta. The sound of your screeching meows in the morning will never leave me, nor will the chew holes in my laptop bag.

Finally, I'd like to thank Vinnie. Despite an office move and a move back to Glasgow, ever since I was placed opposite you at the start of my PhD, I just can't seem to get rid of you... You constantly support and encourage me in everything I do and your silliness never fails to cheer me up on a dull day. You really are a big ray of sunshine in my life.

I dedicate this thesis to my parents, Sue and Craig for always encouraging and supporting my curiosity in the natural world.

I believe I've come a long way since the days of surprise repeated bedtime readings of 'The Very Hungry Caterpillar', stealing water snails from the garden centre, and holding a large variety of innocent invertebrates captive in the Wendy House…

"The Very Hungry Caterpillar story is about hope. You, like the little caterpillar, will grow up, unfold your wings and fly off into the future." – Eric Carle

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Chapter 1

Introduction

1.1 THE EVOLUTIONARY ECOLOGY OF *DROSOPHILA*-PARASITOID INTERACTIONS

1.1.1 *Natural enemies drive host ecological and evolutionary dynamics*

Natural enemies – parasites, pathogens and predators – pose a constant threat of morbidity and mortality to all organisms. As a consequence of their tightly linked antagonistic interactions, natural enemies can have significant impacts on the ecological and evolutionary dynamics of their hosts (Anderson and May, 1978; Holt and Lawton, 1994; Hudson *et al*,. 1998). Natural enemies can impose strong selective forces on hosts to evade attack – leading to the evolution of a wide range of defence strategies. In turn, natural enemies may evolve counter defence strategies to overcome the defence mechanisms of the host, leading to cyclical coevolutionary dynamics between the host and natural enemy (Decaestecker *et al*., 2007; Gomez and Buckling, 2011). The course of such coevolutionary dynamics will depend on many factors, including the extent of genetic variation in traits concerning host resistance and parasite virulence, the costs of defence or attack, the degree of specificity between the host and parasite genotypes, and environmental factors which may influence the outcome of attack.

1.1.2 *Parasitoid wasps represent an important natural enemy of insects*

It is estimated that as many as 350,000 species of parasitoid wasps may exist on earth (Gaston, 1991). Parasitoid wasps lay their eggs on (ectoparasitoids) or in (endoparasitoids) other arthropods, typically insects (Godfray, 1994; Quicke, 1997). After hatching, the parasitoid larvae develop, either singly or gregariously, and feed on their host. Successful parasitoid attack results in the death of the host unless the host can mount a successful immune encapsulation response or defend itself by other means. Initial interest in parasitoid wasps stemmed from their use as biological control agents

in agricultural systems. Their ability to cause heavy host mortality made them a highly suitable tool to control populations of crop pest insects (Waage and Hassell, 1982; Gerling *et al.,* 2001; Boivin *et al.,* 2012). As such, research primarily focussed on aspects of parasitoid biology relating to their use in biological control, such as population dynamics, host detection and acceptance behaviour, and the evolution of reproductive strategies (Vinson and Iwantsch, 1980; King, 1987; van Alphen and Jervis, 1996; Godfray, 1994; Van Alphen and Visser, 1990).

However, over the last few decades the importance of host-parasitoid species as a model system to understand key questions in evolutionary ecology has become increasingly evident. Due to the ever-increasing importance of *Drosophila* as a model system, the majority of research concerning host-parasitoid interactions to date has been developed in the fruit fly, *Drosophila* and its suite of endoparasitoid wasps (Fellowes and Godfray, 2000; Prévost, 2009). At least 42 hymenopterous parasite species are known to infect *Drosophila* (Carton *et al.,* 1986), and the most well studied of these are the larval endoparasitoids of the genus *Leptopilina* and *Asobara*. These wasps represent pervasive parasites of *Drosophila*, commonly laying a single egg inside a host larva. If hosts are unable to successfully defend against attack, the parasitoid feeds on and eventually kills the host. In natural populations, parasitism rates have been shown to vary greatly over different localities, with average attack rates estimated at between 5-40% of flies present (reviewed in Fleury *et al.,* 2009). However, parasitism rates have been observed to exceed 90% in some localities demonstrating that parasitic wasps can exert strong selective pressures on *Drosophila* (Fleury *et al.,* 2004).

1.1.3 *Variation in host resistance and parasitoid virulence exists*

A key determinant of host-parasite evolutionary ecology is variation in host susceptibility to attack, parasite virulence, and also the importance of the interaction of host and parasite genotypes in determining this outcome. Within the *Drosophila*-wasp

interaction, for instance, there is considerable variation in the ability of *Drosophila* to survive wasp attack. This has been demonstrated across several experimental and field assays. Within-population genetic variation in resistance has been observed through comparison of variation between isofemales and through artificial selection experiments (McGonigle *et al.,* 2017; Fellowes *et al.,* 1998; Kraaijeveld and Godfrey, 1997; Hughes and Sokolowski, 1996). Intense selection by wasp attack performed on *D. melanogaster* led to an increase in resistance from 5% to 60% against *A. tabida* and from 0.5% to 45% against *L. boulardi* in only 5 generations (Fellowes *et al.,* 1998; Kraaijeveld and Godfrey, 1997). Variation in parasitoid virulence (ability to overcome *Drosophila* defences) also exists. For instance, there is considerable geographic variation in the ability of *A. tabida* to prevent encapsulation by *D. melanogaster* (Kraaijeveld and van Alphen, 1995), with strains from southern Europe more virulent than those from Northwestern Europe. Variation is also observed for sympatric populations of *L. boulardi* and *D. melanogaster* across its entire geographic range (Dupas *et al.,* 2003).

The genetic basis of parasitic wasp resistance is well described in *Drosophila*. Initially thought to have a complex genetic basis, the ability of *Drosophila* to encapsulate parasitic wasps has been found to be relatively simple, commonly determined by one diallelic locus (Takigahira *et al.,* 2015; Dubuffet *et al.,* 2007; Benassi *et al.,* 1998; Orr and Irving, 1997; Carton *et al.,* 1992). Considering *D. melanogaster* resistance to *L. boulardi*, crosses between resistant and susceptible isogenic lines of flies identified a single major locus, *Rlb* (resistance to *Leptopilina boulardi*) responsible for resistance/susceptibility in this system, with the resistant allele dominant to the susceptible one (Carton *et al.,* 1992). Further characterisation of *D. melanogaster* resistance to *L. boulardi* has focussed on identifying the location of the resistance allele and the genes responsible (Hita *et al.,* 1999, 2006; Poirie *et al.,* 2000). Interestingly, the susceptible fly strain is unable to encapsulate eggs of *L. boulardi,* but is able to encapsulate the eggs of another parasitoid of *Drosophila*, *A. tabida,* demonstrating a degree of specificity within the system, with the

resistant strain able to recognise and encapsulate *L. boulardi* eggs only (Carton and Nappi, 1997; Vass *et al.,* 1993).

Indeed, the outcome of parasitoid wasp attack is not solely determined by host resistance, but rather determined also by its interaction with parasitoid virulence. The genetic bases of interactions between two different lines of *L. boulardi* (ISm and ISy) and two species of *Drosophila* (*D. melanogaster* and *Drosophila yakuba*) were investigated (Dubuffet *et al.,* 2007). Against the ISm *L. boulardi* line, eggs were rarely encapsulated in *D. melanogaster* but always encapsulated in *D. yakuba* lines. In contrast, against the ISy *L. boulardi* line, parasitism success can occur in both *D. melanogaster* and *D. yakuba* depending on the resistance status of the fly strain. Thus, the outcome of attack by the ISm line is dependent on the species of host, whereas the ISy line is dependent on host genotype, demonstrating different levels of specificity within this system. The results of this study demonstrate that the outcome of parasitoid wasp attack is a result of complex interactions between host and parasitoid, species and strains.

1.1.4 Drosophila *defence comes at a cost*

Costs of defence are considered central to the maintenance of genetic variation in resistance within a population and host-parasite coevolution (Agrawal and Lively, 2002). Defence costs can be broadly categorised into two types: inducible and constitutive (Carton *et al.,* 2005; Kraaijeveld *et al.,* 2002). Inducible costs are costs caused by mounting an immune response following parasite attack. Constitutive costs are the costs of forming and maintaining the defensive apparatus irrespective of whether an individual is attacked or not.

Inducible costs are seen in *Drosophila* in the form of costs that are a consequence of parasitoid attack, representing a trade-off between defence and other components of fitness. For instance, *D. melanogaster* which successfully survived attack by *L. boulardi* and *A. tabida* (demonstrated by the presence of a capsule as adult) were smaller in size

(Carton and David, 1983; Fellowes *et al.,* 1999b). Additionally, females which had successfully defended themselves were found to be less fecund compared to un-attacked controls (Carton and David, 1983; Fellowes *et al.,* 1999b).

Constitutive costs are associated with the maintenance of defence mechanisms, and these are evident in *Drosophila*. Populations selected for high resistance to *A. tabida* and *L. boulardi* have reduced survival in a high-competition food environment compared to control populations with low resistance (McGonigle *et al.,* 2017; Fellowes *et al.,* 1998; Kraaijeveld and Godfrey, 1997). Thus, there is a resource trade-off between defence and other components of fitness in *Drosophila*. Lower competitive ability was found to be associated with reduced rates of larval feeding (Fellowes *et al.,* 1999a) and it was later shown that populations selected for high resistance to parasitoids had an increased (almost double) number of circulating haemocytes which are essential for the encapsulation response (Kraaijeveld *et al.,* 2001; McGonigle *et al.,* 2017). This was consistent with previous findings that *A. tabida* resistant *Drosophila simulans* lines had more circulating haemocytes than susceptible lines (Eslin and Prevost, 1996). However, the mechanism by which different species of *Drosophila* increased their number of haemocytes was found to be different. In *D. melanogaster*, it was found that sessile haemocytes were transported into circulation while *D. simulans* and *Drosophila mauritiana*, increased the production of haemocytes (McGonigle *et al.,* 2017).

1.1.5 *Environmental factors can affect the outcome of a* Drosophila-*parasitoid interaction*

Beyond genetic factors, the influence of environmental factors on the outcome of host-parasite interactions has also been considered. Temperature is one environmental factor which has been shown to impact the outcome of parasitoid attack in *Drosophila*. In general, most studies have focussed on the effect of rearing temperature after exposure to parasitoids. For instance, it has been shown that the ability of *Drosophila* to

encapsulate parasitoid eggs increased when they were reared at higher temperatures (Fellowes *et al.,* 1999c; Kraaijeveld and van der Wel, 1994). Temperature can also modify the quality of the host. The survival of *L. heterotoma* in hosts reared at low temperature (14°C) after parasitisation was found to be low regardless of host suitability at other temperatures. However, at higher temperatures (26°C) host suitability depended on the species of host and strain of wasp (Ris *et al.,* 2004). Acknowledging that constant temperature experiments do not necessarily reflect reality in the natural environment, one study also considered the effect of fluctuating developmental temperature on *Drosophila*-parasitoid interactions (Delava *et al.,* 2016). It was found that fluctuating temperatures did not affect the rate of encapsulation by *Drosophila* compared to the constant temperature regime. However, the success of *L. boulardi* parasitism was significantly higher in the fluctuating temperature regimes compared to the constant temperature regime despite infestation rate being unaffected. Collectively, these studies highlight how the effect of temperature on *Drosophila*-parasitoid interactions is highly complex and also depends on the species/strains of host and parasitoid.

Host diet is a further environmental factor that can impact a host's ability to defend against infection. Previous studies have considered the role of dietary yeast on the cellular immune response of *D. melanogaster* against the parasitoid, *L. boulardi*. It was observed that larvae which were moved into food deprived of yeast immediately after exposure to wasps, encapsulated a lower percentage of wasp eggs compared to larvae which were not deprived of yeast. However, when larvae were moved onto food deprived of yeast following 24 hours after exposure to wasps there was no difference in the percentage encapsulated (Vass and Nappi, 1998). In addition to yeast deprivation, the species of yeast can also affect the encapsulation ability of *D. melanogaster* against the parasitoid wasp, *A. tabida* (Anagnostou *et al.,* 2010). It was found that the encapsulation ability of flies was higher in larvae reared on a diet of *Kluyveromyces lactis* and *Metschnikowia pulcherrima* yeast species compared to larvae reared on a diet of

Saccharomyces cerevisiae, *Cryptococcus albidus* and *Pichia toletana* yeast species. The underlying reason for these differences is unknown, but it was not due to differential effects of yeast species on total circulating haemocyte numbers.

1.2 DEFENSIVE SYMBIONTS OF INSECTS

1.2.1 *Most insects harbour maternally inherited symbionts*

Insects are commonly infected with microbial symbionts. Many of these are vertically transmitted (usually maternally), acting as a heritable source of genetic variation alongside the nuclear genome (Cosmides and Tooby, 1981). Traditionally, microbial symbionts of insects fall into two categories: obligate/primary and facultative/secondary. Obligate symbionts predominantly provide nutritional functions for their hosts, allowing insects to utilise food sources which they would not otherwise be able to process. For instance, the obligate symbiont of aphids, *Buchnera aphidicola* synthesises essential amino acids, allowing the host to feed on protein-deficient plant sap (Douglas, 1998). In contrast, facultative symbionts have a much wider range of effects on their hosts ranging from mutualistic to parasitic (although in some cases these are not mutually exclusive) (Xie *et al.,* 2014).

1.2.2 *Facultative symbionts can protect hosts against natural enemies*

It is estimated that over half of all insects carry maternally inherited facultative symbionts (Zug and Hammerstein, 2012; Duron and Hurst, 2013; Weinert *et al.,* 2015). Maternally inherited facultative symbionts are perhaps most well known for their ability to manipulate host reproduction, biasing their own transmission into the next generation via mechanisms such as male-killing, parthenogenesis, cytoplasmic incompatibility and feminization (reviewed in Engelstädter and Hurst, 2009). However, in addition to their parasitic properties, many facultative symbionts can also be mutualistic. The strong association between host and symbiont fitness has led to a range

of beneficial impacts of symbionts on their hosts. For instance, maternally inherited symbionts can protect against heat shock (Chen *et al.,* 2000; Russell and Moran, 2006; Brumin *et al.,* 2011; Heyworth and Ferrari, 2016) and provide protection against natural enemies such as parasitic wasps, nematodes and viruses (Xie *et al.,* 2010; Jaenike *et al.,* 2010; Teixeira *et al.,* 2008; Hedges *et al.,* 2008).

Arguably, protection against natural enemies is the most rapidly evolving areas of symbiosis research. Examples of symbiont-mediated protection in insects was only first described in 2003 in aphids (Oliver *et al.,* 2003), but has since been described across a wide range of taxa against a diverse number of natural enemies (Table 1.1). The drive to study defensive symbioses arises in part from their potential application in natural populations. For instance, in 2008, the maternally inherited symbiont, *Wolbachia,* was demonstrated to reduce the replication of ssRNA viruses within its native *Drosophila* host (Teixeira *et al.,* 2008; Hedges *et al.,* 2008). The ability of *Wolbachia* to reduce viral replication was maintained after artificial transfer into mosquito hosts (Moreira *et al.,* 2009). These findings have resulted in *Wolbachia* being deployed in natural mosquito populations to weaken vector competence and reduce the spread of Dengue and Zika virus across the world (O'Neill *et al.,* 2018; Utarini *et al.,* 2021). Understanding how defensive symbionts contribute to host-parasite evolution is fundamental for predicting the long-term efficacy of symbiont-mediated defence applications.

Table 1.1: Summary of empirical studies finding evidence for symbiont-mediated protection against natural enemies in insects.

Host	Defensive symbiont	Natural enemy	Protection mechanism	References
Fruit fly	*Spiroplasma*	Parasitoids; Nematodes	Exploitative competition, Interference competition	(Xie *et al.,* 2010; Ballinger and Perlman, 2017; Jaenike *et al.,* 2010; Paredes *et al.,* 2016; Hamilton *et al.,* 2016)
	Wolbachia	Arboviruses; Fungi	Immune mediation; Exploitative competition	(Teixeira *et al.,* 2008; Hedges *et al.,* 2008; Caragata *et al.,* 2013; Panteleev *et al.,* 2007)
Aphid	*Hamiltonella defensa; Serratia symbiotica; Regiella insecticola; Enterobacteriaceae*	Parasitoids	Interference competition	(Oliver *et al.,* 2003, 2009; Vorburger *et al.,* 2010; Leybourne *et al.,* 2020)
	Regiella insecticola; Spiroplasma; Rickettsia; Rickettsiella; Enterobacteriaceae	Fungi	Unknown	(Lukasik *et al.,* 2013; Scarborough *et al.,* 2005; Parker *et al.,* 2013)
Mosquito	*Wolbachia*	Arboviruses; Nematodes; Protozoans	Immune mediation; Exploitative competition	(Zélé *et al.,* 2012; Kambris *et al.,* 2009; Glaser and Meola, 2010; Moreira *et al.,* 2009)
Beewolf	*Streptomyces sp.*	Fungi	Interference competition	(Kaltenpoth *et al.,* 2005; Kroiss *et al.,* 2010)

Darkling beetle	*Burkholderia gladioli*	Bacteria	Interference competition	(Flórez *et al.,* 2018; Flórez and Kaltenpoth, 2017)
Psyllid	*Profftella armaturum*	Unknown	Interference competition	(Nakabachi *et al.,* 2013)
Whitefly	*Rickettsia*	Bacteria	Unknown	(Hendry *et al.,* 2014)
Subterrainian termite	*Streptomyces sp.*	Fungi	Interference competition	(Chouvenc *et al.,* 2013)
Dampwood termite	*Unknown*	Fungi	Interference competition	(Rosengaus *et al.,* 2014)
Bumble bee	*Gilliamella*	Protozoa	Unknown	(Cariveau *et al.,* 2014)
Honey bee	*Bombella apis*	Fungi	Interference competition	(Miller *et al.,* 2021)
Leaf-cutting ant	*Actinomycete sp.*	Fungi	Interference competition	(Barke *et al.,* 2010; Mattoso *et al.,* 2012)
Leaf-rolling weevil	*Penicillium herquei*	Unknown bacteria	Interference competition	(Wang *et al.,* 2015)

1.3 THE IMPORTANCE OF DEFENSIVE SYMBIONTS IN HOST-PARASITE INTERACTIONS

1.3.1 *Defensive symbionts can influence key determinants of host-parasite coevolution*

Host-parasite research has typically been considered a pairwise interaction between both host and parasite genetic variants. However, the impacts of symbionts on host and parasite fitness, have led to the belief that defensive symbionts have the ability to strongly influence host-parasite evolutionary dynamics. Indeed, since their discovery, research is continuing to reveal ways in which defensive symbionts can contribute to host-parasite interactions through affecting key determinants of host-parasite coevolution e.g. genetic variation, specificity and costs (reviewed in Vorburger and Perlman, 2018).

First, in order to evolve in response to a parasite, hosts must possess heritable variation for susceptibility to defence. Across many host-parasite systems, there is substantial variation in susceptibility to parasites among hosts (Kraaijeveld and van Alphen, 1995; Ebert *et al*, 1998; Carius *et al.,* 2001; Ferrari *et al.,* 2001; Dubuffet *et al.,* 2007). However, many of these studies were naïve to the contribution of heritable defensive symbionts. It has been shown that defensive symbionts can contribute substantially to the variation in susceptibility observed among hosts. A clear example is provided by the pea aphid. An early study, examining the resistance of pea aphids to parasitoids revealed substantial variation among aphid clones, ranging from complete resistance to high susceptibility (Henter and Via, 1995). However, it later became apparent that much of the resistance in pea aphids was due to variation in the presence/absence of the defensive symbionts, *H. defensa* and *Serratia symbiotica* rather than underlying genetic variation among aphid clones (Oliver *et al.,* 2003; Ferrari *et al.,* 2004; Oliver *et al.,* 2005; Bensadia *et al.,* 2006; Nyabuga *et al.,* 2010). Furthermore, it was shown that selection pressures from natural

enemies can rapidly select for inherited defensive symbionts in populations of the pea aphid (Oliver *et al.,* 2008).

The defensive symbiont, *Spiroplasma* is similar to this. In *Drosophila*, *Spiroplasma* resistance against parasitoids is greater than the observed variation in endogenous resistance in uninfected fly lines (Xie *et al.,* 2010) and the presence of parasitoid wasps rapidly selects for *Spiroplasma*-infected flies (Xie *et al.,* 2015). Similarly, *Spiroplasma* resistance against nematodes is greater than the observed variation in resistance in uninfected flies (Jaenike *et al.,* 2010). Again, the presence of nematodes results in selection for *Spiroplasma* infected flies (Jaenike and Brekke, 2011) and is thought to be responsible for the sweep of *Spiroplasma* in populations of *Drosophila neotestacea* across North America (Cockburn *et al.,* 2013). These studies highlight that the presence/absence of defensive symbionts can provide hosts with substantial variation in the susceptibility to natural enemies which can be inherited across generations.

Secondly, the genetic specificity between a host and parasite is an important factor of host-parasite coevolutionary dynamics. Here, a subset of parasite genotypes are able to successfully parasitize a subset of host genotypes, while a subset of host genotypes are able to successfully resist parasitism from a subset of parasite genotypes. Genetic specificity between host and parasite can give rise to negative frequency-dependent selection and the maintenance of genotypic variation in natural populations (Woolhouse *et al.,* 2002; Schmid-Hempel and Ebert, 2003). Indeed, there is clear evidence for genetic specificity across several systems (Carius *et al.,* 2001; Schulenburg and Ewbank, 2004; Lambrechts *et al.,* 2006; Lambrechts *et al.,* 2009).

Beyond the host and parasite, it has been shown that defensive symbionts can also provide an additional source of specificity to a host-parasite system. For example, in the pea aphid, protection against the entomopathogenic fungus *Pandora neoaphidis* is strongly dependent on the interaction between the symbiont and fungal genotype

(Parker *et al.,* 2017). Defensive symbionts can even be entirely responsible for the observed specificity between host and parasite as described in the example of the black bean aphid and the parasitoid wasp, *Lysiphlebus fabarum*. Here, no genotype-by-genotype interactions were observed between aphids free of defensive symbionts and parasitoid (Sandrock *et al.,* 2010). However, when the experiment was repeated using genetically uniform aphids infected with different strains of the defensive symbiont, *Hamiltonella defensa*, against several lines of parasitoid, G x G interactions between parasitoid and symbiont were observed (Schmid *et al.,* 2012; Cayetano and Vorburger, 2013, 2015). The limited number of studies do reveal that symbionts can contribute to host-parasite specificity. However, whether this specificity can be generalised beyond the aphid systems to other symbiont-mediated defence systems is unknown.

Lastly, heritable variation for resistance can only be maintained in a population when there are costs associated with the resistance mechanism (Agrawal and Lively, 2002). It may be expected that harbouring a population of defensive bacteria is inevitably costly to the host through a trade-off of allocating resources to symbionts and to other functions such as reproduction. Indeed, there is evidence that defensive symbiont infection is costly. For example, in the black bean aphid, the defensive symbiont, *H. defensa* reduces lifespan and lifetime reproduction (Vorburger and Gouskov, 2011). Similarly, coinfections with *Serratia symbiotica* and *H. defensa* reduces fecundity, increases generation times and decreases weights of adult pea aphids (Oliver *et al.,* 2006). Indeed, in the absence of parasitoids, *H. defensa* is lost in populations of pea and cow aphids (Oliver *et al.,* 2006; Dykstra *et al.,* 2014). Costs associated with infection may also manifest as ecological costs. Pea aphids infected with *H. defensa* were observed to express reduced defensive behaviours compared to uninfected aphids, thus suffering increased predation by ladybirds (Polin *et al.,* 2014).

In addition to the cost of harbouring symbionts, further costs can manifest as a consequence of protection. For instance, damage may be endured from defensive toxins or other protective mechanisms (Kwiatkowski and Vorburger, 2012). Indeed, it seems that symbiont-mediated protection does come at a cost for hosts surviving natural enemy attack. For example, protection against viral infection by *Wolbachia* has significant negative impact on the lifespan of *Drosophila* (Chrostek *et al.,* 2013; Martinez *et al.,* 2015). Similarly, protection against parasitoid wasps by *Spiroplasma* reduces the fecundity of adult *Drosophila* (Xie *et al.,* 2011).

1.3.2 *Defensive symbionts can affect the evolution of endogenous defence*

Most hosts have the ability to defend themselves against natural enemies through endogenous defence mechanisms. However, how the presence of defensive symbionts contributes to endogenous defences and alter the path of host-parasite evolution is not well understood. It is likely that in many systems, hosts have the capacity to deploy both endogenous defences and symbiont-mediated defences to protect against natural enemies. Indeed, resistant genotypes of aphids were found in combination with defensive strains of *H. defensa* in field collected pea aphids (Martinez *et al.,* 2014). In addition, *Drosophila* are known to be able to protect against parasitoids through specialised cellular immunity, behavioural defence and through *Spiroplasma*-mediated defence (Salt, 1970; Hwang *et al.,* 2007; Milan *et al.,* 2012; Xie *et al.,* 2010). Defences used in combination may act additively, synergistically or antagonistically.

Empirical studies addressing how symbiont-mediated protection interacts with endogenous defences are limited. To date, there is only a single study example where symbiont-mediated defence has been considered in combination with endogenous defence. The combination of symbiont-mediated defence and endogenous defence in the pea aphid, *Acyrthosiphon pisum* against the common parasitoid wasp, *Aphidius ervi* has

been examined (Martinez *et al.,* 2018). They found that the symbiont, *H. defensa*, conferred additional protection to susceptible aphid genotypes (<10% survival after protection), but rarely conferred additional protection to resistant genotypes (>70% survival after protection). Infection with *H. defensa* was found to be costly across both genotypes in the absence of parasitism. However, in the presence of parasitism, the additional protection of *H. defensa* in resistant genotypes reduced fecundity and survivorship following survival from attack.

The presence of defensive symbionts also has the potential to alter the evolution of endogenous defence mechanisms. Harbouring a defensive symbiont may lead to redundancy of function for host defence genes if both the symbiont genes and host genes are contributing to the same biological function – parasite resistance. The presence of a defensive symbiont may therefore change the strength of selection on host endogenous defences. The presence of a symbiont may relax selection on host defences. As parasites are continually evolving to evade host defences, this relaxation may have important consequences on the susceptibility of a population – creating a population of susceptible individuals. This could leave the host population reliant on symbiont-mediated protection if host defence was lost.

The impact of symbiont-mediated defence on the evolution of endogenous defences has been rarely considered. In a study, populations of *D. melanogaster* were exposed to DCV virus in the presence and absence of the defensive *Wolbachia*. After 9 generations it was shown that the frequency of the resistance allele, *pastrel,* was at a lower frequency in *Wolbachia* infected populations indicating that *Wolbachia* has the ability to relax selection on host resistance genes (Martinez *et al.,* 2016). This study demonstrates that defensive symbionts have the potential to influence the evolution of endogenous defences and the potential to take over as the main line of defence.

1.3.3 *The environment can add additional complexity to the role of defensive symbionts in a host-parasite interaction*

The ability of a defensive symbiont to protect its host against natural enemies can be shaped by environmental factors, which in turn will modulate the coevolutionary dynamics between a host and parasite. One factor which has been extensively studied in relation to its impact on symbiont biology, is the role of the thermal environment. It is expected that the thermal environment may affect symbiont-mediated protection in two ways. First, through indirect effects on host immunity factors or parasite virulence factors which may in turn combine with symbiont-mediated protection. Secondly, through direct effects on the symbiont itself, such as effects on symbiont transmission efficiency or symbiont titre. For instance, *Spiroplasma* has been shown to have reduced transmission efficiency at cooler temperatures in *Drosophila* (Montenegro and Klaczko, 2004; Anbutsu *et al.,* 2008; Osaka *et al.,* 2008).

Indeed, there is ample evidence from the aphid protection systems that symbiont-mediated protection is sensitive to the thermal environment. In the pea aphid, *H. defensa*-mediated protection against *A. ervi* has been repeatedly observed to be negatively impacted, or fail, at warmer temperatures relative to cooler controls (Bensadia *et al.,* 2006; Guay *et al.,* 2009; Doremus *et al.,* 2018; Higashi *et al.,* 2020). Although, interestingly, when pea aphids were coinfected with *H. defensa* and X-type (PAXS), protection remained high despite higher temperatures and even showed higher resistance than aphids singly infected with *H. defensa* (Guay *et al.,* 2009). There is also evidence that symbiont-mediated protection is affected by the thermal environment in *Drosophila*. For instance, *Wolbachia*-mediated protection against *Drosophila* C virus failed when flies were reared through a temperature of 18°C pre-infection compared to flies reared through a temperature of 25°C (Chrostek *et al.,* 2021). Similarly, S*piroplasma*-mediated protection against *Leptopilina heterotoma* in *Drosophila hydei*

completely failed where flies were reared at, and exposed at, the cooler temperature of 18°C compared to 25°C (Corbin *et al.,* 2021).

Another environmental factor which may be likely to impact symbiont-mediated protection is host diet. However, to date there has only been one study considering the effect of diet on symbiont-mediated protection. Host plant species were found to have little effect of *H. defensa* protection against parasitoids in pea aphids. Nevertheless, the costs induced by parasitism challenge did depend on host plant species (Sochard *et al.,* 2019). Although limited in number, this study indicates that environmental factors are likely to directly impact, or at least, impact components of symbiont-mediated protection that in turn, will influence interactions between a host-parasite, adding additional complexity to the role of defensive symbionts in host-parasite coevolutionary dynamics.

1.4 THE STUDY SYSTEM

1.4.1 Leptopilina *wasps are an important parasitoid of* Drosophila

In contrast to predation – where the outcome of the interaction is asymmetric (the prey loses their life if unsuccessful, the predator loses a meal, the life-dinner principle) (Dawkins and Krebs, 1979), parasitism is a strong interaction where both parties either live or die. Further, parasitism is commonly relatively specific – a parasite may have from one to a few host species. This tightly linked interaction between parasitoid and host makes them an ideal system to investigate fundamental questions relating to ecological and evolutionary processes. There are at least 42 hymenopterous parasite species of *Drosophila* (Carton *et al.,* 1986). However, the most well-known of these are the larval endoparasitoid wasps of the genus, *Leptopilina.* Of the *Leptopilina* genus, the most well-researched of these wasps and the two that will be the focus of this thesis are the species *L. heterotoma* and *L. boulardi*. *L. heterotoma* are considered a generalist, with the ability to successfully infect a wide range of *Drosophila* species across the genus, whereas *L. boulardi* are considered a specialist of the frugivorous *Drosophila* including *D.*

melanogaster (Schlenke *et al.,* 2007; Carton *et al.,* 1986). Both species are known to live in sympatry across large portions of their species ranges, but *L. heterotoma* are distributed across the Holarctic region and *L. boulardi* are mainly distributed across Mediterranean and tropical regions (Allemand *et al.,* 2002).

Leptopilina wasps represent a pervasive parasite of *Drosophila.* The larval endoparasitoid wasps lay their eggs inside the host larvae and if the host cannot successfully defend against attack, the parasitoid larva feeds on and eventually kills the host. Parasitism rates in natural populations have been shown to vary greatly across various habitats, climates and localities but average attack rates are estimated at between 5-40% (Fleury *et al.,* 2009). However, in some localities, *Drosophila* parasitism rates have been shown to exceed 90% (Fleury *et al.,* 2004), demonstrating that parasitic wasps can exert a strong selective pressure on *Drosophila* to evade parasitism.

Despite their similarities, the infection strategies of *L. heterotoma* and *L. boulardi* are different at the cellular level. During oviposition, both species inject venom along with the egg to evade the *Drosophila* encapsulation immune response. However, how the venom prevents the encapsulation response of *Drosophila* is different. In *L. heterotoma*, the venom attaches to host lamellocytes subsequently altering their morphology and causing the cells to lyse, preventing them from forming a layer around the wasp egg. However, *L. boulardi* venom appears to block the release of lamellocytes from the lymph gland of *Drosophila*. Additionally, *L. heterotoma* eggs are typically found floating free in the host hemolymph, whereas *L. boulardi* eggs are typically found attached to host tissues, which provides more protection against complete encapsulation (Rizki *et al.,* 1990).

1.4.2 *The maternally inherited bacteria of* Drosophila*,* Spiroplasma*, can protect against* Leptopilina *wasps*

Spiroplasma is a genus of helical, highly motile, secondarily gram-negative bacteria which form part of the *Mollicutes* class. The bacterium was first described in 1973 when it was found to be the causative agent of Citrus Stubborn Disease in citrus plants in California (Saglio *et al.,* 1973). Since its first description, *Spiroplasma* is now known to be a highly diverse and widespread lineage of bacteria infecting a huge variety of organisms including, plants, spiders, crustaceans and insects (Gasparich *et al.,* 2004). Remarkably, highly divergent strains of *Spiroplasma* have recently been discovered in deep-sea invertebrates including jellyfish and sea cucumbers (Viver *et al.,* 2017; He *et al.,* 2018). The only estimate of *Spiroplasma* infection frequencies to date estimates that the bacteria infect ~7% of all terrestrial arthropods (Duron *et al.,* 2008). While many strains appear to be commensal, some strains are considered insect and plant pathogens including *S. melliferum*, *S. apis*, *S. citri*, *S. kunelii* and *S. eriocheiris* (Mouches *et al.,* 1983; Wang *et al.,* 2011; Whitcomb *et al.,* 1986; Clark *et al.,*1985; Saglio *et al.,* 1973).

In the 1950's a maternally inherited factor that caused a highly female skewed sex ratio in the offspring of *Drosophila willistoni* was discovered (Malogolowkin and Poulson, 1957; Malogolowkin *et al.,* 1959). Although originally described as a spirochete, it was later determined to be a member of the *Spiroplasma* genus and given the name *Spiroplasma poulsonii* (Williamson *et al.,* 1999). Very recently it has been shown that *S. poulsonii* is a fast- evolving symbiont, with the highest substitution rates observed of any bacteria (Gerth *et al.,* 2021). The first natural infection of *S. poulsonii* in *D. melanogaster* was found in a collection of flies caught from markets in Campinas, São Paulo State, Brazil (Montenegro *et al.,* 2000, 2005). The strain was given the name 'MSRO' for Melanogaster Sex Ratio Organism and infection prevalence of *Spiroplasma* flies was found to be 2.3% in Recife, Brazil (Montenegro *et al.,* 2005). Only recently has the molecular mechanism underlying *Spiroplasma* male-killing in *D. melanogaster* been uncovered. A *Spiroplasma*-

encoded toxin, *Sp*AID, causes abnormal segregation and breakage of X chromatids in male embryos, subsequently resulting in DNA-damage dependent apoptosis and death (Harumoto and Lemaitre, 2018; Harumoto *et al.,* 2016). *Spiroplasma* are now known to infect at least 16 species of *Drosophila* (Haselkorn *et al.,* 2009). However, not all of these strains cause male-killing, with some effects of strains only becoming apparent more recently.

Beyond the ability to kill males, *Spiroplasma* also has the ability to protect *Drosophila* from natural enemies. In 2010, there were independent reports of protection of drosophilids against nematode and wasp attack. In the North American mushroom-feeding fly, *D. neotestacea,* infection with the sterilizing nematode, *Howardula aoronymphium* is common, and worm prevalence can reach 30% of flies in natural populations (Jaenike *et al.,* 2010; Cockburn *et al.,* 2013). However, it was discovered that females infected with the native 'sNeo' *Spiroplasma* strain, are resistant to *H. aoronymphium* infection (fly fertility is restored, mother-worm nematodes are smaller in size and subsequently produce less juveniles) (Jaenike *et al.,* 2010). The benefit conferred by *Spiroplasma*, has been inferred as the reason for the sweep of *Spiroplasma* across North America which to date is the only example of a protective symbiont acting in a natural population (Cockburn *et al.,* 2013).

In the same year, *Spiroplasma* were also observed to protect *Drosophila* against *Leptopilina* wasps. This mutualism was first described in *D. hydei*, where the *Spiroplasma* strain Hy1 was found to increase larva-to-adult survival of flies attacked by the larval parasitoid wasp, *L. heterotoma* (Xie *et al.,* 2010). Later, in *D. melanogaster*, the male-killing *Spiroplasma* strain MRSO was also observed to improve larval-to-adult survival of flies attacked by *L. heterotoma*, *L. boulardi*, *Leptopilina vicroriae* and *Ganapis xanthopoda* (Ballinger and Perlman, 2017; Paredes *et al.,* 2016; Xie *et al.,* 2014; Mateos *et al.,* 2016).

Collectively, these studies reveal that parasite susceptibility to *Spiroplasma* has evolved at least twice (Mateos *et al.,* 2016).

The mechanisms underlying *Spiroplasma*-mediated protection have also been uncovered. 'Exploitative competition' via competition for lipids and 'interference competition' via toxin production are both likely to be responsible for reduced wasp survival post infection (Paredes *et al.,* 2016; Ballinger and Perlman, 2017). First, *Spiroplasma* proliferation is limited by the availability of lipids (Herren *et al.,* 2014). In addition, some parasitoid wasps including *L. boulardi*, do not have the ability to synthesise lipids and rely on their host, although notably *L. heterotoma* are polymorphic for this capacity (Visser *et al.,* 2010). This dependency of wasps on dietary lipid led to the hypothesis that *Spiroplasma* may be depleting host lipids and preventing the development of the wasp. Indeed, it was shown that depletion of hemolyphic lipids, reduces wasp success in *Spiroplasma*-uninfected *D. melanogaster* (Paredes *et al.,* 2016). Thus, lipid competition between *Spiroplasma* and the developing wasp is thought to be one mechanism underlying *Spiroplasma*-mediated protection in *Drosophila*. Toxins have also been observed to play a major role in *Spiroplasma*-mediated defence. Here, *Spiroplasma* produced Ribosome Inactivating Toxins (RIPs) act upon the developing wasp larvae ribosomes in *Spiroplasma*-infected hosts causing depurination in the α-sarcin/ricin loop of the 28S rRNA soon after the wasp eggs hatch inside the host (Ballinger and Perlman, 2017). There is no evidence to suggest that *Spiroplasma*-induced depurination acts on *D. melanogaster* itself (Alvear *et al.,* 2021).

1.4.3 *The model fruit fly,* Drosophila melanogaster

The frugivorous fruit fly, *D. melanogaster* has been central tool for investigating ecological and evolutionary dynamics. Arguably, it is the most well-studied organism biologically, with many processes relating to genetics and development well

characterised. *D. melanogaster* also lends itself as an ideal model organism to investigate the evolutionary ecology of symbiont-mediated protection. The high selective pressure on *Drosophila* to evade parasitism from parasitoid wasps has led to the evolution of multiple protective defences including nuclear encoded defence and behavioural defence (Salt, 1970; Milan *et al.,* 2012; Hwang *et al.,* 2007). The ability to deploy multiple defences against parasites makes *D. melanogaster* an ideal organism to understand the interaction between symbiont-mediated defence and other protective mechanisms.

Drosophila melanogaster is one of the species of *Drosophila* where the larvae can successfully defend themselves against parasitic wasps by employing a specialised cellular immune response called encapsulation (Salt, 1970). Indeed, there is considerable variation in genetic resistance between *Drosophila* species (e.g. most species within the *obscura*-group are unable to mount a successful immune response against parasitic wasps, showing no evidence of encapsulation (Havard *et al.,* 2009). During the encapsulation process the parasitoid egg is detected as non-self and mature haemocytes are upregulated within the haemocoel and directed towards the egg. An initial layer of plasmatocytes surround the parasitoid egg, which is then further enclosed by lamellocytes. The capsule surrounding the egg then undergoes melanisation, which ultimately kills the unhatched wasp larva directly through asphyxiation (Salt, 1970) or indirectly through the cytotoxic compounds produced (Nappi and Vass, 1998; Nappi *et al.,* 1995). Further to the mechanistic process, the genetic basis of parasitic wasp resistance is well characterised in *D. melanogaster*. The ability to encapsulate the parasitic wasp, *L. boulardi* and *A. tabida* each involve one major gene (Benassi *et al.,* 1998; Carton *et al.,* 1992). The genetic basis of wasp resistance in other species of *Drosophila* however, is limited.

D. melanogaster can also mediate behavioural strategies to protect against parasites. Previous work has shown that *Drosophila* larvae which consume ethanol-containing food

are better protected against parasitic wasps than larvae which consume standard food containing no ethanol (Lynch *et al.,* 2017; Kacsoh *et al.,* 2013; Milan *et al.,* 2012). The consumption of ethanol by *L. heterotoma* infected larvae causes increased death of wasp larvae growing within the hemocoel and consequently, increases fly survival (Lynch *et al.,* 2017; Milan *et al.,* 2012). In addition to this, exposure to ethanol can also reduce parasitic wasp oviposition into *Drosophila* larvae, likely due to wasps becoming sickened by the environment (Milan *et al.,* 2012). This protection allows *Drosophila* to actively use ethanol as a behavioural defence against parasitic wasps in two ways. First, adult flies have been shown to preferentially oviposit into ethanol-containing food in the presence of wasps as a form of kin medication (Kacsoh *et al.,* 2013). Secondly, *Drosophila* larvae have also been shown to self-medicate by actively seeking out ethanol containing food when infected (Milan *et al.,* 2012). However, both findings could not be repeated in subsequent studies following the same experimental methods (Lynch *et al.,* 2017). In addition to ethanol-mediated behaviour, *Drosophila* larvae have also been shown to engage in a rolling behaviour in response to parasitoid wasp attack (Robertson *et al.,* 2013; Hwang *et al.,* 2007). At the onset of wasp attack, the larva rolls towards the wasp which causes the ovipositor to wind around the larva often knocking the wasp off balance and ceasing the attack.

1.5 OUTLINE OF THESIS

This thesis is concerned with understanding the degree of complexity within the *Drosophila-Spiroplasma-Leptopilina* interaction. To date, how symbiont-mediated protection contributes to a host-parasite interaction has been extensively studied within the aphid-parasite model systems. From the aphid-parasite systems it is clear that host, parasite and symbiont genotypes, and the environment can all impact the strength of symbiont-mediated defence and thus, the outcome of natural enemy attack. This thesis aims to extend this knowledge beyond the aphid system to determine whether these

factors can be generalised across to the *Drosophila-Spiroplasma-Leptopilina* system. In doing so, the work presented in this thesis will also extend our knowledge of the *Drosophila-Spiroplasma* system into a model not solely at the mechanistic level, but also in terms of its evolutionary ecology.

1.5.1 *Chapter 2: The effect of wasp strain and environmental ethanol on the strength of* Spiroplasma*-mediated protection*

Previous work on protection of *D. melanogaster* by *Spiroplasma* had utilised a single wasp strain per species tested, which was held to be representative for the wasp species more widely. This chapter examines the effect of *L. heterotoma* strain on the strength of *Spiroplasma*-mediated protection conferred in *D. melanogaster.* This analysis is also conducted in the presence of ethanol, an environmental factor shown to influence the outcome of *L. heterotoma* attack in *Drosophila* larvae (Milan *et al.,* 2012; Kacsoh *et al.,* 2013; Lynch *et al.,* 2017), to determine how *Spiroplasma*-mediated protection is likely to interact with ethanol-conferred protection. The relative strength of each defence combination was considered in terms of individual fly survival and the relative fertility and fecundity of wasp-attacked survivors to produce an overall protection index.

1.5.2 *Chapter 3: The effect of* Spiroplasma *strain on the strength of* Spiroplasma*-mediated protection*

As a continuation of chapter 2, this chapter determines whether the strain of *Spiroplasma* has an effect on the strength of *Spiroplasma*-mediated protection in *D. melanogaster* against *Leptopilina* wasps. Two previous studies reported differences in the strength of *Spiroplasma*-mediated protection against the same wasp strain (Xie *et al.,* 2014; Paredes *et al.,* 2016). One potential cause of the observed difference in protection was the use of different *Spiroplasma* strains (MSRO-Brazil and MSRO-Uganda). To determine whether the results reflected differences in *Spiroplasma* strain or different

protocols/environments I conducted a common laboratory experiment to determine the relative strength of protection provided by the two strains of *Spiroplasma* in *Drosophila* against two strains of *L. boulardi* and two strains of *L. heterotoma*. In line with Chapter 1, I also estimate the overall protection index for each *Spiroplasma* strain-wasp combination.

1.5.3 *Chapter 4: Thermal sensitivity of* Spiroplasma*-mediated protection*

Thermal environments are known to affect symbionts generally and protective symbiosis in particular. In this chapter, I dissect the effect of the thermal environment on *Spiroplasma*-mediated protection. Previous studies have observed that *Spiroplasma*-mediated protection is sensitive to cool temperatures in the *D. hydei*-*L. heterotoma* interaction (Corbin *et al.,* 2021). However, the causal factors underlying this observation are undeterminable from the experiment conducted as temperature may have impacted upon several factors within the experiment. This chapter aims to dissect the effect of the thermal environment on *Spiroplasma*-mediated protection against *L. boulardi* in *D. melanogaster* by examining the effect of temperature before, during and after wasp attack on fly survival and wasp success.

1.5.4 *Chapter 5:* General discussion

I conclude this thesis with a general discussion summarising and synthesising the findings presented within the previous chapters. First, I compare and contrast my findings in relation to results obtained for the aphid-parasite model systems before discussing what this means for the dynamics of *Spiroplasma* in natural populations. Finally, I discuss future directions for the *Spiroplasma*-*Drosophila*-*Leptopilina* symbiosis.

Chapter 2

The effect of wasp strain and environmental ethanol on the strength of *Spiroplasma*-mediated protection

This chapter is published as 'Jones, J. E. and Hurst, G. D. D. 2020. Symbiont-mediated protection varies with wasp genotype in the *Drosophila melanogaster-Spiroplasma* interaction. *Heredity,* **124**, 592–602.'

2.1 ABSTRACT

The ability of an insect to survive attack by natural enemies can be modulated by the presence of defensive symbionts. Study of aphid-symbiont-enemy interactions has indicated that protection may depend on the interplay of symbiont, host and attacking parasite genotypes. However, the importance of these interactions are poorly understood outside of this model system. Here, I study interactions within a *Drosophila* model system, in which *Spiroplasma* protect their host against parasitoid wasps and nematodes. I examine whether the strength of protection conferred by *Spiroplasma* to its host, *Drosophila melanogaster* varies with strain of attacking *Leptopilina heterotoma* wasp. I perform this analysis in the presence and absence of ethanol, an environmental factor that also impacts the outcome of parasitism. I observed that *Spiroplasma* killed all strains of wasp. However, the protection produced by *Spiroplasma* following wasp attack depended on wasp strain. A composite measure of protection, including both the chance of the fly surviving attack and the relative fecundity/fertility of the survivors, varied from a <4% positive effect of the symbiont following attack of the fly host by the Lh14 strain of wasp to 21% for the Lh-Fr strain in the absence of ethanol. I also observed that environmental ethanol altered the pattern of protection against wasp strains. These data indicate that the dynamics of the *Spiroplasma*-*Drosophila*-wasp tripartite interaction depend upon the genetic diversity within the attacking wasp population, and that prediction of symbiont dynamics in natural systems will thus require analysis across natural enemy genotypes and levels of environmental ethanol.

2.2 INTRODUCTION

All organisms face a threat from natural enemies and, in response, are typically able to defend themselves through a variety of protective mechanisms. In many species, the outcome of an encounter may in part be determined by defensive symbionts within the host (and indeed offensive symbionts in the natural enemy) (Brownlie and Johnson, 2009; Oliver *et al.,* 2014; Ballinger and Perlman, 2019). In insects, vertical transmission of bacterial symbionts places heritable symbionts into direct conflict with the natural enemies of their host. This conflict has driven the evolution of host protection in a number of symbiont clades, in a wide range of host species, against a diverse range of enemies. For example, microbial symbionts are known to provide protection against ssRNA viruses (Hedges *et al.,* 2008; Teixeira *et al.,* 2008), nematodes (Jaenike *et al.,* 2010), fungal pathogens (Scarborough *et al.,* 2005; Lukasik *et al.,* 2013) and parasitic wasps (Oliver *et al.,* 2003; Xie *et al.,* 2010, 2014; Mateos *et al.,* 2016; Paredes *et al.,* 2016; Ballinger and Perlman, 2017).

Studies of defensive symbiosis are most well developed in aphid-symbiont-enemy interactions. For example, in the black bean aphid, *Aphis fabae*, the level of resistance conferred against the parasitoid, *L. fabarum*, is dependent on the interaction between the strain of defensive symbiont, *H. defensa*, and the strain of the parasitoid, not the host itself (Schmid *et al.,* 2012; Cayetano and Vorburger, 2013, 2015). Similarly, in the pea aphid, *A. pisum*, protection against the entomopathogenic fungus, *Pandora neoaphidis*, is strongly dependent on the genotype-by-genotype interaction between the parasite and the defensive facultative symbiont, *Regiella insecticola* (Parker *et al.,* 2017). Although these studies have demonstrated the importance of heritable microbes in mediating host-parasite specificity, the generality of these interaction terms is yet to be determined beyond the aphid system.

Regarded as a historically important model system for defence ecology and evolution, symbiont-mediated protection also occurs in the genus *Drosophila*. The facultative endosymbiont, *Spiroplasma*, can protect *Drosophila* against a range of endoparasitoid wasps. In *Drosophila hydei*, the native *Spiroplasma* strain Hy1 protects flies from the endoparasitoid wasp, *L. heterotoma* (Xie *et al.,* 2010), although wasp attack survivors are found to have reduced fertility (Xie *et al.,* 2011). Similarly, in *Drosophila melanogaster*, the *Spiroplasma* strain MSRO protects flies attacked by *Leptopilina boulardi* (Xie *et al.,* 2014; Paredes *et al.,* 2016; Ballinger and Perlman, 2017), *L. victoriae* and *G. xanthopoda* (Mateos *et al.,* 2016). In *D. neotestacea*, *Spiroplasma* confers tolerance against *Howardula* nematode worms, rescuing the fertility of female fly hosts (Jaenike *et al.,* 2010).

Despite their importance as a model system, our understanding of *Spiroplasma*-mediated protection in *Drosophila* is limited in comparison to the equivalent aphid systems. Exploration of evolutionary dynamics is limited to the observation of the sweep of protective symbionts through North American *D. neotestacea* over time (Jaenike *et al.,* 2010). More attention has been given to establishing the extent and molecular underpinnings of the defensive mechanisms. Variation in protective capacity against different parasitoid natural enemies has been observed. For example, whilst *Spiroplasma* strain MSRO is only very weakly able to rescue *D. melanogaster* flies parasitised by *L. heterotoma*, the same symbiont strain increases fly survival by 50% against *L. boulardi* (Xie *et al.,* 2014; Paredes *et al.,* 2016; Ballinger and Perlman, 2017). Defence is considered to occur mechanistically through a combination of RIP toxins secreted by the symbiont, and competition between symbiont and wasp for host lipid reserves (Paredes *et al.,* 2016, Ballinger and Perlman, 2017).

To date, *Spiroplasma* defence of *Drosophila* against attacking natural enemies has commonly been examined in a coarse-grained fashion, with protection against one strain of any particular enemy species being assessed. Parallels with the aphid system indicate

there may be more subtle interactions with enemy genotype, such that measures of protection against one enemy strain do not necessarily reflect the outcome of all interactions with members of that species. Furthermore, symbiont mediated defences have been commonly treated in isolation of other defence systems. Previous work has shown that environmental ethanol is an important determinant of the outcome of parasitoid wasp attack in *Drosophila,* with consumption of ethanol by infected larvae increasing mortality of wasp larvae growing within the hemocoel (Milan *et al.,* 2012; Lynch *et al.,* 2017). This observation implies that the magnitude of protection against wasp attack afforded by symbionts should be measured across a range of environmental ethanol conditions, to improve our ability to predict the outcome of the interaction, and from this, symbiont dynamics.

Understanding the dynamics of symbiont-mediated defence in natural populations thus requires us to determine variation in *Spiroplasma*-mediated protection across enemy strains, and assess how this interacts with other protective mechanisms such as ethanol-mediated protection. Therefore, in this study I assessed whether the variation in *Spiroplasma*-mediated protection previously observed against different wasp species is also reflected in variation in protection against different strains of the same wasp species. Furthermore, I examined whether the degree of protection and specificity to parasite strain is altered by ethanol presence. Within this study, I combine fly survival data with data on the fertility of flies that survived wasp attack to establish a protective index for each combination. This represents the first composite measure of symbiont-mediated protection obtained in any system to date.

2.3 MATERIALS AND METHODS

2.3.1 *Insect strains and maintenance*

Drosophila melanogaster Canton-S flies with and without *Spiroplasma* MSRO-infected Red 42 were used. MSRO-infected Red 42 were originally collected in Brazil in 1997 and maintained in the lab in a Canton-S background in parallel to Canton-S control stock lacking *Spiroplasma*, from which males were derived from each generation for MSRO line maintenance (Montenegro *et al.,* 2000). This strain has previously been shown to kill Lh14 wasps, but produces very weak fly survival (Xie *et al.,* 2014; Ballinger and Perlman, 2017). These stocks both carried *Wolbachia* strain wMel, which occurs naturally and has been observed to provide a weak positive effect on fly larva-to-adult survival against *L. heterotoma* (Lh14 strain) (Xie *et al.,* 2014). It should be noted that all larvae from the *Spiroplasma*-infected treatments are female due to the high efficiency of male-killing. However, there does not appear to be any differences in survival between the sexes against parasitoid wasp attack (Xie *et al.,* 2014). All flies were maintained on Corn Meal Agar (10 g agarose, 85 g sugar, 60 g maize meal, 40 g autolysed yeast in a total volume of 1 L, to which 25 mL 10% Nipagin dissolved in ethanol was added as a fungicide) at 25°C on a 12:12 light:dark cycle.

The *L. heterotoma* used were an inbred strain collected from Sainte Foy-lès-Lyon and la Voulte, France, a strain caught in Madeira, Portugal in March 2017, and the inbred strain Lh14 used in previous studies, initially collected in Winters, California in 2002 (Schlenke *et al.,* 2007). All wasp strains tested positive for *Wolbachia*. Wasp stocks were maintained on second instar Oregon-R larvae at 25°C on a 12:12 light:dark cycle. After emergence, wasps were maintained in grape agar vials supplemented with a flug moistened with honey water and allowed to mature and mate for 7 days prior to exposure to *D. melanogaster* L2 larvae.

2.3.2 *Preparing ethanol food*

The wasp attack assay was performed in fly medium at 0% and 6% ethanol, which is within the normal range experienced by *D. melanogaster* larvae in nature (McKenzie and McKechnie, 1979; Gibson *et al.,* 1981). Medium was prepared by using the standard Corn Meal Agar recipe (above) with the exception of the quantity and concentration of Nipagin added (5 mL 50% w/v / 1 L of medium), to ensure the concentration of ethanol in the experimental vials was close to 0% and 6%. To prevent the evaporation of ethanol during the process, 200 mL of food was dispensed into 250 mL Duran bottles and allowed to cool to 45°C before 12 mL of 100% ethanol was added to the ethanol treatment bottles and homogenised. 6 mL of food was then dispensed into standard *Drosophila* vials and instantly covered with Parafilm to prevent ethanol evaporation before experimental larvae were transferred into the vials.

2.3.3 *Measuring the effect of ethanol and wasp strain on fly survival and wasp success*

To ensure efficient vertical transmission of *Spiroplasma*, MSRO-infected Red 42 females were aged to at least ten days prior to egg laying. Flies were allowed to mate in cages and lay eggs on a grape juice Petri dish painted with live yeast for 24 h. Grape juice Petri dishes were incubated for a further 24 h to allow larvae to hatch. First instar larvae were picked from the grape plate into the experimental vials at 30 larvae per vial. Eight treatments were formed per wasp strain with approximately 10-15 replicate vials per treatment (1) Lh- S- EtOH-, (2) Lh- S- EtOH+, (3) Lh- S+ EtOH-, (4) Lh- S+ EtOH+, (5) Lh+ S- EtOH-, (6) Lh+ S- EtOH+, (7) Lh+ S+ EtOH-, (8) Lh+ S+ EtOH+. Five experienced female wasps and three male wasps were transferred into the wasp treatment vials. Flugs® (Genesee Scientific) were used to bung vials to reduce ethanol evaporation. Adult wasps were allowed to parasitise for 2 days before being removed.

All vials were maintained at 25°C on a 12:12 light:dark cycle. For each vial, the number of pupae, emerging flies and emerging wasps were recorded.

2.3.4 *Measuring the effect of wasp attack and ethanol on fertility*

To determine the degree to which survivors of wasp attack were impacted by wasp attack, the average daily emerged offspring of *Spiroplasma*-infected survivors ("Exposed") and *Spiroplasma*-infected flies which did not undergo wasp attack ("Unexposed") were measured from both the 0% and 6% ethanol treatments. Only fly survivors which underwent attack from the *L. heterotoma* Lh-Fr and Lh-Mad strain were used as there were few survivors from attack of the Lh14 strain of *L. heterotoma* and very low numbers also from the *Spiroplasma*-uninfected wasp attacked group.

To this end, adult female flies from the wasp attack assay were retained on eclosion, and stored in vials containing sugar yeast medium (20 g agarose, 100 g sugar, 100 g autolysed yeast in a total volume of 1 L, to which 30 mL 10% Nipagin w/v propionic acid was added) at mixed ages. A week after emergence commenced, approximately 45 female flies from each of the *Spiroplasma* treatments were placed individually into a vial containing 6 mL of Corn Meal Agar with two Canton-S males with a single yeast ball and allowed to mate. These flies were transferred onto fresh vials each day for five days. Female fertility was measured as the average number of daughters produced over four days (day 2-5), with F1 flies given two weeks to emerge to ensure every fly had emerged before counting. Females which did not produce any daughters were considered infertile.

2.3.5 *Measuring the effect of wasp attack and ethanol on wing size*

Body size as adult measures the stress experienced by flies during development, with many stresses (density, ethanol) resulting in smaller adult flies (Miller and Thomas, 2006; Castañeda and Nespolo, 2013). To determine whether wasp attack affected female

body size, wing size was used as a proxy, as these factors are known to be highly correlated in *Drosophila* (Robertson and Reeve, 1952). To this end, the left wings of individual flies from the experiment above were removed using forceps under a microscope (right wings were used if left wings were damaged) and mounted flat onto a glass microscope slide. A photograph was taken of each wing using a microscope mounted camera using GXCapture-O software (6.9v). Using ImageJ software (1.49v, US National Institutes of Health, USA), the area of the wing was determined by locating the coordinates of the six wing landmarks as defined in Gilchrist and Partridge (2001) and calculating the interior area of the polygon created. A scale slide was used to transform all wing measurements into millimetre square units. All photos where the landmarks were not clearly visible were not measured and excluded from the analysis.

2.3.6 *Measuring wasp oviposition behaviour*

To determine whether the differences in fly survival were due to differences in wasp oviposition behaviour, we compared the number of wasp eggs and larvae per fly larva among the three wasp strains (Lh-Fr, Lh14 and Lh-Mad). In addition, we determined whether wasp oviposition differed between *Spiroplasma* positive and negative fly larvae. To this end, we followed the same protocol as the wasp attack assay, except the no-wasp control and 6% ethanol treatment was omitted. Immediately after wasp removal, approximately 5 fly larvae from each of the five replicate vials were dissected under a microscope to count the number of wasp eggs and/or larvae present.

2.3.7 *Statistical analysis*

All statistical analyses were performed using the statistical software R, version 3.5.0 (R Core Team, 2018). Fly and wasp survival, proportion of flies fertile, and wasp oviposition were analysed by fitting a generalized linear model with binomial, binomial and Poisson distributions, respectively. A Bayesian generalized linear model ('bayesglm' function in

the 'arm' package; Gelman *et al.,* 2018) was used to analyse wasp survival due to extreme separation between symbiont treatments (*Spiroplasma* positive treatments had 0 wasp survival), and for this reason, symbiont interaction terms were additionally excluded from the analysis. The number of daughters produced and fly wing size were analysed using linear models. Wing area measurements were Box-Cox transformed to conform to normality (Crawley, 2007). In all cases, a fully saturated model including all factors and their interaction was reduced to a minimum adequate model through step-wise simplification. Nonsignificant factors are reported as the output of the model comparisons. The effect of significant independent variables are reported from the analysis of the minimum adequate model using the 'car' package (Fox and Weisberg, 2019). The sample size for each experiment conducted in this chapter can be found in Table A.1.

To produce a composite measure of protection, a Protective Index (PI) was calculated by comparing the survival and fecundity of *Spiroplasma*-infected flies in the presence/absence of a given strain of wasp. The PI was calculated as the ratio of p(survival) x p(fertile) x fecundity of fertile individuals for attacked vs unattacked *Spiroplasma*-infected flies and reflects the benefit of *Spiroplasma* in the face of wasp attack. Credible intervals for PI were calculated through simulation. By assuming prior probability distributions for each parameter (Survival probability = beta distribution; Fertility probability = beta distribution; Fecundity = normal distribution), the 'rbeta' and 'rnorm' functions were used to calculate 95% credible intervals for PI. The simulation data was also used to establish the posterior probability of PI differing between attacking wasp strains.

2.4 RESULTS

2.4.1 *Fly survival and wasp success*

In the absence of *L. heterotoma*, mean larva-to-adult fly survival was >69% across all treatments (Figure 2.1). There was no significant effect of *Spiroplasma* ($\chi^2 = 0.990$, d.f. = 1, $P = 0.320$) or ethanol ($\chi^2 = 0.00820$, d.f. = 1, $P = 0.928$), nor a significant interaction between *Spiroplasma* and ethanol on fly larva-to-adult survival ($\chi^2 = 0.0625$, d.f. = 1, $P = 0.803$).

In the presence of *L. heterotoma*, fly *Spiroplasma* infection had a significantly strong and positive effect on fly larva-to-adult survival ($\chi^2 = 223$, d.f. = 1, $P < 0.001$; Figure 2.1). The effect of *Spiroplasma* on fly larva-to-adult survival depended on the strain of attacking parasitoid, which was reflected in a significant interaction between *Spiroplasma* and wasp strain ($\chi^2 = 9.64$, d.f. = 2, $P = 0.008$). *Spiroplasma* provided almost no protection against the Lh14 strain of *L. heterotoma*, increasing fly larva-to-adult survival slightly from <1% to 5.11%. *Spiroplasma* did however, provide strong protection against the Lh-Fr and Lh-Mad wasp strains, increasing fly larva-to-adult survival from <1% to 42.4% and 39.7% respectively. Wasp strain itself had a significant effect on fly larva-to-adult survival ($\chi^2 = 191.02$, d.f. = 2, $P < 0.001$).

The presence of ethanol had a weak, albeit significant positive effect on fly larva-to-adult survival in the presence of wasps ($\chi^2 = 10.3$, d.f. = 1, $P < 0.001$; Figure 2.1). However, the effect of ethanol differed between the strains of attacking *L. heterotoma*, which was reflected in a significant interaction between ethanol and wasp strain ($\chi^2 = 7.82$, d.f. = 2, $P = 0.020$). Specifically, the presence of ethanol in the absence of *Spiroplasma* reduces fly larva-to-adult survival against the Lh14 *L. heterotoma* strain from 0.45% to 0.22%, yet slightly increases fly larva-to-adult survival against the Lh-Fr strain from 0.89% to 3.33% and the Lh-Mad strain from 0.33% to 1.33%. There was also a significant interaction between *Spiroplasma* and ethanol ($\chi^2 = 11.3$, d.f. = 1, $P < 0.001$; Figure 2.1), with the presence of ethanol reducing the effect of *Spiroplasma*-mediated fly larva-to-adult

survival across all three wasp strains (% decrease; Lh-Fr = 22%, Lh14 = 78%, Lh-Mad = 16%). The interaction between *Spiroplasma*, wasp strain and ethanol was not found to be significant ($\chi^2 = 0.365$, d.f. = 2, $P = 0.833$).

Wasp success was strongly negatively affected by fly *Spiroplasma* infection, with the presence of *Spiroplasma* completely preventing the emergence of wasps across all *L. heterotoma* strains in both the presence and absence of ethanol ($\chi^2 = 23.5$, d.f. = 1, $P < 0.001$). In the absence of *Spiroplasma*, the presence of ethanol had a significantly negative effect on wasp success ($\chi^2 = 102$, d.f. = 1, $P < 0.001$; Figure 2.1). However, the effect of ethanol depended on the strain of attacking *L. heterotoma,* reflected in a significant interaction between ethanol and wasp strain ($\chi^2 = 8.42$, d.f. = 2, $P = 0.015$). Ethanol reduced wasp success by 40%, 21%, and 60% across the Lh-Fr, Lh14 and Lh-Mad strains respectively. Wasp success was also significantly affected by the strain of wasp ($\chi^2 = 154$, d.f. = 2, $P < 0.001$).

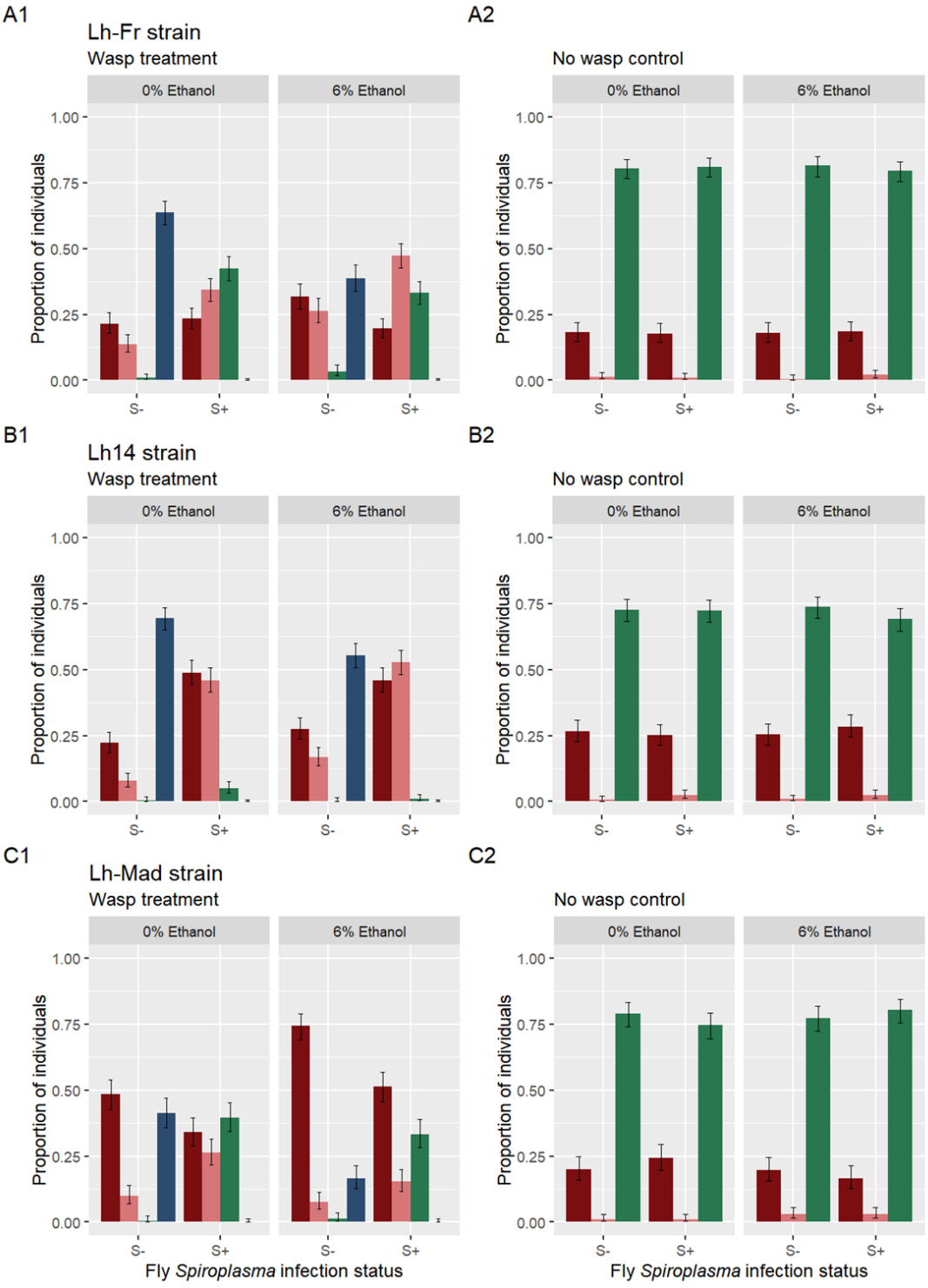

Figure 2.1: Proportion of dead larvae (red), dead pupae (pink), emerging flies (green) and emerging wasps (blue) for *Spiroplasma*-infected and -uninfected *Drosophila melanogaster* attacked by three different *Leptopilina heterotoma* strains in 0% and 6% environmental ethanol. Error bars represent 95% binomial confidence intervals.

2.4.2 *Female fertility*

2.4.2.1 *Proportion fertile*

For both Lh-Fr and Lh-Mad attacking wasp strains, *Spiroplasma*-infected individuals that survived wasp attack were observed to have reduced fertility, measured as the proportion of females able to produce progeny (Figure 2.2).

For attack with the Lh-Fr strain of wasp, there was a significant effect of wasp attack on the proportion of flies which were found to be fertile ($\chi^2 = 19.8$, d.f. = 1, $P < 0.001$; Figure 2.2). The proportion of *D. melanogaster* considered fertile following wasp-attack was reduced by 55% compared to control non-attacked *D. melanogaster*. There was no significant effect of ethanol ($\chi^2 = 3.11$, d.f. = 1, $P = 0.078$), nor a significant interaction between ethanol and wasp attack ($\chi^2 < 0.001$, d.f. = 1, $P = 0.988$).

For attack with the Lh-Mad strain, there was a significant effect of wasp attack on the proportion of flies found to be fertile ($\chi^2 = 28.4$, d.f. = 1, $P < 0.001$; Figure 2.2). The proportion of *D. melanogaster* considered fertile following wasp-attack was reduced by 48% compared to control non-attacked *D. melanogaster*. There was no significant effect of ethanol ($\chi^2 = 3.23$, d.f. = 1, $P = 0.072$), nor a significant interaction between ethanol and wasp attack ($\chi^2 = 0.447$, d.f. = 1, $P = 0.504$).

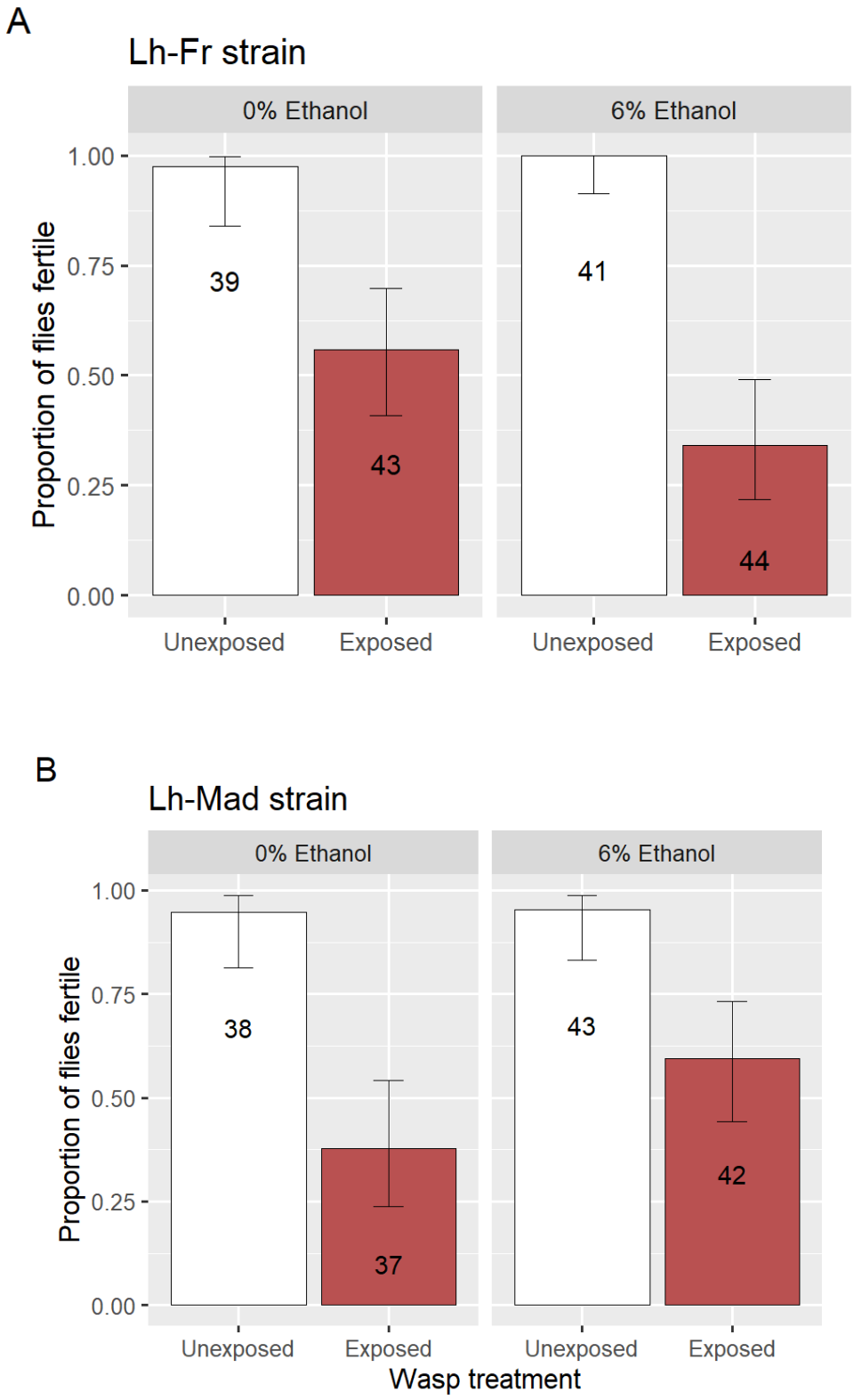

Figure 2.2: The proportion of *Spiroplasma*-infected *Drosophila melanogaster* females considered fertile after exposure to *Leptopilina heterotoma* (A: Lh-Fr and B: Lh-Mad strain) and unexposed controls developed through 0% and 6% ethanol medium. White bars indicate unexposed controls and pink bars represent wasp exposed. Error bars represent 95% binomial confidence intervals.

2.4.2.2 *Number of daughters produced*

In both cases, *Spiroplasma*-infected individuals that survived wasp attack and were fertile were observed to produce fewer daughters compared to fertile, unattacked controls (Figure 2.3).

For attack with the Lh-Fr strain, wasp attack significantly reduced the average number of daughters produced with protected wasp attacked *D. melanogaster* averaging ~39% fewer than control unattacked *D. melanogaster* (Mean ± SE = 10.6 ± 1.44 daughters for attacked flies vs. 17.5 ± 0.969 daughters for control flies; $F = 16.4$, d.f. = 1,116, $P < 0.001$; Figure 2.3). There was no significant effect of ethanol ($F = 1.81$, d.f. = 1,115, $P = 0.181$), nor a significant interaction between ethanol and wasp attack ($F = 1.74$, d.f. = 1,114, $P = 0.190$).

For attack with the Lh-Mad strain, wasp attack also significantly reduced the average number of daughters produced with wasp attacked protected *D. melanogaster* averaging ~40% fewer than control *D. melanogaster* (Mean ± SE = 8.35 ± 1.04 daughters for attacked flies vs. 14.0 ± 0.626 daughters for control flies; $F = 23.9$, d.f. = 1,114, $P < 0.001$; Figure 2.3). There was no significant effect of ethanol ($F = 2.9$, d.f. = 1,113, $P = 0.094$), nor a significant interaction between ethanol and wasp attack ($F = 2.9$, d.f. = 1,112, $P = 0.092$).

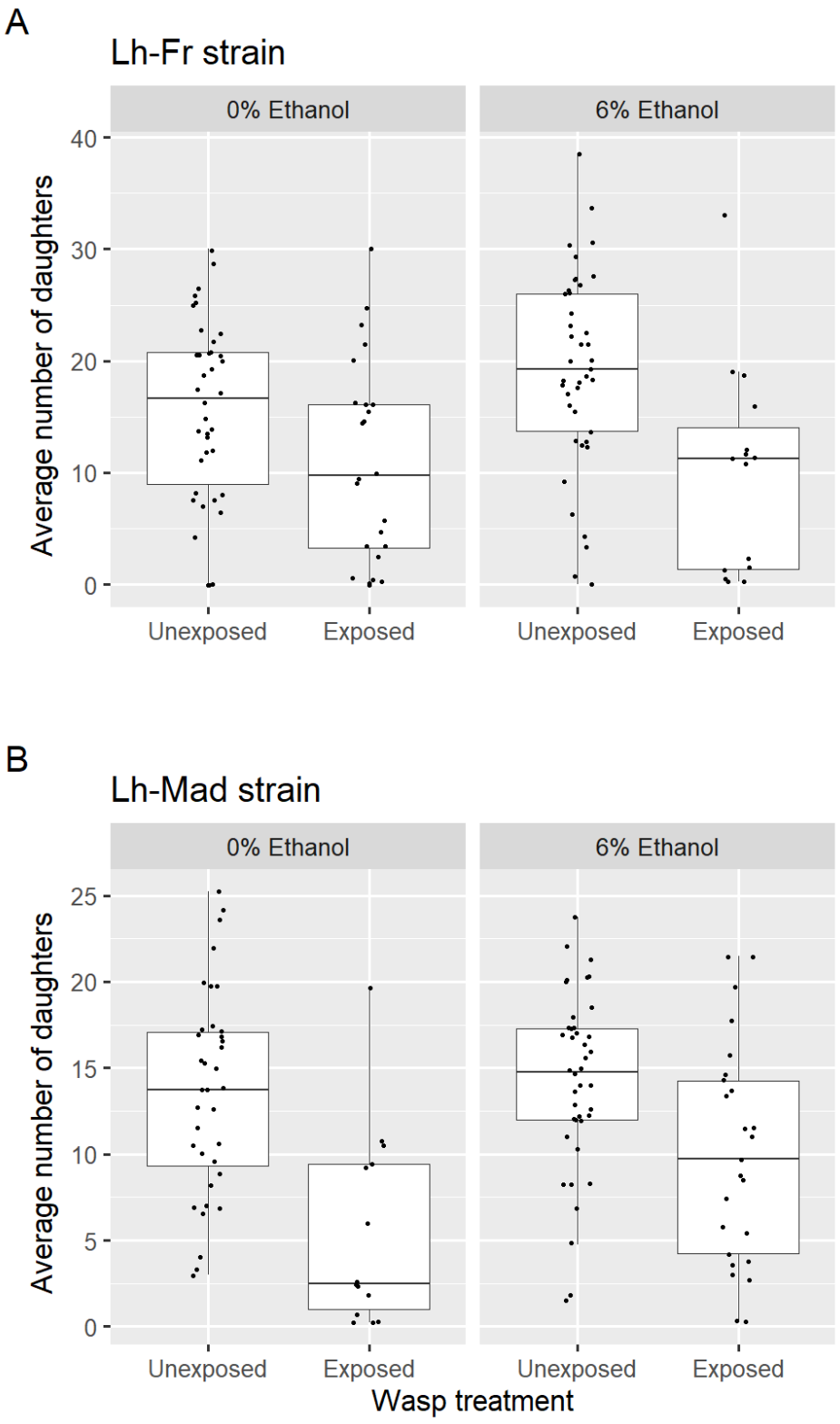

Figure 2.3: The average number of daughters produced by fertile *Spiroplasma*-infected female *Drosophila melanogaster* exposed to *Leptopilina heterotoma* (A: Lh-Fr and B: Lh-Mad strain) and unexposed controls developed through 0% and 6% ethanol medium. The box plots display the upper and lower quartiles, the median and the range. Points represent each measurement obtained.

2.4.3 *Overall protection*

Taking into account the survival, proportion of adults fertile, and the fecundity of wasp attack survivors, compared to unexposed *Spiroplasma*-infected controls, a protection index (PI) was calculated as the product of fly survival x p(fertile) x fecundity of exposed vs unexposed *Spiroplasma*-infected flies (this metric assumes complete mortality from wasps in the absence of *Spiroplasma*, which is approximately true as <1% of individuals tested survived wasp attack). In the absence of ethanol, the estimated protection index was 21%, and 9% against the Lh-Fr and Lh-Mad strains respectively (Table 2.1). The posterior probability that the protection index for *Spiroplasma* against the Lh-Fr strain is greater than the protection index against the Lh-Mad strain was 0.99. In contrast, the PI in the presence of ethanol was 7% and 12% against Lh-Fr and Lh-Mad wasp strains respectively (Table 2.1). The posterior probability that the protection index for *Spiroplasma* against the Lh-Mad strain is greater than the protection index against the Lh-Fr strain in the presence of ethanol was 0.99. With no fecundity measure available for Lh14 (due to insufficient survivors), we assume the estimate of protection to be less than the survival value.

Table 2.1: The overall protection conferred by *Spiroplasma* against the Lh-Fr, Lh14 and Lh-Mad *Leptopilina heterotoma* strains in *Drosophila melanogaster* in the presence (A) and absence (B) of ethanol. Exposed S- = wasp attacked *Spiroplasma*-uninfected flies; Exposed S+ wasp attacked *Spiroplasma*-infected flies; Unexposed S+ *Spiroplasma*-infected flies not attacked. Protective Index is calculated as [p(survival) x p(fertile) x fecundity of fertile individuals] of exposed vs unexposed individuals with credible intervals calculated as given in methods.

A) In the absence of ethanol

Wasp strain	Treatment	Fly Survival (binomial 95% CI intervals (lower, upper))	Proportion fertile (binomial 95% CI intervals (lower, upper))	Fecundity measure ± SE	Estimated protective index (95% Credible interval (lower, upper))
Lh-Fr	Exposed S-	<0.01 (0.0033 - 0.023)	N/A	N/A	
	Exposed S+	0.42 (0.38 - 0.47)	0.56 (0.41 - 0.70)	10.9 ± 1.83	
	Unexposed S+ control	0.81 (0.77 - 0.84)	0.97 (0.84 - 0.99)	15.6 ± 1.31	0.21 (0.12, 0.33)
Lh14	Exposed S-	<0.01	N/A	N/A	
	Exposed S+	0.05	N/A	N/A	
	Unexposed S+ control	0.72	N/A	N/A	<0.036
Lh-Mad	Exposed S-	<0.01 (0.00047 - 0.023)	N/A	N/A	
	Exposed S+	0.40 (0.34 -0.45)	0.40 (0.24 - 0.54)	5.45 ± 1.54	
	Unexposed S+ control	0.75 (0.69 - 0.79)	0.95 (0.81 - 0.99)	13.7 ± 0.985	0.09 (0.033, 0.16)

B) In the presence of 6% ethanol

Wasp strain	Treatment	Fly Survival (binomial 95% CI intervals (lower, upper))	Proportion fertile (binomial 95% CI intervals (lower, upper))	Fecundity measure ± SE	Estimated protective index (95% Credible intervals (lower, upper))
Lh-Fr	Exposed S-	0.03 (0.019, 0.058)	N/A	N/A	
	Exposed S+	0.33 (0.29, 0.38)	0.34 (0.22, 0.49)	9.98 ± 2.41	
	Unexposed S+ control	0.80 (0.76, 0.83)	1 (0.91, 1.00)	19.2 ± 1.38	0.07 (0.033, 0.13)
Lh14	Exposed S-	<0.01	N/A	N/A	
	Exposed S+	0.01	N/A	N/A	
	Unexposed S+ control	0.69	N/A	N/A	<0.007
Lh-Mad	Exposed S-	0.01 (0.0050, 0.035)	N/A	N/A	
	Exposed S+	0.33 (0.28, 0.39)	0.60 (0.44, 0.73)	6.38 ± 1.28	
	Unexposed S+ control	0.80 (0.75, 0.84)	0.95 (0. 83, 0.99)	14.2 ± 0.805	0.12 (0.12, 0.27)

2.4.4 *Wing size*

In both cases, *Spiroplasma*-infected individuals that survived wasp attack, were smaller compared to unattacked *Spiroplasma*-infected individuals (Figure 2.4).

For attack with the Lh-Fr wasp strain, wasp attack strongly reduced wing size, with the wings of wasp attacked female *D. melanogaster* on average 0.04 mm^2 (3%) smaller than unattacked *D. melanogaster* (Mean ± SE = 1.26 ± 0.008 mm^2 for attacked flies vs. 1.30 ± 0.006 mm^2 for unattacked flies; F = 26.7, d.f. = 1,196, P < 0.001; Figure 2.4). Ethanol reduced wing size, with the wing size of *D. melanogaster* reared in ethanol on average 0.02 mm^2 (1.6%) smaller than *D. melanogaster* reared in the absence of ethanol (Mean ± SE = 1.27 ± 0.008 mm^2 for flies reared in 6% ethanol vs. 1.29 ± 0.007 mm^2 for control flies; F = 4.34, d.f. = 1,196, P = 0.038; Figure 2.4). There was no significant interaction between ethanol and wasp attack on wing size (F < 0.001, d.f. = 1,195, P = 0.980).

For attack with the Lh-Mad wasp strain, wasp attack also had a highly significant effect on wing size, with the wing size of wasp attacked female *D. melanogaster* on average 0.05 mm^2 (4%) smaller than control *D. melanogaster* (Mean ± SE = 1.17 ± 0.006 mm^2 for attacked flies vs. 1.22 ± 0.006 mm^2 for unattacked flies; F = 31.9, d.f. = 1,162, P < 0.001; Figure 2.4). Ethanol reduced wing size, with the wing size of *D. melanogaster* reared in ethanol on average 0.02 mm^2 (1.7%) smaller than *D. melanogaster* reared in the absence of ethanol (Mean ± SE = 1.19 ± 0.068 mm^2 for flies reared in 6% ethanol vs. 1.21 ± 0.007 mm^2 for control flies; F = 4.71, d.f. = 1,162, P = 0.032; Figure 2.4). There was no significant interaction between ethanol and wasp attack on wing size (F = 0.227, d.f. = 1,161, P = 0.634).

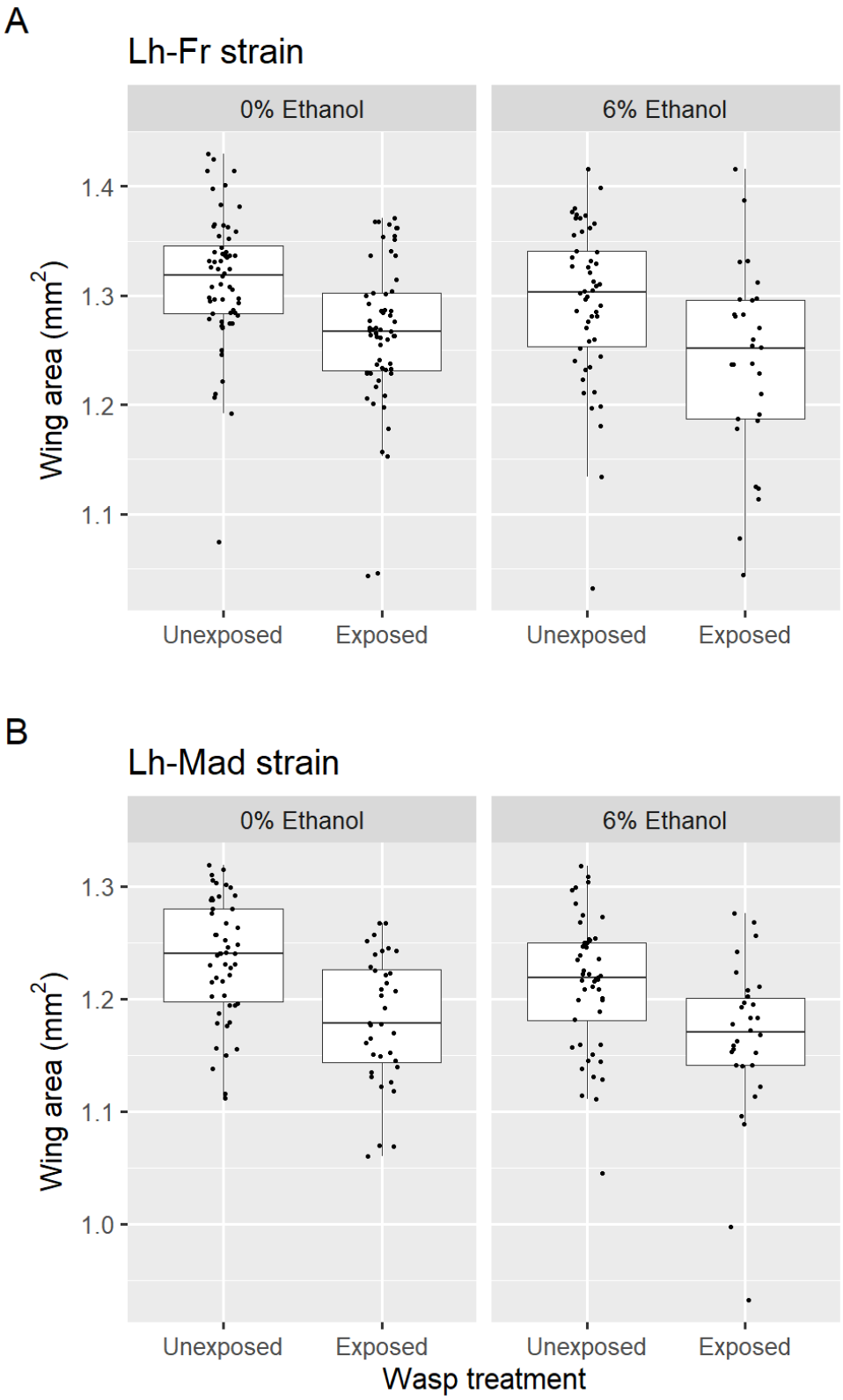

Figure 2.4: The wing area (mm^2) of *Spiroplasma*-infected female *Drosophila melanogaster* exposed to *Leptopilina heterotoma* (A: Lh-Fr and B: Lh-Mad strain) and unexposed controls developed through 0% and 6% ethanol medium. The box plots display the upper and lower quartiles, the median and the range. Points represent each measurement obtained.

2.4.5 *Wasp oviposition behaviour*

The average number of wasp eggs laid into a fly larva across a 48 h period of parasitisation was >1 but <2 for all treatments (Figure 2.5). There was no significant effect of wasp strain ($\chi^2 = 4.94$, d.f. = 2, $p = 0.085$) or fly *Spiroplasma* infection status ($\chi^2 = 1.52$, d.f. = 1, $p = 0.218$), nor a significant interaction between wasp strain and fly *Spiroplasma* infection ($\chi^2 = 0.664$, d.f. = 2, $p = 0.718$) on the number of wasp eggs laid into fly larvae.

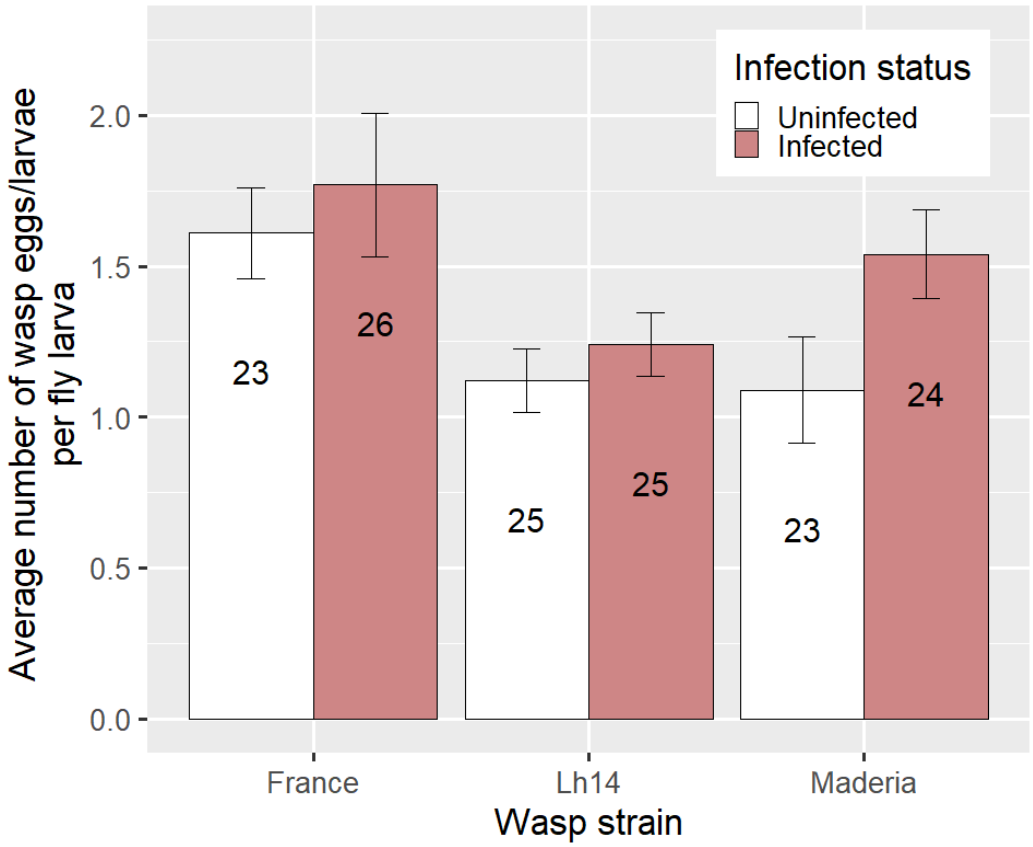

Figure 2.5: The average number of wasp eggs/larvae in *Spiroplasma*-infected and -uninfected *Drosophila melanogaster* larvae following 48 h of parasitisation by three strains of *Leptopilina heterotoma*. White bars indicate *Spiroplasma*-uninfected individuals and pink bars indicate *Spiroplasma*-infected individuals. Error bars depict ± SE.

2.5 DISCUSSION

It is now recognised that the outcome of natural enemy attack can be determined by the presence or absence of defensive heritable symbionts. Beyond their presence, the outcome of these interactions can also depend on the genotypes of all players: symbiont, host and enemy. However, the specificity of symbiont-mediated defence has only been explored within the aphid system. Previous work has found wasp species to be an important component of *Spiroplasma*-mediated protection in *Drosophila*, with *Spiroplasma* able to protect against some wasp species, but not others (Mateos *et al.,* 2016). Protection against *L. heterotoma*, using strain Lh14, for instance, is considered weak or absent in three previous studies (Xie *et al.,* 2014; Paredes *et al.,* 2016; Ballinger and Perlman, 2017). In this study, we examined whether protection against *L. heterotoma* wasps varied with wasp strain. Protection against the Lh14 wasp strains was observed at the low level previously recorded. In contrast, substantial protection was exhibited against the other strains of *L. heterotoma*. The overall protection gained by harbouring *Spiroplasma* against the Lh-Fr, Lh-Mad and Lh14 *L. heterotoma* was approximately 21%, 9% and <4% respectively, measured in the absence of environmental ethanol. Thus, *Spiroplasma* is protective against *L. heterotoma*, but the degree of protection is wasp strain dependent.

The differences in protective index afforded by *Spiroplasma* against different wasps strains arose through both effects on survival in response to wasp attack (Lh14 attack kills flies notwithstanding *Spiroplasma* presence, whereas *Spiroplasma* rescues flies attacked by Lh-Mad/Lh-Fr strains) and through differences in fertility/fecundity (between flies surviving attack by Lh-Fr and Lh-Mad strains). Thus, we conclude the protection afforded by *Spiroplasma* against *L. heterotoma* is dependent on *L. heterotoma* genotype, and that the differences observed are a product of both fly survival and survivor fertility differences. We would note that whilst impacts on the fertility/fecundity of 'protected' survivors of attack is noted in some cases of defensive

symbiosis (Xie *et al.,* 2011; Vorburger *et al.,* 2013), these metrics have not previously been included in models of relative protection against different enemy strains/species. Our data indicate that a complete model of protection dynamics may require measurement and inclusion of these parameters.

The mechanistic processes that determine the degree to which *Spiroplasma* affords protection against different wasp genotypes are uncertain. *Spiroplasma* completely prevented any wasps from emerging in all cases, implying that the symbiont defensive system was always efficient at killing the wasp. However, the degree to which killing the wasp rescued their fly host varied. Flies could be seen developing in the pupal cases in the majority of cases, but it was variation in eclosion to adult that underlies differential fly survival in response to the different genotypes of wasp. Further, we observed variation in surviving fly fertility that implies varying damage from the wasp is carried over beyond the point of wasp death, potentially associated with physical consumption of the fly during parasitisation. From the wasp differential oviposition assay, we can reject the hypothesis that the observed differences are due to differential oviposition behaviour across wasp strains.

The origins of differential fly survival therefore lie within a parasitised host individual. What is it about the wasp-host interaction in the presence of *Spiroplasma* that leads to different outcomes in terms of fly survival? One possibility is that RIP toxins involved in protection differentially affect the wasp strains studied, with Lh14 being less sensitive to RIPs, and thus developing further and/or causing more damage to the fly. A second explanation is variation in the ability of the wasp to synthesise lipids, for which the *Spiroplasma* is thought to compete (Paredes *et al.,* 2016). Intraspecific variation in the ability to synthesise lipids has been observed in *L. heterotoma* (Visser *et al.,* 2018). If Lh14 is unable to synthesise lipids, this could lead to competition between *Spiroplasma* and the wasp for the available lipids within the host, thus leading

to lower survival. A third, non-mutually exclusive, explanation is that the different outcomes are a result of variation in the venom transferred by the wasp strains. Venom is transferred along with eggs to suppress the host immune system and bypass nuclear encoded defences. In this model, a wasp strain delivering more potent venom can develop further (or otherwise causes damage) that prevents fly survival. Intraspecific venom variation amongst *Leptopilina* wasps is known (Colinet *et al.,* 2013). Wasp venom evolution has also been suggested as the target of selection when a wasp is passaged through symbiont-protected aphids (Dennis *et al.,* 2017). This study indicates venom constitution is likely to be important in determining the outcome of a wasp-host interaction in the presence of symbionts. Therefore, two open questions remain. First, what is the aspect of the wasp (sensitivity to RIP toxins, lipid synthesis, venom, other) that is important in producing the variation in protection afforded by *Spiroplasma*? Second, are changes in fly survival associated with longer development of the wasp, or more damage created by certain wasps (with similar total development)? These await further research.

The protection offered by *Spiroplasma* against wasp strains is modified by the presence of environmental ethanol during the larval phase. In contrast to assays where ethanol was absent, protection in the presence of ethanol is strongest against the Lh-Mad strain of wasp, and less strong against the Lh-Fr strain, with protection absent against Lh14. Against the Lh-Fr wasp strain, ethanol had a negative effect on the overall *Spiroplasma*-mediated fly protection, reducing protection from 21% to 7%. In contrast, ethanol had a positive effect on the overall protection against the Lh-Mad wasp strain, increasing overall protection from 9% to 12%, mainly due to the presence of ethanol reducing the negative effect of wasp attack on survivor fertility. In all cases, ethanol was detrimental to fly survival upon wasp attack. These results indicate that the interaction between *Spiroplasma*-mediated protection and ethanol protection is dependent on the genotype of the attacking wasp.

The data presented here have significant implications for the evolutionary and ecological dynamics of the *Spiroplasma-Drosophila*-wasp tripartite interaction in natural populations. From the perspective of the symbiont, the fitness benefit of protection is dependent upon wasp genotype, and thus the degree to which wasp attack drives the symbiont to higher prevalence will depend on the profile of the wasp population. In contrast, the observation that wasp emergence is zero in the presence of the symbiont in all three cases implies that the symbiont will not select upon the wasp population directly, although it will decrease the size of this population. However, a caveat here is that our results are derived from three wasp strains and their interaction with one *Spiroplasma* isolate. It is possible other wasp strains are resistant to *Spiroplasma*, and that there are strains of *Spiroplasma* which are less efficient at killing wasps.

Environmental ethanol, which modulates wasp attack outcome, is likely to be less important than *Spiroplasma*-mediated protection in terms of determining wasp success. In contrast to other lab studies (Milan *et al.,* 2012; Kacsoh *et al.,* 2013; Lynch *et al.,* 2017), here we observed only a small magnitude of protection afforded by ethanol alone. Possible reasons for the disparity include variation in fly strains (Canton-S here, Oregon-R in other studies) and differences in experimental protocols (e.g, the period of exposure). Nevertheless, ethanol did determine the relative protective benefit of *Spiroplasma* against different wasp strains. Thus, the presence/absence of ethanol melds with the genetic makeup of the wasp population to determine protection accorded by *Spiroplasma*, and ultimately therefore is predicted to impact *Spiroplasma* dynamics.

In summary, our work has extended the aphid synthesis to *Drosophila*, and indicates symbiont mediated protection appears generally to depend on the genotype of the attacking wasp species. Further, the environment (in this case ethanol) may modulate protection. More widely, it will be important not to disregard other protective mechanisms and their interaction when predicting the ecological dynamics of symbiont-

mediated protection in this model system. Indeed, how *Spiroplasma*-mediated protection is predicted to interact with *Drosophila's* own innate immunity (and more widely, host genetic background) requires further investigation. Beyond this, parallels with studies of aphids indicate that symbiont genotype and environment should be considered. Thermal environment, for instance, commonly affects symbiotic phenotype, and low temperatures are known ablate *Spiroplasma* male-killing (Anbutsu *et al.,* 2008). Thus, whilst our study indicates the presence of complex interaction terms in this tripartite interaction, the full extent of these awaits resolution.

Chapter 3

The effect of *Spiroplasma* strain on the strength of *Spiroplasma*-mediated protection

This chapter is published as, 'Jones, J. E. and Hurst, G. D. D. 2020. Symbiont-mediated fly survival is independent of defensive symbiont genotype in the *Drosophila melanogaster*-*Spiroplasma*-wasp interaction. *Journal of Evolutionary Biology,* **33**, 1625-1633'.

3.1 ABSTRACT

When a parasite attacks an insect, the outcome is commonly modulated by the presence of defensive heritable symbionts residing within the insect host. Previous studies noted markedly different strengths of *Spiroplasma*-mediated fly survival following attack by the same strain of wasp. One difference between the two studies was the strain of *Spiroplasma* used. Therefore, I performed a laboratory experiment to assess whether *Spiroplasma*-mediated protection depends upon the strain of *Spiroplasma*. I perform this analysis using the two strains of male-killing *Spiroplasma* used previously, and examined response to challenge by two strains of *Leptopilina boulardi* and two strains of *Leptopilina heterotoma* wasp. I found no evidence *Spiroplasma* strain affected fly survival following wasp attack. In contrast, analysis of the overall level of protection, including the fecundity of survivors of wasp attack, did indicate the two *Spiroplasma* strains tested varied in protective efficiency against three of the four wasp strains tested. These data highlight the sensitivity of symbiont-mediated protection phenotypes to laboratory conditions and the importance of common garden comparison. Our results also indicate that *Spiroplasma* strains can vary in protective capacity in *Drosophila*, but these differences may exist in the relative performance of survivors of wasp attack, rather than in survival of attack per se.

3.2 INTRODUCTION

All key players of natural enemy attack have the potential to shape the outcome of the interaction, including the defensive heritable symbionts residing within the host. In the previous chapter, I demonstrated that the strength of *Spiroplasma*-mediated protection was dependent on the strain of attacking parasitoid wasp. Specifically, the strength of *Spiroplasma*-mediated protection was observed to be lower when flies were attacked by the Lh14 strain of *L. heterotoma* compared to the Lh-Mad and Lh-Fr strain. In this chapter, I continue this observation beyond wasp strain by investigating whether the strain of *Spiroplasma* itself can also alter the strength of protection conferred.

The outcome of natural enemy attack has traditionally been considered a function of factors encoded within the genome of the host and infecting parasite. Within this interaction may exist a degree of specificity whereby a subset of parasite genotypes are able to infect a subset of host genotypes and, reciprocally, a subset of host genotypes are able to resist a subset of parasite genotypes (Woolhouse *et al.,* 2002; Lambrechts *et al.,* 2006). Specificity between host and parasite genotypes can lead to negative-frequency dependent selection between players and can contribute to the maintenance of heritable variation for defence and attack factors within a population (Woolhouse *et al.,* 2002; Schmid-Hempel and Ebert 2003).

More recently it has been observed that the outcome of natural enemy attack is not solely determined by host and parasite genotypes, but also by the presence and genotype of defensive heritable microbial symbionts residing within the host (Brownlie and Johnson 2009; Oliver *et al.,* 2009; Ballinger and Perlman 2019). Defensive microbial symbionts have been identified in a wide range of organisms. For example, microbial symbionts are known to provide protection against ssRNA viruses (Hedges *et al.,* 2008; Teixeira *et al.,* 2008), nematodes (Jaenike *et al.,* 2010), fungal pathogens (Scarborough *et al.,* 2005; Lukasik *et al.,* 2013) and parasitic wasps (Oliver *et al.,* 2003; Xie *et al.,* 2010).

Recently, studies have described how microbial strain identity can complement host and parasite genotype as an additional driver of the outcome of a host – parasite interaction. In aphid systems, this is commonly manifested in symbiont strain x host strain x enemy strain interaction terms (Sandrock *et al.,* 2010; Schmid *et al.,* 2012; Cayetano and Vorburger, 2013, 2015; Parker *et al.,* 2017). Beyond the aphid systems, it is known that the strain of infecting *Wolbachia* is an important source of variation in *Wolbachia*-mediated protection against viruses in *Drosophila* associated with different titre achieved by the strains (Osborne *et al.,* 2009; Bian *et al.,* 2013; Chrostek *et al.,* 2013, 2014; Martinez *et al.,* 2017). Similarly, in the bumblebee, *Bombus terrestris*, the defensive gut microbiota type is predominantly responsible for resistant phenotypes against the virulent gut trypanosomatid, *Crithidia bombi* (Koch and Schmid-Hempel, 2012).

The heritable endosymbiont *Spiroplasma*, has been shown to protect *Drosophila* from attack by nematodes and parasitoid wasps (Jaenike *et al.,* 2010; Xie *et al.,* 2010, 2014; Mateos *et al.,* 2016). The ability of *Spiroplasma* to protect *Drosophila* is thought to be orchestrated through a combination of RIP toxin activity (secreted by *Spiroplasma*) and exploitative competition between *Spiroplasma* and the infecting parasite for lipid stores (Paredes *et al.,* 2016; Ballinger and Perlman, 2017). Despite being regarded as an important model system, little is known about the role of host, symbiont and parasite identity in determining the outcome of the interaction. Recent work has revealed that the genotype of attacking parasitoid wasp is important for the degree of protection conferred by *Spiroplasma* (Jones and Hurst, 2020a). It was observed that *Spiroplasma* (MSRO-Br strain) conferred protection of 40% against the Lh-Fr and Lh-Mad *L. heterotoma* wasp strains, contrasting with 5% protection against the Lh14 strain. The reasons underpinning the variation observed is unknown, but intraspecific differences in the toxicity of wasp venom transferred along with the wasp egg during parasitisation may be a factor.

A more general understanding of how symbiont and parasite genotypes are likely to interact is essential for predicting the dynamics of symbionts in natural populations. In this study, we determine whether parasite genotype x symbiont genotype interactions exist within the *Spiroplasma-Drosophila melanogaster* system. Most studies concerning *Spiroplasma*-mediated protection have reported the outcome of experiment in which a single symbiont strain defends against a single enemy strain. Analysis across these studies indicates that the strain of *Spiroplasma* may be an important component of *Spiroplasma*-mediated protection. For instance, survival of flies exposed to the Lb17 strain of the specialist parasitoid wasp *L. boulardi* was recorded at 5% in *D. melanogaster* infected with the MSRO-Br strain (Xie *et al.,* 2014), and 50% in *D. melanogaster* infected with the MSRO-Ug *Spiroplasma* strain (Paredes *et al.,* 2016). One interpretation of these results is that the *Spiroplasma* strains differ in protective capacity in *D. melanogaster*. However, analysis of these two strains within a common experimental design (controlling for potential lab practice, wasp strain and fly strain differences) is required to determine the precise importance of symbiont strain in determining the outcome of the parasite-host interaction.

Here I present an analysis of the capacity of MSRO-Br and MSRO-Ug to defend *D. melanogaster* against wasp attack. This analysis is performed for two strains of the specialist parasitoid *L. boulardi*, and two strains of the generalist *L. heterotoma*. I compare survival following wasp attack, mirroring previous studies, and additionally estimate overall protection combining fly survival data with data on the fertility of flies that survived wasp attack to establish a protective index for each wasp strain by *Spiroplasma* strain combination.

3.3 MATERIALS AND METHODS

3.3.1 *Insect strains and maintenance*

Two strains of *Spiroplasma* were used in this study. The first, Red 42, was originally collected in Campinas, São Paulo State, Brazil in 1997 (Montenegro *et al.,* 2000) and later transinfected and maintained in the laboratory on a Canton-S background. The second *Spiroplasma* strain was collected from Namulonge, Uganda in 2005 (Pool *et al.,* 2006) which was later transferred and maintained in the laboratory on an Oregon-R background. It should be noted that all larvae from the *Spiroplasma* infected treatments are female due to the high efficiency of male-killing. However, there does not appear to be any differences in survival between the sexes against parasitoid wasp attack (Xie *et al.,* 2014). All flies were maintained on ASG corn meal agar vials (10 g agarose, 85 g sugar, 60 g maize meal, 40 g autolysed yeast in a total volume of 1 L, to which 25 mL 10% Nipagin was added) at 25 °C on a 12:12 light:dark cycle.

The *L. boulardi* strains used were the NSRef strain, established from an initial female collected in Gotheron, near Valence, France (Varaldi *et al.,* 2003), and the Lb17 strain, initially collected in Winters, California in 2002 (Schlenke *et al.,* 2007). The *L. heterotoma* strains used were the inbred Lh14 strain also collected in Winters, California in 2002 (Schlenke *et al.,* 2007) and the Lh-Mad strain established from a single female collected in Madeira, Portugal in March 2017 (Jones and Hurst, 2020a). The wasp stocks were all maintained on second instar Oregon-R larvae at 25°C on a 12:12 light:dark cycle. After emergence, wasps were maintained on grape agar vials supplemented with a flug moistened with honey water and allowed to mature and mate for 7 days prior to exposure to *D. melanogaster* L2 larvae.

3.3.2 *Artificial infection of* Spiroplasma

The *Spiroplasma* strains (MSRO-Br and MSRO-Ug) were artificially transferred into a common host background (Canton-S) to remove any effect of host nuclear background

on the level of protection conferred. Canton-S stocks carry the naturally occurring *Wolbachia* strain wMel. *Wolbachia* has been shown to provide a weak positive effect on fly larva-to-adult survival and a negative effect on wasp success in flies attacked against *L. heterotoma* (Lh14 strain) (Xie *et al.,* 2014). Artificial infections were carried out as described by Nakayama *et al.,* (2015). Hemolymph was extracted from the thorax of *Spiroplasma*-infected *D. melanogaster* and mixed with sterile PBS. Virgin female Canton-S were artificially infected by injecting the abdomen with 0.1-0.2 µl of PBS-hemolymph, using a hydraulic positive-pressure microinjection apparatus (Model IM-6, Narushige Ltd, Tokyo, Japan).

3.3.3 *Confirmation of* Spiroplasma *infection status*

Three weeks post injection, the infection status of the artificially infected flies was confirmed via *Spiroplasma*-specific PCR. DNA extraction was carried out using the Wizard® Genomic DNA Purification Kit (Promega). To this end, each injected mother was taken and macerated in 150 µl of Nuclei Lysis Solution and incubated at 65 °C for 30 min. After incubation, 50 µl of Protein Precipitation Solution was added to each sample and then placed on ice for 5 min. Samples were then centrifuged for a further 4 min at 16,000 x g and the supernatant was transferred into a new tube containing 150 µl of isopropanol. Samples were centrifuged for 2 min at 16,000 x g and the supernatant discarded. 150 µl of 70% ethanol was added to each sample and centrifuged for 1 min at 16,000 x g. The supernatant was discarded. Pellets were dried before re-suspending in 25 µl of molecular grade water at 4 °C overnight before use in subsequent PCR assays.

PCR amplifications were conducted using *Spiroplasma* specific primers, SpoulF (5'-GCT TAA CTC CAG TTC GCC-3') and SpoulR (5'-CCT GTC TCA ATG TTA ACC TC-3') (Montenegro *et al.,* 2005). Each reaction was carried out in 15 µl volume containing 7.5 µl of GoTaq® Hot Start Green Master Mix (Promega), 0.5 µl each of the forward and reverse primer, 5.5 µl of Molecular Grade Water and 1 µl of DNA. All reactions were

conducted alongside the positive and negative controls. This included a PCR negative control containing the PCR reaction mixture only (excluding DNA template). The PCR thermal program consisted of an initial denature of 5 min at 95 °C, followed by 35 cycles of 15 s at 94 °C, 1 min at 55 °C and 40 s at 72 °C. The PCR products were electrophoresed in a 1.5% agarose gel at 155 V for 15 min and the products were visualised to confirm *Spiroplasma* infection. Offspring sex ratio of infected mothers were also checked to determine *Spiroplasma* efficiency. Only mothers which were infected with *Spiroplasma* and produced all female broods were used to create new lines.

To confirm the *Spiroplasma* strain status of each artificially injected line of *Drosophila melanogaster*, sequencing was performed on 5 individual flies from each strain. To this end, the DNA of 5 flies from each *Spiroplasma* strain line were extracted using the Wizard® Genomic DNA Purification Kit following the methodology from above. PCR amplifications were conducted using *Spiroplasma* specific primers, Spiro_MSRO_diff_F (5'-TAC GAC CAA TGG CTT GTC CC-3' and Spiro_MSRO_diff_R (5'- CTG GCA TTG CTT TTT CCC CA-3'). The PCR thermal program consisted of an initial denature of 2 min at 94 °C, followed by 35 cycles of 15 s at 94 °C, 1 min at 56 °C and 40 s at 72 °C. To prepare the PCR reaction for sequencing, PCR products underwent an ExoSAP digest clean up to remove excess primers. To this end, 5 µl of PCR product was added to a mixture containing 0.2 µl Shrimp alkaline phosphate, 0.05 µl of Exonuclease I, 0.7 µl 10X RX Buffer and 1.05 µl of molecular grade water. Samples were then incubated for 45 min at 37 °C followed by 15 min at 80 °C and sent for Sanger sequencing. The *Spiroplasma* strain status of the MSRO-Br and MSRO-Ug line were confirmed by the presence of a Guanine and Thymine respectively in position 414193, coding for a type III pantothenate kinase. The expected amplicon size is 509bp. Transinfected fly lines were passaged for >10 generations before experiments were conducted.

3.3.4 *Measuring the effect of* Spiroplasma *strain on fly survival and wasp success*

To ensure efficient vertical transmission of *Spiroplasma*, infected females were aged to at least ten days prior to egg laying. Flies were allowed to mate in cages and lay eggs on a grape juice Petri dish painted with live yeast for 24 h. Grape juice Petri dishes were incubated for a further 24 h to allow larvae to hatch. First instar larvae were picked from the grape plate into the experimental vials at 30 larvae per vial. A fully factorial design was created for each of the four wasp strains described which included *Spiroplasma* strain (MSRO-Br, MSRO-Ug and uninfected control) and wasp (presence or absence). Five experienced, mated female wasps were transferred into the wasp treatment vials. Adult wasps were allowed to parasitise for 2 days before being removed. All vials were maintained at 25 °C on a 12:12 light:dark cycle. For each vial, the number of puparia, emerging flies and emerging wasps were recorded. Experiments using *L. boulardi* and *L. heterotoma* were conducted in separate blocks, one week apart.

3.3.5 *Measuring female fecundity*

Spiroplasma infected flies that survive wasp attack generally have a lower fecundity than *Spiroplasma* infected flies which were not exposed to wasps (Xie *et al.,* 2011; Jones and Hurst, 2020a). To determine whether the wasp attacked survivors were differentially impacted by *Spiroplasma* strain the average daily emerged offspring of *Spiroplasma* infected survivors ("Exposed") and *Spiroplasma* infected flies which did not undergo wasp attack ("Unexposed") was measured for the MSRO-Br and MSRO-Ug strains. The *Spiroplasma* uninfected wasp attacked group was not included due to the extremely low number of flies which emerged, which may have avoided wasp attack all together. After emergence, flies from the wasp attack assay were stored in vials containing sugar yeast medium (20 g agarose, 100 g sugar, 100 g autolysed yeast in a total volume of 1 L, to which 30 mL 10% Nipagin w/v propionic acid was added) at mixed ages. A week after

emergence commenced, a subset of flies from each of the *Spiroplasma* treatments were placed into an ASG vial with two Canton-S males with a single yeast ball and allowed to mate. Approximately 25 replicates per treatment were created. Flies were transferred onto fresh ASG vials each day for five days. Flies were given two weeks to emerge to ensure every fly had emerged before counting. Female fecundity was measured as the average number of offspring produced over four days (day 2-5).

3.3.6 *Statistical analysis*

All statistical analyses were performed using the statistical software R, version 3.5.0 (R Core Team 2018). Fly and wasp survival data were analysed by fitting a generalized linear model with binomial distributions. In all cases, a fully saturated model including all factors and their interaction was reduced to a minimum adequate model through step-wise simplification. Non-significant factors are reported as the output of the model comparisons. The effect of significant independent variables are reported from the analysis of the minimum adequate model using the 'car' package (Fox and Weisberg, 2019). The sample size for each experiment conducted in this chapter can be found in Table A.2.

To produce a composite measure of protection, a Protective Index (PI) was calculated by comparing the survival and fecundity of *Spiroplasma*-infected flies in the presence/absence of a given strain of wasp. The PI was calculated as the ratio of p(survival) x p(fertile) x fecundity of fertile individuals for attacked vs unattacked *Spiroplasma*-infected flies and reflects the benefit of *Spiroplasma* in the face of wasp attack. Credible intervals for PI were calculated through simulation. By assuming prior probability distributions for each parameter (Survival probability = beta distribution; Fertility probability = beta distribution; Fecundity = normal distribution), the 'rbeta' and 'rnorm' functions were used to calculate 95% credible intervals for PI. The simulation

data was also used to establish the posterior probability of PI differing between attacking wasp strains.

3.4 RESULTS

3.4.1 *Fly survival and wasp success*

3.4.1.1 Leptopilina boulardi *experiment*

In the absence of *L. boulardi* wasps, *Spiroplasma* strain had a significant effect on fly larva-to-adult *D. melanogaster* survival ($\chi^2 = 7.74$, d.f. = 1, $P = 0.005$). The mean survival of MSRO-Br infected and MSRO-Ug infected *D. melanogaster* was 72.2% and 83%, respectively (Figure 3.1A). In the presence of *L. boulardi* wasps, there was no significant effect of wasp strain ($\chi^2 = 0.281$, d.f. = 1, $P = 0.596$), *Spiroplasma* strain ($\chi^2 = 0.0008$, d.f. = 1, $P = 0.977$), nor a significant interaction between wasp strain and *Spiroplasma* strain on larva-to-adult survival of *D. melanogaster* ($\chi^2 = 0.284$, d.f. = 1, $P = 0.594$) (Figure 3.1A). There was no significant effect of wasp strain on wasp success ($\chi^2 = 0.121$, d.f. = 1, $P = 0.728$) (Figure 3.1A), and wasps were observed only in the absence of *Spiroplasma*.

3.4.1.2 Leptopilina heterotoma *experiment*

In the absence of *L. heterotoma* wasps, there was no significant effect of *Spiroplasma* strain on fly larva-to-adult survival ($\chi^2 = 0.345$, d.f. = 1, $P = 0.557$). The mean survival of MSRO-Br infected and MSRO-Ug infected *D. melanogaster* was 81.1% and 83%, respectively (Figure 3.1B). In the presence of *L. heterotoma* wasps, there was a significant effect of wasp strain on fly larva-to-adult survival of *D. melanogaster* ($\chi^2 = 34.21$, d.f. = 1, $P < 0.001$). Fly larva-to-adult survival of *Spiroplasma*-infected *D. melanogaster* attacked by the Lh-Mad strain of *L. heterotoma* was approximately double that observed for flies attacked by the Lh14 strain of *L. heterotoma* (Figure 3.1B). Similar to the *L. boulardi* experiment, there was no significant effect of *Spiroplasma* strain ($\chi^2 = 0.740$, d.f. = 1, $P = 0.390$), nor a significant interaction between wasp strain and *Spiroplasma* strain ($\chi^2 = 0.674$, d.f. = 1, $P = 0.412$) on larva-to-adult survival of *D. melanogaster* (Figure 3.1B). There was a significant effect of wasp strain on wasp success ($\chi^2 = 4.805$, d.f. = 1, $P = 0.028$) (Figure 3.1B). The average wasp success of the Lh14 and Lh-Mad wasp strains

were 27.7% and 37.3% respectively. Wasps only emerged in the absence of *Spiroplasma*, with both symbiont strains preventing development of both wasp strains.

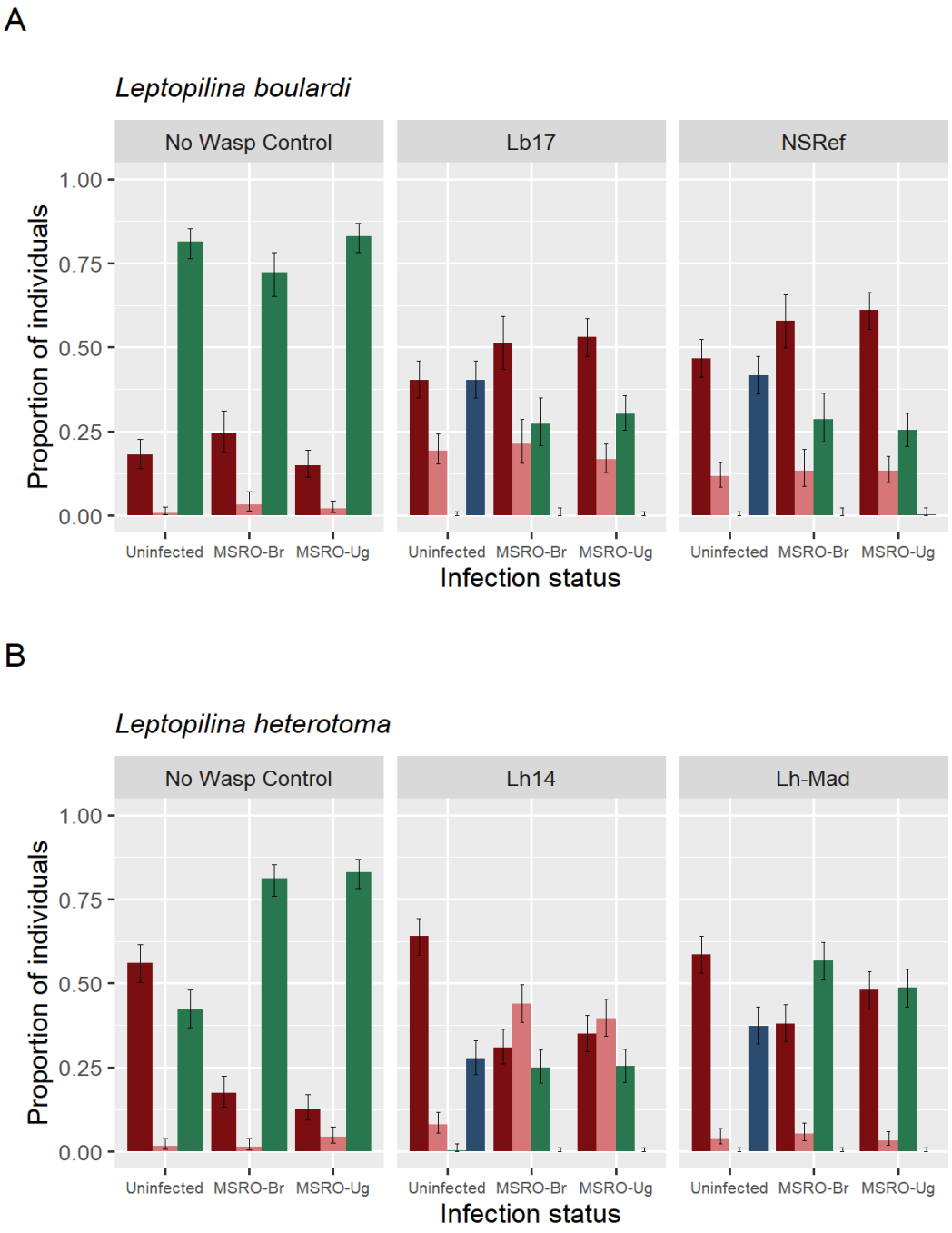

Figure 3.1: Proportion of dead larvae (red), dead pupae (pink), emerging flies (green) and emerging wasps (blue) for *Spiroplasma*-infected (MSRO-Br and MSRO-Ug strains) and uninfected *Drosophila melanogaster* attacked by A) *Leptopilina boulardi* (Lb17 and NSRef strains) and B) *Leptopilina heterotoma* (Lh14 strain and Lh-Mad strains). Error bars represent 95% binomial confidence intervals.

3.4.2 *Overall protection index*

Despite finding no difference between the survival of flies infected with MSRO-Br and MSRO-Ug against each of the four wasp strains tested, previous work has shown that it is also important to consider, in combination with survival, the fertility of wasp-attacked flies compared to non-attacked controls to produce a complete model of protection (Xie *et al.,* 2011; Jones and Hurst, 2020a). Taking into account the survival, proportion of adults fertile, and the fecundity of wasp attack survivors, compared to unexposed *Spiroplasma*-infected controls, a protection index (PI) was calculated as the product of fly survival x p(fertile) x fecundity of exposed vs unexposed *Spiroplasma*-infected flies (Table 3.1). This metric assumes complete mortality from wasps in the absence of *Spiroplasma*, which is approximately true as <1% of individuals tested survived wasp attack. Against the Lb17, NSRef and Lh-Mad strains of wasp, the posterior probability that the protection index for MSRO-Br is greater than the protection index for MSRO-Ug is >0.97 (Table 3.2). However, against the Lh14 strain of wasp, the posterior probability that the protection index for MSRO-Br is greater than the protection index for MSRO-Ug is 0.44 (Table 3.2).

Table 3.1: The overall protection conferred by MSRO-Br and MSRO-Ug *Spiroplasma* strains against A) *Leptopilina boulardi* (Lb17 and NSRef strains) and B) *Leptopilina heterotoma* (Lh14 and Lh-Mad strains) in *Drosophila melanogaster*. Exposed S+ represent wasp attacked *Spiroplasma*-infected flies and unexposed S+ controls represent un-attacked *Spiroplasma*-infected flies. Protective Index is calculated as [p(survival) x p(fertile) x fecundity of fertile individuals] of exposed vs unexposed individuals with credible intervals calculated as given in methods.

a) *Leptopilina boulardi*

***Spiroplasma* strain**	**Treatment**	**Fly Survival (binomial 95% CI intervals (lower, upper))**	**Proportion fertile (binomial 95% CI intervals (lower, upper))**	**Fecundity measure ± SE**	**Estimated protective index (95% Credible interval (lower, upper))**
MSRO-Br	Lb17 exposed S+	0.27 (0.20, 0.35)	0.96 (0.75, 0.99)	15.8 ± 1.31	0.37 (0.25, 0.55)
	NSRef exposed S+	0.29 (0.22, 0.36)	1.00 (0.86, 1.00)	15.6 ± 1.45	0.40 (0.27, 0.59)
	Unexposed S+ control	0.72 (0.65, 0.78)	0.92 (0.72, 0.98)	16.9 ± 1.47	
MSRO-Ug	Lb17 exposed S+	0.30 (0.25, 0.36)	1.0 (0.85, 1.0)	11.0 ± 1.77	0.30 (0.14, 0.32)
	NSRef exposed S+	0.25 (0.21, 0.30)	0.88 (0.68, 0.96)	14.3 ± 1.58	0.20 (0.14, 0.30)
	Unexposed S+ control	0.83 (0.78, 0.87)	0.96 (0.776, 0.99)	19.4 ± 1.49	

b) *Leptopilina heterotoma*

***Spiroplasma* strain**	**Treatment**	**Fly Survival (binomial 95% CI intervals (lower, upper))**	**Proportion fertile (binomial 95% CI intervals (lower, upper))**	**Fecundity measure ± SE**	**Estimated protective index (95% Credible interval (lower, upper))**
MSRO-Br	Lh14 exposed S+	0.25 (0.20, 0.30)	0.79 (0.59, 0.91)	13.2 ± 1.75	0.24 (0.15, 0.39)
	Lh-Mad exposed S+	0.57 (0.51, 0.62)	0.91 (0.71, 0.98)	14.0 ± 1.58	0.68 (0.54, 1.16)
	Unexposed S+ control	0.81 (0.76, 0.85)	0.92 (0.71, 0.98)	14.3 ± 1.72	
MSRO-Ug	Lh14 exposed S+	0.25 (0.20, 0.31)	1.0 (0.82, 1)	12.8 ± 1.14	0.25 (0.18, 0.36)
	Lh-Mad exposed S+	0.49 (0.43, 0.54)	0.91 (0.70, 0.98)	11.0 ± 1.34	0.39 (0.26, 0.56)
	Unexposed S+ control	0.83 (0.78, 0.87)	1.0 (0.82, 1)	15.3 ± 1.58	

Table 3.2: The posterior probability that the estimated protective index for MSRO-Br is greater than the MSRO-Ug for each wasp strain tested.

Wasp strain	**Estimated protective index (95% Credible interval (lower, upper))**		**Posterior probability (EPI MSRO-Br > EPI MSRO-Ug)**
	MSRO-Br	MSRO-Ug	
Leptopilina boulardi			
Lb17	0.37 (0.25, 0.55)	0.30 (0.14, 0.32)	0.97
NSRef	0.40 (0.27, 0.59)	0.20 (0.14, 0.30)	0.99
Leptopilina heterotoma			
Lh14	0.24 (0.15, 0.39)	0.25 (0.18, 0.36)	0.44
Lh-Mad	0.68 (0.54, 1.16)	0.39 (0.26, 0.56)	0.99

3.5 DISCUSSION

Defensive symbionts can contribute to the outcome of a host-parasite interaction. Previous studies in aphids have shown that the strain of symbiont is an important determinant of symbiont-mediated protection across multiple model systems (Schmid *et al.,* 2012; Cayetano and Vorburger, 2013, 2015; Parker *et al.,* 2017). However, whether strains of the *Drosophila* defensive symbiont, *S. poulsonii*, vary in their capacity for protection is unknown. The contrasting levels of fly survival observed between two previous studies on the *Drosophila-Spiroplasma-L. boulardi* interaction suggested that the strain of *Spiroplasma* may be an important determinant of protection capacity in *Drosophila* (Xie *et al.,* 2014; Paredes *et al.,* 2016). I therefore performed an experiment to determine whether the strength of *Spiroplasma*-mediated protection depended on the strain of infecting *Spiroplasma* using two known strains of MSRO *Spiroplasma* (MSRO-Br and MSRO-Ug). I found no evidence that the strength of *Spiroplasma*-mediated fly survival differed between the MSRO-Br and MSRO-Ug strains against any of the four *Leptopilina* wasp strains tested. However, the overall protective index, including the fecundity of survivors of wasp attack, did vary between the two *Spiroplasma* strains for three of the attacking wasp strains.

The strain of *Spiroplasma* did not alter the strength of *Spiroplasma*-mediated fly survival in *D. melanogaster* in our experiment. This result raises the question as to why fly survival following attack differed between the two previous independent studies. Fly survival against the parasitoid wasp, *L. boulardi* (strain Lb17) was observed to vary from 5% with MSRO-Br (Xie *et al.,* 2014), to 50% with MSRO-Ug (Paredes *et al.,* 2016). Comparisons across studies indicate that the strength of symbiont-mediated fly survival appear to be highly variable across laboratory studies. In this study, I found survival of 30% against the *L. boulardi* (Lb17 strain), yet Paredes *et al.,* (2016) found survival of 50% against the same wasp strain despite using the same fly strain. Similarly, I found

fly larva-to-adult survival of 25% against the Lh14 strain of *L. heterotoma*, despite survival of <8% observed in previous studies (Xie *et al.,* 2014, Jones and Hurst, 2020a).

The variability in *Spiroplasma*-mediated survival observed across studies may be the result of variability in wasp success. Whilst wasp attack rate was very high in all cases (with very low fly survival in uninfected controls), wasp success was highly variable across the studies and correlated to some extent with fly survival. Specifically, against the Lb17 wasp strain, Xie *et al.,* (2014) found high wasp success of ~70% and low fly survival of ~5%. In contrast, this study observed reduced wasp success of ~40% and increased fly survival of ~30%. Thus, the variability in *Spiroplasma*-mediated fly survival across studies could be associated with the condition of attacking wasps. Associated with this, it is notable that larval-to-pupa survival following attack is lower in our studies than previously observed, which may potentially explain differences in wasp survival. These studies may highlight the sensitivity of symbiont-mediated protection to husbandry conditions of both fly and wasp.

Several studies have demonstrated that symbiont-mediated survival against natural enemies can be highly sensitive to particular environmental conditions. Temperature is one environmental factor known to impact the strength of symbiont-mediated protection (Corbin *et al.,* 2017). For example, in the pea aphid, higher temperatures can negatively impact *H. defensa*-mediated survival against *A. ervi* (Doremus *et al.,* 2018). Similarly, heat shock also negatively impacts X-type-mediated survival against *A. ervi* wasps in the pea aphid (Heyworth and Ferrari, 2016). Another possibility, raised by studies of the strength of cytoplasmic incompatibility and male-killing exhibited by *Wolbachia*, is that protection strength is influenced by parental *Spiroplasma* titre (Dyer *et al.,* 2005; Layton *et al.,* 2019). It is notable that both thermal environment and age at reproduction are known to affect *S. poulsonii* titre and male-killing strength in *D. melanogaster* (Anbutsu and Fukatsu, 2003; Montenegro and Klaczko, 2004; Anbutsu *et*

al., 2008). Finally, wasp husbandry and attack protocols may vary. Wasp attack success is thought to be higher when wasps are previously conditioned before assays and may also be impacted by the arena in which attack occurs. Wasps attack fly larvae at the surface of the food, and the surface area available for attack, and indeed the medium in which the larvae are feeding, may impact success. The variable strength of protection afforded by symbionts across laboratories may be due to unmeasured differences in stock maintenance/ambient environmental conditions and reinforce the need for common-laboratory experiments when comparing outcomes.

Our experiment nevertheless did indicate differences in protection associated with *Spiroplasma* strain, but these were reflected in the overall phenotype, including the survival and fecundity of wasp-attack survivors. Surviving flies infected with the MSRO-Br strain of *Spiroplasma* had an overall higher protective index against the NSRef, Lb17 and Lh-Mad strains of wasp compared to flies infected with the MSRO-Ug strain. The reasons as to why fly survivors infected with MSRO-Ug had a lower protective index compared to MSRO-Br survivors remains unclear. One possible factor which cannot be ruled out from this study is the effect of *Wolbachia*. Although from the results it does not appear that *Wolbachia* is having an effect on fly survival, it may be possible that the presence of *Wolbachia* is differentially impacting the fertility of wasp-attacked survivors among the MSRO-Br and MSRO-Ug strains tested. Another factor which is difficult to determine is the possibility that a proportion of flies in the *Spiroplasma* treatments were not attacked. Although fly emergence from the *Spiroplasma* negative controls suggests that all larvae were successfully parasitized, this does not exclude the possibility that not all larvae were parasitized in the *Spiroplasma* positive treatments, although past work found no evidence for discrimination by wasps (Xie *et al.,* 2010, Jones and Hurst, 2020a). However, the result that there was no difference in the overall protection between wasp-attacked survivors infected with MSRO-Br and MSRO-Ug against the Lh14 strain of wasp indicates that the reasons for this difference may be a consequence of wasp strain.

This study clearly demonstrates two important features of protection. First, there is a need for common-laboratory experiments to compare levels of protection, because this phenotype has both genetic and environmental drivers. Second, there is a clear distinction between symbiont-mediated survival and symbiont-mediated protection within defensive symbiont studies. Symbiont-mediated protection is often measured as the relative survival of an infected-individual compared to an uninfected individual when faced with natural enemy attack. However, symbiont-mediated protection is not only the ability of an infected-host to survive, but also the relative capacity it has to reproduce compared to un-attacked comparators. Despite finding no evidence that fly survival differed between the two strains of *Spiroplasma* against all four wasp strains tested, differences between *Spiroplasma* strains were observed on the overall strength of symbiont-mediated protection. Assessment of the relative survival and reproductive ability of un-attacked vs. attacked survivors is essential for revealing the true protective capacity of a defensive symbiont.

Chapter 4

Thermal sensitivity of *Spiroplasma*-mediated protection

4.1 ABSTRACT

In insects, the outcome of natural enemy attack can be mediated by the presence of defensive microbial symbionts residing within the host. Beyond their presence, the outcome of the interaction can also depend on genetic and environmental factors. The thermal environment is a key factor known to affect symbiont-mediated traits in insects, including their ability to defend against natural enemy attack. The effect of thermal environment on *Spiroplasma*-mediated protection in *Drosophila* is limited to a single study, whereby *Spiroplasma* protection against *Leptopilina* was ablated at cooler temperatures. However, from this study it is difficult to discern the causal factors underpinning the loss of protection. The experimental temperature remained constant throughout the entire protection experiment, thus temperature may have impacted upon many aspects of protection. Here, I dissect the effect of the thermal environment on *Spiroplasma*-mediated protection against *Leptopilina boulardi* in *Drosophila melanogaster* by examining the effect of temperature before, during and after wasp attack on fly survival and wasp success. I find that the developmental temperature of the mothers, and not the temperature of the offspring during or after wasp attack, strongly determines the protective influence of *Spiroplasma*. When parental flies were reared at 21°C before exposure to wasps, *Spiroplasma*-mediated fly survival of their offspring was found to be ~58% weaker than the offspring of parental flies reared at 25°C. However, there was no effect of thermal environment on protection when mothers were reared at 25°C, and their progeny exposed to 21°C during and after wasp attack. I find evidence that the effect of developmental temperature on *Spiroplasma*-mediated protection is likely mediated by reduced *Spiroplasma* titre and segregation of infection at cooler temperatures. These results indicate that the historical thermal environment is a stronger determinant of protection than current environment, and protection against

wasps is likely influenced by transgenerational effects, mediated by changes in *Spiroplasma* titre of mothers. Reciprocally, the presence of wasps can extend the thermal envelope for which *Spiroplasma* infection can exist, as the segregation of the symbiont that would cause decline is partly balanced by wasp-mediated mortality of the flies that have lost *Spiroplasma*.

4.2 INTRODUCTION

In the previous two chapters, I demonstrated that the strength of *Spiroplasma*-mediated protection can be affected by the strain of parasitoid wasp and the strain of *Spiroplasma* indirectly by reducing the fertility of survivors. However, beyond genetic factors, environmental factors such as temperature have great potential to shape the outcome of symbiont-mediated defence. Temperature can impact upon many factors of a defensive symbiont, including transmission efficiency, titre and phenotype expression, all of which can contribute to the outcome of symbiont-mediated defence. In this chapter, I shift my focus to investigate the effect of temperature, on *Spiroplasma*-mediated protection by investigating its effect on defensive phenotype and titre.

Heritable microbial symbionts are now widely recognised to be key players in the outcome of host-parasite interactions, providing an initial line of defence against attack in insects. The diversity of defensive symbiont associations described has increased dramatically over recent years with symbionts affording protection against a wide range of natural enemies including, parasitoid wasps (Oliver *et al.,* 2003; Xie *et al.,* 2010), fungal pathogens (Scarborough *et al.,* 2005; Lukasik *et al.,* 2013), nematodes (Jaenike *et al.,* 2010) and ssRNA viruses (Hedges *et al.,* 2008; Teixeira *et al.,* 2008). Microbial symbionts and their defensive properties are also important in terms of their application. For instance, the antiviral properties of *Wolbachia* have been utilised in natural populations of mosquitoes to protect humans from diseases such as dengue and Zika (O'Neill *et al.,* 2018; Utarini *et al.,* 2021). Protective traits may also be used to enable beneficial insects. Thus, it is important to gain a general understanding of what factors can impact the protection afforded by defensive bacterial symbionts.

Beyond the mere presence or absence of the defensive symbiont, the outcome of natural enemy attack can depend on the strain of all players in the interaction (Xie *et al.,* 2010;

Schmid *et al.,* 2012; Chrostek *et al.,* 2013; Cayetano and Vorburger, 2013). But, in comparison to host, parasite and symbiont genetics, much less is known about how environmental factors contribute to symbiont-mediated defence. Temperature is a major factor which has the potential to shape the outcome of symbiont-mediated defence in several different ways. Firstly, temperature can affect the outcome of symbiont-mediated protection indirectly, through effects on host immunity factors or parasite virulence factors, which may combine with protection conferred by the symbiont itself. For instance, temperature can impact the immune reaction between *Drosophila* and parasitoid wasps. In addition, it was also observed that temperature altered the venom composition of the wasp, which was found to be partially responsible for the outcome of attack (Cavigliasso *et al.,* 2021).

Secondly, temperature can directly impact factors of the symbiont itself, which may result in consequences for the amount of protection conferred (Corbin *et al.,* 2017). One factor that can be affected by temperature is transmission efficiency. For example, the heritable bacteria *Spiroplasma* has been commonly found to have reduced transmission efficiency at cooler temperatures (Montenegro and Klaczko, 2004; Anbutsu *et al.,* 2008; Osaka *et al.,* 2008). In contrast, *Wolbachia* has been found to have reduced transmission efficiency at warmer temperatures (Jia *et al.,* 2009). In a similar direction, phenotype expression can also be affected by temperature. For instance, the male-killing phenotype of *Spiroplasma* is reduced in *Drosophila* at cooler temperatures (Anbutsu *et al.,* 2008).

A few studies have observed thermal sensitivity of symbiont-mediated protection. In the pea aphid, *H. defensa*-mediated protection against *A. ervi* has been repeatedly shown to be negatively impacted, or fail, at warmer temperatures relative to cooler controls (Bensadia *et al.,* 2006; Doremus *et al.,* 2018; Higashi *et al.,* 2020). Even a modest temperature rise of 2.5°C was enough to reduce the strength of *H. defensa*-mediated protection against *A. ervi* (Higashi *et al.,* 2020). In contrast to the studies above,

Spiroplasma-mediated protection against *Leptopilina heterotoma* in *Drosophila hydei* completely failed at a cooler temperature of 18°C compared to 25°C (Corbin *et al.,* 2021).

Symbiont titre is considered to be an important component of the mechanisms underpinning the thermal sensitivity of symbiont-mediated defence (Corbin *et al.,* 2017). However, it has been observed that symbiont titre cannot always fully explain the differences in protection conferred. Reduction of *Wolbachia* titre in *Drosophila* raised at 18°C compared to 25°C was not enough to fully explain the difference observed in the reduction of *Wolbachia*-mediated protection against *Drosophila* C virus at 18°C (Chrostek *et al.,* 2021). Similarly, the relative titre of *H. defensa* was not found to be a strong indicator of *H. defensa*-protection reduction against *A. ervi* at cooler temperatures (Doremus *et al.,* 2018). Non-mutually exclusive factors should also be considered. Direct effects of temperature on parasite development itself, or direct effects on the defensive products produced by the defensive symbiont in terms of quality or quantity may alter the strength of protection conferred.

However, few studies have considered the timing of experimental thermal conditions on symbiont-mediated defence. In *D. melanogaster*, the effect of temperature on *Wolbachia*-mediated protection against *Drosophila* C virus was considered when flies were exposed to experimental temperatures before and after infection. When flies were exposed to an experimental temperature of 18°C post-infection, *Wolbachia*-mediated protection was observed to be stronger than flies placed at 25°C. This result is considered to be related to lower viral replication at cooler temperatures. In contrast, when flies were reared through a temperature of 18°C pre-infection, *Wolbachia* was found not to protect flies against *Drosophila* C virus regardless of post-infection temperature (Chrostek *et al.,* 2021). Here, there was a clear epigenetic influence of temperature on protection, mirroring epigenetic influences observed in other host-symbiont interactions (e.g. Dyer and Jaenike, 2005; Layton *et al.,* 2019).

In this study, we determine the effect of thermal environment on *Spiroplasma*-mediated protection in *Drosophila melanogaster*. *Spiroplasma* is a facultative symbiont of *Drosophila* and has been shown to protect against parasitoid wasps and nematodes (Xie *et al.,* 2010; Jaenike *et al.,* 2010). Protection is considered to be a result of a combination of *Spiroplasma* RIP toxin production and competition between *Spiroplasma* and wasp for lipids (Paredes *et al.,* 2016; Ballinger and Perlman, 2017). Previous studies on this system have concluded that the strain of wasp, fly and *Spiroplasma* are all important components for the outcome of *Spiroplasma*-mediated protection (Xie *et al.,* 2010; Jones and Hurst, 2020a; Jones and Hurst, 2020b). However, the effect of thermal environment on this system is currently unknown.

Nevertheless, the thermal environment on *Spiroplasma*-mediated protection in *Drosophila hydei* has been investigated. *Spiroplasma*-mediated protection was observed to be 'normal' at temperatures of 25°C, but ablated when flies were exposed to cooler temperature of 18°C for both the generation prior to attack and the generation of attack (Corbin *et al.,* 2021). These data were consistent with other studies showing that *Spiroplasma* is a cool sensitive symbiont (Montenegro and Klaczko, 2004; Osaka *et al.,* 2008). However, as the experimental temperatures were kept constant throughout the entire protection experiment (from the development of parental flies, to the production of F_1 larvae and subsequent wasp attack and F_1 development), it is difficult to determine the causal factors underpinning the loss of the *Spiroplasma* protective phenotype at cooler temperatures.

Here, I dissect the thermal sensitivity of *Spiroplasma*-mediated protection in *Drosophila melanogaster* in terms of cool environment exposure at different timepoints. Specifically, I consider the effect of temperature at three stages: pre-attack (F_1 fly development and production of F_2 larvae), during attack (attack of F_2 larvae) and post attack (development of F_2 larvae into adults). From this, I can identify what components of the protection

interaction are thermally sensitive, which then allows us to determine the degree to which it is thermal environment during parasitism that is important, compared to epigenetic impacts arising before parasitism. Furthermore, I also determine the effect of temperature on the relative *Spiroplasma* titre.

4.3 MATERIALS AND METHODS

4.3.1 *Insect strains and maintenance*

The *Spiroplasma* strain used was the MSRO-Ug strain originally collected from Namulonge, Uganda in 2005 (Pool *et al.,* 2006), which was later transferred and maintained in the laboratory on an Oregon-R background and again transferred onto a Canton-S background in 2020 (Jones and Hurst, 2020b). MSRO-Ug stocks were maintained on corn meal agar (10 g agarose, 85 g sugar, 60 g maize meal, 40 g autolysed yeast in a total volume of 1 L, to which 25 mL 10% Nipagin was added) at 25°C on a 12:12 light:dark cycle.

The *Leptopilina boulardi* used were the inbred Lb17 strain used in previous studies, initially collected in Winters, California in 2002 (Schlenke *et al.,* 2007). Wasp stocks were maintained on second instar Canton-S larvae at 25°C on a 12:12 light:dark cycle. After emergence, wasps were maintained in vials containing sugar yeast medium (20g agarose, 100g sugar, 100g autolysed yeast in a total volume of 1L, to which 30mL 10% Nipagin w/v propionic acid was added) supplemented with a Flugs© (Flystuff, Genesee Scientific) moistened with honey water and allowed to mature and mate for at least 7 days prior to exposure to *D. melanogaster* L2 larvae.

4.3.2 *The effect of temperature on* Spiroplasma*-mediated protection*

4.3.2.1 *Preliminary temperature experiments*

To determine the effect of temperature on *Spiroplasma*-mediated protection, a series of preliminary experiments were conducted to understand the effect of temperature at different stages (attack, attack and protection, and constant) on protection, assessed through fly survival only. The protection experiment was initially split into two stages: the 'attack' stage and the 'attack and protection' stage whereby flies were subject to experimental temperatures of either 21°C, 23°C or 25°C for these periods only (Figure 4.1). The 'attack' temperature regime determined the effect of temperature on

Spiroplasma-mediated fly survival at the point of wasp attack only. The 'attack and protection' temperature regime determined the effect of temperature on *Spiroplasma*-mediated protection at the point of wasp attack and the subsequent development of the F_2 offspring following wasp attack whereby *Spiroplasma* is actively defending against wasp larvae development. An additional experiment was also conducted which examined the effect of a constant experimental temperature of either 21°C, 23°C or 25°C, including the generation before attack as well as the attack period itself (Figure 4.1). The three experiments were not conducted in parallel.

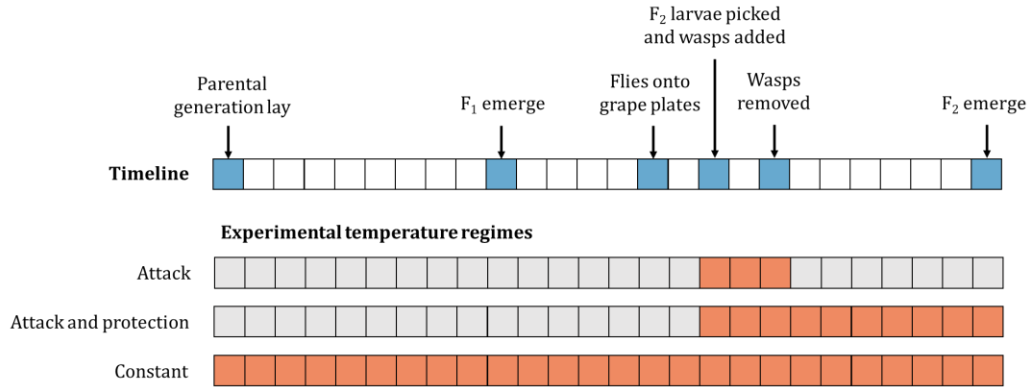

Figure 4.1: Experimental design showing the wasp attack assay timeline and the three experimental temperature regimes conducted for the preliminary experiments. For each regime, orange squares indicate when flies were subject to an experimental temperature of either 21°C, 23°C or 25°C. Grey squares indicate when all flies were subject to a standard 25°C temperature. Timeline squares represent a day based on a fly generation at 25°C.

To this end, MSRO-Ug stocks and uninfected control Canton-S stocks were initiated by placing 3 females and 2 males into ASG vials supplemented with yeast granules and placed at their experimental temperatures to develop (MSRO-Ug females were given 2 Canton-S males to mate). All MSRO-Ug females were 6 days old at the point of egg laying. Adults were removed from vials 4 days after egg laying. As the generation time of *Drosophila melanogaster* increases at lower temperatures (21°C = 15 days; 23°C =

12 days; 25°C, = 10 days) the start of the constant temperature regime was staggered so that parasitisation could be conducted at the same time to control for variation in wasp oviposition.

On emergence, F_1 offspring were allowed to emerge over 3 days before being collected into vials containing sugar yeast medium and supplemented with live yeast paste. MSRO-Ug females were given Canton-S males to mate. After 2 days of mating, flies were transferred into cages and allowed to continue to mate and lay eggs on a grape Petri dish painted with live yeast paste for 24 h at their focal temperature. Grape juice petri dishes were incubated for a further 24 h at their focal temperature to allow larvae to hatch. First instar larvae were picked from the grape plates into the experimental vials at 30 larvae per vial. Twelve treatments were formed in total for each experiment type with 10 replicate vials per treatment (3 temperatures (21°C, 23°C or 25°C) x 2 symbiont treatments (infected/uninfected) x 2 wasp treatments (wasp/no wasp), with the exception of the 'attack' temperature regime which had 6 replicate vials per treatment. Five female wasps were transferred into the wasp treatment vials and allowed 48 h to attack before being removed. All vials were maintained at their focal temperature on a 12:12 light:dark cycle. For each vial, the number of pupae and emerging flies were recorded.

4.3.2.2 *Integrated temperature experiment*

From the preliminary constant temperature experiment, we observed that *Spiroplasma*-mediated fly survival decreased at cooler temperatures. However, this effect was lost when flies were only subject to cooler temperatures at the 'attack' stage or the 'attack and protection' stage. This result suggests that the historical temperature of the fly is more important to the outcome of *Spiroplasma*-mediated fly survival than the ambient temperature at the point of wasp attack and subsequent *Spiroplasma* protection. Thus, an integrated experiment was conducted to include an additional 'pre-attack' stage to

determine whether low temperatures at this stage alone was sufficient to cause the effect seen in the preliminary constant temperature regime experiment alone (Figure 4.2).

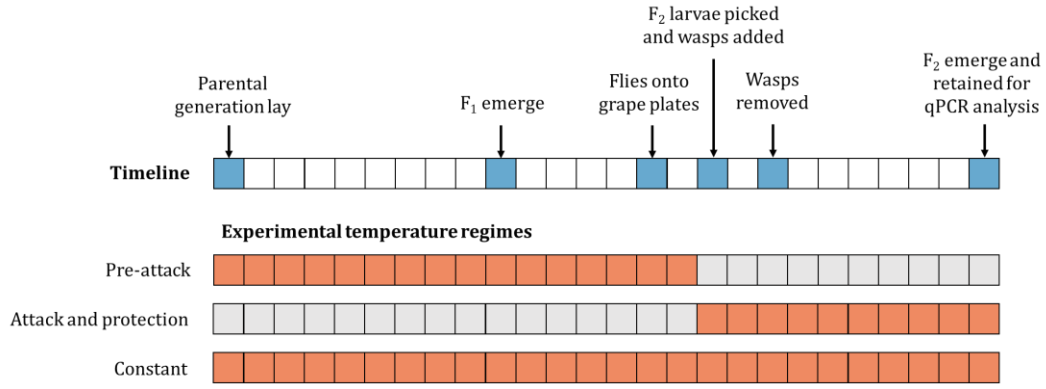

Figure 4.2: Experimental design showing the wasp attack assay timeline and the three experimental temperature regimes conducted in the integrated experiment. For each regime, orange squares indicate when flies were subject to an experimental temperature of either 21°C, 23°C or 25°C. Grey squares indicate when all flies were subject to a standard 25°C temperature. Timeline squares represent a single day based on a fly generation at 25°C.

To this end, the experiment was conducted following the same methods as above. Again, twelve treatments were formed in total for each temperature regime with 10 replicate vials per treatment (3 temperatures (21°C, 23°C or 25°C) x 2 symbiont treatments (infected/uninfected) x 2 wasp treatments (wasp/no wasp). Five female wasps were transferred into the wasp treatment vials and allowed 48 h to attack before being removed. All vials were maintained at their focal temperature on a 12:12 light:dark cycle. For each vial, the number of pupae, emerging wasps and emerging flies and their sex were recorded. *Spiroplasma*-infected wasp attacked and no wasp control flies emerging from the constant temperature regime were collected at 1 day old and frozen in -20°C for subsequent qPCR analysis.

4.3.3 *The effect of temperature and wasp attack on* Spiroplasma *titre*

The effect of temperature and wasp attack on *Spiroplasma* titre was also assessed. DNA template was prepared from individual flies and *Spiroplasma* titre was estimated by quantitative (qPCR). To this end, DNA was extracted from individual surviving female flies on emergence (15-20 flies per treatment) using the Phenol-Chloroform method. In detail, single flies were macerated using a pestle in 25 µl of lysis solution (Tris HCl 0.1 M (pH 9), EDTA 0.1M, SDS 1%) and incubated for 30 mins at 70°C. 20 µl KAc was added to each sample and were incubated on ice for a further 30 mins. Samples were spun down in a centrifuge for 15 min at 13,000 rpm and the supernatant was transferred to a new tube. 125 µl of Phenol-Chloroform was added to each sample and shaken before spinning for 5 mins at 13,000 rpm. This step was repeated and the supernatant moved to a new tube. 75 µl of Isopropanol was added to each tube and shaken before being placed into the centrifuge for 5 mins at 10,000 rpm. The supernatant was removed and the pellet was washed with 500 µl of 70% ethanol before being placed in the centrifuge for 5 mins at 13,000 rpm. The DNA pellet was dried and resuspended in 25 µl of TE buffer. DNA concentrations and quality were determined using NanoDrop ND-1000 spectrophotometer. All samples were stored at -20°C.

Real-time qPCRs were carried out for the *dnaA* gene (*Spiroplasma*) using the primers, DnaA109F (5′-TTA AGA GCA GTT TCA AAA TCG GG-3′), and DnaA246R (5′-TGA AAA AAA CAA ACA AAT TGT TAT TAC TTC-3′) and for the *RPS17* gene (*Drosophila melanogaster* reference) using the primers (Dmel.rps17F 5′-CAC TCC CAG GTG CGT GGT AT-3′ and Dmel.rps17R 5′-GGA GAC GGC CGG GAC GTA GT-3′), using the LightCycler 480 (Roche) (Anbutsu and Fukatsu, 2003; Osborne *et al.,* 2009). Each reaction consisted of 6 µl of PowerUp™ SYBR™ Green Master Mix (ThermoFisher), 0.5 µl of each primer solution at 3.6 µm and 5 µl of diluted DNA, with three technical replicates per reaction. Melting curves were analysed to confirm the specificity of amplified products. Relative amounts of *Spiroplasma* were calculated using the Pfaffl Method (Pfaffl, 2001).

4.3.4 *The effect of temperature on wasp oviposition*

Differences in fly survival in the face of wasp attack may be a consequence of changes in wasp oviposition behaviour. To determine whether wasp oviposition differed with environmental temperature, we compared the number of wasp eggs and larvae per fly larva after a 48 hour exposure period to Lb17 wasps at 21°C, 23°C and 25°C. To this end, the same protocol was followed to obtain fly larvae as the wasp attack assays. Five female wasps were placed into each vial containing 30 L2 *D. melanogaster* Canton-S larvae for 48 hours. Immediately after wasp removal, approximately 5 fly larvae from each of the 6 replicate vials were dissected under a microscope and the number of wasp eggs and/or larvae present were counted.

4.3.5 *Statistical analysis*

All statistical analyses were performed using the statistical software R, version 4.0.2 (R Core Team, 2020). Fly survival data were analysed by fitting a generalised linear model with binomial distributions. A Bayesian generalised linear model ('bayesglm' function in the 'arm' package; Gelman *et al.,* 2018) was used to analyse wasp survival due to extreme separation between symbiont treatments (*Spiroplasma*-infected treatments had low or zero wasp survival). Due to the inclusion of negative *Spiroplasma* titre data resulting from the 21°C no wasp attack control treatment, relative *Spiroplasma* titre data was transformed by adding 1 to account for zero ratios. A generalised model with Poisson distribution was then used to determine the effect of temperature and wasp attack on relative *Spiroplasma* titre. Wasp oviposition data was analysed using the Kruskal-Wallis rank sum test. The sample size for each experiment conducted in this chapter can be found in Table A.3.

4.4 RESULTS

4.4.1 *The effect of temperature on* Spiroplasma*-mediated protection: preliminary experiments*

4.4.1.1 *'Attack' temperature regime*

Mean larva-to-adult fly survival in the absence of wasps was > 70% across all treatments (Figure 4.3). There was no significant effect of temperature (χ^2= 0.0479, d.f. = 1, p = 0.827), or *Spiroplasma* infection status (χ^2= 0.116, d.f. = 1, p = 0.734), nor a significant interaction between *Spiroplasma* infection status and temperature on fly larva-to-adult survival (χ^2= 1.804, d.f. = 1, p = 0.179).

In the presence of wasps, *Spiroplasma* infection had a strong significant effect on fly larva-to-adult survival, increasing fly larva-to-adult survival from 0% to 56.7% at 21°C, <1% to 57.2% at 23°C and 6% to 73.9% at 25°C (χ^2= 85.017, d.f. = 1, p < 0.001; Figure 4.3). Temperature also had a weak significant effect on fly larva-to-adult survival (χ^2= 9.182, d.f. = 1, p < 0.01; Figure 4.3). Specifically, *Spiroplasma*-infected and uninfected fly larva-to-adult survival at 25°C was 49% higher than at 23°C and 52% higher than at 21°C. There was no significant interaction between temperature and *Spiroplasma* infection status (χ^2 = 3.454, d.f. = 1, p = 0.063).

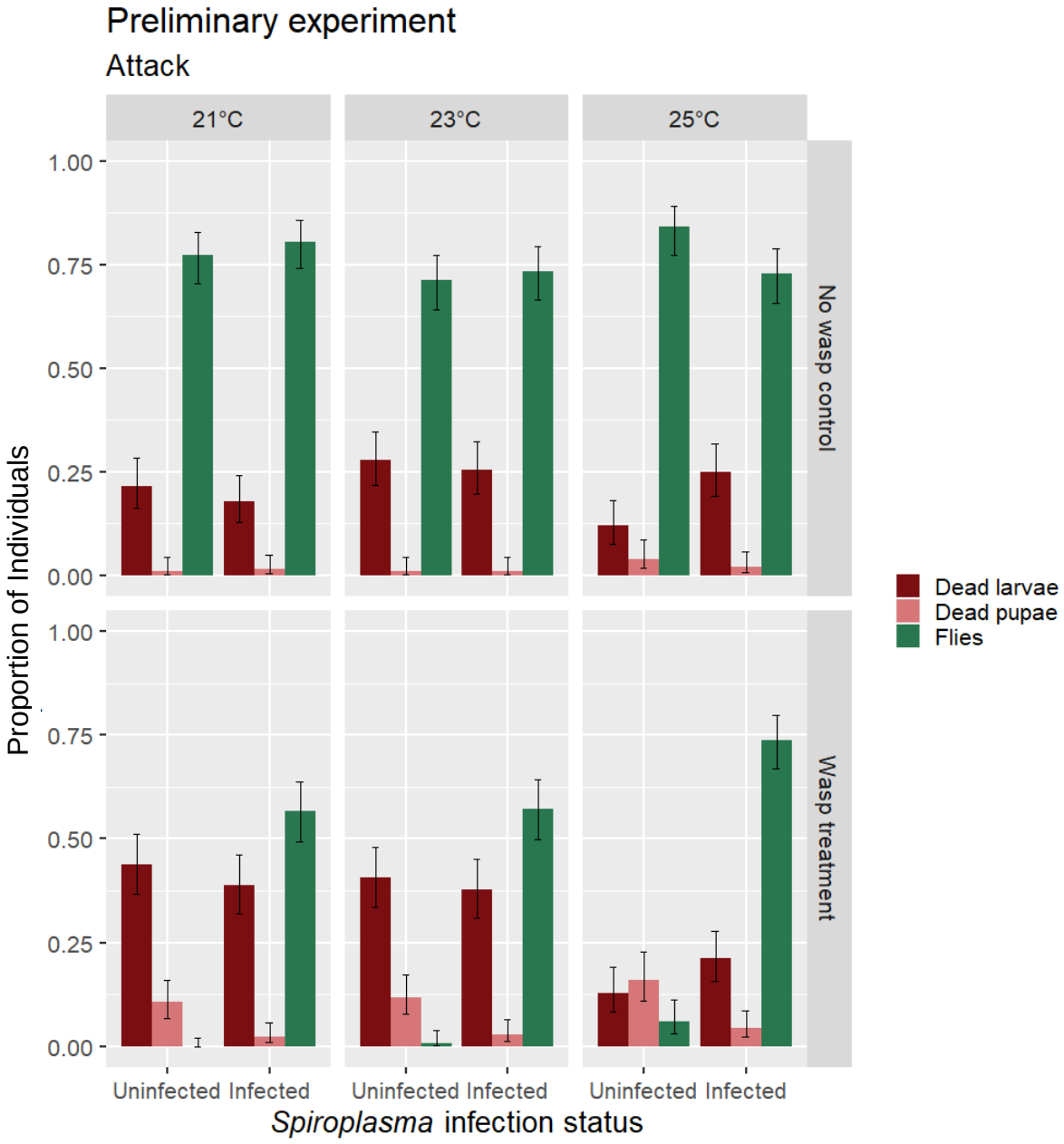

Figure 4.3: Proportion of dead larvae (red), dead pupae (pink) and emerging flies (green) *Spiroplasma*-infected and -uninfected F_2 *Drosophila melanogaster* under the preliminary 'attack' temperature regime. The temperature indicates the temperature during the two days of wasp attack only. Top panels represent no wasp controls and bottom panels represent vials attacked with *Leptopilina boulardi* (Lb17) wasps. Error bars represent 95% binomial confidence intervals.

4.4.1.2 *'Attack and protection' temperature regime*

Mean larva-to-adult fly survival in the absence of wasps was > 69% across all treatments (Figure 4.4). There was no significant effect of temperature (χ^2= 2.369, d.f. = 1, p = 0.124) or *Spiroplasma* infection status on fly larva-to-adult survival (χ^2= 1.862, d.f. = 1, p = 0.172). However, there was a significant interaction between *Spiroplasma* infection status and temperature on fly larva-to-adult survival (χ^2= 16.576, d.f. = 1, p < 0.001). At 25°C fly larva-to-adult survival was 29.56% higher in *Spiroplasma*-infected flies compared to uninfected controls. In contrast, survival of *Spiroplasma*-infected flies was observed to be 2.99% lower at 23°C and 7.76% lower at 21°C compared to uninfected controls.

In the presence of wasps, *Spiroplasma* infection status had a strong significant effect on fly larva-to-adult survival, increasing fly larva-to-adult survival from <5% to 69.7% at 25°C, 0% to 54.7% at 23°C and <2% to 72.7% at 21°C (χ^2= 102.721, d.f. = 1, p < 0.001; Figure 4.4). There was no significant effect of temperature (χ^2= 0.0098, d.f. = 1, p = 0.921) nor a significant interaction between *Spiroplasma* infection status and temperature (χ^2= 2.530, d.f. = 1, p = 0.112).

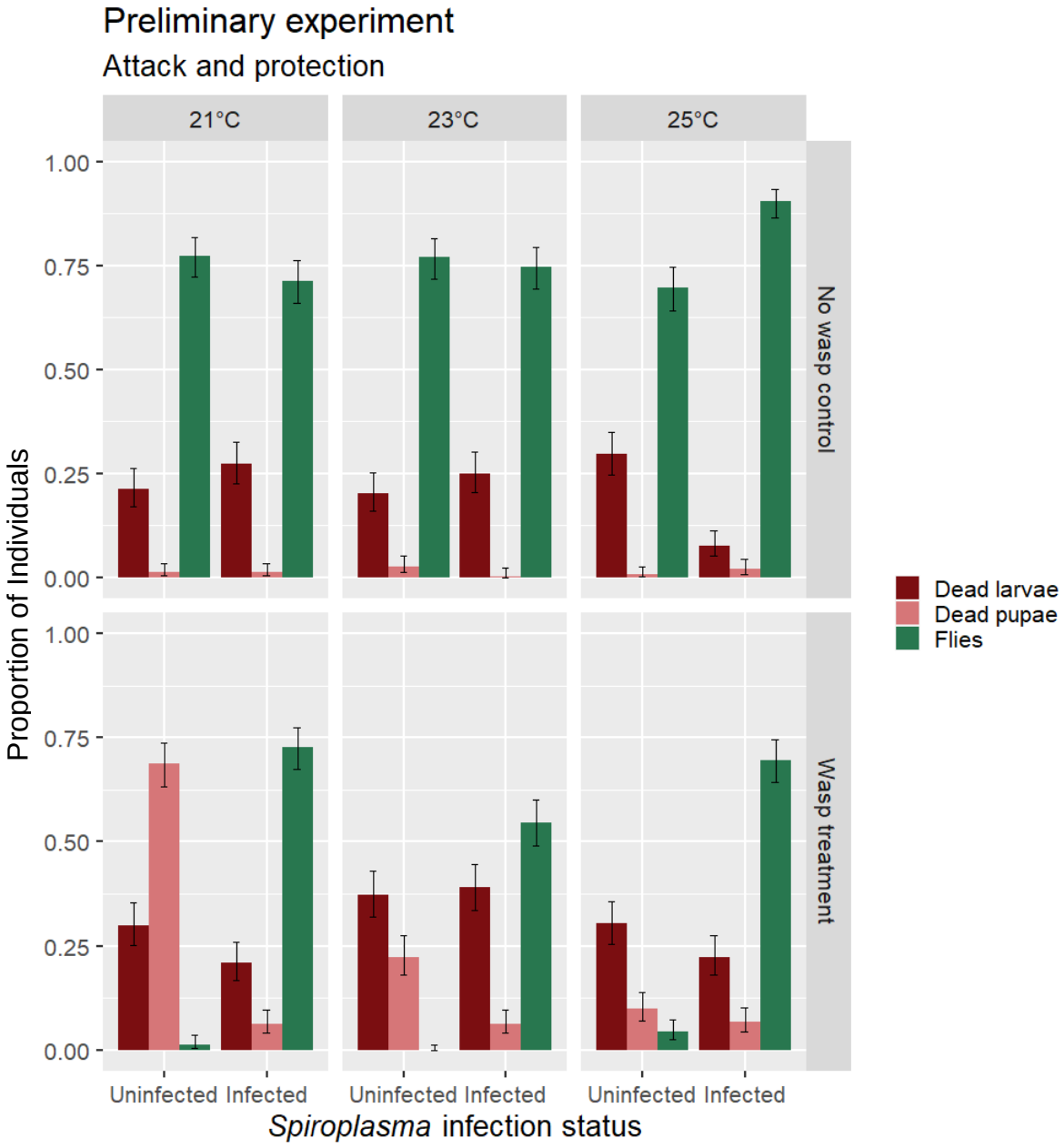

Figure 4.4: Proportion of dead larvae (red), dead pupae (pink) and emerging flies (green) for *Spiroplasma*-infected and -uninfected F_2 *Drosophila melanogaster* under the preliminary 'attack and protection' temperature regime. Flies were reared through a single generation at 25°C before the F_1 larvae were attacked and left to develop at either 21°C, 23°C and 25°C. Top panels represent no wasp controls and bottom panels represent vials attacked with *Leptopilina boulardi* (Lb17) wasps. Error bars represent 95% binomial confidence intervals.

4.4.1.3 *'Constant' temperature regime*

Mean larva-to-adult fly survival in the absence of wasps was >75% across all treatments (Figure 4.5). Temperature had a small but significant effect on fly larva-to-adult survival (χ^2= 4.252, d.f. = 1, p = 0.039). The average survival of *Spiroplasma*-infected and -uninfected flies reared at 21°C was 8.61% lower than flies reared at 23°C and 6.96% lower than flies reared at 25°C. There was no significant effect of *Spiroplasma* infection status (χ^2= 0.457, d.f. = 1, p = 0.499), nor a significant interaction between *Spiroplasma* infection status and temperature (χ^2= 0.680, d.f. = 1, p = 0.409).

In the presence of wasps, *Spiroplasma* infection had a strong significant effect on fly larva-to-adult survival, increasing fly larva-to-adult survival from 3% to 68.7% at 25°C, 0% to 46.3% at 23°C and <1% to 36% at 21°C (χ^2= 65.68, d.f. = 1, p < 0.001; Figure 4.5). Temperature also had a strong significant effect on fly larva-to-adult survival (χ^2= 179.982, d.f. = 1, p < 0.001). Specifically, *Spiroplasma*-infected fly larva-to-adult survival at 21°C was 32.61% lower than at 23°C and 47.6% lower than at 25°C, indicating that cooler temperatures reduce *Spiroplasma*-mediated fly larva-to-adult survival. The interaction between *Spiroplasma* infection and temperature was not found to be significant (χ^2= 2.349, d.f. = 1, p = 0.125).

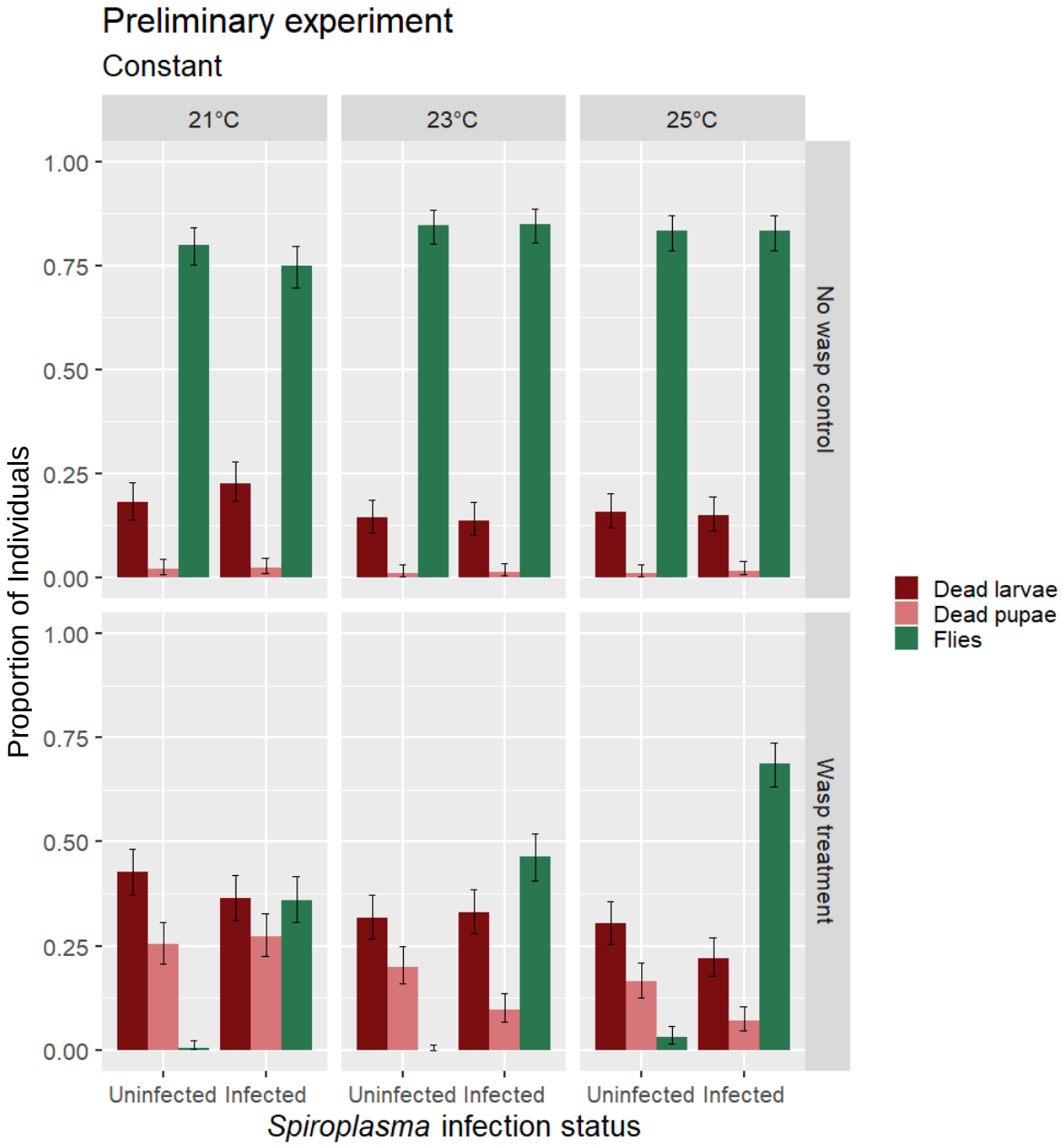

Figure 4.5: Proportion of dead larvae (red), dead pupae (pink) and emerging flies (green) for *Spiroplasma* infected and -uninfected F_2 *Drosophila melanogaster* under the preliminary 'constant' temperature regime. Flies were reared through a single generation and the F_1 larvae were attacked and left to develop at either 21°C, 23°C and 25°C. Top panels represent no wasp controls and bottom panels represent vials attacked with *Leptopilina boulardi* (Lb17) wasps. Error bars represent 95% binomial confidence intervals.

4.4.2 *The effect of temperature on* Spiroplasma*-mediated protection: integrated experiment*

4.4.2.1 *'Pre-attack' temperature regime*

In the absence of wasps, the mean larva-to-adult fly survival was > 68% across all treatments (Figure 4.6). There was no significant effect of temperature (χ^2= 1.528, d.f. = 1, p = 0.216), nor a significant interaction between *Spiroplasma* infection and temperature on fly larva-to-adult survival (χ^2= 0.397, d.f. = 1, p = 0.529). However, there was a significant effect of *Spiroplasma* infection status on fly larva-to-adult survival (χ^2= 16.749, d.f. = 1, p < 0.001). The mean fly larva-to-adult survival of *Spiroplasma*-infected flies was 13% lower than *Spiroplasma*-uninfected flies.

In the presence of wasps, *Spiroplasma* infection had a strong significant effect on fly larva-to-adult survival, increasing fly larva-to-adult survival from <0.4% to 56.7% at 25°C, 0% to 46.3% at 23°C and 0% to 14.8% at 21°C (χ^2= 36.287, d.f. = 1, p < 0.001; Figure 4.6). Temperature also had a significant effect on fly larva-to-adult survival (χ^2= 60.628, d.f. = 1, p < 0.001). Specifically, fly larva-to-adult survival at 21°C was 48.16% lower than at 23°C and 57.67% lower than at 25°C, indicating that cooler temperatures during the pre-attack stage reduce *Spiroplasma*-mediated fly larva-to-adult survival. The interaction between *Spiroplasma* infection and temperature was not found to be significant (χ^2 <0.001, d.f. = 1, p = 0.994).

Fly *Spiroplasma* infection had a strong significant effect on wasp larva-to-adult survival (χ^2= 242.778, d.f. = 1, p < 0.001; Figure 4.6). Across all temperatures, *Spiroplasma* infection decreased wasp larva-to-adult survival by 85% on average. However, the effect of fly *Spiroplasma* infection was lower at 21°C than at 23°C or 25°C resulting in a significant interaction between *Spiroplasma* infection and temperature (% decrease: 63.52% at 21°C; 96.14% at 23°C; 95.62% at 25°C, χ^2= 136.059, d.f. = 1, p < 0.001). Temperature also had a significant effect on wasp larva-to-adult survival (χ^2= 10.500, d.f.

= 1, $p < 0.01$). Average wasp larva-to-adult survival at 25°C was 11.5% lower than at 23°C and 24.7% lower than at 21°C across both *Spiroplasma*-infected and uninfected flies.

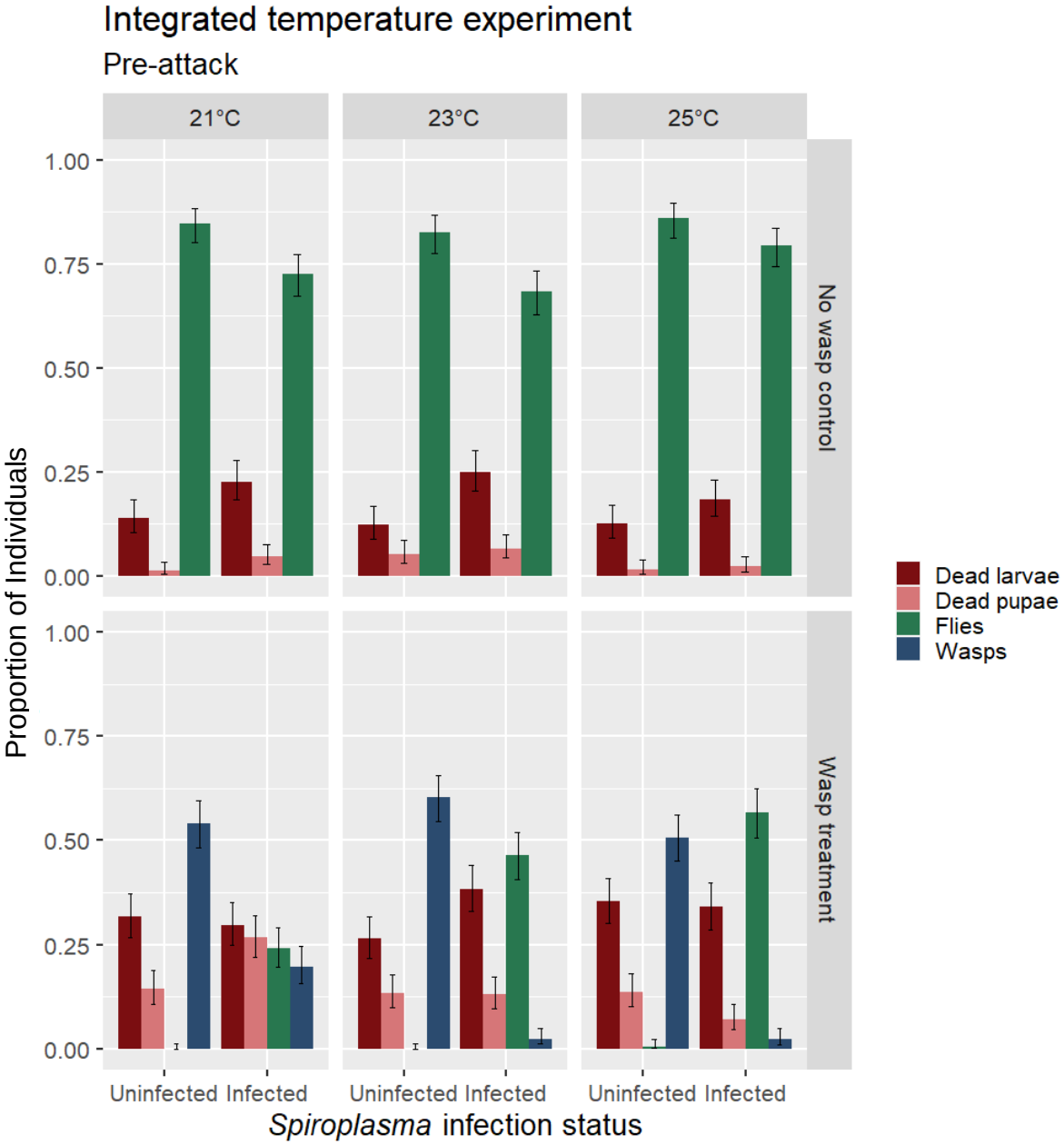

Figure 4.6: Proportion of dead larvae (red), dead pupae (pink), emerging flies (green) and emerging wasps (blue) for *Spiroplasma*-infected and -uninfected F_2 *Drosophila melanogaster* under the 'pre-attack' temperature regime. Flies were reared through a single generation at either 21°C, 23°C and 25°C before the F_1 larvae from all temperature treatments were attacked and left to develop at 25°C. Top panels represent no wasp controls and bottom panels represent vials attacked with *Leptopilina boulardi* (Lb17) wasps. Error bars represent 95% binomial confidence intervals.

4.4.2.2 *'Attack and protection' temperature regime*

In the absence of wasps mean larva-to-adult fly survival was > 68% across all treatments (Figure 4.7). There was no significant effect of temperature (χ^2= 0.069, d.f. = 1, p = 0.793), nor a significant interaction between *Spiroplasma* infection and temperature on fly larva-to-adult survival (χ^2= 0.694, d.f. = 1, p = 0.405). However, there was a significant effect of *Spiroplasma* infection status on fly larva-to-adult survival (χ^2= 5.943, d.f. = 1, p = 0.015). The mean fly larva-to-adult survival of *Spiroplasma*-uninfected flies was 9.4% lower than *Spiroplasma*-infected flies.

In the presence of wasps, *Spiroplasma* infection had a strong significant effect on fly larva-to-adult survival, increasing fly larva-to-adult survival from <3% to 69.5% (χ^2= 95.187, d.f. = 1, p < 0.01; Figure 4.7). There was no significant effect of temperature (χ^2= 2.726, d.f. = 1, p = 0.0987), nor a significant interaction between temperature and *Spiroplasma* infection (χ^2= 0.0324, d.f. = 1, p = 0.857).

Fly *Spiroplasma* infection had a strong significant effect on wasp larva-to-adult survival (χ^2= 157.966, d.f. = 1, p < 0.001; Figure 4.7). Across all temperatures, *Spiroplasma* infection decreased wasp larva-to-adult survival by 94% on average. There was a weak significant interaction between fly *Spiroplasma* infection and temperature (χ^2= 4.023, d.f. = 1, p < 0.001). Specifically, the effect of *Spiroplasma* infection was greater at 25°C reducing wasp larva-to-adult survival from 56.3% to 2.5%, compared to reducing wasp survival from 34.3% to 2.67% at 23°C and from 0.667% to 7% at 21°C. Temperature had a strong significant effect on wasp larva-to-adult survival (χ^2= 143.509, d.f. = 1, p < 0.001). Average wasp larva-to-adult survival at 25°C was 43% higher than at 23°C and 88% higher than at 21°C across both *Spiroplasma*-infected and uninfected flies.

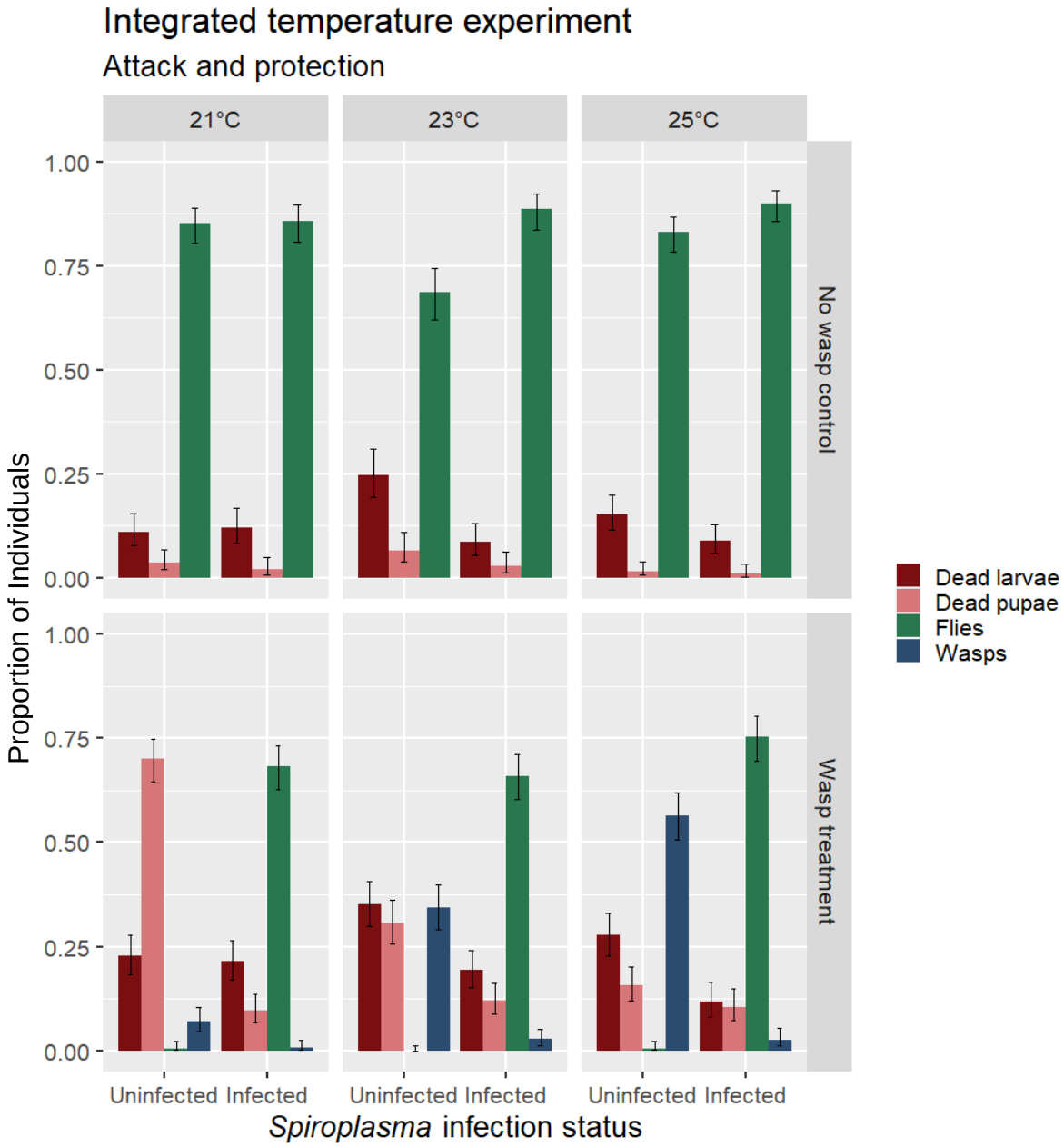

Figure 4.7: Proportion of dead larvae (red), dead pupae (pink), emerging flies (green) and emerging wasps (blue) for *Spiroplasma*-infected and -uninfected F_2 *Drosophila melanogaster* under the 'attack and protection' temperature regime. Flies were reared through a single generation at 25°C before the F_1 larvae were attacked and left to develop at either 21°C, 23°C and 25°C. Top panels represent no wasp controls and bottom panels represent vials attacked with *Leptopilina boulardi* (Lb17) wasps. Error bars represent 95% binomial confidence intervals.

4.4.2.3 *'Constant' temperature regime*

In the absence of wasps, the mean larva-to-adult fly survival was > 70% across all treatments (Figure 4.8). There was no significant effect of temperature (χ^2= 0.007, d.f. = 1, p = 0.931), nor a significant interaction between *Spiroplasma* infection and temperature on fly larva-to-adult survival (χ^2= 1.522, d.f. = 1, p = 0.217). However, there was a small but significant effect of *Spiroplasma* infection status on fly larva-to-adult survival (χ^2= 7.401, d.f. = 1, p = 0.007). The mean fly larva-to-adult survival of *Spiroplasma*-infected flies was 9.1% lower than *Spiroplasma*-uninfected flies.

In the presence of wasps, *Spiroplasma* infection had a strong significant effect on fly larva-to-adult survival, increasing fly larva-to-adult survival from <0.7% to 57% at 25°C, 0% to 39.3% at 23°C and <0.4% to 20.3% at 21°C (χ^2= 85.227, d.f. = 1, p < 0.01; Figure 4.8). Temperature also had a significant effect on fly larva-to-adult survival (χ^2= 80.452, d.f. = 1, p < 0.01). Specifically, *Spiroplasma*-infected fly survival at 21°C was 48.35% lower than at 23°C and 64.39% lower than at 25°C, indicating that cooler temperatures reduce *Spiroplasma*-mediated fly larva-to-adult survival. The interaction between *Spiroplasma* infection and temperature was not found to be significant (χ^2= 0.146, d.f. = 1, p = 0.702).

Fly *Spiroplasma* infection had a strong significant effect on wasp larva-to-adult survival (χ^2= 183.611, d.f. = 1, p < 0.001; Figure 4.8). Across all temperatures, *Spiroplasma* infection decreased wasp larva-to-adult survival by 92% on average. There was a significant interaction between fly *Spiroplasma* infection and temperature (χ^2= 35.705, d.f. = 1, p < 0.001). Specifically, the effect of *Spiroplasma* infection was greater at 25°C, reducing wasp larva-to-adult survival from 62% to 0.3% compared to 23°C (48.7% to 6.3%) and 21°C (11% to 3%). Temperature also had a strong significant effect on wasp larva-to-adult survival (χ^2= 109.250, d.f. = 1, p < 0.001). Average wasp larva-to-adult survival at 25°C was 12% higher than at 23°C and 78% higher than at 21°C across both *Spiroplasma*-infected and uninfected flies.

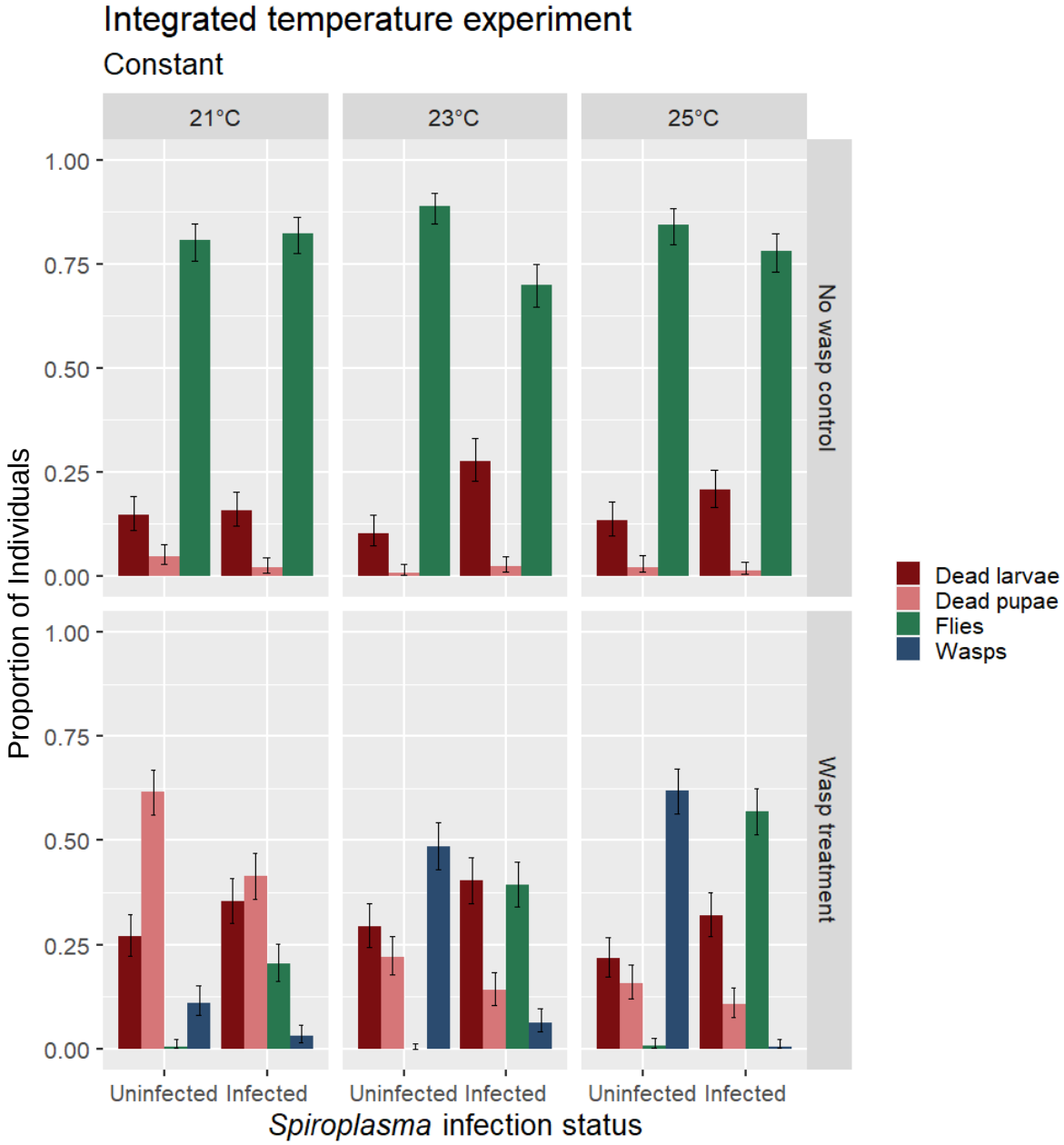

Figure 4.8: Proportion of dead larvae (red), dead pupae (pink), emerging flies (green) and emerging wasps (blue) for *Spiroplasma*-infected and -uninfected F_2 *Drosophila melanogaster* under the 'constant' temperature regime. Flies were reared through a single generation and the F_1 larvae were attacked and left to develop at either 21°C, 23°C and 25°C. Top panels represent no wasp controls and bottom panels represent vials attacked with *Leptopilina boulardi* (Lb17) wasps. Error bars represent 95% binomial confidence intervals.

4.4.3 *The effect of temperature on sex ratio*

The sex of each fly emerging from the *Spiroplasma*-infected 21°C, 23°C and 25°C treatments from each experimental temperature regime was recorded to determine the impact of temperature on the sex ratio. In the constant and pre-attack treatments, male survival increased from <2% at 25°C to 15-19% at 21°C (Table 4.1; Table 4.3). However, in the presence of wasps, male survival was 0% for both 21°C and 25°C, indicating that wasps were killing any *Spiroplasma*-uninfected males produced (Table 4.1; Table 4.3). The same effect was observed in the attack and protection treatment where sporadic males were produced in each temperature treatment but subsequently eliminated by wasps (Table 4.2).

Table 4.1: Sex ratio of *Spiroplasma*-infected *Drosophila melanogaster* emerging from the 21°C, 23°C and 25°C treatments in the 'pre-attack' temperature regime.

Temperature	Wasp treatment	Total fly	Total male	% male	Total female	% female
25°C	Lb-	238	3	1.26	235	98.74
	Lb+	153	0	0.00	153	100.00
23°C	Lb-	205	9	4.39	196	95.61
	Lb+	139	0	0.00	139	100.00
21°C	Lb-	218	41	18.81	177	81.19
	Lb+	72	0	0.00	72	100.00

Table 4.2: Sex ratio of *Spiroplasma*-infected *Drosophila melanogaster* emerging from the 21°C, 23°C and 25°C treatments in the 'attack and protection' temperature regime.

Temperature	Wasp treatment	Total fly	Total male	% male	Total female	% female
25°C	Lb-	243	4	1.65	239	98.35
	Lb+	181	0	0.00	181	100.00
23°C	Lb-	186	3	1.61	183	98.39
	Lb+	198	0	0.00	198	100.00
21°C	Lb-	206	4	1.94	202	98.06
	Lb+	183	0	0.00	183	100.00

Table 4.3: Sex ratio of *Spiroplasma*-infected *Drosophila melanogaster* emerging from the 21°C, 23°C and 25°C treatments in the 'constant' temperature regime.

Temperature	Wasp treatment	Total fly	Total male	% male	Total female	% female
25°C	Lb-	234	1	0.43	233	99.57
	Lb+	171	0	0.00	171	100.00
23°C	Lb-	210	5	2.38	205	97.62
	Lb+	118	0	0.00	118	100.00
21°C	Lb-	247	38	15.38	209	84.62
	Lb+	61	0	0.00	61	100.00

4.4.4 *The effect of temperature and wasp attack on* Spiroplasma *titre*

As expected, *Spiroplasma*-infected flies reared at cooler temperatures were found to have lower *Spiroplasma* titre than flies reared at warmer temperatures (Figure 4.9). Flies reared at 21°C were found to have 1.56× lower *Spiroplasma* titre than flies reared at 23°C and 2.85× lower *Spiroplasma* titre than flies reared at 25°C (χ^2= 46.319, d.f. = 1, $p < 0.01$). There was no significant effect of wasp attack on *Spiroplasma* titre (χ^2= 0.886, d.f. = 1, p = 0.347). However, the effect of wasp attack did depend on temperature, reflected in a significant interaction term between temperature and wasp presence (χ^2= 4.226, d.f. = 1, p = 0.040). At 25°C non-attacked flies were found to have 11% lower *Spiroplasma* titre than wasp attacked flies. However, at 23°C and 21°C non-attacked flies were found to have higher *Spiroplasma* titre than wasp attacked flies (34% and 92% higher respectively). The large increase observed at 21°C was likely due to the number of *Spiroplasma*-negative flies observed in the 21°C non-wasp attacked treatment (21°C Lb-: 8/21 *Spiroplasma* negatives vs. 21°C Lb+: 0/19 *Spiroplasma* negatives), indicating that *Spiroplasma* infection segregates at 21°C, but uninfected individuals created are then killed by wasp attack.

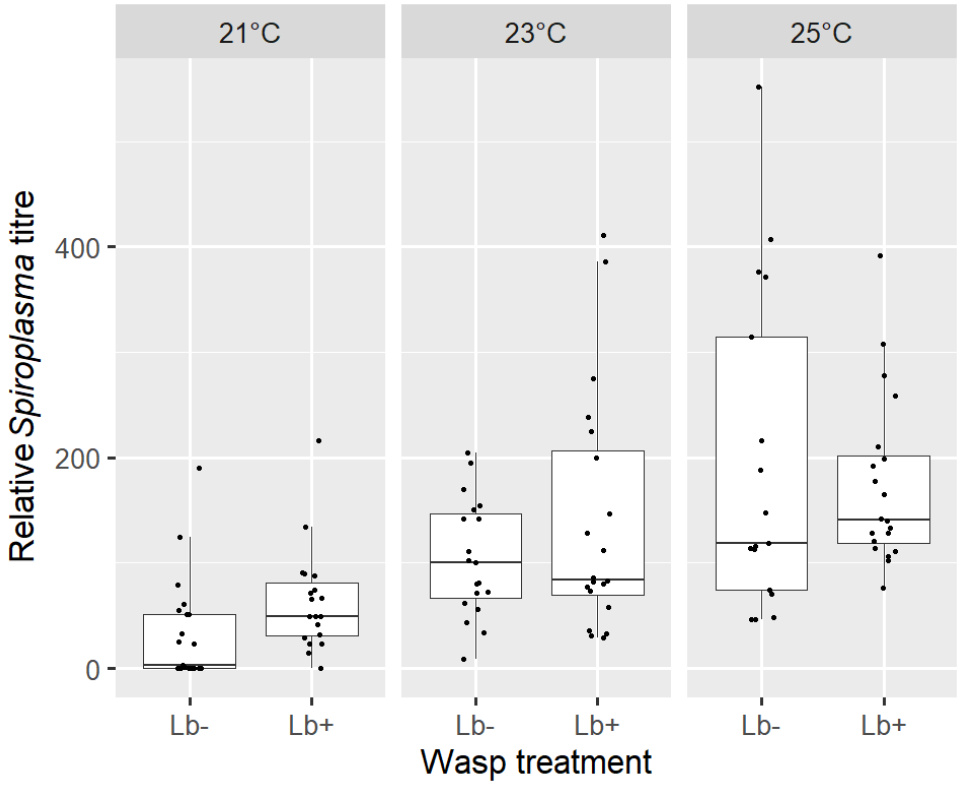

Figure 4.9: Relative *Spiroplasma* titre of wasp-attacked (Lb+) and no wasp attack control (Lb-) *Spiroplasma*-infected *Drosophila melanogaster* emerging from the 21°C, 23°C and 25°C treatments in the constant temperature regime. The box plots display the upper and lower quartiles, the median and the range. Each dot represents a single fly.

4.4.5 *The effect of temperature on wasp oviposition*

The average number of wasp eggs laid into a fly larva across a 48 h period of parasitisation was >3 but <4 for all treatments (Figure 4.10). There was no significant effect of temperature on the number of wasp eggs laid into fly larvae ($\chi^2 = 2.52$, d.f. = 2, $p = 0.284$).

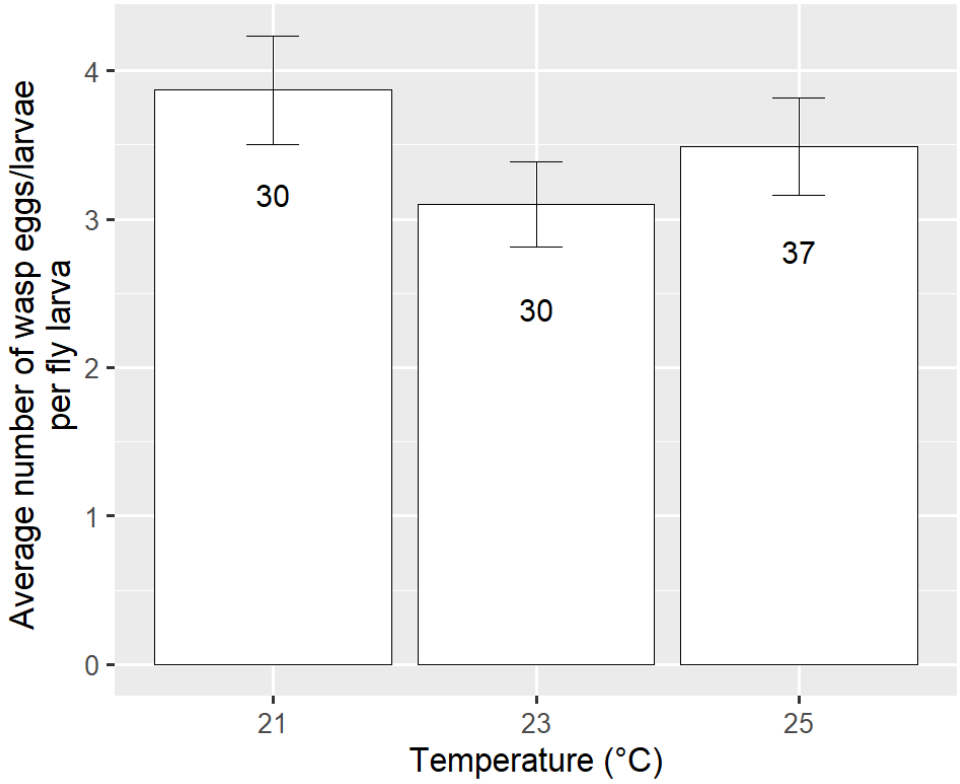

Figure 4.10: The average number of wasp eggs/larvae in *Drosophila melanogaster* (Canton-S) larvae following 48 h of parasitisation by *Leptopilina boulardi* (Lb17) at three different temperatures: 21°C, 23°C and 25°C. Error bars depict ± SE.

4.5 DISCUSSION

The outcome of natural enemy attack in insects can be mediated by the presence or absence of defensive heritable symbionts residing within the host. Beyond symbiont presence, studies have shown that genetic and environmental factors can also mediate the outcome of symbiont-mediated defence (Doremus *et al.,* 2018; Vorburger and Perlman, 2018; Corbin *et al.,* 2021; Chrostek *et al.,* 2021). Thermal environment is of particular importance to symbiont-mediated traits, with minor differences in temperature resulting in major changes in the outcome of the interaction (Higashi *et al.,* 2020). To date, the effect of thermal environment on *Spiroplasma*-mediated protection is limited to a single study whereby protection was ablated at cooler temperatures (Corbin *et al.,* 2021). However, as the experimental temperature was over the entire protection assay, it is difficult to discern the mechanistic process behind the loss of protection, as temperature may have impacted upon many factors in the experiment. In this study, I determined the impact of the thermal environment on *Spiroplasma*-mediated protection in *Drosophila melanogaster* by considering the effect of temperature on fly survival when flies were exposed to an experimental temperature before, during and after wasp attack. I found that *Spiroplasma*-mediated fly survival was ~58% weaker when parental flies were reared through a generation at 21°C, compared to when parental flies were reared through a generation at 25°C before their offspring were exposed to wasps under standard 25°C conditions. However, no effect of thermal environment was observed when the offspring were exposed to a 21°C temperature solely during and after wasp attack. Thus, the thermal environment before, and not during or after wasp attack, determines the outcome of *Spiroplasma*-mediated protection.

The temperature before wasp attack was observed to be most important for the outcome of *Spiroplasma*-mediated fly survival. The absence of any effect of temperature on *Spiroplasma*-mediated fly survival during or after wasp attack suggests that the effect

observed is most likely mediated predominantly by thermal effects on *Spiroplasma* titre and not by properties of the wasp or *Spiroplasma* defence. Indeed, relative *Spiroplasma* titre of no wasp control F_2 adults, reared through 21°C in the constant temperature regime, was found to be ~83% lower than in adults reared through the 25°C treatment. Further, there was evidence that *Spiroplasma* infection was segregating at 21°C demonstrated by the substantial production of males and *Spiroplasma*-uninfected females in the no wasp attack control treatment. Collectively, these results demonstrate that lower *Spiroplasma* titre and loss of infection are predominately responsible for the weak *Spiroplasma*-mediated protection observed at 21°C. This result is perhaps unsurprising given the repeated evidence that *Spiroplasma* is a cool sensitive symbiont, whereby its transmission efficiency and phenotype expression are negatively impacted by lower temperatures (Montenegro and Klaczko 2004; Corbin *et al.,* 2021; Osaka *et al.,* 2008). Nevertheless, it remains possible that temperature may be additionally affecting other properties of *Spiroplasma* defence, as is suspected in cool sensitivity of *Wolbachia* protection against virus attack (Chrostek *et al.,* 2021).

Whilst it is not possible to precisely partition the impact of *Spiroplasma* loss and low titre on the reduced protection phenotype seen when flies are exposed to 21°C at the pre-attack stage, it appears likely that the effect largely derives from *Spiroplasma* loss in flies. Within the 'pre-attack' integrated treatment (Figure 4.6), 41 males and 177 females emerged out of the no wasp control treatment. Assuming males are uninfected and that a similar number of females are also uninfected, 82/218 flies (c. 37%) had likely lost infection at the point of embryogenesis. For wasp survival, controls without *Spiroplasma* showed a wasp survival rate of 54%. Thus, the expected wasp survival rate solely from uninfected individuals in the attacked treatment would be 37% (uninfected flies) x 54% (wasp survival in absence of *Spiroplasma*) = c. 20%. Thus, this value is equal to the actual 20% wasp survival observed, leading to the conclusion that wasps complete development only in *Spiroplasma* uninfected individuals.

We can also perform this analysis for fly survival. Assuming fly survival can only occur in the presence of *Spiroplasma*, the fly survival of 24% should be normalised to 63% (100% - 37% uninfected flies) of flies being infected. Thus, infected flies have a c. 38% chance of surviving attack in the presence of *Spiroplasma*. These data indicate that protection is lowered but to a lesser extent when uninfected flies are removed from the 21°C treatment compared to the other temperature treatments (fly survival of 46% and 57% at 23°C and 25°C respectively during the pre-attack period). In summary, it appears that the presence of *Spiroplasma* is enough to kill all developing wasps at all temperatures. However, although the effect of temperature on *Spiroplasma*-mediated fly survival observed may be largely a consequence of *Spiroplasma* loss, we still cannot rule out a minor effect of low *Spiroplasma* titre. It may be that a low density of *Spiroplasma* is enough to kill developing wasps, but this takes longer, allowing the wasp to cause more damage to the fly resulting in reduced fly rescue.

As the pre-attack treatment was virtually a single generation and only included a single day (egg development) of the second generation, it is most parsimonious to conclude the effect of temperature on *Spiroplasma*-mediated defence is transgenerational. The thermal environment experienced by the mother determines the outcome of *Spiroplasma*-mediated protection in her daughters. This is likely mediated by mothers with reduced *Spiroplasma* titre transmitting lower *Spiroplasma* densities to their offspring, subsequently leading to reduced protection from wasps. Indeed, this has shown to be the case in the *Drosophila*-*Wolbachia* symbiosis. For instance, female *Drosophila innubila* infected with low *Wolbachia* titres, produce daughters with low *Wolbachia* titre which subsequently produce offspring with lower male-killing expression (Dyer and Jaenike, 2005). Although we cannot be completely certain, our results strongly indicate that *Spiroplasma*-mediated protection against wasps is influenced by transgenerational effects, likely mediated by changes in *Spiroplasma* density of mothers. To confirm this, future work should repeat the pre-attack treatment

but exclude the single day of egg development under the experimental temperature regime to confirm whether *Spiroplasma*-mediated fly survival remains weaker at 21°C compared to 25°C. In addition, further work should determine whether the titre of *Spiroplasma* transmitted to offspring is proportional to the *Spiroplasma* titre of the mother. Further, the result raises the possibility that *Spiroplasma* titre is an epigenetic trait which can be selected upon across generations.

The presence of wasps extends the thermal envelope of *Spiroplasma* infection by selecting out *Spiroplasma*-uninfected individuals segregating the infection under cooler temperatures. Within the sample of flies taken for qPCR analysis, 38% of flies were identified as uninfected in the 21°C non-wasp attacked treatment compared to 0% in the 21°C wasp-attack treatment. This result indicates that *Spiroplasma* infection is segregating when flies are reared at 21°C, but the uninfected individuals created are subsequently killed in the presence of wasps. This result was also reflected in the sex ratio of flies emerging out of the 21°C treatments. In the absence of wasps ~20% of flies were found to be male, whereas in the presence of wasps there were no males identified indicating that the presence of wasps selects out uninfected males which are unable to defend against wasp attack. This demonstrates that under cooler temperatures in which *Spiroplasma* infection starts to segregate, the presence of wasps can select for *Spiroplasma*-infected individuals and permit infection to persist over generations at cooler temperatures.

These data apply to presence/absence of infection – wasp attack selectively removes any individuals where the symbiont has been lost through segregation. In addition, it is possible that the presence of wasps may also be selecting for females with higher *Spiroplasma* titre. Where higher titre leads to better protection, it is logical that survivors from the wasp attack treatment would have higher titre than individuals that are infected but not subject to attack. However, despite a strong effect of temperature on *Spiroplasma*

titre, and temperature – protection links, there was no evidence for an effect of wasp attack on *Spiroplasma* titre. One caveat here is that *Spiroplasma* titre was measured in the F_2 adults and not at the point of wasp attack. It could be that infection titre was higher for survivors at the point of attack, but wasp attack itself then reduced titre. This would be possible if, for instance, lipid competition occurred between wasp and symbiont, as has been suggested (Paredes *et al.,* 2016).

In summary, our work has extended our understanding of how the thermal environment affects *Spiroplasma*-mediated protection in *Drosophila*. Our results reveal that the developmental thermal environment of the mother is more important to the outcome of *Spiroplasma*-mediated protection against wasps than the thermal environment of individuals during or after attack. This finding has implications for *Spiroplasma* dynamics in natural populations. Not only should *Spiroplasma* dynamics be considered in relation to the strain of circulating parasitoid wasp strains (as shown to be important in Chapter 2), but also the historical thermal environment they have been exposed to. Indeed, it should be noted that our study was only conducted on one strain of *Leptopilina boulardi* wasp and that more complex interactions between the thermal environment and wasp strain/species may exist (G x G x E interactions). Future work should consider the effect of the thermal environment on other strains of *Leptopilina* wasp to understand whether more complex G x E interactions are important in this system.

Chapter 5

General Discussion

5.1 SUMMARY OF FINDINGS

Defensive symbiosis is a common feature of insects. However, among the known systems, the aphid protective symbioses are by far the most well understood in terms of their mechanistic underpinnings and evolutionary ecology. In aphids, beyond the presence or absence of the defensive symbiont, many factors contribute to the outcome of symbiont-mediated protection (e.g. enemy species/strain; host strain; symbiont strain; environmental factors). The aphid symbioses are interesting both for the sheer diversity of symbioses, and also for the reduced nature of endogenous aphid immune responses, compared to other insects (Gerardo *et al.,* 2010). The aim of this thesis was to extend our knowledge beyond the aphid systems and determine the degree of complexity in the *Drosophila-Leptopilina-Spiroplasma* interaction for which little is understood about its evolutionary ecology. In addition, the dynamics of protection will relate not only to whether flies survive attack, but their onward fecundity and fertility – a factor not examined in most protective symbioses. In answering these questions, I would a) determine whether the complexity of the aphid protective symbiosis was general or specific and b), better understand the *Drosophila* protective symbiosis to develop into a model not solely at the mechanistic level, but also in terms of its evolutionary ecology.

5.1.1 *Chapter 2: The strength of* Spiroplasma*-mediated protection is dependent on the strain of* Leptopilina *wasp*

Previous work on protection of *Drosophila melanogaster* by *Spiroplasma* had utilised a single wasp strain per species tested, which was held to be representative for the wasp species more widely. I presented evidence that the strength of *Spiroplasma*-mediated protection is dependent on the strain of *Leptopilina heterotoma* wasp. I observed that

Spiroplasma-mediated fly survival against the Lh-Mad and Lh-Fr wasp strains was 7 times greater than survival against the Lh14 wasp strain. This analysis was also conducted in the presence of ethanol, an environmental factor known to also protect *Drosophila* from parasitoid wasp attack. However, I found no evidence that ethanol protected against wasp attack in this study.

Following wasp attack, I determined the fertility of female wasp-attacked survivors compared to female flies which had not been exposed to wasps. Wasp attacked survivors were observed to have reduced fertility, measured as the proportion of females able to produce progeny. Additionally, of the females that were able to produce progeny, wasp attacked survivors produced fewer progeny compared to flies which had not been exposed to wasps.

From these data, I produced an overall protection index combining the data on fly survival, fly fertility and fecundity to produce an overall value of the protection afforded by *Spiroplasma* against the different wasp strains. The overall protection afforded by *Spiroplasma* for the Lh14 strain of wasp was estimated to be <4% whereas protection was higher against the Lh-Mad (9%) strain and Lh-Fr strain (21%). Thus, I conclude that the strain of attacking wasp is important for the strength of *Spiroplasma*-mediated protection. With the hypothesis that differential oviposition of the different wasp strains may be leading to the differences observed in *Spiroplasma*-mediated protection, I conducted an experiment to determine the number of eggs laid by each wasp into single larva. I found that each wasp strain laid on average, 2 eggs into each larva in experimental design. Thus, I conclude that differences in wasp oviposition are not responsible for the differences in *Spiroplasma* protection across strains of *L. heterotoma,* which is thus likely to reflect either differences in wasp venom or in the surface properties of the eggs/larvae.

5.1.2 *Chapter 3: The strength of* Spiroplasma*-mediated fly survival is independent on the strain of* Spiroplasma

Spiroplasma-mediated protection has been assayed in different laboratories, and found to differ against the same wasp strain. A potential cause of this difference in protection is the use of different *Spiroplasma* isolates – from Brazil and Uganda. These results may reflect different properties of the *Spiroplasma*, or different protocols/environments in the different laboratories. To reconcile this, I examined protection from these two strains of *Spiroplasma* in a common laboratory environment. I determined the strength of *Spiroplasma*-mediated fly survival and wasp success against two strains of *L. heterotoma* (Lh-Mad and Lh-Fr) and two strains of *L. boulardi* (Lb17 and NSRef), using two strains of *Spiroplasma* (MSRO-Br and MSRO-Ug). I found no evidence to suggest that *Spiroplasma*-mediated fly survival differed between the two strains of *Spiroplasma* against any of the wasp strains tested. Following wasp attack, I determined the relative fertility and fecundity of wasp attacked survivors compared to non-wasp attacked controls to produce an overall protection index of the protection afforded by *Spiroplasma* as in chapter 2. I found that *Spiroplasma*-mediated protection was higher against the Brazilian strain of *Spiroplasma*. However, these differences between *Spiroplasma* strains are not due to differences in survival, but exclusively driven by differences in the relative fertility of wasp attacked survivors compared to their non-attacked comparators.

5.1.3 *Chapter 4: The developmental temperature of mothers, and not the thermal environment of offspring during or after wasp attack, determines the outcome of* Spiroplasma*-mediated fly survival*

Previous work on the protective symbiosis in *Drosophila hydei* indicated a high level of thermal sensitivity to protection, with *Spiroplasma* failing to rescue flies at lower temperatures (18°C). In this chapter, I examined the thermal sensitivity of protection in the *D. melanogaster* system, which also allowed more nuanced examination of how historical and current thermal environments affected protection. Specifically, I found

that the developmental temperature of the mothers, and not the temperature of the offspring during or after wasp attack, strongly determines the outcome of *Spiroplasma*-mediated fly survival. *Spiroplasma*-mediated fly survival was found to be ~58% weaker when parental flies were reared at 21°C before exposure to wasps, compared to parental flies reared at 25°C. However, there was no effect of thermal environment on protection when mothers were reared at 25°C, and their progeny exposed to 21°C during and after wasp attack. From qPCR analysis, I find evidence that the effect of developmental temperature on *Spiroplasma*-mediated fly survival is likely to be mediated by *Spiroplasma* loss and reduced titre at cooler temperatures.

5.2 SYNTHESIS

5.2.1 *Can properties of defensive symbionts be generalised across systems?*

To date, much of the research focussed around the evolutionary ecology of symbiont-mediated protection has been mostly described in aphid systems. However, with the increasing number of newly described protection systems, it is important to consider whether features of symbiont-mediated protection can be generalised across different systems. Understanding general features of symbiont-mediated defence is of particular importance if systems are to be implemented in natural populations for use in biological control or as agents to protect beneficial insects. This thesis aimed to extend much of the research described within aphid systems to the *Drosophila-Spiroplasma-Leptopilina* system. Here, I consider the findings of my thesis in context of other symbiont-mediated defence systems.

Enemy strain identity appears to be a general and important feature across defensive symbiont systems. In chapter 2, I observed that the strength of protection conferred by *Spiroplasma* in *Drosophila* was found to be dependent on the strain of attacking wasp. This finding extends our knowledge beyond the aphid systems, whereby enemy strain

identity is also found to be an important factor for the outcome of symbiont-mediated protection against parasitic wasps and entomopathogenic fungi (Cayetano and Vorburger, 2013, 2015; Parker *et al.,* 2017). This feature of defensive symbiosis has important implications if systems are to be used in natural populations as symbionts may only be able to defend against certain genotypes of enemy.

To date, the source of the variation in symbiont-mediated defence against different enemy strains is currently unknown. Whether the source is mediated through direct effects of enemy factors acting on the symbiont itself or alternatively, through indirect effects of enemy factors acting on the host and interacting with symbiont-mediated protection remains to be tested. Understanding the source of variation may be key to determining how defensive symbionts are likely to evolve with the host and parasite. Given the pressure on parasites to evade host immune systems, it may be likely that the source of variation is arising from indirect effects of enemy factors acting on the host. For instance, to evade the *Drosophila* immune response, wasps of the genus *Leptopilina* transfer venom during oviposition to prevent the encapsulation response of *Drosophila* (Rizki and Rizki, 1990; Labrosse *et al.,* 2003; Morales *et al.,* 2005; Dubuffet *et al.,* 2008). Evidence has shown that strains of the same *Leptopilina* species can vary substantially in their venom composition and thus, their virulence factors (Colinet *et al.,* 2013). It would be interesting to understand how wasp venom itself impacts upon the fitness of the fly without transfer of the wasp egg to disentangle the source of variation seen in *Spiroplasma*-mediated protection against different strains of wasp.

Across defensive symbiont systems, it has been commonly shown that defensive symbiont strain identity is important for the outcome of a host-parasite interaction. For instance, the strain of *H. defensa* in pea aphid protection against parasitoid wasps, the strain of *Regiella insecticola* in the pea aphid protection against pathogenic fungi and the strain of *Wolbachia* in anti-viral protection in *Drosophila* (Sandrock *et al.,* 2010; Schmid

et al., 2012; Chrostek *et al.,* 2013; Cayetano and Vorburger, 2013, 2015; Parker *et al.,* 2017). In contrast to these systems, I did not find any effect of *Spiroplasma* strain on the strength of protection conferred by *Spiroplasma* (see chapter 3). The caveat to this observation here of course, is that only two strains were tested (MSRO-Uganda and MSRO-Brazil). To gain a more reliable assessment as to whether *Spiroplasma*-mediated protection is dependent upon the strain of *Spiroplasma*, more strains of *Spiroplasma* should be tested. However, from analysis of *Spiroplasma* strain sequences, *Spiroplasma* of *D. melanogaster* (MSRO) is found to be most closely related to that of *D. nebulosa* (NSRO) (Montenegro *et al.,* 2005). Given that *D. nebulosa* has only coinhabited with *D. melanogaster* following melanogaster's recent anthropogenic introduction into the New world approximately 300 years ago, it is believed that MSRO is a fairly recent symbiont of *Drosophila melanogaster* (Vilela *et al.,* 1980; ; Tidon-Sklorz *et al.,* 1994; Medeiros and Klaczko, 2004; Montenegro *et al.,* 2005). Hence, it is possible that there has not been enough time following the introduction of *Spiroplasma* into *D. melanogaster*, for substantial divergence of the strains in nature. Nevertheless, *Spiroplasma* has been observed to be an extremely fast evolving symbiont, with substitution rates far greater than for any other bacteria reported (Gerth *et al.,* 2021). Thus, further strains should be tested to verify the importance of *Spiroplasma* strain identity in the outcome of *Spiroplasma*-mediated defence.

Thermal sensitivity of defensive symbionts appears to be a common feature across defensive symbiosis systems. Small changes in temperature can result in substantial changes in the level of protection afforded. For instance, in chapter 4 I observed that just a 4°C decrease in temperature experienced by fly mothers was enough to reduce offspring protection mediated by *Spiroplasma* by approximately 58%. Similarly, a temperature increase of 2.5°C was enough to reduce protection conferred by *H. defensa* against *A. ervi* in the pea aphid (Higashi *et al.,* 2020). Despite our knowledge of their sensitivity, the precise source of this loss of protection is still not fully understood. Given

the vast differences in symbiont defence systems (e.g. host species, parasite species, symbiont species and host life stage at infection), the sources of protection loss at different temperatures are potentially unique to the system and cannot be generalised. Nevertheless, symbiont titre appears to be a major contributing factor affecting protection at different temperatures in *Drosophila*. As in chapter 4, Chrostek *et al.,* 2021 also found that a reduction in *Wolbachia* titre at lower temperatures is at least partially responsible for the reduction in *Wolbachia* mediated anti-viral protection in *Drosophila* at cooler temperatures. However, the differences in *Wolbachia* titre could not fully explain the differences in protection observed suggesting other mechanisms were also acting. In contrast, in the pea aphid system, *H. defensa* titre was not a found to be a strong indicator for the reduction in protection against *A. ervi* at lower temperatures (Doremus *et al.,* 2018). Nevertheless, in the black bean aphid, *H. defensa* titre was observed to increase with host age, as did the level of protection against *A. ervi,* suggesting titre of *H. defensa* may be playing a role in the protection conferred. Indeed, trying to dissect the causes of temperature effects on symbiont-mediated protection is challenging given that temperature is likely to be affecting many aspects of host, symbiont and parasite biology.

The timing of exposure to different thermal environments can also have strong impacts on the outcome of protection. In chapter 4, I demonstrated that the developmental temperature of the mother was most important for the outcome of *Spiroplasma*-mediated protection in her offspring. The result observed in chapter 4 is similar to that observed in Chrostek *et al.,* 2021, whereby the developmental temperature of *Drosophila* was found to impact their outcome to *Wolbachia*-mediated antiviral protection. However, in contrast to my findings, it was also demonstrated that the temperature after infection modulated the outcome of *Wolbachia*-mediated antiviral protection. Here, higher temperatures resulted in higher viral replication, leading to more lethal effects and lower *Wolbachia* conferred protection. In chapter 4, I found no evidence to suggest that the temperature fly larvae were exposed to after infection impacted the outcome of

Spiroplasma-mediated protection. Wasp exposed fly larvae exposed to 25°C were just as well protected as those exposed to 21°C. Thus, direct temperature effects on *Spiroplasma* RIP toxin production appears robust to temperature, and any potential temperature mediated growth effects on wasp development do not appear to result in differences in protection afforded. In general, defensive symbionts are highly sensitive to temperature, but whether they are able to provide protection is likely to depend on the timing of the temperature exposure and prediction of the outcome is likely to be unique to the system depending on individual temperature effects on the host, symbiont and parasite.

5.2.2 *What does this all mean for the dynamics of* Spiroplasma *in natural populations?*

Collectively, the findings of this thesis have broad implications for our understanding of the evolutionary and ecological dynamics of *Spiroplasma* in natural populations. Consideration of *Spiroplasma* dynamics is particularly important for its potential application in biological control programmes (Clark and Whitcomb, 1984; Schneider *et al.,* 2019). In this thesis, I observed that the outcome of *Spiroplasma*-mediated defence is much more complex than the presence or absence of infection. Whether an infected individual survives wasp attack depends on the identity of circulating wasp strains and the thermal conditions of the environment. In turn, the outcome of this interaction will impact upon *Spiroplasma* transmission and thus, the dynamics of *Spiroplasma* in a population.

The mixture of circulating wasp strains is likely to affect the dynamics of *Spiroplasma*. In chapter 2, I observed that the strength of protection conferred by *Spiroplasma* is dependent upon the strain of *L. heterotoma*. Substantial genetic diversity is likely to exist within natural populations of *Leptopilina* wasps. In a population made up of avirulent wasp strains, whereby *Spiroplasma* strongly protects the fly, it would be expected that *Spiroplasma* infection frequencies may rise rapidly. In contrast, in a population made up of highly virulent wasp strains, whereby there is weak protection afforded by

Spiroplasma, it would be expected that *Spiroplasma* infection frequencies may rise slowly, if at all. Previous studies have shown that *Spiroplasma* frequency can rapidly reach fixation in response to selection by wasps which *Spiroplasma* strongly protects against (Xie *et al.,* 2015). It would be interesting to extend this finding to test the predictions on the speed and spread of *Spiroplasma* in the presence of virulent and avirulent wasp strains both in isolation and in combination.

A major distinction from the aphid system is that it is unlikely *Spiroplasma* will select on the balance of wasp strains. The *Spiroplasma* kills the majority of developing wasps, even in the absence of fly rescue (as observed for the Lh14 wasp strain in chapter 2). Thus, eco-evolutionary dynamics, where *Spiroplasma* frequency and wasp genotype both change over short periods of time, are not predicted. Another notable difference is that *Spiroplasma* is also unlikely to cause extinction in populations of *L. heterotoma*, as the wasp is considered a generalist species able to attack a range of *Drosophila* species (Fleury *et al.,* 2009). Thus, the wasp acts as a fixed external force against which the *Spiroplasma* and fly responds. However, the lack of response of wasp population size is likely not to be reflected in *L. boulardi*, which is considered more specialist in its attack. For this wasp, protection of the fly is likely to impact wasp population size and onward attack rates.

The dynamics of *Spiroplasma* will also depend on the thermal environment. In chapter 4, I observed that *Spiroplasma*-mediated protection is a trait sensitive to cooler temperatures. The developmental temperature of mothers dictates the strength of protection against parasitoid wasps in their offspring. Of course, the thermal landscape of *Drosophila* is not likely to be uniform across their habitat range. Rotten fruit and compost heaps will vary in temperature according to their level of decomposition and relative placement to direct sunlight. This creates a patchy thermal landscape in which *Drosophila* adults and fly larvae exist. It is possible that this heterogeneous environment

also generates a population of *Drosophila* with variable *Spiroplasma* titres across the landscape, impacting upon the strength of *Spiroplasma*-mediated protection conferred and dynamics in the next generation, which makes dynamics difficult to predict. In natural populations of *Drosophila melanogaster*, *Spiroplasma* infection frequency is usually observed to be quite low despite its advantages against parasitoid wasp attack and male-killing abilities. In Brazilian populations of *D. melanogaster*, infection frequency was found to be 2.3% (Montenegro *et al.,* 2005), and 1 of 38 individuals were infected in Uganda (Pool *et al,* 2006). The sensitivity of *Spiroplasma* vertical transmission and density to the thermal environment may explain the low frequencies observed in natural populations.

To fully appreciate the impact of the thermal environment on the dynamics of *Spiroplasma*, further work should be conducted to understand whether the differences in the strength of *Spiroplasma*-mediated protection against different strains of *L. heterotoma* are robust to temperature. Resulting G x G x E interactions would have considerable impacts for the prediction of *Spiroplasma* dynamics in nature, making them more complicated and difficult to predict. However, given the high sensitivity of *Spiroplasma* to temperature it seems most likely that protection will be lower at cooler temperatures across all strains of wasp. Indeed, genotype by genotype specificity was observed to be robust to temperature changes in *H. defensa*-mediated protection against *L. fabarum* (Cayetano and Vorburger, 2013). Nevertheless, the developmental temperature of the wasp should also be considered prior to exposure to flies. In a recent study it was shown that venom composition of the wasp varied strongly with developmental temperature which has been suggested to partially explain the observed change in parasitic wasp success (Cavigliasso *et al.,* 2021). Differential venom composition among wasp strains is one of the hypotheses for the observed variation in *Spiroplasma*-mediated protection among different strains of wasps. Thus, it would be interesting to consider the developmental temperature of wasps and whether this

results in G x G x E interactions, adding to the complexity of *Spiroplasma*-mediated defence.

The presence of wasps can also extend the thermal envelope in which *Spiroplasma* infection can persist which will subsequently affect *Spiroplasma* dynamics. In chapter 4 there was evidence that flies from the 21°C 'constant' and 'pre-attack' treatments started to segregate *Spiroplasma* infection. However, in the presence of wasps, any vulnerable *Spiroplasma*-uninfected individuals were subsequently killed leaving only *Spiroplasma*-infected flies albeit at low titre. In areas of cooler temperatures, whereby *Spiroplasma* infection is starting to segregate, the presence of wasps 'selects out' any uninfected which are unable to defend themselves via other defence mechanisms. This leads to wasp-mediated high *Spiroplasma* infection frequencies in the subsequent generation which in turn may recover at higher temperatures.

Altogether, the findings of this thesis demonstrate that protective traits conferred by defensive symbionts and the factors that affect the outcome can have consequences for their dynamics in nature. In general, the *Spiroplasma-Drosophila* system would benefit from more experimental evolution research to understand the dynamics of all players in the interaction overtime. And modelling to predict the outcomes and experimental evolution to test the predictions.

5.3 FUTURE WORK

5.3.1 *How does* Spiroplasma*-mediated defence combine with nuclear-encoded defence in* Drosophila*?*

Within the aphid system, it is known that aphid clone identity is an important determinant of protection, reflecting genetic variation in protection between aphids. In *D. melanogaster*, high selective pressure from parasitoid wasps has led to the evolution of multiple defence mechanisms in *Drosophila melanogaster*. Beyond the help of microbes, *D. melanogaster* are able to mount a specialised cellular response to defend

themselves against wasp attack (Carton *et al.,* 2008). Here, the parasitoid egg is detected as non-self, and mature haemocytes are upregulated within the haemocoel and directed towards the egg. An initial layer of plasmatocytes surround the parasitoid egg, which is then further enclosed by lamellocytes. The capsule surrounding the egg then undergoes melanisation, which ultimately kills the unhatched wasp larva directly through asphyxiation (Salt, 1970) or indirectly through the cytotoxic compounds produced (Nappi *et al.,* 1995; Nappi and Vass, 1998). How *Spiroplasma*-mediated protection combines with nuclear-encoded defence in *Drosophila* is unknown as these protective systems have only been considered in isolation.

Understanding how *Spiroplasma*-mediated defence is likely to interact with nuclear-encoded defence is key to predicting the evolutionary dynamics of *Spiroplasma*. Prediction of their dynamics requires assessment of their combined and individual ability to protect, in addition to their relative fitness costs and benefits beyond immediate protection. For instance, the efficacy of these systems may be additive. Alternately, there may be depreciating returns – a second system adds less protection when applied in addition to an existing one. This is the case inevitably when one system produces complete protection, for instance. Finally, there may be synergistic returns, with a mechanism having greater efficacy in the presence of another. For example, a mechanism that protects poorly on its own may contribute more significantly in the presence of another rescue system. Only when these interactions are understood will we be able to predict the dynamics of *Spiroplasma*. For instance, does the presence of nuclear-encoded resistance prevent or promote the spread of *Spiroplasma*-mediated defence? Prevention is likely where efficacy is lower in combination, and promotion where they are synergistic.

5.3.2 *Transgenerational properties of* Spiroplasma*-mediated protection*

Symbiont traits combine genetic and epigenetic elements. The presence vs absence of *Spiroplasma* is a genetic trait, as is the strain identity of the *Spiroplasma*. However, in addition, the phenotype of the symbiont can be determined in part by titre (or other historical influences) that represent epigenetic traits (phenotype variation that is not underpinned at the DNA sequence level). In chapter 4, I observed that the developmental thermal environment of the mother dictates the outcome of *Spiroplasma*-mediated survival in her progeny, predominantly through complete *Spiroplasma* loss but potentially also through reduction in titre. As the pre-attack treatment was virtually a single generation and only included a single day (egg development) of the second generation, I concluded that the effect of temperature on *Spiroplasma*-mediated defence is transgenerational. This finding raises the possibility that *Spiroplasma* titre itself is an epigenetic trait whereby environmental factors can act upon the protective phenotype which may persist across generations. Thus, selection can act upon both the frequency of infected individuals and the *Spiroplasma* titre within an individual.

The idea that selection can act on the *Spiroplasma* titre of an individual has important implications for the short-term dynamics of resistance. As symbiont titre is both inherited across generations and determines phenotype strength, it may mediate short-term adaptive increases in resistance. When parasitism rates are high, high levels of *Spiroplasma* titre variation within natural populations may allow the evolution of resistance over short timescales. Conversely, adaptive resistance encoded in high titre is expected to degrade rapidly, as titre is impacted by environmental and life history parameters such as the thermal environment or age at reproduction. Thus, adaptive responses mediated through symbiont titre may be expected to create more rapid fluctuations in population level resistance.

Host age at reproduction represents another potential transgenerational property of *Spiroplasma*-mediated protection. Maternally inherited bacteria are likely to experience a transmission bottleneck during their transmission from mother to progeny due to the decrease in population size (Mira and Moran, 2002). Indeed, in *Spiroplasma*-infected *Drosophila*, titre is observed to be low during the larval stages yet increases exponentially during the pupal stage with continued increase for 3-4 weeks after emergence as adults (Anbutsu and Fukatsu, 2003; Herren and Lemaitre, 2011). Although not described for *Spiroplasma*, in *Wolbachia*-infected *Drosophila*, it has been observed that older females have higher *Wolbachia* densities and transmit *Wolbachia* densities proportional to their own (Dyer *et al.,* 2005; Layton *et al.,* 2019). Thus, older *Spiroplasma*-infected mothers may be more likely to transmit higher *Spiroplasma* titre to their progeny which in turn may influence the outcome of *Spiroplasma*-mediated protection. Indeed, host age has been shown to be an important property of *H. defensa*-mediated protection in the black bean aphid against *Aphis fabae,* with protection increasing with host age (Schmid *et al.,* 2012).

Future work should determine the transgenerational effects of host age at reproduction on the level of protection conferred by *Spiroplasma* in the progeny. Does an older *Spiroplasma*-infected mother produce progeny with increased resistance to parasitoid wasp attack mediated through effects on titre? Understanding the effects of host age at reproduction and how these effects may be maintained across generations is important to gain a more accurate picture of the evolutionary ecology of *Spiroplasma*-mediated protection in the wild. To date, the majority of studies concerning *Spiroplasma*-mediated protection in *Drosophila* allow females to mature for approximately 10 days before copulation to ensure high efficiency of *Spiroplasma* transmission from mothers to progeny. However, given that *Drosophila melanogaster* females are estimated to mate, on average, every 27 hours (Giardina *et al.,* 2017), it is unlikely that a fly would remain virgin for 10 days in the wild. Thus, if early reproduction is observed to have a negative

impact on the *Spiroplasma*-mediated resistance of progeny, this would have considerable impacts for the maintenance of *Spiroplasma* in natural populations.

In turn, it would also be important to understand how *Spiroplasma*-mediated protection is affected by the interaction of the thermal environment and age at reproduction in infected mothers. For instance, would progeny of a mother who had developed and was maintained at 21°C before producing offspring at 2 days old (early reproduction), have lower protection than a mother who had developed, and was maintained at 21°C, before producing offspring at 10 days old (late reproduction)? Conversely, does the titre of *Spiroplasma* actually decrease further during the additional 8 days before late reproduction to produce progeny with reduced *Spiroplasma*-mediated resistance compared to mothers reproducing early? Age effects of mothers reared and maintained at 25°C would also be interesting to determine. In the case of *Spiroplasma*, it may be likely that titre reaches a threshold density whereby the maximum protection afforded is reached. If mothers are reared and maintained at 25°C, does early or late reproduction have any effect on the protection afforded, or is the threshold titre already achieved at early reproduction? These questions await further research.

Experimental evidence has revealed that the presence of parasitoid wasps can rapidly select for increased prevalence of *Spiroplasma*-infected flies within a population (Xie *et al*., 2015). Beyond selection relating to the absence or presence of *Spiroplasma* infection, it may be possible that the presence of wasps can also select for flies with higher *Spiroplasma* titre. In the wild, individual flies will vary in *Spiroplasma* titre. For instance, in populations of wild *Drosophila hydei*, titre varies over two orders of magnitude (Osaka *et al.,* 2013). Additionally, like in the case of *Wolbachia*, *Spiroplasma* titre is likely to be heritable, with progeny of high titre females typically having higher titre. If these two conditions are met, it may be expected that high titre individuals will have higher survival following parasitism, and then leave higher titre descendants. Indeed, in chapter

4 I observed some evidence that wasp-attacked *Spiroplasma* fly survivors had higher titre than non-wasp attacked flies in the 21°C and 23°C treatment.

5.4 REFLECTION FOR STUDY OF OTHER DEFENSIVE SYMBIOSES

The epigenetic, and potentially transgenerational, component of the protection phenotype represents an extension of our understanding of protection outcomes beyond the 'aphid model'. With the contemporary report of this phenomenon for *Wolbachia* anti-viral protection, it is clear that study of symbiont phenotype in general will benefit from directed analysis of historical as well as current environments on symbiont phenotype. These analyses are required to understand dynamics in varying, heterogeneous natural environments, both in terms of symbiont dynamics, and also the eco-evolutionary interplay. They will require integrating with seasonality of species in temperate environments, to predict dynamics.

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Appendix

A.1 CHAPTER 2 SUPPLEMENTARY MATERIAL

Table A.1: Replicate identity and number for chapter 2 experiments (Lh- and Lh+: wasp presence and absence; S- and S+: *Spiroplasma* presence and absence; EtOH- and EtOH+: 0% and 6% ethanol).

Experiment	Reference	Replicate identity	Treatment	Number of replicates		
				Wasp strain		
				Lh-Fr	Lh14	Lh-Mad
Survival	Fig. 2.1	Vial of 30 larvae	Lh- S- EtOH-	15	15	10
			Lh- S- EtOH+	13	15	10
			Lh- S+ EtOH-	15	15	10
			Lh- S+ EtOH+	15	15	10
			Lh+ S- EtOH-	15	15	10
			Lh+ S- EtOH+	12	15	10
			Lh+ S+ EtOH-	15	15	10
			Lh+ S+ EtOH+	15	15	10
Proportion fertile	Fig. 2.2	Single female fly	Lh+ S+ EtOH-	43	N/A	37
			Lh+ S+ EtOH+	44	N/A	42
			Lh- S+ EtOH-	39	N/A	38
			Lh- S+ EtOH+	41	N/A	43
Number of daughters produced	Fig. 2.3	Single female fly	Lh+ S+ EtOH-	24	N/A	14
			Lh+ S+ EtOH+	15	N/A	25
			Lh- S+ EtOH-	38	N/A	36
			Lh- S+ EtOH+	41	N/A	41
Wing size	Fig. 2.4	Single female fly	Lh+ S+ EtOH-	60	N/A	36
			Lh+ S+ EtOH+	29	N/A	30
			Lh- S+ EtOH-	60	N/A	50
			Lh- S+ EtOH+	50	N/A	49
Wasp oviposition	Fig. 2.5	Single fly larva dissection	S-	23	25	23
			S+	26	25	24

A.2 CHAPTER 3 SUPPLEMENTARY MATERIAL

Table A.2: Replicate identity and number for chapter 3 experiments (Uninfected: *Spiroplasma* negative, MSRO-Br: Brazilian strain of *Spiroplasma*, MSRO-Ug: Ugandan *Spiroplasma* strain).

Experiment	Reference	Replicate identity	Treatment	Number of replicates		
				Wasp strain		
Leptopilina boulardi				Lb17	NSRef	Control
Survival	Fig. 3.1	Vial of 30 larvae	Uninfected	10	10	10
			MSRO-Br	5	5	6
			MSRO-Ug	10	10	10
Proportion fertile	Table 3.1a	Single female fly	MSRO-Br	23	24	24
			MSRO-Ug	23	24	24
Number of daughters	Table 3.1a	Single female fly	MSRO-Br	22	24	22
			MSRO-Ug	23	21	23
Leptopilina heterotoma				Lh14	Lh-Mad	Control
Survival	Fig. 3.1b	Vial of 30 larvae	Uninfected	10	10	10
			MSRO-Br	10	10	9
			MSRO-Ug	10	10	10
Proportion fertile	Table 3.1b	Single female fly	MSRO-Br	24	23	24
			MSRO-Ug	19	22	19
Number of daughters	Table 3.1b	Single female fly	MSRO-Br	19	21	22
			MSRO-Ug	19	20	19

A.3 CHAPTER 4 SUPPLEMENTARY MATERIAL

Table A.3: Replicate identity and number for chapter 4 experiments (Lb- and Lb+: wasp presence and absence; S- and S+: *Spiroplasma* presence and absence).

Experiment	Reference	Replicate identity	Treatment	Number of replicates
Preliminary experiments				
'Attack' survival	Fig. 4.3	Vial of 30 larvae	25°C Lb- S+	6
			25°C Lb+ S+	6
			25°C Lb- S-	5
			25°C Lb+ S-	5
			23°C Lb- S+	6
			23°C Lb+ S+	6
			23°C Lb- S-	6
			23°C Lb+ S-	6
			21°C Lb- S+	6
			21°C Lb+ S+	6
			21°C Lb- S-	6
			21°C Lb+ S-	6
'Attack and protection' survival	Fig. 4.4	Vial of 30 larvae	25°C Lb- S+	10
			25°C Lb+ S+	10
			25°C Lb- S-	10
			25°C Lb+ S-	10
			23°C Lb- S+	10
			23°C Lb+ S+	10
			23°C Lb- S-	10
			23°C Lb+ S-	10
			21°C Lb- S+	10
			21°C Lb+ S+	10
			21°C Lb- S-	10
			21°C Lb+ S-	10
'Constant' survival	Fig. 4.5	Vial of 30 larvae	25°C Lb- S+	10
			25°C Lb+ S+	10
			25°C Lb- S-	10
			25°C Lb+ S-	10
			23°C Lb- S+	10
			23°C Lb+ S+	10
			23°C Lb- S-	10
			23°C Lb+ S-	10

			21°C Lb- S+	10
			21°C Lb+ S+	10
			21°C Lb- S-	10
			21°C Lb+ S-	10
Integrated experiment				
'Pre-attack' survival	Fig 4.6	Vial of 30 larvae	25°C Lb- S+	10
			25°C Lb+ S+	9
			25°C Lb- S-	9
			25°C Lb+ S-	10
			23°C Lb- S+	10
			23°C Lb+ S+	10
			23°C Lb- S-	9
			23°C Lb+ S-	10
			21°C Lb- S+	10
			21°C Lb+ S+	10
			21°C Lb- S-	10
			21°C Lb+ S-	10
'Attack and protection' survival	Fig 4.8	Vial of 30 larvae	25°C Lb- S+	9
			25°C Lb+ S+	8
			25°C Lb- S-	10
			25°C Lb+ S-	10
			23°C Lb- S+	7
			23°C Lb+ S+	10
			23°C Lb- S-	7
			23°C Lb+ S-	10
			21°C Lb- S+	8
			21°C Lb+ S+	10
			21°C Lb- S-	9
			21°C Lb+ S-	10
'Constant' survival	Fig 4.7	Vial of 30 larvae	25°C Lb- S+	10
			25°C Lb+ S+	10
			25°C Lb- S-	9
			25°C Lb+ S-	10
			23°C Lb- S+	10
			23°C Lb+ S+	10
			23°C Lb- S-	9
			23°C Lb+ S-	10

			21°C Lb- S+	10
			21°C Lb+ S+	10
			21°C Lb- S-	10
			21°C Lb+ S-	10
Spiroplasma titre	Fig. 4.9	Single female fly	25°C Lb-	17
			25°C Lb+	20
			23°C Lb-	19
			23°C Lb+	20
			21°C Lb-	21
			21°C Lb+	19
Wasp oviposition	Fig. 4.10	Single fly larva dissection	25°C	37
			23°C	30
			21°C	30