The evolutionary ecology of an insect-bacterial mutualism

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Abstract

Heritable bacterial endosymbionts are responsible for much phenotypic diversity in insects. Mutualists drive large-scale processes such as niche invasion, speciation and mass resistance to natural enemies. However, to persist, mutualists need to be able to transmit with high fidelity from one generation to the next, to be able to express their beneficial phenotypes, and for the benefits they grant the host to outweigh their costs. The effect of ecologically-relevant environmental temperature variations upon transmission and phenotype is a poorly understood area of endosymbiont biology, as is how the symbiont's cost varies under ecological stress. In this thesis, I examined these parameters for *Spiroplasma* strain hy1, a defensive mutualist which protects the cosmopolitan, temperate fruit fly *Drosophila hydei* from attack by a parasitoid wasp. I detected *Spiroplasma* hy1 in *D. hydei* individuals from the south of the U.K. The bacterium is at low prevalence compared to hy1 in other localities such as North America and Japan, but its presence in this temperate region conflicts with past studies indicating high sensitivity to low temperatures. I first demonstrate that the vertical transmission of *Spiroplasma* hy1 is more robust to the cool temperatures typical of temperate breeding seasons than previously considered, with transmission in a 'permissive passage' experiment occurring at high fidelity for two generations at a constant 18°C and in an alternating 18/15°C condition. Secondly, I demonstrate that the expression of the defensive phenotype is considerably more sensitive to cool temperatures than transmission. *Spiroplasma* hy1 protection ceases at 18°C, suggesting that for much of the *D. hydei* breeding season in areas such as the U.K., hy1 may be selectively neutral in many fly individuals. Finally, I show that hy1 has an unusually low standing cost to its host under starvation stress, contrasting with findings for the related MSRO strain in *D. melanogaster*. Measures of active cost – the fate of survivors of attack – were unclear. These results indicate that sensitivity to cold temperatures could account for hy1's low U.K. prevalence. Small amounts of segregational loss could partially counteract selection upon natural enemy resistance, and loss of phenotypic expression at 18°C almost certainly causes hy1 to be neutral at best for parts of early summer and autumn. Future work should investigate the effects of different temperature on costs of symbiont carriage, and whether cool temperatures could push hy1 from mutualism and neutral commensalism to parasitism, as well as investigate how nuclear-mediated anti-wasp protection might interact and compete with hy1-mediated protection.

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1 Introduction

1.1 The majority of arthropods carry bacterial endosymbionts

Arthropods are the most speciose class of animals living on Earth, representing a hefty slice of the diversity of life. They live in all but the most extreme environmental conditions in terrestrial habitats, filling a variety of niches and deploying an array of strategies to survive and breed. Common amongst them is the habit of forming endosymbioses – long-term associations between two or more organisms (de Bary, 1879), in which one organism lives inside the body or cells of another. These occur most commonly with bacteria, which modify the physiology and behaviour of their hosts and add an extra layer of complexity to the biology of the host individual. Considering only endosymbionts, most of these bacteria come from a handful of genera; *Wolbachia, Spiroplasma, Rickettsia,* and *Cardinium.* Attempts to estimate the commonness of bacterial endosymbiont carriage vary, but according to a recent 2015 estimate based on large-scale screening, 52% (CIs: 48–57) of arthropod species carry *Wolbachia*, 24% (CIs: 20–42) carry *Rickettsia*, and 13% (CIs: 13–55) carry *Cardinium* (Weinert *et al.*, 2015). Other estimates for *Wolbachia* incidence usually fall in the 60-70% range (Hilgenboecker *et al.*, 2008; de Oliveira *et al.*, 2015). *Spiroplasma* meanwhile is present in a variety of arthropods, including 28.4% of ant species (Kautz *et al.*, 2013) and 7 of 19 *Drosophila* fruit fly species tested (Watts *et al.*, 2009). All evidence considered, it is likely that the majority of arthropod species live in association with at least one symbiotic bacterium.

A symbiosis can take many forms. It may be parasitic, in which case one partner derives a benefit at the expense of the other; mutualistic, when both partners gain a net benefit; or commensal, where the relationship is seemingly neutral to all parties. Symbioses can further be divided by whether they are obligate (required by both host and symbiont to survive), or facultative (vital to both partners only under certain conditions). Bacterial symbionts vary in the mechanism of association with their hosts. They may be stored in specialised crypts in the arthropod gut, in the host's haemolymph, or even inside specialised cell organelles. The association can continue across multiple generations, via transmission modes such as inoculation of new offspring through egg-smearing or faecal consumption, and through transovarial transmission, such that the fertilised egg comes pre-packaged with the symbiont.

1.2 Endosymbionts underlie a range of unusual phenotypes in arthropods which don't otherwise make sense

Symbionts are significant in the study of arthropods because they underlie a wide range of unusual phenotypes. Their maternally-inherited nature means that host and symbiont lineages are associated for long periods of time – particularly obligate symbioses, which may last for millions of years (Moran and Wernegreen, 2000) – which couples their fitness and enables selection for host and symbiont to tolerate each other. Amongst the mutualistic interactions, nutritional mutualisms involve the symbiont enabling the host to utilise a food source that it can't otherwise process. An example is *Buchnera aphidicola,* an obligate symbiont of aphids such as the pea aphid, *Acyrthosiphon pisum. Buchnera* synthesizes essential amino acids and so permits the host to utilise protein-deficient plant phloem as its sole food source. Mutualisms can also be defensive, increasing the odds of a host surviving in areas containing natural enemies. Defensive mutualists protect against a range of threats, including predators (Kellner, 2002), viral infections (Hedges *et al.*, 2008; Teixeira *et al.*, 2008), parasites (Jaenike *et al.*, 2010) and parasitoids (Xie *et al.*, 2010, 2013). A third class of mutualism enables hosts to survive at extreme temperatures, buffering it from the effects of heat shock. In aphids, three facultative symbionts have heat shock protective phenotypes, as does *Hamiltonella* in whitefly (Chen *et al.*, 2000; Russell and Moran, 2006; Brumin *et al.*, 2011; Heyworth and Ferrari, 2015).

Meanwhile, reproductive parasitic phenotypes arise from the conflict between what benefits the host and what benefits the symbiont (Hurst and Frost, 2015). The conflict follows from the maternal mode of transmission of many endosymbionts, partly due to the mechanical limitation of egg size (though see **chapter 4** for other hypotheses). Solely matrilineal inheritance means that male offspring are 'useless' to a symbiont in terms of onward transmission. A symbiont may thus increase its fitness by evolving a means of utilising male offspring to increase fitness of infected female offspring. The phenotypic results include the evolution of sex-ratio distortion and cytoplasmic incompatibility. The former steers the host's resources into daughter-production rather than son-production, ensures that daughters never need to compete with their brothers, or literally turns sons into post-eclosion meals for their siblings. The latter sacrifices infected male fitness by ensuring that they cannot form fertile crosses with uninfected females, thus preventing uninfected offspring from existing and competing with infected female offspring.

1.3 Symbionts have impacts on the evolutionary and ecological dynamics of arthropods

1.3.1 Sex ratio skewing, and resulting counter-adaptations to reproductive parasites

Sex ratio skews can have dramatic effects on the demographics of a host population. In the case of U.S. and Hawaiian *Trichogramma,* a *Wolbachia* strain maintains a consistently high female-to-male ratio by inducing parthenogenesis, which is lifted upon antibiotic treatment (Stouthamer *et al.*, 1990). Sex ratio skews can result in very fast selection for counter-adaptations such as suppression. An example is one population of the butterfly *Hypolimnas,* infected with a male-killing *Wolbachia* strain which drove a population level female bias of 100 females/per male for 100 years (Dyson and Hurst, 2004), until a suppressor spread rapidly (Charlat *et al.*, 2007). The same *Wolbachia* also produced cytoplasmic incompatibility, revealed once the male-killing was suppressed (Hornett *et al.*, 2008).

A particularly dramatic example of suppression occurs when host selfish genetic elements act to restore production of males and drive down the symbiont frequency, as is seen in *Trichogramma kaykai. Wolbachia* causes virgin *T. kaykai* females to produce only daughters, but a parasitic B chromosome present in the host population causes only males to be produced, and thus prevents *Wolbachia* frequency from increasing (Stouthamer *et al.*, 2001). Theoretically, it is possible that extinction could result from sex ratio distortion, but due to short timeframes it would be easy to miss it occurring and it has yet to be demonstrated (Kageyama *et al.*, 2012). The reduction in effective population size caused by sex ratio distortions can produce inbreeding and issues associated with it (Kageyama *et al.*, 2012). Models of asymmetric gene flow between subpopulations, resulting from sex ratio distortions in one subpopulation relative to a distorter-uninfected neighbour, have demonstrated that this could hinder local adaptations such as the spread of MK-resistance alleles (Telschow *et al.*, 2006).

1.3.2 Genetic sequences from reproductive parasites can insert into genomes, with consequences including the production of new sex determination systems

Wolbachia sequences sometimes horizontally transfer into arthropod genomes. The adzuki bean beetle, *Callosobruchus chinensis*, is doubly-infected with two strains of *Wolbachia*, plus a distinct *Wolbachia* genome fragment which has transferred to the X chromosome

(Kondo *et al.*, 2002). A *Wolbachia* insert search found a large *Wolbachia* insert (almost the whole genome) in *Drosophila ananassae* from sequence data, verified by further work including FISH (Dunning Hotopp *et al.*, 2007). The insert is present in flies from around the world, and produces some transcripts, though it is not yet known if they are biologically meaningful. Small *Wolbachia* inserts of diverse strains were found in publicly-available sequences of three species of *Nasonia* (Dunning Hotopp *et al.*, 2007).

In one case, *Wolbachia* has effected a change in sex determination system via incorporation of genes into the nuclear genome. Sex determination is widespread amongst organisms, but sex determination systems are variable and have evolved multiple times. The common pillbug, *Armadillidium vulgare*, has a W chromosome which was formed by 3 Mb of a feminising *Wolbachia* genome transferring into the nuclear genome. The new W chromosome is hemizygous (on a haploid region), acts as a female sex-determining region, and is distinct from the native W chromosome, which is likely to have been lost due to an inability to coexist alongside the ancestral *Wolbachia* infection (Leclercq *et al.*, 2016).

1.3.3 Protective mutualists add further complexity to host-parasite dynamics

Dynamics of even simple host-symbiont-enemy systems can become complicated due to their multifactorial nature. In the short-term, protective mutualist prevalence in a population should increase following increases in enemy attack rate. In the longer-term, an evolutionary arms race could arise like that seen for host genetic defences against enemies, in which natural enemies evolve strategies for circumventing protection, and the mutualist evolves counter-adaptations to restore protection. In reality, the dynamics of such a system are likely to be modulated by factors such as cost of symbiont carriage (which could decrease net fitness advantages in attacked insects, and incur a net penalty of symbiont carriage for unattacked insects), the presence or absence of nuclear-encoded enemy resistance, how the nuclear genome of the host animal interacts with the symbiont, and availability of other host or prey animal species for the natural enemy to utilise. Costs and trade-offs can manifest in unusual ways. For example, pea aphids carrying *Hamiltonella defensa*, which protects against parasitoid wasps, are more susceptible to the predator *A. bipunctata* due to a decrease in defensive behaviours. Thus, *H. defensa* frequency in the wild may partly be constrained by the threat from *A. bipunctata* predation (Polin *et al.*, 2014).

Mutualist-enemy ecological dynamics are perhaps studied most intensely in aphids, which harbour a large variety of protective mutualists and thus represent a case in which the causes of dynamics can be especially difficult to dissect out. A field study which tracked symbiont frequencies in wild aphids highlights this, showing that frequencies of protective symbionts could vary quickly and over short periods. *Hamiltonella* and *Regiella* frequencies often correlated with enemy abundance in intuitive ways, seemingly showing that the mutualists and enemies they protect against responded to each other. However, some findings, such as superinfection spikes followed by symbiont frequency crashes, were harder to explain, and the authors proposed infection costs, counter-adaptation, hitchhiking and outside temperature influences as potential causes (Smith *et al.*, 2015).

1.3.4 Symbionts of all classes are capable of driving speciation and cladogenesis

Symbioses are likely to be of import in speciation. In their review, Brucker and Bordenstein argue that speciation and symbiosis are intertwined because 1) microbial symbionts are universally present, 2) there is host-symbiont specificity, and 3) host immune genes evolve rapidly in response to symbionts and often display hybrid incompatibilities (Brucker and Bordenstein, 2012). Considering reproductive parasites, wasp species may be forced into parthenogenesis by *Wolbachia*, becoming female-only, asexual, and thus a separate species when applying the biological species concept (Gottlieb and Zchori-Fein, 2001).

Meanwhile, mutualists may drive speciation through permitting invasion of new niches, if this ultimately results in geographical, pre-mating isolation of the new incipient species from its closest relatives. Nutritional mutualists permit arthropods to utilise resources which are widespread but which would be closed to them without bacterial help. These resources include plant fluids and blood, which may lack essential amino acids, vitamins or cofactors which are necessary for insects. For instance, *Buchnera* permits *A. pisum* to survive on phloem by producing amino acids, compensating for the nitrogen-poor nature of the food source. *Wigglesworthia* in tsetse flies synthesises cofactors and enzymes that are lacking in blood meals, and *Blochmannia* synthesises amino acids so that carpenter ants can survive periods of reliance on honeydew (reviewed in (Zientz *et al.*, 2004)). The sharpshooter species *Homalodisca coagulata* feeds on plant xylem and relies on two primary endosymbionts, *Baumannia cicadellinicola* which produces vitamins and cofactors, and *Sulcia muelleri* which synthesises essential amino acids (reviewed in (Feldhaar and Gross, 2009). The importance of niche-occupation in speciation is evident in aphids, which

have speciated on a variety of different food plants and remain reproductively isolated (Brucker and Bordenstein, 2012).

1.3.5 A host species may evolve dependency on its symbiont and thus be constrained by its needs

A symbiont may evolve to be obligate and thus vital to host survival. For instance, in aphids, *Buchnera* is obligate, as the host cannot survive on plant phloem without it. Perhaps more surprisingly, hosts may also become dependent on reproductive parasites. In European populations, the parasitoid wasp *Asobara tabida* is dependent on one of its three strains of *Wolbachia* for normal reproduction. Cured females either can't produce mature oocytes, or produce few oocytes which hatch into inviable offspring, in a host line-dependent manner. The other two *Wolbachia* strains cause incomplete cytoplasmic incompatibility (Dedeine *et al.*, 2001, 2004, 2005). Parthenogenesis-induction has also independently evolved in a Japanese *Asobara* – *Wolbachia* pair. Cured females in this case can produce mature oocytes, but all their offspring are male (Kremer *et al.*, 2009). As antibiotic-curing of female insects carrying *Wolbachia* is routine, it seems likely that depending on a reproductive parasite for normal reproduction in uncommon, rather than merely infrequently-detected (Dedeine *et al.*, 2001).

A consequence of obligate dependence is that the needs of the symbiont can become limiting to the host. One hypothesis is that symbionts represent a 'thermal weak link' in hosts, if they are more sensitive to environmental conditions than the host. The symbiont may then become the limiting step in expansion of ranges or niches. As a non-microbial example, fungus-cultivating ants can't expand outside tropical environments as their fungal symbiont is susceptible to cold (Mueller *et al.*, 2011). It has been proposed that aphids are limited to temperate climates by their many bacterial endosymbionts (Dixon *et al.*, 1987).

1.3.6 Horizontal transmission of a symbiont into a novel host represents a mechanism of 'fast evolution' in the new host

Symbionts represent a 'pre-evolved' package of genes which have specific functions in their native hosts. If a symbiont becomes introduced into a new host, rather than laboriously evolving a new nuclear-encoded trait from scratch, the new host species could 'obtain' a

ready-made phenotype near-instantly. This transfer of traits represents a potential mechanism of fast evolution. Artificial horizontal transfers through methods such as microinjection are regularly carried out for experimental purposes in the laboratory for facultative symbiont species, and are hypothesised to be possible in the wild through mechanisms such as mechanical damage (reviewed in (Oliver *et al.*, 2010), and see (Haselkorn and Jaenike, 2015) for an example in *Drosophila* and *Spiroplasma*). The near-instantaneous nature of horizontal transfer makes it difficult to catch in the wild. However, cases of rapid increases in symbiont prevalence have been recorded, which are presumed to follow from the horizontal introduction of symbionts into new hosts. For instance, a strain of *Rickettsia* swept into the sweet potato whitefly, *Bemisia tabacii,* in the southwestern U.S. *Rickettsia* is a sex-ratio distorting symbiont which increases female fitness in the whitefly, and its prevalence increased from 1% to 97% in only 6 years. Its prevalence remained at near-fixation 3 years later (Himler *et al.*, 2011). Likewise, a *Spiroplasma* which protects *Drosophila neotestacea* from the sterilising effects of a nematode, *Howardula aoronymphium*, spread from east to west in North America. However, the authors propose that the rapid spread of *Spiroplasma* is probably due to the recent application of selection pressure from the worm, rather than recent acquisition of the symbiont. *Spiroplasma* has two strain variants associated with two host mtDNA variants, and thus may have been present in the host for some time before the sweep (Jaenike *et al.*, 2010).

1.4 Biotic and abiotic factors can change a symbiont's transmission efficiency and phenotype, causing changes in the population biology of the host-symbiont pair

The population biology of a host-symbiont interaction is underlain by three main parameters. If these parameters are altered by outside forces, they can change output variables such as the prevalence, range, and persistence of a symbiont in a population of hosts.

1) Symbiont transmission efficiency: A symbiont will never reach total fixation in a population if its transmission efficiency is below 100%, even if selective forces favour its spread. Segregational loss may occur when symbionts fail to get into the eggs of a female, or in the case of faecal consumption, fail to inoculate the offspring post-hatching. Imperfect transmission efficiency becomes most important in

determining the prevalence and spread of a symbiont in cases where the benefit of a mutualist or the drive of a reproductive parasite are relatively low (Jaenike, 2009; Gundel *et al.*, 2011).

2) Symbiont phenotypic effect, including costs: A mutualist or reproductive parasite will only spread if it confers a net fitness benefit to the transmitting sex compared to uninfected conspecifics. If the symbiont is sufficiently costly to the transmitting sex that this outweighs the benefits, the symbiont should fail to spread.
3) Symbiont titre: The titre of a symbiont may influence the strength of a symbiont's phenotype and its transmission efficiency, and is subject to modulation by external forces.

Outside factors which can influence transmission efficiency and phenotype, often through effects on titre, can be biotic or abiotic. The former includes selective forces such as the frequency of attack by natural enemies (which i.e. may drive selection for protective symbionts), or availability of food sources. Key among the abiotic factors is temperature, and potentially, environmental features associated with temperature such as altitude, latitude, and season.

1.5 Temperature may alter several parameters of evolutionary ecology, but is understudied at ecologically-relevant temperatures

Temperature can influence a symbiont's transmission frequency, titre, and phenotypic expression in the host. Though transmission and titre changes are due simply to changes in the host's body temperature, in the context of the natural environment, temperature fluctuations with season can have complex effects on the fitness benefits or costliness of the phenotype. This is because the seasons will also influence selective biotic forces, such as the presence and abundance of food sources and natural enemy activity. This issue is covered in more depth in chapter 2.

1.6 Cost of symbiont carriage is of interest as a less widely-studied component of symbiont phenotype and as an impact on symbiont evolutionary ecology

Laboratory experiments to investigate symbiont phenotype generally do not attempt to simulate the natural stressors that enable costs of symbionts to manifest. Examples of

ecologically-relevant stressors include nutrient and water limitation (from phenomena such as larval overcrowding on food sources and conspecific competition), immune challenge from natural enemies, heat or cold shock, and mate competition. A sufficiently costly symbiont may die out or fail to spread to high prevalence in a population, even if phenotype experiments demonstrate that they should be fitness-increasing under ideal conditions. Symbiont cost may act as a barrier to horizontal introductions of a symbiont into a novel host species, as laboratory experiments frequently show that new transinfected insect lines can be sickly and difficult to maintain, even under low-stress conditions. For instance, transinfected *Spiroplasma* commonly damage novel host species (Tinsley and Majerus, 2007; Nakayama *et al.*, 2015).

1.7 The study system

1.7.1 Spiroplasma *strain hy1 is protective against a* Drosophila *parasitoid,* Leptopilina heterotoma

The bacteria of the genus *Spiroplasma* fall with the Gram-positive clade, but lack a cell wall. They are helical bacteria in the class *Mollicutes*. A variety of lifestyles are displayed by its members, but a common theme is an association with arthropods (reviewed in (Regassa and Gasparich, 2006)). Early in the history of the study of *Spiroplasma,* it was discovered that some *Spiroplasma* are the causative agents of plant diseases. *Spiroplasma citri* produces citrus stubborn disease, living in the phloem tissues of infected plants and being vectored by sap-sucking insects such as leaf-hoppers (Bové *et al.*, 2003), and *S. kunkelii* causes corn stunt disease and is insect-vectored (Whitcomb *et al.*, 1986). *Spiroplasma* may produce diseases in their arthropod hosts, such as *S. penaei* which infects Pacific white shrimp (Nunan *et al.*, 2005), and *S. apis,* linked to May disease in honeybees (Mouches *et al.*, 1983). It was also noticed early on that some *Spiroplasma* are arthropod endosymbiont sex ratio distorters; the end result of a transovarial, matrilineal inheritance pattern which favours manipulating hosts to invest heavily into daughters at the expense of sons. Examples include the 'SRO' (for 'sex ratio organism') strains in *Drosophila*, such as MSRO in *D. melanogaster,* NSRO in *D. nebulosa,* and WSRO in *D. willistoni* (Montenegro *et al.*, 2005).

Only in more recent years has it been discovered that *Spiroplasma* phenotypic diversity extends beyond the disease-causing/sex ratio distorter dichotomy. Examples of *Spiroplasma*-host mutualism have been unearthed. One example is MSRO, long-known to

be a male-killing sex ratio distorter (Montenegro *et al.*, 2005). Recently, MSRO was discovered to protect *D. melanogaster* against the parasitoid wasps *Asobara* t*abida* (Paredes Escobar, 2014), *Leptopilina heterotoma, L. boulardi* (Xie *et al.*, 2013), *L. victoriae* and *Ganaspis xanthopoda* (Mateos *et al.*, 2016). Some of the assayed braconid and figitid parasitoid wasps of *Drosophila* are not susceptible, and so resistance or susceptibility to MSRO must have evolved at least twice (Mateos *et al.*, 2016). The number of known mutualistic-only spiroplasmas is still relatively small and confined to *Drosophila*. A *Spiroplasma* protects North American *D. neotestacea* from female sterility caused by infection with the parasitic nematode *Howardula aoronymphium* (Jaenike *et al.*, 2010). The protective bacterium has been observed spreading from east to west, and subsequently increasing in prevalence in low-prevalence regions of this range (Jaenike *et al.*, 2010; Cockburn *et al.*, 2013). In addition to providing anti-nematode protection, the *Spiroplasma* of *D. neotestacea* can protect the host against *Leptopilina heterotoma* (Haselkorn and Jaenike, 2015). The other known *Spiroplasma* protective mutualist is found in *Drosophila hydei*, and called haplotype 1 (hy1). Interestingly, all these anti-wasp *Spiroplasma* species in *Drosophila* are in the poulsonii clade, indicating that anti-wasp protection could be ancestral to this clade (Haselkorn and Jaenike, 2015).

The mechanisms of *Spiroplasma*-mediated anti-wasp defence are beginning to be elucidated. Lipid limitation and toxins directed against the parasitoids are both likely to be playing roles, framed in terms of classical ecology as forms of 'exploitation competition' and 'interference competition' respectively by (Mateos *et al.*, 2016). Concerning lipid limitation (investigated in MSRO), it has been demonstrated through transporter knock-down experiments that diacylglyceride (DAG) in host haemolymph is necessary for MSRO to proliferate. Additionally, MSRO-carrying flies die faster under starvation than MSRO-negative flies, likely because MSRO depletes fatty acid reserves (Herren *et al.*, 2014). Subsequent investigations into found that *L. boulardi* and *A. tabida* larvae perform poorly when DAG levels are lowered in non-MSRO-infected flies, suggesting that *Spiroplasma* can kill parasitoid larvae by outcompeting with them for fatty acids (Paredes *et al.*, 2016). Many wasps, including *L. boulardi*, can't synthesise lipids as adults, although *L. heterotoma* can (Visser *et al.*, 2010). Meanwhile, a role for toxins has been demonstrated both in MSRO in *D. melanogaster* and the native *Spiroplasma* 'Sneo' in *D. neotestacea.* When *D. melanogaster* is attacked by *L. heterotoma* and *L. boulardi*, and when *D. neotestacea* is attacked by *L. heterotoma,* ribosome-inactivating proteins (RIP) act specifically upon the parasitoid wasp 28S ribosome, depurinating the α-sarcin/ricin loop. Interestingly, although

wasps are eliminated in all three of these cases, flies are only successfully rescued in the *D. melanogaster*/MSRO/*L. boulardi* combination (Ballinger and Perlman, 2017).

Spiroplasma hy1 is the strain which will be the focus of this thesis. It protects *Drosophila hydei* against the *Drosophila*-generalist parasitoid wasp, *Leptopilina heterotoma,* which lays its eggs inside larval *Drosophila.* The eggs hatch into wasp larvae and feed on fly tissues. If unprotected, the parasitised fly larva remains motile and feeds normally, dies during the pupal stage, and an adult wasp ultimately ecloses from the puparium. However, if hy1 is present, the wasp larva ceases to grow several days after hatching, and the probability of host fly survival increases dramatically, approximately fourfold. *Spiroplasma* hy1 is thus clearly of fitness benefit to the fly under wasp attack, and this is reinforced by population cage experiments, which showed hy1 sweeping in under high wasp attack rates (Xie *et al.*, 2010, 2015).

1.7.2 A temperate fruit fly, **Drosophila hydei**

Drosophila hydei is a species of *Drosophila* in the repleta group of *Drosophila* (Kwiatowski and Ayala, 1999), with a large size and slow development time. It has a mating ecology characterised by delayed sexual maturity and large sperm in males, and promiscuity in both males and females (Markow, 1985). In colder parts of its range in North America, it may overwinter in human dwellings as an adult (Spencer, 1941). *D. hydei* is a temperate and cosmopolitan species, with a global distribution as a human commensal invasive species (Shorrocks, 1972). It is thought to originate from Mexico, and in its North American range, to have spread north from here (Spencer, 1941). In the U.K. it is found commonly in gardens and orchards, and is more commonly caught from July until August (Dyson-Hudson, 1954) though success is also reported in September (F. Jiggins, pers. comm.). Confirmed U.K. captures of *D. hydei* go back to 1935, when a British specimen was deposited in the Natural History Museum, but a record exists of a fly that is almost certainly *D. hydei* captured in a London warehouse in 1930 (Richards and Herford, 1930). *D. hydei*'s abundance appears to be constrained by temperature in the U.K., as it is more common in the warmer south of England and gets scarcer moving northwards and into Scotland (Darren Obbard, pers. comm.; Chris Corbin, pers. obs.).

D. hydei has a huge range, and range expansion may have occurred after it began its association with hy1, as hy1 is found in large areas of North America as well as in Japan. In

contrast, the phylogenetically distinct *Spiroplasma* strain hy2 also found in *D. hydei* has a much more limited range. Thus far, it has only been recorded a few times, all in North America (Mateos *et al.*, 2006).

1.7.3 Leptopilina heterotoma *is a generalist parasitoid on* Drosophila *and is likely to be a significant selective force for* D. hydei

Parasitoid wasps are generally held to be an important cause of fruit fly mortality and can incur heavy losses, and thus stand to be a significant selective force. There is a huge variation in parasitism levels with factors such as type of food source, area, and season; attack rates vary from 5% to 40% in temperate areas of Europe (reviewed in (Fleury *et al.*, 2009)). One of the more prominent larval *Drosophila* parasitoids is *Leptopilina heterotoma*, due to its wide distribution, generalist habit (thought to be partly due to its ability to cope with defences in many host species (Schlenke *et al.*, 2007) and 'host conforming' biology. *Leptopilina heterotoma* has a broad Holarctic distribution (Carton *et al.*, 1986; Hardy and Godfray, 1990), and is present in both North America (Lue *et al.*, 2016) and Japan (Novković *et al.*, 2011), where *Spiroplasma* hy1 in *D. hydei* has been recorded. In northern Europe, where most studies of *Drosophila* parasitoid wasps have been carried out, *Leptopilina heterotoma* is abundant and can colonise Drosophilids on fermenting fruits, sap fluxes and decaying plants (Nordlander, 1980; Carton *et al.*, 1986; Janssen *et al.*, 1988; Hardy and Godfray, 1990; van Alphen *et al.*, 1991; Mitsui *et al.*, 2007).

In England, *L. heterotoma* is one of three common species – alongside *Asobara tabida* and *Tanycarpa punctata* – which attack Drosophilid larvae on fermenting fruit (Hardy and Godfray, 1990), and thus is likely to be in frequent contact with *D. hydei,* which is commonest in gardens and orchards. *Drosophila hydei* in the U.K. is abundant in June to August (Dyson-Hudson, 1954) and September (F. Jiggins, pers. comm.), and therefore should also overlap temporally with *L. heterotoma*, which is active from May to September (Hardy and Godfray, 1990). Because *D. hydei* usually makes up a relatively small proportion of temperate *Drosophila* assemblages, it's likely to be of only minor importance for *L. heterotoma* in terms of raw numbers of wasp production. A study at 21°C on sympatric host species in southeast France showed that *D. hydei* is an 'intermediate quality' host for *L. heterotoma,* with parasite survival in the 40-60% range (Fleury *et al.*, 2009). However, *L. heterotoma* is likely to be an important parasitoid to *D. hydei.* Another wasp, *Asobara tabida*, also uses *D. subobscura* and *D. melanogaster* as its main hosts (Kraaijeveld and Alphen, 1995), and has a similar Holarctic

distribution with reports from the northwest of America (Hoang, 2002), Japan (Mitsui *et al.*, 2007) and Europe (Carton *et al.*, 1986). This raised the issue of how these two wasps might interact in competition with each other on *D. hydei,* but published reports were not found to suggest that *D. hydei* has been tested as a host for *A. tabida*.

Optimal temperatures for *L. heterotoma* seems to deviate from those for its *Drosophila* hosts. The wasp undergoes quiescence as an adult in winter, like *D. hydei* (Eijs and Van Alphen, 1999), undergoing heavy winter mortality but emerging earlier in the spring than species such as *A. tabida*, which could give it a competitive edge early in the season. In temperate areas, *L. heterotoma* can fit up to 4 generations into a breeding season (Fleury *et al.*, 2009). Examined over the temperature range 14-26°C, the wasp has a narrow thermal niche compared to its primary hosts, *D. melanogaster, D. simulans* and *D. subobscura,* and is less tolerant of higher temperatures (Ris *et al.*, 2004).

Temperature interactions with wasp genotype have been documented for *L. heterotoma*, with strains from warmer areas of France showing greater adaptation to warmer temperatures (on *D. simulans*) (Fleury *et al.*, 2009). More generally for parasitoids, temperature may modulate levels of competition between species sharing the same set of hosts. The trait values of parasitoid wasps in a temperate Drosophilid assemblage, including *L. heterotoma* and *Asobara tabida*, overlapped more at higher temperatures, which could potentially produce more competition between the parasitoid species (Le Lann *et al.*, 2014). Interestingly for discussions of how *L. heterotoma* may be able to locally adapt to microclimates, a different study (though focused on a different wasp) found that *L. boulardi* shows local adaptation of life history traits to thermal reaction norms, and these are habitat specific (forest versus orchard) (Moiroux *et al.*, 2013).

1.7.4 Despite being advantageous against L. heterotoma*,* Spiroplasma *hy1 exists at low to intermediate frequencies in* D. hydei

Despite granting a fitness advantage against an important natural enemy, and having a large geographical distribution in its host, hy1's prevalence in the wild generally holds stable at a low-to-intermediate value. Japanese population studies indicate that this prevalence has held steady over several decades, returning prevalence estimates of 34.6% and 36.7% in subsequent years in one modern population survey, 29.4% to 19.3% over three years in a second modern population survey (the fluctuations are likely due to small

sample sizes) (Osaka *et al.*, 2010), a range of prevalences from 26% to 66% across five widely-spaced localities in 2005 (Kageyama *et al.*, 2006), and in 1978, a prevalence of 45.9% in east Japan in 1978 (Ota *et al.*, 1979). In North America, the prevalence in a pool of samples from two populations in Arizona and one population in Mexico was 28.6% (Watts *et al.*, 2009). The lower prevalences suggests that factors may be counteracting hy1's benefits. For instance, previous work demonstrates that hy1 titre in adult flies lowers with temperature, and transmission may decrease at lower temperatures such as 18°C and 15°C (though see Chapter 3's introduction for critique of these data). Much of hy1's range is temperate or experiences shorter-term dramatic temperature fluctuations. Additionally, hy1-protected male survivor flies are thought to suffer from unusual rates of sterility, compared to hy1-protected male flies which never experience wasp attack. This suggests that hy1 may demonstrate costly or incomplete rescue in many male flies. Little is currently known about the cost of hy1 infection to its native host.

1.8 Outline of thesis: what factors could be keeping a 'good mutualist' down?

The factors contributing to low to intermediate prevalence of mutualistic symbionts in insect hosts are poorly understood. This thesis aims to investigate factors which could keep prevalence low in the experimentally tractable *D. hydei/*hy1*/L. heterotoma* system, with a focus on temperature's influence on transmission and phenotype, and how ecologically-relevant stressors may influence the cost of hy1 to its host.

1.8.1 Chapter 2 – A review of temperature's influence on heritable symbionts

To contextualise the temperature-related work included in this thesis, I first present a review of the literature of how temperature interacts with heritable symbionts. The bulk of this considers facultative symbionts, such as *Spiroplasma* hy1. The relationships between symbionts and the thermal environment vary, with some symbionts altering host thermotolerance, and others having their interaction with the host changed by temperature. The review covers what is known about temperature's influence on phenotype (including costs) and vertical transmission efficiency. It finds a variety of patterns; whether a symbiont is sensitive to cold, or instead to heat, varies with host species, and phenotype and transmission can be ablated at different thermal thresholds from each other. Overall, the review highlights the need for evolutionary-ecological

consideration of symbiont–host interactions to assess the interactions for several temperatures in the natural thermal range. Further discussion points include a potential for historical effects of past temperatures, and how temperature constraints may prevent symbionts invading new species after horizontal transfer events, with implications for real-world applications of heritable bacterial symbionts, such as those used for insect vector control.

1.8.2 Chapter 3 (part 1) - Is hy1 present in **Drosophila hydei** *in the U.K.?*

To add to existing knowledge on hy1's range and thus to contribute to whether cooler temperatures may be a factor shaping its prevalence, *D. hydei* individuals from a site in southern England were captured and PCR assayed for *Spiroplasma* hy1. Upon detection, hy1 16S rRNA sequences were obtained to assess similarity of the UK strain to that strain previously found in the U.S.

1.8.3 Chapter 3 (part 2) - How is the transmission of hy1 in **Drosophila hydei** *affected by ecologically-relevant low temperature?*

Spiroplasma strains in *Drosophila* generally have lower phenotypic expression and transmission efficiency at temperatures below the commonly-used laboratory temperature of 25°C, and Jekyll-and-Hyde *Spiroplasma* strains are generally of tropical origin, although *Spiroplasma* mutualists such as in *D. neotestacea, A. pisum,* and *D. hydei* are found in temperate climes. This indicates a possible role for low temperatures in reducing the prevalence of hy1 in *D. hydei,* particularly in more temperate parts of its range.

Transmission of hy1 from female hosts to their offspring could be hampered at lower temperatures. Indeed, prior work by (Osaka *et al.*, 2010) showed significantly attenuated transmission at 18°C and complete ablation of vertical transmission at 15°C compared to 25°C. All of these temperatures would be typical for the U.K. range even in the summer breeding season. However, this study kept flies at their low 'transmission temperatures' for their whole lives, raising concerns that if temperature influences titre, it could also decrease PCR detectability of infection. To address this, an experiment was carried out to assess transmission at a variety of temperatures that used a 'recovery' protocol, where flies destined for assaying were transferred to 25°C after being laid as eggs.

1.8.4 Chapter 4 - How is the phenotype of hy1 in **Drosophila hydei** *affected by ecologically-relevant low temperature?*

Continuing from Chapter 3, in which I investigate a possible role for low temperatures in reducing hy1's prevalence in *D. hydei* via effects on transmission efficiency, I turn my attention to temperature's effects on phenotype. **Phenotype** strength may be weaker at lower temperatures. I carried out an experiment to investigate the effect of a low temperature, 18°C, on the strength of the protective phenotype of hy1 under wasp attack conditions. Results were assessed in terms of effects on fly fitness and effects on wasp fitness.

1.8.5 Chapter 5 - Is hy1 costly to **Drosophila hydei***?*

I was interested to see if a cost existed of hy1 carriage to *D. hydei,* whether this was standing or active, and whether it was masked except under ecologically-relevant stress. Experiments were carried out to investigate the standing (non-wasp-attacked) cost of hy1 to adult flies, firstly under 'ideal' conditions (assayed through wing size) and then under 'costly', starvation conditions (assayed by adult time to death by starvation). To limit confounds caused by infected and uninfected stocks being reared separately, larvae were reared in common garden vials, and their infection statuses recovered post mortem. Additionally, an experiment was carried out to investigate active costs – those which manifest when hy1 has protected its host – following reports in Xie et al. of increased rates of male sterility seen in flies 'rescued' from attack by hy1 (Xie *et al.*, 2011).

1.8.5 Chapter 6 – General discussion

The thesis ends with a discussion in which the results are summarised and synthesized, and the likely impact of environmental variation on symbiont dynamics in the *D. hydei-Spiroplasma* interaction are predicted.

2 Symbiont evolutionary ecology and temperature; a review

2.1 Authorship statement

This chapter is a reproduction of a review paper, 'Corbin et al: Heritable symbionts in a world of varying temperature' (Heredity (2017) 118, 10–20; doi:10.1038/hdy.2016.71). The co-authors are Eleanor R. Heyworth, Julia Ferrari, and Greg Hurst. Greg Hurst edited and helped ensure that sections written by different people were integrated smoothly, and Julia Ferrari proof-read the paper. The remainder of the work on the paper was carried out by me, with the exception of the section on obligate symbionts, 'Obligate heritable microbes commonly represent a thermal 'weak link' for their hosts', which was written by Dr Eleanor Heyworth.

2.2 Abstract

Heritable microbes represent an important component of the biology, ecology and evolution of many plants, animals and fungi, acting as both parasites and partners. In this review, we examine how heritable symbiont–host interactions may alter host thermal tolerance, and how the dynamics of these interactions may more generally be altered by thermal environment. Obligate symbionts, those required by their host, are considered to represent a thermally sensitive weak point for their host, associated with accumulation of deleterious mutations. As such, these symbionts may represent an important determinant of host thermal envelope and spatial distribution. We then examine the varied relationship between thermal environment and the frequency of facultative symbionts that provide ecologically contingent benefits or act as parasites. We note that some facultative symbionts directly alter host thermotolerance. We outline how thermal environment will alter the benefits/costs of infection more widely, and additionally modulate vertical transmission efficiency. Multiple patterns are observed, with symbionts being cold sensitive in some species and heat sensitive in others, with varying and non-coincident thresholds at which phenotype and transmission are ablated. Nevertheless, it is clear that studies aiming to predict ecological and evolutionary dynamics of symbiont–host interactions need to examine the interaction across a range of thermal environments. Finally, we discuss the importance of thermal sensitivity in predicting the success/failure of symbionts to spread into novel species following natural/engineered introduction.

2.3 Introduction

Heritable symbionts—viruses, bacteria, protists or fungal associates that pass from parent to offspring—are found widely in multicellular fungi, plants and animals. It is currently considered that heritable bacteria infect more than half of all arthropod species (Duron *et al.*, 2008), that fungal symbionts are common in both insects and grasses (Clay, 1990; Gibson and Hunter, 2010) and that heritable viruses are widespread in fungi, plants and insects (Roossinck, 2015). Biologically, symbionts such as these represent important modulators of host phenotype and provide heritable variation upon which natural selection acts. Variously, they may provide defence against natural enemies, play a role in host nutrition (through digestive processes, anabolic processes or as farmed symbionts, as in fungal ant gardens) or determine host plant use for insects. These microbes may also modulate the competence of their host for pathogenesis (Bryner and Rigling, 2011) or for vector capability (McMeniman *et al.*, 2012). Maternally inherited symbionts may also act as reproductive parasites, manipulating host reproductive processes towards the production and survival of daughters (Hurst and Frost, 2015). This process is most well recognised in insects, but is also observed in the case of viral-induced male sterility in plants (Grill and Garger, 1981).

The effect of symbiont infection upon host individuals produces further effects at the population and community levels. Sex ratio distorting symbionts affect the reproductive ecology of their host, and may additionally affect population persistence. Those involved in contribution to anabolic function permit their host to exist in nutritional niches that would not otherwise be occupied. Protective symbionts, of course, are likely to impact upon the dynamics of the natural enemies against which they protect (Fenton *et al.*, 2011), and those that affect parasite virulence similarly alter the dynamics of parasite and host. At the community level, plant endophytes alter the pattern of competition between plant species (Clay *et al.*, 1993, 2005; Clay and Holah, 1999), facilitate invasion (Aschehoug *et al.*, 2012) and may change patterns of succession through, for example, reducing herbivory.

In this paper, we examine the sensitivity of these interactions to thermal environment. Thermal environment is well recognised as altering the outcome of host–parasite interactions, both in terms of progression of infection within an individual and in terms of ecological and evolutionary dynamics in populations (Thomas and Blanford, 2003). We examine the thesis that temperature will be an important modulator of heritable symbiont–host interactions. We note that these interactions are distinct from parasite–host

comparators in that they may be either beneficial or parasitic, and the symbiont may on occasions be obligatory for survival. We first outline the evidence that obligate heritable symbionts—those required by their host—form a weak link under thermal stress, potentially limiting the geographic range of their host species. We then outline the interaction between thermal environment and facultative heritable microbes—microbes that are not required, but commonly provide ecologically contingent benefits or act as reproductive parasites or both. We first note heritable symbiont frequency is affected by the magnitude of any benefit they bring to host biology, the physiological cost of carriage of symbionts and the fraction of female offspring that fail to inherit them (segregational loss). We argue that thermal environment affects all of these parameters, and that understanding heritable symbiont dynamics in natural populations requires detailed study across a range of thermal environments.

2.4 Obligate heritable microbes commonly represent a thermal 'weak link' for their hosts

There are many animals (and some plants) in which curing an individual of symbionts through antibiotic, heat or other treatments results in the death or sterility of their host. Dependence upon symbionts is commonly observed in insects (Wernegreen, 2002; Zientz *et al.*, 2004), nematodes (Slatko *et al.*, 2010; Darby *et al.*, 2012) and plants (Rodriguez *et al.*, 2009). In many cases these are coadapted metabolic partnerships where the symbiont provides essential nutrients to the host, allowing the exploitation of nutrient-poor resources or habitats (Baumann, 2005; Douglas, 2009). In others, the microbe gives little metabolic contribution to the host, yet the host has evolved to become dependent on the symbiont, as in the wasps *Asobara* (Dedeine *et al.*, 2001) and *Trichogramma* (Stouthamer *et al.*, 1990) and the plant *Psychotria* (Cowles, 1915).

Removal of the obligate symbiont typically results in the death or sterilisation of its host. Many examples of this come from insects, where the obligate symbionts reside in specialised cells known as bacteriocytes (Sacchi *et al.*, 1993; Montllor *et al.*, 2002). Thermal stress commonly causes the death of bacteriocytes that, once killed, do not regenerate. A model for symbiont studies, the aphid–*Buchnera aphidicola* symbiosis, can be disrupted through exposing the insects to both high (Wilcox *et al.*, 2003; Dunbar *et al.*, 2007) or low temperatures (Parish and Bale, 1991) as the symbiont populations decrease. Indeed,

interclonal variation in the thermal sensitivity of aphids is associated with variation in *Buchnera*, with a single-nucleotide deletion in the heat shock promoter region of the heat shock gene ibpA being associated with reduced tolerance to thermal stress, but improved fitness at normal environmental temperatures (Dunbar *et al.*, 2007; Moran and Yun, 2015). In field cages, aphid clones carrying the reduced heat tolerance strain of *Buchnera* outcompete clones carrying the tolerant strain at low temperatures, but these clones are outcompeted where heat shocks occur (Harmon *et al.*, 2009). Heat treatments in weevils (Heddi *et al.*, 1999) and cockroaches (Sacchi *et al.*, 1993) kill their bacteriocytes in a similar manner. Mealybug symbionts are also killed at elevated temperature, though this only has an impact on survival/fertility if it occurs during pre-adult development (Parkinson *et al.*, 2014).

There are strong evolutionary reasons to believe thermal impacts on obligate symbiont function will be general and widespread. These obligate symbionts are vertically transmitted from the parent to offspring with high fidelity (Bandi *et al.*, 1998; Faeth and Fagan, 2002; Hosokawa *et al.*, 2006, 2012). Indeed, obligate symbionts infecting hosts such as aphids (Shigenobu and Stern, 2013), tsetse flies (Akman *et al.*, 2002), cockroaches (Patiño-Navarrete *et al.*, 2013) and nematodes (Slatko *et al.*, 2010) form close partnerships that have lasted for many millions of years, with congruent host and symbiont phylogenies indicating horizontal transmission of the symbiont is rare. This long coevolution within the protective confines of a host has led to a Muller's ratchet process in the symbiont in which there is accumulation of mildly deleterious mutations, alongside large reductions in genome size as loss of nonessential genes occurs over time (Moran, 1996; Nikoh *et al.*, 2011). The process is likely to lead to the degradation of any systems not under strong selection, such as occasional exposure to high temperature.

The process of mutational decay has a major impact upon thermal tolerance. For instance, extensive genome reduction in *Buchnera* is reflected in this symbiont producing just 5 heat shock proteins, a substantial decrease compared with the 75 produced by its free-living and more thermotolerant relative *Escherichia coli* (Bronikowski *et al.*, 2001; Wilcox *et al.*, 2003; Pérez-Brocal *et al.*, 2006; Liu *et al.*, 2012). More widely, accumulation of deleterious mutations in remaining genes (Moran, 1996) is reflected in weaker secondary and tertiary structure of proteins in *Buchnera* (van Ham *et al.*, 2003), with the result that the function of proteins in obligate symbionts is disproportionately impaired at elevated temperatures compared with proteins encoded in the host genome. It is also notable that chaperonin

genes—that stabilise protein structure under stress—are highly expressed in obligate symbionts at normal temperature. GroEL, for instance, comprises ~10% and 6% of the proteome of *Buchnera* in aphids and *Blochmannia* in ants, respectively, in normal thermal environments (Baumann *et al.*, 1996; Fan *et al.*, 2013). More widely, chaperonins represent 22% of protein abundance in *Buchnera* and 15% in *Blochmannia*. This high level of chaperonin expression is hypothesised to represent a means to cosset proteins that are structurally weak that then fail at elevated temperatures where no further failsafe is possible (Moran, 1996).

The inability of symbionts to cope with temperature stress makes many obligate symbionts into a 'weak link' in host thermal tolerance. Although the services provided by heritable microbes have been credited with allowing early host range expansion by permitting the exploitation of widespread but nutritionally poor resources (Feldhaar and Gross, 2009; Hansen and Moran, 2011), their narrow temperature requirements have been implicated in restricting host spread. Insects such as aphids may be limited to temperate regions by their intracellular symbionts (Dixon *et al.*, 1987), whereas fungus-cultivating ants are restricted to tropical environments by the temperature requirements of their obligate cold-susceptible fungal symbiont (Mueller *et al.*, 2011). To date, there has been no formal comparative test of this hypothesis, in which thermal niche breadth of hosts with and without symbionts are compared. What is clear, however, is that as global temperatures rise (Cox *et al.*, 2000), plants and animals may be required to move ranges to maintain their ideal environment or to adapt to higher temperatures (Walther *et al.*, 2002; Parmesan and Yohe, 2003). The small genomes and lack of horizontal gene transfer in obligate symbionts (O'Fallon, 2008) may mean that the latter process of adaptation is likely to be barred, thus requiring the host to move range rather than adapting *in situ*.

2.5 The interaction between thermal environment and facultative heritable symbionts

Facultative heritable symbionts are those where cured host individuals retain reproduction and fertility. Commonly, bacterial and fungal symbionts are heritable through the female line (but see (Moran and Dunbar, 2006; Watanabe *et al.*, 2014)), whereas viruses are heritable through both parents, although commonly with higher efficiency through egg than sperm. For maternally inherited agents, their capacity to invade populations depends

on their impact on the production, survival and reproduction of female hosts. Minimal models of heritable microbe dynamics thus include two parameters, whose temperature sensitivity will then determine response to thermal environment:

1. The effects the symbiont has upon host fecundity, survival or sex ratio.
2. The vertical transmission efficiency of the symbiont (separated into paternal and maternal components for biparentally inherited agents).

Under this minimal model, a maternally inherited symbiont will spread if, when rare, an infected female leaves on average more infected daughters than an uninfected female leaves daughters. Where the magnitude of improvement in host fecundity/survival/sex ratio is low (that is, an infected female on average leaves a few more infected daughters than an uninfected female leaves daughters), equilibrium prevalence becomes very sensitive to changes in vertical transmission efficiency (Jaenike, 2009; Gundel *et al.*, 2011).

Symbiont-mediated phenotypes that enable facultative heritable microbes to invade populations are very diverse. Some symbionts are reproductive parasites that spread through biasing sex allocation to the production of daughters or inducing incompatibility in uninfected zygotes (Werren *et al.*, 2008). Other interactions are mutualistic and involve benefits to their host that are ecologically contingent—they exist only under particular circumstances, with hosts retaining full function in the absence of symbionts outside these conditions. Symbionts can provide protection from natural enemies (Kellner, 2002; Scarborough *et al.*, 2005; Oliver *et al.*, 2005; Xie *et al.*, 2010; Nakabachi *et al.*, 2013) and disease (Caragata *et al.*, 2013), enhance immune response (Márquez *et al.*, 2007; de Souza *et al.*, 2009) or determine plant host range. They may also be used in offence, as is the case for *Photorhabdus* released from entomopathogenic nematodes into insects on infection, and which then kill the insect (Poinar, 1975).

What then are the likely impacts of thermal environment on the population biology of heritable microbes in natural populations? Associative studies, linking seasonal and spatial variation in symbiont frequency, are limited in power to detect thermal impacts by the presence of multiple covarying factors in natural populations (for example, thermal environment and desiccation) and the presence of spatially varying coevolution. Clinal variation in symbiont prevalence is a more powerful indicator of thermal environment driving symbiont dynamics, and does support temperature–symbiont interactions in a number of cases (see Table 2.7.1). However, these data have multiple potential sources for

the association. Thus, a more precise view can be gained through defined experimental study. At its most powerful, this may involve varying thermal environment within laboratory or caged populations over a number of generations and examining its impact on symbiont dynamics. For instance, (Versace *et al.*, 2014) noted that the *Wolbachia* strain that spread in passage through *Drosophila melanogaster* population cages depended upon the temperature at which the population was maintained (Versace *et al.*, 2014). However, studies such as this are logistically complex for many species. More common are single-generation studies that examine one or more aspects of the host–symbiont interaction under different temperatures. Below we summarise these studies. We first outline evidence that indicate heritable symbionts may directly alter host thermal tolerance. We then outline how phenotypes providing ecologically contingent benefits to their host and reproductive manipulation phenotypes are altered by thermal environment. We then examine data with respect to temperature impacts upon vertical transmission and the direct physiological cost of symbiont infection. We draw this information together to create a generalised picture of the thermal sensitivity of heritable microbe–host interactions.

2.5.1 Direct effects of symbiont presence on host thermal tolerance

Laboratory study indicates that facultative heritable bacteria can affect host thermal tolerance in a number of cases. In aphids, at least three different facultative symbionts increase insect survival or reproduction after heat shock (Chen *et al.*, 2000; Russell and Moran, 2006; Heyworth and Ferrari, 2015). *Hamiltonella* infections in whitefly confer a similar protection (Brumin *et al.*, 2011). The mechanisms behind symbiont-conferred increase in thermal tolerance are not always known, although there are several hypotheses. The ability of *Serratia symbiotica* to permit pea aphids to survive at high temperatures was hypothesised to be due to *Serratia* replacing the amino acid biosynthesis function of the obligate symbiont *Buchnera* (Koga *et al.*, 2003, 2007), but (Burke and Moran, 2011) noted *S. symbiotica* is incapable of this because of deletion or degradation of amino acid biosynthesis pathways, and indeed it may itself be dependent on *Buchnera*. Instead, it seems that *Serratia* protects *Buchnera*, possibly by lysing to release metabolites (Montllor *et al.*, 2002; Burke *et al.*, 2010). Meanwhile in whitefly, the presence of the facultative symbiont increases expression of host-produced stress genes, inadvertently preparing it for thermal stress (Brumin *et al.*, 2011).

Heritable fungal endophytes also impact upon plant heat stress adaptation (Rodriguez and Redman, 2008; Rodriguez *et al.*, 2009). Most notably, endophytes of panic grass permit plant growth on geothermal soils in Yellowstone National Park (Redman *et al.*, 2002; Rodriguez *et al.*, 2008). This is a mutualistic relationship, as in some cases neither plant nor fungus can survive the high temperature without the other (Redman *et al.*, 2002; Márquez *et al.*, 2007). Fascinatingly, the heat tolerance property is determined by a viral heritable symbiont of the endophyte fungus, with the presence of the virus enabling both endophyte and plant persistence. Further to this, endophytes may increase seed germination under thermal stress (Hubbard *et al.*, 2012).

To date, the majority of studies of heritable symbiont impacts on thermal tolerance have investigated the impacts of elevated temperature. We found a single study examining frost resistance in relationship to heritable symbionts in insects, and this revealed no impact of symbiont presence on frost tolerance (Łukasik *et al.*, 2011). However, the presence of nonheritable symbionts with freeze-tolerance phenotypes suggests that similar phenotypes warrant more extensive examination for heritable microbe–host interactions. *Anaplasma phagocytophilum* is acquired horizontally each generation by its tick host *Ixodes scapularis* following blood feeding. Observations and experiments indicate that *Anaplasma* infection protects its host against damage from frost and cold. This occurs through *Anaplasma*-induced induction of anti-freeze protein production by the host individual (Neelakanta *et al.*, 2010). Further to this, nonheritable *Spiroplasma* infections increase corn leafhopper survival during overwintering periods (Ebbert and Nault, 1994), indicating there may be impacts of symbionts on overwinter (freeze) survival.

2.5.2 Impact of temperature on ecologically contingent benefits

We found two studies relating the impact of temperature on protective phenotype in natural infections of insects. In the European beewolf *Philanthus triangulum*, *Streptomyces* heritable symbionts secrete antibiotics that protect the host cocoon from pathogen attack during diapause in the soil. (Koehler and Kaltenpoth, 2013) found thermal environment (from 15 to 25 °C including diurnal variation) had no impact on the quantity of antibiotic produced. In contrast to this, pea aphids carrying *Hamiltonella defensa* were nearly completely resistant to attack by *Aphidius ervi* parasitic wasps at 20 °C, but were susceptible at 25 and 30 °C, postulated to represent thermal sensitivity of

symbiont-mediated protection (Bensadia *et al.*, 2006; Guay *et al.*, 2009). Further work confirmed this result, but additionally showed protection was insensitive to temperature in clones where *H. defensa* co-occurred with PAXS (Guay *et al.*, 2009). Although this would have an impact upon symbiont dynamics, the role of host and symbiont factors in establishing this pattern were not ascertained.

Outside of heritable microbe interactions with insects, temperature modulates the effect of heritable virus infection in the chestnut blight fungus *Cryphonectria parasitica*. In this interaction, viral presence commonly alters fungal growth and sporulation *in vitro*, and produces a hypovirulent phenotype when the fungus is introduced to the chestnut tree. The hypovirulent phenotype associated with virus presence is temperature sensitive, commonly greatest at 24 °C, as compared with 12, 18 and 30 °C. The authors also noted a fungal and viral genotype dependence of the virulence phenotype, and conclude that the coevolutionary dynamics of the system would thus be determined by a complex G × G × E interaction (Bryner and Rigling, 2011).

Studies investigating the impact of thermal environment upon heritable symbiont dynamics have largely focussed on the direct impact of temperature on the phenotype of the symbiont as outlined above. However, the dynamics of heritable microbes may also be altered by changes in the benefit derived from a given phenotype that may be driven by temperature-driven changes in other biotic interactions. For instance, the frequency achieved by a symbiont that protects against natural enemies depends upon the rate of attack by enemies against which the symbiont defends. Thermal environment may alter both individual wasp movement patterns, the density of attackers, their ability to parasitise in the absence of protection and indeed the community of species that do attack. In so doing, it would alter the dynamics of the symbiont even if the transmission and phenotype of the symbiont are temperature invariant. Understanding thermal impacts on this ecological context is a key area for future work.

2.5.3 Impact of temperature on reproductive parasitic phenotypes

Many studies examine the impact of thermal environment on the expression of reproductive parasitic phenotypes in insects (Table 2.7.2). Most commonly, *Wolbachia*-induced male killing, parthenogenesis induction and cytoplasmic incompatibility are ablated at high temperatures. However, the temperature required for the phenotype to be affected

varies—in the temperate species *Drosophila bifasciata*, male killing becomes incomplete above 23.5 °C (Hurst *et al.*, 2000, 2001). Cytoplasmic incompatibility (CI) is commonly less strongly expressed at high temperatures, becoming incomplete in *D. simulans* at 28 °C, and at temperatures of >30 °C in other species (Wright and Wang, 1980; Trpis *et al.*, 1981; Stevens, 1989; Clancy and Hoffmann, 1998; Johanowicz and Hoy, 1998; van Opijnen and Breeuwer, 1999). However, there are a number of cases where phenotype is only affected following multigenerational passage at elevated temperatures. There is also evidence that heat shock (exposure to temperatures exceeding 35 °C for between 30 min and 2 h) alters the expression of CI (Feder *et al.*, 1999). Currently, it is unclear why thermal sensitivity of these traits is so variable, and whether it is associated with host or microbial factors. In contrast to *Wolbachia*-induced phenotypes, *Spiroplasma*-induced male killing is ablated at lower temperatures (Williamson, 1965; Counce and Poulson, 1966; Anbutsu *et al.*, 2008).

As previously discussed with respect to the dynamics of protective symbionts, the impact of temperature on symbiont prevalence may also be affected by the effect of the phenotype on host survival and fecundity. For instance, the drive associated with male killing relates to the intensity of sibling–sibling interactions, with male host death having little impact on symbiont fitness when these interactions are weak (for example, food excess), but are strong when siblings strongly compete (for example, food paucity) (Hurst and Frost, 2015). Thus, external ecological characteristics that may be thermally dependent (for example, aphid supply for ladybirds) are likely to impact upon symbiont dynamics. In contrast, the impact of thermal ablation of phenotype on symbiont prevalence is likely to be much lower for traits like CI, where the effect is not strongly ecologically contingent, and which is under positive frequency-dependent selection. Where CI causing *Wolbachia* are common, nearly all females mate to infected males. If CI strength diminishes by 50%, this remains a very high fitness loss for uninfected females, such that declines in prevalence associated with thermal ablation of phenotype will be small. In contrast, ablation of male killing, which produces only a small (1–20%) impact on female survival, will have a more profound influence, potentially making the symbiont net costly to female host (measured in terms of production/survival of daughters). Thus, theory predicts the impacts to be greater in this case (Jaenike, 2009).

2.5.4 Physiological cost of symbionts at different temperatures

Endosymbionts, which rely on their hosts for nutrition, can impose a cost on their host. For example, the defensive symbiont *H. defensa* can be costly to the hosts *Acyrthosiphon pisum* and *Aphis fabae* (see, for example, (Vorburger *et al.*, 2013; Polin *et al.*, 2014) and references therein). Costs may manifest, or be manifested more dramatically, when the host is under physiological stress. Thus far, there have been few studies examining the physiological cost of symbionts at different temperatures. In *A. pisum*, the endosymbiont *Regiella insecticola* was found to be costly under heat stress, but not when hosts were reared in standard conditions. The cost was observed after 2-day-old nymphs were exposed to a period of heat shock at 37.5 °C. Uninfected heat-shocked aphids were 24% more likely to survive to adulthood than infected heat-shocked aphids, and infected heat-shocked aphids also suffered higher sterility rates (Russell and Moran, 2006).

Study of *Wolbachia*-infected *D. melanogaster* also indicates thermal impacts on the cost of carrying a symbiont. *D. melanogaster* were established in field cages in tropical and temperate areas of Australia during winter. *Wolbachia* effect on the host, relative to uninfected flies, depended on whether the fruit fly nuclear background was tropical or temperate. In tropical cages, infected flies of both backgrounds had lower fecundity than their uninfected counterparts. In contrast, in the temperate cage, the effects of *Wolbachia* depended on the nuclear background, with temperate-background flies experiencing higher fecundity when infected. This example demonstrates that a previously beneficial symbiont might become a liability when local climate is unfavourable (Olsen *et al.*, 2001). More recently, (Kriesner *et al.*, 2016) demonstrated that *Wolbachia* has a particular negative impact upon fecundity in flies that survive through winter. Flies with *Wolbachia* post dormancy have a lower fecundity than flies without the infection (Kriesner *et al.*, 2016).

Outside of insect–bacterium interactions, temperature dependence of heritable viral impacts on fungal growth *in vitro* has also been reported in a number of interactions (see, for example, (Hyder *et al.*, 2013) and references therein). Furthermore, Sigma virus in *D. melanogaster* causes a deleterious CO_2 sensitivity that is highest at low temperatures, with reduced concentrations required to induce death (see (Longdon and Jiggins, 2012) and references therein). Thus, it seems that viral as well as bacterial symbionts show temperature-dependent phenotypes in multiple host species.

2.5.5 Thermal environment and transmission efficiency

Studies of heritable bacteria in insects have concluded that vertical transmission efficiency is sensitive to rearing temperature (Table 2.7.3). In a manner similar to that observed for phenotype, *Wolbachia* vertical transmission efficiency has been observed to be reduced at raised temperature, and *Spiroplasma* vertical transmission efficiency reduced at cool temperatures. However, it is notable that phenotype expression is commonly more sensitive than transmission, with phenotype ablation occurring before loss of vertical transmission in a number of cases.

Few studies examine the impact of overwintering on heritable symbiont transmission. (Perrot-Minnot *et al.*, 1996) note that segregational loss of *Wolbachia* is increased during artificially prolonged (2–6 year) larval diapause. In pea aphids, *R. insecticola* shows segregational loss in sexually produced eggs that persist through winter, but 100% vertical transmission in asexual summer reproduction (Moran and Dunbar, 2006). These observations raise the potential importance of overwinter phases on symbiont transmission, but this requires evaluation over natural diapause periods across a number of symbioses.

One caveat to studies of transmission efficiency is the degree to which we can accurately score infected and uninfected individuals in a standard PCR assay. This is an issue of detectability of low titre infections. For instance, (van Opijnen and Breeuwer, 1999) studied the impact of high temperature (32 °C) passage of laboratory stocks of the red spider mite *Tetranychus urticae* upon the presence of *Wolbachia*. PCR assays were used to detect *Wolbachia* infection, and indicated that prevalence decreased over four generations of exposure to this temperature, with no individual scored as infected in generation 4. However, *Wolbachia* infection was detected in 29% of individuals two generations after restoration of these lines to 25 °C, the permissive temperature. Only after six generations of exposure to 32 °C was *Wolbachia* found to be lost after restoration to the permissive temperature (van Opijnen and Breeuwer, 1999). The most parsimonious explanation for these data is that the symbiont declined in titre during passage, and by generation 4 the titre was sufficiently low that it was undetectable by the PCR methodology used. Care should thus be taken to either use a recovery period before concluding symbiont absence (see examples in Table 2.7.3) or using very stringent quality control with respect to symbiont detectability in PCR assays. Such assays could involve ‘spiking’ of symbiont-

carrying material at varying dilutions into uninfected carrier host DNA to establish the limit to detectability, and also employ quantitative PCR to robustly determine limits to detection.

Outside insect-heritable bacteria interactions, it is known that transmission of sigma virus in *D. melanogaster* is thermally sensitive. Vertical transmission is ablated at high temperatures, with 30 °C passage curing flies. In plants, fungal endophyte vertical transmission in cool season grasses is also known to be affected by temperature. Endophyte fungi commonly transfer on the exterior of seeds. (do Valle Ribeiro, 1993) reviewed the impact of seed storage conditions on the survival of the fungus and its propagation following germination. They concluded that storage time, humidity and temperature of storage affected the likelihood of plants germinating from seeds acquiring the symbiont. Overall, seeds maintained at higher temperatures, at low relative humidity and for longer periods of time were less likely to retain the infection, presumably associated with loss of fungal viability on the seed (do Valle Ribeiro, 1993). However, the impact of temperature is not universal: Oldrup et al. 2010 noted that 80% of locoweed seed maintained in uncontrolled warehouse conditions over 40 years retain *Undifilum* endophyte infection (Oldrup *et al.*, 2010).

Variation in vertical transmission efficiency is thought to be an important driver of endophyte dynamics and equilibrium prevalence, as the 'benefit' from endophyte infection is relatively weak (Afkhami and Rudgers, 2008; Gundel *et al.*, 2008). However, although loss in seed storage argues for a role of temperature in endophyte dynamics, exploration of the whole transmission cycle under natural conditions is required to determine the sensitivity of endophyte dynamics to thermal environment: loss of endophyte infection can occur at any of three stages—from tiller to seed, seed to seedling and during tiller growth (Afkhami and Rudgers, 2008). These authors conclude that vertical transmission variation may be important in determining intraspecific spatial and interspecies differences in endophyte prevalence, and the role of the environment in generating vertical transmission variation warranted investigation. However, they note that variation in transmission and prevalence of infection may be additionally associated with the frequency with which the drought tolerance phenotype is induced (Davitt *et al.*, 2011), or may derive from coevolutionary interactions between host and fungus affecting transmission efficiency.

2.6 A generalised view of thermal impacts on facultative heritable symbionts

The above account creates a few clear messages. The first of these is that many aspects of heritable symbiont phenotype and transmission are thermally sensitive. Although our review is biased to heritable bacteria–insect interactions, thermal sensitivity was noted in a wide range of interactions (bacteria–insect, fungus–plant, virus–plant, virus–insect), and is likely to be general. However, the pattern of thermal sensitivity (chill vs heat; threshold for thermal impact) varies greatly across interactions. Thus, it is clear that although thermal environment is very likely to affect facultative symbiont dynamics in many systems, the way in which it does so will vary greatly.

A second observation is that different aspects of the host–symbiont interaction have different thermal sensitivities. One commonly measured 'linking' variable is symbiont titre—the number of symbionts resident in a host. Thermal environment impacts upon titre, and phenotype ablation and segregational loss during reproduction is commonly associated with low titre. Commonly, phenotype ablation occurs before high levels of segregational loss, as attested by the recovery of phenotypes after passage through permissive temperature regimes. Indeed, studies of paternal inheritance of bacterial symbionts indicate as few as four bacterial cells are sufficient to establish infection in the new generation (Watanabe *et al.*, 2014).

The underpinning of phenotype and transmission by titre is important as it indicates that the impact of thermal environment is not simply associated with the current thermal regime, but will have strong historical influences (see, for example, (Jaenike, 2009)). Temperature previously experienced in life impacts upon current titre, and thus on the expression of phenotype and vertical transmission rate. Indeed, thermal impacts in a number of systems have been shown to be transgenerational, with symbioses taking a number of generations to recover to maximum expression following return to the permissive temperature. An important property of a symbiont–host interaction, therefore, is the rate at which symbiont titre is affected by temperature, both in terms of reduction and recovery. A practical consequence of this short-term evolution is that laboratory passage conditions may produce rather rapid changes in this aspect of host biology. For *Drosophila*, the simple act of maintaining a *Spiroplasma* stock at 18 °C may cure the host of heritable symbiont infection. Changing thermal environment may more subtly alter symbiont titre in other cases that may take time to recover. Overall, the heritable symbiont element of a host may be inadvertently (and in the case of curing) permanently altered by

simply placing stocks at a different temperature during maintenance, or during an experiment. The heritable symbiont component of an organism is much less fixed in the creation of isofemale lineages than is nuclear genetic variation.

The centrality of titre in expression of phenotype and vertical transmission further suggests that thermal sensitivity of host–symbiont interactions may affect the success/failure of heritable symbionts in novel host species. Facultative symbiont incidence in host communities is partly a function of their movement into, and subsequent propagation through, new host species (Zug *et al.*, 2012; Longdon *et al.*, 2014).
Furthermore, *Wolbachia* transinfected into novel host species is in applied usage as a means to interrupt vector competence of focal species. It is notable that when symbionts are placed into novel hosts they may attain a different titre from the native host (Kageyama *et al.*, 2006), and this is likely to be reflected in changes to the thermal sensitivity of the host–symbiont interaction. Thermal sensitivity of phenotype in novel hosts has been investigated in two mosquito species transinfected with *Wolbachia* from *D. melanogaster* as a means of altering vector competence. Studies show that the impact of wMel on reducing *Aedes aegypti* competence for dengue virus transmission is insensitive to environmental temperature (Ye *et al.*, 2016). In contrast, the impact of *Wolbachia* strain wAlbB on *Plasmodium* proliferation in *An. stephensi* is temperature sensitive (Murdock *et al.*, 2014). *w*AlbB reduced mosquito potential to transmit *Plasmodium* at 28 °C but had no effect at either 20 or 24 °C. Thus, although focal traits can be robust to thermal variation on transinfection, this characteristic must be determined on a case-by-case basis, and this is an important biosafety and efficacy consideration with respect to releases. It also indicates that temperature may affect the ability of an infection to propagate through a novel host species.

Overall, linking laboratory measures with field data remains a challenge. In part, this is because (as discussed above) impacts can be historical. As noted previously, the presence of latitudinal clines in symbiont prevalence in focal species supports a link between thermal environment and symbiont dynamics in nature (Table 2.7.1). Furthermore, broad between-species surveys indicate latitudinal patterns that indicate general patterns. For instance, *Wolbachia* is generally rare in butterflies from high latitudes, both in terms of more commonly being absent, and where present, more commonly being at low prevalence (Ahmed *et al.*, 2015). Determining the role of thermal environment in creating these patterns is complicated by temperature being one of a number of abiotic, biotic and

coevolutionary factors that affect symbiont–host dynamics. There are, however, examples where the pattern is consistent with experimental data. For instance, *Wolbachia* in *D. melanogaster* is costly in the context of overwintering, and *Wolbachia* is less common in temperate populations than tropical populations of this species. For male-killing *Spiroplasma* in *Drosophila*, experiments indicate symbiont phenotype and vertical transmission are ablated at low temperatures. Consistent with this, male-killing *Spiroplasma* are recorded commonly in drosophilids from tropical biomes (Williamson and Poulson, 1979; Montenegro *et al.*, 2005, 2006; Pool *et al.*, 2006), but not in temperate species/temperate parts of species range (see (Haselkorn, 2010)). This is unlikely to be a study bias, as male-killing *Wolbachia* have been isolated from temperate flies following observation of female-biased sex ratios produced by individual females (Hurst *et al.*, 2000; Sheeley and McAllister, 2009; Unckless and Jaenike, 2012)). Furthermore, although male-killing *Spiroplasma* strains have been isolated from South American and Sub-Saharan African *D. melanogaster*, no records exist from *D. melanogaster* from temperate biomes. Given that the intensity of collection and study is biased toward temperate collection, it is fair to conclude that male-killing *Spiroplasma* show a tropical bias in *Drosophila*, consistent with the observed thermal sensitivity of this symbiotic interaction.

The review above also highlights a variety of areas for future study. The impact of overwintering environment on symbiont survival, and reciprocally of symbionts on host survival overwinter, are both very poorly researched. There are good reasons (outlined above) to believe diapause/overwinter period may be an important contributor to symbiont dynamics, and these factors should be studied both in the field and laboratory. Furthermore, laboratory experiments on thermal impacts should adopt greater realism, incorporating diurnal temperature cycles in addition to investigating impacts of static temperatures. These may also benefit from adding in covarying factors such as day length, in case host/symbionts thermal behaviour has photoperiodic sensitivity. Furthermore, effects in a number of systems are known to be genotype dependent. Thus, prediction of dynamics may require a G × G × E framework. Finally, the impact of particular symbiont phenotypes of fitness (rather than their expression) is also likely to be thermally sensitive, and will require detailed examination of the wider ecological context in which the host exists. It is likely we will only get a predictive picture of thermal impacts when these aspects of natural environment complexity are incorporated.

The thermal sensitivity of heritable-microbe interactions begs two further questions. First, is host behaviour in terms of selecting thermal environments ever an adaptation to symbionts? Many organisms exhibit behavioural thermoregulation (Feder *et al.*, 1997; Anderson *et al.*, 2013)). The possibility is that species carrying beneficial symbionts will be selected for temperature optima that cosset their symbionts, and may indeed be constrained in using behavioural fever as a means of curing pathogen infections. Reciprocally, presence of parasitic heritable symbionts may lead to selection for adopting temperatures that reduce the impact and transmission of the symbiont. Secondly, are the patterns of thermal impact on symbionts that we observe ever adaptive for the symbiont? Certain phenotypes (for example, natural enemy resistance) are only beneficial at particular times of year (when the natural enemy is active). If the expression of high titre to gain the phenotype is associated with a physiological cost, then titre may be expected to evolve as a thermally plastic trait of the symbiont, elevating only under the conditions present when the enemy is active. Microbial pathogens are well known to alter behaviour with temperature; for example, *Listeria* pathogenicity determinants are expressed at 37 °C in association with ingestion by a mammal (Leimeister-Wächter *et al.*, 1992). Thus, the machinery for microbial adaptive thermal plasticity clearly exists. Whether it is employed by heritable symbionts is an interesting question.

In conclusion, laboratory studies have revealed that symbiont presence may in part determine host thermal tolerance, and that many aspects of host–symbiont interactions are thermally sensitive such that thermal environment will likely alter the prevalence of heritable symbionts and the strength of phenotype observed in interactions. However, there commonly remains a research disconnect between laboratory measures and field dynamics. All laboratory measures in essence create hypotheses about how phenotype and transmission may be affected in the field, as the experimental study simplifies systems for purposes of experimental control. Furthermore, the ecological context will alter the benefits of particular phenotype in ways that are not easily predictable from the laboratory, but are likely to be thermally sensitive. These, and the degree to which thermal sensitivity is part of an adapted symbiosis, as opposed to an uncontrollable biological constraint, remain major questions for future research.

2.7 Tables

Table 2.7.1 Studies showing geographical variation in symbiont prevalence which may be attributable to temperature differences

Host	Symbiont	Locality	Pattern	References
Acyrthosiphon pisum	*Regiella insecticola*	Japan	Higher prevalence in colder north and east. Significant correlation with temperature, as well as precipitation and host plant. There was no temperature correlation for *Serratia, Rickettsia,* or *Spiroplasma*, though the latter two are found only in the southwest at low frequency.	(Tsuchida *et al.*, 2002)
Adalia bipunctata	*Spiroplasma*	Sweden	*Spiroplasma* absent north of 63°N in 2011-2013. The northernmost limit was 61°N in 2000-2002.	(Tinsley, 2003; Pastok, 2015)
Culicoides imicola	*Cardinium*	Israel	Prevalence declines with increasing maximum daytime temperature in locality and increases with increasing minimum night-time temperature.	(Morag *et al.*, 2012)
Curculio sikkimensis	*Sodalis, Rickettsia* and *Wolbachia*	Japan	Higher prevalence of three symbionts in warmer areas to the south-west. Significant correlation with temperature. No correlation for *Spiroplasma*.	(Toju and Fukatsu, 2011)
Drosophila melanogaster	*Wolbachia*	Eastern Australia	Higher prevalence in tropical regions of Australia compared to subtropical and temperate regions. Pattern stable over 20 years. Similar, weaker pattern observed in North America.	(Hoffmann *et al.*, 1986; Kriesner *et al.*, 2016)

Table 2.7.2 Thermal effects on the phenotypes of natural reproductive parasites of insects

Host	Symbiont	Nature of symbiosis	Assay type	Impact of temperature on phenotype	Source
Aedes polynesiensis	*Wolbachia*	CI	Phenotype, cytology	CI eliminated by 32-33°C exposure as larvae for 5-7 days. 30-32°C did not eliminate CI. Larva dies above 33°C.	(Wright and Wang, 1980)
Drosophila equinoxalis	ESRO *Spiroplasma*	MK	Phenotype	MK reduced by embryonic heat-treatment with various temperatures and durations between 34°C and 40°C.	(Malogolowkin, 1959)
D. nebulosi	NSRO *Spiroplasma*	MK	Phenotype, qPCR	Highly penetrant MK at 25°C. At 18°C there is loss of fully-female broods at generation 2. At 28°C, gradual loss occurs until at generation 8, 1/8 strains show strong female-bias.	(Anbutsu *et al.*, 2008)
D. willistoni	WSRO *Spiroplasma*	MK	Phenotype	No effect of embryonic heat-treatment, at various temperatures and durations between 34°C and 40°C.	(Malogolowkin, 1959)
D. bifasciata	A-group *Wolbachia*	MK	Phenotype, cytology	Phenotype lost between 23.5°C and 25°C.	(Hurst *et al.*, 2000, 2001)
D. melanogaster	wMelPop *Wolbachia* (may not exist in wild)	Premature host death	Phenotype	No mortality effect at 19°C. At 25°C, wMelPop induces early mortality, with effect increasing at 29°C.	(Min and Benzer, 1997; Reynolds *et al.*, 2003)

D. simulans	wRi *Wolbachia*	CI	Phenotype, cytology	Ageing and rearing males at elevated temperature (27°C) reduces incompatibility; larval thermal environment critical.	(Clancy and Hoffmann, 1998)
D. simulans	*Wolbachia*	CI	Phenotype	CI suppressed in crosses between two unidirectionally-incompatible fly strains exposed to 28°C in early life.	(Hoffmann *et al.*, 1986)
D. simulans	*Wolbachia*	CI	Phenotype	Larval heat shock at 36°C (1 hour) reduced CI in adult male flies. Egg mortality was 90% rather than 45%. Heat shock didn't influence survival or fertility.	(Feder *et al.*, 1999)
Nasonia vitripennis	*Wolbachia* strain A	CI	Phenotype, qPCR	Positive correlation between density and CI penetrance within temperature groups. However, density and CI were decoupled between groups. Temperature may change the density threshold required for CI.	(Bordenstein and Bordenstein, 2011)
Ostrinia scapulalis, adzuki bean borer moth	*Wolbachia*	MK	Phenotype, PCR	Exposing larval female moths to 63°C for 20-30 minutes suppresses phenotype. 40 minutes has a greater effect but causes high lethality. 53°C not efficient at non-lethal exposure times. 34-38°C for long periods doesn't fully suppress MK.	(Sakamoto *et al.*, 2008; Sugimoto *et al.*, 2015)
Tribolium confusum	*Wolbachia*	CI	Phenotype	Suppression of CI with exposure to 37°C for 12 days in larval stage. Number of individuals lacking the phenotype increases with exposure time.	(Stevens, 1989)

Trichogramma cordubensis	*Wolbachia*	Induces thelytoky	Phenotype with 'permissive passage'	Thelytoky reduced over 4 generations at 30°C, significant during generations 2-4. Recovery with 4 generations of passage at 23°C.	(Girin and Boulétreau, 1995; Pintureau *et al.*, 1999)
Tetranychus urticae	*Wolbachia*	CI	Phenotype, PCR with 'permissive passage'	High loss of phenotype after 4 generations at 32°C (threshold at 31-32°C). Development time was reduced, and many heat-cured lines died out.	(van Opijnen and Breeuwer, 1999)

Table 2.7.3 Thermal effects on the vertical transmission of natural bacterial symbionts of insects

Host	Symbiont	Nature of symbiosis	Assay type	Impact of temperature on vertical transmission	Source
Acyrthosiphon pisum	*Regiella insecticola*	Parasitoid protection	PCR	Segregational loss in sexually produced eggs that persist through winter, but 100% vertical transmission in asexual summer reproduction.	(Moran and Dunbar, 2006)
Aedes kesseli males crossed with *Ae. polynesiensis* females	*Wolbachia*	CI *(Ae. polynesiensis* females have *Wolbachia*)	Cytology	Loss from ovaries with a heat treatment of 32.5°C (versus 27°C). This also killed the host.	(Trpis *et al.*, 1981)
Drosophila hydei	hy1 *Spiroplasma*	Parasitoid protection	qPCR	Blocked at 15°C, impaired at 18°C (2/5 broods had imperfect transmission), near-perfect at 25°C and 28°C.	(Osaka *et al.*, 2008)
D. melanogaster	MSRO *Spiroplasma*	MK	Phenotype after 'permissive passage'	Transmission loss at 16.5°C between F1 and F2. No phenotype recovery in non-MK lines returned to permissive temperature.	(Montenegro and Klaczko, 2004)
D. nebulosa	NSRO *Spiroplasma*	MK	Phenotype, qPCR	Rapid loss at 18°C (by generation 2). Stable maintenance at 25°C. Gradual loss at 28°C over several generations.	(Anbutsu *et al.*, 2008)
D. bifasciata	A-group *Wolbachia*	MK	Phenotype, cytology	Estimated at 92.9% at 25°C, compared to c. 100% at 18°C.	(Hurst *et al.*, 2000, 2001)

Liposcelis tricolor	*Wolbachia*	Increases fertility and fecundity	PCR	Complete elimination of *Wolbachia* over 6 generations at 33°C. Base population had 100% infection.	(Jia *et al.*, 2009)
Metaseiulus occidentalis	*Wolbachia*	CI	Phenotype, PCR after 'permissive passage'	After passage at 33°C for at least 8 generations, 0/10 tested females were infected. At 24°C, 12/20 tested females were infected. Males were also heat-cured.	(Johanowicz and Hoy, 1998)
Nasonia vitripennis	*Wolbachia* (2 strains)	CI, various	Phenotype, PCR, cytology, Southern hybridisation	AB Double-infected wasps lose strains A and/or B in diapause.	(Perrot-Minnot *et al.*, 1996)
Ostrinia scapulalis	*Wolbachia*	MK	Phenotype, PCR	Some cured progeny (shown by PCR) were derived from the 63°C-treated females, indicating transmission loss. Males uninfected, females/sexual mosaics infected.	(Sakamoto *et al.*, 2008; Sugimoto *et al.*, 2015)
Tetranychus urticae	*Wolbachia*	CI	Phenotype, PCR after 'permissive passage'	29% of mites remain infected after 4 generations at 32°C (threshold at 31-32°C). Undetectable by PCR until passaged at 23°C for 2 generations. Complete cure with 6 generations at 32°C.	(van Opijnen and Breeuwer, 1999)

Chapter 3: Temperature's effect on transmission, and a UK survey of *Spiroplasma* hy1

Abstract

Temperature potentially alters the population biology of many maternally-inherited endosymbionts by modulating the strength and type of a symbiont's phenotype, the cost of the symbiont for the host, and the efficiency of vertical transmission, likely via effects on titre. When vertical transmission efficiency is reduced in particular thermal environments, consequences can include a limit to the ecological range of a host-symbiont pairing, and reduced prevalence or loss of even an advantageous symbiont. For *Drosophila hydei* and its protective mutualist, *Spiroplasma* strain hy1, previous work has reported transmission losses at low temperatures, with a significant reduction in transmission efficiency within one generation at 18°C and total loss of transmission at 15°C. This data predicts that *Spiroplasma* would not be found in temperate countries such as the U.K. I first present evidence that contrary to this temperature sensitivity, hy1 *Spiroplasma* was observed in 15% of U.K. *D. hydei* from Tunbridge Wells in 2015 (n = 183). I then present a multi-generational experiment to investigate the thermal tolerance of two fly-*Spiroplasma* isolines, one from the U.K, the other from Mexico. To reveal transmission events which might otherwise be obscured by low bacterial titre, a 'recovery' protocol was used. In the F3 generation, infection prevalence remained high for all temperature groups except for the 15°C treatment, in which it dropped to 27.9% for the U.K. isoline. The study shows that *Spiroplasma* is more tolerant to low ambient temperature than previously recognised, which widens the climate envelope in which symbiont could persist in nature to include temperate countries such as the U.K. The experiments also support the use of a 'recovery' protocol in experiments, as a methodological improvement that will provide clearer measures of symbiont prevalence and transmission in this species.

3.1 Introduction

The prevalence of a heritable endosymbiont is the proportion of individuals in a wild host population which carries that symbiont. As such, prevalence is an output variable which is determined by the symbiont's vertical transmission efficiency, the nature and strength of the phenotype it causes in the individual hosts which carry it, and the cost to the host of carrying the symbiont. These in turn are modulated by biotic and abiotic factors such as temperature, natural enemy attack probability and severity (for a protective mutualist), and food abundance. Thus, the prevalence of a symbiont can give clues as to its phenotype, particularly one in a system which isn't already well-characterised (Ferrari and Vavre, 2011). If tracked over time, prevalence may reveal whether a symbiont is spreading through, or is being selected out of a population, and thus can indicate whether a phenotype is actually of detectable benefit or cost in the wild, cutting through the confounding factors of the natural environment. For example, the defensive anti-nematode *Spiroplasma* of *Drosophila neotestacea* in its eastern North American range experienced an increase in prevalence from 0-0.14 to 0.8 in less than 20 years, thought to be due to selection on its beneficial phenotype (Jaenike *et al.*, 2010). A functional role may also be responsible for a prevalence cline in *Arsenophonus* in the red gum lerp psyllid, *Glycaspis brimblecombei*, which is invasive in California. There was no link between prevalence and average temperature. However, there is a correlation with parasitism pressure applied by a parasitoid of the psyllid, *Psyllaphaegus bliteus,* consistent with a protective role for *Arsenophonus* (Hansen *et al.*, 2007).

However, a key weakness of prevalence as a means to infer differences in dynamical properties of particular parameters (such as transmission efficiency) is that multiple factors affect prevalence. This makes the output variable of 'prevalence' hard to interpret, especially when a symbiont might be maintained by a frequency-dependent selection mechanism with a delayed response, or when several symbionts exist in a population. One large aphid study followed the prevalence of several endosymbionts in two aphid races and two geographical regions, over the course of a field season (Smith *et al.*, 2015). Symbiont frequencies were observed shifting quickly over just a few generations. The clearest pattern, seen within one population, was that of defensive symbiont prevalence increasing in response to enemy pressure. However, other correlations were less clear or were counter-intuitive. *Serratia symbiotica* prevalence was higher in the warmer region than in the cooler one – though paradoxically, across the four populations examined, it showed a negative overall correlation with temperature – but there was no temporal pattern

matching the seasonal temperature changes. This finding contradicted earlier work showing summertime increase in *Serratia* prevalence (Montllor *et al.*, 2002). Complexity in this system is proposed to emerge from factors such as competition, host plant, predation, enemy counter-adaptation, and hitchhiking superinfections.

The multifactorial nature by which prevalence is determined is probably the reason why there is no strong evidence for an obvious link between wild prevalence and proxies for temperature (such as geographical cline, altitude, and season). The prevalence of *Regiella insecticola* in Japanese *A. pisum* is higher in the colder north and east of the country, and higher prevalence is associated with low temperature, low mean annual precipitation, and *Trifolium repens* rather than *Vicia sativa* as a host plant. However there are no correlations with temperature for *Serratia, Rickettsia* and *Spiroplasma* in the same species (Tsuchida *et al.*, 2002). In the chestnut weevil *Curculio sikkimensis* in Japan, *Sodalis, Rickettsia* and *Wolbachia* have a higher prevalence in warmer areas to the south-west, but there is no link for *Spiroplasma* (Toju and Fukatsu, 2011). *Spiroplasma* in Swedish *Adalia bipunctata* was absent north of 63°N in 2011-2013, representing a northward shift of 2° since 2000-2002, which could be due to increasing average temperatures globally (Tinsley, 2003; Pastok, 2015). *Cardinium* in the biting midge *Culicoides imicola* has a prevalence which declines with increasing maximum daytime temperature across different localities, and increases with increasing minimum nighttime temperature (Morag *et al.*, 2012). *Wolbachia* in the pale grass blue butterfly, *Zizeeria maha*, was not observed to change in prevalence with geographical cline or season, but does show seasonal fluctuations in titre. The *Wolbachia* density in the host is highest in spring and early summer, and then declines through late summer and early autumn (Sumi *et al.*, 2017).

As an approach, wild prevalence studies may be most powerful when combined with controlled laboratory study and predictive models, as seen with the discovery of a possible need for mutualism in *Wolbachia* in East Australian *D. melanogaster*. Here, *Wolbachia* was found to have a higher prevalence in tropical regions compared to subtropical and temperate, but to be at intermediate frequencies generally. The pattern was stable over 20 years, and a weaker pattern was observed in North America. The knowledge of these wild clines plus laboratory-derived information about the fitness effects of the *Wolbachia* was incorporated into a theoretical model to help explain the symbiont's intermediate frequency (Hoffmann *et al.*, 1986; Kriesner *et al.*, 2016).

3.1.1 Temperature can influence endosymbiont prevalence by changing the transmission efficiency

Whether a symbiont persists in the population or not is determined by its phenotype, its cost to the host, and its vertical transmission efficiency (see Chapter 2 for examples). Vertical transmission efficiency is a factor which sets symbiont-associated phenotypes apart from those traits encoded on the host's nuclear genome; when transmission is imperfect, even a low-cost, highly-beneficial symbiont will fail to fix in a population because segregational loss will continue to generate uninfected insects. Transmission efficiency of a symbiont is modulated by abiotic factors such as temperature, even over the course of a single generation. The effect of a change in temperature on titre may also persist for several generations. Mechanistically, it is likely that temperature influences a symbiont's proliferative ability and thus its titre in host tissues, with titre peaking at an optimal temperature for the symbiont and reducing to zero at extreme temperatures. Because temperature has considerable temporal and geographical variation, it has the potential to change the fate of many host-symbiont pairings. However, symbiont cost, titre, phenotype, and vertical transmission are rarely studied under ecologically-relevant temperatures in the laboratory, and so the details of wild host-symbiont interactions are poorly understood. Using realistic temperatures in controlled experiments investigating these factors can only increase our understanding of the evolutionary ecology of host-symbiont systems.

3.1.2 hy1 is at intermediate prevalence in much of its natural range, and it may be sensitive to temperature

Drosophila hydei is naturally infected with *Spiroplasma* strain hy1 in Japan, the U.S., and Mexico. Current data demonstrates that hy1 tends to exist at intermediate infection frequencies in these populations. A study which followed two Japanese populations over the summer seasons of 2006-2008, found that the infection frequency was stable between 34.6% and 36.7% in one population. In the second population, it changed from 29.4% to 19.3% over three years; the authors propose that this could be due to smaller sample sizes and more-sporadic sampling in this population (Osaka *et al.*, 2010). Older studies in Japan found a prevalence of 45.9% in Ito, in east Japan, in 1978 (Ota *et al.*, 1979), and a range of prevalences from 26% to 66% across five Japanese localities, spaced from east to west near the south coastal regions, in 2005 (Kageyama *et al.*, 2006). In North America, the

prevalence in a pool of samples from two populations in Arizona and one population in Mexico was 28.6% (Watts *et al.*, 2009).

Regarding factors which could produce this intermediate prevalence, some data already exists on temperature and transmission for *Spiroplasma* strain hy1 in *D. hydei,* in Japanese host-symbiont combinations. Over the course of one generation, it was found that transmission was near-perfect in the 25°C control condition, but decreased significantly at 18°C. Additionally, transmission failed immediately and entirely at 15°C (Osaka *et al.*, 2008). This thermal sensitivity is of interest because exposure to these temperatures is expected to occur in *D. hydei's* natural range (Osaka *et al.*, 2008). So far, temperature-prevalence correlations for wild hy1 populations have not been found; a Japanese study found no correlation between prevalence and average temperature in the 'active' spring-summer season of *Drosophila hydei* across three years and two populations (Osaka *et al.*, 2010), and in a North American study, it was noted that the hotter, desert site had a lower prevalence than the cooler site, with the authors speculating that higher temperatures could also limit prevalence (Watts *et al.*, 2009).

A methodological limitation of the existing hy1 temperature-transmission study is that transmission was detected via PCR-assay of flies which were reared and kept at the experimental temperature. More recent studies have demonstrated that 'permissive passage' – allowing transmission to occur at the experimental temperature, and then rearing at a 'permissive' temperature to increase detectability – can reveal transmission events even in cases where the titre is highly reduced (Montenegro and Klaczko, 2004). For example, *Wolbachia* in the mite *Tetranychus urticae* becomes undetectable by PCR after 4 generations of unfavourable-temperature passage, but becomes PCR-detectable again after two generations of permissive passage (van Opijnen and Breeuwer, 1999). This ability to persist at low titre has interesting implications for evolutionary ecology. A vertically-transmitted symbiont might be able to 'invisibly persist' under unfavourable environmental conditions for a few generations, by transmitting with a hidden phenotype and possibly even hidden costs.

The possible temperature sensitivity of hy1 is particularly of interest in the U.K. context. In the U.K., *D. hydei* is an introduced species, and the differences in temperatures across the seasons tend to be less extreme than Japan or the south of the North American continent. From Table 3.1.1, winters in the southern U.K. town of Tunbridge Wells are somewhat milder than those in sites in Japan and North America, but maximum temperatures in the

breeding months in the U.K. rarely exceed 22°C, while in the non-U.K. sites, the maximum temperatures are in the low 20°Cs/early 30°Cs in July and August, and are in the mid/high 20°Cs in September, when the U.K. maximum rarely exceeds 18°C. It should also be noted that these maxima are also present alongside cooler night-time temperatures.

The data presented previously predicts that *Spiroplasma* would be absent in the UK, as the thermal environment would produce high levels of segregational loss. In this chapter, I first test this hypothesis by examining the prevalence and identity of *Spiroplasma* in a southern U.K. population of *D. hydei*. Having established (contrary to hypothesis) that the *Spiroplasma* is present, I then determine the degree to which vertical transmission is temperature sensitive, using a more sensitive assay approach for *Spiroplasma* presence than used previously.

Locality	Jul, min	Jul, max	Aug, min	Aug, max	Sep, min	Sep, max	Coldest min month/min/ max	Source
Mansfield, Ohio, US (near Wooster)	16.3	27.6	15.6	26.7	11.4	22.9	Jan/-7.6/0.4	(National Centers for Environmental Information, 2015)
Tenancingo de Degollado, Mexico	12.6	26.8	12.6	26.7	12.5	25.6	Jan/4.3/25.3	(Coordinación General del Servicio Meteorológico Nacional) (60 year)
Tunbridge Wells, UK	12.8	21.2	12.7	21.5	10.7	18.8	Feb/1.8/7.6	(Met Office)
Tokyo, Japan	21.8	29.2	23.0	30.8	19.7	26.9	Jan/0.9/9.6	(Japan Meteorological Agency)
Nagoya, Japan	23.0	30.8	24.3	32.8	20.7	28.6	Jan/0.8/9.0	(Japan Meteorological Agency)
Takamatsu, Japan	23.6	31.2	24.4	32.4	20.7	28.4	Jan1.6/9.4	(Japan Meteorological Agency)

Table 3.1.1: Average maximum and minimum temperatures for selected localities sampled for hy1 prevalence Data shows 30-year averages from 1981-2010 unless otherwise specified. U.S. data converted from Fahrenheit. Mansfield chosen for proximity to Wooster, site of *D. hydei* studies by (Spencer, 1941). Tenancingo included as the origin site of the hy1-carrying Mexican *D. hydei* used in these studies. Tunbridge Wells included as the origin site of the U.K. *D. hydei* tested for hy1 prevalence. Sites from Japan chosen for being major climate stations near *Spiroplasma* prevalence sampling sites in (Kageyama *et al.*, 2006), including Tokyo (near Matsudo and Tsukuba), Nagoya (near Iwata), and Takamatsu (near Matsuyama).

3.2 Aims

1. To assess the prevalence of *Spiroplasma* in *D. hydei* in the U.K. in two different years.
    a. Determine whether prevalence differs by sex.
    b. Determine whether prevalence varies by year.
2. To ascertain the strain of the U.K. *Spiroplasma* through sequencing.
3. To determine transmission efficiency for hy1 in *D. hydei* under ecologically-relevant temperature conditions.
    a. Use 25°C as a baseline for comparing a variety of stable temperatures, as well as a fluctuating 'day-night' temperature.
    b. Use a 'permissive passage' method, making transmission events more likely to be picked up by PCR assay.
    c. Compare the transmission efficiencies of a *Spiroplasma*-infected Mexican fly isoline, and a naturally *Spiroplasma*-infected isoline established from the southern U.K.

3.3 Methods

3.3.1 Prevalence of Spiroplasma *in U.K. wild flies*

Collecting wild *D. hydei* for U.K. prevalence estimation: Wild *D. hydei* specimens were collected in Royal Tunbridge Wells, southern England, in July 2014 and August 2015 (51.09 N, 0.16 E), generously sent as by-catch from Prof. Darren Obbard's fly collections. I had initially planned to sample flies from Liverpool, as a more northern U.K. locality as a comparison point, but had limited success, capturing only two *D. hydei* specimens.

Adult flies were caught with sweep nets over fruit bait and transferred to vials containing sugar-yeast (SY) food (see Appendix). Flies were sexed based on phenotype, kept alive in a CT room at 25°C for 2 weeks to increase PCR detectability, before being frozen at -80°C. The nearest climate station to the collecting site is 'Herstmonceux West End'. At this location, from the 30-year averages, the highest average maximum temperature occurred in the month of August, and was 21.5°C. The average minimum temperature during August was 12.7°C (Met Office).

DNA extraction and *Spiroplasma* diagnostic PCR: DNA was extracted by homogenising whole flies with a pestle and then using the Promega Wizard DNA extraction kit (Promega), quartering the recommended amounts for animal tissue. PCR reactions were carried out using GoTaq Hot Start Green Master Mix (Promega). 'SpoulF' and 'SpoulR' primers were used to test for *Spiroplasma* infection (after (Montenegro *et al.*, 2005)). 'CO1' primers were used to amplify host DNA, as a quality-control test for successful DNA extraction (Folmer *et al.*, 1994); failure to amplify host DNA was taken as an indicator that DNA extraction had failed, and these samples were excluded from analysis (see Table 3.3.1). 6 μl of each PCR product was run on 1.5% agarose gels, using Midori Green Nucleic Acid Staining Solution (Nippon Genetics Europe) to visualise and Hyperladder I (Bioline) to confirm product length alongside positive and negative control PCR assays.

Diagnostic for	Primer name	Sequence	PCR conditions
Spiroplasma	SpoulF SpoulR	5'-GCT TAA CTC CAG TTC GCC-3' 5'-CCT GTC TCA ATG TTA ACC TC-3'	Initially: 94˚C (150s) 35 rounds of: 94˚C (15s); 55˚C (60s); 72˚C (40s)
Host	HCO LCO	5'-TAA ACT TCA GGG TGA CCA AAA AAT CA-3' 5'-GGT CAA CAA ATC ATA AAG ATA TTG G-3'	Initially: 94˚C (120s) 35 rounds of: 93˚C (15s); 52˚C (60s); 72˚C (60s)

Table 3.3.1 Primers used in diagnosing *Spiroplasma* hy1 infection of *Drosophila hydei*

Statistics: The presence of heterogeneity in prevalence between host sexes and collection years was tested. All statistical analyses were carried out in R version 3.0.2 (R Core Team, 2013). The glm() function was used to carry out a binomial GLM on prevalence data from Tunbridge Wells. The maximal model contained sex, year, and their interaction as factors,

and the functions drop1() and update() were used to refine the model. 95% confidence intervals were calculated with the package binom() (Doraj-Raj, 2014) using the exact (Pearson-Clopper) method.

3.3.2 Identifying the strain of Spiroplasma *in U.K. flies*

Obtaining sequences from the U.K. wild-derived *Spiroplasma*: Sequencing of a variety of *Spiroplasma* genes was carried out for *Spiroplasma*-positive samples collected from Tunbridge Wells in the summer of 2015, to determine whether the U.K. wild *Spiroplasma* was similar to either of the already-known haplotypes, hy1 (previously detected in the southern U.S., Mexico, and Japan) and hy2 (previously detected only in Mexico). The primer pairs used and their associated PCR programs are given in Table 3.3.2 and are as described by (Haselkorn *et al.*, 2009).

DNA from twenty-six individuals collected from Tunbridge Wells in 2015, as well as the foundress of the 2013 Cambridge isoline used in the temperature-transmission experiment, underwent hot-start GoTaq PCRs with two sets of 16S primers, labelled 'A' and 'B' in Table 3.3.2. This locus permits differentiation of hy1 from hy2 (Mateos *et al.*, 2006; Haselkorn *et al.*, 2009). In addition, one of the Tunbridge Wells 2015 individuals (F7), plus the foundress of the Cambridge line (FA34E2), underwent PCR with 3 primer sets, as shown in Table 3.3.2. The purpose of this was to establish more accurate relationships of U.K. *Spiroplasma* to others, and to more precisely describe the isolate used in onward experiments. Each PCR product was cleaned of unincorporated primers and nucleotides by adding 2µl of product to a mix of 0.05 µl of Exonuclease I, 0.20 µl of Shrimp alkaline phosphatase, 0.70 µl of x10 shrimp reaction buffer, and 1.05 µl of water (New England Biolabs). The mixes were incubated at 37°C for 45 minutes, followed by a heat-kill at 80°C for 15 minutes. The product was then sent for Sanger sequencing for each strand using GATC's LightRun service, which uses ABI 3730xl DNA Analyzer systems and KB basecalling (version KB 1.4.1.8).

Producing consensus sequences: Sequence traces were viewed in Chromas version 2.6.4 (Techelysium) and used to guide trimming of primers and low-quality regions at the ends of sequences. Low-quality sequence was defined as regions where the KB quality score is below 0.35 for 3 or more nucleotides in a row, at sequence ends where there isn't better-quality sequence on the opposite strand to help build a consensus. To produce consensus sequences, forward and reverse-complemented reverse strands for each primer/individual

were aligned in MEGA 6 (Tamura *et al.*, 2013), using the MUSCLE algorithm with a gap opening penalty of -400 and a gap extending penalty of -8 (Edgar, 2004), and visually checked and edited where base calls disagreed. For samples which were successfully sequenced with both primer set A and primer set B, the 16S rRNA sequences were joined together, as they overlap sufficiently to permit this.

Alignments and identifying strains: MUSCLE (Edgar, 2004) was used to produce an alignment of the U.K. sequences for each primer pair in MEGA 6, again with a gap opening penalty of -400 and a gap extending penalty of -8. These penalties are higher than default, to prevent the algorithm from opening gaps on shorter sequences by aligning terminal bases with the first matching base of the longer, more-complete sequences. This process revealed that the U.K. *Spiroplasma* samples are all identical for the in-common parts of the 16S rRNA sequences. The 16S sequence obtained for Tunbridge Wells sample F163 – which provided one of the longest 16S sequences out of all the samples tested – was used to query Haselkorn et al. 2009's '*Spiroplasma* endosymbiont of *Drosophila hydei*' dataset on NCBI, with nucleotide BLAST using the default parameters for highly-similar sequences (Altschul *et al.*, 1990). Sequence obtained for the other 3 primer sets, from samples F7 from Tunbridge Wells and FA34E2 from Cambridge, were queried in the same way.

				PCR Conditions (°C, s)					
Set	**Primer**	**Sequence 5'-3'**	**Product**	**Denature**	**Cycles**	**Melt**	**Anneal**	**Touchdown**	**Extend**
A	23F	CTCAGGATGAACGCTGGCGGCAT	16S rDNA, partial	94, 180	35	94, 30	65-48 TD, 45	Lower 1 per cycle for 15, then keep 48	72, 45
A	TKSSsp	TAGCCGTGGCTTTCTGGTAA							
B	16STF1	GGTCTTCGGATTGTAAAGGTCTG	16S rDNA, partial	94, 180	35	94, 30	65-48 TD, 45	Lower 1 per cycle for 15, then keep 48	72, 45
B	16STR1	GGTGTGTACAAGACCCGAGAA							
E	RpoBF1	ATGGATCAAACAAATCCATTAGCAGA	RNA polymerase B	94, 180	35	94, 30	63-53 TD, 45	Lower 1 per cycle for 10, then keep 53	72, 45
E	RpoBR4	CTTTGTTTCCATGGCGTCCAGCC							
F	ParEF2	GGAAAATTTGGTGGTGATGG	DNA topoisomer-ase	94, 180	35	94, 30	63-53 TD, 45	Lower 1 per cycle for 10, then keep 53	72, 45
F	ParER2	TGGCATTAATCATTACATTAATTTCT							
H	FruF	GTCATAATTGCAATTGCTGG	Partial fructose operon	94, 180	35	94, 30	58-48 TD, 45	Lower 1 per cycle for 10, then keep 48	72, 45
H	FruR	CAATGATTAAAGCGGAGGT							

Table 3.3.2 Primers used to identify the U.K. *Spiroplasma* strain Primer sequences and conditions from (Haselkorn *et al.*, 2009).

3.3.3 Laboratory temperature-transmission experiment

Stock flies used: The Cambridge *D. hydei* stock isoline, CAM001b, was established from a single, hy1-positive mated female caught in Cambridge in September 2013, and maintained in ASG cornmeal agar vials (see Appendix for composition) at 25°C until early 2014, when it was transferred to ASG bottles to increase numbers. The female tested *Spiroplasma*-positive based on SpoulF & SpoulR primers, and the *Spiroplasma* appears to be related to hy1 (see results for wild fly data). The nearest climate station to the collecting site is 'Cambridgeniab', where (averages 1981-2010) the highest average maximum temperature occurred in the month of July, at 22.8°C. The average minimum temperature during July was 12.4°C (Met Office).

The Mexican *D. hydei* stock (TEN104-106) was originally established from a single, hy1-infected female in 2004 (Mateos *et al.*, 2006) and maintained at 25°C. An uninfected stock was subsequently produced through tetracycline-curing. A new infected line was generated approximately a year before the temperature-transmission experiment began, in case the infected and cured lines had diverged through mutation and drift. Haemolymph was drawn up from adult female donors of the original infected line, and injected into recently-eclosed adult female flies from the cured line. Injections were carried out with pulled capillary needles fixed to a Hamilton syringe via narrow paraffin-filled tubing (Hutchence, 2011). The injected flies were bred on ASG vials. Offspring from females which tested positive for hy1 at PCR (see 'DNA extraction and *Spiroplasma* diagnostic PCR') were used to re-establish the infected line.

Temperatures used: The control condition was a constant 25°C. This temperature was also used as a 'permissive' condition to increase the PCR detectability of infections in non-breeding flies. Experimental conditions were constant 18°C, constant 15°C, and day/night alternating 18°C/15°C. All temperature conditions were exposed to a 12-hour photoperiod between 10am and 10pm, except for 18°C, which was exposed to constant light due to a malfunction in the room settings. 25°C and 18°C vials were kept in constant-temperature rooms, and 15°C and 18°C/15°C vials were kept in incubators (Sanyo MLR-351).

Obtaining similarly-raised generation Parental (P) flies: To homogenise rearing conditions of the Cambridge and Mexican flies prior to the experiment, at 25°C, the mothers of generation P laid eggs in large ASG vials containing a mature adult male. After two days, the

flies were tipped into a new vial for an additional two days, before vial-mothers were individually frozen at -80°C. This laying time was chosen because it keeps the density of *D. hydei* larvae relatively low, preventing competition between the larvae. The mothers underwent DNA extraction (Promega Wizard kit) and were tested for *Spiroplasma* using hot-start PCR with Spoul primers. Only vials from infected mothers were kept and matured. The progeny of these infected mothers, generation P, were matured for 13-17 days at 25°C.

Obtaining larvae for the F1: For each line, two population cages were established at 25°C to generate F1 larvae. Each cage contained 50 female and 10 male generation-P flies on a grape-juice agar plate 'painted' with live yeast paste, which was replaced daily. Once first instar larvae were successfully obtained on plates, 20 breeding females from each cage were frozen at -80°C for later verification of infection status. After ageing for a day, larvae were picked from plates into small ASG vials containing approximately 7ml food at a density of 25 larvae per vial. 16 ASG vials were picked from each plate, giving 32 vials per line. Vials were randomly-assigned to one of four temperature conditions using the shuffle function in Python (Python Software Foundation), such that 8 vials were in each temperature condition for each line. Therefore, F1 larvae were all laid as eggs at 25°C, but placed at experimental temperature very early in their lives. Larvae vials were watered and shuffled in their storage tray twice a week.

Collecting F1s and establishing generations F2 and F3: The lines were reared to eclosion, which took approximately 2 weeks at 25 degrees, 3 weeks at 18 degrees, and 4-5 weeks at 18/15°C and 15°C. Flies were sexed shortly after eclosion and maintained until sexual maturity on SY food (see Appendix for composition). At sexual maturity, the experimental-temperature adult females from each vial of origin were mated to stock *Spiroplasma*-negative 25°C males and left to lay eggs in individual vials at their experimental temperature. The laying females were tipped onto a new vial so that they produced two vials of eggs in total. Vial 1 was kept at experimental temperature. Once eclosed and mature, F2s at experimental temperatures were bred from. F3 larvae obtained from F2 experimental temperature crosses were picked into vials, and exposed to the 25°C control temperature during the remainder of rearing, to ensure any inherited bacteria reached PCR-detectable titre. Upon eclosing, they were virginized and kept for 5-7 days on SY, as *Spiroplasma* titre continues to increase in the female for several days after maturity (Kageyama *et al.*, 2006), then frozen for *Spiroplasma* assay. The minimum times given for each step at the different experimental temperatures are given in Table 3.3.3. Non-25°C

timings were derived from a mix of pilot experiments (not shown) and from information on *D. hydei* life cycle lengths at 18°C as described by Shorrocks (Shorrocks, 1972).

Temperature /°C	Picking-eclosion /days	Eclosion-female maturity /days	Laying time given /days
25	12	3	2
18	24	7	4
18/15	30	7	5
15	36	9	6

Table 3.3.3 Minimum times (in days) for each step of the temperature-transmission experiment

DNA extraction and *Spiroplasma* diagnostic PCR: To speed up the overall diagnostic process, DNA was first extracted using the fast Chelex method prior to PCR testing for *Spiroplasma*. If a sample tested negative for *Spiroplasma,* it was then cleaned using a modified Promega Wizard kit protocol (see wild fly methods section) before re-testing *Spiroplasma*. Samples that remained negative were then tested with host DNA, CO1 primers. If they amplified with CO1 primers, they were taken to be genuinely *Spiroplasma*-negative samples. If they didn't produce an amplicon, this was a sign that DNA extraction had failed, and the sample was discarded.

In the Chelex method, DNA was extracted by homogenising whole flies in 50μl of molecular water containing 5% w/v Chelex 100 (BioRad) and 0.4mg/ml Proteinase K (Bioline), incubating at 37°C overnight, then centrifuging to produce DNA-containing eluate. This material was then boiled for 10 minutes to inactivate proteinase K. PCR assays and gel electrophoresis were carried out as described in the wild fly methods section.

Statistics: A GLM was carried out on the temperature-transmission experiment, to compare F3 'recovery' condition flies descended from the different experimental temperatures. The temperatures were encoded as ordered categories with the labels '1' (25°C), '2' (18°C), '3' (18/15°C) and '4' (15°C). The original maximal model contained sex, temperature, isoline (Mexico or Cambridge) and the interactions between these factors. The model was refined with drop1() and update(), which caused sex to be dropped as a factor, producing the minimal adequate model : Infected ~ Temperature + Isoline + Temperature:Isoline. Graphs were drawn in R using the package plotrix() (Lemon, 2006). Confidence intervals for graphing error bars were calculated with the Wilson interval using the R package binom()

(Doraj-Raj, 2014) as this is a method recommended for when mean values approach the extremes of 1 or 0, as occurred for data at warmer temperatures (Brown *et al.*, 2001).

3.4 Results

3.4.1 Summertime prevalence of Spiroplasma *in* D. hydei *in the south of England, 2013-15*

Tunbridge Wells prevalence data, July 2014 and August 2015

In July 2014 (see Table 3.4.1 for raw numbers and Table 3.4.2 for prevalence values with confidence intervals), 9 out of 60 successfully DNA-extracted wild-caught flies from Tunbridge Wells were positive in the PCR assay for *Spiroplasma* presence*,* to give an estimated prevalence of 15.0%. By sex, prevalence was 6/30 for males and 3/30 for females. In August 2015, 27 of 183 (14.8%) successfully DNA-extracted wild-caught flies from Tunbridge Wells were positive for *Spiroplasma*. By sex, prevalence was 15/93 for males and 12/90 for females.

A binomial GLM indicated that there was no statistically significant effect of sex, year, or a sex/year interaction on prevalence, and sequentially dropping terms did not improve the model fit. Thus, the data can be amalgamated, giving an overall infection prevalence of 36/243, or 14.8% (the lower 95% confidence interval is 10.6%, and the upper CI is 19.9%).

	2014		2015		TOTAL
	Males	**Females**	**Males**	**Females**	
Infected	**6**	**3**	**15**	**12**	**36**
Uninfected	**24**	**27**	**78**	**78**	**207**
TOTAL	**30**	**30**	**93**	**90**	**243**

Table 3.4.1 *Spiroplasma* raw prevalence data from Tunbridge Wells, U.K., broken down by year and sex

Year	Sex	Prevalence	Upper CI	Lower CI
2014	Male	0.200	0.386	0.077
	Female	0.100	0.265	0.021
2015	Male	0.161	0.252	0.093
	Female	0.133	0.221	0.071
Total		0.148	0.199	0.106

Table 3.4.2 *Spiroplasma* prevalence data from Tunbridge Wells, U.K., as percentages with confidence intervals, broken down by year and sex

Prevalence in Cambridge, 2013

Spiroplasma infection was detected by PCR assay in 6 of 14 flies collected in Cambridge in the August of 2013. The small sample size precludes further analysis.

3.4.2 The Spiroplasma *detected in U.K.* D. hydei *is likely to be strain hy1, as found in North America and Japan*

Sequences for 16S rRNA amplified using primer sets A and B were successfully obtained for 25 of 26 Tunbridge Wells individuals. Full coverage (both primer sets) was obtained for 23 of 26 Tunbridge Wells samples. For the non-16S primer sets E, F, and H, sequences were obtained from Tunbridge Wells sample F7. For the Cambridge isoline foundress, sequence was obtained for the primer sets B and H, but not A, E, and F. This may be due to the old age of the sample, and how it was derived through clean-up of a Chelex-extraction carried out after a year of -80°C freezer storage.

All of the Tunbridge Wells 16S sequences were identical to each other when aligned. The same was true for the Cambridge foundress sequence obtained with primer set B. Therefore, at the 16S rRNA locus, there is no *Spiroplasma* diversity in this set of U.K. flies.

For 16S rRNA sequence from sample F163, nucleotide BLAST searches were carried out against the '*Spiroplasma* endosymbiont of *Drosophila hydei*' dataset, from (Haselkorn *et al.*,

2009). The results and inferences are shown in Tables 3.4.3. Once unidentified ('N') bases are taken into account, F163's 16S rRNA sequences are identical to those identified as haplotype 1, the *poulsonii* group *Spiroplasma* which provides parasitoid wasp protection in *D. hydei.* Meanwhile, when aligned against the strains in the BLAST results which are known to be hy2, the UK sequence has 18-19 single-nucleotide differences. This indicates that the strain detected in the UK is hy1.

The data from non-16S loci (data not shown) support the 16S results, in that they match completely against previously curated hy1 sequences but not against sequences from hy2.

BLAST outputs								Inferences		
Description	**Max score**	**Total score**	**Query cover**	**E value**	**Identity**	**Identity (%)**	**Accession**	**Subject's haplotype**	**# N bases**	**Identity (without Ns)**
Haplotype 1	2390	2390	98%	0	1296/1298	99%	DQ412090.1	1	2	1296/1296
Isolate FC806117	2386	2386	98%	0	1292/1292	100%	FJ657183.1	1	0	1292/1292
Isolate FC806115A	2383	2383	98%	0	1291/1292	99%	FJ657182.1	1	1	1291/1291
Haplotype 2	2300	2300	99%	0	1283/1302	99%	DQ412089.1	2	0	1283/1302
Isolate mag4	2287	2287	98%	0	1274/1292	99%	FJ657238.1	2	0	1274/1292
Isolate OPNM0407A4	2278	2278	98%	0	1272/1292	98%	FJ657237.1	2	1	1272/1291

Table 3.4.3 BLAST results for F163 sequence for 16S rRNA sequence The BLAST is against the dataset of (Haselkorn et al., 2009). The queries are 16S rRNA partial sequences.

3.4.3 Transmission of hy1 was significantly reduced at 15°C, but was still occurring after two rounds of breeding in both isolines

A binomial GLM was used to compare infection levels amongst F3 generation 'permissive condition' flies from the different temperature groups. These flies had experienced two generations of transmission at experimental temperatures. In the final model, temperature ($P = 5.63e-05$), isoline ($P = 0.007435$) and the temperature-isoline interaction ($P = 0.000868$) all had P-values below 0.001. This indicates that temperature, isoline and the interaction term are factors which influenced hy1's prevalence in this experiment.

Considering both the Mexico and Cambridge isolines individually (see Figure 3.4.1), the confidence intervals for the 25°C, 18°C and 18/15°C groups all overlap each other's prevalence values, while this is not the case for the prevalence at 15°C. Thus the significance of temperature in the model is likely due to the prevalence differences in the 15°C condition. When comparing the isolines, prevalence tends to be similarly high at 25°C, 18°C and 18/15°C. However, the Cambridge isoline has a significantly lower prevalence at 15°C than the Mexico isoline.

Overall, lowering the temperature to 18°C or 18/15°C had no significant effect on hy1's prevalence in the experimental populations, relative to the optimal temperature 25°C population, even after two complete transmission events (F1 -> F2 and F2 -> F3) at the focal temperatures. Meanwhile, the temperature of 15°C had a significant effect on ultimate prevalence, with the Cambridge line experiencing a lower prevalence than the Mexico line.

Temperature/°C and isoline	Prevalence	Upper CI	Lower CI	N
25, Mexico	0.9231	0.9667	0.8322	65
25, Cambridge	1.0000	1.0000	0.8865	30
18, Mexico	1.0000	1.0000	0.9059	37
18, Cambridge	1.0000	1.0000	0.8794	28
18/15, Mexico	1.0000	1.0000	0.8830	29
18/15, Cambridge	0.9048	0.9735	0.7109	19
15, Mexico	0.7778	0.9100	0.5479	18
15, Cambridge	0.3778	0.5237	0.2511	45

Table 3.4.4 *Spiroplasma* prevalence in generation F3 of the temperature-transmission experiment, by temperature and isoline

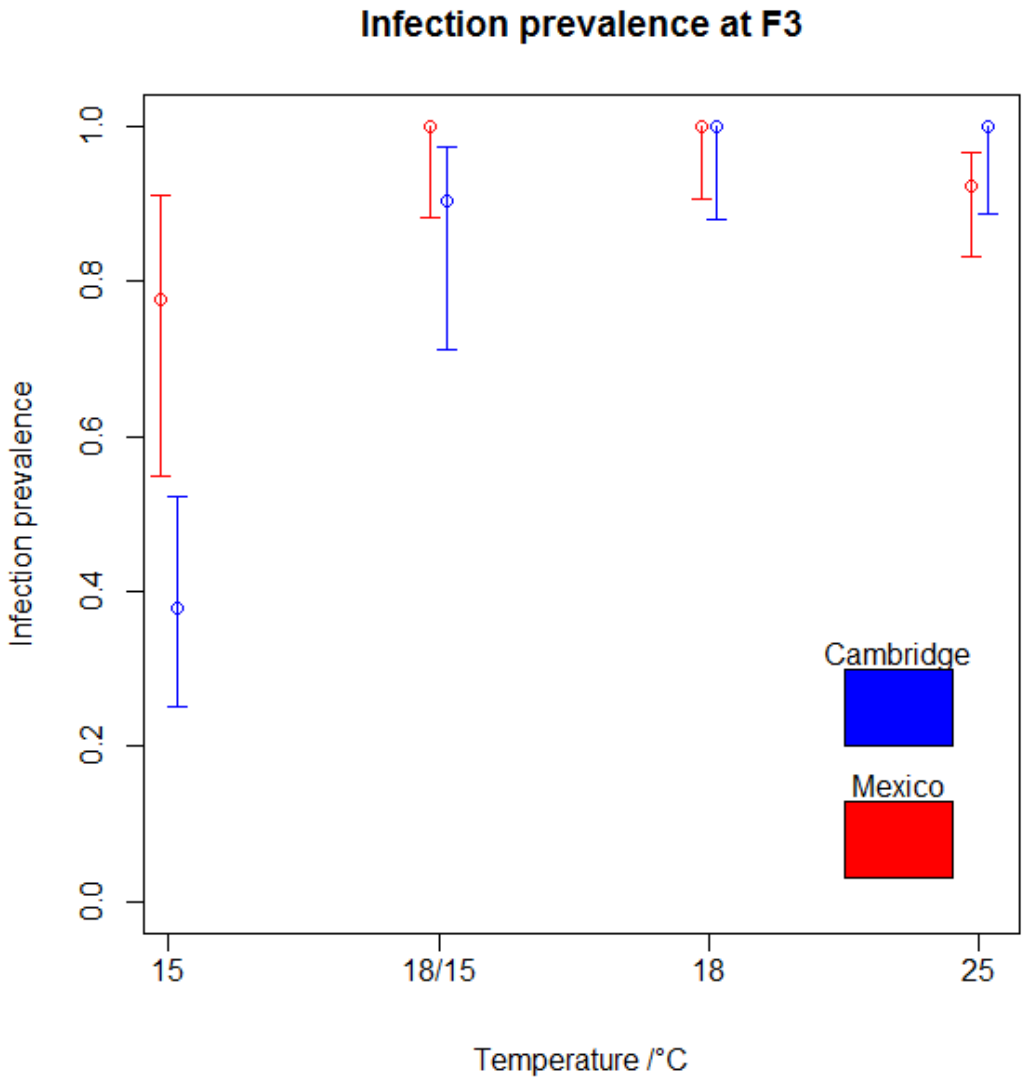

Figure 3.4.1 The prevalence of infection in generation F3 in the two different isolines

Where two temperatures are given, these represent day/night environments respectively. Error bars represent the 95% confidence intervals, calculated using the Wilson method.

3.5 Conclusions

3.5.1 U.K. prevalence

In late summer in 2014 and 2015, the prevalence of *Spiroplasma* infection in *D. hydei* is 14.8% in the south of England (confidence intervals are 10.6% - 19.9%). This prevalence is lower than usually recorded in North America, where it averages 28.6% across Arizona and Mexico (Watts *et al.*, 2009), and Japan, where it ranges from 26% to 66% across several well-sampled localities (Ota *et al.*, 1979; Kageyama *et al.*, 2006). UK prevalence is comparable to the lowest value recorded in Japan, 19.3%, from a population which fluctuated over three years. The authors hypothesised that the low value and the fluctuations could be due to sporadic sampling and low sample sizes (Osaka *et al.*, 2010). In contrast to this, the U.K. infection prevalence was the same in 2015 as in 2014, and based on a large sample. Whether prevalence is stable at this value, or fluctuates and just tends to be this value in late summer, is a matter for further investigation, though it is worth noting that the average prevalence tends to be stable over decades in hy1's Japanese range (Kageyama *et al.*, 2006). Prevalence is also the same for both sexes, consistent with no male-killing activity and either no or limited sex-specific mortality costs. It is of note that in my experiment, the prevalence after two generations at 15°C dropped until the low end of the range approached 0.2, not far off the prevalence seen from wild U.K. *D. hydei*. It's not hard to imagine that being non-stop exposed to fluctuating and relatively cool U.K. temperatures, wild *D. hydei* would end up with a low *Spiroplasma* prevalence.

3.5.2 U.K. Spiroplasma *strain*

The strain of *Spiroplasma* found in the U.K. is identical to the North American hy1 at the 16S rRNA locus. North American hy1 is identical at the 16S rRNA locus to the *Spiroplasma* strain found in Japanese *D. hydei* (Kageyama *et al.*, 2006). The global distribution of the strain in *D. hydei* suggests that it hasn't been eliminated across multiple geographical invasions. There is debate about whether losses of symbiont infections in arthropods often accompany invasions, with the case for *Wolbachia* summarised in (Nguyen *et al.*, 2016). Loss with geographical invasion is recorded in several invasive ant species (Tsutsui *et al.*, 2003; Reuter *et al.*, 2005). This includes *Solenopsis* fire ant species introduced from South America to the U.S. which have undergone severe genetic bottlenecks, in which case the authors propose uninfected foundresses, or loss of *Wolbachia* upon invasion by selection or

drift, as likely culprits (Shoemaker *et al.*, 2000). Invasion-associated loss has been recorded with Australian tephritid fruit flies in invasions from tropical to temperate regions (Morrow *et al.*, 2014, 2015). An Australian thrip *Pezothrips kellyanus,* introduced to the Mediterranean and New Zealand in separate events, has lost *Wolbachia,* but not *Cardinium*. Again, this is proposed to be due to stochasticity, selection against costs if these are higher with *Wolbachia* than with *Cardinium*, or interestingly, selection against higher costs of *Wolbachia* caused by changed environmental conditions (Nguyen *et al.*, 2016).

In contrast to cases of invasive loss, the North American fruit fly *Rhagoletis cingulata* invaded Europe and then horizontally acquired the *Wolbachia* of a native congeneric species (Schuler *et al.*, 2013). There is also a pattern of *Wolbachia* retention in *Drosophila melanogaster* invasions, with *Wolbachia* present throughout the host's range (Riegler *et al.*, 2005). This is despite experimentally-verified changes in cost with changing environment being reported for weakly-CI-causing *Wolbachia* in *D. melanogaster*. Temperate nuclear background Australian flies in a temperate setting benefitted from increased fecundity with *Wolbachia,* but the same flies suffered a cost of infection when placed in a tropical environment (Olsen *et al.*, 2001).

The retention of *Spiroplasma* hy1 by *D. hydei* across multiple geographical invasions seems to mimic the case with *Wolbachia* in *D. melanogaster.* It is difficult to resolve why this is, especially as the current pool of evidence contains more information about *Wolbachia* than about *Spiroplasma.* It may be the case that due to its human commensal nature, small size, and ability to stow away easily in food plants, *Drosophila* species aren't as subject to bottle-necks as they experience frequent and continuous introductions, rather than rare introductions with small effective population sizes.

3.5.3 Temperature and transmission

hy1 *Spiroplasma's* transmission is more robust to temperature than previously suspected. The transmission experiment's result would seem to support Osaka's previous work on temperature, in that cooler temperatures do indeed reduce the prevalence of infection in the population. However, the reduction in infection prevalence is much less dramatic than that which was apparently seen in the prior work, with transmission retained at high levels over three generations at 18°C and 18/15°C treatments, and at lower (but still non-zero levels) at 15°C.

The difference in findings between this study and that of Osaka *et al.* could be due to the use of the 'permissive passage' technique raising the *Spiroplasma* titre above the PCR detection threshold, creating a more robust assay for symbiont presence. This adds to the body of knowledge suggesting that 'permissive passage' is important for reducing the rate of false negative assay results, and thus accurately gauging transmission efficiency. However, the possibility that Japanese hy1-host combinations are less robust to low temperatures cannot be ruled out.

From the perspective of *Spiroplasma* in *Drosophila*, hy1's transmission's robustness is noteworthy. Male-killing *Spiroplasma* strains found in *Drosophila* species tend to have a tropical bias in distribution, which may be linked to low-temperature sensitivity in *Spiroplasma* generally (Williamson and Poulson, 1979; Montenegro *et al.*, 2005, 2006; Pool *et al.*, 2006; Haselkorn, 2010). However, the protective anti-nematode *Spiroplasma* of *D. neotestacea* ('sNeo') is distributed in temperate North America, and is spreading in these regions under strong selection (Jaenike *et al.*, 2010; Cockburn *et al.*, 2013). 'sNeo' clusters with other *Spiroplasma poulsonii* strains, and is a sister to the clade that includes hy1 and tropics-dwelling MSRO (Haselkorn and Jaenike, 2015). Therefore, the tolerance to temperate climates isn't necessarily shared by the protective *Spiroplasma* strains due to close relatedness.

One interesting finding is that transmission at 18/15°C, in both isolines, tends to act more similarly to 18°C than to 15°C. This could suggest that the 12 hours a day spent at the higher temperature is more influential over titre and transmission than the 12 hours spent at the lower temperature. Consequently, peak daytime temperatures could be more important in driving symbiont dynamics than night-time temperatures. In environments such as North American deserts, where maximum temperature in the *D. hydei* breeding season reaches the 30°Cs but minimum temperatures are about the same as those seen in Tunbridge Wells, this could mean that transmission efficiency will still tend to be higher than in Tunbridge Wells.

Focusing on those localities from which fly isolines used in transmission experiments originated – Tenancingo in Mexico and Cambridge in the U.K. (shown in Table 3.1.2) – minimum temperatures are similar for both localities, but average summer temperatures are higher for Tenancingo. Additionally, Tenancingo in its coldest month (January) has a much higher average maximum temperature than Cambridge in its coldest month (February), at 25.3°C compared to Cambridge's 7.7°C. The average temperature in

Tenancingo's coldest month is 14.8°C, exceeding Cambridge's average maximum. Indeed, the average maximum temperature in Tenancingo remains above 25°C in every month of the year (Coordinación General del Servicio Meteorológico Nacional). Therefore, Tenancingo tends to be warmer than Cambridge in the 'temperate climate' breeding season of *D. hydei*, and the temperatures may be high enough to permit a longer breeding season.

Locality	Jul, min	Jul, max	Aug, min	Aug, max	Sep, min	Sep, max	Coldest month/min/max	Source
Tenancingo, Mexico	12.6	26.8	12.6	26.7	12.5	25.6	Jan/4.3/25.3	(Coordinación General del Servicio Meteorológico Nacional)
Cambridge, U.K.	12.4	22.8	12.4	22.6	10.4	19.3	Feb/1.3/7.7	(Met Office)

Table 3.5.1 Average maximum and minimum temperatures for original localities of experimental isolines Averages for Cambridge are from 1981-2010. Averages for Tenancingo are from 1951-2010.

In the transmission experiment, Cambridge and Mexican isolines only perform differently at 15°C. Cambridge has a lower prevalence than Mexico at the coldest temperature. This result seems paradoxical, because the U.K. is cooler overall during the breeding season, which would seem a prime environment for selecting for more cold-robust transmission. However, *Drosophila hydei* is invasive in the U.K.; the timing of the introduction(s) is not known, but the earliest recorded catch of *D. hydei* in the British Isles is from a London warehouse in 1930 ((Richards and Herford, 1930), though note that *D. hydei* wasn't taxonomically described until 1921, by Sturtevant). If a founder effect is in place, this could produce a relatively low genetic diversity in the host fly and/or the symbiont, reducing the ability of the two genomes to respond to selection for cold-tolerance. Alternatively, if

individuals are routinely introduced from other ranges, as was proposed for why *Spiroplasma* hasn't been lost during geographical invasions, this could act to slow the spread of cold-resisting alleles. Regardless, this would not necessarily explain why Cambridge has a lower tolerance, rather than an identical one. It could be that the ancestors of the Cambridge *D. hydei* were from a region of the U.S. with greater temperature extremes than Mexico. Investigation into the mitochondrial haplotypes of the U.K. flies could provide some insight as to the original source and founder effect's impact on the population.

The 'permissive passage' findings, which suggest that titre and thus PCR detectability are restored by warm temperatures, mean that an adult that overwinters could have its titre restored to pre-winter titre before it breeds in late summer. This is less likely to be a mechanism in flies from Tenancingo, where even the cooler months have relatively high minimum temperatures. However, the breeding ecology of Cambridge fruit flies is likely to be similar to that suspected in Ohio, where flies cease to breed – thus ceasing transmission of symbionts – and overwinter in the adult stage in sheltered areas (Spencer, 1941). Experimental testing of this through artificially overwintering many *D. hydei* in a refrigerator for several months, then transmission-testing flies at different timepoints after restoring to 25°C or 18°C, would make an interesting extension to this investigation.

3.5.4 Prevalence and transmission considered together

As discussed earlier, prevalence is an output variable influenced by a symbiont's transmission efficiency, strength of and selection on the symbiont phenotype, and symbiont cost. Temperature is potentially able to influence all of these, and thus is a strong candidate for shaping prevalence. In considering why U.K. prevalence is particularly low, it's worth comparing temperature conditions. The U.K. has relatively mild winters, comparable more to the Japanese sampling sites than to some of the northern American ones. However, the maximum temperatures in July, August and September, which data from the fluctuating-temperature condition suggests may be most important in setting prevalence, tend to be 5-10°C lower in the U.K.

Investigating the overwintering ecology of fruit flies in Britain, both through observation of wild flies and experimental manipulation, would provide more insights into the year-to-year persistence of *Spiroplasma* hy1. Spencer (1941) found that *D. hydei* in Wooster, Ohio, are

human commensals, with towns in the northern U.S. acting like 'island' populations, and that adults overwinter in restaurants and cellars as they are unable to survive overwintering outside in the northern latitudes. He noted that in the lab, *D. hydei* can go from egg to adult in 2 weeks, plus 2 days (females) and 4 days (males) to reach sexual maturity, but in the wild in Wooster, a generation was one month to six weeks long. Interestingly, this is approximately the time taken for *D. hydei* in this chapter's experiment to reach maturity at 18°C (1 month) or 15°C (6 weeks), suggesting a semi-realistic simulation of wild temperature conditions.

The presence of a positive 'drive' phenotype will be important in the wild in the U.K., to enable stable maintenance of the hy1 symbiont under wild, suboptimal-for-transmission temperature conditions. There is limited data on *Drosophila* parasitoid pressure in the U.K. Data exists from France, which is likely to be climatically similar to the U.K., suggesting that parasitoid wasp attack can be very important as a selection pressure on flies, causing losses of 5-40% in some *Drosophila* species (Fleury *et al.*, 2009). Under artificial population cage conditions, hy1 sweeps in rapidly under very high wasp pressure (Xie *et al.*, 2015). A U.K. survey to examine the importance of wasp attack on fly mortality would aid in parameterising the maintenance of the symbiont, especially performed alongside seasonal *Spiroplasma* prevalence-tracking, to monitor for co-fluctuations in wasp population and *Spiroplasma* prevalence.

3.5.5 Conclusions summarised

1. *Spiroplasma* strain hy1 is found in the south of the U.K. This observation is despite average summer temperatures regularly being cooler than that needed for high transmission frequencies, as demonstrated in previous studies.
2. *Spiroplasma* strain hy1 is more tolerant to cool temperatures than previously suspected. A methodological difference – using a 'recovery temperature' protocol, rather than rearing flies at the transmission temperature – probably underlies the difference in results.
3. This tolerance of cool temperatures partly explains the persistence of *Spiroplasma* strain hy1 in the U.K. environment, but nevertheless segregational loss will occur, and indicates the presence of a benefit to the host from infection even in the U.K.

4 Temperature's effect on phenotype and titre

Abstract

The evolutionary ecology of an arthropod bacterial symbiont depends heavily on the type and strength of the phenotype conferred by the symbiont. Whilst environmental temperature has been shown to alter the strength of the phenotype in reproductive parasitic symbionts, the effect of thermal environment on protective phenotypes is poorly understood. The phenotype of *Spiroplasma* hy1's phenotype has, to date, been studied at 25°C. However, 25°C is on the higher end of the temperatures that the host will typically experience in temperate parts of its range, so the results may not be informative of a 'typical' temperature for this system. Here, an experiment was performed on a fly-and-hy1 symbiosis from Mexico to determine the strength of hy1's protective phenotype at 18°C, which mimics the mean of the average maximum and minimum temperatures for Tenancingo in Mexico in September. Fly fitness measurements at 25°C under wasp attack were in line with previously-recorded values. However, there was no evidence that hy1-protected fly fitness was greater than uninfected fly fitness at 18°C. Wasp fitness was lowered in hy1-protected groups at both temperatures, suggesting that fly fitness loss is primarily due to an increase in pupae dying without eclosing, rather than successful conversion into wasps. The results indicate that a 'silent co-existence' of hy1 with its host could be ongoing in cooler times in the season, and particularly in cooler parts of the host range, easing selection for the symbiont and resulting in lower prevalence. They further highlight the general importance of examining protective symbiont–host interactions across the range of temperatures encountered by the host.

4.1 Introduction

The prevalence of an insect mutualistic endosymbiont is the product of transmission efficiency, phenotype strength, and cost. Environmental temperature has the potential to influence these three factors. In the previous chapter, I demonstrated that environmental temperature influences *Spiroplasma* transmission efficiency in the *D. hydei-Spiroplasma* hy1 symbiosis. A significantly reduced prevalence was observed over two generations of transmission at 15°C, but with relatively little effect at 18°C and at an alternating 18/15°C regime. In this chapter, I follow this investigation by investigating whether expression of the symbiont's phenotype was also influenced by temperature.

4.1.1 Temperature can affect the strength of the phenotype produced by an insect endosymbiont, and phenotype may be more temperature-sensitive than transmission

There is evidence for temperature's ability to influence the strength of symbiont-mediated phenotypes. Most of the evidence is from *Wolbachia* reproductive parasites or 'Jekyll-and-Hyde' symbionts with mixed mutualism/parasitism phenotypes, rather than from mutualists. The degree of temperature sensitivity varies strongly by host and symbiont species and strains (summarised in chapter 2/(Corbin *et al.*, 2017)).

There are a few examples of temperature-phenotype effects in *Drosophila*/*Spiroplasma* symbioses, particularly in the reproductive-parasite 'sex ratio organism' *Spiroplasma* species. A variety of responses are seen to high versus low temperature. NSRO in *D. nebulosa* sees a rapid decrease in the male-killing (MK) phenotype over two generations at low temperatures, and a slow decrease in the strength of the MK phenotype at high temperatures over eight generations (Anbutsu *et al.*, 2008). ESRO in *D. equinoxalis* sees a decrease in the MK phenotype with high-temperature treatment of embryos; however, this is not observed in the case of WSRO in *D. willistoni* (Malogolowkin, 1959). Even in this limited set of samples, variation is observed in whether higher or lower temperatures than optimal ablate the phenotype, and how quickly. A further complexity is that experiments rarely decouple transmission efficiency and phenotype strength. For instance, in Anbutsu et al. 2008, the transmission efficiency of a sex ratio distorter was also changed by temperature, and increasing male frequencies could be due to transmission loss as well as or instead of weakened phenotype in still-infected mothers (Anbutsu *et al.*, 2008).

One common observation is that suboptimal temperatures seem to eliminate symbiont phenotype before they begin to strongly affect the transmission. Evidence for this comes from several experiments which demonstrate the phenotype recovering after 'recovery temperature' passages are carried out (detailed in chapter 3). The impact of recovery temperatures is almost certainly mediated through symbiont titre. *Spiroplasma* titre, for instance, is frequently observed to be repressed by cool temperatures (see (Osaka *et al.*, 2008) for *Spiroplasma* in *D. hydei,* and (Anbutsu *et al.*, 2008) for NSRO in *D. nebulosa*). The extent to which this is true may depend on the type or mechanism of the symbiont-mediated phenotype, with mechanisms often being unclear for many symbioses. For instance, if *Spiroplasma* hy1 protects the host by releasing parasitoid-specific toxins into the parasitoid upon being consumed, titre could affect phenotype in two ways. Firstly, there may be a threshold titre of hy1 for there to be any growth-stunting effect, and if the parasitoid doesn't consume this threshold number of hy1 cells, it will survive. Secondly, a higher symbiont titre beyond this threshold may result in faster death of the parasitoid.

When temperature effects on titre are producing changes in phenotype strength, this could result in 'historical' effects, in which titre and phenotype remain depressed over several generations after exposure to the suboptimal temperature (see (Jaenike, 2009) for an example). However, this phenomenon would itself depend on how density affects the number of transmitted bacteria getting into the offspring, and how the size of this inoculation influences later titre in the insect. The symbiont transmission bottleneck may be evolutionarily important for other reasons, as a 'narrow' bottleneck can influence mutation-accumulation, drift, clonal structure and selection in the endosymbiont population (summarised in (Mira and Moran, 2002)).

4.1.2 Possible implications of temperature effects for mutualistic phenotypes in their ecological context

One key potential implication of a seasonal temperature effect on phenotype strength is that the strength of selection for a mutualist will be higher around the optimal temperature, and reduce as the temperature becomes suboptimal, and cease when the phenotype is ablated. Consequently, a switch back-and-forth between selection and drift may occur as the seasons change.

The impact of temperature on symbiont dynamics becomes more complicated if a reduction in phenotype strength is produced through a decrease in symbiont titre. This is because a reduced titre may also lower the cost of bearing a symbiont, especially if, with falling temperature, costs reduce more quickly than benefits. If the cost is low, a null-phenotype mutualist may simply drift in frequency (if transmitted with perfect fidelity), with the possibility of loss depending on start-frequency and population size. If the cost of the mutualist is still sizable and the mutualistic phenotype is ablated, the mutualist may be selected out of the population. The result is that cooler seasons may produce the loss of a mutualist which would have been useful in the warmer ones. An additional complication is the effect of temperature on biotic selective forces. In the case of *D. hydei*, where selection pressure is provided by parasitoid wasps, this adds a layer of complexity, as general wasp biology – and wasp attack rate – can be influenced by temperature, too.

The literature and thesis work presented thus far about *Spiroplasma* hy1 in *Drosophila hydei* suggests that as temperatures decreases to 18°C, it tends to experience repressed titre in adult flies, though transmits with high fidelity for up to two generations. As the temperature lowers further to 15°C, segregational loss begins to become common. Therefore, I hypothesise that at 18°C, *Spiroplasma* hy1 will experience a loss of its protective phenotype, associated with reduced titre.

4.2 Aims

1. To determine the effect of low temperature on *Spiroplasma's* protective phenotype in *D. hydei.*
    a. Determine whether *Spiroplasma* confers a protective phenotype upon its fly host at 18°C, in the same manner as recorded previously at 25°C.
    b. Similarly, compare the effect of *Spiroplasma* on wasp fitness at different temperatures.

4.3 Methods

4.3.1 Temperature's effect on the strength of the protective phenotype

Blocks: Due to constraints on the numbers of fertile female *L. heterotoma* available at any one time, the experiment was split into two blocks, Block A and Block B, repeated 2-3 months apart in the same incubators and under the same conditions. The block design was incorporated into later statistical analyses.

Generating parents of experimental larvae: The *D. hydei* stocks used were the hy1-infected and hy1-uninfected versions of the Mexican isoline, TEN104-106 (Mateos *et al.*, 2006), as described in chapter 3. Parental stocks to produce the phenotype-testing F1 larvae were established through a complete generation at the experimental temperatures. To this end, hy1-positive stock and hy1-negative grandparent stocks were allowed to lay in separate bottles at both 25°C and 18°C (4 bottles total, one of each temperature/hy1 status combination). Each bottle used ~50 females and ~20 males. Flies laid eggs for 2 days at 25°C and 4 days at 18°C, then adults were disposed of to prevent generations mixing. Parent stocks were reared in 12 hour/12 hour light/dark cycle incubators at the focal temperature (Sanyo MLR-351), sexed on their eclosion days, and the females stored at their birth temperatures on SY food (see Appendix for composition).

Verifying infection status of mothers of experimental flies: Mothers of phenotype-tested larvae were homogenised with a pestle, extracted using the Promega Wizard kit, then tested for *Spiroplasma* infection status with 'Spoul' primers using host CO1 primers used to test for successful DNA extraction. DNA was visualised by gel electrophoresis. Details are as given under 'DNA extraction and *Spiroplasma* diagnostic PCR' in the methods section of chapter 3. Only larvae from verified-infected mothers were included in the experiment.

Generating experimental larvae: After reaching sexual maturity (day 2 at 25°C and day 4 at 18°C) the female flies created above were placed in individual population cages over a small yeast-painted grape juice agar plate, each with two hy1-negative Mexican isoline males which were at least 6 days old. Females were permitted to mate and lay eggs for one day at 25°C and two days at 18°C, and tipped onto new plates after this period, repeating until sufficient larvae were obtained. Three days after laying commenced at 25°C, and 6 days after laying commenced at 18°C, L1 larvae were picked with hooks onto small ASG food vials (see Appendix for composition), such that the larvae in a vial all came from one known mother. Target larval density was 15 larvae per vial, but due to laying rate constraints,

some vials contained 7 or 8. Mothers of vials were frozen at -80°C for later infection status verification by PCR assay.

Attacking experimental larvae: After picking, larvae were immediately exposed to *L. heterotoma* wasps. The wasps had previously been matured to at least 7 days of age at 22°C on grape agar vials, with honey available for nutrition, and then given three days of oviposition experience on L1-L2 *Drosophila melanogaster* (Oregon R). Five female wasps and three males were transferred to each picked vial of larvae; pilot attempts (data not shown) had used three female wasps and three male wasps per vial, but this had been insufficient for a good attack rate. The wasps were left to attack larvae for three days at 25°C and 6 days at 18°C.

Phenotype assay: Vials were monitored daily. The numbers of eclosing flies and wasps were counted for each vial. Typically, at 25°C, fly emergence began at day 14 and wasp emergence at day 21. These times were approximately doubled at 18°C. Observations continued until 30 days after picking at 25°C, and 60 days after picking at 18°C, at which time any remaining full, dark-coloured puparia ('closed puparia') were counted as dead. In addition to counting emergences, successfully-eclosed empty puparia ('open puparia') were counted for each vial. This enables fly fitness to be assessed in terms of the number of flies surviving the pupal stage. It also allows double-checking of the number of emergences, as adult *D. hydei* at 18°C seems adept at escaping by crawling between the cotton wool bung and the vial wall. Pupal fate can be determined by examining the exit hole of the puparium. Adult flies eclose at the end of the puparium which bears the respiratory filaments. This leaves a lifted flap-like structure. Wasps in contrast chew through either end of the puparium, leaving a small circular hole and no lifted flap.

Dissection of failed puparia: Following termination of the phenotype assay stage, 'closed puparia' (those assumed dead) were opened under a dissecting microscope (Leica) using needle-nosed forceps and a mounted needle. The contents were visually examined and classified either as 'closed wasp', 'closed fly' or 'closed unidentifiable'. 'Closed wasps' were puparia where the contents were clearly identifiable as adult wasps from the head and thorax, though specimens varied in whether they had pale bloated abdomens or looked identical to post-eclosion adult wasps. Similarly, 'closed flies' were clearly identifiable as flies from the head and thorax, again with some variation in the appearance of the abdomen. 'Closed unidentifiable' was a category for all other puparia, which varied from pale and larva-like through to white, grey or black fluids or granular masses.

Statistics: All statistics were carried out in R version 3.0.2 (R Core Team, 2013). Data from the eight experimental groups was encoded in terms of fly fitness (number of flies eclosing, versus number from which wasps emerged plus pupal deaths) and wasp fitness (number of pupae from which wasps eclosed, versus number of puparia from which flies emerged plus pupal deaths). The non-wasp-attacked control group data was included in statistical analysis for the flies, but not for the wasps, for whom it would have been uninformative. The glm() function was used to carry out a binomial GLM on fly fitness and wasp fitness. The maximal model contained temperature, infection, attack (fly fitness data only), the interactions between these three factors, plus block. The functions drop1() and update() were used to refine the model. For fly fitness, the minimal model was Fitness ~ Temp + Inf. + Attack + Temp:Inf. For wasp fitness, the minimal model was Fitness ~ Temp + Inf. + Block.

4.4 Results

4.4.1 Temperature has a significant effect on the strength of the protective phenotype

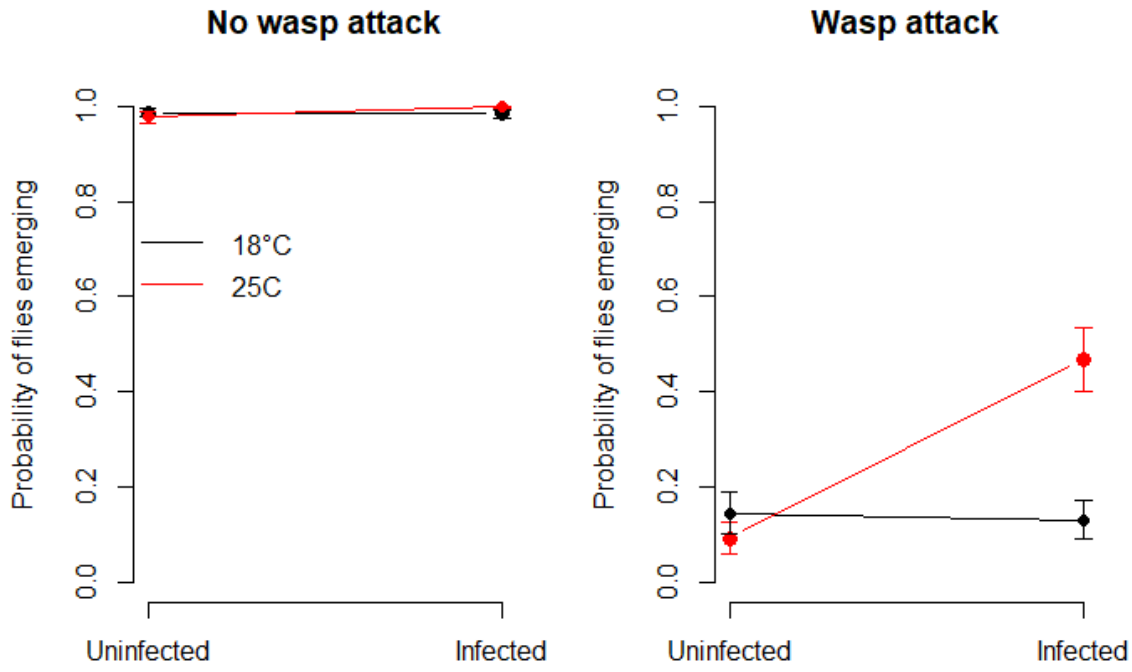

Figure 4.4.1 Probabilities of fly emergence by temperature, infection status, and attack. Values predicted from the final GLM. Error bars show the standard error of the predicted values. Sample sizes, in the format 'total number of pupae (total number of replicate vials)': **18°C S- Lh-**, 83 (8); **18°C S+ Lh-**, 95 (8); **18°C S- Lh+**, 56 (8); **18°C S+ Lh+**, 57 (8); **25°C S- Lh-**, 84 (7); **25°C S+ Lh-**, 94 (8); **25°C S- Lh+**, 64 (9); **25°C S+ Lh+**, 54 (9).

For fly fitness, the minimal model was Fitness ~ Temperature + Infection + Attack + Temperature:Infection. The significant terms in the model were infection ($p = 0.006155$), the temperature*infection interaction ($p = 0.001063$) and wasp attack ($p < 2 \times 10^{-16}$). Temperature alone was not significant, but was left in the model because of its interaction with infection. Block was removed during the model-refining process as this didn't significantly increase the model AIC.

The significance of infection is likely to be due to how strongly it modulates wasp attack at 25°C, as Figure 4.4.1 shows that infected flies don't differ from their uninfected counterparts under any conditions other than wasp attack at 25°C. Attack is highly significant, with Figure 4.4.1 showing flies doing worse under wasp attack than without it in every condition. The root of the temperature*infection interaction's significance may be due to the difference between fly fitness in the infected group at 25°C and fly fitness in the infected group at 18°C. In the former, infection provides a significant fitness boost relative to uninfected flies. In the latter, survival in infected flies is no different from uninfected flies, indicating that hy1's phenotype is depressed at the cooler temperature. No three-way interaction between infection, attack and temperature is seen, which seems paradoxical, due to the visually striking fitness increase of attacked/infected flies at 25°C but not 18°C. However, it is due to how at each temperature, the graph lines for both attacked and unattacked flies show the same trends. At 25°C, non-attacked, infected flies have higher survival than uninfected flies (mimicking the case in the attacked flies, though with a much smaller slope in the graph), but at 18°C, non-attacked, uninfected flies have higher survival than infected flies (mimicking the case in the attacked flies). Therefore, both the attacked and non-attacked lines trend upwards at 25°C, while both the attacked and non-attacked lines trend downwards at 18°C. This means that no attack/infection/temperature interaction emerges in the model, but instead, only an infection/temperature interaction.

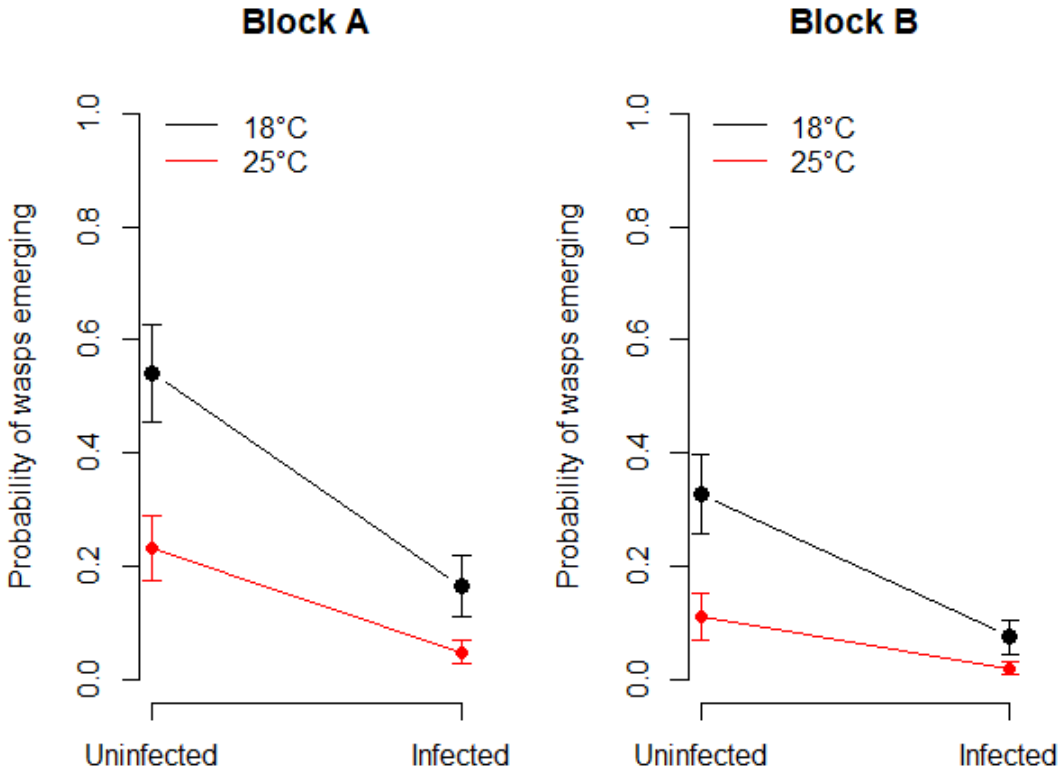

Figure 4.4.2 Probabilities of wasp emergence in wasp-attacked vials by infection status, temperature, and block Values predicted from the final GLM. Error bars show the standard error of the predicted values. Sample sizes, in the format 'total number of pupae (total number of replicate vials)' for Block A: **18°C S-**, 22 (3); **18°C S+**, 29 (3); **25°C S-**, 41 (5); **25°C S+,** 36 (5). Sample sizes for Block B: **18°C S-**, 34 (5); **18°C S+**, 28 (5); **25°C S-**, 21 (4); **25°C S+,** 18 (4).

For wasp fitness, the minimal model was Fitness ~ Temp + Inf. + Block. The Temperature*Infection interaction term was dropped to increase model stability. Infection was a significant factor, which is expected due to hy1's protective phenotype (p=2.41e-05). The second significant factor was temperature (p=0.000641), and the third was block (p=0.024946). From Figure 4.4.2, the proportion of wasps emerging is higher at 18°C than at 25°C across both infected and uninfected conditions, indicating that this is a temperature which suits the wasp's biology better, though the infected/uninfected difference is also larger at 18°C.

4.4.2 The 'double-death' phenotype seen in wasp-attacked pupae shows a variety of failure states for wasps and **Spiroplasma**

In this experiment, the double-death pupae were dissected to observe how far along in development they tended to fail, with proportionate data shown in Figure 4.4.3. The proportion of identifiable pupae did not vary much by wasp attack or infection status. However, within the wasp-attacked condition, there seems to be a noticeable difference between *Spiroplasma*-protected and *Spiroplasma*-uninfected groups at both temperatures. *Spiroplasma*-protected groups are more variable; they contain a higher proportion of recognisable flies, making up half or more of all recognisable pupae. Meanwhile, *Spiroplasma*-uninfected groups rarely contain recognisable flies, and wasps make up almost all of the recognisable pupae. There may be a slight increase in the proportion of recognisable dead wasps versus dead flies in the 18°C wasp-attacked/infected condition than in the 25°C version of the same set-up, but this has not been statistically tested due to small sample sizes. Unrecognisable pupal contents are the largest group in each condition, taking up ~0.65 to ~0.80 of the dissected pupae.

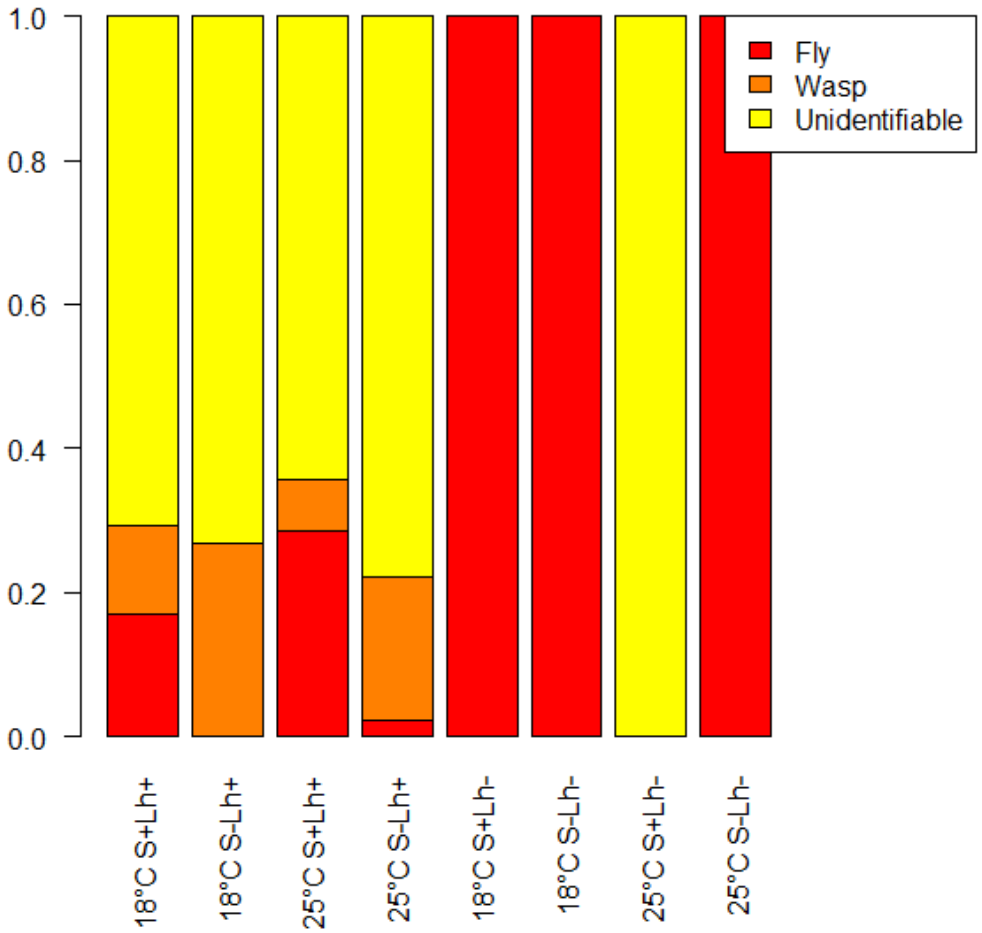

Figure 4.4.3 Proportions of closed puparia in each of 3 death states, by experimental group 'S' denotes infection status (+ is infected, - is uninfected) and 'Lh' denotes wasp attack status (Lh+ is attacked, Lh- is unattacked). Note extremely low sample sizes for Lh- groups. Sample sizes and replicate number in each group, left to right: 41 closed pupae (8 vials); 26 (8); 28 (9); 45 (9); 1 (8); 2 (8); 1 (8); 1 (7).

4.5 Conclusions

The strength of a symbiont's phenotype influences its prevalence in the host population, and indeed it ability to invade or persist. The expression of symbiont phenotype is known to be modulated by host genetic variation, but less attention has been given to abiotic factors such as environmental temperature. *Spiroplasma* hy1 in *D. hydei* is found in the U.K., a temperate environment, yet studies on its phenotype have been exclusively carried out at 25°C, which is warmer than average for the U.K. even in summer. To address how phenotype might vary within ecologically-relevant temperature ranges, I carried out an experiment to see if hy1's ability to protect against wasp attack is stronger, weaker, or the same at 18°C as at 25°C. I found that while the symbiont is protective following attack at 25°C, similar to that recorded in earlier studies, at 18°C, fly fitness in the hy1-infected flies was no different from fly fitness in hy1-uninfected flies. This suggests that at 18°C, the protective hy1 phenotype is absent.

4.5.1 Spiroplasma *hy1 does not protect fly fitness at 18°C*

At 18°C, there is no significant difference in fly fitness (manifesting as fly pupation-to-eclosion survival rates in the presence of wasps) between *Spiroplasma*-infected and uninfected groups. The proportion of pupae eclosing into flies is approximately 0.15 in each of these groups. This contrasts with 25°C, where *Spiroplasma*-mediated protection causes proportion of pupae eclosing into flies to increase from ~0.1 to ~0.45. The overall effect, then, is that *Spiroplasma's* fly protective phenotype is ablated at 18°C.

At 25°C, results seen in this experiment are consistent with those seen in (Xie *et al.*, 2010), suggesting that wasp line used in my experiment was comparable to that used previously (Xie et al. used strain Lh14, and this experiment used a line from France, as import of wasps into the EU was not possible). The previous work reported that *Spiroplasma* infection can change pupal-to-adult survival under the 'ideal' non-wasp-exposed laboratory conditions in some fly lines. In the line experimented on here, fly fitness remains consistently high across non-attacked conditions, even at 18°C. We report a higher fly pupa-to-adult survival in unattacked conditions than Xie et al., who experienced an average survival of ~90% across all infection conditions, while our GLM model-predicted survival is almost 100%. This may be due to this experiment being run with a lower density of flies than in Xie et al., using 15 larvae per vial rather than 30.

Compared to Xie et al., the protective effect of hy1 at 25°C was less dramatic. Xie et al. reported an average survival of 7.17% for uninfected flies and an average survival of 47.30% for infected flies, an almost 7-fold increase in survival. In contrast, I found that the model-predicted fitness of attacked, infected flies at 25°C in this experiment is approximately five times that seen in attacked, uninfected flies. Considering that Xie et al. reported differences by fly strain in endogenous fly resistance to wasp attack, and in the degree of survival benefit under wasp attack conferred by *Spiroplasma*, it is likely that this is partially to do with fly genetic background.

4.5.2 Despite hy1 not rescuing flies at 18°C, wasp fitness still decreases when hy1 is present, regardless of temperature

The fitness of wasps is always lower in *Spiroplasma*-carrying hosts than it is in *Spiroplasma*-free hosts, regardless of the rearing temperature. Wasps always do better at the cooler temperature of 18°C than at the corresponding *Spiroplasma* status at 25°C. That *Spiroplasma* presence kills wasps and decreases their fitness at both temperatures, but doesn't improve fly fitness at the cool temperatures, supports the hypothesis that the wasp-killing phenotype remains at 18°C even though the fly-protective phenotype is lost.

The lack of a stable Temperature*Infection interaction supports the idea that the fly fitness 'losses' in the 18°C infected condition are not all converted into wasp fitness 'gains'. It could be due to the 'double-death' phenotype disproportionately claiming wasp pupae rather than fly ones.

4.5.3 There is evidence of residual **Spiroplasma** *hy1 protection – insufficient to increase fitness above that seen in uninfected/attacked flies – from dissected 'double death' pupae*

The 'double-death' phenotype – in which a proportion of wasp-attacked pupae die and become blackened in appearance without eclosing as either a fly or wasp – was previously noted in *Spiroplasma*-infected *D. hydei* by Xie et al. (Xie *et al.*, 2011). 'Double-death' pupae were seen to be produced under wasp attack in this experiment which matches the description seen by Xie.

The dual fitness loss could result from a failure of *Spiroplasma* to 'fully rescue' the host at 18°C, perhaps by killing the wasp after it has already inflicted lethal damage on the fly.

Dissections of double-death pupae show that more of the 'near-adult' insects are developing as 'sick' flies in the *Spiroplasma*-infected groups compared to uninfected groups, supporting the hypothesis that *Spiroplasma* often fails by killing wasp larvae too late to save the fly. There is only a little variation by temperature; although a higher proportion of the recognisable double-death pupae appear to be wasps at 18°C than at 25°C, supporting the hypothesis that *Spiroplasma* takes longer to kill off the wasp at 18°C Additionally, the majority of the 'double death' pupae in the wasp-attacked conditions were unidentifiable, suggesting that a drawn-out failure of *Spiroplasma* is not the most common failure mode.

4.5.4 Disproportionate hyperparasitism at 18°C but not 25°C is unlikely to be a factor in these results

Due to wasps appearing to do better at 18°C than 25°C, there was concern that hyperparasitism at 18°C but not 25°C could account for the observed differences in *Spiroplasma's* protective phenotype, by overwhelming the protective ability of *Spiroplasma* with multiple attacks. This does not appear to be the case, as flies in the uninfected/wasp attacked control groups suffer equally low fitness at both temperatures. There was also concern that the 'double death' phenotype could be from hyperparasitism. However, if this equally effects all experimental groups, this shouldn't be a concern for interpreting the data.

A disadvantage of this experimental approach is that the fly larvae and wasps were not supervised, but instead left together for a few days. A supervising approach was not chosen due to time limitations, but would have been more appropriate for reducing the risk of hyperparasitism. It would also have reduced any effect from larval age, which could potentially have varied from L1-L3 over the chosen time span.

4.5.5 The **Spiroplasma** *hy1/***D. hydei/L. heterotoma** *system may exhibit a mismatch of parasite-host optima*

In our laboratory experiments, wasps appear to be generally fitter at 18°C than at 25°C. In the uninfected group, where *Spiroplasma* protection or lack thereof isn't a factor, wasp emergence is significantly higher at 18°C. An interaction between a host genome, a natural

enemy genome, and the environmental temperature is called a G x G x E interaction. Mismatches can occur in the temperatures at which hosts and their natural enemies function best, producing complex response profiles for the system as a whole (reviewed in (Thomas and Blanford, 2003)). The *D. hydei* system may be an example of this, as the phenotype data shows an apparent mismatch between the optimal temperature for Spiroplasma's phenotypic expression in its fly host, and the optimal temperature for wasp development inside the fly.

One set of studies which found an effect of temperature on protective phenotype in natural infections of insects, was carried out in pea aphids carrying *Hamiltonella defensa.* Aphids were nearly completely resistant to attack by *Aphidius ervi* parasitic wasps at 20°C, but were susceptible at 25°C and 30°C. This may represent thermal sensitivity of symbiont-mediated protection (Bensadia *et al.*, 2006; Guay *et al.*, 2009). Protection became temperature-insensitive in clones which were doubly-infected with *H. defensa* and PAXS (Guay *et al.*, 2009).

4.5.6 If **Spiroplasma** *coexists 'silently' with* **D. hydei***, this could leave a protection gap to be covered by nuclear-mediated defence*

The loss of protective phenotype at 18°C sits in contrast to hy1's transmission at 18°C, which is known to persist for several generations. Consequently, 'silent' coexistence of hy1 and *D. hydei* may be occurring at cooler times of year in temperate parts of the range, such as in the U.K. The temperature of the larval medium (generally fallen fruit) could be highly heterogeneous, depending on age of the food, wind exposure, and whether it sits in sunlight or shade (Feder, 1997). By reducing selective forces on hy1 so that it becomes more prone to genetic drift, temperature heterogeneity could drive down hy1's prevalence, relative to its prevalence if it consistently expressed a phenotype. In investigating the importance of seasonal temperature to hy1's prevalence, a useful next step would be to ascertain how well the warmer, phenotype-expressing times of year match up with peak *L. heterotoma* activity, and whether the times or temperatures at which hy1 protection occurs coincide with when the fly host could most benefit from the phenotype.

A corollary of this is that flies may not be able to rely on symbionts as sole means of protection unless attack is restricted to 'high season'. This heterogeneity in protection will

be evolutionary significant, producing 'space' for other resistance mechanisms to spread as the *Spiroplasma* protection is incomplete.

4.5.7 The importance of temperature in protection may mean that **D. hydei** *could increase its fitness through behavioural means*

Studies demonstrate that flies in the genus *Drosophila* change their behaviour to alter their adaptive thermal niche. For instance, some species such as *D. immigrans* and *D. curviceps* undergo seasonal migrations between high and low altitudes to maintain an ideal temperature (Kimura and Beppu, 1993), and *D. melanogaster* females avoid ovipositing on warm fruit to prevent their offspring from dying from heat shock as feeding larvae (Fogleman, 1979; Schnebel and Grossfield, 1986). Additionally, 'thermal curing' of pathogens has been shown in other insect systems (Blanford *et al.*, 2000). Therefore, it might be possible that hy1-infected *D. hydei,* or other insects carrying bacterial mutualists, select thermal niches that favour the health of their symbionts. In the case of hy1-infected *D. hydei* this may mean that hy1-carrying females preferentially oviposit on warmer materials, which could be tested through a choice experiment. Although ovipositing on warm media may be useful to *D. hydei* generally because it shortens development time, a 'trade-off' against larval heat-shock risk may be at work, with the heat-shock risk needing to be larger to deter hy1-carrying *D. hydei.* Factors which may work against the evolution of thermal niche modulation in this system include a) a lack of reliable signals of future temperature being available to ovipositing females (Feder *et al.*, 1997) and limited chances for larvae to modulate their own environments through moving within their food source (Feder, 1997), and b) a low prevalence of hy1 in *D. hydei* preventing selection and spread of a thermal modulation behaviour in response to hy1 infection.

4.5.8 Future work

In the temperature-phenotype experiment discussed in this chapter, I examined the effect of low temperature over a protracted period – simulating an average lower-temperature over the period of several weeks – rather than the effect of low temperature for periods immediately during and after a wasp attack event. This means that multiple components are influenced by low temperature in this experiment, including effects on titre, plus effects on the defence mechanism linked to hy1 infection at the actual point of attack. It may be

useful to carry out experiments to separate these two components of the phenotype, particularly to find out what happens in scenarios in which temperature varies over the 18-25°C range within a relatively short period.

5 Standing and active costs of hy1 infection in *D. hydei*

Abstract

The cost of a symbiont to its host can influence both its frequency and ability to persist. Costs of carrying a symbiont make protective symbioses potential labile across the parasitism-mutualism continuum, moving from mutualists where the need for protection is common, to parasitic where they are absent. As such, they form an important source of loss or decline of host-symbiont interactions. Aside from observations of delayed male fertility in *Spiroplasma*-protected, wasp-attacked flies, relatively little is known about the costs of *Spiroplasma* hy1 to *D. hydei.* In this chapter, several experiments were carried out to investigate the passive and active costs to *D. hydei* of carrying *Spiroplasma* hy1. Common-garden-reared hy1-positive and hy1-negative flies were tested for a passive cost under ecologically 'ideal' conditions, using wing size as a proxy, and the same flies were tested for a passive cost under the ecological 'stress' condition of starvation. An active cost of hy1 was investigated by attacking hy1-positive and hy1-negative flies with *L. heterotoma,* and examining whether onset of reproduction related behaviours differed relative to non-wasp-attacked controls. The data indicate *Spiroplasma* hy1 is a 'cheap' mutualist with regards to passive cost, as neither wing size nor starvation survival times reduce with infection. These data contrast with those observed in the MSRO/ *D. melanogaster* symbiosis. For active cost, results were unclear. Onset of mating behaviour is no different amongst hy1-carrying wasp survivors than in any other group. The onset of offspring production did show a greater variance in the male, hy1-positive, wasp surviving group, but this was not as dramatic as the delayed reproduction seen in earlier work. The results show that hy1 is relatively low-cost to its host, which may help preserve it in wild populations during times when hy1 is less beneficial. To obtain a better understanding of how cost dynamics work in the hy1/*D. hydei* system, future work should test these parameters over a wider temperature range.

5.1 Introduction

5.1.1 Both endogenous and symbiont-mediated defence can be costly to the host, resulting in trade-offs

Insects are assailed by many natural enemies, including parasites, parasitoids, and predators. Accordingly, most insects have some form of defence response. Many species possess innate immunity, consisting of humoral and cellular defences. The cellular arm can carry out phagocytosis and encapsulation (Strand, 2008), the latter being important in responses to parasitoids in some *Drosophila* (reviewed in (Lynch *et al.*, 2016)). Some insects, such as flies in the *Drosophila melanogaster* subgroup, have behavioural immunity to threats like parasitoid wasps (Lynch *et al.*, 2016). Beyond nuclear-gene encoded traits, some insects have protective mutualistic endosymbionts, which are generally maternally-inherited. The nuclear and symbiont-mediated defences may interact with one another, but with the exception of some recent insights from aphids (Parker *et al.*, 2017), this interaction is still poorly-understood.

Nuclear-encoded immunity generally comes at a cost. Costs may be standing or active. Standing costs are those borne by the insect even when the immune system is not being challenged, and active costs are those which are incurred only when the immune system is challenged (McKean and Lazzaro, 2011). Costs arise because standing and active defence uses energy, because defence may trade off against other traits, or because resistance for one threat may reduce the resistance to a different kind of natural enemy (Cayetano *et al.*, 2015). Costs may sometimes be proportional to the strength of the immunity phenotype, as seen in aphids (Vorburger *et al.*, 2008) and flies (Hoang, 2002).

Importantly, the costs of a trait such as immunity may not always manifest in cossetted laboratory populations, where organisms are kept in close-to-optimal conditions. It may take ecological stress – such as food limitation or suboptimal temperatures – for costs to be revealed. An example comes from bumblebees, which show an active cost of nuclear-mediated defence, but only when starved, not well-fed (Moret and Schmid-Hempel, 2000). Similarly, *D. melanogaster* lines selected for resistance to the parasitoid *Asobara tabida* show a cost of resistance relative to unselected control lines, but only under larval food competition (Kraaijeveld and Godfray, 1997).

Defensive symbionts in insects can also be costly, and costs can be standing or active. Most defensive symbiosis data is from aphids, which harbour a variety of defensive, secondary

mutualists in addition to their obligate nutritional symbiont *Buchnera*. Some aphid symbionts are costly only under suboptimal circumstances; *Hamiltonella defensa* and *Serratia symbiotica* protect *A. pisum* under heat-shock, but *Regiella insecticola* becomes costly, with uninfected adults being 24% more likely to survive to adulthood. Neither *H. defensa* nor *R. insecticola* have costs or benefits to *A. pisum* reared constantly at 18°C, though *S. symbiotica* slightly accelerates development (Russell and Moran, 2006). Standing costs of several isolates of *H. defensa* under non-stressful conditions are evident in *Aphis fabae*, as the isolates reduce lifetime reproductive success, with the level of cost showing a symbiont genotype-by-host-genotype interaction (Vorburger and Gouskov, 2011). Regarding active costs, unusually, symbiont-infected aphids which are attacked by the parasitoid and survive have increased longevity and lifetime reproduction compared to their infected-unattacked counterparts, whereas uninfected-attacked aphids suffered a reduction of longevity and reproduction after resisting an attack. This suggests that there is no induced cost of the symbiont-conferred resistance phenotype (Vorburger *et al.*, 2013). Though costs may be proportional to strength of the protective phenotype, this isn't always the case (Cayetano *et al.*, 2015).

The magnitude of costs are important in evolutionary ecology because high costs may constrain trait evolution. Costs might cause a resistance phenotype to be selected against, especially when natural enemies are rare or attack has little impact on fitness. Further, symbiont-encoded and nuclear defences may not always successfully coexist in the same animal when defence is costly, as cost of defence may increase without an equal increase in strength of defence, as is seen in aphids (Cayetano *et al.*, 2015). Additive costs, with non-additive benefits, can result in a polymorphic population, as can fluctuating selection from changing levels of a natural enemy.

Even when the costs and benefits of an isolated immune response are well-understood, due to the complexity of the real world – with multiple host background genotypes, levels of nuclear immunity, strains and coinfections of secondary symbionts, and various strains and species of enemy – the resulting portrait of immunity in a population may be complicated or beyond current understanding. For example, there is an ecological cost to pea aphids carrying *Hamiltonella defensa*, which is an anti-parasitoid mutualist. Infected aphids express less defensive behaviour against the predator *A. bipunctata*, and thus are eaten more. Thus, *H. defensa* frequency in the wild may ultimately be determined both by level of parasitoid attack, and to the threat from *A. bipunctata* predation (Polin *et al.*,

2014). A study which tracked symbiont frequencies in wild aphid populations on two host plants, across several localities in two US states, found that the microbiome was very dynamic even over short, seasonal timescales (Smith *et al.*, 2015). Though *Hamiltonella* and *Regiella* often had seemingly-intuitive correlations between symbiont frequency and enemy abundance, other findings were harder to explain. For instance, superinfections varied over time, and symbiont frequencies dropped 3 weeks after a superinfection spike across many localities. The authors proposed infection costs as a potential cause of instability, alongside other factors such as symbiont-symbiont hitchhiking, enemy counter-adaptation and alternative environmental forces such as temperature.

5.1.2 Different sexes can bear different costs of immunity

Defence can have differential costs to each sex. In nuclear-mediated defence, this can be due to life history trade-offs, with males preferentially allocating resources into finding mates and producing many 'cheap' gametes, while females who have 'expensive' gametes invest in their immune systems instead. This is seen in some insect species, although some female insects preferentially mate with males showing indicators of higher immune health (see summaries in (Kurtz *et al.*, 2000; Kraaijeveld *et al.*, 2002)). Meanwhile, in symbiont-mediated defence, differences in cost are primarily driven by the 'mother's curse' effect.

A 'mother's curse' (MC) is when the sex which transmits cytoplasmic elements is favoured by the element's phenotype, and the non-transmitting sex is disfavoured (Gemmell *et al.*, 2004). In most animals, the transmitting sex for microbial symbionts is the female. MC was first described when considering mitochondria, and occurs here because purifying selection on mitochondria that is restricted to the trait in males cannot produce an evolutionary response, due to exclusively matrilineal transmission (Bonduriansky and Chenoweth, 2009). Paradoxically, female-only transmission is widespread in animals, despite the risk of a mother's curse developing. Proposed mechanisms for vertical transmission persisting despite the threat of MC include host-nuclear compensation, surviving males helping their sisters, inbreeding, competitive coexistence of symbionts and pathogens, and the mutational exclusivity of membership in the maternally provisioned microbiome (Wade, 2014).

Sex-ratio distortion is a well-documented phenomenon in maternally-inherited symbionts, and is produced by a symbiont forcing the host to invest in female offspring over male

offspring, so that female offspring can better compete with non-symbiont-infected conspecifics (Hurst and Frost, 2015). There are also 'Jekyll and Hyde' symbionts, which cause both sex-ratio distortion and mutualistic phenotypes. For instance, native strains of *Wolbachia* in *Culex pipiens* protect the blood-feeding female host from the avian malaria parasite *Plasmodium relictum*, while harming male host fitness through cytoplasmic incompatibility (Zélé *et al.*, 2012). Many Jekyll and Hyde symbionts are found in flies with *Wolbachia* being the most heavily-studied genus. Several wMel strains in multiple *D. melanogaster* genetic backgrounds protect against RNA viruses while causing CI (Hedges *et al.*, 2008; Teixeira *et al.*, 2008). Jekyll and Hyde *Wolbachia* strains are also found in *D. simulans* (causes CI and protects against C and Flock House viruses) and *D. innubila* (causes male-killing and protects against Flock House virus) (Osborne *et al.*, 2009; Unckless and Jaenike, 2012). *Spiroplasma* is also represented; MSRO in *D. melanogaster* weakly protects the fly against the parasitoid *L. heterotoma* and strongly protects against *L. boulardi*, and kills male flies (Xie *et al.*, 2013; Paredes Escobar, 2014).

Despite the above collection of Jekyll and Hyde phenotypes, there is limited evidence for mother's curse effects in mutualistic symbionts which reduce male fitness **without** distorting the sex ratio. This absence of evidence occurs even though selection to reduce cost should be non-existent in the male line, leading to the passive accumulation of male-harming traits. The absence of a mother's curse phenotype could be due in part to the mechanisms given above in Wade. Whilst helping behaviour from live siblings and inbreeding seem unlikely to be important factors in wild human-commensal *Drosophila*, because of the often-large population sizes, a third condition reducing selection for MC may be met: there is evidence that female *D. melanogaster* preferentially mate with brothers over unrelated flies (Loyau *et al.*, 2012). A lack of costly non-distorters could also be a consequence of producing an equal sex ratio being an unstable strategy – for both host and symbiont – under circumstances where one sex is usually fitter (Fisher, 1930). In part, the lack of evidence may be because outside of aphids, costs are not well-studied for insect-mutualist systems. Subtler effects on fitness that do not produce a sex ratio distortion, or only manifest under ecological stress, are less likely to be spotted in a laboratory stock. Frequently, studies also focus only on costs in female animals, and this prevents comparison of the sexes; indeed in the case of aphids, most study focuses on the asexual stage. Finally, there is also the possibility that symbiont cost in males and females are mechanistically 'tethered together', such that selection for lower costs in females tends to produce lower costs in males.

5.2 Aims

Earlier work in this thesis demonstrates that temperature may constrain the *D. hydei* mutualism in temperate habitats, through reducing the transmission efficiency and lowering the strength of the protective phenotype. This chapter examines whether the cost of hy1 could also be acting as a potential constraint on its frequency.

The aims are:

1. Compare wing sizes of hy1-infected and uninfected wild *D.hydei*, to determine whether infection exerts a noticeable cost (active and standing combined) in terms of body size in a complex wild environment. Wing size would reflect physiological costs in the larval/pupal phases.
2. Experimentally determine the passive costs of hy1 on body size in males and females, using stock Mexican flies (TEN 104-106) reared in a 'common-garden' environment:
    a. Before ecological stress: flies are reared to adulthood under non-stress conditions, then wing size is measured as a proxy for body size and other fitness correlates (Partridge *et al.*, 1987; Santos *et al.*, 1992; Pitnick and Markow, 1994).
    b. After ecological stress: flies from the above experiment undergo starvation, with survival time measured as a proxy for cost. This technique revealed a strong cost of harbouring the 'Jekyll-and-Hyde' *Spiroplasma* MSRO in *D. melanogaster* (Herren *et al.*, 2014), permitting comparison with other *Drosophila* systems. Stress resistance is correlated with body size in *D. melanogaster* (Djawdan *et al.*, 1998), and thus decreased resistance to starvation can result from smaller body size (Kraaijeveld *et al.*, 2002), which must be borne in mind when interpreting the results.
3. Experimentally determine the active costs of hy1 on reproductive fitness in males and females, using stock Mexican (TEN 104-106) flies. Previous work characterising *Spiroplasma* hy1 protection noted that many attack-surviving males did not reproduce during days 10-13 of life, even though males tend to become reproductively mature by day 6 (Xie *et al.*, 2011). Both onset of mating behaviour and onset of offspring production will be assayed, to differentiate between behavioural and other (e.g. gonadal damage) forms of infertility.

5.3 Methods

5.3.1 Surveying phenotypic differences in a wild fly population

Obtaining wild *D. hydei*: Wild *D. hydei* specimens were collected in Royal Tunbridge Wells, southern England, in August 2015 (51.09 N, 0.16 E), generously sent as by-catch from Prof. Darren Obbard's fly collections. As detailed in chapter 3, adult flies were caught with sweep nets over fruit bait and transferred to vials containing sugar-yeast (SY) food, sexed visually, then kept alive in a CT room at 25°C for 2 weeks before being frozen in 95% ethanol at -80°C.

Wing size assays: To obtain wings, each fly was removed from ethanol storage, the body held down by the thorax with blunt forceps with the dorsal side facing upwards, and the wing removed whole by grasping the wing base with needle-nosed forceps and pulling in the direction of the fly's head, with a motion parallel to the bench. The remainder of the fly's body was then placed into an individual Eppendorf vial and returned to -80°C. Forceps were cleaned between each fly by 30 seconds of immersion in 50% bleach, followed by two sets of 30 second immersions in molecular water, and each fly was handled on a new piece of paper towel to prevent cross-contamination of *Spiroplasma*-containing haemolymph. The right wing was preferentially removed, but left wings were used when the right wing was too damaged for measuring.

The wing was mounted for measurements by placing it on the adhesive surface of clear sticky-tape, then smoothing the tape adhesive-side-down onto a glass microscope slide. Photographs of the wings, plus a photo of a standard measure taken on the same magnification, were captured using LAS software (Leica). In ImageJ, the standard was used to calibrate distance, then the lengths of the proximal and distal sections of wing vein IV were measured for each wing (Schneider *et al.*, 2012).

DNA extraction and *Spiroplasma* diagnostic PCR: Because fly bodies were kept in ethanol prior to wing-removal, they were dried prior to DNA extraction. The Eppendorf tubes were opened and *dried on a 65°C heat block for 30 minutes, to evaporate off the ethanol.* As detailed in chapter 3, DNA was extracted by homogenising whole flies with the Promega Wizard DNA extraction kit (Promega), quartering the recommended amounts for animal tissue. PCR reactions were carried out using GoTaq Hot Start Green Master Mix (Promega). 'SpoulF' and 'SpoulR' primers (see chapter 3 for primer sequences and details) were used to test for *Spiroplasma* infection (after (Montenegro *et al.*, 2005)). 'CO1' primers were used to

amplify host DNA, as a test for successful DNA extraction; failure to amplify host DNA was taken as an indicator that DNA extraction had failed, and these samples were excluded from analysis. 6 μl of each PCR product was run on 1.5% agarose gels, using Midori Green Nucleic Acid Staining Solution (Nippon Genetics Europe) to visualise amplicons and Hyperladder I (Bioline) to confirm product length alongside positive and negative control PCR assays.

Statistics: The data were fitted to a GLM in R (version 3.4.1) using the function glm(). A Gaussian distribution was chosen because Q-Q plots demonstrated that the data was normally distributed. The total length of wing vein VI was obtained by summing the length of the proximal and distal sections for each fly. The total length was modelled in terms of sex, infection, and the interactions between these. drop1() with chi squared tests was used to refine the model, and demonstrated that including the side of the body did not improve the model. The final, minimal model included only sex. Q-Q plots of the model residuals confirmed that a Gaussian distribution was the correct choice.

To obtain the means of each sex/infection group for graphing, the aggregate() function was used. To obtain the two-tailed 95% confidence intervals for graphing, I used the function ciMean() from the package lsr (Navarro, 2015).

5.3.2 Measuring standing cost in an experimental fly population

Obtaining 'common garden' experimental flies: The Mexican *Spiroplasma* hy1-infected *D.hydei* stock (TEN104-106) and its uninfected counterpart, were generated and maintained as described in chapter 3 of this thesis (Mateos *et al.*, 2006). All flies were maintained at 25°C on a 12 hour/12 hour light/dark cycle in an incubator (Sanyo MLR-351). To obtain experimental larvae, population cages were assembled of 30 adult females and 20 males, which were at least six days old and segregated by stock infection status. Flies laid eggs for one day on grape juice agar painted with yeast paste. Three days after onset of egg-laying, L1 fly larvae were picked with hooks into thirty, mixed-infection-status common garden ASG food vials (see Appendix for composition) for maturation. Eight infected-stock larvae and eight uninfected-stock larvae were picked into each replicate vial. To prevent gut flora effects on fitness acting as an experimental confound, the day after picking, larvae were inoculated with two drops per vial of gut flora filtrate. Filtrate was made by mixing 2 g of old fly food from each parent-stock bottle in 10 ml of molecular water, and filtering the

homogenate through filter paper in a Buchner funnel under vacuum pressure. Larval vials were shuffled within the incubator tray twice a week. The adult flies eclosed 12-15 days after being picked, and were sorted into SY adult storage vials based on sex, vial of origin, and eclosion date. The flies remained in storage until they reached day ten post-eclosion, at which point female *Spiroplasma* titre is relatively stable (Haselkorn *et al.*, 2013) and males have usually reached sexual maturity.

Starvation assays: After ageing up to day 10, non-anaesthetised flies were moved with a pooter into individual, 1.5% w/v agar-bottomed plastic vials, which were closed with Parafilm (Bemis Company, Ltd.) to stop the agar desiccating. Each fly was given a unique identifier linking it to records of its emergence date, starvation start date, sex, and vial of origin. The starvation vials were stored in the same 25°C incubator, and the trays housing them turned and rearranged daily. Vials were checked for starvation deaths every eight hours. When flies were found dead or no longer capable of standing upright or walking, the hour of the observed death was recorded and the fly was preserved in a 95%-ethanol-filled screw-cap vial for later estimation of infection status.

Wing size assays: Wings were collected from flies and measured, following the same protocol as for wild flies (section 5.3.1). As flies were individually stored in ethanol vials to prevent cross-contamination, they were placed back into their ethanol vials after wing removal, rather than immediately into a new empty Eppendorf tube.

Recovery of infection status: The infection status of each fly was recovered post hoc by PCR assay. Flies were transferred to individual Eppendorf tubes. As with the wild flies (section 5.3.1), they were dried on heat blocks, the DNA extracted with the Promega Wizard extraction kit (Promega), and underwent PCR assays with Spoul for *Spiroplasma* detection and CO1 primers for extraction efficacy quality control (as detailed in chapter 3). The infection status of each fly was then paired to its wing size data and its starvation data.

Statistics for wing size data: The total length of wing vein VI was obtained and fitted to a GLM in R with a Gaussian distribution, using the same method as for the wild fly wing size data. The total length was modelled in terms of sex, infection, the side of the body that the wing was taken from, and the interactions between these. drop1() with Chi squared tests was used to refine the model, and demonstrated that including the side of the body did not improve the model. The final, minimal model included sex, infection, and the interaction term. Q-Q plots of the model residuals confirmed that a Gaussian distribution was the appropriate choice.

The means of each sex/infection group, and two-tailed 95% confidence intervals for graphing, were obtained using the same functions detailed for the wild fly data.

Statistics for starvation data: The data were analysed with the function survreg() in the package 'survival' (Therneau and Lumley, 2013) in R version 3.0.2 (R Core Team, 2013), using a Weibull accelerated failure time model. The Weibull model was chosen over a Cox model, because analysis using the cox.zph() function demonstrated that the data violated the assumption of proportional hazards. Additionally, the Weibull function permits different scale and shape functions to be fitted to the data by sex, which was necessary as the male and female distributions were different shapes. The maximal model was the survival function in terms of sex, strata(sex) (which tells the model to fit shape and scale parameters separately for each sex), infection, and the interactions. The model was refined using anova() to compare simpler models to the maximal. The final, minimal model included sex and strata(sex), but not infection. The Weibull scale function for the male distribution and the female distribution, which gives the time to death for the 63rd percentile, was obtained from the model using the unique(predict()) functions from R package rms (Harrell Jnr, 2017).

5.3.3 Measuring active cost in an experimental fly population

Obtaining larvae for attacking: The flies used were from an infected stock and an uninfected stock of Mexican *D. hydei*, of the same genetic background (see details for Mexican flies as described for the temperature and transmission experiment). All flies were maintained at 25°C on a 12 hour/12 hour light/dark cycle in a controlled-temperature room. Two population cages were set up per hy1 infection status, each containing 50 female flies with 10-20 male flies of the opposing infection status, to ensure homogeneity of the nuclear

genetic background. In these cages, the females laid eggs on grape agar plates painted with yeast paste, which were changed once a day. Three days after laying began, L1 larvae were picked from plates into small ASG vials. Vials were segregated by infection status. Twenty-five vials of 15 larvae each were picked for each infection status.

Attacking larvae with wasps, and collecting adult flies for assay: Fourteen vials of hy1 infected fly larvae, and 14 vials of hy1 uninfected fly larvae, were randomly selected for attack by *L. heterotoma*. A control group of 11 hy1 infected and 11 hy1 uninfected larval vials were kept unattacked. Adult wasps were aged to at least 10 days old to ensure sexual maturity had been reached. Wasps were given oviposition experience and mating opportunities by keeping them on vials of *Drosophila melanogaster* (Oregon R) larvae for three days prior to use. On the day that *D. hydei* larvae were picked from cages, five female and three male wasps were transferred to each attack vial and left for four days, spanning the L1 and L2 larval period. After wasps were removed, fly larvae were fed filtered gut flora homogenate using the method detailed for the standing-cost experiment. Vials were rearranged twice weekly to even out temperature effects. Vials were checked daily for eclosing flies. Eclosing flies were collected onto SY food storage vials (see Appendix for composition) and separated by sex, replicate, and emergence day.

Adult mating behaviour assays: Mating behaviour observation assays ran on day 1, 2 and 3 for females, and days 5, 6 and 7 for males. Voice recording was used during the assays to enable the experimenter to note assay start times, end times, and observations without needing to look away from the flies, because mating is a brief process in *D. hydei*. Experimental flies were transferred by pooter into individual ASG vials containing a 'tester' fly of the other sex, which was derived from the *Spiroplasma*-uninfected stock, and known to be at the age of sexual maturity. Assays ran for 1 hour. During the assay time, failed and successful courtship (male pursuit and licking behaviours) and copulation attempts were noted. If an experimental fly was observed to mate, it was removed from the mating-observation assay early, left with its tester fly, and the next day, entered the offspring-production assay. If an experimental fly did not mate during the 1 hour assay, it was separated from the tester fly. The next day, if the fly was still within the age bracket for mating behaviour observations, the assay was repeated. If the fly was now outside the age bracket, it passed into the offspring production assays.

Adult offspring production assays: Flies entered offspring-production assays after they mated or left the age bracket for the mating behaviour assays. Each day, the experimental

fly was transferred to a new ASG test vial with a new tester fly, and left unobserved. This continued until day 7 of the offspring-production assay, after which flies that had produced no offspring were switched to an every-two-days schedule instead (e.g. day 8, day 10, and so on). When experimental females were moved to a new vial, the previous vial's tester male was disposed of. When experimental males were moved to a new vial, the previous vial's test female was left in the vial for an extra day, to ensure that she had time to lay fertile eggs if she had been fertilised. The test vials were checked daily for larvae for at least 3 days after the experimental fly was removed. The day of offspring production onset was recorded as the day of the earliest assay vial with larvae inside it. Once they'd produced larvae or reached day 18 of adult life, experimental flies were stored frozen at -80°C in molecular water.

Statistics for adult mating behaviour assays: Mating behaviour of each fly assayed within the 3-day period was encoded as a binary trait (1 = mating, 0 = no mating) and a binomial GLM carried out in R (version 3.4.1). The maximal starting model was Mated ~ Sex * Attack * Infection. The functions drop1() and update() were used to refine the model. The end, minimal model was Mated ~ Sex + Infection + Attack.

Statistics for offspring production assays: Time to production of larvae for each adult fly, encoded as number of days from entry into the assay, was put into a survival model. Some samples were right-censored from adult flies escaping early or 'ageing out' of the assay without ever reproducing, and censored/non-censored status was entered into the survival object for each sample. As with the starvation data, a Weibull accelerated failure time model was used with the function survreg() in the package 'survival' (Therneau and Lumley, 2013) in R version 3.0.2 (R Core Team, 2013). The maximal model was time to larvae production in terms of sex, strata(sex), wasp attack, *Spiroplasma* infection status, and their interaction terms. The model was refined using anova() to compare more-reduced models, but the final, minimal model included every term and set of interactions except strata(sex).

5.4 Results

5.4.1 Wild data: wing size differs by sex but not infection status in a wild population of D. hydei

To investigate whether a cost of *Spiroplasma* infection is strong enough to be evident in wild flies, a population of *D. hydei* was sampled from Tunbridge Wells, southern England. For each fly, a wing was removed and measured, and the rest of the corpse was PCR assayed for *Spiroplasma* infection.

An initial GLM was constructed of wing size in terms of sex, infection status and their interactions. The final, minimal model contained only sex as a significant factor explaining variance in wing size ($p = 1.54x10^{-6}$), both the infection-sex interaction and infection being removed to improve the model during model-testing. Females are on average larger than males, which is expected and seen in many *Drosophila* species (Ashburner, 1989).

As can be seen on Figure 5.4.1, the data on wild fly wing size was variable and the confidence interval on size are large. This is due in part to the relatively small sample size of symbiont-infected individuals. This is a consequence of low prevalence (~15% for this population). Wing removal must be performed prior to DNA extraction and PCR testing; thus obtaining large numbers of infected flies becomes a limiting step. The large error bars are also likely to be due to the inherent 'noisiness' of the natural environment of the flies, which will act to obscure more-subtle effects, and is the reason for performing the 'common garden' experiment.

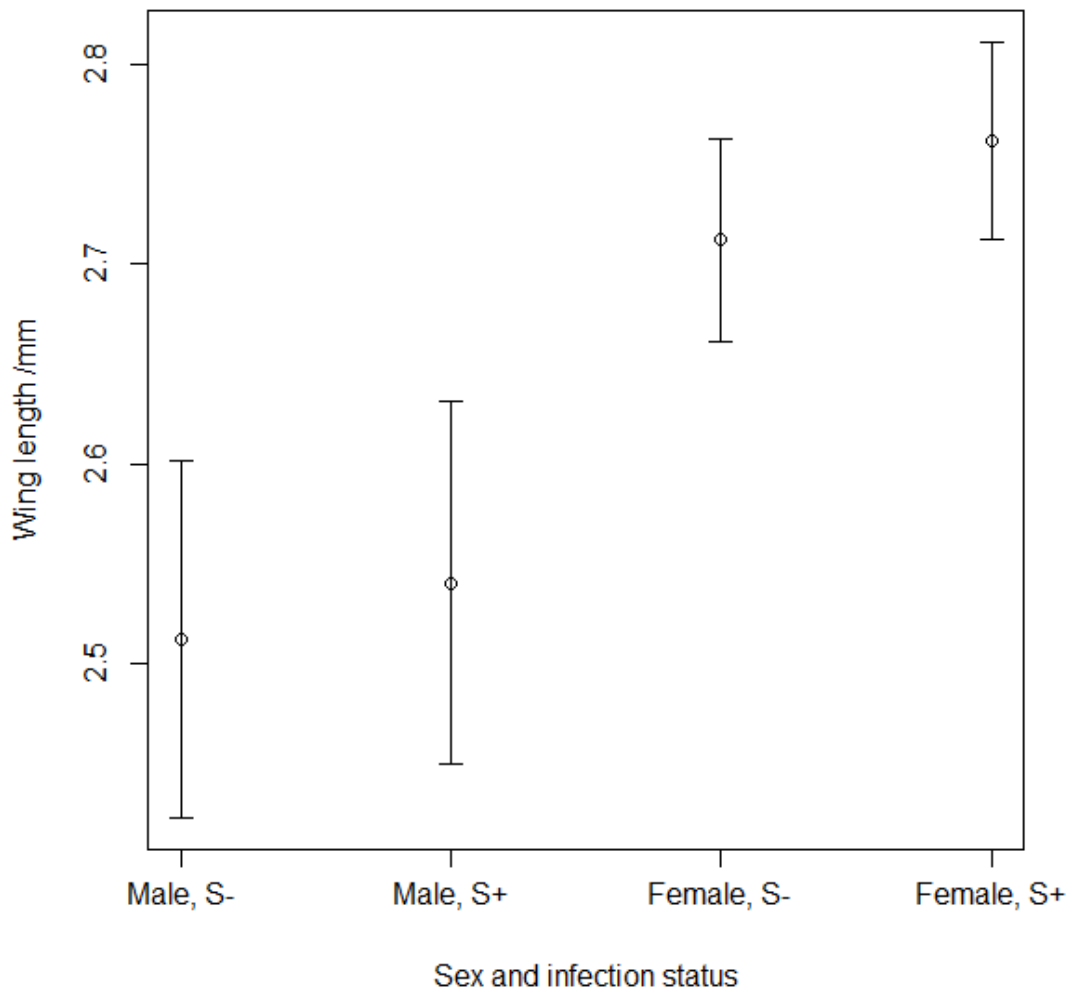

Figure 5.4.1 Average wing size of wild flies, divided by sex and infection status Error bars show the 95% confidence intervals. hy1-infected flies are denoted by S+, hy-uninfected flies by S-. Samples sizes: Male, S- = 15; Male, S+ = 14; Female, S- = 9; Female, S+ = 10.

5.4.2 Standing cost: wing size differs by sex, hy1 infection status, and their interaction in D. hydei reared in a 'common garden'

A common garden experiment was carried out to investigate whether *Spiroplasma* carriage imposes a standing cost under good rearing conditions. For the 'common garden' reared flies, the final minimal model for wing vein IV length contained sex ($p = <2 \times 10^{-16}$), infection status ($p = 0.0168$) and a sex-infection interaction ($p = 0.0361$) all of which are significant to the $p = 0.05$ level.

Females tend to have larger wings than males, regardless of infection status. From examining the graph (Figure 5.4.2), the infected and uninfected males do not significantly differ from each other in wing length, but there is a difference between infected and uninfected females, explaining the significance of the sex/infection interaction term in the model.

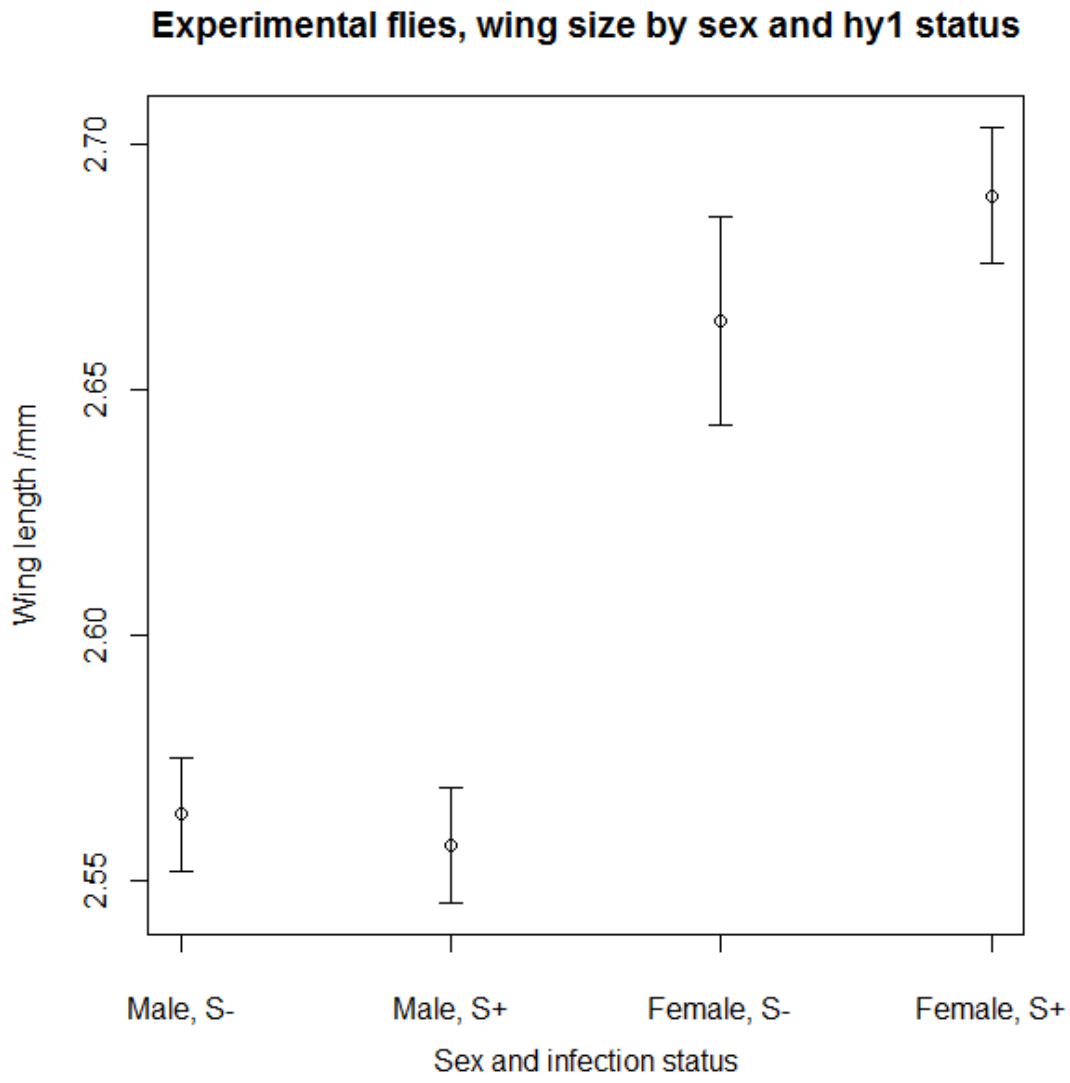

Figure 5.4.2 Average wing size of experimental flies, split by sex and infection status Error bars are the 95% confidence intervals. hy1-infected flies are denoted by S+, hy-uninfected flies by S-. Sample sizes: Male, S- = 83; Male, S+ = 72; Female, S- = 75; Female, S+ = 83.

5.4.3 Standing cost: starvation time of 'common garden' reared flies doesn't differ by infection status

Flies reared in a common garden – some of which were *Spiroplasma*-infected, and some of which were not – underwent starvation as adults. Starvation time is a measure of standing *Spiroplasma* cost which may be more relevant than wing size when considering the metabolic effects of *Spiroplasma*.

Infection was dropped as a factor during ANOVA model testing, as it made no improvement to the model. Thus, infection does not significantly change the time taken for flies to starve. However, the effect of sex is highly significant ($p = 2.62 \times 10^{-70}$). For females, the Weibull scale function (which gives an impression of the characteristic lifespan) to 3 significant figures is 185 hours, while for males, it is 140 hours (Figure 5.4.3). This reflects the much higher longevity of females compared to males under starvation, which itself is probably the result of larger average body size in females.

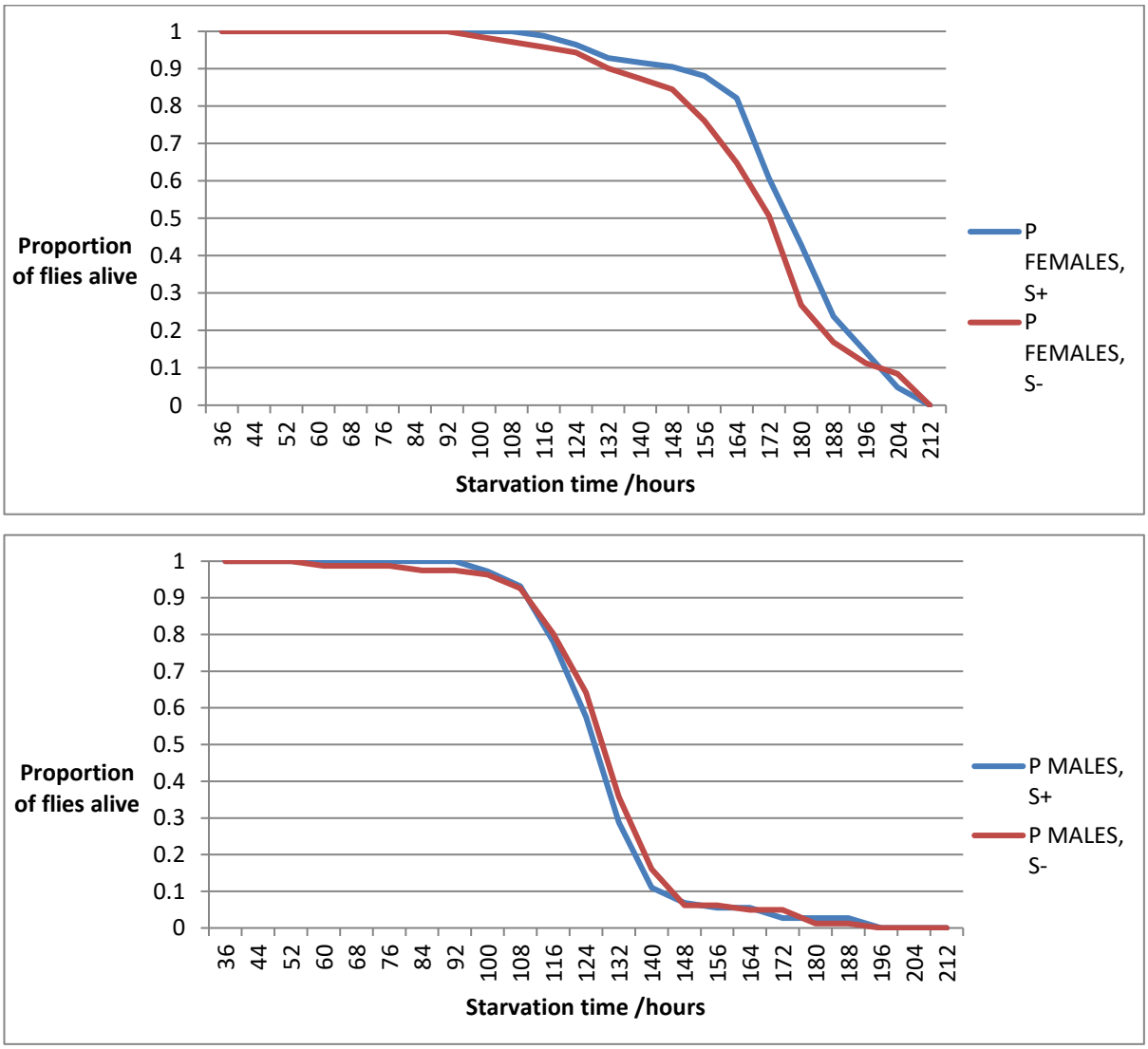

Figure 5.4.3 Graph showing the proportion of females (top) and male (bottom) alive over time (given in hours). Infected flies are in blue, uninfected flies in red. Sample sizes: Male, S- = 81; Male, S+ = 73; Female, S- = 71; Female, S+ = 84

5.4.4 Active cost and mating attempts: there is no difference in mating attempts between infected/surviving and other males

Costs of *Spiroplasma* were assayed by observing the time to onset of mating behaviour in male and female flies. Flies were of *Spiroplasma*-infected and *Spiroplasma*-uninfected conditions. Additionally, some flies had undergone attack by *L. heterotoma,* making this an assay of active costs; those incurred by the symbiont carrying out its protective function. Whether males experience greater or lesser active costs than females is of interest, because the mother's curse hypothesis states that maternally-inherited agents should be evolutionarily 'indifferent' to male survival.

The minimal model in the binomial GLM for the mating assay was Mated ~ Sex + Infection + Attack. None of the terms in this model are significant to p = 0.05, and there are no interaction terms. Mating attempts don't differ significantly between the combinations of sex (p = 0.0523), wasp attack (p = 0.9940), and protection (p = 0.9949).

5.4.5 Active cost and larvae production: the interactions between sex, infection, and wasp attack status influence time to successful production of larvae

Active costs of *Spiroplasma* were also assayed by observing the onset of larval production. The minimal model in the Weibull survival analysis was Sex * Infection * Attack. Only strata(sex) was dropped from the initial maximal model, for not significantly improving the model in anova model tests. All factors and interactions were significant (see Table 5.4.1).

From Figure 5.4.4, it can be seen that although individuals in most groups tended to produce larvae within the first 2 days of the larvae production assay, the most variance is seen in 'M.1.1', the male, wasp-attacked, *Spiroplasma*-infected group. There are also a greater number of individuals in this group which never bred, either ageing out or escaping from the assay relatively late (post day 10).

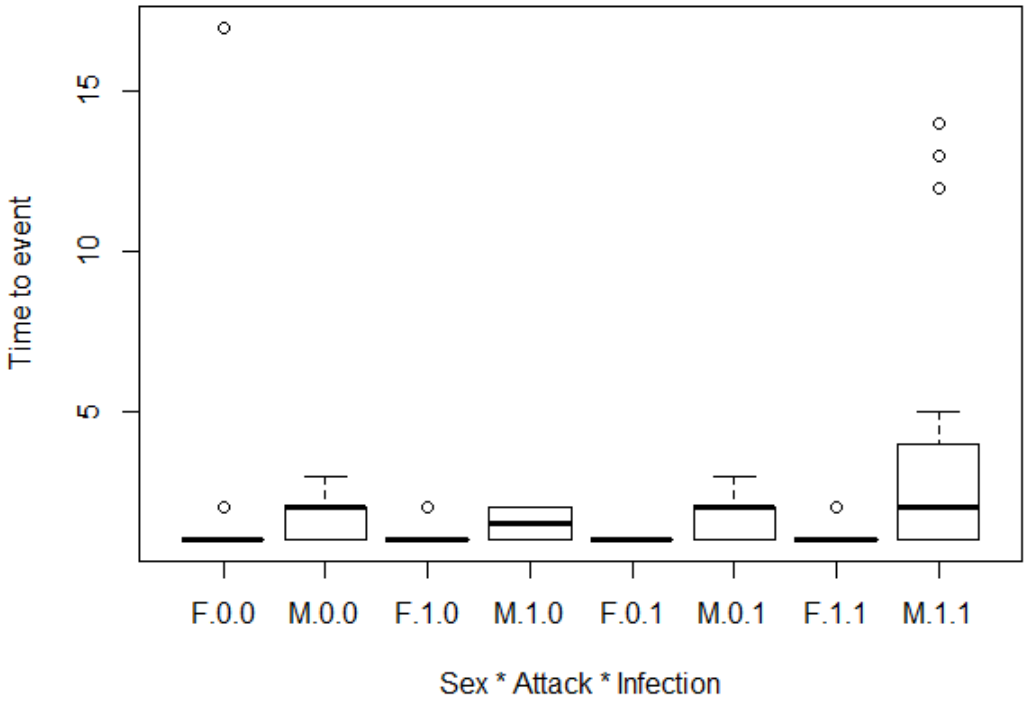

Figure 5.4.4 Time in days to larval production or censorship for the 8 different groups For x axis labels, the initial letter denotes sex, the first number attack status (1 = attacked, 0 = not attacked), and the second number denotes *Spiroplasma* status (1 = infected, 0 = not infected). Note the greater variance in males compared to females, and particularly in the male/attacked/infected group. **Sample sizes (total = 162)**: F.0.0 = 20; M.0.0 = 27; F.1.0 = 13; M.1.0 = 8; F.0.1 = 17; M.0.1 = 29; F.1.1 = 21; M.1.1 = 27.

Weibull model parameter	**P value**
Sex	4.80×10^{-22}
Lh	2.81×10^{-07}
S	4.02×10^{-10}
Sex * Lh	1.19×10^{-11}
Sex * S	3.23×10^{-05}
Lh * S	1.95×10^{-06}
Sex * Lh * S	7.27×10^{-01}

Table 5.4.1 The p values for each parameter of the Weibull model for time-to-larvae-production

5.5 Discussion

In order to determine the standing and active costs of *Spiroplasma* hy1 to its host, I first measured wing size for wild-caught, known-infection flies. Then, I reared *Spiroplasma* infected and uninfected flies in a common garden environment and measured time to starvation and wing size. Finally, to investigate active costs, I carried out mating assays on infected fruit flies which had been reared in the presence of wasps.

5.5.1 There is no evidence for a standing cost of Spiroplasma *infection in wild or experimentally-reared flies, either to wing size or to starvation survival time*

For wild flies, no correlation was observed between wing size and infection in either sex. The lack of an association could be partly due to the small sample size, combined with the noise inherent from living in the wild, such as different early-life rearing conditions and wasp attack histories. For instance, environmental temperature during rearing is a key determinant of body size (James *et al.*, 1997). However, even when flies were reared in a common-garden environment, there was no evidence of a wing size cost.

Considering the starvation data, there is a difference in starvation time by sex – likely due to body size – but there is no evidence for a difference by infection status. This is despite the experiment being held at 25°C, which should be optimal for the symbiont's transmission, phenotypic expression and titre. Flies at this temperature contain many thousands of *Spiroplasma* within their hemolymph, but these apparently pose little standing cost. This apparently non-existent cost even in the face of extreme resource limitation is marked, particularly when compared to *Spiroplasma* MSRO in *D. melanogaster,* a Jekyll-and-Hyde male-killer which provides protection against *Leptopilina boulardi* and *L. heterotoma* (Xie *et al.*, 2015). MSRO infection makes no difference to survival of hosts on nutrient-poor food, but reduces survival time under starvation by more than 25% (Herren *et al.*, 2014). The authors propose that MSRO's growth is coupled to the host's nutritional state via a dependence on haemolymph lipids, preventing host-harming over-proliferation and providing the mechanism for anti-wasp protection. However, lipid limitation is not a likely mechanism of protection for *Spiroplasma* hy1 in *D. hydei,* as *Leptopilina heterotoma* is capable of synthesising its own lipids rather than relying on the host haemolymph (Visser *et al.*, 2010). It would be interesting to follow up on *Spiroplasma* hy1's titre at different levels of nutrient restriction, as was measured for MSRO by Herren et al., to see whether

Spiroplasma hy1 experiences titre suppression under starvation and if this is what permits it to be low-cost. It would also be interesting to compare costliness of *Spiroplasma* hy1 in *D. hydei* with the protective *Spiroplasma* of *D. neotestacea*, to observe whether an apparent low cost is a general feature of protective, non-sex ratio distorting mutualists in *Drosophila.*

My results which show a low standing cost of *Spiroplasma* are consistent with the limited prior work on this system. A study on wasp selection pressure's influence on hy1's frequency, by Xie et al. 2015, used 7 mixed-infection, non-wasp-attacked replicate bottles as controls. In these bottles, hy1's frequency drifted over 10 generations, consistent with hy1 not being selected against due to cost and a lack of segregational loss. However, the authors note that larval competition probably varied over time, as shown by oscillating female fecundity in each replicate, making it difficult to tell the degree of ecological stress on these replicates (Xie *et al.*, 2015).

5.5.2 Interestingly, there is evidence for a benefit of Spiroplasma to female flies under wasp-free conditions

Rather than *Spiroplasma* merely being non-costly when *L. heterotoma* was absent, females in the infected group tended to be larger than those in the uninfected group. This was not the case for males.

It could be the case that larger female size is an example of a non-protective, sex-spsecific advantage provided by hy1 in *D. hydei*. One hypothesis is that a larger size could come with a link to fecundity, as there is more abdominal space for ovaries. However, previous work by Xie et al. 2011 doesn't support this. Non-wasp-attacked *Spiroplasma*-carrying flies had the same larva-to- adult survival as unattacked, uninfected flies, suggesting neither cost nor advantage of *Spiroplasma* to the fly using this metric (though this included survival data from both sexes). Additionally, female egg-laying did not differ between these groups, and neither did two metrics of male fecundity (Xie *et al.*, 2011).

An alternative hypothesis is that larger female size could provide more room for sperm storage and thus competition. Females run out of sperm relatively quickly in *D. hydei*, and they remate frequently, though rematings within a single day do not increase female reproductive output and have been hypothesised instead confer sperm competition benefits (Markow, 1985; Markow and O'Grady, 2008). However, these genetic benefits are unfortunately difficult to demonstrate in an experimental setting.

A third hypothesis is that the larger adult size of infected females could be a consequence of these flies being big as larvae. Larger larvae could be able to eat faster, giving them an advantage when they are in more-competitive environments. This could be investigated by repeating the experiment under larval competition, but looking instead at correlates of larval success, such as development time.

5.5.3 The extent of any active cost of Spiroplasma *is still uncertain, but is not due to differences in mating activity*

Mating activity is not significantly different by *Spiroplasma* infection status or wasp attack status in *D. hydei*. This observation indicates that if there is any difference in reproductive success in *Spiroplasma*-infected, wasp-attacked flies, due to a mother's curse, it is not derived from behavioural difference between these flies and other groups. Instead, factors such as mechanical damage to the gonads are more likely.

When investigating the latency to larvae production, there was a significant effect of sex, wasp attack, *Spiroplasma* infection, and their interactions. This is likely to be due to the greater variance in onset of offspring production in the male, *Spiroplasma* carrying, wasp attacked group. Some individuals in this group were observed never to produce offspring, up to 2 weeks of assaying (day 18 since eclosion). This is consistent with Xie's findings that some males remained sterile days past the usual age of male onset of reproduction (Xie *et al.*, 2011). However, in addition to those males which never bred, the majority of male flies in the *Spiroplasma*-infected wasp-attacked group successfully produced offspring.

Complications of this experiment included losses of adult flies from assays, as daily handling increased the risk of escape or damage over time. There was a lack of a reliable assay to tell attack-surviving flies apart from flies in the 'attacked' condition who merely escaped attack. Whether active cost is important to males – and whether a mother's curse exists – is therefore still uncertain. There is a small possibility is that *L. heterotoma* may have transmitted *Spiroplasma* between larvae in the common garden environment. I have not personally observed signs of horizontal transmission in non-wasp conditions and believe this to be low-risk, but low rates of transmission cannot be completely ruled out. Whether *L. heterotoma* might accidently carry around the cure to its own offspring is also an interesting consideration, although I suspect under wild conditions, titre would be too low

and delivered too late to protect the attacked larva, and likely be lost when the host is destroyed.

5.5.4 Overall, **Spiroplasma** *hy1 appears to be a relatively 'low cost' symbiont, although further investigation is required*

When examining wing size under favourable larval conditions, no passive cost of *Spiroplasma* manifests in *D. hydei.* Additionally, *Spiroplasma* hy1 has a low cost to its host under starvation conditions, even though starvation produces dramatic and significant costs for *D. melanogaster* bearing another *Spiroplasma,* MSRO (Herren *et al.*, 2014). Further investigations into an active cost of *Spiroplasma* hy1 on its host are still needed, as although there appears to be a higher incidence of reproductive maturity delay in male, hy1-carrying survivors of wasp attack, the variance is higher than the picture of complete sterility hinted at in the earlier observations of Xie et al (2011).

5.5.5 The low cost of **Spiroplasma** *hy1 could mean that costliness doesn't work against retention of the symbiont in the wild*

If *Spiroplasma* hy1 is low cost, this should act to favour its retention in the wild as absence of wasp attack will cause it to move from beneficial to neutral, rather than to costly and parasitic. Further, less intense selective pressure will be required from wasp attacks to maintain it, and in the absence of wasp pressure, infection is likely to remain in the population for longer without purifying selection removing it.

However, the behaviour of *Spiroplasma* cost at different temperatures is yet to be tested, including at those lower temperatures which are known to decrease phenotypic strength and transmission efficiency. For instance, if lower temperature produces lower costs of hy1 (e.g. through reducing symbiont titre) then purifying selection against hy1 will decrease. This low-phenotype, low-cost scenario would encourage hy1 to drift in the population. Alternatively, if lower temperature produces a higher hy1 cost (e.g. through the host being less tolerant of symbionts at less-optimal developmental temperatures), then purifying selection will be more of a factor at low temperatures. The result could be a 'snowball' effect, as higher cost is combined with a reduced protective phenotype.

5.5.6 Final observations

Defence against natural enemies commonly comes at a cost; both a standing cost of systems such as haemocytes or microbes held in preparation for an attack, and active costs of inducible defences following attack. For symbiont systems, an individual possesses quite a large standing load of microbes. For *Spiroplasma*, many thousands of bacteria can be seen on haemocyte smears, and others in ovarian tissues. Thus, a metabolic cost of symbiont carriage would seem highly likely. Nevertheless, no impact on stress traits such as starvation tolerance were observed, in experiments with sufficient power to detect quite small effects. Thus, unusually, this form of protection against natural enemies is low cost despite the presence of bacteria. One interesting possibility is that the microbe, whilst having costs associated with metabolism, has additional, as yet unidentified, physiological benefits that counterbalance these costs.

6 General discussion

The impact of host genotype and symbiont genotype on symbiont transmission and expression of phenotype in symbiosis are well recognised. In comparison, environmental impacts on these key parameters have been less well researched. This is particularly true for protective symbiosis. The primary aim of this thesis was to establish the factors which may contribute to the low-to-intermediate prevalence and persistence of *Spiroplasma* hy1 in its global range. A motivating feature was the initial discovery of *Spiroplasma* strain hy1 in a southern England sampling site at low prevalence (~15%), consistent across two consecutive summers. First, the thesis addressed the effect of ecologically-relevant temperature on symbiont vertical transmission and protection phenotypes, as this is an abiotic factor with strong variability on a variety of timescales. Secondly, the thesis examined whether *Spiroplasma* hy1 was costly to its fly host, assaying passive cost under ideal conditions and under starvation stress, then active cost under ideal conditions. Partly, this was to see if a 'mother's curse' effect (in which costs affect males but not females) existed in this system.

The effect of temperature on the vertical transmission efficiency and hy1's anti-parasitoid protective phenotype were tested. Transmission was found to be relatively robust for both a Mexican fly/hy1 line and a Cambridge fly/hy1 line, with PCR-detected prevalence in 'recovery' condition flies dropping significantly after 2 full rounds of transmission at 15°C, but not at 18°C, when compared to a 25°C control. Meanwhile, phenotype (investigated in the Mexican fly/hy1 line) was less robust to cooler temperatures. The fitness of hy1-infected, wasp-attacked flies at 18°C was no higher than their hy1-uninfected, wasp-attacked counterparts at the same temperature. Additionally, the parasitoid wasp, *L. heterotoma,* appears to have better fitness at 18°C than at 25°C both when fly hosts are infected and when they are uninfected, suggesting a potential host/parasitoid mismatch in optimal temperatures. Considered together, it is likely that hy1 'persists silently' at cooler times of the breeding season and in individuals placed in cooler larval food sources, allowing drift to influence hy1's prevalence.

The hy1/*D. hydei* relationship was tested for the presence of symbiont-associated passive cost at 25°C. A link was found for wing size and infection under 'ideal' conditions, but rather than infected flies being smaller, or only males being smaller under infection (consistent with a mother's curse effect), females were significantly larger when infected with hy1. This indicates that hy1 is not obviously deleterious in the absence of ecological

stress, and may even be slightly beneficial for females. When these flies were placed under the stress of starvation and their survival assayed as another fitness measure, there was no significant effect of infection on time to death. This observation is in stark contrast with the costliness of MSRO, the *Spiroplasma* symbiont that is resident in *D. melanogaster.* Overall, hy1 appears low-cost to the host, at least when wasps are not present.

I now present summaries of each thesis chapter, an outline of interesting issues remaining in this system, and some general questions stemming from this thesis.

6.1 Summary of findings

6.1.1 Chapter 3: Spiroplasma *hy1 has reduced transmission over 2 generations at 15°C but is stable at 18°C*

In this chapter, I first presented evidence that *Spiroplasma* exists in the U.K. at a Tunbridge Wells (south of England) sampling site. *Spiroplasma* has a prevalence of ~15%, which remained the same in two consecutive years with samples taken in late summer/early autumn. Adult flies were kept at 25°C for 2 weeks before DNA extraction and PCR to increase *Spiroplasma* detectability. This hints that the *Spiroplasma* prevalence may be stable at a low frequency in the U.K., or alternatively that ~15% is typical for late summer/early autumn. A multiple-timepoint trapping protocol over the course of one season would elucidate which is the case. Sequencing of the 16S rRNA gene demonstrated that the *Spiroplasma* strain had 100% similarity to hy1, rather than the rare hy2 strain additionally documented in North America. The discovery of hy1 in the U.K. means that it must have been retained throughout the geographical invasion process, like *Wolbachia* and its persistence across *D. melanogaster's* host (Verspoor and Haddrill, 2011). I hypothesise that this could be due to frequent re-introductions of *D. hydei* which could reduce the severity of bottlenecks.

Secondly, I presented data on hy1's transmission in two isolines, a Mexican *D. hydei* line with a natural infection of hy1 (TEN104-106) and a Cambridge *D. hydei* line also carrying a natural hy1 infection (CAM001b). The experimental temperatures investigated were 18°C, 18°C /15°C alternating, and 15°C, compared to a 25°C control. Transmission was carried out at the experimental temperatures, but after being laid as eggs, the flies destined to produce the next generation were kept at their experimental temperature, while the flies destined for PCR assay to determine infection prevalence were raised to adulthood at 25°C (referred to as 'permissive passage'). Two full generations of transmission at focal temperatures were obtained. At the end of the two generations, *Spiroplasma* transmission appears more robust to cool temperatures than suspected from earlier work. Prevalence in the populations was at 100% (or very close to it) for both lines at 25°C, 18°C, and the 18/15°C fluctuating condition. However, the prevalence was significantly lower at 15°C than at the other temperatures, at ~0.78 for the Mexican line and ~0.38 for the Cambridge line. The contrast to previous studies is probably associated with the 'permissive passage' technique, which revealed hy1 infections which were obscured by low, PCR-undetectable titres in (Osaka *et al.*, 2008), though the possibility that fly strain/*Spiroplasma* strain

variation is the reason for the difference cannot be excluded. That prevalence at 18°C /15°C was closer to that seen at 18°C than that seen at 15°C suggests that daytime 'peak' temperature may be more influential than night-time temperature in allowing a symbiont to persist. The ability of 'permissive passage' to boost PCR detectability – likely resulting from an increase in bacterial titre in the host – suggests that those *D. hydei* which overwinter as adults could preserve their infections at very low titre, then restore the titre with the warming seasons before they breed in the summer.

Considering the prevalence and transmission data together, it seems highly likely that there is segregational loss in natural populations, and thus that selection for the symbiont must be ongoing in order to maintain hy1 in *D. hydei* populations in the U.K.

6.1.2 Chapter 4: The phenotype of Spiroplasma *hy1 is vulnerable to temperature, with hy1-infected fly survival at 18°C being indistinguishable from uninfected controls*

I conducted a phenotype experiment in which pupal-to-adult fly fitness – measured as proportion of pupae which emerged as flies – was investigated at 18°C against a 25°C control for Mexican (TEN104-106) flies. Wasp fitness (proportion of pupae emerging as wasps) was also measured. With the aim of determining the stage at which death tended to occur under each condition, pupae which failed to eclose – the 'double death' phenotype – were dissected to see if the contents were recognisable as one species or the other.

While 25°C wasp-attacked hy1-infected flies had measures of survival within the range seen in earlier hy1 phenotype studies, 18°C attacked infected flies performed no better than their uninfected counterparts at the same temperature (significant temperature*infection interaction in the GLM for fly fitness, $p = 0.001063$). Therefore at 18°C, flies are effectively not protected by hy1, and thus protection provides little or no 'drive' to maintain the symbiont during cool seasons (or even cool summers).

Ecological context is likely to be very important in determining the temperature at which flies and larvae are situated, and thus the dynamics of infection. Fly larvae are likely to exist in a thermally patchy landscape, where food sources such as fallen apples or compost heaps vary in temperature due to varying levels of decomposition or exposure to direct sunlight. The result could be a 'selected-for' subset of hy1-infected flies at the optimal temperature, coexisting alongside 'neutral' hy1-infected flies at a cool temperature. Weak

selection alongside gradual segregational loss could partly explain the low prevalence seen in the U.K. The imperfectly-protecting nature of hy1 also leaves room in the *D. hydei* population for other forms of immunity to evolve and cover the vulnerability. Xie et al. found that there appear to be between-*D. hydei*-strain differences in innate immunity with and without hy1. An investigation into whether there is temperature-sensitivity of the innate immunity of these strains would be interesting, as it could be that the optimal ranges of host-mediated and hy1-mediated immunities complement each other.

In contrast to the eclosion results in flies, wasp fitness was reduced by the presence of hy1 infection regardless of the temperature. That the loss of wasps with hy1 infection at 18°C is not being 'converted' into a fitness advantage for hy1 infected flies at 18°C suggests that incomplete rescue is at work at 18°C, i.e. the rescue mechanism hasn't been fully ablated even though hy1's fitness benefit to flies has ceased. It is not possible to glean much about this from 'double-death' pupae, as from all wasp-attacked groups, the majority of pupal contents are not visually recognisable as near-eclosion insects. It is possible that through causing a decrease in wasp fitness, hy1 may still grant an indirect, kin selected fly fitness benefit to relatives feeding in the same patch by decreasing the number of nearby *L. heterotoma* to parasitise them; however, this seems unlikely, as *L. heterotoma* is a generalist and thus its population size probably isn't constrained by *D. hydei*, and the adult is also mobile between fly-feeding patches. Interestingly, in all groups, wasps always perform better at 18°C than at 25°C. Therefore, this system is an example of a host-parasite mismatch in temperature optima, and thus a G x G x E interaction.

6.1.3 Chapter 5: **Spiroplasma** *hy1 is a low-cost symbiont at 25°C*

In many cases, the carriage of many thousands of microbial symbiont individuals within a host imposes a metabolic, and occasionally pathological burden that is reflected in lower survival or fecundity of infected individuals. In chapter 5, I measured two metrics – body size and starvation tolerance - which reflect potential fitness costs of carrying a symbiont. Wing vein IV length (as a proxy for body size and fitness) was measured for a sample of wild flies captured in Tunbridge Wells in 2015, to examine whether infection was sufficiently costly to introduce wing size differences even in a wild population. Secondly, a passive cost experiment was conducted in which Mexican TEN104-106 flies of mixed hy1 infection status were reared under 'ideal', 25°C, common-garden conditions as larvae. After eclosing

and rearing to adulthood, they were placed under the ecological stress of starvation in individual vials, permitting time of death of each fly to be tracked. After death, the flies had their wings removed and measured, and their infection statuses were recovered through DNA extraction and PCR. Time to death by starvation was used as an assay for hy1 cost to fitness under ecological stress, while wing length (which is fixed in early development) was used as an assay for hy1 cost without ecological stress.

For the wild fly data, infection did not contribute significantly to wing size variation, used as a proxy for body size and fitness. The only significant factor was sex ($p = 1.54x10^{-6}$), with females being larger than males, as is expected for most *Drosophila* species including *D. hydei.* In the common garden experiment, sex ($p < 2 \times 10^{-16}$), infection ($p = 0.0168$), and the sex/infection interaction ($p = 0.0361$) were all significant. From examining the graph of the data (Figure 5.4.2), hy1 infection is neutral in males and is associated with increased body size in females. This suggests that under 'good' environmental conditions, hy1 isn't costly to either sex (and thus there isn't a 'mother's curse' effect in males), and may be linked to slightly improved fitness in females (in as far as body size and fecundity are associated). However, existing data does not back up the hypothesis that hy1 increases female fly fitness; in unattacked fly groups in (Xie *et al.*, 2011), larva-to-adult survival and egg-laying didn't differ between infected and uninfected females. An alternative hypothesis is that larger female body size could produce fitness benefits through larger spermathecae, more sperm storage space, and thus greater sperm competition. Sperm competition has already been proposed to be important in *D. hydei*, as females mate multiply with no apparent increase in reproductive output (Markow, 1985; Markow and O'Grady, 2008).

For the starvation tolerance assay, sex was significantly associated with time-until-starvation death ($p = 2.62x10^{-70}$) with females generally living almost two days longer than males. However, neither infection nor the sex/infection interaction was significant. In this case, the sex difference is probably due to a body size difference, with the larger size of infected females not being sufficient to give them a significantly increased lifespan under starvation than uninfected females. The lack of an effect of *Spiroplasma* infection on starvation tolerance in *D. hydei* contrasts with *Spiroplasma* MSRO in *D. melanogaster,* which provides protection against *Leptopilina boulardi* and *L. heterotoma,* and is costly under starvation(Xie *et al.*, 2015). Potential further avenues of investigation include following hy1 titre under starvation, to see if becomes low-cost due to titre suppression following removal of lipid (as seen in (Herren *et al.*, 2014)), and comparing cost of the

Spiroplasma protective symbiont of *D. neotestacea* to see if low-cost is a general feature of protective non-distorter Spiroplasmas.

To investigate active costs of *Spiroplasma* protection, I carried out an experiment measuring reproductive onset in *Spiroplasma* infected, wasp-attacked flies relative to their uninfected and unattacked counterparts. The results were unclear, as although mating behaviour doesn't differ between groups, a statistical model shows that infection, attack, sex, and their interactions are important in modulating the time to offspring production. Most of this seems to be due to a greater variation in time to offspring production in male, hy1-positive flies which experienced wasp attack, although the effect isn't as clear-cut as indicated in earlier informal observations by Xie et al. Ideally, future work should test costs over a wider temperature range, because as transmission and phenotype change with temperature, so too will trade-offs with costs if these manifest.

6.2 Outstanding issues from this system

6.2.1 Unexplored temperature regimes: overwintering, early versus late season studies, and patchiness

The work in this thesis largely focused on daily average temperatures realistic for the U.K. *D. hydei* breeding season, but this is only one aspect of thermal variation which the fly is subjected to. Other topics worthy of further study include hy1 transmission/phenotypic behaviour after exposure to cool overwintering temperatures; early season temperature effects and hy1 prevalence compared to late-season equivalents. In addition, study of how microclimatic 'patchiness' could affect hy1 prevalence in the wild would be worthwhile.

Overwintering effects are understudied in insect-symbiont interactions generally, including in *Drosophila.* Studies thus far include one on segregational loss in diapause, in which the diapause period was artificially long (Perrot-Minnot *et al.*, 1996), and a study finding that *R. insecticola* in aphids shows segregational loss in overwintering eggs (produced by sexual reproduction) but not in asexual summer reproduction (Moran and Dunbar, 2006). Finally, *Wolbachia* was observed to have a fecundity cost in post-diapause *D. melanogaster* in Australia (Olsen *et al.*, 2001). *D. hydei* is suspected to overwinter as an adult in human dwellings in cooler parts of its range (Spencer, 1941), so the species within the U.K. would be subjected to cooling then rewarming within a single adult generation, which subsequently breeds in the spring/summer. Therefore, it would be interesting to investigate whether symbiont loss occurs often in the adult fly in this overwintering time, or whether instead, titre collapses to very low levels and then recovers in time to permit high transmission and expression levels in the larvae.

If overwintering does affect hy1 titre in adult flies, historical effects might come into play in the first post-winter generation of flies. This particularly could be the case for phenotypic expression, which seems a more cold-sensitive phenotype than transmission efficiency. Consequently, with other factors being equal, hy1-infected wild *D. hydei* in the U.K. might be more vulnerable to *L. heterotoma* attack early in the breeding season. As a result, hy1 might be more prone to drift early in the season. Even disregarding the potential for an overwintering effect, temperature changes within a season could alter phenotypic expression and segregational loss levels from one generation to the next. Therefore, a study sampling the full activity period of *D. hydei* in each of several capture sites would give hints as to whether overwintering and within-season temperature variations change

symbiont prevalence. Ideally this would run for multiple years to see whether variance in prevalence is strongest in the cooler times of year, consistent with drift due to a weak phenotype. Unfortunately, one potential issue with this approach is that sample sizes will probably be smaller early in the season, when temperatures are less optimal for the fly anyway.

Another temperature consideration is the 'patchiness' of the thermal environment. *Drosophila hydei* prefers environments such as orchards (Shorrocks, 1972), where it can lay eggs on fallen fruit. Pieces of fruit on the ground can be highly variable in temperature. Therefore, a single piece of fruit can represent its own microclimate. A qualitative study by Feder (Feder, 1997) on fallen fruit temperatures, carried out in the summer in the U.S. when the daytime air temperature in the shade was around 30°C during the sample period, found that fruit in the shade of trees tended not to increase much above air temperature, but that fruit in full sun between orchard rows were often 10°C above air temperature. For some fruit, a temperature excess of 20°C was recorded. Other factors which alter fruit temperature include colour and mass of the fruit. The study did not follow fallen fruit over extended periods of time, so changes may occur with decomposition stage. Within a single piece of fruit temperature varied 3-5°C, and as *Drosophila melanogaster* larvae behaviourally thermoregulate in the laboratory (McKenzie and McKechnie, 1979) there is scope for larvae to partially buffer themselves against the effects of high temperatures. Female *D. melanogaster* avoid ovipositing on sites which are excessively warm at oviposition time (Fogleman, 1979; Schnebel and Grossfield, 1986) but do not avoid fruit that has previously been heated to larvae-lethal 45°C or that has heat-killed larvae on it (Feder *et al.*, 1997). Consequently, even at the same time of year and within the same generation, hy1 may be low-phenotype in larvae in one piece of fruit, and high-phenotype in larvae in a more-sun-exposed piece of fruit.

Finally, the intersection of temperature with cost requires further investigation. This thesis focused on cost only at 25°C to keep experiment sizes manageable. At this temperature, standing costs were not detectable, and active costs manifested following attack were unclear. At cooler temperatures, it is likely that titre will be lower, and this means the symbiont may still not impose a standing cost on *D. hydei*. However, cooler temperatures are not optimal for fast development of *D. hydei*, and so the host's ability to tolerate symbionts without a cost might be impaired as temperatures reduce. Active costs are unlikely to be a factor at reduced temperatures due to resistance failing at 18°C regardless.

6.2.2 The interaction of wasps with the hy1 system at different temperatures in the wild

Questions remain about how *L. heterotoma* interacts with *D. hydei* and hy1 in the wild context. Many wild *L. heterotoma* studies focus on the interaction of the wasp with other *Drosophila* host species, which are generally more abundant than *D. hydei* and thus likely to be more important to maintaining *L. heterotoma* populations. From the perspective of the fly, however, *L. heterotoma* attack is likely to be a significant fitness-limiter across hy1's known range (Fleury *et al.*, 2009).

One important consideration in speculating on the fate of hy1 is whether *D. hydei* larvae with functioning hy1 protection, and *L. heterotoma* adult females, coincide during most of their breeding seasons. *Drosophila hydei* in the U.K. is abundant in June to August (Dyson-Hudson, 1954) and September (F. Jiggins, pers comm), while *L. heterotoma* is active from May to September (Hardy and Godfray, 1990), getting an 'early start' relative to many other frugivorous *Drosophila* parasitoids by overwintering as an adult (Eijs and Van Alphen, 1999). Therefore, the species coincide temporally. However as discussed previously, little is known about whether hy1's phenotype would be active early in the season. If wasp pressure is relatively low early in the season after spring – which is likely, due to heavy *L. heterotoma* losses being reported over winter (Fleury *et al.*, 2009) – it is unlikely that a lack of expression of hy1 would pose an issue for those flies carrying it.

A lot of studies into temperature effects on *L. heterotoma* focus on how temperature modulates its competitive ability against other wasps, mostly against *L. boulardi* which is absent in the U.K., and *Asobara tabida*, which is sympatric with *D. hydei* but may not attack *D. hydei,* or at least was not recorded as such in (Van Alphen and Janssen, 1981). *Spiroplasma* hy1 may not have many wider impacts on the drosophilid parasitoid community in the U.K., but this is hard to determine due to a lack of data. However, it would be interesting to investigate whether hy1 is present, and how prevalent it is, in parasitoid communities where *L. heterotoma* is already under well-documented competitive pressure. For instance, *L. boulardi* outcompetes *L. heterotoma* at warmer temperatures in France south of 45N (Fleury *et al.*, 2004). Although *D. hydei* is not a primary host for *L. heterotoma*, it may serve as a 'refuge' for *L. heterotoma* in areas where the more-specific *L. boulardi* outcompetes it on their shared hosts. This refuge could be

compromised when hy1 is at high prevalence and expressing its phenotype, further limiting the range of *L. heterotoma.*

6.2.3 How might other protection mechanisms, such as host nuclear and host behavioural mechanisms, interact with hy1?

Symbiont-mediated protection is one of a number of defence mechanisms employed by *Drosophila*, and it is worth exploring how the presence of one system may impact on the evolutionary ecology of others. Relatively little is known with respect to endogenous, nuclear-encoded wasp defences, or about potential behavioural defences, in *D. hydei*. Because hy1 provides 'imperfect' protection, suffering from segregational loss at 15°C and phenotype ablation at 18°C, there is scope for other forms of anti-wasp defence to be selected for in this system to cover the protection gaps.

Regarding nuclear defence, *D. hydei* was not seen to melanotically encapsulate any parasitoid eggs in laboratory experiments, – although some melanisation was seen – and non-cellular mediated parasitoid defence seems to be at work. However, the fly line investigated was not tested for the presence of a symbiont (Kacsoh, 2012). In the paper which established a wasp-protective effect for hy1, Xie reported differences by fly strain in endogenous, non-hy1-infected fly resistance to wasp attack, as well as strain differences in the hy1-infected wasp-attacked condition (Xie *et al.*, 2010). This indicates the existence of genetic variance between *D. hydei* strains for wasp protection. In *D. melanogaster,* variation in endogenous fruit fly immunity is well-characterised (Lazzaro *et al.*, 2004; Salazar-Jaramillo *et al.*, 2017). Perhaps endogenous protection is greater in those *D. hydei* populations where low hy1 prevalence or phenotypic expression coincides with *L. heterotoma* activity? A correlative study using recently-established fly lines from known-attack-level locations could provide answers.

Because temperature is important in hy1 phenotypic expression, behavioural thermoregulation is present in insects generally (Heinrich, 2003) and fruit flies have been recorded using thermal cues for other purposes (e.g. *Drosophila melanogaster* avoids ovipositing on overheating food sources, (Fogleman, 1979; Schnebel and Grossfield, 1986)), it raises the question of whether symbiont-carrying *Drosophila hydei* larvae might have evolved the strategy of locomoting towards feeding areas at temperatures which better-suit the symbiont's phenotypic expression, in effect cosseting their partner. Previous work

on fallen fruit suggests that individual fruits only vary from 3-5°C (Feder, 1997). However, it only takes a temperature change within a 7°C to go from hy1-protection to no hy1-protection, so even small-magnitude adjustments could drastically change the fate of a fly larva. Because only a few flies have hy1 and hy1 has a relatively weak phenotype, there may not be sufficient selective pressure to drive the evolution of this trait. A provisional experiment to investigate where hy1-infected *D. hydei* larvae choose warmer environments than hy1-uninfected ones, would be to provide larvae with food on a temperature gradient, and establish if *Spiroplasma* affected oviposition preference. Adding a second group where parasitoid pressure is present might reveal whether this behaviour is chosen in response to external cues (as is considered likely for alcohol selection in *D. melanogaster*; but see (Lynch *et al.*, 2017)). A potential complication might be that all flies choose their optimal temperature, and hy1's optimal temperature is the same as the *D. hydei* optimal temperature, in which case the investigation is moot.

6.3 General perspectives arising from the thesis

6.3.1. How many symbionts are doing what we think they are?

A general issue emerging in this thesis is that the laboratory behaviour of a symbiont does not always match up with that which is observed in an ecological context. It highlights the need to field-verify symbiont phenotypes after they are discovered to be at work in the laboratory, using field cage set-ups, and through adopting greater realism in laboratory experiments, including ecologically relevant temperature variations and possibly situations which can induce symbiont costs. This is particularly important when a phenotype is pre-spread or already at equilibrium, without a selective sweep which is observed as-it-happens or through analysis of collected samples, as was the case in the *Spiroplasma* of *D. neotestacea* (Jaenike *et al.*, 2010).

Field cages can be an excellent set-up in which to examine the realistic behaviour of a symbiont. Cytoplasmic incompatibility-causing and mutualistic symbionts can be more cryptic than straight-forward sex ratio distorters, which if present in significant numbers in the wild should produce a sex ratio bias detectable upon sampling, but field experiments allow an assessment of how symbiont behaviour differs between ecologically realistic conditions compared to 'ideal' laboratory environments. The field/laboratory mismatch was demonstrated by (Hoffmann *et al.*, 1998), who demonstrated both that the fidelity of *Wolbachia* transmission was lower in the field than in the lab in *D. melanogaster,* and that the cytoplasmic incompatibility phenotype was weaker. Additionally, they demonstrated a spread of the symbiont with high larval density (a condition which can induce costs in some systems) which could indicate a previously-unknown benefit of *Wolbachia* infection to the host (Hoffmann *et al.*, 1998). Field cage experiments can then be followed up with laboratory experiments to manipulate the environment on a finer scale and dissect out precise components of the symbiont-host-environment interaction. In some cases, however, it may be best to use an experimental incubator set-up to investigate realistic diurnal and seasonal combinations of temperatures, to discover whether combinations are truly 'unworkable' before investing in larger-scale field cage experiments. This may be the case when symbionts have been artificially introduced into new hosts, or when the economic stakes are particularly high, as with some vector control projects.

6.3.2 How could insects be mixing their defensive strategies?

In this thesis, the emerging picture seems to be that hy1 has a low standing cost, but may only be functioning as a protective mutualist for those flies which both inherit the symbiont (only a low percentage in the U.K.) and also live at the correct temperature as larvae. This leaves a 'protection gap' which could be filled by other forms of protection. Multiple forms of protection may coincide within an organism, including symbiont-mediated, but little is known about how these interact for most systems, or whether different mechanisms evolve in different populations of the same organism.

As an illustrative case, *D. melanogaster* has multiple forms of anti-wasp protection. First, there is nuclear-mediated protection, as the fly exhibits melanotic encapsulation of wasp eggs (Lemaitre and Hoffmann, 2007). Standing genetic variation exists in this trait in the wild (Gerritsma *et al.*, 2013). Secondly, ethanol can protect larvae against parasitoid wasps, which is more effective against *L. heterotoma* than *L. boulardi* (Milan *et al.*, 2012), and female flies will preferentially oviposit on media with ethanol concentrations which are dangerous to wasps but tolerable to flies if adult wasps are present (Kacsoh *et al.*, 2013). Additionally, *D. melanogaster* can also be protected by MSRO (Xie *et al.*, 2013), which is present in Brazil and Uganda (Montenegro *et al.*, 2005; Pool *et al.*, 2006). Studies have found that temperate-environment *Drosophila melanogaster* have higher ethanol-resistance and a greater preference for laying on ethanol, than tropical-environment *D. melanogaster* (Zhu and Fry, 2015). Initially, this appears as if *D. melanogaster* has obtained different additional non-nuclear defence mechanisms in different areas, using MSRO in Afrotropical regions and ethanol in the Holarctic. However, Zhu and Fry note that most *D. melanogaster* ecological studies are from the temperate zone and thus it isn't known whether temperate flies suffer from higher rates of wasp parasitism. Additionally, as *L. heterotoma* is broadly Holarctic and heat-sensitive it is probably complementary in distribution to the symbiont, suggesting that MSRO-mediated protection against *L. heterotoma* may be a shared-derived characteristic rather than the symbiont's main source of drive in *D. melanogaster* populations.

An experiment to test how defence strategies interact, would first require the characterisation of non-symbiont defence systems in *D. hydei,* for instance by producing a high-genetic-resistance line from a diverse lab population under wasp selection. Then symbiont infected flies could be tested for survival against endogenously protected flies, and also against flies with a mix of the two strategies. Characterisation of cost would also be necessary in seeing how likely one strategy would be to outcompete the other in the wild.

6.3.3 Climate change could have unpredictable effects on the spread of facultative mutualists

The effects of global climate change are difficult to predict on the fine geographical scale. If average and extreme temperatures change in the range of *D. hydei*, these may influence transmission fidelity of hy1, and its phenotypic expression. A result could be to cause a northwards shift in occurrence of *D. hydei* and hy1, as is seen for a *Spiroplasma* strain in ladybirds (Pastok, 2015). However, costs of hy1 may instead manifest under heat stress, and in the southernmost parts of hy1's range in *D. hydei*, protective phenotypes may no longer be relevant if high temperatures reduce the range of *L. heterotoma.*

Protective mutualism represents a form of symbiosis which is sensitive to the environment. Consequently, study of the evolutionary ecology of host-mutualist systems requires a deep understanding of the natural context, including how factors such as temperature vary over time. Integrating how the environment acts upon the mutualist's transmission, phenotype and cost, with knowledge of the behaviour of the natural enemy applying selective pressure under these same conditions, helps provide a clearer picture of when a mutualist can persist in the wild.

Appendix

ASG (corn meal) food composition

Ingredient	Quantity per litre
Agar	10 g
Sugar	85 g
Maize meal	60 g
Yeast	20 g
Water	1000 ml
Nipagin, 10% w/v in ethanol	25 ml

SY (sugar yeast) food composition

Ingredient	Quantity per litre
Yeast	100 g
Agar	20 g
Sugar	100 g
Water	1000 ml
Nipagin, 10% w/v in ethanol	30 ml
Propionic acid	3 ml

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