Insect facultative endosymbionts: phenotypic effects and competitive interactions

Eleanor Rose Heyworth

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University of York

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Abstract

Facultative endosymbionts are ubiquitous in insect populations, and can affect a wide range of ecological and life-history traits. Symbiont infections, therefore, have the potential to affect insect responses to natural enemies and climate warming. Aphids are a model for endosymbiont research, and the work in this thesis uses pea aphids to explore symbiont effects and interactions.

We manipulate symbiont infections in clonal genotypes of aphids to investigate the phenotypic effects of microbial infection and expose the insects to a range of stress and fecundity tests. We find that a single species of symbiont (known as X-type) can provide multiple ecological benefits to a host, but that there is a fitness cost to infection. We also discover that symbiont-mediated protection to heat is caused by two species of facultative symbiont protecting the obligate symbiont.

Although many symbiont communities involve multiple species, much previous research has focused on individual infections. We create superinfections of symbionts to explore interactions between the microbes, and how these may affect host effects. We find that infections of two closely related symbionts can lead to loss of superinfections and that the microbes have different responses to competition.

Our work suggests a dynamic, diverse and complex pool of symbiont effects and interactions, and that the symbiont-mediated effects can depend strongly on host and symbiont genotype. As a result, benefits caused by facultative symbionts may vary depending on host population, and determine how vulnerable insect communities are to disturbance and natural enemies. Loss of symbiont infection can also correspond to trait loss in aphid populations. Our work highlights that aphids are an ideal system for studying insect symbiosis and a simple model for more complex free-living or symbiotic microbial communities.

Table of Contents

List of Figures

List of Tables

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Author's Declaration

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Chapter 1

General Introduction:

Endosymbiosis and insects

Introduction to endosymbiosis

Origins of endosymbiosis

Endosymbiosis is defined as a long-term relationship between two organisms where one lives inside the other (Brownlie and Johnson 2009; Feldhaar 2011), and it has been a vital part of evolutionary history. Although the advent of genomic techniques is only now exposing how common, widespread and important endosymbiotic partnerships are, they have had an unimaginable effect on the current diversity of life. Both mitochondria and chloroplasts are thought to be derived from ancient endosymbiotic events where co-existence between a cellular host and symbiont persisted due to metabolic advantages (Whatley et al. 1979; Martin and Russell 2003). But although both of these events probably happened just once (Dyall et al. 2004), endosymbiotic relationships today are abundant and can vary greatly in terms of evolutionary history, functional mechanism and ecological impact.

One of the most rapidly expanding fields in endosymbiosis is microbe-insect infections. Insects are found in ecosystems worldwide (Samways 1993; Hansen and Moran 2011) with their spread facilitated, at least in part, by microbial endosymbionts. The vast majority of insect species are infected with at least one species of symbiotic microbe in close, long-term and stable relationships which can affect various aspects of host ecology (Hilgenboecker et al. 2008; Moran et al. 2008; Feldhaar 2011). The dominance of insects and their ecological roles are a vital part of the wider ecosystem, and the interactions between host and microbe affect these interactions. The organism and all of its associated microbes is known as a 'holobiont' and is the unit of selection that evolution works upon (Rosenberg et al. 2010). When studying insect ecology, it is important to appreciate how the different organisms that comprise the holobiont interact, and understanding the dynamics of insect-symbiont interactions is vital to understanding insect ecology.

Types of insect endosymbiont

Insect endosymbionts can be roughly divided into two categories: obligate and facultative. The best studied are the obligate symbionts. These microbes are long-term intracellular associates of their hosts and are required by the insects for reproduction or survival (Moran et al. 2008). In many cases they are highly-specialized metabolic partners, allowing their insect hosts to survive on nutritionally unbalanced diets such as plant sap, wood or blood (Zientz et al. 2004) while residing in specialist cells known as 'bacteriocytes'. These long-term infections have in many cases lasted for millions of years (Dale and Moran 2006) and the combination of vertical transmission and obligate intracellular lifestyles has led to high levels of genome degradation (Wilcox et al. 2003; Zientz et al. 2004). In fact, obligate insect endosymbionts have the smallest genomes discovered (Wernegreen 2002; Moran 2007), coding mainly for essential pathways and lacking genes found in their free-living relatives (Moran 1996). As they are transmitted vertically, these microbes pass through a bottleneck at each generation, leading to homogeneous populations that can only survive in the protective confines of their insect host (Moran 1996; Baumann et al. 1997).

The second type of insect endosymbiont is found in non-obligate, facultative infections. They differ from the obligate symbionts in several key ways. Primarily, they are not required by the host for survival, and are found at varying frequencies in insect populations (Hilgenboecker et al. 2008; Oliver et al. 2010; Sirviö and Pamilo 2010; Toju and Fukatsu 2011; Kopecky et al. 2013). Although many are still unable to survive outside of a host, they are not restricted to vertical transmission and can be spread horizontally between insect lineages (Dale and Moran 2006). This leads to less specialized relationships between host and symbiont (Oliver et al. 2010), and as the insect is not reliant on these bacteria for survival or reproduction, the microbe must enable their own transmission through different means. As a result, facultative symbionts can also be categorized into two main, occasionally overlapping groups: reproductive manipulators and beneficial mutualists (Duron et al. 2008; Hilgenboecker et al. 2008; Oliver et al. 2010; Feldhaar 2011; Kaltenpoth and Engl 2014).

Parasitic reproductive manipulators promote their own vertical transmission by altering the reproductive mechanisms of their hosts, and one common example is *Wolbachia* (Hiroki et al. 2004; Duron et al. 2008; Hilgenboecker et al. 2008). Mechanisms of reproductive disruption include inducing parthenogenesis, feminization, male killing or cytoplasmic incompatibility (CI), where infected females only produce healthy offspring if they mate with a similarly infected male (Hiroki et al. 2004; Werren et al. 2008). These manipulations promote the rapid increase of the microbe in host populations, but can also alter sex ratios and affect community ecology.

Beneficial mutualists have a very different way of ensuring their spread. Instead of hijacking reproductive mechanisms, these facultative endosymbionts raise the fitness of their hosts (Oliver et al. 2010; Feldhaar 2011). As they are predominantly spread vertically, a fit host is more likely to outlive and outcompete uninfected insects, producing more infected offspring. Their effects can range from facilitating new host plant shifts (Henry et al. 2013), to protection against natural enemies (Brownlie and Johnson 2009; Cockburn et al. 2013; Kaltenpoth and Engl 2014) and abiotic stresses (Montllor et al. 2002; Koga et al. 2003). It is even possible for insects to attain resistance against insecticides through symbiont infection (Kikuchi et al. 2012). The different species of potential facultative symbionts can act almost as a horizontal gene pool for their insect hosts, allowing the insects to acquire whole metabolic pathways in one event (Kikuchi et al. 2012; Clay 2014). Found at varying frequencies in populations, these facultative symbionts can be vital to the success of their insect hosts.

The phenotypic effects of endosymbiosis

Studies across many different insect taxa are beginning to uncover the wide diversity of effects conferred by beneficial facultative symbionts, and to explore the mechanisms behind their ecological phenotypes. In several insect species symbionts can increase resistance to natural enemies, including parasitoid wasps (Oliver et al. 2005, 2014; Hurst and Hutchence 2010; Xie et al. 2010), fungi (Kaltenpoth et al. 2005; Scarborough et al. 2005; Cardoza et al. 2006; Six 2013,Łukasik et al. 2013b) and insect

pathogens (Haine 2008; Hedges et al. 2008; Jaenike et al. 2010; Bian et al. 2013; Kaltenpoth and Engl 2014) as well as climate stresses (Montllor et al. 2002; Neelakanta et al. 2010; Brumin et al. 2011). In population level assays, frequencies of insects infected with protective symbionts increase when there is high selection pressure for their use (Oliver et al. 2008; Kikuchi et al. 2012), illustrating how these traits can spread through and dominate populations depending on natural enemy frequencies. As well as protection, facultative symbionts can affect insect spread and dispersal by affecting resource use. Symbionts can affect insect ability to survive on different host plants (Douglas 2009; Tsuchida et al. 2011) and have facilitated invasions in some populations (Brown et al. 2013; Ferrater et al. 2013; Henry et al. 2013).

More recent research has exploited the phenotypes conferred by symbionts in terms of insect survival, reproduction or behaviour (Hosokawa et al. 2008; Dion et al. 2011) and employed them in the more applied areas of crop protection and even human health. Manipulating either natural or artificial symbiotic relationships has implications in curtailing insect spread of horizontally transmitted pathogens by targeting hosts that vector diseases (Dale and Welburn 2001; Walker et al. 2011; Wang et al. 2012; Hughes et al. 2014). The interactions between the symbiont and pathogen have been shown to decrease vector transmission efficiency, making symbiotic relationships relevant not only in terms of insect ecology but in these more applied fields as well. Symbionts have also been shown to protect economically important insects, such as bees, from pathogens (Koch and Schmid-Hempel 2012) and may affect insect responses to climate change (Wernegreen 2012). Investigating the phenotypic effects and traits conferred by symbionts will be vital in understanding and manipulating insect ecology in future.

Mechanisms behind the phenotypes

Although research is continually uncovering new phenotypes conferred by endosymbionts, little is yet known about many of the mechanisms behind these traits. Much of the current evidence has focused on the most well studied protective symbionts, such as the parasitoid protection conferred by *Hamiltonella defensa*, found in the model system of the pea aphid. In this case the symbiont carries a phage on its

genome (APSE, for *Acyrthosiphon pisum* secondary endosymbiont) which secretes toxins that may target parasitoid nutritional cells (Oliver et al. 2009; Nyabuga et al. 2010). Loss of this phage results in loss of protection in aphid individuals (Weldon et al. 2013). Little is known of parasitoid protection mechanisms in other aphid symbionts (von Burg et al. 2008; Hansen et al. 2012) but in other insects resistance to pathogens, parasitoids or temperature extremes may be conferred through 'priming' of the immune system from beneficial symbionts (Evans and Lopez 2004; de Souza et al. 2009; Brumin et al. 2011; Kuechler et al. 2013), comparable to an inoculation. Resistance to microbial pathogens and viruses may also be due to competition for resources in the insect host (Bian et al. 2013; Caragata et al. 2013). To fully understand and employ facultative symbionts in future requires much more investigation of the mechanisms behind symbiont infection. In particular, knowledge about which phenotypes are broad-spectrum, conferred by multiple species of symbiont and which are specific to microbial strain will lead to greater understanding about the evolution and ecology of these ubiquitous relationships.

Establishment of symbiont infections

Facultative endosymbionts can be gained in several ways by their insect hosts. They may be acquired from the environment regularly (Kikuchi et al. 2012), but are generally transmitted vertically, either through close contact with parents (Kaltenpoth et al. 2005, 2012) or through the germline (Oliver et al. 2010; Vorburger 2014). Horizontal transmission of many facultative symbionts occurs less commonly (Russell et al. 2003; Anderson et al. 2012) and symbionts must successfully establish in a novel lineage, avoiding or tolerating any immune response (Kaltenpoth and Engl 2014; Sicard et al. 2014). Potential hosts already infected with symbionts complicate the invasion (Vautrin and Vavre 2009), as the symbiont must contend with competition (Oliver et al. 2006) and potentially heightened host immunity (Evans and Lopez 2004; Laughton et al. 2013).

Multiple endosymbiont infections

The number of insects being scanned for symbionts is increasing, and correspondingly the number of known symbiont species is growing rapidly. As molecular techniques allow for more accurate identification and quantification of microbial populations, the prevalence of multiple species of coexisting facultative symbionts within a single host individual is also becoming apparent. Communities of multiple species of bacteria are ubiquitous in nature, and best studied in certain environments such as biofilms (Yang et al. 2011) and general microbiomes (Martin et al. 2007; Turnbaugh et al. 2007). Insect-associated communities are more specific combinations of microbes found in insect guts, hemolymph, secondary bacteriocytes or other cells known as sheath cells (Montllor et al. 2002; Cloutier and Douglas 2003; Sakurai et al. 2005). It is often unknown how long double infections of symbionts have coexisted, but the combinations of facultative symbionts raise overarching questions about how different species in the same host coexist and spread vertically together.

Theoretically, the maintenance of multiple strains or species of different symbionts inside a single insect is unlikely. In many species, vertically transmitted symbionts are passed through the oocyte and there is space for relatively few cells (Mira and Moran 2002). Both symbionts must be at high enough densities to be transferred to the next generation together through a bottleneck (Mouton et al. 2003; Vautrin and Vavre 2009). In cases of beneficial facultative symbionts, the cost of hosting two different species must not be high enough to devastate the fitness of the host through depletion of its own resources through high densities of infection or antagonistic interactions between the bacterial species (Kondo et al. 2005). In this case, it would be advantageous for beneficial symbionts in double infections to provide complementary or increased benefits above and beyond either single infection, control their proliferation so as not to affect the host, or be found only as transient infections which cannot be maintained (Vautrin and Vavre 2009).

One of the few scenarios which actively selects for multiple infections is the mechanism of symbiont-mediated cytoplasmic incompatibility (CI) (Vautrin et al. 2008). In this reproductive manipulation, mating between infected males and females

only produces viable offspring if the female has the same infection as the male. This system favours infections of multiple endosymbionts in females as it increases the probability of a compatible match with a higher proportion of males (Vautrin et al. 2008). Experimentally, super-infections of multiple strains of the reproductive manipulator *Wolbachia* strains spread rapidly through insect populations affected by endosymbiont-mediated CI, a strong ecological benefit to the microbe (Sinkins et al. 1995; Kittayapong et al. 2002). Yet multiple infections of symbionts are common even when CI is not a factor in the maintenance of both species.

In such cases of symbiont 'superinfections', questions arise about the dynamics and potential for interaction between the different species. In such a confined area, where competition for space and resources is high, the endosymbionts are likely to come into contact. Free-living bacteria are known to interact with their own and other species through quorum sensing, where the bacteria continually secrete chemical compounds. They are able to measure the concentrations of these compounds in the surrounding environment, and so can detect and react to the density of nearby bacterial cells (Crespi 2001). Vertically transmitted symbiosis is a prime system for adaptive quorum sensing, as it is one of the few situations where the ecological fitness of one species directly impacts another (Keller and Surette 2006). In fact, at least one symbiont species, *Sodalis glossinidius*, has genes linked to quorum sensing (Pontes et al. 2008). This symbiont produces proteins that upregulate the insect oxidative stress response, meaning that infection with the symbiont increases host tolerance to stress, but also that the bacteria may be able to detect and respond to other bacterial species (Pontes et al. 2008). Indeed, it may be beneficial if different species were at least aware of each other's presence in order to avoid overexploitation of the host resulting in a 'tragedy of the commons' (Hardin 1968), where different organisms overexploit and deplete common resources at a detriment to all.

Because of CI actively selecting for multiple infections, much of the experimental literature on the topic features the reproductive manipulator *Wolbachia*. In one case, bean beetles infected with two different *Wolbachia* strains had a lower overall endosymbiont density than in the single infections of either (Kondo et al. 2005). This suggests that the bacteria either suppress each other, control their own proliferation

when a competitor is present (Kondo et al. 2005), or host regulation of bacterial densities (Login et al. 2011; Kim et al. 2013). But there are also studies which have found that overall microbial densities are higher in multiple infections, including with double strains of *Wolbachia* in wasps (Mouton et al. 2003) and moths (Ikeda et al. 2003) and endosymbionts in ants (Sirviö and Pamilo 2010). In each of these cases, the density of each strain did not vary whether it was in a single or double infection, suggesting independent regulation of the bacteria. This superinfection can cost the insects – in a parasitoid study, the hosts weighed less and died earlier corresponding with an increase in the diversity of its endosymbionts (Mouton et al. 2004).

When two completely different species of symbiont are involved, however, there is some evidence for bacterial interactions. In a study using *Drosophila* infected with *Wolbachia* and another common endosymbiont, *Spiroplasma*, there was an asymmetrical antagonistic interaction. The density of *Wolbachia* was negatively affected by the competitor while *Spiroplasma* densities were unchanged (Goto et al. 2006). There is little other evidence about interactions between multiple species of symbionts, yet it is likely that complex community interactions between different species of facultative symbiont can affect insect ecology and distribution of symbiont-conferred traits.

Aphid endosymbiosis

This thesis aims to explore overarching questions about symbiont-conferred phenotypes, the mechanisms behind them and the dynamics of symbiont interactions in multiple infections. The pea aphid is an ideal model system for this study.

Aphid ecology

To study endosymbiotic infections and interactions, aphids are commonly used as a model system. These small, phytophagous insects are ubiquitous in temperate regions worldwide with over 4000 species described so far (Dixon et al. 1987). They show strong host plant specificity (Ferrari et al. 2008; Gauthier et al. 2015) and are commonly found in crop fields, gardens and greenhouses. As a result, aphids are agriculturally important crop pests and vector nearly half of insect-borne plant

pathogens (Hogenhout et al. 2008; Dedryver et al. 2010) as well forming the basis of food webs as the diet of birds and many other species of insects.

Pea aphids (*Acyrthosiphon pisum*) are ideal as a scientific model system due to their cyclical parthenogenesis and rapid generation times. In controlled long-day light conditions, aphids are asexual. A single female, through telescopic parthenogenesis, can produce over a dozen genetically identical offspring daily, leading to the rapid population growth that characterises aphid field populations. In an experimental setting, large, genetically-identical populations can be created rapidly and aphid lines maintained in stock cultures indefinitely. This, adding to their ease of keep and the ability of nearly all pea aphid strains to thrive on *Vicia faba* (broad bean) plants (Ferrari et al. 2008), makes the pea aphid a common and useful scientific study system.

Pea aphid symbiosis

Aphids are fully phytophagous, feeding on plant phloem for their entire lives. This unbalanced diet is high in carbohydrates but low in essential amino acids (Douglas 1998; Akman Gündüz and Douglas 2009; Hansen and Moran 2011) and nearly all aphids rely on their obligate nutritional symbiont *Buchnera aphidicola* to survive. *Buchnera* has coevolved with aphids for ~180 million years (Shigenobu et al. 2000) showing strict vertical transmission throughout (Baumann et al. 1997). As a result, it has a much reduced genome (Fares et al. 2002; Gómez-Valero et al. 2007), one of the smallest yet sequenced, with less than 15% of the metabolic genes found in the close free-living relative *Escherichia coli* (Pérez-Brocal et al. 2006). The intricate metabolic partnership between host and *Buchnera* has allowed aphids to utilize one of the most widespread but nutritionally unbalanced diets available, enabling their spread.

But alongside *Buchnera*, pea aphids can also be infected with up to eight known facultative symbionts. These comprise of five gamma proteo-bacteria – *Hamiltonella defensa, Serratia symbiotica, Regiella insecticola,* a species currently known as X-type and *Rickettsiella viridis*, alongside a *Spiroplasma*, a *Rickettsia* and potentially *Wolbachia* (Oliver et al. 2010; Gauthier et al. 2015). As pea aphids are a commonly used insect for endosymbiont studies, the range of traits conferred by facultative symbiont infection is relatively well known, but biased towards certain species. The

infection phenotypes of the various symbionts are characterised to varying extents in previous studies, generally through the use of fitness assays under different experimental conditions (Ferrari and Vavre 2011).

The aphid model is valuable in that genetically identical lines of aphid genotypes can be kept indefinitely under lab conditions, and symbionts can be relatively easily manipulated to be both introduced and removed stably from the lines. This allows for testing of symbiont-mediated effects in a common genetic background and has formed the basis for much of the aphid endosymbiosis literature.

Effects of facultative symbionts

In most cases, one species of symbiont is linked with a single ecological benefit which is then explored in detail. One of the best examples of this is *Hamiltonella defensa*. *Hamiltonella* is known for providing resistance to parasitoid wasps through a phage (APSE) carried on its genome which targets parasitoid egg nutritional cells using specific toxins (Oliver et al. 2003, 2005, 2009; Moran et al. 2005; Degnan and Moran 2008). The protected insect is able to survive the attack and produce offspring, spreading the symbiont vertically into the next generation and benefitting both partners of the symbiosis (Oliver et al. 2003). Once this effect was discovered, routes opened into looking at how this valuable protection affects other aspects of the parasitoid/aphid/symbiont relationship. Aphids infected with *Hamiltonella* are less likely to exhibit evasive tactics against the wasps (Dion et al. 2011) and release less alarm pheromone (Oliver et al. 2012). The wasps themselves are more likely to superparasitise infected aphids to overcome their symbiont-mediated defence (Oliver et al. 2012). Despite this protection, levels of *Hamiltonella* are generally not fixed in aphid populations (Ferrari et al. 2012; Russell et al. 2013) and fitness trade-offs to infection have been found in some cases (Simon et al. 2011; Vorburger and Gouskov 2011) but not others (Oliver et al. 2008; Cayetano et al. 2015).

Other benefits have been found. *Regiella* is known for providing fungus resistance (Scarborough et al. 2005) and *Serratia* for protecting against heat stress (Montllor et al. 2002; Koga et al. 2003). In reality, these beneficial effects are not as specific as they appear. Fungus resistance can also be provided by *Rickettsia*, *Rickettsiella* and

Spiroplasma (Łukasik et al. 2013b), parasitoid protection by some strains of *Serratia* and *Regiella* (Oliver et al. 2006; Hansen et al. 2012) and heat protection by *Hamiltonella* (Russell and Moran 2006). It is likely that at least some of these effects are general effects of infection, and others are more specific to strain, and even host genotype. The mechanisms behind heat shock protection and fungus resistance are largely unknown.

Multiple endosymbiont infections in aphids

Facultative symbionts are usually found at intermediate levels in aphid populations, (Sakurai et al. 2005; Koga et al. 2007; Ferrari et al. 2012; Russell et al. 2013). Double and even triple infections are relatively common, with up to 25% of all infections containing more than one facultative symbiont (Frantz et al. 2009; Ferrari et al. 2012; Russell et al. 2013), although the frequencies of multiple infections vary geographically and temporally (Smith et al. 2015). Multiple infections among pea aphid facultative endosymbionts could be maintained through complementary or increased benefits to the host or through reproductive manipulation during annual sexual reproduction, where a second symbiont follows and spreads along with the manipulator (Vautrin and Vavre 2009). *Rickettsia* and *Spiroplasma* are known to manipulate reproduction in multiple insect species (Kageyama et al. 2012) and *Spiroplasma* is known to kill males in aphids (Simon et al. 2011), but the aphids' predominantly asexual reproduction makes this trait less ecologically relevant throughout most of the year.

Correspondingly, some combinations of facultative symbionts are more common in aphids than others. One study found that just three combinations represented 90% of double infections, two of which contained *Spiroplasma* and one *Rickettsia* (Frantz et al. 2009), *Regiella-Spiroplasma*, *Hamiltonella-Spiroplasma* and *Serratia-Rickettsia*. Another corroborated this, with *Serratia-Rickettsia* combinations representing over half surveyed (Simon et al. 2003). No increased benefits have been observed with this combination in manipulated aphids (Montllor et al. 2002). If this is the case, the high frequencies of these combinations could be the result of low relatedness between the symbionts, reducing competition for space and resources. Consistently, combinations of multiple infections of the more closely-related gamma proteo-bacteria, specifically

Hamiltonella-Regiella and *Regiella*-X-type, appear less frequently than expected (Ferrari et al. 2012; Russell et al. 2013). The phylogenetic relationships between the aphid symbionts are yet to be fully understood, but *Regiella* and *Hamiltonella* are closely related (Hosokawa et al. 2006) and preliminary exploration of the X-type genome indicates it is a sister species to *Regiella* (Degnan et al. 2010, A. McLean, C. Godfray, J. Ferrari, pers. comm.).

Aphids are split into plant-specific biotypes (Ferrari et al. 2012) that are often linked to symbiont infection. As a result, it is likely that the effects of aphid biotype and host plant may be the most important factors in symbiont frequencies in the aphid system (Leonardo and Muiru 2003; Ferrari et al. 2004).

There have been studies suggesting that facultative symbiont interactions can potentially increase benefits to the pea aphid host. One study using a natural *Hamiltonella-Spiroplasma* superinfection found that the aphids had higher resistance to wasps than *Hamiltonella* alone (Cloutier and Douglas 2003) and a natural X-type-*Hamiltonella* infection maintained high wasp resistance under heat shock (Guay et al. 2009). In the cases of these natural infections, it can be difficult to extract symbiont-driven traits from phenotypes caused by the aphid genetic line itself, but there have been similar results with manipulated clones using injected aphid lines. Oliver *et al.* 2006, created a *Hamiltonella-Serratia* double infection and found, alongside a significant reduction in fecundity, a higher resistance to wasps than *Hamiltonella* alone. This could be due either to synergistic effects or to the noticed increase in the density of Serratia in double infections (Oliver et al. 2006). This leads to additional questions about how and whether aphid-associated bacteria can communicate and respond to each other inside an insect host.

As well as the facultative symbionts, every aphid is infected with the obligate symbiont *Buchnera* (Moran 2008). With its highly reduced genome and intracellular location, *Buchnera* is a poor candidate for interactions with non-obligate symbionts, but they do occur. It appears that certain facultative bacteria repress the density of *Buchnera*, either due to competition between the two species, or the facultative symbiont reducing the fitness of the aphid. This has been seen in aphids infected with *Rickettsia*

(Sakurai et al. 2005) and *Serratia* (Lamelas et al. 2011). *Cinara cedri*, the conifer aphid, is infected with the of *Buchnera* species with the smallest genome (Pérez-Brocal et al. 2006). In this species, *Buchnera* and *Serratia* appear to form a 'metabolic collaboration' where they coexist in order to provide the aphid with essential nutrients that *Buchnera* itself can no longer provide (Lamelas et al. 2011). But one study found that *Serratia* actually outcompetes *Buchnera* for space when both are present and is housed in its own bacteriocytes in *Cinara cedri* aphids (Gómez-Valero et al. 2004). It appears as though in these aphids, the coexistence of the two endosymbionts may be an evolutionary temporary measure as *Serratia* gradually becomes the primary symbiont.

Purpose of thesis

The remainder of this thesis sets out to explore facultative symbiont dynamics of the pea aphid. My initial aim was to explore the interactions between non-obligate symbionts in multiple infections, yet background work was necessary first to characterise one of the relatively newly-discovered symbionts that I used. As a result, Chapter 2 is a characterisation of the facultative pea aphid symbiont X-type (also known as PAXS). My assays explored whether X-type, like other aphid facultative symbionts (Oliver et al. 2010) can provide protection against natural enemies and abiotic stresses. Testing one symbiont for several different traits aimed to investigate the range of effects a single strain or species of symbiont can confer while providing phenotypic information useful for later experiments.

Using aphid lines artificially cured of X-type infection to detect symbiont effects across identical aphid genetic backgrounds, I exposed aphids to a suite of stress and fitness tests. I tested aphids for resistance to parasitoid wasps, pathogenic fungi and heat stress, and for fitness costs on the universal host *Vicia faba* and the field hosts *Trifolium pratense* and *Medicago sativa.* This is one of the first studies to explore the beneficial traits that X-type gives to its aphid host, and one of the first to test a single species of aphid symbiont for an extensive range of potential traits. I found that infection with X-type (in coinfections with another symbiont, *Spiroplasma*) can increase aphid resistance to heat, pathogenic fungi and parasitoid wasps, yet imposes

a strong fecundity cost on its host. This range of effects makes X-type one of the only pea aphid symbionts to be implicated in such a broad spectrum of beneficial effects. The characterisation of X-type was necessary to fully understand the symbiont before artificially creating double infections, and led to questions about the mechanisms behind these phenotypes.

In chapter 3 I report on a follow-up experiment to the previous chapter. Previous to my work, the only study looking at X-type effects found that in natural double infections with *Hamiltonella defensa*, aphids benefitted from an increased resistance to parasitoid wasps, which was maintained under heat stress (Guay et al. 2009). As X-type is one of the few symbionts where it is known that a single strain can give diverse benefits, including to both biotic and abiotic stresses, I tested the hypothesis that single infections of X-type can also provide increased resistance to multiple stresses simultaneously and investigate the dynamics between the different types of resistance. Exposing X-type infected aphids to heat stress either the day before or the day after attack by a parasitoid wasp, this chapter attempted to explore how resistance to this parasitoid can be affected by different stresses and tested the resistance in a more stressful and ecologically-relevant environment. I found complex costs of X-type infection and no evidence that the symbiont can maintain parasitoid resistance under heat stress.

In chapter 4 I aimed to investigate the mechanism behind one of the phenotypes of infection found in Chapter 2, the ability of X-type to recover the reproduction of heat shocked aphids. Using aphids infected with X-type, *Regiella insecticola* or *Hamiltonella defensa*, I conducted a further heat shock experiment, measuring the densities of the facultative symbionts and the obligate symbiont *Buchnera* under heat stress. I found that heat shock killed *Buchnera* cells, corroborating other studies (Wilcox et al. 2003; Chen et al. 2009) but that infection with X-type or *Regiella* led to increased population densities of the obligate symbiont under heat, corresponding to an increased number of offspring produced. I found no effect of *Hamiltonella* on temperature protection. This partly contrasts with a previous study which found no effect of *Regiella* on fecundity after heat shock (Russell and Moran 2006) but also no effect of *Hamiltonella*

(Russell and Moran 2006). This implies a strain-specific effect, but a general phenotype that is common across different symbiont species.

Double infections of symbionts are common in pea aphid populations (Ferrari et al. 2012; Russell et al. 2013) yet little is known about the interactions between the microbial partners, and the dynamics of infection. In chapter 5 I explored the competitive dynamics of two facultative symbionts, X-type and *Regiella insecticola*, by creating novel double infections in different aphid lines and measuring population densities using qPCR. Although I expected this infection to persist, I found the complete and consistent loss of the double infection due to competition between the symbionts. I measured the establishment success of the horizontal transfers, the maintenance of the resulting double infections and the titres of both facultative symbionts through several generations. The constant loss of the infection could be a mechanism for loss of symbiont-conferred traits in field populations of aphids and raises further questions about communication and competition between extracellular endosymbionts.

My final chapter is a discussion about the results presented in this thesis, and how they contribute to our understanding of endosymbiosis in aphids and other insects. It draws together the connections between the different chapters with regards to facultative and obligate symbiont interactions, and how they may explain ecological phenotypes. It reflects on how the importance of symbiosis and the traits conferred by facultative symbionts affect aphid populations and outlines the next steps in exploring these interactions. It explores how the results found in this thesis may be applicable to systems outside of the aphid sphere, and how they may need to be taken into account when considering insect ecology in future.

Chapter 2

A facultative endosymbiont in aphids can provide diverse ecological benefits

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Abstract

Ecologically important traits of insects are often affected by facultative bacterial endosymbionts. This is best studied in the pea aphid *Acyrthosiphon pisum*, which is frequently infected by one or more of eight facultative symbiont species. Many of these symbiont species have been shown to provide one ecological benefit, but we have little understanding of the range of effects that a single strain can have. Here, we describe the phenotypes conferred by three strains of the recently discovered bacterium known as X-type (Enterobacteriaceae), each in their original aphid genotype which also carries a *Spiroplasma* symbiont. All comparisons are made between aphids at that are coinfected with *Spiroplasma* and X-type and aphids of the same genotype that harbour only *Spiroplasma*. We show that in all cases, infection with X-type protects aphids from the lethal fungal pathogen *Pandora neoaphidis*, and in two cases resistance to the parasitoid *Aphidius ervi* also increases. X-type can additionally affect aphid stress responses – the presence of X-type increased reproduction after the aphids were heat stressed. Two of the three strains of X-type are able to provide all of these benefits. Under benign conditions the aphids tended to suffer from reduced fecundity when harbouring X-type, a mechanism that might maintain intermediate frequencies in field populations. These findings highlight that a single strain of a facultative endosymbiont has the potential to provide diverse benefits to its aphid host.

Introduction

Symbiosis between animals and microbes is remarkably common, and in the last decade it has emerged that most insect species are infected by bacterial endosymbionts (Hilgenboecker et al. 2008; Duron and Hurst 2013). For the majority of species the effect on the host is unknown but it is clear that the symbionts can be divided into three overlapping functional groups (Moran et al. 2008). Primary or obligate symbionts tend to occur in insects feeding on unbalanced diets and supply the hosts with nutrients that are deficient in the diet (Douglas 1998). Facultative symbionts can be divided into two groups: reproductive manipulators that affect the hosts' reproduction in order to maximize their own transmission and mutualists that can affect a wide range of life-history and ecologically important traits (Oliver et al. 2010; Feldhaar 2011; Ferrari and Vavre 2011).

The symbionts are typically transmitted from mother to offspring, although horizontal transfer occurs at lower frequencies (Moran and Dunbar 2006; Gehrer and Vorburger 2012; Ahmed et al. 2013; Henry et al. 2013). The predominantly vertical transmission suggests that host and symbiont share their fate and mutualistic relationships are likely to evolve (Lively et al. 2005). Indeed, facultative symbionts confer a wide range of benefits to their hosts, for example protection from natural enemies (Oliver et al. 2003; Scarborough et al. 2005; Teixeira et al. 2008; Xie et al. 2010), protection from extreme temperatures (Montllor et al. 2002; Neelakanta et al. 2010) and the ability to use a greater diversity of resources (Tsuchida et al. 2004; Hansen and Moran 2014). In some cases horizontal transfer of the symbionts between insects can lead to the instant acquisition of a beneficial ecological trait (Tsuchida et al. 2011; Kikuchi et al. 2012), and the symbiont may rapidly spread through the population (Ferrari and Vavre 2011; Himler et al. 2011; Cockburn et al. 2013).

Aphids and specifically the pea aphid, *Acyrthosiphon pisum*, are among the best studied insect-symbiont systems. The pea aphid alone is known to host at least eight different facultative symbiont species (Sandström et al. 2001; Tsuchida et al. 2010; Russell et al. 2013), with typically zero to four species within the same aphid individual (Ferrari et al. 2012; Henry et al. 2013; Russell et al. 2013). Several of these species are

well known for one particular ecological effect. For example *Hamiltonella defensa* carries a lysogenic bacteriophage that protects the aphid from the parasitoid wasp *Aphidius ervi* (Oliver et al. 2003, 2009; Degnan and Moran 2008), whereas *Regiella insecticola* is known for providing resistance to a fungal pathogen (Scarborough et al. 2005), a benefit that other species of endosymbionts, including *Spiroplasma*, have also been shown to confer (Łukasik et al. 2013b).

However, at present little is known about the breadth of ecological effects that a single strain of symbiont can have, predominantly because experiments on different traits tend to be performed by different research groups. Exceptions are a strain of *H. defensa* and a strain of *Serratia symbiotica* that can both improve resistance to a parasitoid and increase tolerance to heat shock albeit to different degrees (Oliver et al. 2003; Russell and Moran 2006). Understanding the diversity of effects that a single genotype can have is important for explaining the distribution of symbionts in insect populations. A possible explanation for the typically intermediate frequencies is that different symbionts provide different benefits, and are therefore selected for in divergent ecological scenarios (Oliver et al. 2014). To test this hypothesis a more complete characterization of single host-symbiont combinations, as well as of the variation within symbiont species is needed.

An alternative explanation for variable frequencies is that carrying a symbiont comes at a cost. Evidence for such costs is extremely variable between studies, with some documenting no costs under benign conditions and others a reduction in fitness of about 10-20% compared to uninfected hosts (Sakurai et al. 2005; Simon et al. 2007; Chandler et al. 2008). In particular, many but not all *H. defensa* strains cause a reduction in longevity and therefore also lifetime fecundity (Simon et al. 2011; Vorburger and Gouskov 2011; Tsuchida et al. 2013).

Here, we employ the recently discovered symbiont known as X-type or PAXS (pea aphid X-type symbiont) (Enterobacteriaceae)(Guay et al. 2009) and provide a representative overview of its effects on the aphid phenotype, namely resistance to two natural enemies from two different kingdoms, resistance to heat shock and fitness under benign conditions. X-type is found commonly in field populations, with

prevalence of up to 45% (Ferrari et al. 2012; Russell et al. 2013) and has also been found in other aphid species (Lamelas et al. 2008; Henry et al. 2015). The first study published on its effects correlates natural double infections of X-type and *H. defensa* with a high level of physiological defence against the parasitoid *Aphidius ervi* under heat stress (Guay et al. 2009).

The aphid genotypes used in this study were all naturally coinfected with X-type and a *Spiroplasma* symbiont. Coinfections of multiple symbionts are common in aphids (Ferrari et al. 2012; Smith et al. 2015) and the presence of one species has the potential to alter the other species' effect on the host's phenotype (Oliver et al. 2006). For example, it is possible that carrying two symbiont species increases the costs imposed on the host (Oliver et al. 2006).

Here, we used three pea aphid genotypes that are naturally infected with X-type and *Spiroplasma*, and cured them of X-type, giving us six aphid lines in total. This allowed comparison of X-type mediated effects within each of the aphid genotypes. We tested the following hypotheses: (i) X-type can provide multiple ecological benefits to the host (resistance to heat shock, a parasitoid and a lethal fungal pathogen). (ii) There is genetic variation in the degree to which these benefits are conferred by X-type, which is due to variation between X-type strains or an interaction between X-type and the genotype of the host or other symbionts. (*iii*) Carrying X-type is costly for the aphid under benign conditions.

Material and methods

Aphids

The pea aphid (*Acyrthosiphon pisum* (Harris)) reproduces parthenogenetically throughout spring and summer, allowing genetically identical individuals to be maintained indefinitely in the laboratory. Pea aphids feed on a variety of different legume species, but individuals are highly specialized on single plant species (Via 1991; Ferrari et al. 2008) and populations found on different plants are genetically differentiated (Peccoud et al. 2009; Ferrari et al. 2012). Despite this specialization, almost all pea aphids perform well on broad bean, *Vicia faba* (L.)(Ferrari et al. 2008). For this study three pea aphid clonal genotypes were used (codes 217, 322, 324), each collected from the UK and naturally infected with X-type and *Spiroplasma*, a bacterial species that cannot yet be cured and so was a consistent background across all lines. The aphids were also screened for *H. defensa*, *R. insecticola, S. symbiotica*, *Rickettsia* sp. and *Rickettsiella viridis* using the diagnostic PCR protocols described in Ferrari *et al.* (2012) and in Tsuchida *et al.* (2010) for *R. viridis*; none of these symbionts were detected.

The aphids were cured from the X-type infection by feeding on leaves placed in an antibiotic cocktail of 1% Ampicillin, 0.5% Gentamicin and 0.5% Cefotaxime (McLean et al. 2011), leading to a total of six aphid lines. Aphids were left for at least six months after curing and retested for endosymbionts regularly to ensure that both the natural infection and the cured lines were stably maintained. To check for contamination between lines, the aphid genotype was regularly confirmed by screening four microsatellite loci (Ferrari et al. 2008).

Our choice of aphid lines requires some caution in the interpretation of our results. Each pair of uninfected and infected lines consisted of a different aphid genotype and potentially a different genotype of the primary symbiont *Buchnera aphidicola* or the *Spiroplasma* symbiont (for simplicity we will refer to these as “aphid genotype” in the results). Any variation that we observe between the three pairs in the phenotypic effects of X-type may therefore be due to genetic variation between X-type strains or a genotypic interaction between X-type and the other three species. Based on the

sequences of six household genes, there is little genetic variation between strains of X-type and no variation has been found for the three strains used here (Henry et al. 2013).

Genotypes 322 and 324 were collected in 2008 from *Trifolium pratense* L. and genotype 217 in 2010 from *Medicago sativa* L., all in the South of England. Unless otherwise noted, experiments were performed at 20°C and long-daylight conditions of 16h:8h light:dark with a relative humidity of 40 ± 15%. Aphids were reared on *Vicia faba* cv. "The Sutton" leaves or seedlings. Aphids were kept in simultaneously refreshed cultures and to reduce maternal effects were raised in smaller groups prior to use in experiments.

Performance under benign conditions and after heat shock

Fecundity is a basic measure of fitness, and we tested the difference between cured lines and those infected with X-type under benign conditions and after exposure to heat shock. Heat stress can render aphids essentially incapable of reproduction (Montllor et al. 2002) but some facultative endosymbionts have been shown to maintain aphid survival and reproduction after heat shock (Montllor et al. 2002; Russell and Moran 2006).

For each line, groups of ten two-day old aphids that had been reared at 20°C were put in cages formed by placing a plastic, vented 2l cage over a pot containing four *V. faba* seedlings. The replicates were then divided into two treatment groups, one group to be tested at 20°C and the other exposed to heat shock. In the heat shock treatment the temperature rose consistently from 20°C to 38.5°C over a period of two hours; this temperature was maintained for four hours and then decreased to 20°C over another two hours. All plants, including those of the controls, were exchanged on the day after the heat shock to ensure consistent plant quality. The proportion of surviving aphids was counted seven days after the heat shock. One surviving apterous aphid per pot was put on a petri dish containing a broad bean leaf and its number of offspring recorded daily for eighteen days, at which point the vast majority of aphids were dead or had stopped reproducing. For survival measures there were seven to nine

replicates for each aphid line, for fecundity there were between five and nine (mean 6.9) across two temporal blocks.

Performance on the original collection plant species

In addition to measuring fecundity on *V. faba*, we looked at whether infection with X-type affects the reproduction and survival on the plant that the aphids were specialized on (assumed to be the species that the aphids were collected from). Young adult aphids were put on Petri dishes containing leaves from the plant species that the aphids were originally found on (*Trifolium pratense* for 322 and 324*, Medicago sativa* for 217) placed in 2% agar. On the following day their offspring were used to create groups of five one-day old aphids on Petri dishes containing the same plant species. Nine days later the number surviving from each group was counted. From each group, one apterous individual was placed on its own *T. pratense* or *M. sativa* plate and offspring counted daily for eighteen days. The dishes were exchanged regularly. The number of replicates was six per aphid line for survival and four to five for fecundity.

Genotype 217 performed very poorly on *M. sativa*, suggesting that it might have been a migrant or hybrid specialized on a different plant species. It was collected from a field where both *M. sativa* and *T. pratense* were grown and we therefore repeated the experiment with 217 on *T. pratense* as well as *M. sativa*. We pooled the *M. sativa* data for both experiments.

Resistance to the parasitoid Aphidius ervi

Parasitoid wasps are a major source of aphid mortality (Müller and Godfray 1998; Schmidt et al. 2003). The wasps oviposit into aphid nymphs and their larvae develop inside the living aphid. The larvae pupate after approximately one week, killing the aphid. The aphid then forms the so-called mummy, out of which the adult parasitoid eventually emerges. *Aphidius ervi* Haliday (Hymenoptera, Braconidae) attacks a range of aphid species and is commonly found on pea aphids. To investigate whether X-type affects the parasitoid resistance of its hosts, aphids were exposed to *A. ervi* females (Ferrari et al. 2001). Thirty four-day old aphids from each aphid line were placed on *V. faba* plants enclosed in a clear plastic, vented cage. A single *A. ervi* female that had

emerged up to 24 hours earlier was added to each cage for nine hours to forage for aphids and to oviposit. After ten days, mummies had formed from aphids that had been successfully parasitised and were counted along with any surviving aphids. This type of parasitism assay is routinely used to assess physiological resistance to parasitoids in aphids (Henter and Via 1995; Ferrari et al. 2001; Oliver et al. 2003). It is possible that there are differences in parasitoid oviposition behaviour between aphid lines, but typically this is found in choice rather than no-choice situations such as the assay used here (Henter and Via 1995; Oliver et al. 2003,Łukasik et al. 2013a). The total number of replicates for each aphid line varied between four and ten (with a mean of 6.8), spread between two temporal blocks. The blocks were pooled for the statistical analysis since no significant difference was found between blocks.

Resistance to the fungal pathogen Pandora neoaphidis

Under warm and humid conditions, entomopathogenic fungi are a further major cause of aphid mortality. These fungi grow in the aphid's body, and like parasitoids, kill the host after a few days. We assessed aphid resistance to the pathogenic fungus *Pandora neoaphidis* (Remaudière & Hennebert) Humber (Zygomycetes; Entomophorales) (isolate reference X4, Rothamsted Research collection). To produce the experimental aphids, young adult aphids were placed on Petri dishes with *Vicia faba* leaves and left overnight before being removed. Once the offspring were ten days old, they were assembled in groups of twenty. Each group was exposed to a pair of adult fungus-killed aphids that had been placed on damp filter paper suspended from the lid of a Petri dish and been left overnight at 20ºC and high humidity to start the fungus sporulating. These aphid cadavers created a spore shower over a small plastic tube (height 50mm, diameter 15mm) that contained the group of 20 aphids. After 90 minutes the sporulating cadavers were removed and the populations placed on fresh two-week old *Vicia faba* plants. For the next two weeks plants were checked every few days and sporulating aphids removed and counted.

As all six aphid lines are infected with *Spiroplasma*, which can confer resistance to *P. neoaphidis* (Łukasik et al. 2013b), we included a fourth genotype, known as 145. This genotype does not carry any known facultative symbionts and is susceptible to this

strain of *P. neoaphidis*. It was included as a control to ensure the protocol worked, in case infection with *Spiroplasma* made the target aphids fully resistant.

Statistical analysis

Data were analysed using the R package 3.2.1 (R Core Team, 2012). Survival data were analysed using analysis of deviance, assuming a quasibinomial error distribution. All other data (except susceptibility to the fungal pathogen) were subjected to an analysis of variance and the data were transformed when necessary to meet model assumptions (square root transformation for fecundity, arcsine square root transformation for susceptibility to the parasitoid). Aphid genotype, presence of X-type and their interaction were the explanatory variables plus heat shock treatment and its interactions in the heat shock experiment. The model assumptions were checked with Shapiro-Wilk normality tests and Levene's test for homogeneity of variances. When an explanatory variable with more than two levels or an interaction was significant, we performed post-hoc tests using Holm's correction for multiple testing in the package "phia" in the R software. The data for fungus susceptibility could not be transformed to meet ANOVA assumptions and were therefore analysed using non-parametric tests (Mann-Whitney U test for presence of X-type and Kruskal-Wallis test for aphid genotype).

Results

Performance under benign conditions and after heat shock

As expected, the heat stressed insects had significantly lower seven-day survival than the control aphids ($F_{1, 87} = 40.39$, $P < 0.001$), showing that the heat stress had a negative effect on the aphids. Survival was not significantly affected by any other factor, including the presence of X-type ($F_{1, 87} = 0.20$, $P = 0.65$), aphid genotype ($F_{2, 87} = 1.07$, $P = 0.35$) or their interaction ($F_{2, 87} = 2.17$, $P = 0.12$).

Heat shock also reduced the fecundity of the aphids (Fig. 2.1; $F_{1, 68} = 47.65$, $P < 0.001$) and the three aphid genotypes differed intrinsically in their fecundity irrespective of symbiont infection ($F_{2, 68} = 3.43$, $P = 0.04$). There was no overall effect of infection with X-type on fecundity ($F_{1, 68} = 1.34$, $P = 0.25$), but it differed between treatments (heat shock × infection with X-type: $F_{1, 68} = 12.19$, $P < 0.001$): aphids infected with X-type had higher fecundity after heat shock but tended to have lower numbers of offspring in the control treatment. This effect did not differ significantly between the three aphid genotypes (heat shock × infection with X-type × aphid genotype: $F_{2, 68} = 2.37$, $P = 0.10$), even though Fig. 2.1 suggests that a cost of carrying X-type under benign conditions occurred in genotype 217.

Performance on the original collection plant species

The aphids' performance was tested on the *Trifolium pratense*. Genotype 217 was additionally tested on *M. sativa*, see methods. On *T. pratense*, survival was high with little variation, but there was a small effect of aphid genotype with genotype 322 having poorer survival overall (see Appendix Fig. A2.3; $F_{2, 28} = 4.49$, $P = 0.02$). There was neither a significant effect of X-type on survival ($F_{1, 28} = 3.13$, $P = 0.09$) nor an interaction of X-type with aphid genotype ($F_{2, 28} = 0.36$, $P = 0.70$). For genotype 217, the presence of X-type also had no effect on survival on *M. sativa* (Fig. S2a; $F_{1, 31} = 1.81$, $P = 0.19$).

The fecundity on *T. pratense* did not differ between aphid genotypes (Fig. Appendix Fig. A2.2a; $F_{2, 24} = 1.90$, $P = 0.17$). It was overall reduced by the presence of X-type ($F_{1, 24} = 11.37$, $P = 0.003$), a cost that did not occur in genotype 324 (aphid genotype ×

infection with X-type: $F_{2, 24} = 3.42$, $P = 0.049$). The presence of X-type also reduced the fecundity of genotype 217 on *M. sativa* (see Appendix Fig. A2.2b; $F_{1, 27} = 9.91$, $P = 0.004$).

Resistance to the parasitoid Aphidius ervi

We tested whether X-type provides resistance to the parasitoid *A. ervi*. Overall, X-type caused a significant decrease in susceptibility to the parasitoid (Fig. 2.2b; $F_{1, 34} = 11.61$, $P = 0.002$) and there was a clear difference between aphid genotypes ($F_{2, 34} = 61.95$, $P < 0.001$) with genotype 217 being overall highly resistant. There was a significant interaction between genotype and infection status ($F_{2, 34} = 5.02$, $P = 0.01$) since X-type had no effect on resistance in genotype 324.

Resistance to the fungal pathogen Pandora neoaphidis

Aphid genotype 145 was highly susceptible to *P. neoaphidis*, showing that the spore shower protocol worked (Fig. 2.2c). The other aphid genotypes were more resistant to the pathogen, suggesting that *Spiroplasma* may be providing a degree of resistance to these aphids. Aphids infected with X-type were significantly more resistant to the fungus ($W = 268.5$, $P < 0.001$), which was also found in separate tests on each aphid genotype (217: $W = 38.5$, $P < 0.01$; 322: $W = 25.5$, $P < 0.05$; 324: $W = 24.0$, $P = 0.05$). There were no significant differences between the three aphid genotypes (Kruskal-Wallis $X^2_2 = 0.24$, $P = 0.89$).

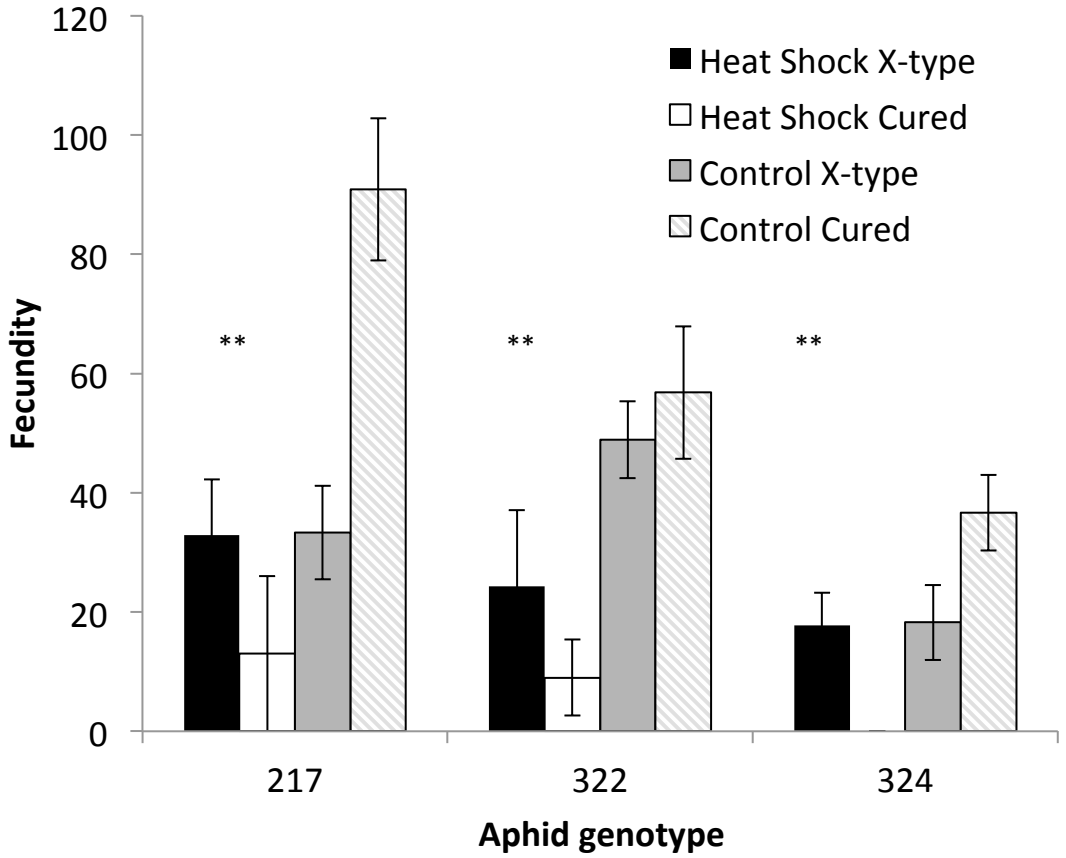


Figure 2.1. The effect of infection with X-type on the fecundity of three pea aphid genotypes feeding on *Vicia faba*. The figure shows a comparison between aphids that are naturally infected with X-type and *Spiroplasma* (black and dark grey bars) or cured from X-type, but still infected with *Spiroplasma* (white and light grey bars). The black and white bars show fecundity after a heat shock treatment; dark and light grey bars show fecundity at 20°C. Means and standard errors are shown. The asterisks indicate the results of post-hoc tests: there is a significant difference between aphids infected with X-type and cured aphids in the heat shock treatment, but not under control conditions (**: $P < 0.01$).

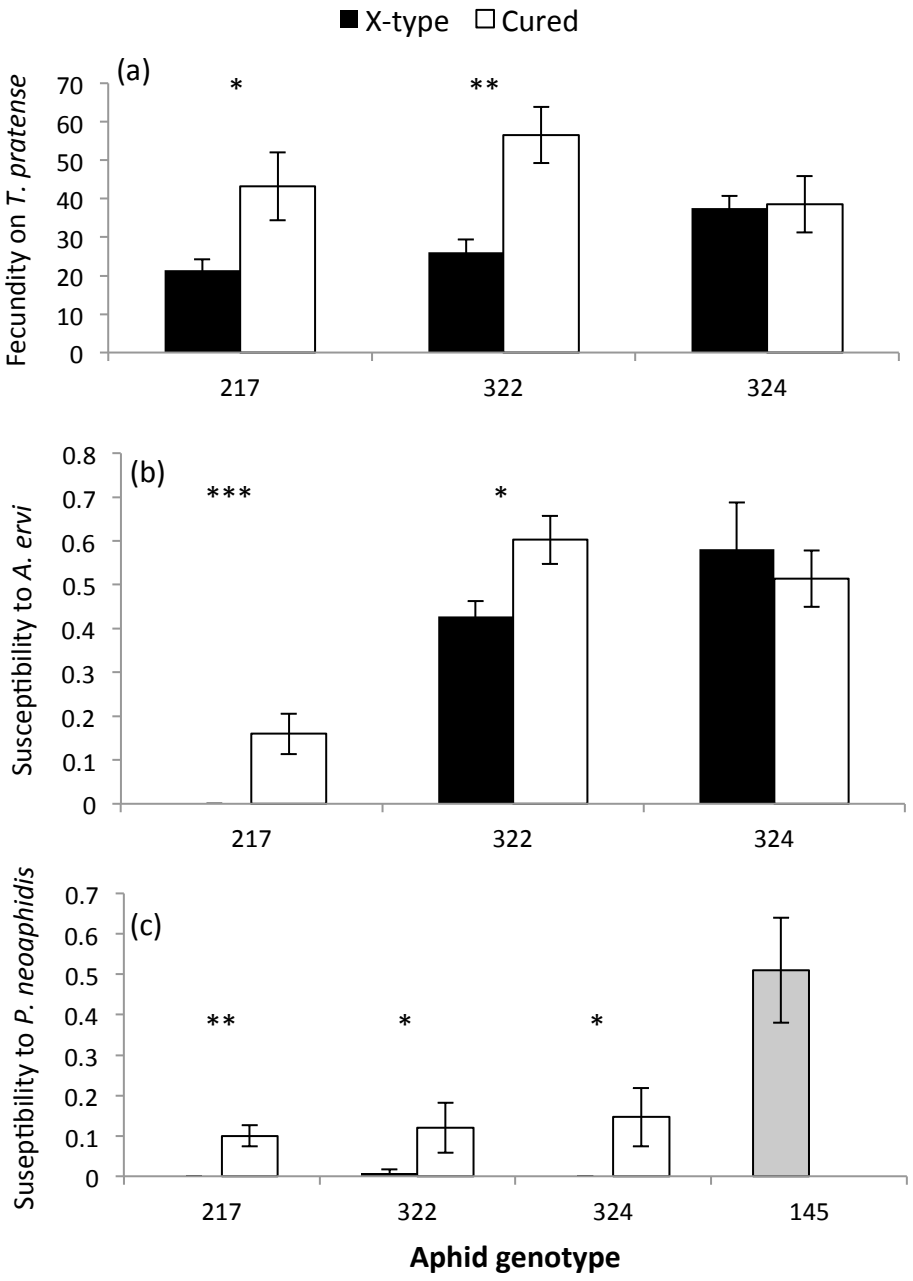


Figure 2.2. The effect of infection with X-type on three pea aphid genotypes. (a) Number of offspring produced by aphids raised on *T. pratense*. (b) Aphid mortality after attack by the parasitoid *A. ervi*. (c) Aphid mortality after exposure to the fungal pathogen *P. neoaphidis*, which includes a control uninfected aphid genotype that is known to be susceptible (genotype 145, grey bar). All panels show a comparison between aphids that are naturally infected with X-type and *Spiroplasma* (black bars) or cured from X-type, (white bars). Means and standard errors are shown. The asterisks denote significant differences between lines infected with X-type and cured from X-type within aphid genotypes (*: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$).

Discussion

We found strong evidence that infection with the endosymbiont X-type can provide multiple and ecologically diverse benefits, which include tolerance to heat shock and resistance to natural enemies from two different kingdoms. However, while some strains of X-type appear to be able to confer all of these benefits, others provide only a subset. Harbouring X-type under benign conditions can carry a cost in terms of aphid fecundity, especially on the original host plant species.

All of the benefits observed in this study have previously been found for other insect-endosymbiont combinations (Montllor et al. 2002; Oliver et al. 2003; Scarborough et al. 2005) but to our knowledge no single insect-symbiont genotype combination has been tested for all of these (or a similar range of other benefits) and only one strain of *H. defensa* and one strain of *S. symbiotica* have been shown to provide more than one benefit (Oliver et al. 2003; Russell and Moran 2006). A possible explanation for the diversity of endosymbiont species in insect populations is that separate species provide different benefits and that the host is selected for carrying the symbiont most suited to its environmental conditions (Oliver et al. 2014). Given that our study is one of the very few examples where a single strain has been tested for multiple benefits, our results suggest that it may be relatively common that a symbiont provides multiple benefits and therefore it might be adaptive to carry the same symbiont in a wide range of conditions.

We have also shown that not all X-type strains employed here show all of the benefits and there is functional variation between the three combinations of genotypes. These differences between X-type strains are unexpected because there are no differences in six household genes of the three X-type strains (Henry et al. 2013) and two of the aphid genotypes (322 and 324) are closely related and were collected in close proximity, but the simplest explanation of this result is that there is variation in functional genes between the three X-type strains. However, caution must be taken when interpreting this result, because each X-type strain was tested in its original host genotype, with additional potential genetic variation between the primary symbiont *Buchnera* and the *Spiroplasma* strains present. It is therefore possible that the

observed strain variation is actually due to an interaction between X-type genotype and the genotype of at least one of the other three players in the system, and future experiments are needed to address this directly. Whether the observed variation is due to interactions between genotypes or not has implications for the dynamics of the system, in particular if the symbiont is horizontally acquired by a different aphid genotype (Russell et al. 2003).

Similarly, the presence of *Spiroplasma* in both the original lines and those cured of X-type complicates the interpretation. The most straightforward explanation of the observed patterns is that X-type alters the host's phenotype directly without interacting with *Spiroplasma*. It is possible that X-type is merely enhancing an effect that is actually caused by *Spiroplasma* rather than providing direct benefits, or other interactions between the symbionts are impacting the host. For example, *Spiroplasma*'s only known benefit, providing resistance to the fungal pathogen *P. neoaphidis* (Łukasik et al. 2013b), may be increased by the presence of X-type. Experiments with single infections of X-type are required to clarify this issue, but unfortunately we were unable to find such natural lines before performing our experiments. However, the difference between the cured lines and their counterparts infected with X-type will be caused by the presence of X-type, whether this is a direct or indirect effect.

The presence of X-type used here exerted a fecundity cost on their host, with a reduction of fecundity of up to 50% on the natural host plant species *T. pratense*. Costs on this magnitude have previously only been reported for artificial host-symbiont associations (Tsuchida et al. 2013) which may be because of incompatibilities between particular genotypes. Natural infections usually appear to come at a lower cost (Sakurai et al. 2005; Simon et al. 2007; Chandler et al. 2008) or a cost that can only be detected under more stressful conditions (Oliver et al. 2006; Dykstra et al. 2014). These high costs are likely to outweigh some of the benefits provided by X-type and if these are representative of costs suffered under field conditions it is surprising that X-type is maintained at relatively high frequencies in aphid populations. It is also possible that the high cost is a result of the coinfection with *Spiroplasma* since

coinfections may require more resources from the host than single infections (Oliver et al. 2006).

In conclusion, we have shown that a single endosymbiont can confer a wide range of ecological benefits to its host at a considerable cost. This study further highlights the variability between strains or genotypic interactions with the host or other symbionts demonstrating the difficulty of predicting the dynamics in natural populations.

Appendix: Supplementary information

Supplementary figures

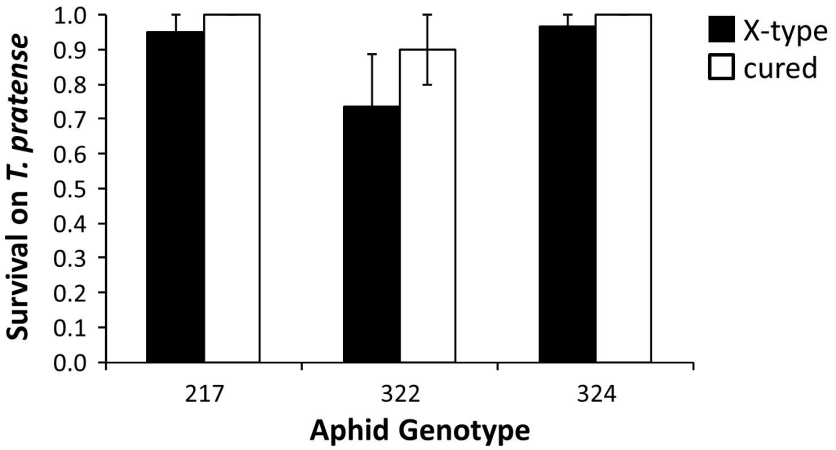


Figure A2.3. The effect of infection with X-type on the survival of three pea aphid genotypes feeding on *Trifolium pratense*. The figure shows a comparison between aphids that are naturally infected with X-type and *Spiroplasma* (black bars) or cured from X-type, but still infected with *Spiroplasma* (white bars). Means and standard errors are shown.

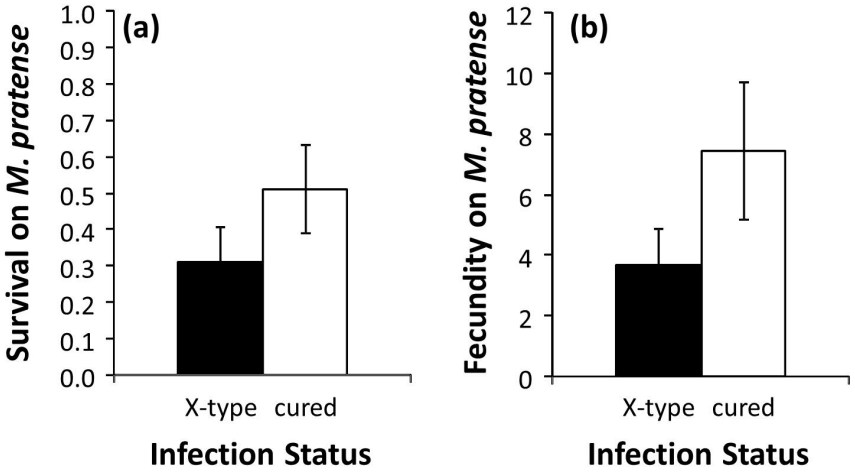


Figure A2.4. **The effect of infection with X-type on (a) survival and (b) fecundity in pea aphid genotype 217 on when feeding on *Medicago sativa*.** The figure shows a comparison between aphids that are naturally infected with X-type and *Spiroplasma* (black bars) or cured from X-type, but still infected with *Spiroplasma* (white bars). Means and standard errors are shown.

Chapter 3

Heat stress affects facultative symbiont-mediated protection against a parasitoid wasp

Abstract

Insects face a range of threats to their survival, and relatively few individuals successfully reproduce. They can be aided by mutualistic facultative symbionts, which provide host benefits including resistance to natural enemies and abiotic stresses. Facultative symbionts can protect their hosts from a variety of different dangers in isolation, but little is known about how their beneficial phenotypes are affected when threats occur simultaneously. The pea aphid (*Acyrthosiphon pisum*) is a model system for exploring symbiont effects due to its well-characterised range of symbionts. We have shown that the symbiont X-type, in aphids infected with a second symbiont *Spiroplasma*, can protect against both parasitoid wasps and heat stress in separate assays (Chapter 2). We now use the same aphids infected with or cured from X-type to investigate the ability of the symbiont to cope with simultaneous threats; heat stress and attack by parasitoid wasps. Typically, experiments designed to look at these traits are performed under otherwise benign conditions, and our aim is to explore how robust symbiont protection may be outside of such controlled environments. We subject aphids to heat shock either before or after attack by parasitoid wasps and score aphid susceptibility to parasitoids, aiming to explore the effect of temperature stress on symbiont-mediated parasitoid protection. Under a benign temperature regime we find parasitoid protection conferred by the presence of X-type, which disappears when aphids are heat shocked before or after parasitoid attack. We find that if previously heat-stressed aphids are parasitised any protection otherwise provided by symbionts is lost, whereas if parasitised aphids are later heat shocked susceptibility decreases overall, regardless of infection. The results demonstrate how resistance to parasitoid wasps can be strongly environment-dependent and that a beneficial phenotype under controlled conditions in the laboratory does not necessarily equate to a consistently useful effect in natural populations.

Introduction

Insects face many threats to their survival, ranging from the challenges of extreme temperatures to a wide range of natural enemies. Predators, parasitoids and pathogenic fungi are common natural enemies, but are rarely encountered in isolation, and the interactions between these different stresses can be the determining factors in insect survival. The ecology and efficiency of natural enemies can be affected by temperature and precipitation (Muller and Schmid-Hempel 1993; Steinkraus 2006; Hance et al. 2007; Diehl et al. 2013), while the interactions between different types of predators affect the success of both (Ingels and De Clercq 2011). Insect host plant can also affect tritrophic interactions between natural enemy and insect (Steinkraus 2006; Pineda et al. 2013; Babikova et al. 2014). As the surrounding environment affects natural enemy efficacy, it changes the selection pressures on insect populations.

Many insects depend on their facultative symbionts to increase their survival against common threats, and an increasing number of mutualistic microbes have been shown to provide protection (Moran et al. 2008; Feldhaar 2011; Ferrari and Vavre 2011). With many such symbionts vertically transmitted, host and microbe fitness are closely linked, and increased insect survival benefits both partners. As a result, selection pressures in these systems act upon the unit known as the 'holobiont', comprising the insect and all of its associated endosymbionts (Rosenberg et al. 2010).

Facultative symbionts across many taxa can guard against natural enemy attack (Kaltenpoth et al. 2005; Xie et al. 2010; Kaltenpoth and Engl 2014) and temperature extremes (Neelakanta et al. 2010; Oliver et al. 2010) and reciprocal dynamics between threat and symbiont cause them to impact the frequencies of each other. High predation pressure or extreme temperatures may select for insects harbouring certain symbionts, and in turn, symbionts can potentially affect the strength of the stress to the insect population (Montllor et al. 2002; Scarborough et al. 2005; Oliver et al. 2010; Feldhaar 2011).

When characterising the effects of facultative endosymbionts on their hosts, it is important to consider that studying symbiont effects on just one threat may miss more

complex interactions. In this study we use the pea aphid (*Acyrthosiphon pisum*) as a model species to investigate how temperature can affect known symbiont-mediated protection against parasitoid wasps.

Pea aphids can be infected with at least eight different species of facultative endosymbionts (Russell et al. 2013; Gauthier et al. 2015). Several of their symbionts have been shown to ameliorate the negative effects of heat on their hosts (Chapter 2; Chen et al. 2000; Montllor et al. 2002; Russell and Moran 2006), increasing insect reproduction and so symbiont spread into the next generation. Others can protect against parasitoid wasp mortality (Chapter 2; Oliver et al. 2006; Nyabuga et al. 2010; Vorburger et al. 2010). Typically, each species of aphid endosymbiont is known for providing a specific benefit to its host; for example *Hamiltonella* increases resistance to parasitoid wasps (Oliver et al. 2003), *Regiella* protects against fungus (Scarborough et al. 2005) and *Serratia symbiotica* increases reproduction and survival after heat stress (Montllor et al. 2002). In reality, all of these symbiont species have been shown to provide multiple benefits, with potentially more to be found (Oliver et al. 2006; Russell and Moran 2006; Vorburger et al. 2010).

The symbiont known as X-type is one of the more recently discovered symbionts (Guay et al. 2009), and is unusual in that a single strain has been shown to provide more than one advantage (Chapter 2), with individual genotypes improving both host resistance to heat shock and parasitoid wasps. As such, it is an ideal symbiont to study how interactions between these abiotic and biotic dangers affect infected aphids.

Previous studies have found that parasitoid protection provided by *Hamiltonella* can often (but not universally) fail under moderate heat stress (Bensadia et al. 2006; Guay et al. 2009; Cayetano and Vorburger 2013). Interestingly, a correlative study involving naturally infected with *Hamiltonella* and X-type aphids found that resistance to a parasitoid was maintained under heat while single infections of *Hamiltonella* strains lost their protection (Guay et al. 2009). It is therefore possible that parasitoid resistance provided by X-type is resistant to temperature.

We investigate how protection against parasitoids is affected by heat stress to illustrate how symbiont-conferred phenotypes may differ under non-benign

conditions, and to explore the ecological relevance of lab-based symbiont assays. We investigate whether infection with X-type affected susceptibility to the parasitoid *Aphidius ervi* when the aphids had experienced heat stress a day before or a day after being parasitised compared to a constant temperature. The aphids used were also infected with *Spiroplasma*, as before (Chapter 2), and based on Guay et al.'s (2009) observations and our results in Chapter 2 showing that X-type improves tolerance to heat shock in the presence of *Spiroplasma*, we hypothesized that the level of resistance to *A. ervi* provided by X-type under a benign temperature regime would be maintained under heat stress.

Methods

Aphids

Pea aphids (*Acyrthosiphon pisum* (Harris)) reproduce asexually under long-day light conditions, allowing genetically identical clonal lines to be maintained in the laboratory. For this study the same three pea aphid genotypes were used as in Chapter 2 (codes 217, 322 and 324), each collected from the UK naturally infected with X-type and *Spiroplasma*. Genotypes 322 and 324 were collected in 2008 from *Trifolium pratense* and genotype 217 from *Medicago sativa* in 2010, all in Southern England.

The aphids were cured from X-type by feeding them on *Vicia faba* leaves placed in an antibiotic solution of 1% Ampicillin, 0.5% Gentamicin and 0.5% Cefotaxime (McLean et al. 2011), leading to a total of six aphid lines. These were maintained for at least six months before the start of the experiment. *Spiroplasma* is unable to be cured by this method, so was maintained in all lines. The aphids were also tested for the pea aphid symbionts *Hamiltonella defensa*, *Regiella insecticola*, *Serratia symbiotica*, *Rickettsia* and *Rickettsiella viridis* using symbiont-specific PCR (Tsuchida et al. 2010; Ferrari et al. 2012), and none of these were detected. Lines were retested regularly to confirm infection and avoid contamination.

Each pair of uninfected and infected aphid lines of a different genotype potentially also contains a different strain of *Buchnera* and *Spiroplasma*, and for simplicity we refer to this combination as 'aphid genotype'. Any effect of X-type infection may therefore be an effect of X-type itself, or of an interaction between the facultative symbiont and one or more of the other genotypes involved. Based on the sequences of six household genes, there is little variation between strains of X-type and no variation has been found for the three strains used here (Henry et al. 2013).

Despite strong host specificity in the field (Via 1991), pea aphids of different genotypes generally perform well on *Vicia faba* (broad bean) plants (Ferrari et al. 2008) and these experiments were all performed using *V. faba* ("The Sutton") leaves or seedlings. Unless otherwise noted, experiments were performed at 20°C and long-day light

conditions of 16h:8h light:dark with a relative humidity of 40 ± 15%. Aphids were kept in simultaneously refreshed cultures and were raised in smaller groups prior to use in experiments to reduce maternal effects.

Heat shock/parasitism assay

To investigate the effects of heat on symbiont-mediated protection against parasitoids, groups of aphids were heat stressed either before or after being exposed to parasitoid wasps. Dividing the aphids into three groups (heat shocked before parasitism, heat shocked after parasitism and non-heat shocked populations) allowed us to compare how heat shock affects parasitoid resistance at different stages, both before the aphid was attacked and once the parasitoid eggs were laid.

Young adult aphids were placed on *V. faba* leaves in Petri dishes (Sterilin, 90mm) overnight to produce offspring of a standardized age. For each of the six aphid lines, populations of 30 offspring were exposed to one of the three treatments. Aphids in all treatments were kept at 20°C, 40 ± 15% relative humidity, and a 16h light: 8h dark regime, except on the day they were heat stressed. On the day of the heat shock treatment, the aphids were exposed to a temperature that steadily rose from 20°C to 37°C over a period of two hours, was stable at 37°C for four hours and then decreased over another two hours back to 20°C. Aphids in the "heat shock before parasitism" treatment experienced this regime when they were 48-72h old and aphids in the "heat shock after parasitism" treatment when they were 96-118h old. A third group never experienced the heat treatment, and were kept at 20°C as a control. In all three treatments, the aphid populations were exposed to a female *Aphidius ervi* parasitoid wasp for 9 hours at 20°C when they were 72-96h old. All aphids from all three experimental and control groups were transferred to fresh plants on the day after the second heat treatment to minimize the effects that the heat might have had on plant quality.

Successful parasitism involves the parasitoid wasp larvae internally consuming the aphid. While the aphid is consumed its cuticle is transformed into a protective casing for the developing wasp, the distinctive brown 'mummy'. The number of mummies was counted 10 days after exposure to wasps and susceptibility calculated as the

number of mummies formed out of the total numbers of aphids found (alive or mummified). Dead aphids were not counted as it was unclear if heat, parasitism or random effects had caused death. There were between four and six repeats for each of the six aphid lines in each of the three treatments.

Statistical analysis

The proportion of mummified aphids was analysed in a single binomial general linear model. Model explanatory variables were block, treatment, aphid genotype, symbiont presence and all possible interactions between treatment, aphid genotype and symbiont presence. Residuals were checked using Levene's test for homogeneity of variance. Data were not transformed for analysis.

Results

The high temperature treatments had a significant effect on the aphids' susceptibility to *A. ervi* ($F_{2, 70} = 8.47$, $P < 0.001$, Table 3.1); aphids that were kept at benign temperatures, or those that were heat shocked on the day before parasitism (Figure 3.1) were more susceptible to the parasitoid than those that were heat shocked after parasitism (Figure 3.2).

There was also variation in resistance between the three aphid genotypes ($F_{2, 70} = 23.62$, $P < 0.001$) with genotype 217 being the most resistant to the parasitoids. Infection with X-type had no overall effect on susceptibility to *A. ervi* ($F_{1, 70} = 0.04$, $P = 0.83$), but had contrasting effects in the different heat treatments (heat treatment × infection with X-type: $F_{2, 70} = 4.55$, $P < 0.01$): In the control treatment, X-type increased resistance to *A. ervi*, whereas it had no effect on susceptibility when the aphids were parasitised after or before heat shock. There was no three-way interaction between aphid genotype, X-type and treatment ($F_{4, 70} = 0.61$, $P = 0.65$) and no two-way interaction between treatment and aphid genotype ($F_{4, 70} = 1.10$, $P = 0.36$).

Table 3.1 Aphid mortality after parasitism summary.

Aphid line	Treatment	Repeats	Max	Min	Mean	Standard error
217X	Heat shock before	5	63.16	0.00	32.63	10.15
	Control	5	22.73	0.00	4.55	4.55
	Heat shock after	5	11.11	0.00	4.73	2.13
217 cured	Heat shock before	5	53.33	0.00	13.35	10.11
	Control	4	22.73	0.00	43.42	25.63
	Heat shock after	5	21.74	0.00	5.60	4.21
322X	Heat shock before	5	83.33	27.27	62.11	15.32
	Control	5	86.36	54.55	60.49	12.77
	Heat shock after	5	66.67	37.50	46.23	5.32
322 cured	Heat shock before	4	100.0	25.00	63.50	10.15
	Control	4	88.24	54.55	71.32	7.50
	Heat shock after	4	88.89	14.29	54.27	15.32
324X	Heat shock before	6	94.44	10.00	62.89	11.52
	Control	6	88.89	30.43	63.91	8.53
	Heat shock after	5	75.00	8.33	22.66	6.46
324 cured	Heat shock before	6	64.29	18.18	38.55	10.32
	Control	5	95.45	27.76	69.76	12.28
	Heat shock after	6	36.84	0.00	22.66	6.46

	Residual DF	Residual Deviance	DF	Deviance	F	P value
Null	88	644.51				
Block	87	639.86	1	4.64	1.16	0.28
Treatment	85	572.29	2	67.57	8.47	**<0.001**
Aphid genotype	83	383.87	2	188.42	23.62	**<0.001**
Symbiont	82	383.70	1	0.18	0.04	0.83
Treatment * Genotype	78	366.11	4	17.59	1.10	0.36
Treatment * Symbiont	76	329.78	2	36.33	4.55	**<0.05**
Genotype * Symbiont	74	320.93	2	8.85	1.11	0.34
Genotype * Symbiont * Treatment	70	311.14	4	9.79	0.61	0.65

Table 3.2 General linear model test results.

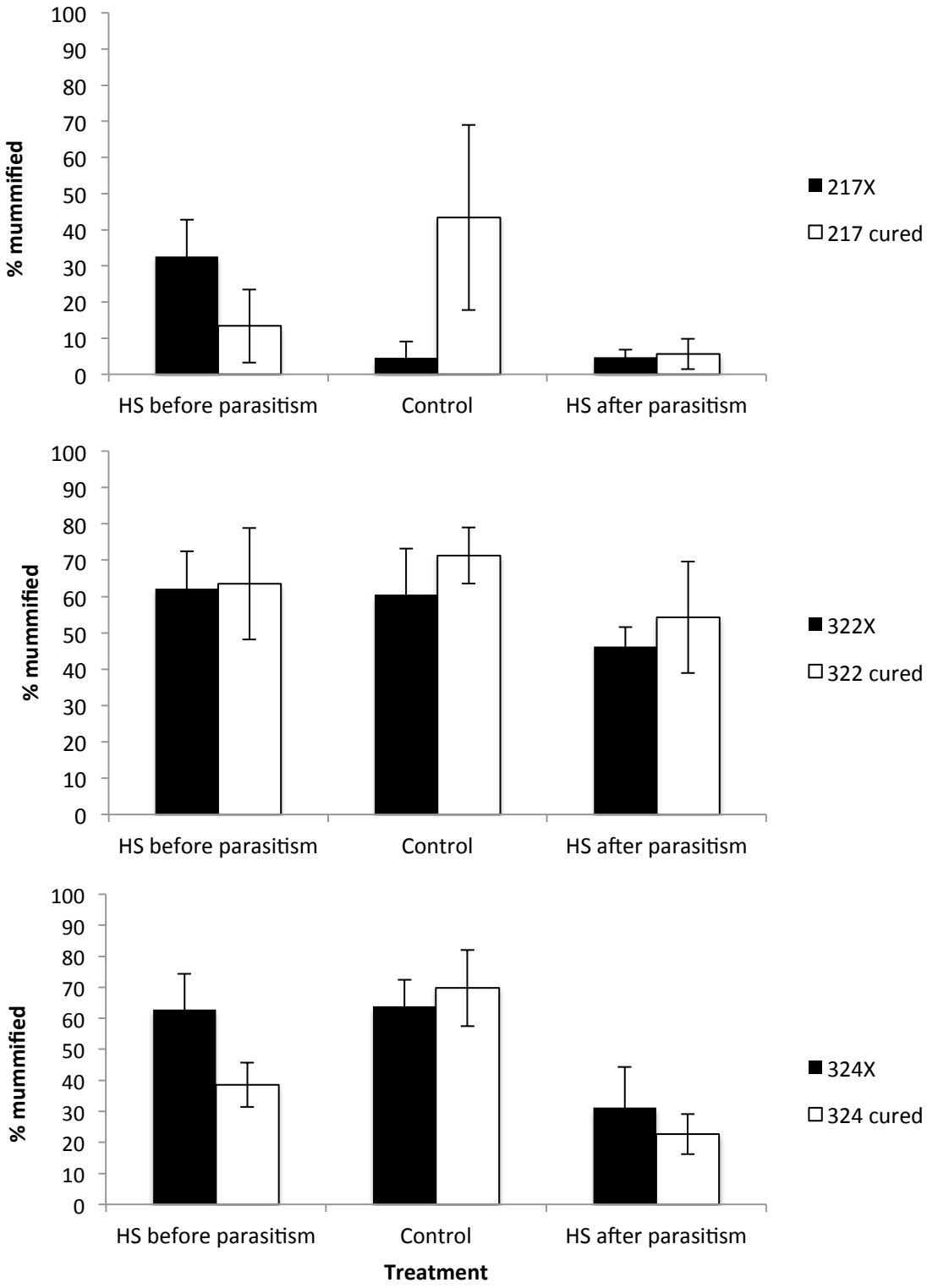


Figure 3.1. The average susceptibility to the parasitoid *Aphidius ervi* ± SE of three pea aphid genotypes infected with or cured from X-type. All lines are also infected with the symbiont *Spiroplasma*. Error bars are standard error.

Discussion

We find that multiple factors can affect an aphid's susceptibility to parasitism, including heat stress and aphid genotype. Aphids that were heat shocked before being parasitised tended to increase in susceptibility compared to controls, whereas aphids that were heat shocked after parasitism showed higher resistance to mummification. The facultative symbiont X-type has been shown to protect its aphid host against parasitoids and heat in separate laboratory assays (Chapter 2), and here we find that this parasitoid protection can be affected by different temperatures.

When aphids are heat stressed before being parasitised there is no protection conferred by X-type infection. X-type population densities do initially decrease when measured a day after heat stress (Chapter 4), at the time when these aphids were being exposed to the parasitoids. It is possible that this slight decrease in symbiont population level is responsible for the decrease in protection, and this is one hypothesis for the mechanism behind why parasitoid resistance decreases after heat stress. Parasitoid resistance provided by *Hamiltonella defensa*, another aphid symbiont, is conferred by a phage (APSE, for *Acyrthosiphon pisum* secondary endosymbiont) carried on the symbiont genome (Oliver et al. 2009). APSE produces toxins that target eukaryotic tissue and may attack parasitoid nutritional cells (Degnan and Moran 2008; Degnan et al. 2009; Oliver et al. 2009). Strains of X-type and *Regiella insecticola* have also been found to provide parasitoid resistance (Chapter 2; Vorburger et al. 2010) but no evidence of the phage has yet been found (Hansen et al. 2012). Finding the mechanism behind this resistance, and understanding how it may interact with heat stress, would be an interesting avenue for future research.

The trend in a slight increase of susceptibility in X-infected aphids compared to control may in turn be due to the maintenance of obligate symbiont *Buchnera aphidicola* densities in X-type infected aphids under heat stress (Chapter 4). *Buchnera* is sensitive to heat and cells die under high temperatures (Dixon et al. 1987; Dunbar et al. 2007), and infections with facultative symbionts including X-type can protect obligate symbiont populations (Chapter 4; Montllor et al. 2002). Parasitoid wasps depend on the nutrients provided by *Buchnera* for the development of their eggs (Pennacchio et

al. 1999) and a lack of the symbiont in heat-stressed aphids could decrease the ability of parasitoid wasp larvae to survive and develop after oviposition (Pennacchio et al. 1999; Cloutier and Douglas 2003). If X-type is protecting *Buchnera*, it may be actually increasing aphid susceptibility to parasitism under high temperature conditions. Our results are in contrast to a study that found lower susceptibility of Hamiltonella-infected aphids after extreme heat shock (39°C) (Cayetano and Vorburger 2013) although this may be because the more extreme heat treatment used led to high aphid mortality and potential avoidance of poor quality aphids by the parasitoids (Cayetano and Vorburger 2013).

When aphids were heat stressed after being parasitised aphids tended to increase in resistance regardless of symbiont infection. At this stage of parasitism the parasitoid egg is developing inside the aphids and instead of the aphids becoming more resistant under heat shock, it is far more plausible that the egg is being detrimentally affected by the temperature spike, leading to a decrease in successful development. Parasitoid fitness reduces at higher temperatures (Malina and Praslicka 2008) and the developing egg may have been killed outright, leading to an observed pattern of high aphid resistance.

These results contrast with a correlative study on X-type effects after heat shock, where parasitoid protection after heat was tested in natural superinfections with the highly parasitoid protective symbiont *Hamiltonella defensa* (Guay et al. 2009). We found no maintenance of parasitoid resistance conferred by X-type in aphids heat shocked either before or after parasitism, suggesting that this may be an attribute of the double infection, an interaction between the symbionts and aphid genotype that we do not see or an effect of the strain of *Hamiltonella* or aphid genotype used (Guay et al. 2009).

We found that aphid genotype also affects an aphid's susceptibility to parasitism, corroborating previous work which shows the specific genotype x genotype interactions that can affect aphid susceptibility to parasitoid mortality (Cayetano and Vorburger 2014). In our study, aphid genotype effects are unable to be uncoupled from the strain of *Buchnera* and *Spiroplasma* in each genotype, potentially also

conferring protection. There may also be interactions between X-type and *Spiroplasma* that result in the decrease or increase of protection under different heat treatments, which we cannot explore in this study.

These results have implications for the understanding of how complex interactions may occur in field populations. There, parasitoid wasps are a common natural enemy (Muller et al. 1999; Boivin et al. 2012), but aphids and other insects must also face a range of simultaneous threats to their survival. Temperature and precipitation can affect the strength of pathogen and predation pressures, even as they affect the insect itself (Malina and Praslicka 2008; Brumin et al. 2011). Facultative symbionts add another layer of complication to the interactions (Montllor et al. 2002; Bensadia et al. 2006; Shan et al. 2014). We find that symbiont-mediated parasitoid protection can be decreased or rendered obsolete under different temperature regimes, with implications for how the effects of symbiont infection may be studied in future. This work illustrates further that the balance of costs and benefits of harbouring facultative symbionts can be strongly dependent on the environment and that complex and potentially quite specific interactions can affect the value of a symbiont to an insect host. As facultative symbionts may drive rapid adaptation in host populations due to their protective effects (White 2011), understanding more about how robust symbiont-mediated protection is under different temperature conditions is also vital to understanding how insect populations may be affected by changes in climate in future.

Chapter 4

Facultative symbionts shield nutritional mutualists from heat stress

Abstract

Insects are subject to extremes of temperature and a changing climate, and the ability of species to adapt to different environmental conditions will affect their future success. Aphids are currently a common temperate pest, but are much rarer in tropical regions. This incongruity is linked with their obligate nutritional endosymbiont, *Buchnera aphidicola*, which is susceptible to heat. Aphids can also be infected with non-obligate symbionts, of which several species have been shown to protect their hosts from high temperatures. We use three common aphid facultative endosymbionts to investigate the mechanism behind this protection. After heat stress, we measure aphid fitness using fecundity counts, and measure population densities of the obligate and facultative symbionts using qPCR. We find that infection with *Regiella insecticola* or X-type confers resistance to heat stress, with heat shocked infected aphids having a higher lifetime fecundity than those infected with *Hamiltonella defensa* or uninfected with facultative symbionts. Correspondingly, we find that the heat shocked aphids infected with *Regiella* or X-type have higher population levels of *Buchnera* eleven days after heat shock compared to genetically identical uninfected lines. We hypothesize that the maintenance of *Buchnera* in infected lines allows these aphids to reproduce even after heat stress, giving aphids infected with X-type or *Regiella* a strong ecological advantage in high temperature environments.

Introduction

Both temperature and climate affect insect ecology (Dixon et al. 1987; Dill et al. 1990; Hance et al. 2007; Diehl et al. 2013) and any change in environmental conditions is likely to have a strong ecological impact. The current climate models predict a steady rise in global temperatures in the coming decades (Cox et al. 2000; Risbey et al. 2014), altering ecosystems worldwide. This general increase in temperature is already affecting insects in terms of range shifts (Parmesan and Yohe 2003), phenology (Walther et al. 2002) and interactions with predators and parasitoids (Harrington et al. 1999; Schmitz and Barton 2014), with these trends likely to continue in future.

Facultative symbionts are found at intermediate frequencies in insect populations (Feldhaar 2011; Duron and Hurst 2013) and have the potential to affect insect responses to heat (Brumin et al. 2011; Wernegreen 2012) either through directly protecting their hosts from extremes of temperature (Montllor et al. 2002; Neelakanta et al. 2010; Brumin et al. 2011) or through the indirect effects of interactions between symbiont and temperature on the insect hosts themselves (Chen et al. 2009; Bordenstein and Bordenstein 2011). As facultative symbionts can spread horizontally between insect lineages (Russell et al. 2003; Ferrari and Vavre 2011) their presence in field populations acts as a 'horizontal gene pool' of ecological phenotypes with the ability to alter insect responses to changing environmental factors (Wernegreen 2012).

The mechanisms behind symbiont-mediated thermal protection are unknown, although there are several plausible hypotheses. As species of symbionts across several insect taxa have been shown to provide protection (Montllor et al. 2002; Russell and Moran 2006; Neelakanta et al. 2010; Brumin et al. 2011), it is possible that the beneficial phenotype is a by-product of infection with a wide range of symbiont species. Aphids infected with facultative symbionts produce more immune cells than those that are uninfected (Laughton et al. 2013) and such constitutively high immunity levels may confer protection against multiple different stresses. For example, the presence of *Rickettsia* in whiteflies increases survival of the insect under heat shock due to constitutive host production of cytoskeletal genes in *Rickettsia*-infected insects

(Brumin et al. 2011). The stress response to the symbiont itself leaves its host prepared for additional challenges that arise, known as 'priming'.

Another possible mechanism is a more direct form of protection. Many beneficial symbionts predominantly spread vertically from parent to offspring (Haine 2008; Goodacre and Martin 2012) and so depend on the fitness of their host for their own spread. It is plausible that symbiont-mediated protection against heat shock is an adaptation of the microbes to provide benefits to their host and to ensure their own maintenance in populations under stress. This mechanism could involve protecting either the insect itself or the obligate, heat-susceptible symbionts that many insects rely on for survival (Montllor et al. 2002; Dunbar et al. 2007; Wernegreen 2012).

We use the pea aphid (*Acyrthosiphon pisum*) to investigate the mechanisms behind symbiont-conferred thermal protection. Pea aphids are an ideal model system to investigate interactions between environmental conditions and symbionts due to their protective facultative symbionts (Oliver et al. 2010) and intolerance to heat (Dixon et al. 1987). Rapid, asexual reproduction results in clonal lines of aphids that can be kept indefinitely under long-day conditions, leading to easy manipulation of their bacterial endosymbionts through antibiotic curing and hemolymph injections. As a result, several of the eight known facultative pea aphid symbionts are relatively well characterised and infection can affect the hosts in a wide variety of ways, including increasing resistance to natural enemies (Oliver et al. 2003; Scarborough et al. 2005) and abiotic stresses (Chen et al. 2000; Burke et al. 2010). Several of the previous studies looking at such stresses have focused on heat protection, where infection with symbionts has been shown to increase aphid survival or improve reproduction after heat shock (Chapter 2; Montllor et al. 2002; Koga et al. 2003; Russell and Moran 2006). All aphids are also infected with a nutritional symbiont, *Buchnera aphidicola*, which synthesises essential amino acids for its insect host (Douglas 1998). This obligate mutualist provides aphids with the nutrients they require to thrive on their homogenous phytophagous diet (Whitehead and Douglas 1993). *Buchnera* has a highly reduced genome (Moran 1996; Gómez-Valero et al. 2007) and is susceptible to high temperature: under heat stress, just five protective heat shock proteins are deployed (Wilcox et al. 2003) compared to over 80 for its free living relative

Escherichia coli (Carruthers and Minion 2009) and during severe heat shock *Buchnera* can be killed (Dunbar et al. 2007; Chen et al. 2009). Some strains of *Buchnera* are more resistant to heat than others, but such protection comes at a fitness cost (Dunbar et al. 2007) and aphids without *Buchnera* altogether suffer from strongly reduced fitness and fecundity (Koga et al. 2007).

The first facultative aphid endosymbiont discovered that confers resistance to heat shock is *Serratia symbiotica* (Montllor et al. 2002). When aphids infected with *Serratia* are heat shocked, there is evidence that they are able to reproduce while uninfected aphids are sterilized (Montllor et al. 2002) and heat-shocked infected aphids can also benefit from higher initial survival and faster development times (Russell and Moran 2006). Other symbiont species can provide similar benefits when tested. Strains of *Hamiltonella defensa* can increase survival of aphids under heat (Russell and Moran 2006) and X-type increases reproduction after heat shock compared to uninfected controls (Chapter 2).

There are two plausible hypotheses that we investigate for the mechanism behind heat shock protection in aphids. The first is that the beneficial facultative symbiont protects *Buchnera*, potentially through production of heat shock proteins or metabolites that the obligate symbiont lacks. It is possible that these are produced constitutively, up-regulated by heat stress or released by lysis of the symbiont; exposing *Serratia*-infected aphids to high temperatures can kill the facultative symbiont but maintain densities of *Buchnera*, in contrast to uninfected controls (Montllor et al. 2002; Burke et al. 2010). This mechanism would result in a maintained or increased density of heat-sensitive *Buchnera* when infected with facultative symbionts.

It is also possible that facultative symbionts, instead of protecting *Buchnera* through lysis or constitutive or heat-triggered release of metabolites, may be replacing the obligate symbiont. The *Buchnera*-aphid partnership is ancient (Baumann et al. 1997; Wernegreen 2012) but not irreplaceable. Infection with *Serratia* can allow aphids to reproduce if *Buchnera* is removed using antibiotics, with the facultative symbionts moving into the bacteriocytes usually occupied by the obligate symbiont (Koga et al.

2003, 2007). It is possible that a similar mechanism allows facultative symbionts to replace *Buchnera* spatially and mechanistically when the obligate symbiont dies through heat stress, resulting in a lower population level of *Buchnera* but increased reproduction in aphids infected with the protective symbionts.

We explore whether three common facultative pea aphid endosymbionts, *Hamiltonella defensa*, *Regiella insecticola* and X-type, affect host fitness after heat stress and explore the mechanism behind any protection by measuring symbiont population densities using qPCR. We investigate the hypotheses that facultative symbionts can protect their insect hosts from extremes of temperature, and that *Buchnera* densities after heat shock are affected by the presence of facultative symbionts.

Methods

Aphids and endosymbionts

Two aphid genotypes were used for this study (Table 4.1). Genotype 218 was collected naturally infected with X-type and *Hamiltonella,* and was cured over a year before this study by feeding young aphids on broad bean leaves suspended in a tube of antibiotic solution (0.5% Gentomicin, 1% Ampicillin, 0.5% Cefotaxime in distilled water) over 4 days (McLean et al. 2011). Genotype 200 was collected naturally infected with no facultative symbionts. Aphids were confirmed free of symbionts using symbiont-specific PCR primers (Table 4.2) and were rechecked regularly to avoid contamination. The PCR mix comprises 6.25µl BioMix (Bioline), 0.1µl (20µM) of forward and 0.1µl (20µM) reverse primer, 5.55µl distilled water and 1.0µl sample DNA. There are two reverse primers for *Hamiltonella* due to polymorphism between strains, and both are used in half measures in the protocol. The PCR program comprised 94°C for 2 minutes, followed by 35 cycles of: 94°C for 30 seconds, 55°C for 30 seconds and 72°C for one minute. It concluded with 6 minutes at 72°C and then cooled the sample to 4°C. PCR products were run on a 1% agarose gel and the presence of a band confirmed the presence of the symbiont.

To introduce the selected infections of *Hamiltonella*, *Regiella* and X-type we used hemolymph injections from infected donor aphids (Table 4.1). Hemolymph was extracted from donor aphids under a microscope using small glass needles and surviving aphids raised to adulthood. Glass needles were pulled from Kwik-Fil™ borosilicate glass capillaries (1B100-4, World Precision Instruments, 1mm diameter) using a model P-97 Flaming/Brown micropipette Puller (Sutter Instrument Co.), program 4. The offspring of the surviving aphids were tested for the successful establishment of the novel infection, and were retested regularly to ensure continual vertical maintenance. All injected lines had been maintained in the laboratory for at least a year before being used for experimental assays. These strains of symbiont were chosen because preliminary results indicated that they were likely to provide heat shock protection.

Five aphid lines were used in the experiment, two uninfected with facultative symbionts (200 and 218) and the remainder infected singly with one of three facultative symbionts (200R, 218X, 218H). As a result, genotype 200 is used twice, either uninfected or infected with *Regiella*, and genotype 218 used three times, uninfected or infected with X-type or *Hamiltonella*. These five aphid lines comprise three pairwise comparisons between uninfected and infected aphids, with the uninfected line of 218 used in two comparisons, and this design aimed to compare host fitness and symbiont densities in each pair across the same aphid genetic background. All aphid lines used were screened for *Hamiltonella defensa*, *Regiella insecticola*, *Serratia symbiotica*, X-type, *Spiroplasma*, *Rickettsia* and *Rickettsiella viridis* (Tsuchida et al. 2010; Ferrari et al. 2012) to ensure that they had the appropriate symbiont infection and no additional endosymbiont infections.

Table 4.1: Experimental lines used during the experiments and injected symbiont information.

Experimental lines used to compare symbiont infection across the same genetic backgrounds				**Donor symbiont lines**		
Code	Symbiont	Collection host plant	Collection location	Donor code	Collection host plant	Collection location
200	None	*Medicago sativa*	Lincoln, 2012 53.23°N, 0.53°W			
200R	*Regiella insecticola*			30100	*Trifolium pratense*	Windsor, 2008 51.48°N, 0.61°W
218	None (previously *Hamiltonella defensa* and X-type)	*Medicago sativa*	Eling, 2010 52.47°N, 1.25°W			
218X	X-type			23800	*Medicago sativa*	Beaconsfield, 2010 51.60°N, 0.63°W
218H	*Hamiltonella defensa*			20700	*Medicago sativa*	Lincoln, 2012 53.23°N, 0.53°W

Heat shock protocol

This assay was designed to investigate the mechanisms behind symbiont-conferred heat tolerance by measuring aphid fecundity and symbiont population density of both obligate and facultative symbionts after heat shock. Aphids were exposed to either a single peak of high temperatures or were maintained at a steady, control temperature to measure responses.

To produce age-controlled populations of each of the five lines, groups of young adults were placed into petri dishes that contained a single broad bean (*Vicia faba*) leaf, placed in 2% agar to delay desiccation. *V. faba* is a host plant that most otherwise host-specific pea aphids accept, and it is commonly used in pea aphid experiments (Ferrari et al. 2008). The adults were left to reproduce overnight at 20°C, and offspring produced put onto two-week old *V. faba* plants in groups of 50 and enclosed in a vented, transparent 2l cage. On the following day (aphid age 24-48hrs) the populations were either exposed to heat stress or left at 20°C as a control.

While the control treatment was left at 20°C, the heat treatment subjected the aphids to a single spike in temperature. The temperature was increased from 20°C to 38.5°C steadily over the course of 2 hours, held at 38.5°C for four hours, and then decreased back to 20°C over a further two hours. Surviving aphids from both treatments were moved onto fresh two week old plants on the day after heat shock to mitigate any temperature effects on the plant itself.

Aphids were removed to measure symbiont density at two timepoints, the first 24-26 hours after the peak heat shock period, and the second eleven days post heat shock. For the first timepoint five surviving young aphids were removed, and for the second a single, adult aphid was taken. Aphids for symbiont density measures were flash frozen using dry ice and kept at -80°C until DNA extraction. In addition, one surviving apterous individual from each group was placed on a *V. faba* petri dish (as above) to measure offspring produced. These dishes were refreshed every 3-4 days to ensure healthy *V. faba* leaves. Offspring counts continued until all aphids had died, measuring total lifetime fecundity. There were 5-6 replicates for fecundity counts and symbiont density for each of the five aphid lines at each treatment.

qPCR protocol

DNA was extracted from the aphids after samples were defrosted at room temperature. Aphids were homogenised in a 200µl 5% Chelex solution made in distilled water. 10µl of proteinase K (10mg/ml) was added per sample, and samples were incubated for 6 hours at 56°C to facilitate digestion. They were then centrifuged at 16,200 x g for 3 minutes and the supernatant containing the DNA pipetted into a clean 1.5ml Eppendorf tube which was stored at -20°C until use. One aphid sample was generated by each repeat of the five lines at both of the timepoints, 24-26 hours after heat shock (aphids were 3 days old) and eleven days later (aphids were 14 days old), approximately six days after an aphid would usually begin reproducing.

Extracted DNA samples were frozen at -20°C until use and then run in duplicate using Syber® Green reagent on a StepOnePlus™ Real Time PCR machine (Applied Biosystems). Each well consisted of 10µl FAST SYBR 2x mastermix (ABI), 1µl forward primer (7µl), 1µl reverse primer (7µl), 6µl nuclease-free water and 2µl DNA sample. qPCR primers for *Regiella,* X-type, *Hamiltonella* and the aphid housekeeping gene Ef1α were tested for efficiency and successful single-peak amplification by running a melt curve to ensure they only bound once to the target (please see Table 4.2). Cycling conditions were 95°C for 20 seconds, followed by 40 cycles of: 95°C for 3 seconds and 60°C for 30 seconds. A melt curve was also run for each plate, involving a further 95°C for 15 seconds, 60°C for one minute and then a gradual increase to 95°C over 15 minutes. Melt curves were used to confirm primer efficiency and accuracy. Each 96-well qPCR plate was analysed using StepOne Software v2.2.2 (Applied Biosystems) and Ct values were obtained by comparing each primer sample to a single standard curve of known concentration and using identical threshold and baseline levels for each primer target across plates. Standard curves were created by amplifying positive control samples using PCR, calculating DNA concentrations using a High Sensitivity DNA Assay on a2100 Bioanalyzer system (Agilent), and then serially diluting the sample 1:10 with distilled water to create a 5-sample curve comprising known concentrations decreasing from 10pmol/ml. Samples with Ct values over 30 were classed as negative, confirmed by our negative controls. This corresponds to a copy number of <52 for all primers, and is below the threshold of detection. There were 5 to 11 biological repeats

(mean 7.6) for each of the aphid lines at each treatment. Samples were run in duplicate, resulting in two technical repeats. Technical repeats with >1.5 Ct difference were rerun or discarded.

DNA concentration was calculated for each sample by comparing sample Ct values back to the equation of the standard curve. DNA copy numbers were then calculated by multiplying the molar concentration by Avogadro's constant (the number of particles in one mole of substance). To control for aphid size and extraction efficiency, copy numbers for each sample were normalised to a housekeeping aphid gene as a control, giving a final value in terms of bacterial to aphid cell ratio.

Standard PCR primers					
	Forward	**Sequence**	**Reverse**	**Sequence**	**Reference**
X-type	10F	5' - AGTTTGATCATGGCTCAGATTG - 3'	X420R	5' - GCAACACTCTTTGCATTGCT - 3'	Ferrari et al. 2012
Regiella	10F	5' - AGTTTGATCATGGCTCAGATTG - 3'	U433R	5' - GGTAACGTCAATCGATAAGCA - 3'	Ferrari et al. 2012
Hamiltonella	10F	5' - AGTTTGATCATGGCTCAGATTG - 3'	T419R	5' - AAATGGTATTCGCATTTATCG - 3'	Ferrari et al. 2012
			T0419R	5' - AAATGGTATTGGCATTTATCG - 3'	Ferrari et al. 2012
qPCR primers					
	Forward	**Sequence**	**Reverse**	**Sequence**	**Reference**
X-type	gyrB-X397F	5' - TGG ATT GGC TGG TGA AAG AAT - 3'	gyrB-X466R	5' - TTC ATC TCT CCC AAA CCT TTA TAG - 3'	J. Ferrari pers. comm.
Regiella	Regiella gyrB F	5' - CGT CGC TTA GCT GTT GAC AGT T - 3'	Regiella gyrB R	5' - CGC GCC GAG ACT CTT TTA CT - 3'	J. Ferrari pers. comm.
Hamiltonella	H1165F	5' - CGT GAA ATG ACA AGA CGT AAA GGT - 3'	H1241R	5' - TCA CGC TCC TGG CAA TCC - 3'	J. Ferrari pers. comm.
Buchnera	B arg G 2F	5' - GGT GCT ACT GGG AAA GGA AAT G - 3'	B arg G 2R	5' - CGG TGC AAT TAC ATT TAA ATT CGG A - 3'	Edited from Wilson et al., 2006
Ef1alpha	EF1a 107F	5' - CTG ATT GTG CCG TGC TTA TTG - 3'	EF1a 246R	5' - TAT GGT GGT TCA GTA GAG TCC - 3'	Laughton et al. 2013

Table 4.2: Primers used for PCR and qPCR.

Statistical analysis

Data were analysed using R (R Core Team 2012). The data were then split into the three pairs of facultative symbiont uninfected/infected lines, and analysed separately. As a result, each dataset comprises the infected aphid line compared to the genetically identical uninfected line (200 for *Regiella* and 218 for X-type and *Hamiltonella*) and the data for the uninfected line 218 was used twice, paired with 218 infected with *Hamiltonella* or X-type. The datasets were also analysed separately by timepoint.

Offspring count data were analysed using a general linear model, with Offspring+1 as the response variable, and block, line, treatment and the interaction between line and treatment as explanatory variables. Offspring is the measure of lifetime fecundity, and one was added to adjust for large numbers of zeros in the data. Data were checked for homogeneity of variance using Levene's Test, and then run on a quasipoisson model to account for overdispersion. To confirm the effect of temperature, Welch's two-sample t-tests were run on the heat shock and control counts within each aphid line.

qPCR data for *Buchnera* were analysed using general linear models with a quasipoisson distribution to account for overdispersion. Data were split into the three pairs of lines, and analysed separately for each timepoint, with block, treatment, symbiont presence and the interaction between treatment and symbiont as explanatory variables.

qPCR data for the three facultative symbiont densities were non-parametric and could not be transformed to meet parametric model assumptions. These data were separated by timepoint and species of symbiont and analysed using Wilcoxon signed-rank tests to investigate the variance of the means.

Results

Effects of facultative symbionts on fecundity after heat shock

We found that infection with a facultative symbiont can affect the ability of an aphid to reproduce after heat stress. In four of the five lines heat shock caused a significant decrease in offspring produced (200: t_5=40.79, p<0.001, 200R: $t_{6.5}$=3.63, p<0.01, 218: $t_{5.2}$=5.2, p<0.01, 218H: t_5=5.5, p<0.001, Figure 4.1, Table 4.3) whereas there was a non-significant effect of treatment in the 218X line ($t_{0.96}$=9.9, p=0.36). Infection with X-type exerts a strong fitness cost under benign conditions, leading to a similar total offspring measure under both heat shock and control experiments.

In two of the three pairwise comparisons between uninfected and infected lines on the same genetic background, there was a significant interaction between symbiont and treatment driven by the ability of the symbiont to increase fecundity under heat stress. This was significant for infection with X-type ($F_{1,19}$=9.5, p<0.01) and *Regiella* ($F_{1,19}$=13.0, p<0.01), but the interaction in the *Hamiltonella* lines was non-significant ($F_{1,19}$=0.14, p=0.71). Both X-type and *Regiella* allowed aphids to produce significantly more offspring than the uninfected genotypes, whereas *Hamiltonella* did not.

There was no main effect of infection with X-type ($F_{1,21}$=0.23, p=0.63), *Regiella* ($F_{1,21}$=0.29, p=0.59) or *Hamiltonella* ($F_{1,21}$=0.06, p=0.81) on overall fecundity. There was also no main effect of heat shock treatment on reproduction in the X-type comparisons ($F_{1,20}$=3.42, p=0.08) due to low reproduction in the X-type-infected line regardless of temperature, but there was an effect of treatment in the *Regiella* ($F_{1,20}$=60.03, p<0.001) and *Hamiltonella* ($F_{1,20}$=19.26, p<0.001) due to lower reproduction in heat shocked aphids. Because the interactions between treatment and line were pronounced for the *Regiella* and X-type lines, caution must be taken when interpreting the main effects of temperature and line.

Genotype	Symbiont	Treatment	Sample size	Max value	Min value	Mean	Standard error of the mean
200cu	None	*Heat shock*	6	0	0	0.00	0.00
		Control	6	122	105	110.17	2.70
200R	*Regiella*	*Heat shock*	5	89	0	33.20	20.23
		Control	6	104	62	95.67	6.74
218cu	None	*Heat shock*	6	75	0	19.17	12.93
		Control	6	110	84	101.33	3.96
218X	X-type	*Heat shock*	6	104	0	65.83	16.79
		Control	6	105	2	49.00	14.27
218H	*Hamiltonella*	*Heat shock*	5	114	0	22.80	22.80
		Control	6	133	103	117.83	3.91

Table 4.3. Summary statistics for heat shock/control offspring counts.

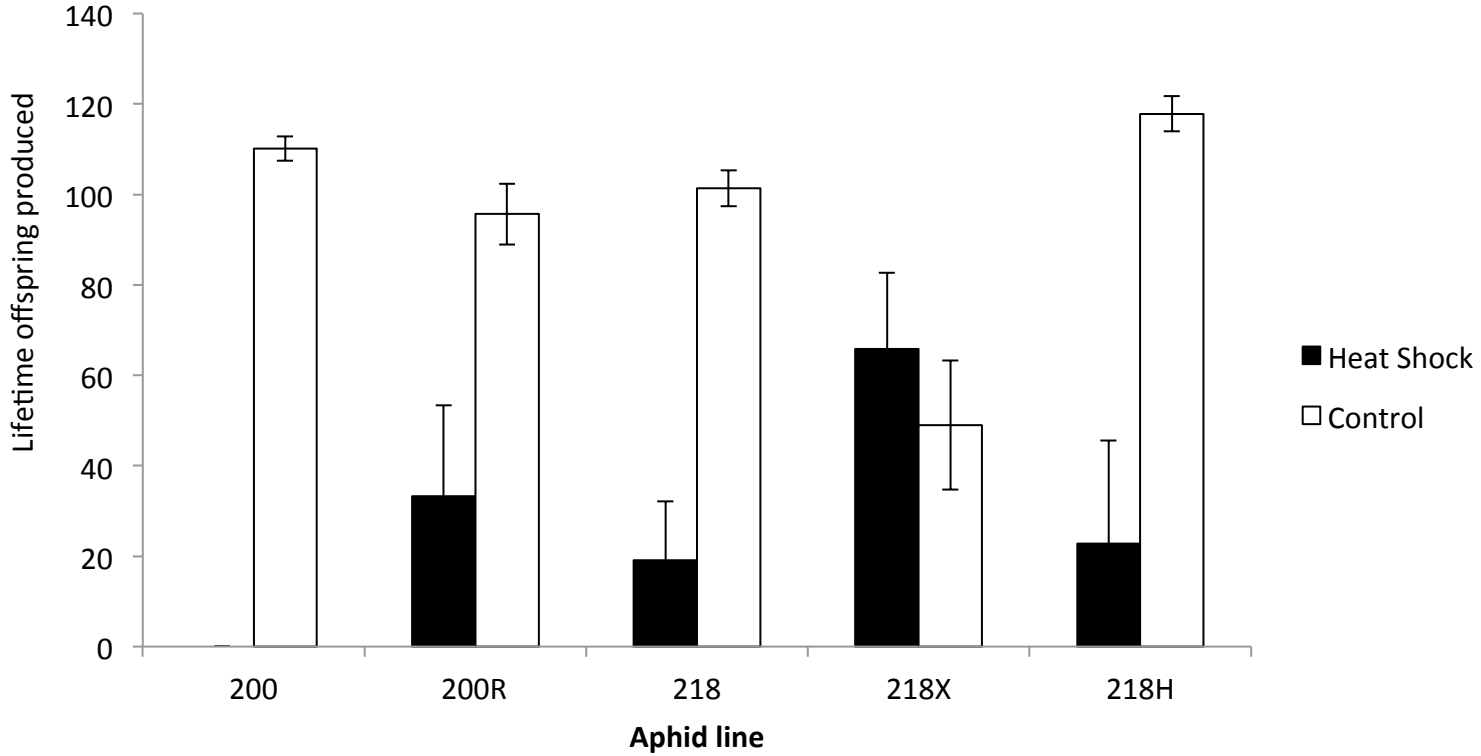


Figure 4.1. Offspring produced from heat shocked or control aphids, and the effect of infection with *Regiella*, X-type or *Hamiltonella*. In analysis, aphid lines are compared in pairs of uninfected or infected aphids. Data shown are means and error bars show standard errors. (*: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$).

Facultative symbiont densities under heat shock

We measured the densities of the three facultative symbionts at two timepoints after exposure to heat; 24-26 hours or 12 days post-heat shock (Figure 4.2). We found that compared to non-heat shocked controls the densities of two of the symbionts, X-type and *Hamiltonella*, are lower the day after heat shock (X-type: W = 30, p<0.01, *Hamiltonella*: W = 28, p<0.05, Table 4.5), whereas densities of *Regiella* are unaffected (W = 28, p=0.13, Table 4.4., Table 4.5).

By the second timepoint, taken when the aphids are young adults, there is no difference between population densities in heat stressed or control aphids for any of the three facultative symbionts (X-type: W =14, p=0.93, *Hamiltonella*: W = 14, p=0.84, *Regiella*: W = 9, p=0.90), suggesting that heat does not have long-term effects on facultative symbiont population.

24 days post heat stress		**Biological repeats**	**Maximum**	**Minimum**	**Mean**	**Standard error**
X-type	Heat shock	5	0.57	0.35	0.47	0.04
	Control	6	1.24	0.9	1.06	0.05
Regiella	Heat shock	6	2.92	1.44	2.17	0.27
	Control	6	3.23	2.68	2.87	0.08
Hamiltonella	Heat shock	5	0.27	0.2	0.24	0.01
	Control	6	0.49	0.26	0.3	0.02
12 days post heat stress		**Biological repeats**	**Maximum**	**Minimum**	**Mean**	**Standard error**
X-type	Heat shock	6	3.42	0.87	1.6	0.41
	Control	5	1.99	0.87	1.27	0.21
Regiella	Heat shock	5	7.61	1.88	5	1.21
	Control	4	10.32	2.16	4.48	1.96
Hamiltonella	Heat shock	5	3.45	0.47	1.36	0.54
	Control	5	1.38	0.69	1.03	0.13

Table 4.4. Relative densities of facultative symbionts post heat stress.

24 days post heat stress	*Mean of Heat shock*	*Mean of Control*	*W value*	*P value*
X-type	0.47	1.06	30	**<0.01**
Regiella	2.17	2.87	28	0.13
Hamiltonella	0.24	0.3	28	**<0.05**
12 days post heat stress	*Mean of Heat shock*	*Mean of Control*	*W value*	*P value*
X-type	1.6	1.27	14	0.93
Regiella	5	4.48	9	0.90
Hamiltonella	1.36	1.03	14	0.83

Table 4.5. Results of the Wilcoxon signed-rank tests performed on the relative facultative symbiont densities.

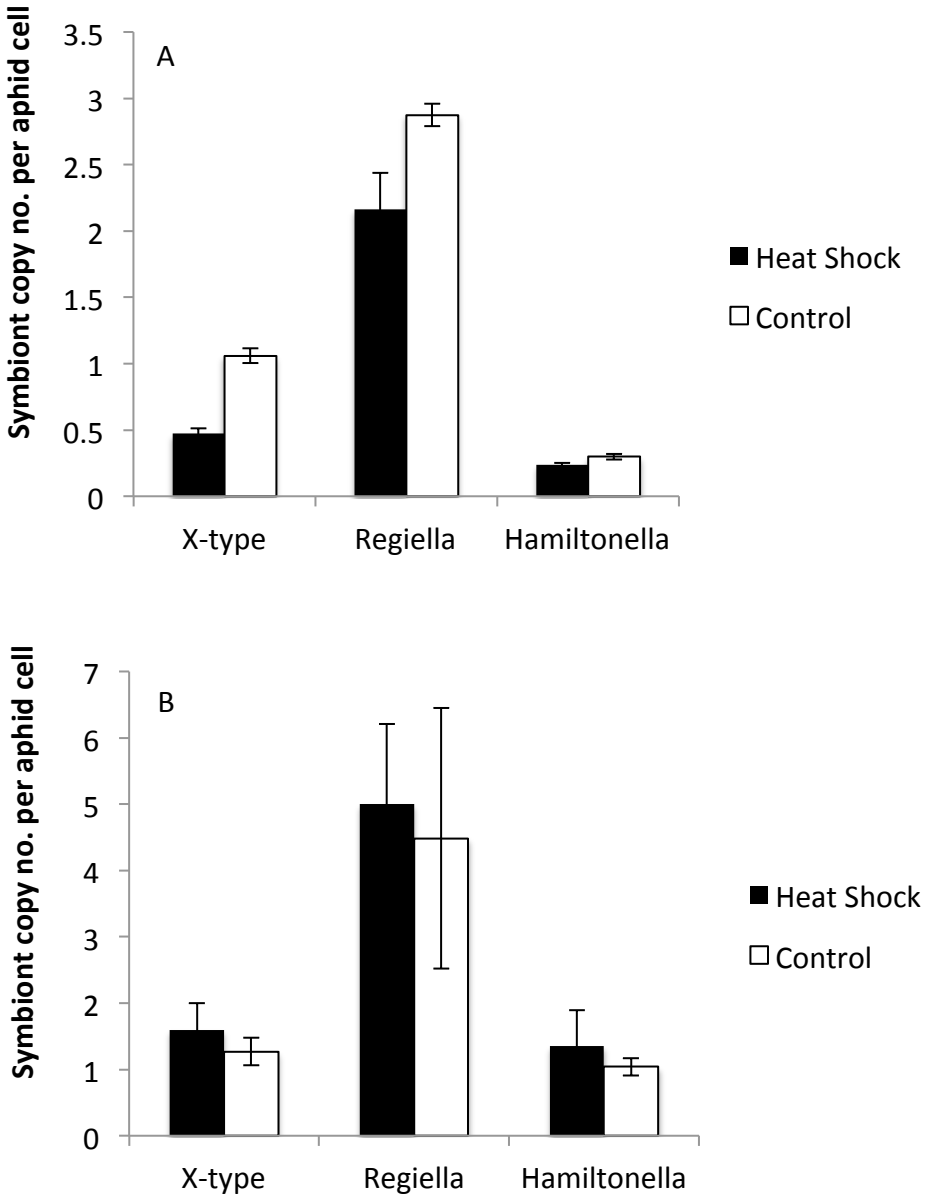


Figure 4.2: Facultative symbiont copy number per aphid cell in heat shocked and control aphids. Charts show data either 24-26 hours after heat shock (A) or twelve days after heat shock (B). Data shown are means with standard error bars.

Obligate symbiont densities under heat shock

We found that on the day after heat shock, densities of *Buchnera* were decreased in each of the three pairs of lines, regardless of facultative symbiont infection (Figure 4.2, Table 4.6, Table 4.7). In two cases there was a significant effect of symbiont presence, regardless of treatment, in *Buchnera* densities, driven by higher *Buchnera* population levels in the X-type and *Hamiltonella* lines that was not seen in the *Regiella* lines (Table 4.7). In no case was there a significant interaction between symbiont presence and treatment at this early timepoint (Table 4.7).

At the later timepoint, when aphids were young adults, there were contrasting results. Once again, temperature affected *Buchnera* densities; this was only marginally significant in the X-type lines but highly significant for the *Regiella* and *Hamiltonella* lines. X-type presence also significantly affected the density of *Buchnera* regardless of treatment, driven by the high levels of *Buchnera* in heat shocked aphids, but this was not found for *Regiella* or *Hamiltonella*. There was a significant interaction between symbiont infection and temperature when aphids were infected with X-type or *Regiella* compared to the uninfected pair, which was not seen for *Hamiltonella.* This is a result of X-type- and *Regiella*-infected aphids increasing the population densities of the obligate symbiont *Buchnera* after heat stress relative to the uninfected lines.

24 hours post heat shock							
Line	*Symbiont*	*Treatment*	*Biological repeats*	*Maximum*	*Minimum*	*Mean*	*Standard error*
200 cured	None	Heat shock	10	16.84	9.21	12.58	0.82
		Control	11	30.03	13.64	21.19	1.21
200R	*Regiella*	Heat shock	11	17.83	8.00	12.92	0.73
		Control	11	32.57	18.42	23.07	1.3
218 cured	None	Heat shock	6	18.73	11.7	13.23	0.83
		Control	6	18.74	11.7	14.34	1.07
218X	X-type	Heat shock	5	13.99	5.89	11.38	1.47
		Control	6	26.79	20.64	23.48	0.94
218H	*Hamiltonella*	Heat shock	5	13.49	7.65	11.61	1.18
		Control	5	13.49	7.65	11.61	1.24
12 days post heat shock							
Line	*Symbiont*	*Treatment*	*Biological repeats*	*Maximum*	*Minimum*	*Mean*	*Standard error*
200 cured	None	Heat shock	9	7.89	0.08	1.07	0.85
		Control	10	19.46	12.16	17	0.72
200R	*Regiella*	Heat shock	9	20.39	0.16	10.45	2.74
		Control	8	19.4	9.36	14.71	1.22
218 cured	None	Heat shock	7	15.21	0.1	3.06	2.44
		Control	7	22.84	12.32	18.74	1.92
218X	X-type	Heat shock	7	33.22	1.09	19.67	4.34
		Control	7	29.05	14.76	19.28	2.09
218H	*Hamiltonella*	Heat shock	6	20.22	0.41	7.32	3.97
		Control	6	18.99	12.11	15.25	1.44

Table 4.6. Summary statistics for *Buchnera aphidicola* obligate symbiont relative densities

24 hours post heat shock					
Lines					
X-type		Df	Residual Df	F value	P value
	Block	1	21	0.2	0.66
	Treatment	1	20	62.78	**<0.001**
	Clone	1	19	29.15	**<0.001**
	Treatment:Clone	1	18	2.09	0.17
Regiella					
	Block	1	22	6.7	**<0.05**
	Treatment	1	21	71.95	**<0.001**
	Clone	1	20	0.06	0.81
	Treatment:Clone	1	19	1.9	0.18
Hamiltonella					
	Block	1	21	0.32	0.68
	Treatment	1	20	54.98	**<0.001**
	Clone	1	19	23.79	**<0.001**
	Treatment:Clone	1	18	0.89	0.36
12 days post heat shock					
Lines					
X-type		Df	Residual Df	F value	P value
	Block	1	22	0.6	0.45
	Treatment	1	21	4.37	0.05
	Clone	1	20	5.06	**<0.05**
	Treatment:Clone	1	19	9.71	**<0.01**
Regiella					
	Block	1	20	1.58	0.23
	Treatment	1	19	16.71	**<0.001**
	Clone	1	18	1.34	0.26
	Treatment:Clone	1	17	8.28	**<0.05**
Hamiltonella					
	Block	1	20	0.56	0.46
	Treatment	1	19	13.96	**<0.01**
	Clone	1	18	0.13	0.72
	Treatment:Clone	1	17	1.78	0.2

Table 4.7. Statistics table for general linear model of *Buchnera aphidicola* densities in different lines, and under different treatments. Df = degrees of freedom.

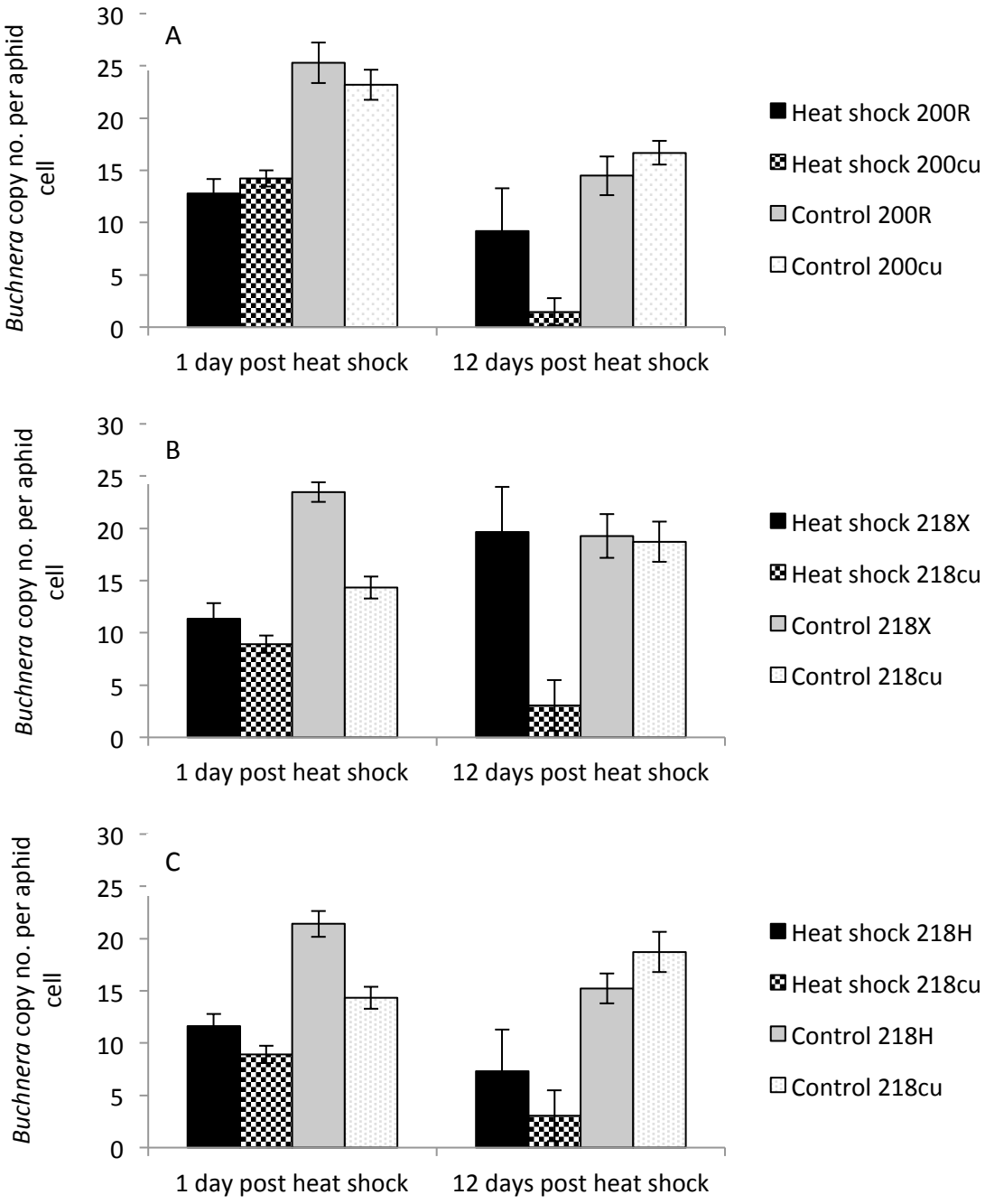


Figure 4.3. Densities of *Buchnera* in pairs of aphid lines infected with or free from each of the three facultative symbionts. A: *Regiella*, B: X-type and C: *Hamiltonella.* Data shown are mean *Buchnera* copy number per aphid cell, with standard error bars.

Discussion

We find that two aphid endosymbionts, X-type and *Regiella insecticola*, protect the obligate aphid endosymbiont *Buchnera aphidicola* from heat stress and increase host fitness compared to uninfected controls. This adds to evidence that the mechanism behind symbiont-mediated temperature resistance involves protection of the obligate symbiont, and has implications for the spread of insects and symbionts under warming environmental conditions.

Obligate endosymbionts in insects have among the smallest bacterial genomes known, a consequence of long term, high fidelity vertical transmission in the confines of their host (Moran 1996; Dale and Moran 2006). Populations of these nutritional mutualists tend to decrease after heat stress (Sacchi et al. 1993; Montllor et al. 2002; Prado et al. 2010; Lu et al. 2014), often leading to a decrease in fitness of the host (Prado et al. 2010). Facultative symbionts in aphids have previously been shown to increase host reproduction after heat shock (Montllor et al. 2002; Koga et al. 2003; Russell and Moran 2006) and here we find evidence that this phenotype is due to the survival of *Buchnera* in aphids infected with protective facultative symbionts.

Exposing aphids to heat stress had a detrimental effect both on aphid fecundity and population density of the obligate symbiont *Buchnera* in the uninfected aphid lines, as expected from previous research (Montllor et al. 2002; Koga et al. 2003; Burke et al. 2010). Both X-type and *Regiella* allowed aphids to produce more offspring after heat stress than the uninfected genetically identical controls, a protective phenotype that was not provided by *Hamiltonella.* This corresponded with increased densities of *Buchnera* in heat shocked adult aphids infected with X-type or *Regiella* compared to uninfected controls, but not *Hamiltonella*.

The higher densities of *Buchnera* in X-type- and *Regiella*-infected lines show that these facultative symbionts are not providing heat shock resistance by replacing the metabolic function of *Buchnera* after obligate symbiont death. Nor is there widespread lysis of X-type and *Regiella*, providing metabolites that sustain *Buchnera*, as seen in the other protective symbiont, *Serratia* (Burke et al. 2010). Although densities of X-type are decreased the day after heat stress, this is not evident in

Regiella, which maintains population levels comparable to control aphids throughout. Instead, it appears that these facultative symbionts may provide constitutively or heat-produced metabolites or heat shock proteins that keep *Buchnera* or the bacteriocytes that house it from dying. Another possibility is that infection with these symbionts stresses the aphids, leading to higher constitutive immunity in infected aphids (Laughton et al. 2013) and a host indirectly prepared for further challenges. The third symbiont tested, *Hamiltonella,* and indeed, other strains of *Regiella* (Russell and Moran 2006) do not confer protection, suggesting that although this result increases the number of symbiont species known to provide heat tolerance, it is not a universal attribute of infection. To explore the mechanism further, metabolomics or transcriptomics of the symbiont and host under heat stress could identify any differentially expressed heat shock candidate genes or metabolites.

As well as the protective effect of X-type and *Regiella*, the symbiont data raise some interesting points. Once again (see Chapter 2), we find a fecundity cost to X-type, but the densities of *Buchnera* confirm that this is not due to suppression of the obligate symbiont leading to a reduction in fitness (Koga et al. 2003). Surprisingly, infection with X-type or *Hamiltonella* actually leads to an increase of *Buchnera* population levels in younger aphids. As these two symbionts are both infections in a single aphid genotype (218), there are two possible explanations. This genotype was originally collected with, and cured of, a double infection of X-type and *Hamiltonella*, so there may be a coevolved tripartite interaction between aphid genotype, facultative symbiont and obligate symbiont. This could result in the aphid up-regulating *Buchnera* densities in response to symbiont infection. Another possibility is that the *Buchnera* strain in this background responds to the presence of a facultative symbiont by increasing its population size in young aphids. In either case, the density of *Buchnera* cells in infected aphids is comparable to uninfected aphids once the aphids are adult, and we do not know what, if any, the ecological effect of this pattern may be.

One caveat to our study is the use of DNA densities to measure symbiont populations. DNA extractions show the presence and copy number of symbiont genes, but do not guarantee that the microbes are still alive at time of sampling. Using RNA would create a more accurate measure of symbiont vitality, and as a result our decreased

populations of *Buchnera* may actually overestimate the numbers of live symbiont cells. In addition, there may be more than one copy of the *Buchnera* genome per cell, again overestimating the number of *Buchnera* cells in these samples (Martinez et al. 2014). This would be consistent across all our samples, but it is important to note that one gene copy in *Buchnera* does not necessarily equate to one symbiont cell.

The ability of facultative symbionts to protect obligate nutritional endosymbionts from heat stress has implications for the spread of both secondary symbionts and insects themselves under changing climactic conditions. Differing ecological pressures can affect facultative symbiont frequencies in insect populations, a fact most clearly seen in symbionts that protect against natural enemies such as parasitoid wasps. Levels of protective symbionts rise when there are high densities of parasitoids (Hansen et al. 2007; Oliver et al. 2008). Temperature and climate do also have the potential to affect symbiont frequencies (Tsuchida et al. 2002) and, correlatively, levels of the heat-protective symbiont *Serratia* are high in aphid populations in the Southern USA (Chen and Purcell 1997; Montllor et al. 2002). Such trends can be difficult to extract from genotypes and locations as aphid races tend to be strongly linked to specific host plants (Tsuchida et al. 2002; Ferrari et al. 2012; Brady et al. 2014), and symbiont infection to aphid race (Leonardo and Muiru 2003; Ferrari et al. 2012), meaning that although external stresses may be affecting symbiont frequencies, any patterns may be obscured by strong host plant specificity. Future climate change may, however, give additional advantages to symbiont-infected aphids and drive insect spread under less benign conditions.

Parasitism, temperature and host plant can all potentially drive symbiont frequencies in field aphid populations, but in general all of the symbiont species are at intermediate levels and aphid populations often contain diverse assemblages of symbionts (Ferrari et al. 2012; Smith et al. 2015). Populations generally contain multiple different combinations of the eight symbiont species, suggesting that, on a population level, the aphids have the ability to respond rapidly to different external biotic and abiotic pressures (Russell et al. 2013). As the climate warms, these insects may take advantage of their diverse array of symbionts to adapt rapidly to changing climatic conditions, facilitating their own survival and spread.

Chapter 5

Competition and loss in superinfections of aphid endosymbionts

Abstract

The success and spread of many insects is facilitated by their partnerships with endosymbiotic microbes. Non-obligate vertically-transmitted bacteria are found at intermediate levels in insect populations, and can protect their hosts against environmental stresses. Many insects are infected with more than one species of symbiont, yet the interactions between them are poorly understood. We use pea aphids (*Acyrthosiphon pisum*) to investigate interactions between multiple symbionts. Manipulating three common aphid endosymbionts, X-type, *Spiroplasma* and *Regiella insecticola*, we use hemolymph transfers to create novel superinfections and follow the aphids through eight generations of vertical transmission, using qPCR to measure symbiont population densities. We find an unstable partnership where there is competition between, and subsequent loss, of symbionts. Repeated loss of X-type and *Regiella* suggests that, despite superinfections being common in the field, rapid competition may occur between some combinations of symbiont species. Population densities of *Regiella* increase in the presence of X-type, corresponding with the loss of the double infection and showing that symbionts can respond to the presence of a competitor in a way which could affect symbiont spread in aphid populations. This suggests a mechanism that could drive loss and replacement of symbionts in the field, altering population frequencies and consequently the spread of ecologically relevant traits.

Introduction

Host-associated communities of symbionts are common across plants (Berendsen et al. 2012; Turner et al. 2013) and animals; including vertebrates (Martin et al. 2007; Ley et al. 2008; Peterson et al. 2009) and invertebrates (Fraune et al. 2009; Thurber et al. 2009; Duron and Hurst 2013). These communities are termed 'microbiomes', and are assemblages of microbes closely associated with their host (Peterson et al. 2009). They can affect host development (Montgomery and McFall-Ngai 1994; Pradeu 2011), immunity (Mazmanian et al. 2005), behaviour (Markov et al. 2009; Sharon et al. 2010; Heijtz et al. 2011), survival (Oliver et al. 2010) and reproduction (Sinkins et al. 1995; Frank 1998; Markov et al. 2009). In many cases, information is lacking about how stable these communities are over time, how similar they are within and between populations, and the effect of extinction and invasion on community dynamics and host-associated effects (Fierer et al. 2012; Lozupone et al. 2012; Sanders et al. 2014). Understanding how these microbes form communities, and how the host and the microbes interact, is vital to understanding the evolution and ecology of organisms across all taxa. One of the best studied groups for microbe-host interactions are the insects and other arthropods, in part because of their ease of study and relatively simple microbial communities (Brownlie and Johnson 2009; Kikuchi 2009; Goodacre and Martin 2012). Insects are commonly infected with several facultative microbial endosymbionts (Kikuchi 2009) which can confer ecologically important traits (Oliver et al. 2010). These bacteria are generally transmitted vertically, from parent to offspring, but can also be transmitted horizontally between insect individuals and lineages. While vertical transmission often has a success rate of nearly 100% (Darby and Douglas 2003; Degnan and Moran 2008; Vorburger 2014), horizontal transmission is a riskier method of spread (Darby and Douglas 2003).

Horizontal transmission allows symbionts to spread into different insect lines, and even different species (Kyei-Poku et al. 2005; Fukatsu et al. 2007; Simon et al. 2011; Anderson et al. 2012; Patot et al. 2012; Karimi and Darsouei 2014). It correspondingly gives host insects access to a wide gene pool of potential endosymbionts, conferring the ability to pick up whole metabolic pathways and ecological phenotypes rapidly

(Moran 2007; Kikuchi et al. 2012). These facultative symbionts can affect host survival against natural enemies (Haine 2008; Oliver et al. 2008; Teixeira et al. 2008; Xie et al. 2010; Kaltenpoth and Engl 2014) and abiotic stresses (Montllor et al. 2002; Neelakanta et al. 2010), as well as performance on specific host plants (Ferrater et al. 2013; Hansen and Moran 2014) and even insect behaviour (Hosokawa et al. 2008; Dion et al. 2011; Goodacre and Martin 2012). Horizontal transmission of endosymbionts in many insects has occurred recently and repeatedly (Russell et al. 2003; Ahmed et al. 2013; Duron and Hurst 2013) and allows beneficial infections to sweep through populations according to the prevalent selection pressures (Oliver et al. 2008; White 2011).

But despite this dynamic picture of insect symbiont frequencies and dynamics, little is known about the actual process of horizontal transmission, what factors affect its success, and how stable symbiont infections and communities are formed (Moran and Dunbar 2006; Anderson et al. 2012). There are several plausible hypotheses for mechanisms of horizontal transmission, with varying levels of support. There is some evidence that symbionts can transfer through parasitoid wasps and predators (Kyei-Poku et al. 2005; Jaenike et al. 2007; Ahmed et al. 2013; Vorburger 2014), close proximity or host plant sharing (Patot et al. 2012) and sexual reproduction (Moran and Dunbar 2006; Vorburger 2014). Once the endosymbiont successfully invades a novel host, it must establish itself to spread vertically into the next generation. Insect immune systems can target endosymbionts, controlling their proliferation (Feldhaar and Gross 2008; Login et al. 2011; Laughton et al. 2013). Symbionts must successfully adapt to their new environment (Feldhaar and Gross 2008) or evade insect immunity (Gottlieb et al. 2008) in order to establish.

Insect facultative symbionts are ubiquitous, with frequencies of up to 100% in some populations (Koga et al. 2007; Martin et al. 2013; Russell et al. 2013; Karimi and Darsouei 2014), and so it is likely that horizontally transferred microbes may find themselves contending with a resident endosymbiont. If the invasive symbiont successfully establishes the result is a symbiont superinfection: a community of host-associated microbes. These multiple infections are relatively common in insect populations, with individuals infected with dynamic communities of symbionts (Ferrari

and Vavre 2011; Martin et al. 2013; Russell et al. 2013; Smith et al. 2015). The interactions between multiple species of symbiont are likely to have implications for the strength and direction of symbiont-mediated host phenotypes. As such, ecological selection acts on the 'holobiont', comprising the insect and all associated microbes, and interactions between coexisting symbionts and the host affect the strength of selection on the insect. Despite superinfections being common in insect populations (Mouton et al. 2003; Duron et al. 2008; Sirviö and Pamilo 2010; Toju and Fukatsu 2011; Martin et al. 2013), little is known about the interactions between the different symbionts, and how these themselves may affect the host (Vautrin et al. 2008; Vautrin and Vavre 2009; May and Nelson 2014).

Theoretically, these multiple infections are difficult to maintain through vertical transmission as all species present must spread together to reach the next generation as a stable co-infection (Mira and Moran 2002). To ensure maintenance, infections must provide benefits to the host that negate any negative effects of competition, antagonism or resource depletion caused by the superinfections (Vautrin et al. 2008; Vautrin and Vavre 2009). The ability of a microbe to respond to competing symbionts may avoid overexploitation of host resources and benefit all parties involved (Keller and Surette 2006; Alizon et al. 2013).

Aphids such as the pea aphid, *Acyrthosiphon pisum*, are a model species for studying endosymbiosis in insect systems. Eight facultative symbiont species are found at intermediate frequencies in aphid populations (Oliver et al. 2010; Ferrari et al. 2012; Russell et al. 2013; Smith et al. 2015), and superinfections are common (Ferrari et al. 2012; Russell et al. 2013). Several of their symbionts are relatively well characterised in terms of host-associated ecological effects and can affect insect protection against natural enemies and other stresses (Oliver et al. 2010; Ferrari and Vavre 2011; Montllor et al. 2002). Pea aphids are an ideal model insect due to their rapid asexual reproduction, easy manipulation of symbionts, and because the well-understood system is unlikely to be infected with as-yet undiscovered complicating microbes (Gauthier et al. 2015). Their known symbionts include five gamma-proteobacteria (*Hamiltonella defensa*, *Regiella insecticola*, *Serratia symbiotica*, *Rickettsiella* sp. and a

species known as X-type), one *Spiroplasma* and a *Rickettsia* and there is evidence of widespread transfer of symbionts between different aphid lineages (Russell et al. 2003; Oliver et al. 2010; Henry et al. 2013; Smith et al. 2015). The mechanisms of horizontal transmission could include transfer through the plant (Caspi-Fluger et al. 2011), sexual reproduction (Moran and Dunbar 2006) and spread by parasitoid wasps through a 'dirty needle' effect (Gehrer and Vorburger 2012) but evidence is so far lacking for the potentially frequent gain and loss that supports dynamic endosymbiont populations (Smith et al. 2015). Aphids are commonly infected with multiple species of symbiont (Ferrari et al. 2012) and there is evidence for interactions between the species that can affect host-conferred traits (Cloutier and Douglas 2003; Oliver et al. 2006; Guay et al. 2009).

We explore horizontal transmission, competition and the maintenance of multiple infections using pea aphids and three of their common facultative endosymbionts, *Regiella*, *Spiroplasma* and X-type. All three symbionts are found at intermediate infection frequencies in aphid populations, ranging from 0-68% (Ferrari et al. 2012; Russell et al. 2013). In terms of ecological effects, the presence of X-type in aphids infected with *Spiroplasma* can improve aphid resistance to parasitoid wasps, fungi and heat shock, but impose a heavy fitness cost on its host (Chapter 2). *Regiella* is well known for providing resistance to fungal pathogens (Scarborough et al. 2005) but some strains are also implicated in heat shock protection (Chapter 4), parasitoid resistance (Vorburger et al. 2010) and performance on *Trifolium pratense* (Tsuchida et al. 2011). X-type and *Regiella* are gamma-proteobacteria; phylogenetically sister species (Degnan et al. 2010, A. McLean, C. Godfray, J. Ferrari, pers. comm.) which confer similar ecological effects (Chapter 2; Chapter 4; Scarborough et al. 2005). X-type's protection against heat shock and fecundity cost are confirmed in Chapter 4, although its other effects are so far only shown when X-type is in co-infections with *Spiroplasma* (Chapter 2). Less is known about the phenotypes conferred by *Spiroplasma* in isolation, but some strains can protect against fungal pathogens (Łukasik et al. 2013b) and affect reproduction by initiating male-killing in sexual populations (Simon et al. 2011).

We investigate interactions between different symbiont species in a single aphid host by creating superinfections of aphid symbionts to test the hypothesis that competition between symbiont species can affect symbiont transmission. We use hemolymph transfers to mimic horizontal transmission, track symbiont loss through vertical generations and measure symbiont population densities after infection using quantitative qPCR. We hypothesise that i) establishment success will be lower when creating superinfections due to the presence of a pre-infection symbiont, ii) that superinfections will be less stable than symbiont single infections and iii) that symbiont population levels will correspond to symbiont loss or maintenance. We aim to explore the success of establishment and long-term stability of superinfections in the pea aphid model to gain understanding about how such mechanisms may be potentially affecting host-associated microbial communities in other, more complex systems.

Methods

Aphid lines

For all experiments three aphid genotypes were used, codes 200, 301 and 322. Aphids reproduce asexually under summer conditions, and each of these lines is descended from a single individual collected in England from *Medicago sativa* (200) or *Trifolium pratense* (301, 322). Genotype 200 is naturally uninfected with facultative symbionts, genotype 301 is naturally infected with *Regiella insecticola* and genotype 322 is naturally infected with X-type and *Spiroplasma*. The strains of the three symbionts used are the natural infections from genotypes 322 and 301 (Table 5.1). Aphid lines were also tested for presence of *Hamiltonella defensa*, *Serratia symbiotica*, *Rickettsia* and *Rickettsiella viridis* (Tsuchida et al. 2010; Ferrari et al. 2012) and we did not detect any of these symbionts. All experiments were conducted on *Vicia faba* ("The Sutton") leaves or seedlings. Pea aphids are highly specialised on different host plants (Peccoud et al. 2009; Ferrari et al. 2012), but lines from different host races all tend to perform well on *V. faba*, and it is often used as a universal host (Ferrari et al. 2008).

Natural genotypes 301 and 322 were cured of *Regiella* and X-type respectively by feeding juvenile aphids on *V. faba* leaves suspended in an antibiotic solution of 1% Ampicillin, 0.5% Gentamicin and 0.5% Cefotaxime (McLean et al. 2011). Cured lines were maintained for over a year before being used for experiments. *Spiroplasma* cannot be removed by this method, so genotype 322 is always infected with this symbiont.

Aphids were maintained on *V. faba* leaves with stalks placed in 2% agar inside plastic petri dishes (90mm, Sterilin Ltd.) for all experiments. Conditions were 20°C and long-day light conditions of 16h:8h light:dark with a humidity of 40 ± 15%.

Table 5.1. Natural aphid line used for manipulation experiments. Lines were kept in the lab for at least a year before starting experiments and are descended from a single individual.

Genotype	Natural symbiont infection	Collection location and date	Year cured	Collection host plant
200	Uninfected	Lincoln, 2012 53.23°N, 0.54°W	N/A	*Medicago sativa*
301	*Regiella insecticola*	Windsor, 2010 51.48°N, 0.61°W	2012	*Trifolium pratense*
322	X-type + *Spiroplasma*	Oddington, 2008 51.83°N, 1.20°W	2011	*Trifolium pratense*

Artificial transfer of symbionts

We created novel infections of different combinations of the symbionts to explore establishment success into 'uninfected' or 'infected' host backgrounds. 'Uninfected' refers to aphids not infected with the facultative symbionts X-type or *Regiella*, the two gamma-proteobacteria. We employ it for simplicity, as all aphids used are infected with the obligate symbiont *Buchnera*, and genotype 322 is always also infected with the symbiont *Spiroplasma*, which cannot be cured. 'Infected' hosts were infected with either X-type or *Regiella* prior to injection, and as well as *Buchnera* and potentially *Spiroplasma*.

We hypothesised that establishment success would be lowest when creating superinfections, due to high densities of symbiont already in the host and competition for space and resources. Horizontal transfer of symbionts was accomplished using hemolymph transfers from infected aphids.

Manipulating endosymbiont infections across genetically identical aphid lines is a common method of characterising symbiont infections (Koga et al. 2003, 2007; Leonardo 2004; Simon et al. 2007). In a laboratory setting, horizontal transfer of symbionts is imitated using hemolymph transfers from endosymbiont-infected aphids to establish new infections in novel hosts (Koga et al. 2007; Tsuchida et al. 2011). Generally, aphids acquire these infections with relative ease (Russell and Moran 2005), although success can vary by aphid genotype or symbiont injected (Russell and Moran 2005).

Glass needles were created using a Flaming/Brown micropipette puller (Sutter Instrument Co. model P-97) and Kwik-Fil™ borosilicate glass capillaries (1B100-4, World Precision Instruments, 1mm diameter) on program no. 4. Symbiont-infected donor aphids were immobilised upside-down in a petri dish using masking tape, and hemolymph was extracted, using the needles, under a microscope. This was immediately injected into 4-day old recipient aphids. Successfully injected aphids were kept in groups on *V. faba* leaves suspended in 2% agar in petri dishes until 3 days after injection. Surviving juveniles were then separated into individual dishes for maturity

and the start of reproduction, and one of their later progeny (after more than 10 offspring had been produced) was moved into its own dish and the original adult discarded (Generation 0). The young aphid (Generation 1) was then allowed to mature, and once it had produced offspring it was tested for the injected symbiont (X-type or *Regiella*) using symbiont-specific PCR (Table 5.2, see figure 5.1). Samples were extracted using the DNeasy Blood and Tissue Kit (Qiagen) according to manufacturer's instructions with these additions: add 2.5µl RNAse A (Qiagen, 100mg/ml) after the 56°C incubation step and incubate at room temperature for 2 minutes, and the final spin cycle was lengthened to 2 minutes instead of 1 minute. PCR and gel electrophoresis was run as in Chapter 4. Generation 1 aphids which tested positive for the symbiont were counted as successful transfers, and the proportion of horizontal transmission calculated from these aphids. A subset of successfully-injected aphids across the different injections was also tested for *Spiroplasma*. Not all aphids were tested for the symbiont as initial tests on 15 aphids showed high transfer and maintenance (6/6 positive first generation transfers alongside X-type into genotypes 301 or 200, 5/5 *Spiroplasma* infections maintained through injection of *Regiella* into 322 backgrounds and 4/4 infections maintained after the loss of X-type). During later tests, we found that all aphids of genotype 322 were consistently positive for *Spiroplasma* (19/19 samples), and that the symbiont transferred alongside X-type into genotypes 301 and 200 90% of the time (18/20 samples).

There were twelve different types of infections: injections of either X-type and *Spiroplasma* or *Regiella* into each of the three lines that were uninfected with the other symbiont species and the same injections into each of the three genotypes pre-infected with the competing symbiont. The donor aphids for X-type and *Spiroplasma* were either the 322X or 200X lines, and the donor aphids for *Regiella* were either the 301R or 200R lines (Table 5.3), although in both cases the same strain of each symbiont was being transferred. Artificially created infections into uninfected backgrounds were left for a minimum of eight generations before being used as donor or recipient aphids. Aphids were tested in the first generation after injection (Generation 1), so the recipient aphid must have survived the hemolymph transfer and

produced offspring which reached adulthood. One of their later (after 10 offspring produced) offspring was tested. Sample sizes for horizontal establishment were between 35 and 93 injections per aphid recipient genotype/symbiont combination (i.e. a minimum of 140 tested injections for *Regiella* or X-type and *Spiroplasma* into either uninfected or infected backgrounds, see Table 5.3 for details).

Table 5.2. PCR primers used for symbiont detection.

PCR primers					
	Forward	**Sequence**	**Reverse**	**Sequence**	**Reference**
X-type	10F	5' - AGTTTGATCATGGCTCAGATTG - 3'	X420R	5' - GCAACACTCTTTGCATTGCT - 3'	Ferrari et al. 2012
Regiella	10F	5' - AGTTTGATCATGGCTCAGATTG - 3'	U433R	5' - GGTAACGTCAATCGATAAGCA - 3'	Ferrari et al. 2012
Spiroplasma	10F	5' - AGTTTGATCATGGCTCAGATTG - 3'	TKSSsp	5’ – TAGCCGTGGCTTTCTGGTAA – 3’	Fukatsu et al. 2001

Table 5.3. Aphid lines and symbiont combinations created through injecting. Aphids in the first generation after injection were used; the original aphid (Generation 0) must have survived hemolymph transfer and produced offspring (Generation 1), one of which was tested. N is the number of Generation 1 aphids which were positive for successful transfers, out of the total tested giving a proportion of successful transfers. 322 lines are also infected with *Spiroplasma*, and this symbiont commonly transfers along with X-type (see methods).

Recipient Genotype	**200**	**301**	**322**
Natural symbiont	None	*Regiella*	X-type and *Spiroplasma*
Target Symbiont Status			
None	Name: 200 *Natural*	Name: 301 *Cured*	Name: 322 *Cured*
X-type	Name: 200X Injections X-> 200 *N = 22/35 (63%)*	Name: 301X Injections X-> 301 *N = 29/74 (39%)*	Name: 322X Injections X-> 322 *N = 9/50 (18%)*
Regiella	Name: 200R Injections R->200 *N = 22/37 (60%)*	Name: 301R Injections R-> 301 *N = 28/59 (47%)*	Name: 322R Injections R-> 322 *N = 14/47 (30%)*
X-type and *Regiella*	Name: 200XR Injections X-> 200R *N = 15/93* Injections R-> 200X *N = 9/69 (13%)*	Name: 301XR Injections X-> 301R *N = 10/84* Injections R-> 301X *N = 1/38 (3%)*	Name: 322XR Injections X-> 322R *N = 2/60* Injections R-> 322X *N = 10/53(19%)*

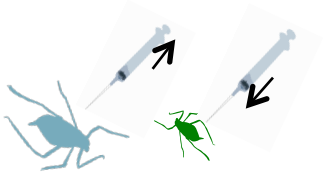
Maintenance of new infections

After creating artificial superinfections of X-type, *Regiella* and *Spiroplasma*, we followed the infected aphids through eight vertical generations to look at the stability of the infections. We hypothesised that these superinfections would be less stable than single symbiont infections, which have an essentially 100% vertical transmission rate (Darby and Douglas 2003; Vorburger 2014). New superinfections were kept for eight generations, and a single aphid tested each generation once it had reached maturity and produced offspring. As expected, we noted no loss of the injections involving X-type or *Regiella* alone or with just *Spiroplasma* and we did not track these infections past three generations. Sample sizes for vertical transmission were 23 when X-type was injected into aphids infected with *Regiella*, and 15 when *Regiella* was the injected symbiont. This is the number of superinfected lines that successfully survived for eight generations after injection, although the superinfections themselves were often lost rapidly, and data were not further split by aphid recipient genotype due to low sample sizes.

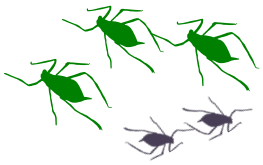
Figure 5.1. Flow diagram of methods.

1. Aphids injected with hemolymph from a donor aphid

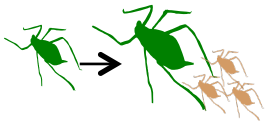
Generation 0



2. Surviving aphids split onto individual dishes

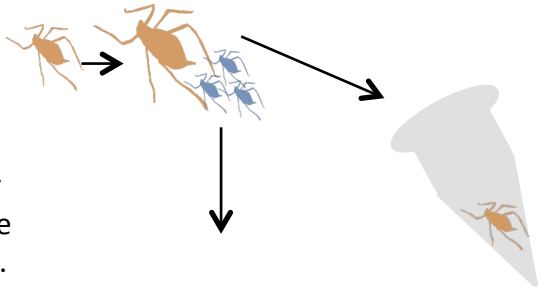


3. Aphids allowed to grow and to reproduce



Generation 1

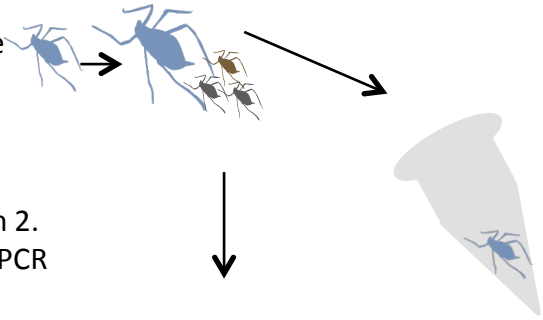
4. One later offspring taken, allowed to grow and reproduce



5. Adult tested for symbiont presence and kept for qPCR.

Generation 2

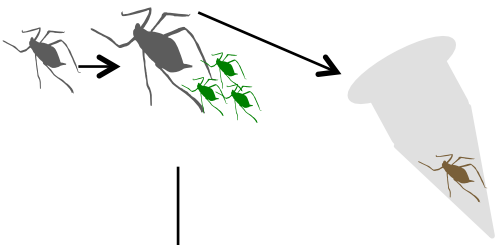
6. If adult tests positive, keep one offspring until reproduction



7. Test Generation 2. Sample kept for qPCR

Generation 3

8. Test Generation 3. Sample kept for qPCR



9. Keep superinfections. Test each generation for symbionts until Generation 8. Discard.

Generations 4-8

Symbiont population densities inside individual aphids

To look at the dynamics of the symbiont species when faced with a competitor compared to in isolation, we measured symbiont population densities under different types of infection. We hypothesised that symbiont population sizes would correspond to loss or maintenance of the infection. Adult aphids were taken for DNA extraction in the first three days after reproduction. Samples were extracted using the DNeasy Blood and Tissue Kit (Qiagen), as explained above. DNA was diluted 1:4 with distilled water and stored at -20°C until use. Samples were taken for the first three generations after infection, and symbiont densities measured for up to three generations, or until one generation after loss, whichever came first. Samples were run in duplicate using Syber® Green reagent on a StepOnePlus™ Real Time PCR machine (Applied Biosystems) as in Chapter 4. For this comparison, identical threshold and baseline levels were set across plates. A single standard curve was used per primer because of rapid degeneration of diluted standard samples between runs; some samples were run across multiple plates to ensure consistency. Samples with Ct values over 30 were classed as negative, confirmed by our negative controls, as this is below the threshold of copy number detection. Technical duplicate repeats with >1.5 Ct difference were discarded.

Final DNA concentration per sample was calculated by comparing sample Ct values to the equation of the standard curve of known concentrations. Avogadro's constant (the number of particles in one molar concentration of a solution) was used to calculate absolute DNA copy numbers. Facultative symbiont copy numbers were normalised to an aphid control gene to account for sample extraction efficiency, and aphid size giving final results in terms of bacteria to aphid cell ratio. Injections into the three different aphid genotype recipients were pooled for qPCR analysis due to low numbers of positive infections in some genotype/symbiont infection combinations, and total sample size is 10-15 aphids over three vertical generations. Four samples of the two original unmanipulated aphid genotypes (322 and 301) were also tested to compare natural and artificial infection symbiont densities. There are no data for the samples where symbionts were replaced by the invasive symbiont as this work was

only planned when these aphids were no longer available, and because symbiont replacement always happened after the three generations that were sampled for qPCR.

Table 5.4. qPCR primers used.

qPCR primers					
	Forward	**Sequence**	**Reverse**	**Sequence**	**Reference**
X-type	gyrB-X397F	5' - TGG ATT GGC TGG TGA AAG AAT - 3'	gyrB-X466R	5' - TTC ATC TCT CCC AAA CCT TTA TAG - 3'	J. Ferrari, pers.comm.
Regiella	Regiella gyrB F	5' - CGT CGC TTA GCT GTT GAC AGT T - 3'	Regiella gyrB R	5' - CGC GCC GAG ACT CTT TTA CT - 3'	J. Ferrari, pers.comm.
Spiroplasma	ApDnaAqF2	5' - TTA ATG AAG TTT CAA AAT CTG GTG -3'	ApDnaAqR2	5' - GTT AAC AAA CAA AAA CAA ATT GTT ATT AC - 3'	J. Hrcek, pers. comm. 2014
Ef1alpha	EF1a 107F	5' - CTG ATT GTG CCG TGC TTA TTG - 3'	EF1a 246R	5' - TAT GGT GGT TCA GTA GAG TCC - 3'	Laughton et al. 2013

Statistical analysis

Data were analysed using the R package 2.13.1 (R Core Team 2012). Establishment data consisted of the proportion of Generation 1 aphids that tested positive for the injected symbiont, out of the total number of Generation 1 aphids tested, and were analysed using binomial general linear models. Data were initially analysed in a single model, which showed that there was a significant difference between establishment successes between injections into uninfected and infected backgrounds. For the initial model, main factors of type of infection (into uninfected or infected backgrounds), recipient genotype and symbiont injected were the explanatory variables. For further analysis the infections were split by recipient infection status, and donor genotype was removed as it was not significant in the original model. The separate models included recipient genotype and symbiont injected as explanatory variables, as well as the pairwise interaction.

To explore the maintenance of the superinfections, the proportional loss of the X-type or *Regiella* was noted at each generation. Data were analysed using Kaplan-Meier survival curves with the symbiont injected as the explanatory variable and amount of loss at each generation compared to amount of loss over eight generations as the formula. Significance was attributed using a G-rho test on the Kaplan-Meier estimates of survival.

The qPCR data were separated by symbiont into three data sets, densities of X-type, *Regiella* or *Spiroplasma* and analysed using ANOVA tests. The explanatory variable in all cases was the type of symbiont infection ('natural' unmanipulated infections, 'artificial' infections into uninfected backgrounds, 'superinfection' with X-type or *Regiella* injected, or the first generation after loss). Data were transformed to meet ANOVA assumptions by square rooting (X-type and *Regiella*) or cube-rooting (*Spiroplasma*) the data. Models were checked by confirming normality of residuals. When there was a significant effect of infection type on symbiont density, posthoc Tukey tests were performed to extract differences between individual types of infections. They could not be compared between symbionts due to different primer

efficiencies, so all analysis investigates symbiont densities within one species, depending on type of infection.

Results

Horizontal establishment

We measured the establishment success of new symbiont infections using hemolymph injections of X-type and *Regiella*. These injections were split into two types: injections into an uninfected background and injections into a background pre-infected with the other. We found no differences in overall establishment success between the symbiont species (χ^2=685.17, p=0.34) but the infections into a pre-infected background were significantly less likely to establish than those into an uninfected background (Figure 5.2. χ2=686.09, p<0.001), suggesting competition or exclusion. For sample sizes and summary statistics please see Table 5.3.

To test whether there was a difference in establishment success between different recipient aphid genotypes, data were split into 'injections into uninfected backgrounds' or 'injections into infected backgrounds' (superinfections) for analysis. Overall, there was a significant effect of recipient genotype during injections into an uninfected background (χ2=376.45, p<0.001) as some aphid genotypes were more receptive to new infections than others, (Figure 5.3). The aphid line with the lowest successful establishment was 322, also the only line infected with the symbiont *Spiroplasma*. There was no effect of injected symbiont (χ2=375.47, p=0.32) and no interaction between recipient genotype and injected symbiont (χ2=372.78, p=0.10). This shows that while there was an effect of aphid recipient genotype on successful symbiont establishment, neither symbiont was inherently more successful at horizontal transfer.

When injecting into the infected background, there was no significant effect of recipient genotype (χ2=292.08, p=0.23) or injected symbiont (χ2=292.06, p=0.88) and no significant interaction between them (χ2=290.49, p=0.21, data not shown).

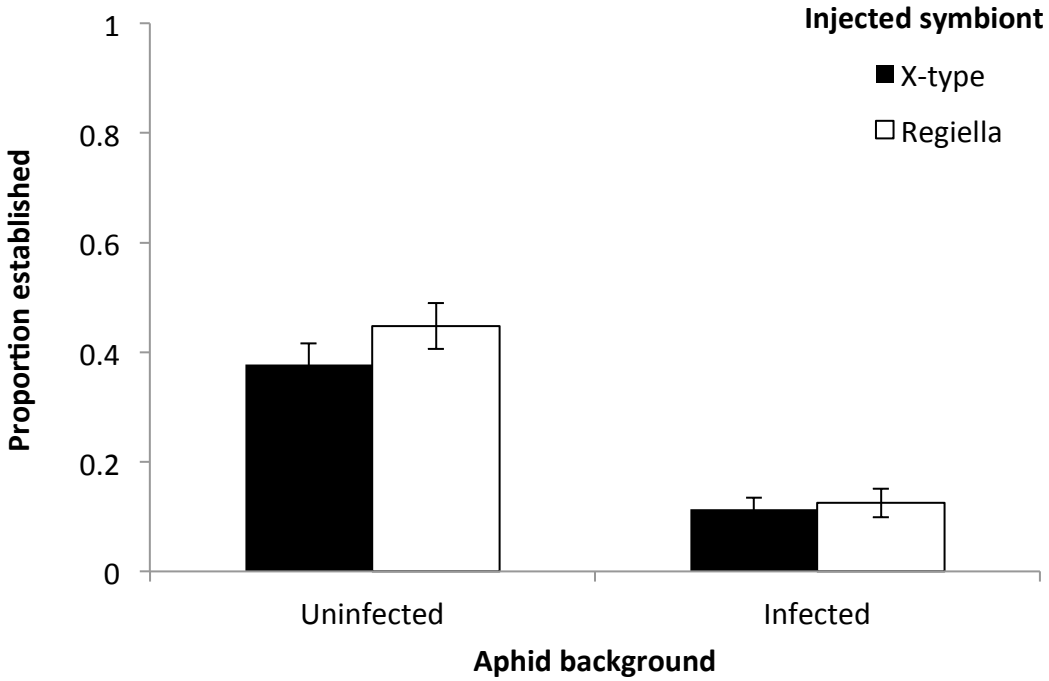


Figure 5.2. Establishment frequencies of X-type and *Regiella* into uninfected or infected aphid lines. *Spiroplasma* commonly transfers along with X-type (see methods). Error bars are binomial errors.

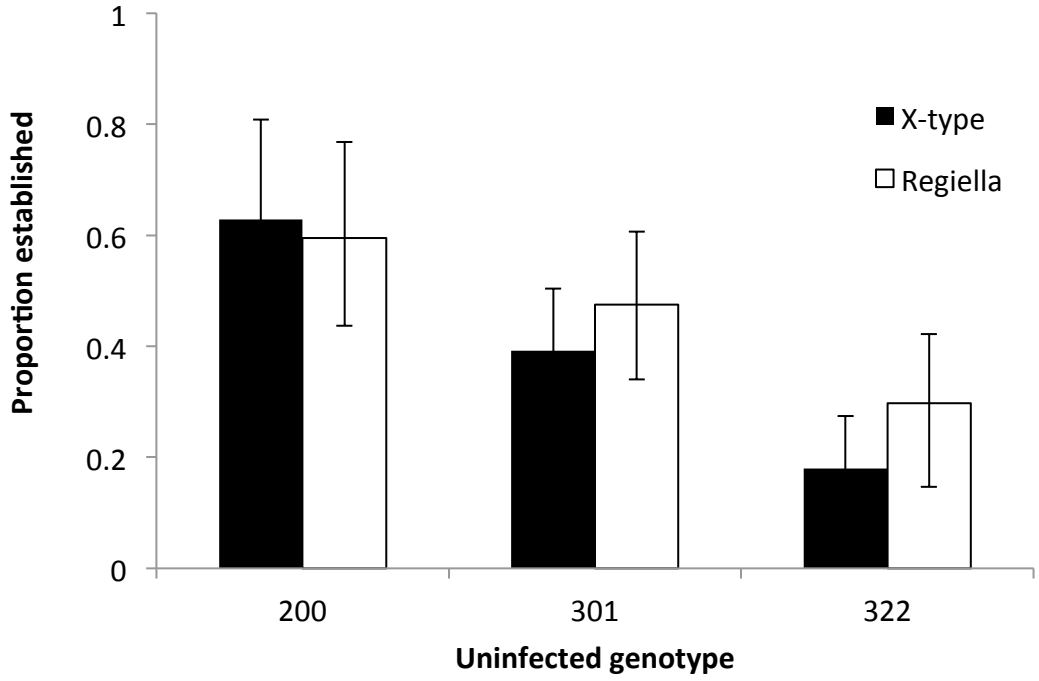


Figure 5.3. Establishment frequencies of X-type and *Regiella* into uninfected lines. *Spiroplasma* commonly transfers along with X-type (see methods), and line 322 is always infected with *Spiroplasma*. Error bars are binomial errors.

Vertical transmission and maintenance of superinfections

Vertical transmission of single symbiont infections is virtually 100% in lab populations (Darby and Douglas 2003; Vorburger 2014) and any loss of symbionts from superinfections is most likely caused, directly or indirectly, by the presence of the other. We tested vertical transmission of superinfections of X-type, *Spiroplasma* and *Regiella* and hypothesised that competition for space and resources may lead to lower levels of vertical transmission.

We found that while there was no difference in horizontal establishment success between X-type and *Regiella*, over the next few vertical generations there was a difference in outcome depending on which symbiont was the resident and which was horizontally introduced. The injected species of symbiont had a significant effect on the length of the superinfection ($\chi 2=4.9$, $p<0.05$, Figure 5.4), so data were then split by injected symbiont.

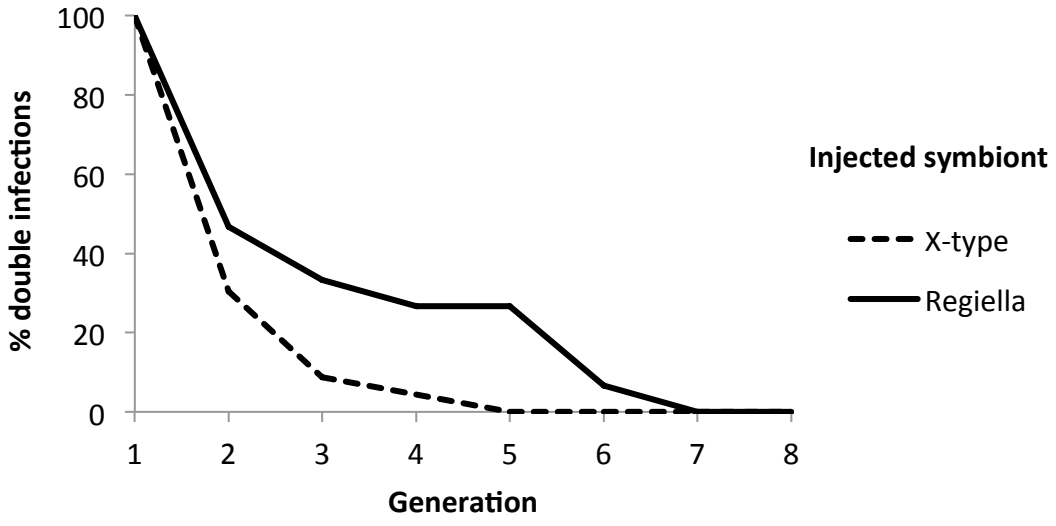


Figure 5.4. Maintenance of superinfection over eight generations when X-type or *Regiella* is the injected symbiont.

Superinfections were tracked from the first generation after successful establishment, ensuring that at Generation 1 all aphids had triple infections of X-type, *Spiroplasma* and *Regiella*. When X-type and *Spiroplasma* invaded a host that was already infected with *Regiella*, 69% of aphids lost X-type in the first generation, and the median number of generations to loss was one. All losses were of X-type; *Regiella* was maintained in all cases. A subset of these aphids was tested for *Spiroplasma*, and all were found to be consistently positive. The results are more complex when *Regiella* was the invader, although once again all double infections with X-type and *Regiella* were lost within eight generations. In the first generation 53% of double infections were lost, all due to the loss of invasive *Regiella*, but by the end of the experiment *Regiella* managed to displace X-type in 40% of cases. Once again *Spiroplasma* was present in all aphids tested. The median time to loss of the superinfection when *Regiella* was the invader was one generation; one generation if *Regiella* was lost but four generations if the loss was of X-type. The data show that if *Regiella* is not immediately displaced from the aphid, it manages to replace X-type in the later generations (Figure 5.5).

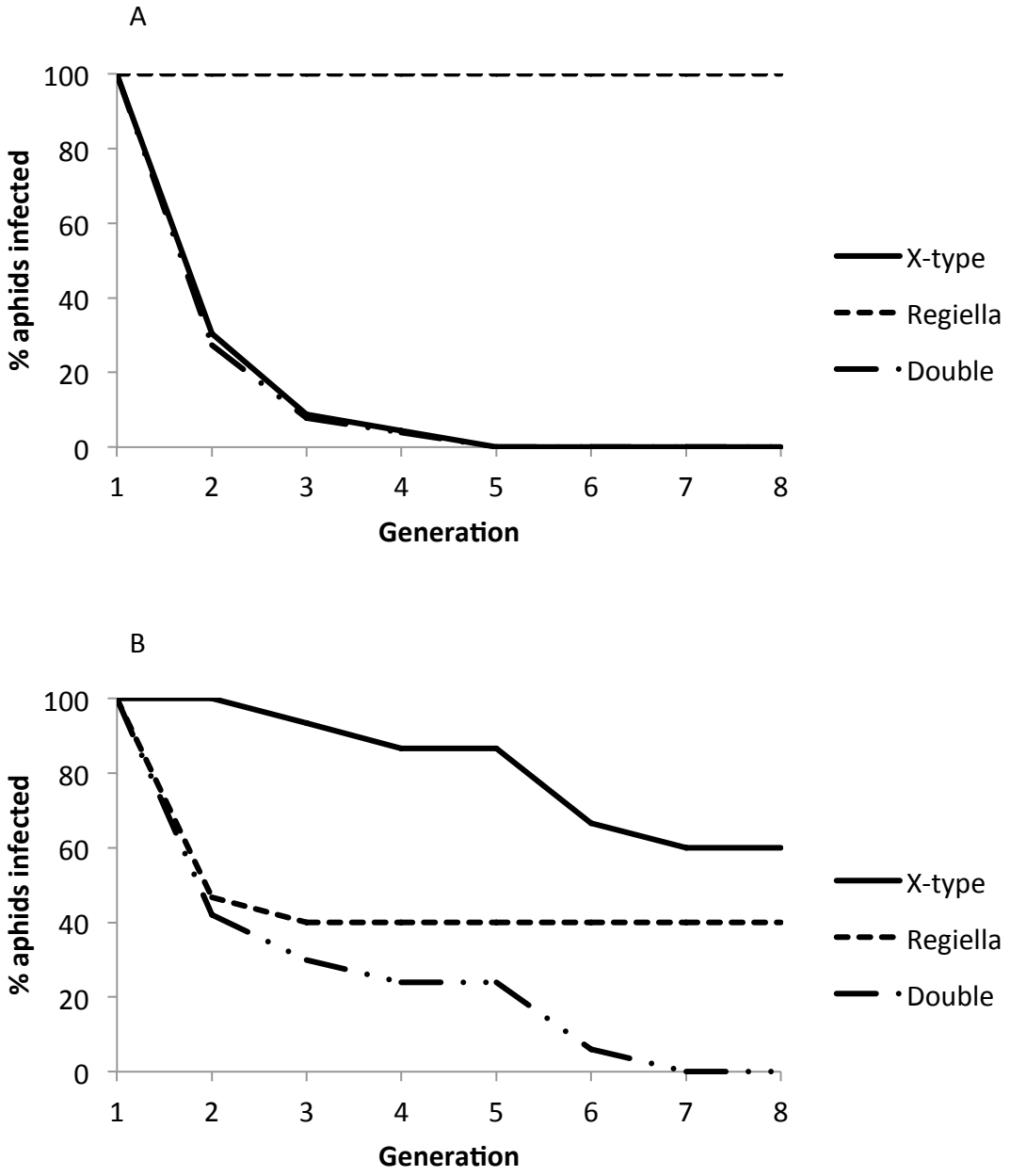


Figure 5.5. Maintenance of X-type, *Regiella* and the superinfection when X-type (A) or *Regiella* (B) invades an infected background. The dashed lines show double infections, while the solid and dotted lines show the frequencies of X-type and *Regiella* respectively. The double infection line is slightly offset to ensure visibility.

Endosymbiont population densities

We measured the population densities of the facultative symbionts X-type, *Regiella* and *Spiroplasma* in natural infections and artificial injections into an uninfected or infected background to explore the effects of symbiont density of dynamics of loss. We hypothesised that *Regiella* population levels would be higher than X-type in superinfections, and that there would be no effect of type of infection on *Spiroplasma* population sizes. Symbiont densities were measured in the first generation after injection and the first generation after loss of invader (Figure 5.6).

We found that different types of infection significantly affected the densities of both X-type and *Regiella* (X-type: $F_{4, 40} = 4.34$, $p<0.05$, *Regiella*: $F_{5, 46} = 3.00$, $p<0.05$) whereas it did not affect the densities of *Spiroplasma* ($F_{5, 47} = 1.53$, $p=0.20$). For X-type population densities, post-hoc Tukey tests showed that there was a significant difference between X-type densities in superinfections depending on whether X-type or *Regiella* was the invader; densities of X-type were lower when it was the invasive symbiont. There were also significant differences between X-type densities in superinfections when it was the invader compared to when it was injected into an uninfected background, showing that there was a significant effect of *Regiella* presence. It was also lower as an invader than in unmanipulated, natural infections. There were no significant differences between levels of X-type in natural and artificial single infections.

For *Regiella*, there was a significant difference to the $p<0.05$ level when it was the invader in superinfections compared to the artificial infections into an uninfected background, driven by high densities in the superinfections. This is the opposite trend to X-type. There were no other significant differences between individual types of infection. As *Spiroplasma* showed no difference in density according to type of infection, post-hoc tests were not performed.

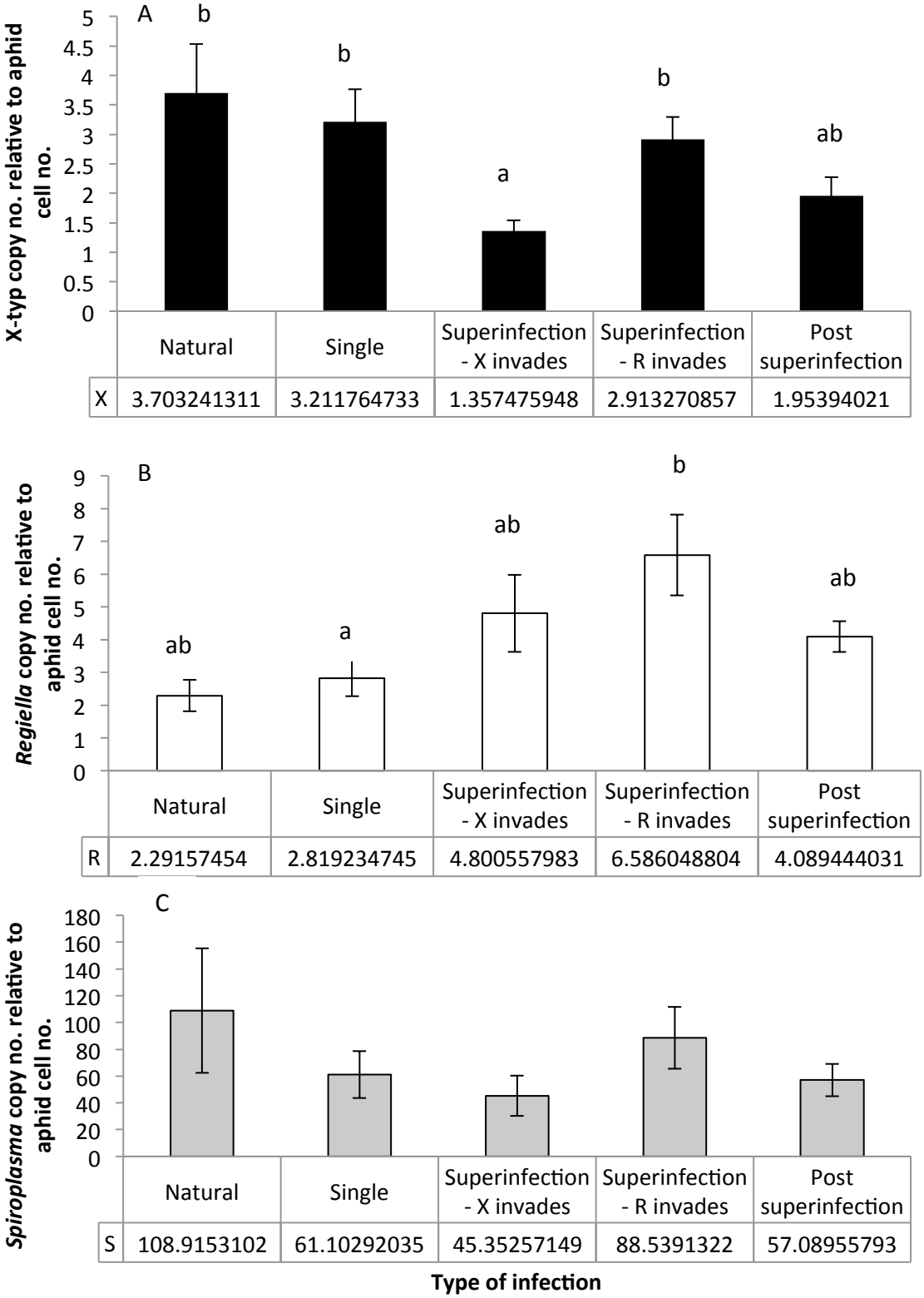


Figure 5.6. Copy numbers of symbionts relative to aphid cell copy number for X-type (A), *Regiella* (B) and *Spiroplasma* (C) in different infections. 'Natural' aphids show unmanipulated infections, while 'single' infections refer to injections into uninfected backgrounds. Single and superinfections were measured in the first generation of successful establishment. Post-superinfection titre was measured in the first generation after loss. Significance between bars was determined through posthoc Tukey tests, and error bars are standard error.

Discussion

We find that the successful establishment of new endosymbiont infections is decreased by the presence of a competitor and that superinfections can be lost through interactions between symbionts. This exclusion and competition is restricted to the two gamma-proteobacteria tested, X-type and *Regiella*, and does not appear to involve the third symbiont, *Spiroplasma*.

Horizontal transmission of facultative symbionts is necessary for establishment in new aphid lineages, and, if successful, is followed by generations of vertical transmission. Recent work implies that horizontal transmission is widespread in aphid field populations (Smith et al. 2015), leading to a dynamic pool of symbiont gain and loss. We find that there is no difference in the overall establishment success between the two aphid symbionts tested, X-type and *Regiella*, but that both symbionts were much less likely to successfully establish in hosts already infected with the other. It may be that there is competition for resources inside the host and that the resident microbe impedes the routes of vertical transmission (Mira and Moran 2002) through niche exclusion. It may also be that the infected host is 'primed' through symbiont infection, leading to higher levels of defence against the invading microbe (Laughton et al. 2013; Hamilton et al. 2014).

In injections into uninfected backgrounds, there was a significant effect of recipient genotype not seen in the superinfection. Each of the three aphid genotypes tested had a general level of susceptibility to horizontal establishment, and interestingly, the genotype infected with *Spiroplasma* was the least receptive to new infections. It may be that this symbiont also affects establishment. In this study it is impossible to decouple *Spiroplasma* presence from aphid genotype, but this would be an interesting angle for future work.

We found no evidence that X-type or *Regiella* established more easily in the aphid genotype that they were cured from originally, supporting the hypothesis that these are not long-term infections, and that there is no extensive coevolution between symbiont and aphid. Although both X-type and *Regiella* were able to establish in new

lineages, both had lower success when introduced to an infected aphid, and all superinfections of these two symbionts were lost within eight generations of vertical transmission. X-type was lost rapidly in every case when it was the invader, whereas *Regiella* was lost in just over half of cases when it invaded. *Spiroplasma* was also present in every superinfection, but we noted no cases of loss. In the field superinfections of aphid symbionts are common, with the average aphid infected with 1.4 facultative symbionts (Ferrari et al. 2012; Russell et al. 2013), although most superinfections only contain zero or one species of gamma-proteobacteria (Simon et al. 2003; Frantz et al. 2009). It is possible that having two symbionts that are closely related may increase pressure for space and resources due to similar niches, or that there is functional redundancy of traits that results in only one being maintained.

The loss of redundant double infections may be ecologically advantageous to the aphid. Both X-type and *Regiella* cause similar ecological phenotypes in their hosts by increasing tolerance to heat stress (Chapter 4) and fungal pathogens (Chapter 2, Scarborough et al. 2005), although infection with X-type imposes a high fecundity cost (Chapter 2) that is not consistently seen in *Regiella*-infected aphids (Russell and Moran 2005, 2006; Ferrari et al. 2007; Vorburger et al. 2010). As a result, aphids infected with both symbionts may not gain any synergistic benefits from the superinfection, and may incur costs. In this experiment we were unable to explore the aphid fitness and phenotypic effects of the superinfection due to the rapid rate of loss, but previous work using a *Serratia-Hamiltonella* double infection found high fitness costs to harbouring two symbionts (Oliver et al. 2006) and it is plausible that there may be a similar effect here.

The instability of this superinfection raises questions about how stable multiple infections of symbionts are in field populations, and about how rapidly they are gained and lost from aphid lineages. Facultative aphid symbionts confer a range of different traits including resistance against natural enemies (Oliver et al. 2003; Scarborough et al. 2005) and abiotic stresses (Montllor et al. 2002; Koga et al. 2003). They can also affect performance on different host plants (Tsuchida et al. 2004), meaning that loss of symbionts can have strong implications for the survival of their hosts when faced with

different environmental pressures. Competition and niche exclusion as seen in these experiments may be a common form of symbiont loss, leading to trait loss, in aphid populations.

By looking at facultative symbiont densities using qPCR, we found that there was no significant difference in populations of X-type and *Spiroplasma* or *Regiella* in unmanipulated natural infections compared to artificial infections. There was no difference in *Spiroplasma* density across any of the different types of infection, but we did find that the population densities for X-type and *Regiella* differed across type of infection. Populations of X-type were significantly decreased in superinfections where it was the invader, whereas populations of *Regiella* were significantly increased, compared to invasions into uninfected backgrounds. The decrease in X-type densities made the population size when it invaded an infected background lower than when it invaded an uninfected background, or in natural infections. This decreased density of X-type correlated with the rapid loss of this symbiont in vertical transmission, whereas the increased population of *Regiella* aligned with its ability to displace X-type frequently when it invades. The changes in density suggest that the loss of symbiont through vertical transmission may be due to niche exclusion through the maternal bottleneck, where relatively few cells can pass (Mira and Moran 2002). This increase in symbiont density was also seen in a previous study looking at another pair of the proteo-gamma bacteria, *Serratia symbiotica* and *Hamiltonella defensa*. In this case, densities of *Serratia* were significantly higher when in co-infections than single infections, whereas the densities of *Hamiltonella* did not differ (Oliver et al. 2006). Unlike in our study, they noted no loss of the superinfection and were able to find increased resistance to parasitoid wasps in superinfected insects, as well as lower fecundity (Oliver et al. 2006).

In our study, *Regiella*'s increase in density correlates to the loss of X-type, and has implications for loss of symbiont-associated traits and potentially aphid fitness. It also raises questions about bacterial communication inside the confines of a host, and how the ability to respond to competing symbionts may be a useful adaptive trait. Free-living bacteria are known to interact with their own and other species through quorum

sensing. Quorum sensing is a communication tool where bacterial species secrete chemical compounds into their environment, allowing cells to detect and react to the density of nearby bacterial cells (Crespi 2001). One species of symbiont *Sodalis*, in tsetse flies, has already been found to have quorum sensing genes (Pontes et al. 2008) and using genetic analysis to identify potential quorum sensing genes may show whether *Regiella* has this ability.

There are limitations to the methods we used to investigate these symbiont superinfections. We only used one strain of each of the three symbionts, and although the results were similar across all three aphid genotypes, additional work would be needed to generalise these interactions across more strains of the symbionts involved. Artificial infections may also not replicate sufficiently how symbionts establish and form communities in more natural populations (Gottlieb et al. 2008), and until more is known about the frequencies and methods of natural horizontal transmission in the field, we cannot confirm that our methods mimic natural symbiont transfers. The qPCR data do show that, there was no difference in density between natural infections and artificial infections into an uninfected background for any symbiont, which suggests that our methods are, at least in part, not drastically disrupting symbiont colonization.

The interactions and loss of these symbionts has implications for how microbes interact in more complex microbiomes and the effects this may have on host phenotype. The discovery that symbionts react differently when invading infected or uninfected novel hosts may be common across other taxa, and affect horizontal establishment and invasion in other symbionts communities.

The aphid model is ideal for studying these types of interaction, and the insect can essentially model a community invasion on a microscopic scale. Invasion dynamics are notoriously difficult to measure in larger ecosystems (Bøhn et al. 2007) due to complex logistics, long timescales and lack of replication, but here we show repeated loss of invasive and resident species depending on initial species composition. This system is also a potential model for community stability and functional redundancy. In wider

ecosystems, diversity of species and a degree of functional redundancy (Walker 1992; Naeem 1998) is important for ecosystem stability and functioning (Naeem 1998), especially in the face of threats (Peterson et al. 1998; Díaz and Cabido 2001). In this example we see loss of trait redundancy and species diversity through community interactions, and it is important to consider that endosymbiont communities are all, to some degree, mediated by the host. Host fitness is plausibly highest when associated with a low-diversity community of symbionts, leading to higher specificity and closer partnerships between host and microbe.

The microbiome is defined as the community of microorganisms associated with a host organism (Fierer et al. 2012). Understanding how the interactions between these symbionts can affect microbiome composition and function is vital to understanding the resilience and stability of the community, and could lead to changes in host ecology (Fierer et al. 2012). Here we show evidence for competition and exclusion between symbionts in an single host, illustrating that interactions between microorganisms can lead to species loss, and so trait loss, in insect lines. These interactions demonstrate the possibility of bacterial competition in larger microbial consortia, and show that even vertically-transmitted symbionts retain the ability to compete with other species to ensure their own transmission.

Chapter 6

General Discussion

Endosymbiosis – complex interactions and ecological implications

Overview

Infections with non-obligate endosymbionts have ecological implications for the survival and spread of insect individuals and populations. This thesis aims to further current understanding of symbiotic relationships by using pea aphid endosymbionts to explore infection phenotypes, their causative mechanisms, and the interactions between the microbes themselves.

I explored symbiont effects through the characterisation of a newly discovered symbiont, X-type, discovering a wide range of protective phenotypes balanced by a high fecundity cost to infection. Focusing on the mechanism behind heat shock resistance, I investigated how the presence of facultative symbionts can protect populations of the obligate endosymbiont, *Buchnera aphidicola* from being killed by heat stress; finding a broad protective phenotype caused by at least two different symbiont species. I finally looked at how two related species of symbiont interact inside a host insect, discovering competition that leads to the loss of the superinfection. I now draw together the overall results from these experimental studies to make more general conclusions about the dynamics and effects of symbiont infections and implications for future research.

Implications of results

The four experimental chapters of this thesis introduce new understanding of endosymbiont interactions, corroborate existing knowledge of endosymbiont effects and open up new avenues for exploration in the future. They confirm and reinforce the fact that endosymbiont infections can be complex: not only affecting insects in variable ways depending on host and symbiont genotype but also contingent on interactions with other microbes and the external environment.

One species, multiple phenotypes

Facultative symbionts in many insect species have been implicated in affecting host reproduction (Werren et al. 2008), behaviour (Dion et al. 2011) and resource use (Hansen and Moran 2014), but a large amount of research has focused on their protective abilities. Symbionts can increase host resistance to fungus (Currie et al. 1999; Kaltenpoth et al. 2005), parasitoids (Xie et al. 2010) and temperature extremes (Neelakanta et al. 2010; Brumin et al. 2011), and, as they are widespread in insects, can therefore have important impacts on population ecology (Hilgenboecker et al. 2008; Feldhaar 2011).

Although several of the eight facultative endosymbionts of pea aphids are relatively well characterised, each species is usually linked to a single ecological host phenotype. *Hamiltonella defensa* is known for conferring resistance to parasitoid wasps (Oliver et al. 2005, 2009; Vorburger et al. 2009), a phenotype that affects parasitoid and aphid behaviour (Dion et al. 2011; Oliver et al. 2012) as well as providing within-host protection (Moran et al. 2005a; Degnan and Moran 2008). *Serratia symbiotica* is known for leading to increased survival (Russell and Moran 2006) and reproduction (Montllor et al. 2002; Russell and Moran 2006) after heat stress, and *Regiella insecticola* for protecting against fungal pathogens (Scarborough et al. 2005). But other work has shown that these phenotypes are less species-specific than perhaps thought. Some strains of *Hamiltonella*, X-type and *Regiella* can also protect against heat shock (Chapter 2; Chapter 4; Russell and Moran 2006), *Serratia* and *Regiella* against parasitoids (Oliver et al. 2003, 2006; Vorburger et al. 2010) and fungus

protection can be conferred by strains of at least five different species of endosymbiont (Chapter 2; Scarborough et al. 2005,Łukasik et al. 2013b). It is clear from Chapter 2 that the symbiont "X-type" has the potential to confer resistance to parasitoid wasps, fungal pathogens and heat stress. The strength of protection varies depending on the symbiont strain, the genotype of the aphid and the interactions between them. This is the first time that a single strain of symbiont has been shown to affect multiple aphid phenotypes, but this is likely to be an artefact of a lack of testing rather than a feature unique to X-type. It is plausible that while some phenotypes are conferred by specific species through adaptation and coevolution between host and symbiont, others may be beneficial by-products of symbiont infection, and confer a more general effect. When testing symbiont-conferred effects in insect hosts, it is essential to consider the broad range of phenotypes that may be caused by infection, and that one symbiont may affect multiple insect traits, affecting the spread of infection through field populations.

Symbiont costs are complex

Facultative symbionts are found at intermediate levels in aphid populations (Ferrari et al. 2012; Russell et al. 2013; Smith et al. 2015) yet often confer beneficial traits to their insect hosts (Oliver et al. 2010). This lack of fixation suggests a trade-off to hosting, which is commonly sought in terms of lifespan, growth rate or fecundity (Koga et al. 2003; Sakurai et al. 2005; Russell and Moran 2006; Laughton et al. 2013). Costs to infection are sometimes found (Chen et al. 2000; Fukatsu et al. 2001; Tsuchida et al. 2013) but are not always evident under laboratory conditions (Russell and Moran 2006; Oliver et al. 2008; Tsuchida et al. 2013). In this thesis I find a fecundity cost to X-type infection across multiple strains of the symbiont, illustrating a potential trade-off to the benefits it provides (Chapter 2; Chapter 4). The aphid lines used for these assays in Chapter 2 were also infected with the *Spiroplasma* symbiont, meaning that the results found could be an interaction between the two facultative symbionts. However, the pairs of lines used in Chapter 4 were free of *Spiroplasma*, and X-type still showed heat shock protection, balanced by a fecundity cost to infection (Chapter 4), suggesting that these traits at least are an effect of this symbiont alone.

I also find loss of symbiont-conferred benefits under more variable conditions. When testing X-type-mediated parasitoid protection under high temperatures (Chapter 3), the parasitoid protection conferred by infection under benign conditions was not maintained. In some cases infected aphids actually tended to be more susceptible to the wasps if they endured heat treatments before parasitism. This may be due to the heat shock protection mechanism explored in Chapter 4, where X-type protects the obligate symbiont *Buchnera aphidicola.* *Buchnera* is an obligate nutritional symbiont responsible for synthesising essential amino acids and nutrients to supplement its host's homogenous diet (Baumann 2005; Feldhaar and Gross 2009). Aphids without *Buchnera* undergo a reduction in fitness and fecundity (Pennacchio et al. 1999; Koga et al. 2003, 2007), but *Buchnera* is susceptible to heat and cells die under high temperatures (Chapter 4; Wilcox et al. 2003; Dunbar et al. 2007). Infection with X-type keeps *Buchnera* populations alive under heat stress (Chapter 4).

Parasitoid wasps require *Buchnera* in the aphids they consume, diverting the essential amino acids to larval growth (Cloutier and Douglas 2003). Protection by X-type under heat shock may be maintaining high population levels of *Buchnera*, allowing the parasitoids to develop more successfully than in uninfected aphids (Chapter 3; Chapter 4). This protective mechanism could contribute to the lack of parasitoid protection in heat-stressed aphids by maintaining the levels of *Buchnera* that the parasitoids require (Chapter 3; Chapter 4).

This result suggests that some symbiont costs can be found under complex environmental scenarios, and that costs and benefits found in the lab may not globally translate to field populations. It is important to consider that there may be as-yet undiscovered detrimental effects to symbiont infections that are only found under certain conditions, helping to explain the observed frequencies in field populations when costs to infection are not found in laboratory assays (Russell and Moran 2006; Oliver et al. 2008; Tsuchida et al. 2013).

Mechanisms behind the phenotypes

The fecundity cost found with X-type infection (Chapter 2; Chapter 4) is not unique (Chen et al. 2000; Koga et al. 2003; Sakurai et al. 2005; Cayetano and Vorburger 2013), and it is generally assumed that the resources consumed by symbiont infection decrease overall insect fitness. One plausible mechanism is that the symbiont suppresses or competes with the obligate symbiont *Buchnera* (Koga et al. 2003; Sakurai et al. 2005), leading to a decrease in aphid reproduction. But as shown in Chapter 4, population densities of *Buchnera* in X-type infected aphids were comparable to uninfected aphids under benign conditions, and any interactions between them seem to be mutualistic (Chapter 4). Further work would be necessary to find out how the symbiont is impacting aphid reproduction, potentially exploring its role in diverting resources from developing embryos or the aphid itself.

It was a natural progression to also investigate the mechanisms behind the variety of benefits caused by infection with X-type, as so little is known about how many symbiont-mediated phenotypes function. Previous research has uncovered the phage that gives *Hamiltonella* its parasitoid protective phenotype (Oliver et al. 2009), but this is one of the few cases where the mechanism of a symbiont-conferred trait is relatively well understood. Chapter 4 begins to explore the mechanisms behind heat shock protection, an effect that can be conferred by strains of at least four pea aphid endosymbionts, X-type, *Regiella insecticola*, *Hamiltonella defensa* and *Serratia symbiotica* (Chapter 2; Chapter 4; Koga et al. 2003; Russell and Moran 2006), although in not all cases (Chapter 4). I found that strains of *Regiella* and X-type increased reproduction compared to uninfected controls after heat shock, and that *Buchnera* levels in these infected aphids were also significantly higher than the uninfected counterparts. Similar results have been found with *Serratia symbiotica* (Montllor et al. 2002) but we found no evidence of long-term lysis of X-type or *Regiella* populations (Burke et al. 2010). In other insects temperature resistance can be caused by an increase of host stress genes from symbiont infection (Neelakanta et al. 2010; Brumin et al. 2011). In this case, future work could focus on the systems that allow the symbionts to protect *Buchnera*; with alternative plausible hypotheses including release

of heat shock proteins or protective metabolites, and transcriptomic techniques may help to explore this mechanism further.

Symbionts in field populations

The frequencies of symbiont infections in aphid populations are likely linked to the balance between the costs and benefits of infection. The fact that symbionts can affect more than one phenotypic trait will affect the spread of the microbes, as interactions between different abiotic and biotic threats will determine their effectiveness in populations. If horizontal transfer is common, the symbionts essentially comprise a 'horizontal gene pool' (Henry et al. 2013) of traits that can be acquired by the insects. Indeed, protective symbionts can sweep through populations when exposed to natural enemy pressure (Oliver et al. 2008; Cockburn et al. 2013) and can even mediate rapid adaptation of pests to engineered pesticides and resistant crops (Kikuchi et al. 2012; Ferrater et al. 2013). While evolution of the pathways required for resistance would take the insects many generations, acquiring a protective symbiont can accomplish the same goal much faster (Kikuchi et al. 2012). It is now not only agriculturally relevant that some natural symbionts can protect against parasitoids (Oliver et al. 2003), but that insects harbouring those symbionts will have a strong ecological advantage if the parasitoids are overused. This could potential lead to an increase of the symbiont frequency in the populations (Oliver et al. 2008) and decrease in the efficiency of the biological control. Symbionts may also be important in spread and survival of their hosts under climate warming (Walther et al. 2002) as they can increase insect resistance under heat (Chapter 2; Chapter 4; Montllor et al. 2002) and affect insect dispersal (Leonardo and Mondor 2006).

Recent work suggests that symbiont frequencies in field populations are variable, with the number of symbiont species within the same aphid lineages fluctuates widely within a year (Russell et al. 2003; Vorburger 2014; Smith et al. 2015). The swift loss of symbionts seen in Chapter 5 corroborates the hypothesis that symbiont populations are dynamic, and provides evidence for loss that could affect population frequencies (Oliver et al. 2008). A picture of rapid gain and loss suggests horizontal transmission of

symbionts between insects, and little is known about the mechanisms involved. Although there are plausible hypotheses involving parasitoid spread (Gehrer and Vorburger 2012), host plant vectoring (Caspi-Fluger et al. 2011) and sexual reproduction (Moran and Dunbar 2006), there is a lack of convincing evidence for high frequencies of horizontal transmission (Russell et al. 2003; Henry et al. 2013; Peccoud et al. 2014; Smith et al. 2015). This is a topic for future exploration, and it will be vital to understand how population symbiont frequencies respond to selection pressures on the 'holobiont', the unit comprising the insect and its associated microbes.

Invasions and establishment, a model for community ecology

For successful horizontal transmission, a symbiont must invade a novel host, adapt to any pre-existing microbes, contend with the insect immune system and position itself for further transmission and spread. I found that establishment into a new aphid lineage was more successful if the insect was uninfected with competing related symbionts (Chapter 5). While invasions into uninfected hosts were relatively easy to achieve, establishing a superinfection was much more difficult.

There are several plausible mechanisms that may mediate this effect. Insects infected with facultative symbionts produce more immune cells than uninfected insects (Kim et al. 2013; Laughton et al. 2013; Gerardo and Parker 2014). This may result in host immunity targeting newly invasive microbes more successfully if they are already infected with a different species. The presence of a pre-existing symbiont could also result in exclusion or competition between the microbes in the early stages of infection, either directly antagonistic or through indirect competition for resources.

The aphid symbiont system provides an interesting and novel model for studying niche dynamics and invasions in empirical systems, traditionally rare due to the practical, and occasionally ethical, difficulty of creating invasion scenarios (Bøhn et al. 2007). Aphids are commonly infected with several species of symbiont (Smith et al. 2015), with average infections per individual in a population ranging from 1.4 species per aphid to 3.7 (Ferrari et al. 2012; Smith et al. 2015). With at least eight different species of symbionts (Gauthier et al. 2015), this is a large range of potential combinations of

species, which could be a useful model for both studying interactions between different taxa of symbionts and how they affect a host. The X-type-*Regiella* interactions were repeatable and simple to create, and are an illustration of invasion dynamics in a relatively simple system.

Host-associated microbes are commonly found in complex communities, which can comprise dozens of different species. They may include symbionts from a range of taxa, which may be obligate, facultative, parasitic or mutualistic to their host. The invasion of new species, the loss or gain of certain taxa and the competition and interactions between them could have strong implications for host health, development and survival (Kikuchi et al. 2012; Blaser 2014). Using a system with a few, controllable species to explore how microbes are able to interact, the mechanisms they use to react to competitors and the stability of insect systems may reveal the types of interactions found in much more complicated communities, and the aphid model is ideal for this work.

Maintenance of symbiont superinfections

The consistent and complete loss of the X-type-*Regiella* superinfection (Chapter 5) was surprising considering that superinfections of different combinations of aphid symbionts are relatively common in the field (Ferrari et al. 2012; Smith et al. 2015). However, multiple infections of the gamma-proteobacteria are found less frequently than expected (Russell et al. 2013), possibly explained by their redundancy of traits, high relatedness and potential competition for limited resources (Chapter 5; Russell et al. 2013). The two gamma-proteobacteria species used, X-type and *Regiella*, are closely related (A. McLean, C. Godfray, J. Ferrari, pers. comm.) and we found that densities of *Regiella* increase in the presence of the competitor, leading to a frequent displacement of X-type. This was not seen for the more distantly related species; we found no effect of competitors on the density of *Spiroplasma* populations in singly or doubly infected hosts and there was no loss of *Spiroplasma* in superinfections with X-type or *Regiella*. Exploring the mechanism behind the interactions between X-type and *Regiella* may explain how symbionts and other bacteria can communicate in

different systems. There are three main hypotheses for the competition and loss we observe, and those are direct antagonism, a cheating mechanism or host-mediated control of redundant symbionts.

Players in endosymbiont communities are strongly affected by the presence and success of other members (Keller and Surette 2006) and it is advantageous for species to be able to react to competitors. Some species of bacteria can detect nearby cells of the same and different species through quorum sensing, where cells constitutively secrete compounds, then measure concentrations in the surrounding environment (Crespi 2001). Quorum sensing genes have been found so far in just one species of symbiont, *Sodalis glossinidius* of tsetse flies (Pontes et al. 2008), but it is possible that *Regiella* is able to detect X-type through this type of interaction. In response, it may secrete antimicrobial compounds that target the competitor (Hibbing et al. 2010) or increase in density to monopolise the most advantageous niche (Hibbing et al. 2010) and block the bottleneck to the next generation (Mira and Moran 2002).

Another hypothesis is that the ecology of the two species involved causes *Regiella* to increase in density through a type of 'cheating' seen in mixed populations of bacterial species (West et al. 2007). It is possible that X-type is producing a type of public good, such as iron-scavenging siderophores, which benefit all surrounding microbes (Brockhurst et al. 2008). In this case, being surrounded by clonal kin is beneficial for the producer, but it incurs costs of production in mixed populations, resulting in an increase in the non-producing ("cheating") species (West et al. 2007). In a confined host environment, where both symbionts are present in the hemolymph, this mechanism may cause the density patterns seen.

X-type and *Regiella* have similar known functions in terms of fungus, parasitoid and temperature resistance, and one final hypothesis is that high relatedness and similarity of phenotypic effects leads to loss. Functional redundancy occurs when more than one species in a community performs the same ecological function, and it helps ecosystems to retain stability in the face of climatic change and other disturbances (Naeem 1998; Peterson et al. 1998). The superinfection of X-type and *Regiella* is an

example of a redundant community, but with the addition of a potential mediator; the aphid host. In the interactions between host and symbiont, a single-species, high-specificity relationship generally results in closer partnerships and less costs for the insect (Visick and McFall-Ngai 2000; Sicard et al. 2004). Diverse communities of bacteria, and the interactions between them, may negatively impact host fitness (Mouton et al. 2004; Oliver et al. 2006). As a result, superinfections will only be maintained if the synergistic effects of the symbionts involved are greater than the fitness decrease, otherwise there may be host selection for a single strain. In this case, where benefits conferred by both symbionts are similar loss may be beneficial for the host.

Application of symbiont infections

The field of symbiosis continues to expand as symbionts are recognized as not only ubiquitous in ecosystems but also drivers of many ecological functions. One of the newest angles of symbiont research is exploring how symbionts can be used to improve food security and global health, either by harnessing the natural effects of infection, or as a target for genetic engineering.

Exploring the interactions between symbionts such as X-type and *Regiella* begins to investigate symbiont community communication, and also raises questions about how symbionts could respond to other microbes infecting their host. *Regiella* responds to the presence of the competitor by increasing in density, in many cases resulting in the loss of X-type. Correspondingly, a decrease in the population size of X-type often heralds its loss from the superinfection. Contrastingly, superinfections containing *Spiroplasma* in this aphid system appeared to be stable. Such interactions are now being studied for practical applications. Recent work has found that symbionts including *Wolbachia* and *Spiroplasma* can decrease transmission of malaria (Wang et al. 2012) and dengue virus (Bian et al. 2013; Caragata et al. 2013) in mosquitoes through decreasing the pathogen load (Hussain et al. 2013). It appears that competition between symbiont and pathogen result in lower amounts of the disease vectored by the insect, a result which can be achieved by injecting *Wolbachia* strains

from flies (Bian et al. 2013; Caragata et al. 2013) or by genetically manipulating facultative mosquito symbionts (Wang et al. 2012). Research in this direction may lead to breakthroughs in how vector-spread human diseases are managed, with symbionts taking a vital role in any new technology.

Other research investigating the endosymbionts of whiteflies have found that *Rickettsia* and *Hamiltonella* can both actually increase the spread of pathogens (Su et al. 2013; Kliot et al. 2014) due to an increase in viral titres in symbiont-infected insects (Kliot et al. 2014). As aphids vector over half of all insect-borne plant viruses (Dedryver et al. 2010), there is scope for investigating how symbionts may affect the vector effectiveness of their aphid hosts, with potential for development of future control technology focusing on the microbial interactions.

Symbionts in insect populations not only affect disease vector efficiency, but also how their hosts are themselves affected by different control methods. Symbiont-mediated protection against parasitoids (Oliver et al. 2006) is a clear example. Infected aphids are much less susceptible to parasitoid wasps, a common biological control method (Boivin et al. 2012) and overuse of the control is likely to result in an increase of infected aphids as they gain a fitness advantage (Oliver et al. 2008). This in turn could potentially affect insect vectoring of plant pathogens in a complex web of interactions all mediated, to some degree, by the microbial community.

Symbiont communities and microbiomes are found across all taxa, and understanding the interactions between different species of symbionts is also important in other systems. Plants, for example, are commonly associated with complex microbial networks that increase soil fertility (Van Der Heijden et al. 2008), impacting plant productivity, pathogen defence and community structure (Jeffries et al. 2003). These symbionts are essential for plant growth, and as a result, for the production of crops and maintenance of ecosystems. Common farming practices can disrupt these communities through the use of chemicals and regular soil disturbance, decreasing symbiont diversity and changing community structure (Oehl et al. 2004). Understanding the stability, resilience and communication between these soil

symbiont communities may be vital for increasing food security and plant productivity in future.

Finally, symbiont communities are also, of course, important to human health, with implications for immunity (Kau et al. 2011), nutrient assimilation and obesity (Turnbaugh et al. 2006) and a variety of other diseases (Cho and Blaser 2012). Understanding what a healthy microbiome is, and how it can be created or maintained will be useful in future medical techniques. Antibiotics are commonly used to treat disease in humans, but may perturb healthy microbiomes by reducing diversity and causing extinctions (Blaser 2014). Conversely, transplants of human gut microbiome member *Helicobacter pylori* can cure severe *Clostridium difficile* infections in human patients, stabilising the community (Blaser 2014). Human microbiomes may also affect how medicines and drugs are metabolised in the gut, and even control our immune system responses (Blaser 2014). It is clear that there is much more to discover about how microbiomes and other symbiont communities mediate human, plant and animal health, and understanding in some small way the capabilities of symbionts, the resilience of their communities and exactly how they interact is likely to be vital for both food security and human health in future.

Summary and conclusions

Insects face a tumultuous life at the mercy of the environment, natural enemies and a changing climate. But their interactions can be mediated and modulated by their infections with facultative bacterial endosymbionts. With a high diversity of phenotypic benefits conferred by multiple species of symbionts, including resistance to natural enemies and to environmental stresses, symbionts can affect insect survival, reproduction and behaviour.

My work shows that a single endosymbiont can have multiple phenotypic effects on its host, and that interactions between symbionts, as well as selection pressures, can affect symbiont frequencies in host populations. I start to explore the mechanism behind one common symbiont phenotypes, and find that the dynamics of facultative and obligate symbionts can affect insect survival and phenotypic traits, in complex and

rapid interactions. Symbionts are a “horizontal gene pool” in the environment that insects pick up that may facilitate insect spread (White 2011; Henry et al. 2013), and adaptation to environmental changes or natural enemy pressures (Oliver et al. 2008; Feldhaar 2011). Using aphids as a model species, so much has been discovered about how symbionts infect and affect their hosts, but, in both aphids and beyond, there is so much potential for exploration into how infection with different microbes impacts their hosts, each other, and even more pathogenic invaders, with implications for agricultural and medical technologies in future.

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