FRIEND OR FOE?: CHARACTERIZATION OF THE X-TYPE SYMBIONT IN THE PEA APHID, *ACYRTHOSIPHON PISUM*

By

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(Under the Direction of Kerry M. Oliver)

ABSTRACT

Insects and other animals commonly form symbioses with heritable bacteria. These symbioses often have profound impacts on their hosts including providing protection against natural enemies. The pea aphid, *Acyrthosiphon pisum*, is a model organism for studying heritable symbioses and is associated with seven symbionts known or suspected to provide resistance to abiotic and biotic stresses. However, effects of infection with a common symbiont called X-type remain unclear. Using genetically controlled experimental lines, I found that X-type does not confer any of the established benefits associated with aphid symbioses, including protection against parasitoids, pathogens and thermal stress, yet is costly to aphids. Interestingly, X-type rarely occurs as a single infection and instead typically co-infects aphids with *Hamiltonella defensa*, which protects against parasitoids. Moreover, observed costs are ameliorated in co-infected aphids. Together these findings suggest that X-type is maintained in nature by the novel strategy of 'hitchhiking' with *H. defensa's* benefits.

INDEX WORDS: Symbiosis, heritable bacteria, co-infection, microbial ecology, natural enemies

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DEDICATION

I dedicate this thesis to my mother, Ellen Doremus, for her continuous support and advice (and patience while I babble on about insects), and my sister, Caitlin Castro, who in addition to being a tremendous inspiration and wonderful sister, somehow corralled her brother to spend his spring breaks helping her in the lab – a truly impressive feat that exposed me to a real-world lab setting and further sparked my own aspirations in research. Finally, I also dedicate my thesis to my father, Malcolm Doremus, who I wish could have seen this work.

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CHAPTER 1

LITERATURE REVIEW AND INTRODUCTION

1.1 Introduction

Eukaryotic organisms evolved and persist in a microbial world. Their biology has been profoundly shaped by repeated interactions with diverse microbial species, which frequently results in the formation of intimate and persistent associations called symbioses. These symbiotic interactions are ubiquitous among multicellular taxa and can be grouped into general categories: environmental microbes which colonize the epidermis of the host (Johnson et al. 1997), or the guts of animals (Engel and Moran 2013), and maternally-transmitted (heritable) microbes, which are largely restricted to invertebrate animals, and often persist intracellularly within the hemocoel (Buchner 1965). The interactions between these microbes and their eukaryotic hosts can range from mutualistic to parasitic (Fukatsu and Hosokawa 2002, Oliver et al. 2003, Stavrinides et al. 2009, Graham et al. 2012). For brevity, this review will focus on the gut and heritable bacteria of insects, ignoring the fascinating interactions occurring in other host (e.g. mammals and plants) and symbiont (e.g. fungi and viruses) taxa (Johnson et al. 1997, Mueller et al. 2005, Luan et al. 2013). In the past two decades, the increased availability of molecular techniques, particularly Polymerase Chain Reaction (PCR) and DNA sequencing, has allowed for a more thorough study of these microbes, particularly the heritable and more specialized enteric varieties, many of which are uncultivable by

standard techniques (Buchner 1965). Since the development of these techniques, the bacterial associates of insects have been relatively well studied (Buchner 1965, Huw Davies et al. 1987, Chapela et al. 1994, Moran et al. 2008, Engel and Moran 2013). Here I will describe the general patterns and characteristics of gut and heritable intracellular bacteria, then discuss the current knowledge concerning a model symbiosis system, and introduce the focus of the novel research in this thesis and briefly discuss its importance to the field.

1.2 Environmental Gut Microbiota of Insects

Among the environmental microbes that animals encounter is a subset of symbionts that are specialized to inhabit the animal gut (Ley et al. 2008, Engel and Moran 2013). These enteric microbes are found across animal taxa, but are becoming better-studied in insects, due to the less diverse assemblage of gut microbiota relative to mammals and the ease of using insects as models (Engel and Moran 2013). Within insects, enteric microbiota infect most taxa, although the number of microbial species, titers, and degree of specialization varies among insect species. Some, including the sap-sucking members of the Sternorrhyncha (Hemiptera), have little gut microbiota diversity and typically house small, or nonexistent, populations (Buchner 1965, Engel and Moran 2013). Most insect taxa exhibit an intermediate degree of association with their gut microbes; symbionts within these insect species typically consist of a variety of species that variably infect portions of the mid- and hindgut (Engel and Moran 2013). Other insect groups, including higher termites, honey bees, and some true bugs (Hemiptera: Heteroptera) have suites of specialized microbiota as well as specialized structures within

the gut which act as fermentation chambers for the sheltering of their symbionts (Fukatsu and Hosokawa 2002, Kikuchi et al. 2007, Hosokawa et al. 2012, Engel and Moran 2013).

Just as the degree of specialization between host and gut microbiota differs among insect taxa, so too does the acquisition/transmission routes of these gut microbes. Many insect species obtain gut microbes from the environment, typically from their diet, however in more specialized symbioses the microbes can be transferred in a direct manner (Engel and Moran 2013). Direct transfer of gut microbiota is commonly seen in social insects, such as termites and honey bees, which inoculate themselves with their symbionts during early life stages, typically via trophallaxis in termites, and social interactions in honey bees (Hongoh 2010, Martinson et al. 2012). Other non-social insects, particularly fluid-feeding true bugs, also exhibit direct transfer of gut symbionts. The transfer method differs among host taxa, but in some species, including the bean plataspid, *Megacopta punctatissima*, transmission involves a 'symbiont capsule' which the mother deposits near a batch of eggs. Upon hatching, the young nymphs immediately eat the capsule, inoculating themselves with the symbiont (Fukatsu and Hosokawa 2002). Other Hemipterans obligatorily obtain their gut microbes from the environment, as seen in the broad-headed bug, *Riptortis clavatus*, which postnatally obtains its *Burkholderia* gut symbiont from the soil every generation (Kikuchi et al. 2007). While modes of transmission can differ drastically, from chance infection via dietary ingestion to direct transfer of specific bacterial species, higher degrees of specialization (gut crypts), symbiont specificity, and fidelity of transmission modes often highlight associations in which microbes play essential roles in their host's biology.

Gut microbiota can have important impacts on the ecology of their hosts, with interactions between the host and gut microbes spanning the continuum from mutualistic to antagonistic. (Ben-Yakir 1987, Fukatsu and Hosokawa 2002, Dillon et al. 2005, Stavrinides et al. 2010, Coon et al. 2014). The microbial specificity of these symbioses also range from involving a single, highly specific symbiont species to a range of general symbiotic partners (Ben-Yakir 1987, Fukatsu and Hosokawa 2002, De Gaio et al. 2011, Coon et al. 2014). On the mutualistic side, in return for a stable environment and steady source of nutrients in the host, many gut microbiota provide essential amino acids or vitamins to the host or aid host growth rate through a variety of means. First, a portion of the microbes themselves may be digested by the host, directly providing the host with nutrients; although this is the most basic of the nutritional interactions, and may not represent a true mutualism (Engel and Moran 2013). Many gut symbionts also aid in the processing of difficult to digest components of the host diet. A classic example is seen in the gut communities of many termite species, which are capable of degrading cellulose, a primary carbon source in wood which is usually not accessible to the termite host (Engel and Moran 2013). Similarly, lignin-degrading fungi and bacteria may aid Cerambycid beetles including the Asian longhorned beetle, *Anoplophora glabripennis*, and the linden borer, *Saperda vestita*, in digesting their wood-based diet (Schloss et al. 2006, Geib et al. 2008). Gut symbionts of other host species, particularly those with nutrient deficient diets, can also provision the host with nutrients lacking in the insect diet. A notable example of such nutrient provisioning is seen in *Ishikawaella capsulatus*, the gut symbiont of *M. punctatissima*, which provides its host with amino acids lacking in its plant-sap diet (Nikoh et al. 2011). Hosts lacking this symbiont show highly decreased

growth rates, suggesting the host requirement of this gut symbiont for proper development (Fukatsu and Hosokawa 2002).

Along with mutualistic and neutral microbes, insects routinely ingest pathogens capable of colonizing the gut, often causing host mortality (Damgaard et al. 1997, Brown et al. 2003, Vodovar et al. 2005, Stavrinides et al. 2010). In addition to aiding in host nutrition, mutualistic gut symbionts can protect insect hosts from a variety of these pathogens, including trypanosomes in honey bees and pathogenic bacteria in locusts (Engel and Moran 2013). This protection can occur through a variety of mechanisms, including colonization resistance where gut symbionts reduce the likelihood of the establishment of pathogenic microbes by competitive exclusion (Dillon et al. 2005), or via immune priming where, in response to symbiont infection, an upregulated immune system also increases host resistance to pathogenic bacteria (Sadd and Schmid-Hempel 2006). Mechanisms likely differ depending on host species and microbial symbionts, but in both honey bees and locusts, resistance to the focal pathogen increased with more complex gut communities, which may reflect either a more competitive landscape or increasing immune upregulation with a more diverse gut microbiota (Dillon et al. 2005, Koch and Schmid-Hempel 2011). Alternatively, if gut symbionts are also providing nutrients to the host, then a more diverse complement of gut bacteria may result in a healthier host, which may also bolster the immune response (Engel and Moran 2013).

1.3 Heritable symbiosis in insects

The most specialized and ancient terrestrial host-microbe associations involve heritable bacterial symbionts infecting nematodes and arthropods, and these are best-studied in insects (Stouthamer et al. 1999, Moran et al. 2008). Heritable bacterial

symbionts are those that undergo highly efficient transmission from mother to offspring. They are incapable of surviving outside of their host, and in many cases, the host cannot survive without the symbiont. From the host's perspective, heritable symbionts can vary from being obligate, i.e. required for host survival, or facultative and not required for host survival but often conditionally beneficial (Moran et al. 2008). That heritable facultative symbionts reach new hosts primarily through maternal transmission, combined with their inability to survive outside the host, results in the linkage of the symbiont and host fitness. This in turn has resulted in the evolution of a variety of strategies for heritable symbionts to spread and persist to within host populations (Oliver et al. 2014) including providing a range of beneficial services and manipulating host reproduction. The latter strategy contributes to symbiont spread by increasing the number of infected daughters in a population at the expense of males, which are dead-end hosts, or uninfected females (Stouthamer et al. 1999).

Heritable nutritional obligate symbionts represent the most ancient and highly-specialized of the insect-microbe interactions, with estimates placing the origin of the *Sulcia muelleri* symbiont of the Auchenorrhynca at 260 mya (Moran et al. 2008). The ancient interactions of obligate bacteria and host have resulted in host adaptations for housing these symbionts including the formation of organs called bacteriomes that are made up of specialized cells called bacteriocytes devoted to housing and regulating these bacteria, often within host-derived vesicles (Baumann 2005). The obligate symbionts are confined to these cells for most of the host lifespan, although symbionts are often released during periods of vertical transmission, as seen in aphids, where *Buchnera* cells are released from bacteriocytes and packaged into blastulae when undergoing vertical

transmission; however other obligate symbionts may undergo different pathways to enter the offspring (Baumann 2005, Koga et al. 2012). Obligate symbionts perform nutritional roles for their hosts and, as such, are required in hosts limited to feeding on poor substrates (phloem, xylem, and blood) and common in those with more varied, yet nutrient poor diets (Douglas 1992, Baumann 2005);(Akman et al. 2002, McCutcheon and Moran 2007). These symbionts provision the host with nutrients, such as amino acids in the case of *Buchnera* and aphids (Douglas 1992), and B vitamins in the case of *Wigglesworthia* and the obligate blood-feeding tsetse flies, *Glossina* (Akman et al. 2002). Removal of these obligate symbionts from the host, either by antibiotics or abiotic stress, results in reduced fecundity, growth rate, and often mortality prior to adulthood (Moran et al. 2008). This level of dependence is not seen with facultative symbionts, however, and because facultative symbionts are not restricted to hosts with nutrient-poor diets, facultative symbionts are often much more widespread than obligate symbionts and confer a wide array of effects on their insect hosts (Moran et al. 2008).

Obligate symbionts undergo strict vertical transmission and display patterns of codiversification between symbiont and host. These patterns suggest that the ancestral acquisition of nutritional symbionts opened previously unavailable niches for resource acquisition, including plant phloem and xylem, with subsequent diversification (Moran et al. 2005c). Obligate symbionts are commonly members of the γ-Proteobacteria (i.e. *Buchnera*) or Bacteroidetes (i.e. *Sulcia*), although other lineages are also represented (Moran et al. 2008). These long-term, host-restricted associations have resulted in highly reduced symbiont genomes compared to free-living bacteria (Moran et al. 2008). In extreme cases, the bacterial genomes encode the basic pathways for sustained bacterial

life, complete pathways for host-required products, and little else (McCutcheon and Moran 2007). Remarkably, in several cases the obligate symbiont has been lost or become partially obsolete and is replaced or interdependent with a second, complementary symbiont (Moran et al. 2005c, McCutcheon and Moran 2007, Lamelas et al. 2011, McCutcheon and von Dohlen 2011).

1.4 General characteristic of heritable facultative symbionts

Facultative symbionts, while not required for survival of their host, do share some commonalities with their obligate cousins. Both typically undergo vertical transmission with high fidelity and exhibit genome reduction compared to free living bacteria; however many facultative symbionts retain some semblance of their pathogenic past, including the Type III secretion system, and are capable of horizontal transmission (Degnan et al. 2009, Degnan et al. 2010, Oliver et al. 2010, Henry et al. 2013). Facultative symbionts are also not restricted to bacteriocytes; they are often found infecting various organs in the insect, including the bacteriomes, as many persist extracellularly throughout the hemolymph (Moran et al. 2008). Additionally, facultative symbionts have evolved more diverse strategies for maintenance in host populations. These traits are often ecologically important and may change the sex ratios of their hosts (reproductive manipulators) (Stouthamer et al. 1999, Zchori-Fein et al. 2004) or confer conditional benefits including resistance against abiotic (Montllor et al. 2002, Russell and Moran 2006) and biotic stress (Oliver et al. 2003, Oliver et al. 2005, Scarborough et al. 2005, Teixeira et al. 2008, Jaenike et al. 2010, Łukasik et al. 2013b). Moreover, the ability of facultative symbionts to undergo occasional horizontal transmission, indicates that ecologically important traits encoded by symbiont genomes may jump laterally

within and among host lineages providing the instantaneous acquisition of traits that can influence host fitness (Oliver et al. 2010).

1.5 Heritable manipulators

Bacteria species implicated in reproductive manipulation are among the most widespread of symbiotic microbes, with the estimated frequency of *Wolbachia* – infected species alone ranging from 30 to 40% of all insects (Duron et al. 2008, Zug and Hammerstein 2012). In addition to *Wolbachia*, several other reproductive manipulators are commonly in Arthropods including *Cardinium* and *Spiroplasma* (Zchori-Fein and Perlman 2004, Duron et al. 2008), all of which can induce a biased sex ratio using a variety of mechanisms (Stouthamer et al. 1999). Of the four known manipulative strategies, cytoplasmic incompatibility (CI) is the best studied, and is a common phenotype induced by *Wolbachia* (Stouthamer et al. 1999) as well as *Cardinium* spp (Hunter et al. 2003). CI results in incompatible crosses between infected males and uninfected females; crosses between uninfected males and infected females result in viable, symbiont-infected offspring (Stouthamer et al. 1999) resulting in the increased fitness of symbiont-infected females relative to uninfected females (Stouthamer et al. 1999). Though the exact mechanism is still unclear, it appears to involve the degeneration of paternal chromosomes post-fertilization. This results in haploid offspring which are inviable in diploid species and the production of all males in haplodiploid species, such as the Hymenoptera (Stouthamer et al. 1999). The reigning hypothesis suggests that sperm chromosomes from symbiont-infected males are modified and "rescued" by symbiont-infected eggs post-fertilization, a process which, in *Wolbachia*, may involve the

symbiont-associated wsp protein and bacteriophage, WO (Werren 1997, Stouthamer et al. 1999, Bordenstein et al. 2006). Further complicating matters, this rescue effect seems to be specific to manipulator species and strain, with combinations of two different CI symbiont species or strains typically failing to rescue each other (Stouthamer et al. 1999, White et al. 2009).

As a means of maintaining vertical transmission, reproductive manipulators can induce female-biased sex ratios through other mechanisms, including parthenogenesis induction, feminization, and male-killing; the mechanisms for these strategies are less understood (Engelstädter and Hurst 2009). In the case of the maternally inherited symbiont, increasing production of female progeny via parthenogenesis induction and feminization increases the number of hosts capable of vertically transmitting the symbiont. The benefit of male-killing is assumed to come in the form of reduced competition between female and male offspring or the increase in available nutrients for female offspring (in cannibalistic species), resulting in higher fitness of the female and also the symbiont (Engelstädter and Hurst 2009). The type of manipulation varies by bacterial strain, species, and host species with some notable symbionts, like *Wolbachia* and *Cardinium*, exhibiting multiple manipulation strategies (Engelstädter and Hurst 2009). There is also a growing appreciation that classic manipulators, particularly *Wolbachia*, are often mutualists rather than reproductive parasites (Hamilton and Perlman 2013). For example, in *Drosophila melanogaster*, *Wolbachia* confers higher resistance to RNA viruses (Teixeira et al. 2008). Strains of another known manipulator, *Spiroplasma*, also act as defensive mutualists in *D. melanogaster*, increasing resistance against a prominent nematode natural enemy (Jaenike et al. 2010), as well as in the pea aphid,

Acyrthosiphon pisum, where it increases aphid resistance against a fungal pathogen (Łukasik et al. 2013b). Interestingly, these strains exhibit a weakly or nonexistent manipulative phenotype in these species and have made a complete transition to the second propagative strategy: conferral of mutualistic effects symbiont (Jaenike et al. 2010, Łukasik et al. 2013b). While it is currently unknown how common mutualistic strains of *Wolbachia*, *Spiroplasma*, and other manipulators are in nature, many more examples of similar mutualisms should be expected in the future.

1.6 Heritable mutualists

Many facultative symbionts are not known to manipulate host reproduction and yet often occur at moderate to high frequencies in natural populations (Toju and Fukatsu 2011, Ferrari et al. 2012, Russell et al. 2013). In several cases, facultative symbionts have rapidly spread through natural populations, increasing from relative rarity to near fixation (Cockburn et al. 2013, Cass et al. 2015). Infection frequencies of facultative symbionts often vary seasonally and with regards to host plant use, natural enemy pressure, and temperature (Montllor et al. 2002, Toju and Fukatsu 2011, Russell et al. 2013, Smith et al. 2015). These variable distributions, which seem to be affected by abiotic and biotic pressure, suggest that facultative symbionts play important roles in mediating host ecology in natural populations; the frequencies of these symbionts are thus expected to respond to these pressures, either by increasing in a population when providing a benefit, or decreasing in a population when they are unnecessary or detrimental.

Increased protection against biotic and abiotic threats are the most commonly observed effects of mutualistic facultative symbiont infection (Montllor et al. 2002, Oliver et al. 2003, Oliver et al. 2005, Scarborough et al. 2005, Russell and Moran 2006,

Teixeira et al. 2008, Jaenike et al. 2010, Łukasik et al. 2013b). Defensive symbionts are expected to be common in insects, but are not well documented outside of pea aphids and *Drosophila*, which have become model systems for the study of these interactions (Oliver et al. 2014).

Within the pea aphid system, three facultative symbionts within the Enterobacteriaceae (γ-Proteobacteria) are of particular notoriety. The first, *Serratia symbiotica*, increases aphid resistance to high temperatures, which is known to have detrimental effects on aphid fecundity (Montllor et al. 2002, Russell and Moran 2006). This detrimental effect of temperature is caused by the loss of bacteriocytes and the obligate symbiont of the aphid, *B. aphidicola*, which has low thermal tolerance (Montllor et al. 2002). Additionally, *B. aphidicola*, is prone to a mutation in the promoter of a gene encoding a heat-shock protein that decreases the already weak ability of *B. aphidicola* to survive heat stress, but which improves host fecundity at lower temperatures (Dunbar et al. 2007); such a mutation further reduces the ability of the aphid host to survive thermal stress. The mechanism of *Serratia*-conferred resistance is hypothesized to involve the lysing of *S. symbiotica* within bacteriocytes at high temperatures, releasing heat shock proteins that protect *B. aphidicola* cells and improve host fecundity (Burke et al. 2010). Field surveys have found that while *S. symbiotica* frequencies do not correspond to seasonal changes in temperature, they do increase in warmer geographic regions, reinforcing the role of this symbiont as a thermal protector in the pea aphid (Montllor et al. 2002, Smith et al. 2015).

In addition to low thermal tolerance, pea aphids generally have a weak immune response to specialized pathogens and parasitoid wasps (Gerardo et al. 2010, Laughton et

al. 2011). Instead, facultative symbionts often assume the role of protecting the pea aphid against natural enemies, including the fungal pathogens (Moran et al. 2005b, Scarborough et al. 2005, Łukasik et al. 2013b, Parker et al. 2013). *Regiella insecticola* was the first symbiont found to confer an anti-fungal effect, protecting pea aphid hosts from an important aphid fungal pathogen, *Pandora neoaphidis* (Moran et al. 2005b, Scarborough et al. 2005). Little is known concerning the mechanism of defense, but genomic studies have revealed the presence of several genes encoding putative toxins (Degnan et al. 2010). A more recent study revealed that *R. insecticola* also increases pea aphids resistance to a second specialized aphid fungal pathogen, *Zoophthora occidentalis*, but not to a generalist entomopathogenic fungus, *Beauveria bassiana* (Parker et al. 2013). Symbiont-conferred fungal resistance in aphids seems to exhibit specificity towards aphid pathogens, however it remains to be seen if this a general trend of symbiont-conferred resistance in other systems. Another study found similar anti-fungal effects in three additional species of aphid symbionts (Łukasik et al. 2013b), suggesting that increasing aphid resistance to fungal pathogens may be a common strategy of aphid symbionts. Alternatively, fungal resistance may be a general result of symbiont infection by way of competition or immune priming, yet all aphid symbionts do not confer protection against specialized fungal pathogens.

Pea aphid receive protection from parasitoids via infection with *Hamiltonella defensa,* which confers varying degrees of resistance to the dominant pea aphid parasitoid, *Aphidius ervi* (Oliver et al. 2003, Oliver et al. 2005). The mechanism of this defense is not known, but is thought to involve a *H. defensa*-infecting bacteriophage, APSE, that encodes putative eukaryotic toxins (Moran et al. 2005a, Degnan and Moran

2008, Oliver et al. 2009). The variability of *H. defensa*-conferred protection correlates strongly with APSE phage strain, and aphids infected with *H. defensa* strains that have lost the APSE phage exhibit a complete loss of protection and incur costs associated with higher symbiont titers (Oliver et al. 2009, Weldon et al. 2013). *H. defensa*-conferred protection may be lost at warmer temperatures and wasps have strategies, including selective superparasitism (> 1 egg) of infected hosts, that can improves odds of successful infection (Bensadia et al. 2006, Oliver et al. 2012). Costs of maintaining the symbiosis in the absence of parasitism, combined with reduced benefits at higher temperatures and when wasps employ counter-responses, likely inhibit the spread of this symbiont and explain observations of intermediate frequencies in natural populations, despite its protective benefits (Russell et al. 2013, Oliver et al. 2014). Another facultative symbiont, X-type (a.k.a. PAXS), which often coinfects individual aphids along with *H. defensa*, has been implicated in the rescue of this loss of protection in both warmer temperatures and superparasitism; unfortunately confounding variables, such as host and symbiont genotype, as well as additive effects of symbiont coinfection, make it difficult to elucidate the role of X-type or *H. defensa* in these "rescue" phenotypes (Guay et al. 2009, Russell et al. 2013, Donald et al. 2016).

The scarce studies concerning the *H. defensa*-X-type coinfection, and indeed, defensive symbiont coinfection in general, highlights an area in need of further research (Oliver et al. 2006, Guay et al. 2009, Donald et al. 2016). Little is currently known about coinfection of two or more symbionts in pea aphids, despite the model status of the organism and the high frequency of individuals infected with multiple symbionts in natural populations (Russell et al. 2013). One study showed an increased cost to the host

with infection with multiple symbionts, however it is unclear if elevated cost is a general trend of coinfection (Oliver et al. 2006). Other coinfection studies, particularly those concerning X-type, have suggested synergistic benefits of defensive symbiont coinfections, including rescue of lost benefits (Guay et al. 2009, Donald et al. 2016) and increased levels of resistance to natural enemies (Heyworth and Ferrari 2015). Unfortunately, as previously mentioned, these studies contain various confounding variables and it is unclear whether coinfection or symbiont/host genotype is the source of the benefit. In order to effectively determine the cost and benefits of coinfection, studies will have to include full complements of infection status (i.e. hosts with both symbionts, symbiont A, symbiont B, neither), preferably in multiple host genotypes. Coinfection studies are a logical next step for defensive symbiosis in pea aphids, as we gain a better understanding of the influence of single symbiont infections on host biology.

1.7 Conclusions from Literature Review

Insects form associations with a multitude of microbial partners, which can span a continuum of interactions from defensive and nutritional mutualisms (Six and Klepzig 2004, Baumann 2005, Moran et al. 2008, Engel and Moran 2013, Oliver et al. 2014) to parasitic manipulators of host biology (Stouthamer et al. 1999, Duron et al. 2008). Individual symbiont species and genera can even fall into multiple categories, depending on their genotype, host species and environmental factors (Hosokawa et al. 2010, Lamelas et al. 2011, Graham et al. 2012, Hamilton and Perlman 2013, Weldon et al. 2013, Cass et al. 2015). These microbes are often environmental but there is a growing appreciation for the diversity and importance of obligatory enteric and inherited symbionts (Engel and Moran 2013, Oliver and Martinez 2014). The latter in particular

can have a profound effect on the biology and ecology of their host, including host diet, range, reproduction, and interaction with natural enemies (Oliver and Martinez 2014). Understanding the dynamics and diversity of these symbiont-host interactions has far reaching implications not only in the study of ecology and evolutionary biology, but in agricultural, medical, and conservational fields. As the need for sustainable practices in agriculture and vector control increases, the study of bacterial symbiosis in insects can aid our implementation of more sustainable control practices. Perhaps most importantly, the study of symbiotic interactions between eukaryotes and their microbial partners can reveal the extent to which these symbionts shape eukaryotic evolution and biology.

1.8 Purpose of the present study

As well developed as the pea aphid model system has become, there is still much to study, including the major roles of some common facultative symbionts. While there is growing body of work on the remaining symbionts, including the identification of traits that influence host ecology, only three (*S. symbiotica*, *H. defensa*, *R. insecticola*) of the seven known symbionts have received substantial study (Oliver et al. 2005, Scarborough et al. 2005, Russell and Moran 2006, Oliver et al. 2009, Burke et al. 2010, Parker et al. 2013). As mentioned previously, the effects of coinfecting symbionts on the host phenotype, as well as interactions between these symbionts, are also understudied, despite evidence of a propensity for coinfections in nature (Oliver et al. 2006, Łukasik et al. 2013a, Russell et al. 2013).

Perhaps the least understood of the pea aphid symbionts is the X-type bacterium, which commonly coinfects hosts along with *H. defensa* (Russell et al. 2013). This bacterium thus represents a perfect opportunity to gain insight both on the effects of X-

type infection on the pea aphid, as well as investigate the interactions between commonly coinfecting symbionts, and the effect of this coinfection on the host. While there have been several prior studies involving X-type (Guay et al. 2009, Heyworth and Ferrari 2015), the current investigation will utilize a full complement of infection statuses to effectively determine the effect of this symbiont, as a single and coinfection, on its host. In doing so, this study will provide novel information on the X-type bacterium of pea aphids, including its interactions with another facultative symbiont, *H. defensa*, and the broader implications of this symbiosis.

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CHAPTER 2

CHARACTERIZATION OF THE X-TYPE SYMBIONT IN THE PEA APHID, *ACYRTHOSIPHON PISUM*[1,2]

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2.1 Abstract

Symbioses between terrestrial eukaryotes and heritable intracellular bacteria are ubiquitous in nature, particularly among insects. These symbiotic bacteria are typically unable to survive outside their host, and thus the symbiont fitness is tied to that of the host organism. Heritable facultative symbionts (HFS), which are not typically required for host survival and reproduction, invade and persist in host populations via increasing host fitness by providing benefits or manipulating of host reproduction. Among insects, the pea aphid, *Acyrthosiphon pisum*, has emerged as a model for the study of heritable symbioses. In addition to the obligate symbiont, *Buchnera*, aphids are usually infected with one or more of seven species of HFS, most of which provide increased protection against natural enemies. For example, infection with *Hamiltonella defensa* confers protection against parasitoids. One pea aphid HFS, the X-type symbiont, remains understudied, and here I use differentially infected sublines sharing host genotype to characterize the biology of this HFS which commonly superinfects N. American pea aphids along with *H. defensa*. Unexpectedly, I did not find that X-type confers protection against parasitoids, fungal pathogens or thermal stress as previously reported. Instead, I found that X-type infection carried a severe fitness cost, which was somewhat mitigated in superinfections, especially after exposure to heat shock. The high cost and lack of benefits from X-type infection, combined with the rarity of X-type single infections, suggests that this virulent symbiont is largely maintained in field populations by 'hitchhiking' along with the defensive benefits of *H. defensa*.

2.2 Introduction

Heritable symbionts are ubiquitous in arthropod species occupying terrestrial ecosystems and associations between insects and bacterial symbionts are particularly well studied (Moran et al. 2008). Heritable symbionts are maternally-transmitted and cannot survive outside of host tissues and consequently have limited strategies for reaching new hosts (Werren and O'Neill 1997, Jaenike 2012, Oliver et al. 2014). Given that host and symbiont fitness are intimately linked, symbionts can invade host populations by providing net fitness benefits, often by mediating resource acquisition or providing protection against natural enemies (Oliver and Martinez 2014). Alternatively, as males are dead ends, heritable symbionts can invade populations by manipulating host reproduction to favor infected females at the expense of uninfected females or males (Duron et al. 2008, Werren et al. 2008). Naturally, instances of heritable pathogens are expected to be exceedingly rare (Moran et al. 2008), yet under some conditions, a symbiont that was providing neither conditional benefits nor manipulating host reproduction could spread by 'hitchhiking' alongside a beneficial symbiont (Smith et al. 2015). The hitchhiking symbiont could even harm host fitness as long as conditional benefits outweighed these costs.

The pea aphid, *Acyrthosiphon pisum* (Hemiptera: Aphididae), has emerged as a model organism for studying the consequences of infection with heritable symbionts. This is due primarily to the diverse assemblage of pea aphid symbiont species combined with clonal reproduction and the relative ease in manipulating heritable symbiont communities (Oliver et al. 2014). All aphids carry the obligate symbiont, *Buchnera aphidicola,* which provides essential nutrients not found in sufficient quantities in their

phloem diet (Buchner 1965, Douglas 1992). Individual pea aphids are also usually infected with one or more of up to seven species of heritable facultative symbionts (HFS) which confer diverse protective benefits to the aphid host (Russell et al. 2013, Oliver et al. 2014). For example, the common HFS, *Hamiltonella defensa*, confers resistance to the dominant parasitoid of the pea aphid, *Aphidius ervi* (Oliver et al. 2003, Oliver et al. 2005). The mechanism of resistance is not known, but requires that the bacterial symbiont be infected with the bacteriophage APSE, which encodes eukaryotic toxins hypothesized to harm *A. ervi* larvae developing within the aphid (Moran et al. 2005b, Degnan and Moran 2008, Oliver et al. 2009). Other facultative symbionts commonly found infecting pea aphids and associated with protective roles include *Serratia symbiotica*, which confers resistance to thermal stress, and *Regiella insecticola*, *Spiroplasma, Rickettsia, and Rickettsiella viridis*, which protect against an aphid-specific fungal pathogen, *Pandora neoaphidis* (Montllor et al. 2002, Scarborough et al. 2005, Russell and Moran 2006, Burke et al. 2010, Łukasik et al. 2013, Russell et al. 2013).

Despite the pea aphid being among the best characterized models for heritable insect symbiosis, little is known about a symbiont called X-type (aka PAXS) commonly infecting North American pea aphid populations (Russell et al. 2013). Sequences of X-type 16S rRNA indicate its placement in the Enterobacteriaceae (γ-Proteobacteria), along with other aphid symbionts, including *Buchnera* and *H. defensa*, as well as notable human pathogens, such as *Salmonella* (Guay et al. 2009). Interestingly, population-level studies show that X-type almost never occurs as a single infection like its fellow facultative symbionts, but rather is typically found 'superinfecting' (i.e. > 1 symbiont) individual pea aphids with other symbionts, especially *H. defensa* (Russell et al. 2013).

Superinfections with multiple HFS are expected to carry higher costs, yet infections of multiple HFS appear common in aphids and other insect hosts (Oliver et al. 2006, Chiel et al. 2007, Toju and Fukatsu 2011, Ferrari et al. 2012, Russell et al. 2013). Host-level selection can select for the maintenance of particular combinations of synergistic HFS (e.g. multiple distinct benefits conferred), or when costs (relative to single-infected and uninfected aphids) are impacted by co-residence and may occur over competition for space or resources within the host (Oliver et al. 2006, Vautrin and Vavre 2009, Toju and Fukatsu 2011, Jaenike 2012, Russell et al. 2013). Non-selective factors may also be important as vertical transmission rates are expected to be lower in superinfections relative to single infections (Sandström et al. 2001, Oliver et al. 2006, Chiel et al. 2007, Toju and Fukatsu 2011, Russell et al. 2013). The propensity of X-type to occur as a superinfection thus suggests it may act synergistically by enhancing the performance of co-infectors or, alternatively, may deploy a cheating strategy and 'hitchhike' in association with the benefits conferred by a 'sucker' symbiont (Jaenike 2012, Smith et al. 2015).

Building on prior research indicating that *H. defensa*-conferred protection against the parasitoid *A. ervi* likely fails at warmer temperatures (25-30 °C) (Bensadia et al. 2006), a subsequent study found a possible synergistic role for X-type, as symbiont-based protection was not lost at higher temperatures in aphids infected with both X-type and *H. defensa* (Guay et al. 2009). Unfortunately, these studies did not control for variation in aphid and symbiont genotypes, which potentially contributed to the observed results. Furthermore, aphids were dissected prior to parasitoid pupation such that effects caused by strains of *H. defensa* (e.g. those with APSE2) that kill wasps late in development

(Martinez et al. 2014b) may not have been observed. Most recently, in European pea aphids X-type has been implicated in protection against thermal stress and the fungal pathogen, *P. neoaphidis* (Heyworth and Ferrari 2015). In this case, X-type was not isolated from the co-infecting symbiont *Spiroplasma*, which also provides protection against *P. neoaphidis* (Łukasik et al. 2013) and possibly contributed to the observed protective phenotype. In N. American pea aphid populations, *Spiroplasma* is rare and is not commonly associated with X-type (Russell et al 2013, Doremus & Smith, unpublished).

In summary, despite claims that X-type provides various protective roles to pea aphids, these have not been unambiguously demonstrated and separated from co-infecting symbionts. Here I examine roles of X-type as a single infection, and superinfection with its most common partner *H. defensa*, by establishing experimental lines sharing aphid genotype, yet differing in the four possible symbiont infection types: uninfected with HFS, *H. defensa*-only, X-type-only and superinfected. Using these lines I examine 1) the relative constitutive infection costs in the absence of natural stress; 2) resistance to the parasitoid *A. ervi* at both 'normal' and 'high' temperatures, 3) resistance to a second parasitoid, *Praon pequodorum,* 4) resistance to the fungal pathogen, *P. neoaphidis*; 5) effects of thermal stress on aphid fitness, and 6) whether superinfection influences the within aphid abundance of either facultative symbiont or *Buchnera*. I also examine X-type strain variation using a multi-locus typing approach and examine the phylogenetic placement of N. American X-type based on several housekeeping genes of related bacteria. In doing so, I hope to deliver a robust account of X-type induced effects

on the aphid phenotype in an effort to determine the propagation strategy of this common heritable symbiont.

2.3 Methods

2.3.1 Study organisms

Pea aphids are phloem-feeding herbivores that consume a range of herbaceous legumes. Due to their parthenogenetic lifecycle, asexual cultures can be maintained in the laboratory via the use of an extended photoperiod. Pea aphid lines were maintained in small cages containing a fava bean plant, *Vicia fava*, within Percival Biological Incubators at 20°C with 70% humidity under 16h light/ 8h dark conditions, unless otherwise noted. *V. fava* plants were periodically replaced as needed. The primary aphid line (5D) used in these experiments was collected from alfalfa (*Medicago sativa*) from Dane County Wisconsin in 2012. A single parthenogenetic female was placed in a Petri dish with *V. fava* leaves to establish the line. A subset of her offspring were screened for all known pea aphid HFS using diagnostic PCR (for primers specific for pea aphid HFS see Henry et al. 2013, Martinez et al. 2014b) as well as any 'unexpected' symbionts using universal primers with DGGE (Martinez et al. 2014a). Line 5D was found to be naturally superinfected with *H. defensa* and the X-type symbiont. The APSE strain in this line (APSE8) is structurally similar to APSE3, but encodes the *cdtB2* toxin, and is functionally more similar to APSE2 as it provides a moderate level of protection against *A. ervi* (Weldon et al in prep).

The parasitoid, *A. ervi* (Hymenoptera: Braconidae), is the dominant parasitoid attacking the pea aphid in N. America (Angalet and Fuester 1977). The *A. ervi* in this study consisted of a mixture of field caught (ND and WI) and commercially produced

(Syngenta) wasps reared on a mixture of susceptible pea aphid genotypes. Adult wasps were provided honey and water, and females were at least three days old, and mated, prior to all parasitism studies. Another Aphidiine braconid, *Praon pequodorum* (Hymenoptera: Braconidae), which is the second most common wasp attacking pea aphids in N. America, was field-collected from ND and reared under the same conditions.

The fungal pathogen, *Pandora neoaphidus* (Zygomycota: Entomophthorales), another common natural enemy of the pea aphid, is often used along with *A. ervi* for the biological control of pea aphid populations. The *P. neoaphidis* (genotype ARSEF 2588) used in this study originated from the USDAARS Collection of Entomopathogenic Fungal Cultures. *P. neoaphidis* was maintained on the susceptible aphid line, WI-48 (no HFS), with desiccated fungal cadavers held at 4°C with low humidity for no more than two months prior to use in experiment (see Fungal assay section for additional information).

2.3.2 Creation of experimental lines

A full complement of differentially infected aphid experimental lines was created, consisting of the naturally superinfected line (XH), a line singly infected with *H. defensa*-APSE (H), a line singly infected with X-type, and a line uninfected with either symbiont (AB – antibiotically cured). These experimental lines were then used in all experiments in this study. To create the X-only and uninfected lines, aphids were selectively cured by allowing second and third instar nymphs to feed on aphid artificial diet spiked with an antibiotic cocktail containing 150 µg/mL of ampicillin, gentamycin, and cefotaxime for three days (Douglas et al. 2006). At this point, surviving aphids were placed on fresh fava bean (*Vicia faba*) plants and monitored until adulthood. Adults were separated, moved to

individual Petri dishes containing a fresh fava leaf that was replaced every 1-2 days with a fresh leaf, and allowed to reproduce. Offspring (2^{nd}-3^{rd} instar) were collected and underwent DNA extraction. Nymph samples were squished in 20-30 µL of lysis buffer (depending on size) with 1% proteinase K and incubated using the following protocol: 38˚ C for 35 minutes, 95˚ C for 2.5 minutes, and held at 4˚ C or on ice for immediate use. Samples were screened for symbiont infection using the aforementioned diagnostic PCR protocols (Degnan and Moran 2008). If the AB-fed mother produced offspring with the desired infection status, her subsequent offspring were reared to adulthood and separated into individual Petri dishes where second generation aphids were screened for infection status. Once confirmed, a single parthenogenetic female was used to establish experimental line. I was unable to generate the *H. defensa*-only line through selective symbiont curing, so to create the *H. defensa*-only line, 3^{rd} instar aphids cured of both symbionts were microinjected in the abdomen using glass capillary needles with hemolymph collected from superinfected aphids; briefly, hemolymph containing *H. defensa* was collected by homogenizing several superinfected 5D aphids in 40 µL of 1X PBS buffer in a 0.5 mL tube, which was then centrifuged at 7000xg for fifteen seconds (Oliver et al. 2005, Martinez et al in press). Post-microinjection, aphids were placed on fresh fava plants and underwent the same screening process as the antibiotic-exposed aphids until I confirmed infection with only *H. defensa*. Pea aphid experimental lines were maintained for at least six generations prior to use in experiments and lines were screened for HFS infection status periodically and prior to experiments using diagnostic PCR primers for *H. defensa* and X-type (Henry et al. 2013).

2.3.4 Fitness assays

Defensive symbionts can incur costs in the absence of enemy challenge (Russell and Moran 2006); a cost that may be amplified in cases of superinfection (Oliver et al. 2006). While costs of many pea aphid HFS have been difficult to detect (e.g. Russell and Moran 2006), casual observations of X-type infected aphids suggested that this symbiont was quite costly to the host (Doremus, personal observation). The cost of X-type on aphid fitness in the absence of natural enemies was further characterized by performing two fitness assays to determine the effect of this HFS on aphid development and fecundity.

Aphid developmental assays

The first assay measured aphid developmental time as the period from birth to first reproduction (TFR), fresh weight at first reproduction, and adult dorsal body area (i.e. a 2-D representation of body area) of the four experimental sublines (X-type, *H. defensa*, both symbionts, and neither symbiont). Adult aphids where allowed to reproduce on fresh fava plants for 24 hours. The offspring were collected and transferred to fresh plants in cohorts of five aphids. Six replicates were used, with each cohort of five aphids representing a replicate, resulting in a total of thirty aphids/line (= 120 aphids). Aphids were monitored every two days until day eight (near adulthood), at which point aphids were monitored every three hours until first reproduction. Upon first reproduction, the time and date were recorded (TFR) and the adult was removed and weighed using a microscale. All offspring were also removed and discarded to avoid confusion with those aphids that had not yet reproduced. Additionally, the body size of adults was estimated by using an Olympus SZX16 stereoscope to photograph the dorsal view of each adult aphid. The dorsal circumference of the body, excluding antennae and legs, was traced to

estimate the dorsal body area of the aphid from each photo using the measurement function in cellSens Standard. Using SAS JMP Pro 11. TFR was analyzed using the non-parametric multiple Wilcoxon Rank Sum tests with Benjamini-Hochberg corrected significance values, as the TFR data had a non-normal distribution even after transformation attempts. Fresh weight and adult body size were compared using Dunnett's test with the uninfected (AB) line as the control.

Aphid fecundity assays

The second fitness assay was designed to measure aphid survival and fecundity. Prior to the experiment, adult aphids were placed onto fresh *V. fava* plants, allowed to reproduce for 24 hours, and offspring were collected and transferred to a fresh *V. fava* plant in cohorts of five aphids, as in the developmental assays. Again, six replicates were used for a total of thirty aphids/line (= 120 aphids). Aphids were checked every three days for 30 days, at which point most aphids had died for stopped reproducing. The number of surviving and dead aphids were counted at each date and, upon aphid reproductive maturation, any offspring produced were also removed and counted. The sum of offspring at day 30 was interpreted as lifetime fecundity and analyzed using Dunnett's test with the AB line as the control. Lifetime survival data was fit to a lognormal distribution to estimate 0.5 survival time.

2.3.5 Parasitism assays

A single, mated female *A. ervi* was placed in a large Petri dish and allowed to parasitize 2^{nd}-3^{rd} instar aphids. To reduce the chance of superparasitism, which can overcome symbiont-based resistance, aphids were removed from the arena as they were parasitized (Oliver et al. 2012). Post-parasitism, sixteen replicates of aphids per line were

placed onto fresh *V. fava* in cohorts of twenty aphids (= 1280). For each line (XH, H, X, AB) replicates were randomly divided into one of two temperature treatments post-parasitism (eight replicates/treatment). The first treatment was held under 'normal' conditions at 20°C, while second treatment was held at an elevated temperature cycle of 30°C day/ 24°C night. The decreased night temperature in the elevated temperature treatment was used to mimic natural temperature fluctuations between day and night and to reduce dual mortality of aphid and wasp caused by elevated temperatures. Nine days later, the proportion of aphids surviving (resistant), mummified (susceptible), and dead (dual mortality of aphid and parasitoid) were counted. As survival, mummification, and mortality for each line in either temperature treatment had non-normal distributions, the data were arcsin transformed and the distributions were checked for normality using the Goodness-of-fit test. The results were then compared between aphid lines as well as temperature treatment lines using logistic regression, as well as ANOVA with Tukey's HSD *post-hoc* test to compare means.

A previous study found neither *H. defensa*-based protection (APSE2, APSE3) nor aphid encoded protection against the parasitoid, *A. ervi*, extended to a related parasitoid *P. pequodorum* (Martinez et al. 2016). Thus, X-type persistence could be explained by providing protection against this less common enemy. To test this I conducted a second parasitism assay using *Praon pequodorum* (Hymenoptera: Braconidae) at 20°C to determine the effect of single- and super-infecting X-type + *H. defensa* on aphid resistance to this second parasitoid natural enemy. Cohorts of 15 2^{nd}-3^{rd} instar aphids infected with either X-type, *H. defensa*, X-type + *H. defensa*, or lacking both symbionts were placed onto a fresh *V. fava* plant and given 2 hours to settle. A mated, female, *P.*

pequodorum was then introduced into the cage and, after visual confirmation that the wasp was viable, allowed to parasitize for 24 hours after which point it was removed. Nine days later surviving, mummified, and dead aphids were counted and analyzed as in the first parasitism.

2.3.6 Fungal assays

Prior to the experiment, corpses of aphids killed by *P. neoaphidis* were stored post-death but pre-sporulation at 4°C for no more than twelve weeks within a plastic screw cap container along with a desiccator bag. To rehydrate corpses and initiate sporulation, two corpses were centrally placed onto a freshly made 1.5% tap water agar plate. The plates were then sealed with Parafilm and held in the dark at 20°C for 14-16 hours to encourage sporulation. Sporulation was visually confirmed by the presence of large spore showers around the corpses, and corpses that failed to sporulate were discarded and not used in the experiment. Sixteen cohorts of ten young apterous adults (= 160, 10 ± 1 days old aphids) from each line (5D-X, H, XH, AB; = 640) were placed in a 35 mm diameter deep Petri dish with a fungal agar plate (with two corpses) inverted over them, thus mimicking a natural spore shower. A slide cover slip was placed under the aphids to ensure sporulation occurred post exposure. Additionally, four replicates (= 40) of an aphid line (WI27-HR) superinfected with *H. defensa* and *Regiella insecticola* and highly resistant to *P. neoaphidis*, were included as a control in this assay. Fungal plates were rotated every fifteen minutes between replicates of similar infection status to normalize the number of spores released. This procedure was repeated for a total exposure time of one hour and thirty minutes, at which point each cohort was placed onto a fresh *V. fava* plant at 100% humidity (via an unvented cup lid), 20°C, and normal long-

day light conditions for 24 hours. At this point, the unvented lid was then replaced with a vented lid (fungal assay modified from Weldon et al. in press, Ferrari et al. 2001, Łukasik et al. 2013, Parker et al. 2013). Aphids were checked every twenty-four hours for ten days post-exposure for aphid survival, dual mortality (aphid and pathogen), and sporulation; any offspring produced were removed. As the Aphid survival, sporulation, and mortality had non-normal distributions, the data were arcsin transformed and the distributions were checked for normality using the Goodness-of-fit test. The results were then compared between aphid lines using logistic regression, as well as ANOVA with Tukey's HSD *post-hoc* test to compare means. Additionally, a lognormal distribution was used to estimate 0.5 survival as in the fecundity assay.

2.3.7 Heat Shock Assay

Adult aphids were placed on fresh plants and allowed to reproduce for 24 hours, after which point the offspring were collected and placed onto fresh *V. fava* in cohorts of 10 newborn nymphs. Twelve replicates of cohorts in each line were divided into one of two temperature treatments, modified from Russell and Moran (2006). The control treatment consisted of four replicates (= 160 aphids) kept at a standard temperature of 20°C for the duration of the experiment, while the remaining eight replicates (= 320 aphids) were maintained at 20°C for two days (~2nd instar), at which point the temperature was gradually increased over the span of 2 hours to 37.5°C (± 1°C). The temperature was maintained at 37.5°C for an additional two hours, then reduced gradually to 30°C (± 1°C) and maintained for another hour. Finally, temperature was reduced to 20°C for the remainder of the experiment, with heat shocked aphids transferred to fresh plants the day after heat shock to reduce any temperature effects on

the host plant. Aphids were monitored every two days until day eight, as in the original developmental assay, at which point they were monitored twice a day (morning and afternoon) for reproduction. Upon reproduction, the TFR was recorded and the first five aphids to reproduce in each cohort were individually transferred to a fresh *V. fava* sprout to record fecundity. The reproductive output of this subset of aphids was monitored every three days until day nine post-TFR, at which point most X-type infected aphids either died or stopped reproducing. Offspring were removed, counted, and discarded. The number of aphids surviving to adulthood was compared between aphid line and temperature treatment using logistic regression. TFR had a non-normal distribution, so the non-parametric Wilcoxon-rank sum test with a Bonferroni corrected significance level was used to compare developmental time between lines and treatments. To compensate for adult mortality at the later time points, fecundity data was treated both as rate of reproduction (offspring/day) and summed as total fecundity and compared using ANOVA with Tukey's HSD.

2.3.8 Symbiont titers

Symbiont titers in uninfected (only *B. aphidicola*), single (H or X), and superinfected (XH) aphids were estimated from whole aphid extractions of aphids collected at five time points representing 2^{nd} - 4^{th} instars, as well as early and older adults (i.e. 48, 96, 144, 192, 288 h) in replicates of eight aphids per time point. DNA was extracted as previously described, except in the case of the 288h old aphids, which were extracted using the E.Z.N.A. Tissue DNA kit (OMEGA Bio-Tek). Fragments of the single-copy bacterial genes, *dnaK* from *H. defensa* and *gryB* from X-type, along with the polyploid *dnaK* gene from *B. aphidicola*, were amplified using RT-qPCR (Moran et al.

2005a, Wilson et al. 2006, Weldon et al. 2013, Martinez et al. 2014b). The X-type primers new to this study were: forward (ACG GAG GTG AGT ACC CGA AAA) and reverse (ATC AGC GTT CAT CTC TCC CA). 10 µL PCR reactions were performed on a Roche LightCycler 480 II using Roche LightCycler SYBR Green I Master chemistry and 0.5 µM of forward and reverse primers. A standard PCR cycle was used for all primers: 95˚C for 5 min; 45 cycles of 95˚C for 10 sec, 68-56˚C touchdown for 13 cycles, then 55˚C for 32 cycles, each cycle for 10 sec; 72˚C for 10 sec, as in (Weldon et al. 2013). Symbiont titers were estimated using external standard curves of each gene previously created with serial dilutions of $1x10^2$ to $1x10^9$ with duplicate standards of $1x10^7$ (Oliver et al. 2006). Additionally, to correct for differing extraction efficiency between DNA samples, the aphid *Ef-1 α* gene was amplified for all samples. A correction factor was determined by dividing the highest *Ef-1 α* by the *Ef-1 α* of each sample for each time point; symbiont estimates were then multiplied by this factor. The symbiont titers were log-transformed, and the distributions of symbiont titers in each experimental line at each time treatment were checked for normality using the Goodness-of-fit test. Transformed titers of each symbionts were then compared (when applicable) between aphid lines and time using ANOVA with Tukey's HSD.

2.3.9 X-type strain variation and phylogeny

To examine strain variation of N. American X-type, I used primers and reaction conditions developed in Henry et al 2013 for sequencing five X-type housekeeping genes (*accD, hrpA, murE, recJ, rpoS*). As the *accD* gene was most effective at revealing X-type strain variation (Russell, personal communication), *accD* sequences from 146 aphid samples from Wisconsin, collected from 2012-2015 were amplified using PCR, with

amplification confirmed after running samples on 1% agarose gel. PCR product was sent to Eurofins MWG Operon for sequencing after enzyme cleaning with 0.3 µL exonuclease I and 0.5 µL FastAP under the following protocol: 35˚C for 15 min; 85˚C for 15 min; hold at 10˚C. A subset of these samples were also used to generate additional *rpoS* (= 19) and *hrpA* (= 21) sequences, as these housekeeping genes were also determined to be effective at determining X-type strain variation. Using Geneious v8.1.5, gene sequences were then aligned with reference sequences from Henry et al, as well as sequences obtained from Jake Russell (Drexel), with ClustleW using MUSCLE and trimmed manually. The same protocol was used to investigate the strain diversity of *H. defensa* found superinfecting with X-type, focusing on the *ptsI* and *recJ* genes of *H. defensa* (primers and reaction from Degnan and Moran 2008, Russell personal communication). A total of 89 samples (65 from superinfections) collected from Wisconsin over 2011-2015 were sequenced and aligned as previously described. The tendency for a particular strain of *H. defensa* to associate with X-type was analyzed using Fisher's Exact Test with Bonferroni correction.

To create a phylogeny of X-type within the Enterobacteriaceae, two X-type housekeeping genes (*accD, gyrB*) and the 16s rRNA gene were amplified from strain 5D. I also obtained representative sequences of these same housekeeping genes and 16s genes of several other Enterobacteriaceae, including other pea aphid HFS and free-living bacteria (*H. defensa*, *R. insecticola, Serratia* spp, *Yersinia* spp*, Klebsiella pneumoniae, Erwinia amylovora, Enterobacter cloacae, Esherichia coli, Shigella flexneri, Salmonella enterica, Pantoea agglomerans, Proteus vulgaris,* and the outgroup *Vibrio cholerae*) downloaded from Genebank. Concatenated sequences were aligned in Geneious as

above, and the Akaike information criteria (AIC) was calculated using MEGA v6.0 and used to predict the best fit model of evolution. Additionally, concatenated sequences underwent DualBrothers recombination analysis. The best fit model, Tamura-Nei with gamma distribution and transition/transversion bias, was used along with PHYML in Geneious to generate Maximum likelihood trees, which were run with 1000 bootstrap iterations.

2.4 Results

2.4.1 Cost of X-type on host fitness in the absence of stress

Fecundity

Using the established complement of infections in the controlled genotype, 5D, I found substantial reductions in aphid fecundity for aphids harboring X-type relative to uninfected controls (**Figure 1**, fecundity significance values in **Table 1**). Surprisingly, while *H. defensa* infection resulted in significant increases in fecundity compared to controls, superinfections with X-type not only eliminated this benefit but significantly

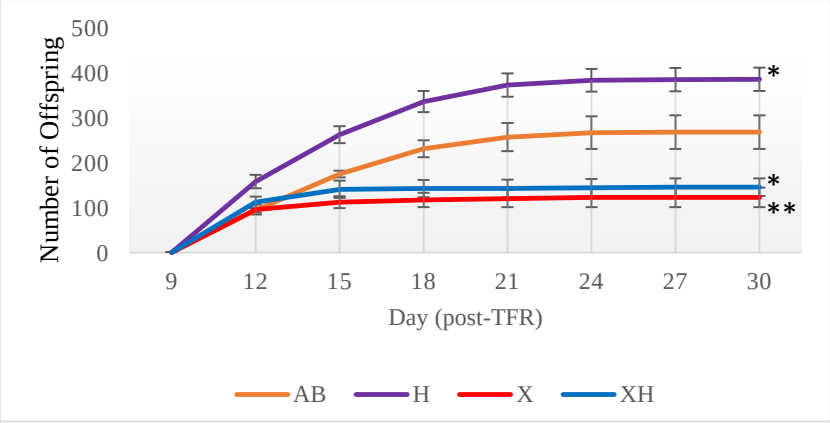

Figure 1 Cumulative fecundity in the absence of natural enemies. Bars represent standard error. Total fecundity at day 30 was compared among lines using Dunnett's test with AB line as control. Significant differences between lines are marked in **Table 1**.

decreased fecundity almost to levels seen in X-type single infections (**Fig 1**). X-type-infected aphids (X and XH) appear to reproduce normally for the first 3-6 days of adulthood, at which point nearly all reproduction stops (**Fig 1**). This abrupt cessation of reproduction seen with X-type infection is not due to shorten lifespan, as X-type-infected

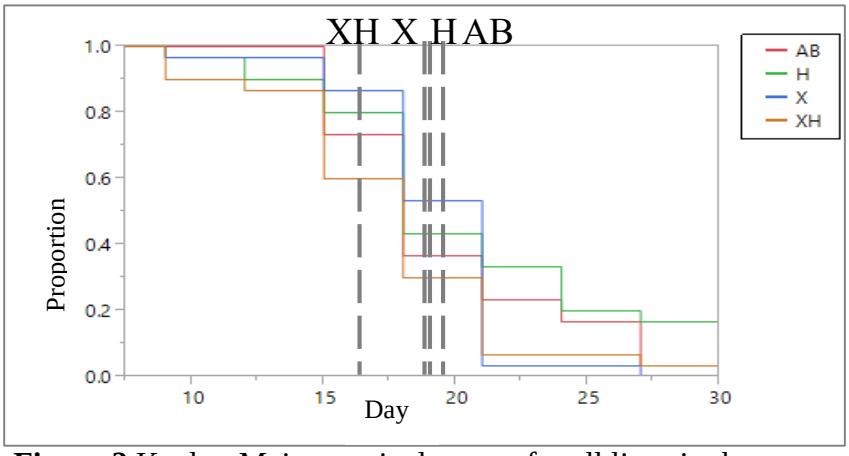

Figure 2 Kaplan-Meier survival curves for all lines in the absence of natural stress. Dashed lines represent estimated 0.5 survivorship, $\alpha = 0.05$. Exact values in **Table 1**.

aphids had a similar estimated time to 0.5 survival as *H. defensa* and uninfected aphids (**Fig. 2**; **Table 1**); the superinfected line did suffer a reduction in survival, suggesting that early mortality may have depressed the total fecundity of superinfected aphids in this assay. Interestingly, this reproduction cessation (3-6 days post adulthood) coincides with a severe phenotypic change seen in X-type infected aphids (both X and XH), in which the aphids become extremely engorged resulting in the complete loss of reproductive capabilities, although they occasionally produce small, developmentally incomplete and inviable offspring (Doremus, personal observation). Dissections of these large, bloated aphids revealed only a few malformed offspring, while uninfected aphids of the same age contained ~15-20 properly developed offspring.

Development effects

Single infection with X-type resulted in significantly faster aphid development than both uninfected and *H. defensa*-infected lines (**Table 1**). Although superinfection resulted in slightly faster developmental time, and single infection with *H. defensa* resulted in delayed development, neither effect was significant relative to uninfected controls (line AB) (**Table 1**). Aphids infected with X-type (both X and XH) were significantly larger and heavier at adulthood (measured as TFR) than uninfected and *H. defensa*-infected aphids (AB and H; **Table 1**). *H. defensa*-infected aphids did not differ in either weight or body size from the uninfected lines, nor did X-type and superinfected lines differ from each other in weight and body size (**Table 1**). The decreased developmental time, as well as the larger size and heavier weight of adult X-type-infected aphids suggests a general fitness benefit with X-type infection, while no discernable fitness costs (aside from delayed reproduction) or benefits were found for infection with *H. defensa.*

Table 1 Aphid development measured as time from birth to first reproduction (TFR) analyzed with multiple Wilcoxon Rank Sum tests (with Benjamini-Hochberg Corrected $\alpha = 0.0214$), only comparisons to uninfected control line (AB) included. Fresh weight, body size, and fecundity compared using Dunnett's test with uninfected line (AB) as the control. Survival data was fit to a lognormal distribution for estimates of 0.5 survivorship, $\alpha = 0.05$

Line	**Time to First Reproduction** (hr) ± SE, (p-value)	**Fresh Weight** (mg) ± SE, (p-value)	**30 Day Fecundity** Offspring ± SE, (p-value)	**0.5 Survival** (N = 30)
AB	237.13 ± 2.49	3.13 ± 0.16, (1.0)	268.5 ± 37.23, (1.0)	19.15
H	246.66 ± 3.61, (0.2166)	3.11 ± 0.092, (0.9983)	386.17 ± 26.07, (**0.0153**)	19.58
X	227.62 ± 2.36, (**0.0196**)	3.63 ± 0.10, (**0.0163**)	123 ± 21.44, (**0.003**)	18.91
XH	230.67 ± 2.54, (0.0399)	3.65 ± 0.14, (**0.0111**)	145.83 ± 19.79, **(0.0118)**	16.87

2.4.2 Effects of X-type infection and temperature on aphid resistance to parasitoid natural enemies

Using the same experimental lines as the fitness assays (genotype 5D), I investigated both the protective phenotypes of X-type and *H. defensa*-APSE8 as single infections as well as the proposed rescue synergism of lost symbiont-based resistance at warm temperatures, previously observed in *H. defensa*-X-type superinfections (Guay et al 2009). Consistent with prior *H. defensa* parasitisms, pea aphids infected with *H. defensa*-APSE8 enjoyed moderately reduced rates of mummification compared to their uninfected counterparts when held at 20 °C (**Fig. 3**, LRE Y = -0. 26 – 0.598^{XH} - 0.60^{H} – 0.007^{X}; LRT $F_H = 7.18$, P = 0.0073). Additionally, pea aphids superinfected with *H. defensa* + PAXS showed a similar reduction in mummification (LRT $F_{XH} = 8.06$, P = 0.0045); however, as a single infection, X-type had no effect on mummification at 20°C (LRT $F_X = 0.002$, P = 0.968). Consistent with previous assays observing the effect of temperature on *H. defensa*-based protection, the protection conferred by *H. defensa* failed at the warmer

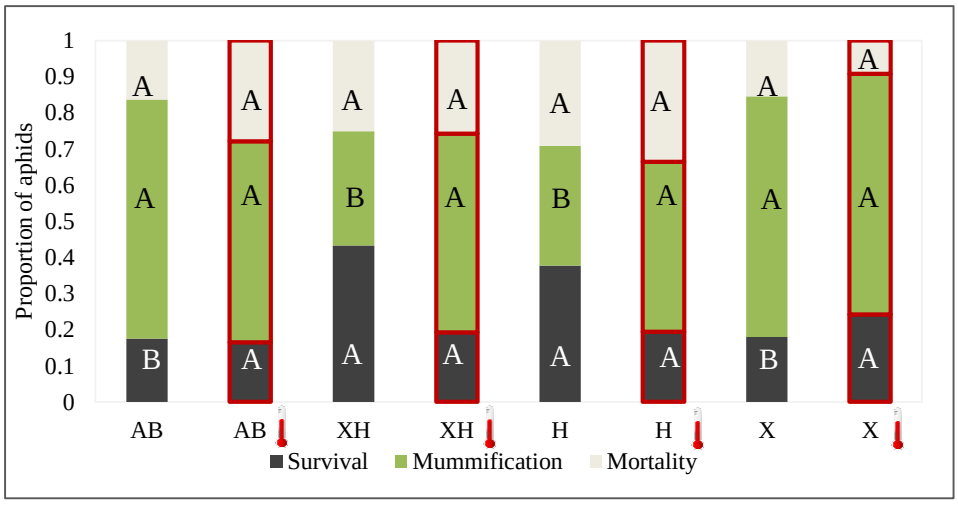

Figure 3 Mean survival, mummification, and mortality post-parasitism. Red-bordered bars represent 30°C/24°C treatment, bars lacking border represent 20°C treatment. Different letters represent significant differences among lines, within each treatment determined by Tukey's HSD after arcsin transformation (α = 0.05).

temperature cycle (30°C day/24°C night); however this protection was lost not only in the *H. defensa* singly infected line (**Fig. 3**, LRE Y = -0.26 + 0.539^{XH} + 0.496^{H} - 0.130^{X} – 0.021^{AB}; LRT F_H = 4.37, P = 0.0365), but also in the line superinfected with both *H. defensa* + X-type (LRT F_{XH} = 6.15, P = 0.0131), showing that X-type does not rescue the loss of symbiont-conferred protection at elevated temperatures. I also found no effect of X-type as a single infection on mummification at elevated temperatures (**Fig. 3**, LRT F = 0.487, P = 0.485), nor any effect of elevated temperature on the rate of mummification in aphids lacking facultative symbionts (**Fig. 3**, LRT F = 0.012, P = 0.9129). X-type alone does not confer any protection to the pea aphid against *A. ervi*, nor did temperature itself effect the success of *A. ervi* mummification in uninfected aphids, reinforcing the observation that the increased mummification rates observed at elevated temperatures were due to the loss of *H. defensa*-based protection.

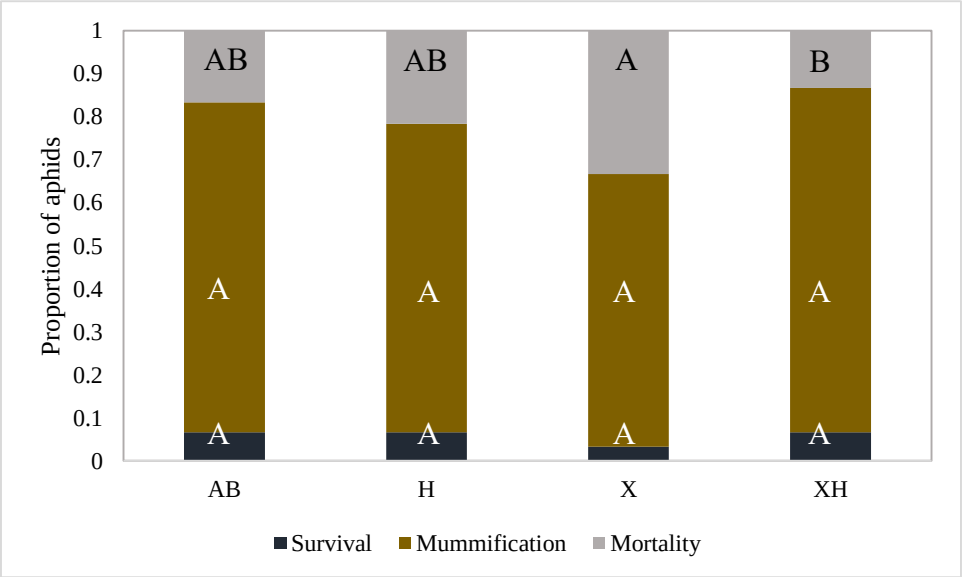

Figure 4 Mean aphid survival, mummification, and mortality after parasitism by *P. pequodorum*. Different letters represent significant differences determined by ANOVA with Tukey's HSD test after arcsin transformation of data (α = 0.05)

Additionally, superinfections with X-type did not affect aphid mummification when attacked by a second parasitoid species, *P. pequodorum* (**Fig. 4**; LRE Y = -0.17 - $0.03^{XH} - 0.03^{H} - 0.28^{X}$; LRT $F_{XH} = 0.0061$, P = 0.9376), nor did *H. defensa*-APSE8 (Fig. 4; LRT $F_{H} = 0.011$, P = 0.9155) or X-type (**Fig. 4**; LRT $F_{X} = 0.935$, P = 0.3335) increase aphid resistance to *P. pequodorum* as a single infection. Neither X-type, nor *H. defensa*-APSE8, offers any protection to the pea aphid host against *P. pequodorum.*

2.4.3 Effect of X-type on aphid resistance to a fungal pathogen

To investigate the protective effects of X-type on yet another common enemy, aphids were exposed to the specialized fungal pathogen, *P. neoaphidis*, with aphid survival, sporulation (pathogen survival), and dual-mortality measured over a period of 10 days post-fungal incubation (Łukasik et al. 2013). Additionally, an aphid line infected with *R. insecticola* was used as a control, as it has been previously determined to be resistant to *P. neoaphidis* when infected with *R. insecticola* (Weldon 2015, unpublished).

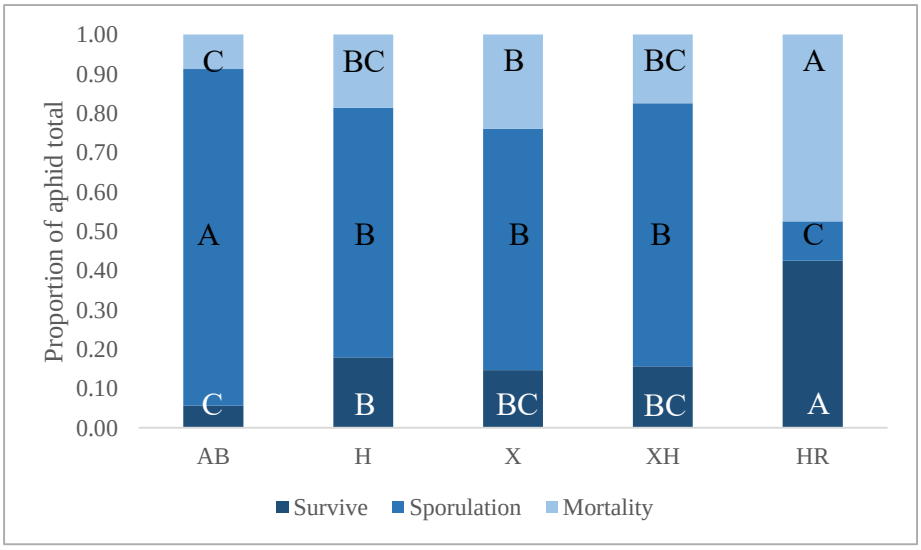

Figure 5 Mean aphid survival, sporulation, and mortality after exposure to *P. neoaphidis*. Different letters represent significant differences determined by ANOVA with Tukey's HSD using arcsin transformed data ($\alpha = 0.05$).

Neither single infection with X-type, nor superinfection with both HFS, increased aphid survival when exposed to *P. neoaphidis* (**Fig. 5**; LRE $Y = -1.10 + 0.09^{H} + 0.07^{X} + 0.07^{XH} + 0.79^{R}$; LRT $F_X = 0.67$, $P = 0.4118$; LRT $F_{XH} = 0.69$, $P = 0.4062$). Infection with *H. defensa* resulted in a small, albeit significant, increase in survival, suggesting a minor protective effect of *H. defensa*-APSE8 against *P. neoaphidis* (**Fig. 5**; LRT $F_H = 1.07$, $P = 0.3004$). A more sizable increase in survival was seen with *R. insecticola* infection (LRT $F_R = 10.76$, $P = 0.001$).

Surprisingly, both X-type and *H. defensa* single infections resulted in decreased fungal sporulation compared to the uninfected aphid line (**Fig. 5**; LRE $Y = 0.27 - 0.637^{H} - 0.571^{X} - 0.561^{XH} - 1.28^{HR}$; LRT $F_H = 11.56$, $P = 0.0007$; LRT $F_X = 8.33$, $P = 0.0039$). Additionally, superinfection resulted in a similar, minor decrease in sporulation compared to the uninfected line, suggesting that superinfection with X-type and *H. defensa* does not provide any additive benefits in this interaction (LRT $F_{XH} = 8.53$, $P = 0.0035$). Aphids infected with *R. insecticola*, which is known to increase aphid resistance to *P. neoaphidis*, showed greatly decreased sporulation compared to uninfected controls consistent with that of other *R. insecticola* strains, as well as other HFS known to protect against *P. neoaphidis* (**Fig. 5**; LRT $F_R = 29.55$, $P = <0.0001$; Weldon et al, in press, Lukasik et al 2013). HFS infected lines also showed increased host mortality after pathogen exposure which may be responsible for the observed reduction in sporulation (**Fig. 5**; LRE $Y = -0.29 + 0.43^{H} + 0.51^{X} + 0.34^{XH} + 0.84^{R}$). Additionally, average time of sporulation was delayed in all lines infected with HFS, compared to the line lacking HFS (**Fig. 6**; Multiple Wilcoxon $P = <0.001$ for all comparisons, Bonferroni-corrected $\alpha = 0.0125$).

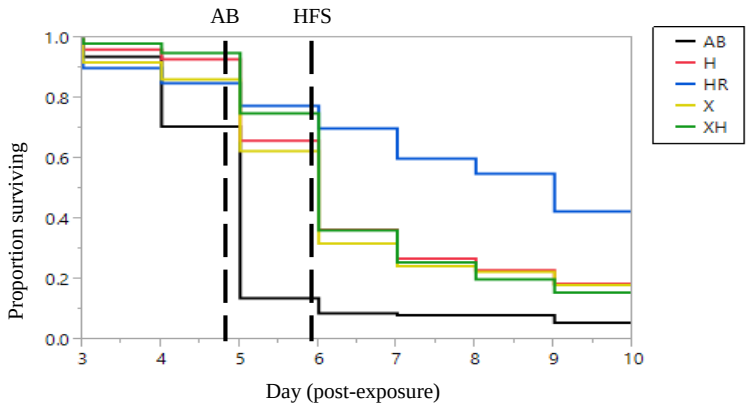

Figure 6 Kaplan-Meier plot of aphid survival post exposure to *P. neoaphidis*. Dashed lines represent estimated time of 0.5 sporulation for aphids lacking facultative symbionts (AB) and those with symbionts (HFS) ($\alpha = 0.05$).

2.4.4 Effect of X-type on aphid thermal tolerance

I compared developmental time, measured as the time from birth to first reproduction (TFR), across our four experimental lines when exposed to heat shock both within lines (heat shock or not) or among lines using the uninfected line (AB) as a control. Consistent with results of the first fitness assay conducted only at 20°C, aphids infected singly with X-type reached developmental maturity faster than uninfected or *H. defensa* singly infected aphids both in the presence and absence of heat stress (**Table 2**). Superinfected aphids also developed faster in both temperature treatments, although not significantly so in the 20°C treatments (**Table 2**). Heat shock delayed reproduction in all lines compared to their 20°C counterparts, with the X-type singly infected line exhibiting the longest delay while the delay was slightest for superinfected aphids. Infection with HFS (X-type or *H. defensa*) as a single or superinfection, as well as heat shock, had no

Table 2 Aphid development time as TFR analyzed at control and heat shock conditions with multiple Wilcoxon Rank Sum tests (Bonferroni corrected $\alpha = 0.0083$), comparisons to AB line (among lines) and 20°C (within lines; p-values only) included. Daily fecundity compared using Dunnett's test with AB as control, $\alpha = 0.05$.

Line	Temperature Treatment	Time to First Reproduction hr ± SE, (p-value)		Daily Fecundity offspring/day ± SE, (p-value)	
		Compared to AB	Compared to 20°C	Compared to AB	Compared to 20°C
AB	Control (20°C)	242 ± 3.18, (1.0)	0.1626	5.00 ± 0.31, (1.0)	0.6951
AB	Heat Shock (37.5°C)	251.9 ± 4.52, (1.0)		5.16 ± 0.29, (1.0)	
H	Control (20°C)	243.35 ± 4.6, (0.782)	0.1555	4.65 ± 0.34, (0.4638)	0.7202
H	Heat Shock (37.5°C)	251.44 ± 3.82, (0.8125)		4.50 ± 0.20, (0.1081)	
X	Control (20°C)	228.03 ± 5.57, (**0.0063**)	**0.0055**	1.99 ± 0.22, (<**0.0001**)	**0.0296**
X	Heat Shock (37.5°C)	245.93 ± 4.69, (0.4075)		2.64 ± 0.18, (<**0.0001**)	
XH	Control (20°C)	234.56 ± 3.64, (0.1338)	0.8738	3.89 ± 0.29, (**0.0097**)	**0.0029**
XH	Heat Shock (37.5°C)	236.74 ± 2.92, (**0.0083**)		5.10 ± 0.26, (0.9960)	

effect on aphid survival to reproduction, although there was a non-significant reduction in survival in all aphid lines in the heat shock treatment and in singly infected aphids (both HFS species) in both temperature treatments (LRE $Y = -0.059 + 0.085^{XH} - 0.299^{X} - 0.152^{H} - 0.154^{Temp}$; LRT $F_{XH} = 0.202$, $P = 0.653$; $F_X = 2.27$, $P = 0.132$; $F_H = 0.611$, $P = 0.435$; $F_{Temp} = 1.13$, $P = 0.287$).

To account for the increased mortality that can affect fecundity at later time points, reproductive output was analyzed both in terms of cumulative fecundity and reproduction rate (offspring of each aphid/day). As above, I also compared fecundity among our four experimental with the uninfected line (AB) serving as a control, and within each line (heat shock vs. control). In the within line comparisons, heat shock had no effect on total fecundity (Student's T-test $T_{AB} = 0.332$, $P = 0.742$; $T_H = 0.11$, $P = 0.91$) nor rate of

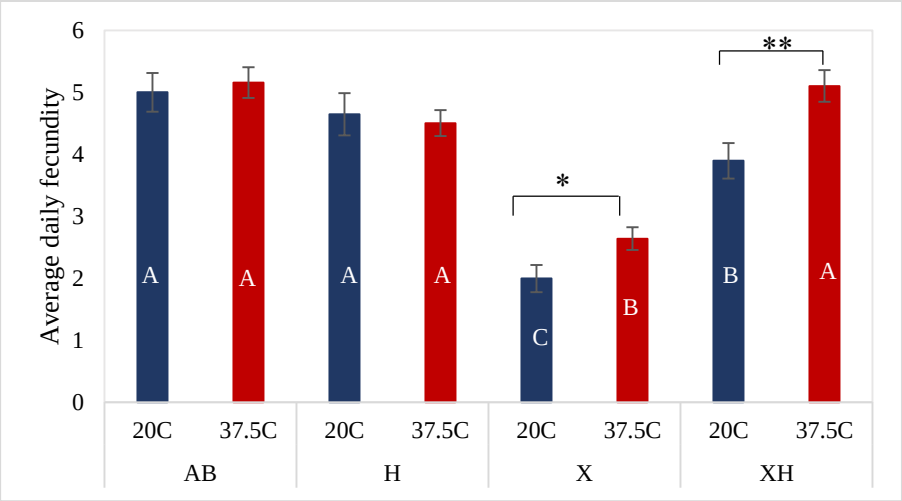

Figure 7 Daily reproduction per aphid at 20°C (blue bars) and 37.5°C heat shock treatments (red bars). Asterisks represent significant differences between heat shock and control for each experimental line (Student's T-test). Different letters represent significantly different values compared to the uninfected control line,within temperature treatments (AB; Dunnett's test, see **Table 2** for significance values) (α = 0.05)

reproduction (**Fig 7**; T_{AB} = 0.395, P = 0.695; T_{H} = -0.361, P = 0.72) of uninfected and *H. defensa*-infected aphids compared to 20°C controls. Surprisingly, aphids infected with X-type or superinfected with both X-type and *H. defensa* exhibited higher total fecundity (T_{X} = 1.81, P = 0.0384; T_{XH} = 4.28, P = 0.0001) and rate of reproduction (T_{X} = 2.24, P = 0.0148; T_{XH} = 3.14, P = 0.0015) after heat shock compared to 20°C controls (**Fig 7**). In among lines comparisons, *H. defensa* infection did not impose a cost to total fecundity or reproductive rate in either treatment compared to the uninfected control (Fig 7), but I also did not see fecundity increases as seen in the first assay. In contrast, X-type only aphids again exhibited greatly reduced fecundity and reproductive rate compared to uninfected (AB) controls; thus despite increased reproductive output post heat shock, these aphids still suffered a severe reduction in fecundity relative to uninfected controls (**Fig 7**). Finally, superinfected aphids also exhibited a reduction in fecundity and reproductive rate

at 20°C, but interestingly, this cost was eliminated in the heat shock assay where the reproduction of superinfected aphids was on par with that of controls (**Fig 7**).

2.4.5 HFS titers and impact on *Buchnera*

Buchnera genomic copies varied over time within each experimental line and each line exhibited a general decrease in *Buchnera* during the nymphal instars, followed by increases upon reaching adulthood (**Fig. 8a**). However, there were no consistent effects of single or superinfections with HFS on *Buchnera* titers, although titers were generally higher in the superinfected line. *H. defensa* titers increased during single and superinfected nymphal development, but decreased or leveled off upon reaching adulthood (**Fig. 8b**). X-type titers in single infections generally increased as the aphid

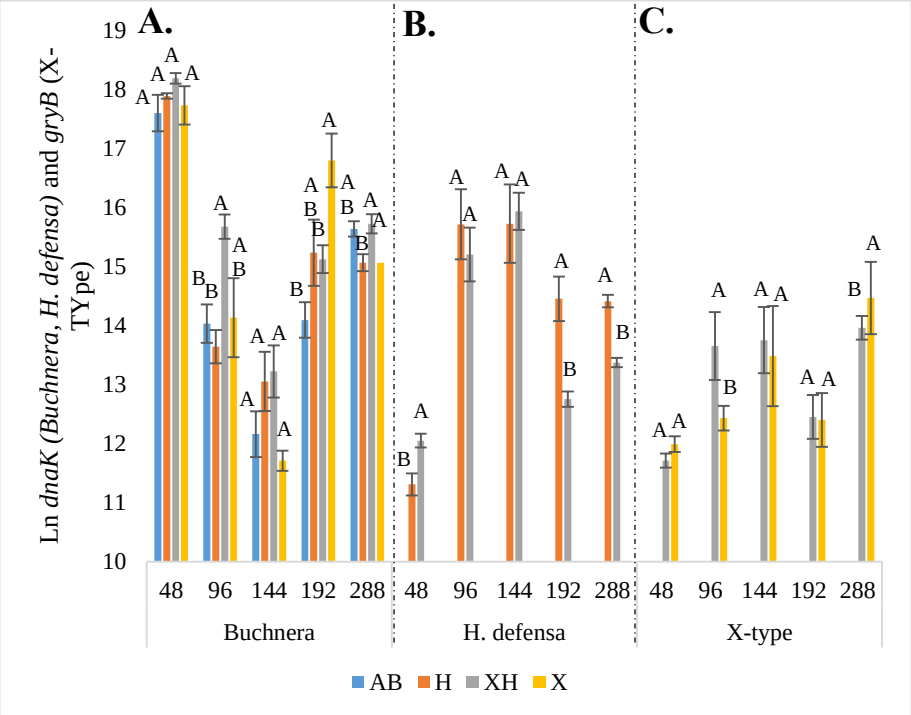

Figure 8 Natural log values of mean estimated gene copies (or genome for *Buchnera*) within aphid across five time points. A) *Buchnera* genomic copies of *dnaK* in all four experimental lines, compared with ANOVA using Tukey's HSD. B) Mean *H. defensa dnaK* copies in single and superinfections, compared using Student's T-test. C) Mean *gyrB* copies in single and superinfections, compared using Student's T-test. Letters represent significant differences, $\alpha = 0.05$ for all comparisons.

aged, while X-type titers in superinfections remained steady throughout aphid development (**Fig. 8c**) In single infections, *H. defensa* is generally found at higher titers than X-type consistent with previous studies comparing *H. defensa* superinfections with *R. insecticola* (Weldon et al, submitted), and *S. symbiotica* (Oliver et al. 2006). These studies also found that superinfection did not affect *H. defensa* titers, but the titer of the second superinfecting HFS species (*R. insecticola* or *S. symbiotica*) was elevated. Contrary to this previous trend, this study found that *H. defensa* titers decreased significantly in adult aphids (e.g. 192 and 288h) superinfected with X-type, while X-type titers were either uninfected (192h) or increased (288h) in superinfections, suggesting a

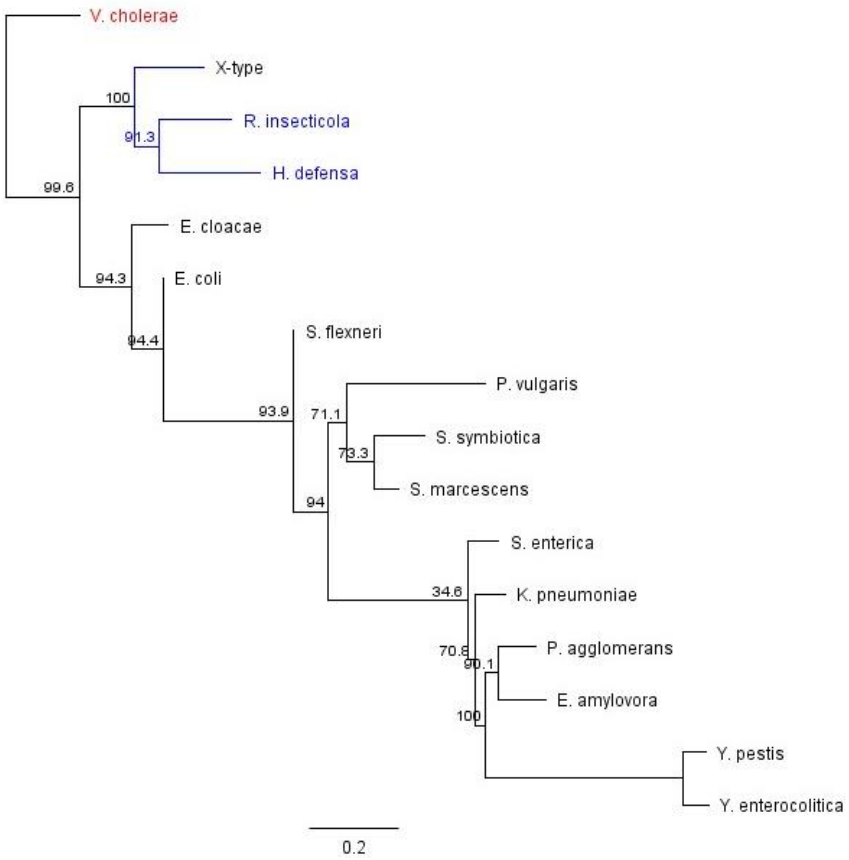

Figure 9 TN93+G+I ML PHYML tree of concatenated bacterial *accD*, *gyrB*, and *16S* sequences. *V. cholerae* (red) was used as an outgroup. Clade containing X-type highlighted in blue. The bar represents substitutions per site.

possible antagonism between X-type and *H. defensa*. At the latest time point (288h), where observable fitness costs emerge in X-type infected aphids, X-type titers began to surpass those of *H. defensa* in the superinfected line (XH) and rose dramatically in the single infected line (X), suggesting the presence of a detrimental effect of increasing X-type titers on aphid fecundity beginning at approximately 12 days of age.

2.3.6 HFS strain variation and X-type phylogenetic position within Enterobacteriaceae

Phylogenetic analysis of X-type within the Enterobacteriaceae, including several other known pea aphid HFS, revealed X-type is most closely related to the sister species HFS *H. defensa* and *R. insecticola*, but more sampling is needed to confirm whether the three form a symbiont-only clade (**Fig. 9**). I also identified very little strain variation in the X-type symbiont. I found 100% nucleotide similarity in two housekeeping genes (*hrpA*, *rpoS*) sequenced for X-type across our WI samples or those from Russell or Henry et al 2013 and among 146 X-type infected aphids collected across a four year span from Wisconsin, I found a single SNP in the *accD* loci from a single sample. While our sampling is most intense in Wisconsin, comparisons with samples from New York and Pennsylvania strongly suggest that X-type diversity is very limited and the strain used in this study appears to be the dominant strain in North America (Russell, personal communication).

As X-type is typically found superinfecting aphids with *H. defensa*, I also sequenced several housekeeping genes (*ptsI*, *recJ*) of *H. defensa* in X-type-*H. defensa* superinfections. Remarkably, I found that 58 of the 65 total sequenced superinfected samples contained identical *H. defensa* strains. Thus, X-type primarily associates with a

single strain of *H. defensa*, suggesting the presence of more complex interactions between not only HFS species, but strains within those species (Fisher's Exact P = <0.0001).

2.5 Discussion

Controlling for genetic background, I examined effects of infection with the X-type symbiont, both as a single-infection and as a co-infector with its most common partner, *H. defensa*, on the protective phenotype of pea aphid against three natural enemies and thermal stress. I found that X-type as a single or co-infection does not contribute to resistance to parasitism by either *A. ervi* or *P. pequodorum*, the two parasitoids commonly attacking this aphid in North America. While I found that *H. defensa*-based resistance is lost at higher temps, consistent with earlier reports (Doremus in prep, Bensadia et al 2006), I did not find that X-type rescues this loss as previously reported (Guay et al. 2009). I also did not find roles for X-type, as single or superinfection, in providing protection against fungal pathogens or thermal stress as reported for European strains (Heyworth and Ferrari 2015). Instead, I identified large fitness costs of infection for aphids infected with only X-type in the form of substantially reduced fecundity; costs which are often ameliorated when X-type is found superinfecting with *H. defensa*, especially following heat shock. Contrary to the observed fecundity costs, X-type infection resulted in faster developmental time compared to uninfected aphids; however, aphids can undergo faster development in stressful situations, and it is possible that the observed aphid developmental effects resulted from X-type-induced stress (Altincicek et al. 2008, Barribeau et al. 2010). Given that X-type occurs almost exclusively as a superinfector combined with very limited strain diversity

of X-type detected in North America pea aphid populations, the results of this study suggest that X-type may persist in pea aphid populations only in superinfected hosts by hitchhiking with a beneficial heritable partner.

Contrary to a previous study concerning X-type's role in aphid resistance to parasitoids, I found no evidence that X-type protects the pea aphid against the dominant parasitoid, *A. ervi*, either through acting alone or via the synergistic rescue of *H. defensa*-based resistance at warm temperatures (Guay et al. 2009). The previously described rescue effect may be the result of innate aphid resistance to *A. ervi*, which is not lost at high temperatures (Doremus in prep.) rather than the presence of X-type. Additionally, X-type did not protect aphids against a second wasp species, *P. pequodorum*, which is also unaffected by the *H. defensa*- or host-based resistance that is effective against *A. ervi* (Martinez et al. 2016). X-type also does not confer benefits in response to the specialized aphid fungal pathogen, *P. neoaphidis*, or thermal stress, the two other common protective benefits currently attributed to pea aphid HFS (Russell and Moran 2006, Łukasik et al. 2013, Oliver et al. 2014, Heyworth and Ferrari 2015). Although X-type single and superinfections did decrease *P. neoaphidis* sporulation compared to uninfected controls, these infections did not result in significantly increased aphid survival, rendering an anti-fungal benefit unlikely for X-type. While X-type may provide some yet untested benefit, such as resistance to bacterial or viral pathogens attributed to another common HFS *Wolbachia* (Hamilton and Perlman 2013), the lack of expected benefits identified here suggests that X-type may not be a conditional mutualist like *H. defensa* and other common aphid HFS, and as suggested in the literature. It is also unlikely that X-type invades pea aphid populations as a reproductive manipulator, as pea aphids primarily

reproduce asexually, although some reproductive manipulation has been seen with *Spiroplasma* infected pea aphids during their sexual phase (Simon et al. 2011).

Despite not finding any of the expected defensive benefits, X-type commonly is found in North American pea aphid populations at intermediate infection frequencies (~3 – 40% infection rate; Russell et al 2013, Smith et al 2015, Doremus unpublished), which is even more surprising considering X-type infections impart a severe cost to host fecundity in single infections (**Fig. 1, 8**). Preliminary experiments found no instances of horizontal (i.e. infectious) transmission, which combined with vertical transmission assays (data not shown) suggests that X-type is primarily vertically transmitted like other common pea aphid facultative symbionts (Doremus, unpublished, Moran and Dunbar 2006). Interestingly, superinfections seem to mitigate the cost of harboring X-type, particularly under conditions of heat stress, where I found no difference in fecundity between super- and uninfected aphids (**Fig 8**). The cause of this mitigation is unclear, but may involve reduction of symbiont titers, possibly through antagonist interactions between HFS (**Fig. 9b**). Heat shock has also been shown to decrease HFS titers (Burke et al. 2010). Reduced costs of superinfections may allow for the maintenance of *H. defensa* and X-type superinfections when parasitism pressure is high, an effect that may be enhanced when the host population is also exposed to thermal stress.

Of course, there are numerous cases of heritable symbionts carrying high infection costs, including strains of *H. defensa* in black bean and pea aphids (Vorburger and Gouskov 2011, Weldon et al submitted), but these costs are likely offset by strong conditional benefits. There are also virulent *Wolbachia* strains, including *w*MelPop, yet these HFS also likely spread via protective benefits and/or reproductive manipulation

(Min and Benzer 1997, Chrostek et al. 2013). The phenotype conferred by X-type infection shares particular similarity with that of *w*MelPop; both carry severe costs that develop in adult hosts, likely due to over-replication of the virulent symbiont (Min and Benzer 1997, Reynolds et al. 2003, Chrostek and Teixeira 2015).

Theory and empirical work have revealed two primary strategies for the spread and maintenance of heritable symbioses: providing net fitness benefits and manipulating host reproduction (Werren and O'Neill 1997, Moran et al. 2008, Jaenike 2012, Oliver et al. 2014). With the caveat that another, yet unidentified, synergistic benefit may underlie the maintenance of *H. defensa*-X-type superinfections, this marks, to our knowledge, the first empirical evidence for a heritable bacterium apparently 'hitchhiking' via the benefits provided by a second beneficial symbiont (Smith et al. 2015). This appears to be a tenuous strategy; X-type requires a beneficial partner (*H. defensa*), and pressure from a specific natural enemy to provide selection for the partner symbiont. Yet aphids singly infected with *H. defensa* should still be expected to outperform superinfected aphids implying that that susceptible hosts are also required for the maintenance of this superinfection. While X-type appears to be an atypical case, superinfections are common in many insect species (e.g. Gottlieb et al. 2008, Toju and Fukatsu 2011, Russell et al. 2013) rendering it possible that cheating symbionts are more common than previously thought. Unfortunately, confirming hitchhiking symbionts will remain troublesome, due to the difficulty in 'proving a negative,' as there is always the possibility that the hitchhiker confers some unknown conditional benefit. However, as X-type does not confer any expected benefit common to pea aphid HFS, imposes greater costs as single-

infections, and is rarely found in single infections in NA pea aphid populations, it is likely utilizing a cheater strategy.

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CHAPTER 3

CONCLUSIONS

Terrestrial Arthropods, including insects, often form symbiotic relationships with heritable bacteria, which can have profound effects on host biology. As heritable symbionts propagate primarily through vertical transmission from mother to offspring most have lost the ability to survive *sans* hosts, hence their fitness is typically intimately tied to that of the host. This requirement for a host restricts their ability to propagate, and has led to the development of various strategies to increase stable transmission. This is especially true for heritable facultative symbionts (HFS), which are widespread in nature, yet are not generally required by the host for survival or reproduction and therefore have devised fascinating means to increase the odds of their own success. The most obvious, and likely common, strategy is that HFS spread to new hosts by increasing host net fitness, usually by providing one or more benefits, such as increased resistance to natural enemies. A second general route is that since males are 'dead-end' hosts for HFS, they can spread by manipulating host reproduction in ways that increase the fitness of infected females at the expense of males or uninfected females. Infection with HFS can also incur costs, which depending on their strength, can limit spread or remove HFS from host populations. In my thesis, I suggest the possibility of a third route of symbiont maintenance in host populations, which is that commensal or even pathogenic HFS can be stably maintained by hitchhiking alongside a beneficial symbiont.

The pea aphid, *Acyrthosiphon pisum*, has emerged as a model system for studying heritable symbioses, especially effects of infection on host biology. The parthenogenetic lifecycle and ability to manipulate the diverse menagerie of pea aphid HFS has afforded researchers the ability to alter symbiont infection in genetically controlled experimental lines. The seven species of pea aphid HFS are largely thought to act as heritable mutualists, and several species has established benefits, namely increasing aphid resistance to parasitoid (*Hamiltonella defensa*) or pathogenic (*Regiella insecticola*) natural enemies and increasing aphid thermal tolerance (*Serratia symbiotica*). Despite the model status of the pea aphid, much is still unclear concerning the effects of other HFS species, including the X-type symbiont (γ-Proteobacteria), which has recently been found to commonly superinfect N. American pea aphids with *H. defensa*.

X-type was previously reported to act as a defensive mutualist, with various benefits attributed to X-type infection including the rescue of lost symbiont-based parasitoid resistance at warm temperatures, protection against a fungal pathogen, and increased thermal tolerance; unfortunately it was difficult to parse the effect of X-type from the confounding variables of host/symbiont genotype and superinfection in previous studies. By using a full complement of experimental lines, I determined that X-type does not confer any benefit to the aphid host in response to parasitism, pathogen exposure, or thermal stress, which are all known protective benefits of aphid HFS. I also found no evidence for the previously described synergistic rescue effect of lost symbiont-based resistance in X-type - *H. defensa* superinfections. Rather, I found that X-type infections carry a high cost to the host, which is partially mitigated in superinfections with *H. defensa* under standard conditions and fully mitigated in superinfections at warmer

temperatures. Additionally, by sequencing X-type housekeeping genes, I found low strain variation of X-type in N. America, increasing the likelihood that the results of this study are generalizable to N. American X-type symbionts.

Theory predicts that the virulent X-type should be lost from natural populations, and indeed X-type is rarely found as a single infection; however superinfections of *H. defensa* and X-type are surprisingly common. The lack of an identified benefit combined with this pattern of superinfection reveals a third strategy for HFS propagation: cheating symbionts (either commensals or pathogens) hitchhiking off the benefit of a partner HFS. Co-opting *H. defensa*-based protection against a common aphid natural enemy, the parasitoid *Aphidius ervi*, allows X-type to persist as a heritable symbiont despite its virulent nature. With the caveat that another, yet unidentified, benefit that functions only in concert with *H. defensa* may explain the persistence of superinfections, this study is, to my knowledge, the first case of a successful hitchhiking cheater symbiont. Although, as superinfections are common in nature, there are likely other symbionts employing this strategy.

The antagonistic interaction between X-type and *H. defensa* likely indirectly depresses the frequencies of its partner symbiont, *H. defensa*, in the absence of parasitoid pressure. Additionally, as high temperatures mitigate the cost of X-type superinfections, X-type prevalence may be higher in warmer regions or increase with warming global temperatures. This research further develops our knowledge of the pea aphid-symbiont model system, particularly by contradicting prior reported roles of X-type as a defensive symbiont, and of symbiosis in general, by providing evidence consistent with the

phenomenon of exploitative hitchhiking where a cheater symbiont takes advantage of a mutualistic partner symbiont as a means to locate new hosts.