MATRYOSHKA MUTUALISMS: DEVELOPING THE BACTERIOPHAGE APSE-*HAMILTONELLA DEFENSA-ACYRTHOSIPHON PISUM* SYSTEM AS A MODEL FOR TRIPARTITE SYMBIOSES

by

STEPHANIE RAY WELDON

(Under the direction of Kerry M. Oliver)

Viruses are the most abundant, most diverse, and least understood organic structures on earth. While the best-studied viruses are pathogenic, many viruses engage in commensal or even mutualistic relationships with eukaryotes. In the pea aphid, *Acyrthosiphon pisum,* a facultative bacterial mutualist, *Hamiltonella defensa*, provides anti-hymenopteran parasitoid defense only when it is itself infected by virus APSE (*A*cyrthosiphon *p*isum Secondary Endosymbiont). While this is the only known instance of mutualism-by-proxy, the prevalence of mobile genetic elements in symbionts makes it unlikely that it will be the last. At present, however, the diversity, prevalence, and effects of viruses infecting insect-associated bacteria are largely unknown. I conducted four studies to elucidate the biology of the tripartite aphid/*H. defensa*/APSE interaction. Firstly, within-host bacterial abundance can affect conferred phenotypes and rates of transmission. All stable beneficial heritable symbiont infections must have sufficient titer to produce the beneficial phenotype and ensure vertical transmission to progeny, without engaging in over-replication that might affect host fitness. Using quantitative-PCR and standard fitness tests, I determined that in the absence of APSE *H. defensa* replicates at rates detrimental to its host's development and reproduction. Secondly, previous sampling of North American APSEs in *A. pisum* was limited to a handful of samples from two New York counties and one in Utah. I tested more than 1100 aphids from six states for *H. defensa* presence and phage strain identity: in the process I identified a new strain of APSE, called

APSE-8, and I present its associated defensive phenotype here. Thirdly, most studies examine effects of single heritable symbiont infection, but ~25% of individual aphids carry multiple facultative symbiont lineages despite previous reports of lower fidelity in the transmission of double infections and additional costs to hosts of superinfection I created artificially differentially-infected clonal lineages using *H. defensa* and its sister species, the anti-fungal defender *Regiella insecticola,* and determined that, under most conditions, coinfection is actually beneficial. Finally, I present the first pass at parsing aphid transcriptional responses to wasp parasitism in the presence and absence of aphid genotypic resistance and *H. defensa*-induced resistance, finding little-to-no evidence for the mobilization of innate immunity.

INDEX WORDS: Symbiosis, bacteriophage, parasitoid, natural selection, microbial ecology

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DEDICATION

I dedicate this dissertation to my parents, Walt and Cindy Weldon: without their near-three decades of support, the odds that I would have stuck it out in science are slim. They taught me to prioritize the process of learning, while also exhorting me to put down the books and get outside on occasion. I still prefer my insects mashed in TRIzol at a lab bench, but my parents ensured I would never forget that my work is part of a wider world.

I would also like to dedicate this work to my younger brother, Jon, who is terrified of insects. I took up entomology primarily to taunt him.

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INTRODUCTION AND LITERATURE REVIEW

1.1 Summary

1.1.1 Dissertation structure. This dissertation seeks to address the fundamental biology of the APSE-*Hamiltonella defensa*-*Acyrthosiphon pisum* interaction, a tripartite heritable mutualistic system associated with the defense of aphids from parasitoid wasps. The below introduction (modified from an unpublished planned book chapter: Weldon S, Oliver K., projected 2016, Chapter 7: Bacteriophage dynamics in heritable symbioses. In Hurst C (Ed.),*The Mechanistic Benefits of Microbial Symbionts*. Springer, included with permission) functions as a literature review of this system, with comparisons made to the other major phage-heritable symbiont system in insect hosts, WO-*Wolbachia* spp. In the first major data chapter, **§2,** I use quantitative PCR to study the effects of APSE loss on titers in *H. defensa* over aphid lifetime (in short: drastic increase), and additionally apply standard component measures of aphid fitness in the absence of natural enemies, finding that APSE loss is extremely detrimental to aphid fecundity; this chapter is a slightly-modified reproduction of Weldon S, Strand M, Oliver K. (2013) Phage loss and the breakdown of a defensive symbiosis in aphids. Proc Roy Soc B 280. In **§3**, I provide a survey of *H. defensa* infections across North American pea aphids collected on alfalfa, and determined the diversity of their *H. defensa* and APSE strains using multi-locus sequencing typing for *H. defensa* and a combination of phage structural gene allele typing and variable toxin cassette region typing for APSE: on this basis I identified a novel strain of APSE, and I also present its associated defensive phenotype in this chapter. In **§4**, I used three artificially-infected clonal lineages of aphids to determine the fitness and titer effects on *H. defensa*-infected aphids of coinfection by *H. defensa*'s sister species *Regiella insecticola* and

found that, contrary to expectations from field surveys, coinfection was generally beneficial to the host aphid. The effects on intrahost symbiont densities, however, were in line with those found in the only prior study of coinfection, between *H. defensa* and *Serratia symbiotica.* In **§5**, I provide an early summary of a sizeable transcriptome study looking at expression changes following parasitism in three aphid lines; one without any form of anti-parasitoid defense, a second with genotypic resistance to parasitoids, and third without genotypic resistance but with a protective APSE-*H. defensa* infection. I find little evidence for the involvement of canonical innate insect immune genes in the interaction. The overall intent of this project is to help develop the APSE-*H. defensa-Ac. pisum* system as a model for tripartite mutualisms, and the contribution of these studies and key follow-ups are addressed in the concluding section, **§6**.

1.1.2 Introduction abstract: Diverse bacteriophage roles in an aphid-bacterial defensive mutualism. Despite the well-described importance of bacteriophages to bacterial pathogens, little is known about their influence on the many bacterial species that form beneficial symbioses with eukaryotes. Most insect species, for example, are infected with one or more maternally inherited symbionts, which provide nutritional and defensive services in exchange for housing. The pea aphid, *Acyrthosiphon pisum,* harbors at least seven common heritable symbionts that mediate a range of ecological interactions, and has emerged as a model for studies of beneficial symbionts. One common pea aphid defensive symbiont, *Hamiltonella defensa*, protects against parasitic wasps, which are important natural enemies. The bacterium is itself infected by temperate bacteriophages, called APSEs (*Acyrthosiphon pisum* Secondary Endosymbiont), which are necessary for *H. defensa*-mediated protection. This represents the first known instance of a bacteria-insect mutualism requiring a viral partner. APSEs play other key roles in the regulation and maintenance of *H. defensa*: APSE loss results in high titers of the bacterial symbiont, which is correlated with severe fitness costs to the aphid host. These costs to the aphid incurred by phage loss likely lead to phage-free *H. defensa* infected aphids being rapidly

removed from the population, thus limiting the invasion potential of *H. defensa*. Below we review the roles of APSEs in the *H. defensa*-aphid defensive symbiosis and suggest a framework for future studies. We then make comparisons with another well-studied phage-symbiont interaction (*Wolbachia*-WO) and consider the roles of bacteriophages in the evolution of heritable symbioses and how they may influence other insect-associated bacteria, such as the gut microbiota.

1.2 Introduction

1.2.1 Mutualistic viruses. Viruses are the most abundant and genetically diverse segment of the biosphere (Weinbauer 2004; Hatfull 2008). Due to their obligate reproductive parasitism, viruses have historically been associated with pathogenicity, yet they are now known to participate in mutualisms with most major organismal groups, including bacteria, fungi, plants, and animals. While the provision of pathogenicity loci to their bacterial hosts is a well-known attribute of phage infection, viruses may also be conditional or even obligate mutualists of eukaryotes. In some eukaryotic hosts, for example, infection by nonpathogenic viruses interferes with the establishment of pathogens via a number of mechanisms, including resource competition and immune system priming (Barton et al. 2007; Strive et al. 2010; Tillmann et al. 2001). Viruses may also mediate key ecological interactions for their hosts: persistent viruses confer cold and drought tolerance to plants, while insect-associated viruses improve food-plant quality for herbivores (reviewed in: Roossinck 2011). In a highly-specialized obligate mutualism, many parasitic wasps employ viruses to overcome the defenses of the insects they attack. In this interaction, the polydnavirus is stably integrated into the wasp genome, and replicates only in specialized cells in the female wasp's reproductive tract. The encapsidated form of the virus is injected into the wasp's host along with the wasp egg, and suppresses the host's immune response to allow for the successful development of the wasp offspring (reviewed in: Strand 2010). Viruses thus engage in diverse beneficial interactions, which vary in complexity and

specialization, and most are involved with providing hosts with a range of nutritional and defensive services.

Virus-mediated symbioses also exist at one remove: bacteriophages can infect insect-associated bacteria with important consequences for the insect host. Insects are easily the most diverse and abundant group of animals, and they have varied and dynamic interactions with microbes, especially bacteria. Insect guts, for example, often contain microbial communities (including bacteria, archaea, fungi, and protists) that assist in breaking down difficult to digest materials, such as cellulose (reviewed in: Engel and Moran 2013). These animal-microbe holobionts are therefore major players in nutrient recycling and other ecosystem services. Gut microbiota, which persist extracellularly and are often environmentally transmitted, have also been shown to detoxify plant allelochemicals, changing the range of permissible herbivore diets, and to modulate insect innate immunity and confer protection against pathogens and parasites (Dillon and Dillon 2004, Engel and Moran 2013). The majority of insect species are also likely infected with maternally-transmitted symbionts, a phenomenon that is uncommon in mammals (Moran et al. 2008; Zug and Hammerstein 2012). Maternally transmitted symbionts contribute an additional source of heritable genetic variation that can be acted on by natural selection, so many invade and persist in natural populations by conferring net benefits relative to uninfected individuals (Oliver et al. 2010; Dillon and Dillon 2004; Hedges et al. 2008; Xie et al. 2010). Though bacteriophages are the most abundant organisms on earth, and are present as prophage in the majority of sequenced bacterial genomes (Bordenstein and Wernegreen 2004), little is known about their associations with the diverse and ecologically important array of insect symbionts described above. Recent findings, however, indicate the potential for a wide assortment of ecologically and evolutionarily important roles for these proxy mutualists.

1.2.2 Heritable symbionts profoundly influence insect ecology and evolution. The acquisition of heritable microbial infections has allowed insects to exploit and specialize on

nutrient poor diets, including plant sap and vertebrate blood (Gibson and Hunter 2010; Douglas 1998). Insects, like other animals, cannot synthesize essential amino acids and must acquire them from their diet or other sources. In aphids, which are small, soft-bodied herbivores, an ancient infection with the gamma-proteobacterium *Buchnera aphidicola* allowed its host to subsist on only plant phloem: a sugar rich, but nitrogen poor diet. This ancient symbiosis (160-280 MYA) allowed aphids to first exploit a previously unavailable resource, and subsequently specialize and diversify (ca. 4400 spp.) with flowering plants (Moran et al. 1993). More generally, at least ten percent (> 100,000 spp.) of extant insect species are hosts to one or more obligate nutrient-provisioning bacterial symbionts, demonstrating their importance in the evolutionary success of insects (Wernegreen 2002). While most obligate symbionts characterized perform a nutritional role, and those not explicitly examined are expected to, given that they occur in hosts specialized on restricted diets (Moran and Wernegreen 2000; Baumann 2005), obligate symbionts may possibly provide other, or additional, mutualistic benefits to their insect hosts, such as defense (Nakabachi et al. 2013).

Obligate nutritional symbionts cannot survive or reproduce outside of their host insects, and the insects themselves cannot reproduce, and generally do not survive, after antibiotic removal of nutritional symbionts (Douglas 1989). This inextricable mutual dependence results in a number of key characteristics shared among obligate symbionts. Insects control obligate symbionts by cordoning them off in specialized cells known as bacteriocytes, which are often grouped into organs called bacteriomes. Their strict maternal inheritance renders the bacteria's fitness directly dependent on the host's reproductive success (Vautrin et al. 2008), and insect hosts and obligate symbionts typically show patterns of cospeciation (Thao et al. 2000; Sauer et al. 2000; Clark et al. 2000). These domesticated bacteria also exhibit many of the smallest, most A-T rich genomes yet sequenced, and have lost entire functional gene groups (including

those involved in DNA repair), yet retain pathways critical for essential nutrient provisioning (Moran and Wernegreen 2000; McCutcheon and Moran 2012).

The facultative symbionts of insects are even more common, likely infecting most species, and they exert far more varied effects on hosts (Feldhaar 2011). Relative to obligate symbionts and their free-living relatives, facultative symbionts tend to exhibit intermediate genome sizes and A-T richness, but have a higher percentage of mobile genetic elements than either (Burke and Moran 2011; Degnan et al. 2009; Degnan et al. 2010; Newton and Bordenstein 2011; Belda et al. 2010). Some facultative symbionts confer ecologically important traits valuable to the host organism, including heat protection, defense against natural enemies and host-plant specification (Ferrari et al. 2007; Koga et al. 2003; Montllor et al. 2002; Scarborough et al. 2005; Tsuchida et al. 2004). Others are reproductive parasites, manipulating the host's reproduction to favor infected female hosts at the expense of males or uninfected females to ensure their own propagation (Werren et al. 2008). The line between parasite and mutualist is often blurred: some bacterial clades are manipulators or beneficial depending on the host, and others are both at once. For example, *Wolbachia* spp., which may infect 40% of arthropod species (Zug and Hammerstein 2012), are generally considered reproductive manipulators, but many strains also provide protection against a range of pathogens (reviewed in: Hamilton and Perlman 2013). In addition, many facultative symbionts confer benefits to infected individuals under some conditions, but costs under others (Oliver et al. 2008). Tissue tropism of facultative symbionts is variable, depending on symbiont and host species, but they are not typically isolated in the bacteriocytes and hence are more likely, relative to obligate symbionts, to interact with other bacteria and their mobile elements. However, the limited diversity of bacterial taxa found in tissues generally frequented by facultative bacteria constrains their bacteriophage exposure relative to free-living bacteria (Gottlieb et al. 2008).

While the prevalence of bacteriophages in insect facultative symbionts is not known, many widespread symbionts, including *Wolbachia, Arsenophonus, Spiroplasma* and *Hamiltonella* are associated with active bacteriophages, and there are remnants of inactivated prophage in other species, including *Regiella insecticola* and *Sodalis glossinidus* (Bordenstein et al. 2006; Darby et al. 2010; Belda et al. 2010). One of the best studied phage-facultative symbiont interactions, and the only currently known instance of mutualism by proxy, involves the pea aphid, *Acyrthosiphon pisum*, its bacterial symbiont *Hamiltonella defensa,* and its temperate phage APSE (*Acyrthosiphon pisum* Secondary Endosymbiont). Below we will review the diverse roles attributed to APSE in the maintenance and regulation of a defensive symbiosis, in which infected aphids are protected against a common natural enemy, the parasitic wasp *Aphidius ervi* (Oliver et al. 2009). We will also draw comparisons to the other well-studied tripartite interaction between insects, *Wolbachia* and the temperate phage WO.

1.2.3 Introduction to pea aphids, their symbionts their natural enemies. The pea aphid, *Acyrthosiphon pisum*, is a cosmopolitan pest of cultivated legumes, including clover and alfalfa. In addition to the obligate nutritional symbiont *Buchnera aphidicola* (**§1.2.2**), individual pea aphids are usually infected with one or more facultative symbionts (Russell et al. 2013). This aphid has emerged as a model organism for identifying the phenotypic effects of infection with heritable symbionts, due to its cyclically parthenogenetic reproduction and the relative ease with which facultative symbionts can be manipulated among clonal lines. At present, seven facultative symbionts are known to frequently infect pea aphids, and all seven facultative symbionts are known or suspected to mediate important ecological interactions including heat shock protection, host-plant specification, and protection from natural enemies (Oliver et al. 2010, Lukasik et al. 2013b).

One of the most prevalent natural enemies of the pea aphid in North America is the hymenopteran parasitic wasp *Aphidius ervi* (Braconidae). A female wasp injects an egg into the aphid hemocoel and the resulting wasp larva feeds and develops within a still living aphid. When larval development is complete, the wasp kills and consumes the remaining contents of

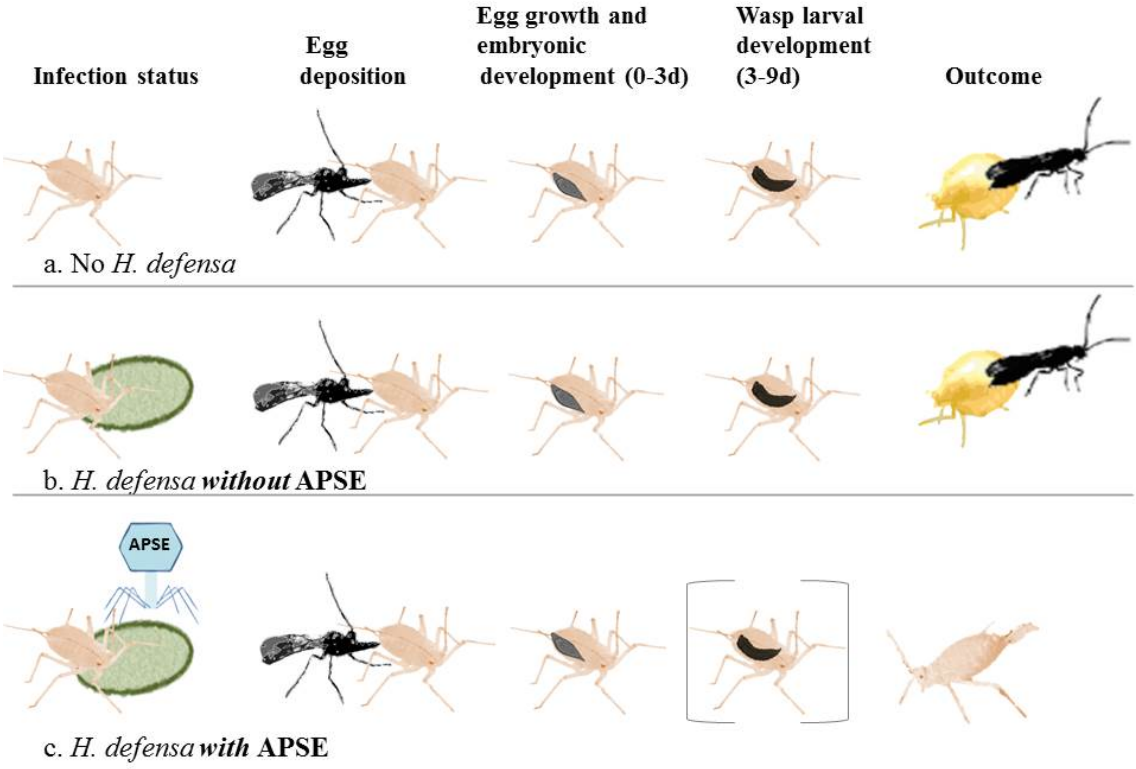

Figure 1.1 Developmental trajectories of pea aphids (*Acyrthosiphon pisum*) parasitized by the wasp Aphidius ervi in the presence and absence of facultative endosymbiont *Hamiltonella defensa* and its associated phage, APSE (*Acyrthosiphon pisum* Secondary Endosymbionts). Development times assume a 16L:8D light cycle and constant 20C temperature. a.) *H. defensa* free aphid: Wasp injects a single egg into a pre-adult aphid and embryonic development occurs from 0-3 days after oviposition. Wasp larval development continues from 3-9 days post-parasitism. The wasp larva pupates at approximately 9 days, killing and 'mummifying' the aphid. Single adult wasp emerges from aphid 'mummy' 14-15 days post parasitism (He et al. 2008). b.) *H. defensa*-infected aphid without APSE: wasp development proceeds as in '1a.' c.) H. defensa-infected aphid with phage APSE: wasp mortality at either egg or larval stage, depending on APSE strain (brackets indicate that larval development stage may or may not occur) (Martinez et. al. 2013). Aphids survive to reproduce (Oliver et al. 2009).

the aphid and pupates within the desiccated and hardened cuticle, called a “mummy” (**Figure 1.1**) (Oliver et al. 2005). The parasitoid wasp manipulates the aphid to support the wasp larva's stage-specific nutritional needs (Caccia et al. 2005); as part of these manipulations *Ap. ervi* injects a venom that degrades the aphid's reproductive system, which, depending on timing of attack, can completely castrate the aphid (Digilio et al. 2000). Aphid embryos that survive venom castration may then be degraded by teratocytes, large polyploid cells that dissociate from the developing wasp’s extraembryonic membrane (Falabella et al. 2009; Digilio et al. 2000). Parasitoids may also specifically manipulate the nutritional symbiosis. In susceptible aphids uninfected by facultative symbionts, parasitism results in an increased abundance of *Buchnera,* as well as the number and mass of bacteriocytes relative to unparasitized controls (Cloutier and Douglas 2003). Parasitism influences the type and quantity of amino acid provisioning performed by *Buchnera,* presumably to the parasitoid's benefit (Rahbe et al. 2002); wasps fail to thrive in aposymbiotic (*Buchnera*-free) aphids (Falabella et al. 2000).

1.2.4 Bacteria-mediated defense against wasp *Aphidius ervi.* An early study indicated that pea aphid clonal lines varied tremendously in susceptibility to the wasp *Ap. ervi*, with some lines entirely resistant and others highly susceptible (Henter and Via 1995). Originally, it was assumed that the basis for this variation was encoded in the aphid genome, although this was surprising given that aphids, including *Ac. pisum*, lack a strong encapsulation response, i.e., the typical insect innate cellular immune response to internal parasites (Carver and Sullivan 1988; Laughton et al. 2011). Shortly after this, several facultative symbionts were characterized in this aphid (Chen and Purcell 1997; Fukatsu et al. 2000; Sandstrom et al. 2001; Darby et al. 2001), and two of these symbionts, *Hamiltonella defensa* and *Serratia symbiotica*, were found to contribute to variation in susceptibility to parasitism (Oliver et al. 2003). Subsequent studies confirmed that resistance to wasps was largely due to infection with *H. defensa*, and in the absence of facultative symbionts most *Ac. pisum* are highly susceptible to *Ap. ervi* (Oliver et al.

2005; Oliver et al. 2009), although some aphid-genotype-based variation in susceptibility also occurs (Martinez et al. 2014a). Furthermore, it was shown that symbiont-mediated resistance was due to intra-aphid factors: wasps readily attack infected and uninfected aphids but are far less likely to complete development in infected aphids (Oliver et al. 2003). *H. defensa* also rescues the reproductive capacity of parasitized aphids, which are normally castrated by parasitoid venoms: the fact that resistant aphids not only survive, but reproduce, demonstrates a direct, heritable, benefit to *H. defensa* infection in the presence of parasitoid wasps (Oliver et al. 2008).

H. defensa is estimated to infect 14% of aphid species, and occurs in other hemipteran insects (Oliver et al. 2010). In addition to *Ac. pisum*, an anti-parasitoid role for *H. defensa* has only been demonstrated in the black bean aphid, *Aphis fabae*, and the cowpea aphid, *Aphis craccivora* (Schmid et al. 2012; Asplen et al. 2014). In the grain aphid, *Sitobion avenae,* and the blackberry-cereal aphid, *Sitobion fragariae*, *H. defensa* provides no or very low protection from either *Aphidius ervi* or another parasitoid wasp, *Ephedrus plagiator* (Lukasik et al. 2013a; Lukasik et al. 2015 in prep). However, in the cowpea aphid, it appears that *H. defensa's* protection is specific to parasitoid identity, as a single strain conferred complete protection against two *Binodoxys* species, but no protection against two other species in the same subfamily (Aphidiinae) (Asplen et al. 2014).

1.3 The role of phage in an aphid defensive mutualism.

Given the clear benefits of protection owing to infection with *H. defensa,* the next question emerged: what mechanisms underlie symbiont-based protection? Unfortunately, like most heritable bacterial symbionts, *H. defensa* is uncultivable and hence not amenable to standard microbiological assays. In order to identify potential mechanisms underlying symbiont-based protection Moran *et al*. produced a preliminary sequence of the *H. defensa* genome (2005). This effort reported a number of putative toxins and pathogenicity loci, but also

confirmed the presence of an intact prophage called APSE, which has proven to be highly influential in the beneficial heritable symbiosis between aphid *Ac. pisum* and *H. defensa*: APSE is, in fact, the first active phage known to be required by an insect mutualism. As in heritable bacteria, the lack of horizontal escape routes in isolated animal hosts renders phage fitness dependent on the vertical transmission of their host bacterial line to the next generation of insect hosts (Vautrin and Vavre 2009). Phages in mutualistic symbioses may therefore encode factors critical to protective or nutritional services, which could act alone or in conjunction with those of bacterial origin (Moran et al. 2005). On the other hand, phage effects on symbiont abundance may reduce the effectiveness of conferred benefits and limit symbiont and phage invasion into insect host populations. Finally, increasing the number of required players may generally lead to instability as each will have their own biotic and abiotic optima, such as overlapping but distinct ideal temperature ranges, resulting in a mutualism that functions well only under restricted conditions.

1.3.1 Phage can provision functions important to bacteria-insect mutualisms. APSE was first reported in a European line of *Ac. pisum* (van der Wilk et al. 1999), and it was subsequently determined that its host was *H. defensa* (Sandstrom et al. 2001). APSEs are dsDNA viruses with capsid morphology similar to the Podoviridae and share sequence similarity with P22 (Degnan and Moran 2008b; van der Wilk et al. 1999). Synteny and nucleotide identity are conserved among APSE haplotypes with the exception of a virulence cassette region (VCR), which varies in length and contains the toxic effector molecules hypothesized to provide or support the defensive symbiosis (Moran et al. 2005; Degnan and Moran 2008b, a). Seven APSE variants (APSE1-7) have been designated on the basis of the assortment of putative eukaryotic toxins and bacterial lysis genes; their genomes vary in length from 36-39 kbp (van der Wilk et al. 1999; Degnan and Moran 2008a). The presence of toxin-encoding APSEs in a

defensive symbiont strongly suggested its involvement in the protective phenotype (Moran et al. 2005).

APSE variants are each associated with one of three different eukaryotic toxins: cytolethal-distending toxin subunit B homolog (CdtB; APSEs 2, 6 & 7), a Shiga-like toxin (Stx; APSEs 1, 4 & 5), and a putative toxin containing a YD repeat – a motif characterized by a highly conserved tyrosyl-aspartate dipeptide (Ydp; APSE3) (Degnan and Moran 2008a; Moran et al. 2005; van der Wilk et al. 1999). In North American pea aphids, where only APSE2 and APSE3 have been reported in the literature, aphids infected with *H. defensa* containing either APSE variant receive protection against parasitoids and the phage variant generally correlates with the intensity of the defensive phenotype: APSE2s are associated with moderate protection, while APSE3s are associated with high to complete protection from the wasp *Ap. ervi;* a correlation that bolstered the hypothesized phage role in defense (Oliver et al. 2005). It was next found that some descendants of a pea aphid clonal lab line infected with *H. defensa* and APSE3 spontaneously lost their bacteriophage (more on this below) while retaining the bacterial symbiont. This allowed the creation of experimental lines that shared the same pea aphid and *H. defensa* genotype, with or without APSE3. After it was confirmed that components other than APSE3 were not lost from the *H. defensa* chromosome, these experimental lines were used to determine the contribution of APSE to the protective phenotype. As previously reported, pea aphids with neither *H. defensa* nor APSE were susceptible to parasitism, while those with *H. defensa* and APSE were highly resistant; however, the line infected with *H. defensa* without APSE was just as susceptible to the wasp as the *H. defensa*–free line (**Figure 1.1**) (Oliver et al. 2009). This same study confirmed that this result was not a quirk of either the particular *H. defensa* strain or aphid clonal genotype, as numerous additional lines that lost APSE also lost resistance to wasps. Together, the experimental and correlation-based evidence makes a

strong case that infection with APSE3, and probably APSE2, is required for *H. defensa* to produce the protective phenotype.

Some APSE-associated toxins have been found in pathogenic bacteria where their functions have been characterized. The APSE2-associated CdtBs (also found in APSE6 from the aphid *Chaitophorus* and APSE7 from the whitefly *Bemisia tabaci*), for example, were first identified in *E. coli* (Johnson and Lior 1987). CdtB is the DNase I subunit of the cyclomodulin cytolethal distending toxin, which disrupts actively dividing eukaryotic cells (Ohara et al. 2004). Homologs of the Shiga-like toxin, found in APSE1 (*Ac. pisum* from the Netherlands), APSE4 (the cowpea aphid, *Aphis craccivora*) and APSE5 (aphid *Uroleucon rudbeckia*) are also found in other enteric pathogens, including *E. coli,* where they were shown to prevent protein synthesis by cleaving ribosomal RNA via N-glycosidase action (Endo et al. 1988). The Ydp encoded by APSE3 is associated with the *H. defensa* strains that confer the most protection, but they are understood least at the functional level: however the YD dipeptide motif in the reading frame (ORF) is associated with binding carbohydrates and at least one Ydp is associated with eukaryotic toxicity (Degnan and Moran 2008a). It is important to remember that the inability to culture *H. defensa* and APSEs limits the tools available for functional studies, so whether phage toxins, acting alone or in concert with *H. defensa* or aphid-encoded factors, cause harm to wasps is not yet known.

The potential contribution of other APSE variants to the protective phenotype has received little attention. APSE4 (Stx) wielding *H. defensa* protect their native cowpea aphid host against some, but not all parasitoids (Asplen et al. 2014), but an APSE4/*H. defensa* strain in the grain aphid *Sitobion avenae* provided no protection from either of the two parasitoid species assayed, although it may confer protection against other wasps attacking this aphid (Lukasik et al. 2013a; Lukasik et al. 2015 in prep). Some *H. defensa* strains may be maintained in host

populations by conferring thermal protection or other benefits rather than defense against parasitoids (Russell and Moran 2006).

APSE also commonly infects the widespread insect symbiont *Arsenophonus* (Enterobacteriaceae), though less is known about the phage's roles in these hosts. That *Arsenophonus*-associated APSEs exhibit much greater genetic diversity, and *H. defensa*-infecting APSEs form a distinct branch within the larger *Arsenophonus*-APSE phylogenetic tree, suggests that *Arsenophonus* was APSE's original host (Duron 2014). While *Arsenophonus* has not been found in pea aphids, it has been reported in other aphids and is widespread in some *Ap. craccivora* populations (Nováková et al. 2009; Brady and White 2013; Duron 2014), where *Arsenophonus* is known to influence dietary breadth (Wagner et al 2015). Eukaryotic toxins homologous to an open reading frame (called ORF D) in *H. defensa*-APSE's variable cassette region have been found in wasp-associated *Arsenophonus*-APSEs, where they may contribute to a male-killing reproductively-manipulative phenotype, but whether any potential eukaryotic toxins are found in hemipteran-infecting *Arsenophonus*-APSEs is unknown (Wilkes et al. 2010).

1.3.2 Role of phage in horizontal transmission of ecologically important traits. Phages are well known vectors of lateral gene transmission among bacterial lineages, contributing functional pathways that profoundly affect host ecology and evolution (Ochman et al. 2000). In heritable symbionts, phage may move traits that influence not only the bacterial host, but also the animal host (Moran et al. 2005). Phylogenetic studies reveal that APSEs move extensively among *H. defensa* strains and hence likely move traits important in aphid protection (Degnan and Moran 2008b). The high G+C content of the virulence cassette region, where the putative toxins are found, suggests that it is foreign to both APSE and *H. defensa*, further highlighting the potential for lateral exchange of genes relevant to defensive symbioses among phages (Degnan and Moran 2008a). Phylogenetic evidence and transfection experiments show that facultative symbionts, including *H. defensa*, also move horizontally within and among arthropod species,

transferring ecologically significant bacterial or phage encoded traits in the process (Oliver et al. 2010). In the lab, an experimental transfer of APSE4-infected *H. defensa* from cowpea aphids (*Ap. craccivora*) to pea aphids (*Ac. pisum*) resulted in the instant acquisition of increased resistance to parasitism (Oliver et al. 2005). The observation that phages move traits among heritable symbionts, and symbionts among arthropods, has led to speculation of a reservoir of ecologically important traits that are shared among communities of interacting species (Henry et al. 2013; Jaenike 2012; Moran 2007), However, aphid species which share host plants (e.g. pea aphids and cowpea aphids) are associated with distinct *H. defensa* strains and APSE variants, which suggests that there may be factors limiting the spread of both bacteria and phage between insect species (Degnan and Moran 2008a; Dykstra et al. 2014; Weldon et al. 2015 in prep).While the historical evidence for lateral phage movement between *H. defensa* strains is strong, experimental movement of APSEs in the laboratory has not been reported.

1.3.3 Phage roles in the regulation and maintenance of heritable symbiosis. Intra-host bacterial symbiont density can be important for the maintenance and performance of heritable symbionts and their insect hosts. Within-host bacterial abundance can affect conferred phenotypes (e.g., Noda et al. 2001; Ikeda et al. 2003), rates of horizontal transfer, the establishment of novel infections (Chafee et al. 2010), and the maintenance of tripartite symbioses (Jaenike 2009; Bordenstein and Bordenstein 2011). All stable beneficial heritable symbiont infections must be coordinated between host and symbiont to strike a balance between a sufficient titer to produce the beneficial phenotype and ensure vertical transmission to progeny, and an excessive titer that might deplete host resources and negatively affect both host reproduction and the bacterium's chances of reaching the next generation of insects (Jaenike 2009).

Virulent phages are predacious on their hosts and hence capable of influencing bacterial abundance. Temperate phages capable of integrating into the host genome can also influence

bacterial abundance, either through excision and lysis, or by influencing the ecology of the bacterial host, such as by modifying reproductive rates relative to uninfected cells (Lin et al. 1977). Phage-bacteria interactions are often only considered in terms of integrated prophages and lytic behavior, but a wide range of complex interactions exists, including persistent infections distinguished by, for example, episomal prophage or phage shedding or budding (Weinbauer 2004; Calendar and Abedon 2005). As these different phage lifestyles will differentially influence bacterial abundance, it will be important to understand the basic biology of these interactions.

1.3.4 APSEs are temperate, with variable lifecycles and no known lytic triggers. Within-aphid *H. defensa* populations are heterogeneous for APSE prophage integration (Degnan and Moran 2008a), and APSEs tend to outnumber their bacterial hosts 10-100 fold within the pea aphid hemolymph, suggesting that most are not maintained as lysogens (Moran et al. 2005; Martinez et al. 2014b; Oliver et al. 2009). The lytic triggers or density-dependent factors that control APSE's interactions with its host-limited bacterial population are unknown, though there is some evidence wasp parasitism may trigger APSE-induced lysis of *H. defensa* cells (Degnan and Moran 2008a; Martinez et al. 2014b). Lysis of a subset of *H. defensa* may be a prerequisite to the release of phage-encoded toxins into the aphid hemolymph to disrupt wasp development (Moran et al. 2005; Martinez et al. 2014b); this has not, however, been examined mechanistically, and *H. defensa* retains potential transporters for phage-encoded toxins that would render lysis superfluous (Degnan et al. 2009). Lytic activity may not be a specialized response to wasp parasitism, but rather APSE's general reaction to stressors acting on the host insect. Several APSE genes have been proposed as orthologs to lysogenic cycle control genes found in other lambdoid phages: ORF1 (or *P1*) is homologous to lambdoid phage HB19's C1 protein, based both off of sequence similarity and genomic placement; ORF2 (or *P2*) has been identified as an ortholog to the cro-like gene in lambdoid phage 434 (van der Wilk et al. 1999).

Sequencing results suggest that some APSE variants may now be inactivated prophage, though none of these variants are present in the pea aphid defense mutualism and their biology is largely unknown (Degnan and Moran 2008a). In laboratory–reared pea aphid clones, *H. defensa* is vertically transmitted at rates approaching 100% (Oliver et al. 2010). The highly-protective APSE3 phage infections are also transmitted with very high fidelity, but can be spontaneously lost at very low rates as described above (Oliver et al. 2009). Moderately protective APSE2's, however, have never been lost from lab-held *H. defensa* lines (Weldon et al. 2013). The underlying basis for the differential persistence of APSE2 and APSE3 across aphid generations is currently unclear, but may be influenced by differential rates of phage integration between variants. Further, if not all *H. defensa* cells within an individual aphid carry APSEs, then intraspecific competition between APSE-infected and uninfected *H. defensa* could reduce the proportion of infected bacteria, making it more likely that only uninfected symbionts will pass through the tight trans-generational bottleneck.

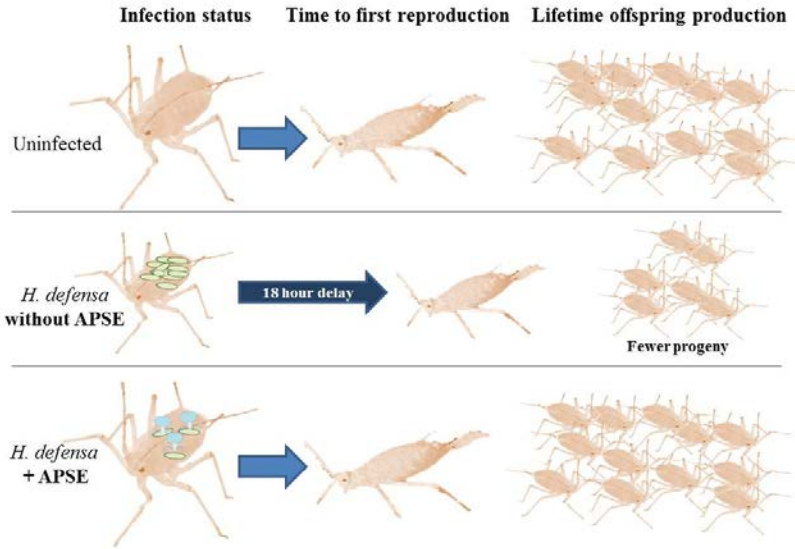

Figure 1.2 Aphids infected with APSE-free *H. defensa* weigh ~20% less at adulthood, first reproduce on average 18 hours later, and produce half as many total offspring as their uninfected clone-mates. Aphids infected with *H. defensa* + APSE show no significant component fitness assay detriments relative to uninfected clonemates (Weldon et al. 2013, Oliver et al. 2006).

1.3.5 APSE3 loss is associated with consistent and immediate increases in *H. defensa* titer. One study has examined the effects of APSE on within-host *H. defensa* abundance, and reports results consistent with phage roles in the regulation of the protective symbiont (Weldon et al. 2013). That APSE3s are often lost from *H. defensa* allows for cross-line comparisons between APSE infected and uninfected lines, and in all cases APSE-free aphid lines carried significantly higher *H. defensa* titers than aphid lines with related *H. defensa* strains that maintained APSE3 infections. Similarly, APSE losses makes it possible, although difficult due to the rarity of the event, to establish experimental lines comprised of the same aphid host clonal lineage and identical *H. defensa* genotypes, but varying with respect to APSE infection status, to experimentally show the effects of phage loss on symbiont abundance. In this case, estimates of symbiont abundance revealed that phage-free pea aphids contained significantly more *H. defensa* than aphids with APSE3 at all examined ages in aphid development. The increase in *H. defensa* abundance following APSE loss was dramatic, up to nine-fold at its maximum. This, however, was not the gradual result of *H. defensa* equilibrium divergence over the course of many generations reared apart. Some phage-harboring aphid mothers produced a mix of phage-infected and phage-free offspring, and comparisons among their offspring found that siblings without phage had significantly higher titers of *H. defensa* than their phage-harboring clonal sisters. While the other common North American pea aphid-associated phage variant, APSE2, has proven too stably infective to naturally produce phage-free identical *H. defensa* strains, the same study reported an inverse correlation between APSE2 numbers and *H. defensa* titers consistent with phage effects on bacterial abundance (Weldon et al. 2013).

1.3.6 APSE3 loss is severely deleterious to pea aphid fitness. The dramatically increased *H. defensa* titers associated with the loss of APSE3 suggested potentially harmful effects for the aphid host. In component fitness assays costs to infection with *H. defensa* + APSE have generally been small or difficult to show (Oliver et al. 2006; Russell and Moran 2005; Oliver et

al. 2008). *H. defensa*, however, relies on *B. aphidicola* for some of the same nutrients it provides its aphid host (Degnan et al. 2009), and at higher *H. defensa* densities, competition with aphid tissues for *B. aphidicola*-provisioned resources may increase. To assess the effects of phage loss on aphid performance, three component fitness parameters (fecundity, development time, and fresh weight at adulthood) were examined in phage-free and phage-harboring pea aphids (Weldon et al. 2013). In each instance, it was found that the absence of APSE3 significantly increased fitness costs to the aphid host (**Figure 1.2**). On average, aphids lacking APSE3 reproduced 18 hours later, weighed 20% less at adulthood, and produced roughly half as many total offspring over the course of their lifespans compared to clonal aphids with APSE3 (Weldon et al. 2013). Thus, APSE loss resulted in a huge fitness deficit for genetically identical aphids. The negative effects associated with high *H. defensa* abundance, combined with the loss of protection from parasitoids (**§1.3.1**), essentially convert a heritable mutualist, *H. defensa,* into a heritable pathogen.

1.3.7. APSEs may influence the maintenance of *H. defensa* diversity in natural populations. As noted above, vertically transmitted symbionts, such as *H. defensa*, provide heritable variation that can be acted on by host-level selection. While more work is needed in field populations, laboratory-based population cage studies show that aphids infected with *H. defensa* and APSE3 increase rapidly in the population when parasitism pressure is present, but decrease in the absence of natural enemies (Oliver et al. 2008). Thus, benefits in the presence and costs in the absence of parasitism likely partially explain why field populations exhibit varying infection frequencies that never reach fixation (Russell et al. 2013). The recurrent loss of APSE3 furthermore produces aphids that suffer doubly from the loss of anti-parasitoid protection and the introduction of additional and quite severe costs to *H. defensa* infection (Weldon et al. 2013). Thus negative (i.e. purifying) selection at the host level likely rapidly removes these phage-free, *H. defensa* infected aphids from the population. This would explain

the infrequency of phage-free *H. defensa* in field surveys (Oliver et al. 2009, Weldon et al. 2015 in prep). It may also explain the maintenance of APSE2 *H. defensa* strains, which are more prevalent in field populations despite being inferior defenders (Weldon et al. 2013; 2015 in prep). Hence, there may be a tradeoff between defensive ability (APSE3 > APSE2) and stability of the interaction (APSE2 > APSE3), such that APSE3 *H. defensa* strains are only maintained in populations with heavy parasitism pressure. Temporal variation in parasitism pressure may lead to cyclical replacement of highly protective strains with highly stable strains or vice versa.

1.4 WO phage of *Wolbachia* spp.

1.4.1 *Wolbachia* are associated with a group of bacteriophages known as WO. Roles for viruses in heritable symbiosis have been substantially investigated in one other tripartite interaction: the Rickettsiales symbiont *Wolbachia*, its temperate phage WO, and their diverse insect hosts. The *Wolbachia* group comprises both facultative strains with the capacity for host-swapping (primarily infecting arthropods) and obligate mutualist strains (mostly infecting filarial nematodes) which undergo far stricter vertical inheritance (Bordenstein and Reznikoff 2005; Kent and Bordenstein 2010; Foster et al. 2005; Masui et al. 2000). Facultative *Wolbachia* strains are associated with both beneficial defensive phenotypes, such as improved resistance to microbial pathogens (reviewed in: Hamilton and Perlman 2013), and reproductive parasitism (reviewed in: Stouthamer et al. 1999; Werren et al. 2008). Most facultative strains are infected with one or more active temperate phages, called WO, which can move laterally among *Wolbachia* strains occupying different phylogenetic supergroups (Bordenstein and Wernegreen 2004; Baldo et al. 2006; Chafee et al. 2010; Masui et al. 2000; Tanaka et al. 2009). Given that WO has primarily been studied in parasitic rather than beneficial strains, and has been reviewed recently (Kent and Bordenstein 2010; Metcalf and Bordenstein 2012), we will limit our coverage to summarizing major findings and making comparisons with APSE.

1.4.2 WO and *Wolbachia* density interactions affect host phenotype, and are in turn affected by host genotype and abiotic factors. *Wolbachia* has been described as the "master manipulator" of arthropod reproduction (Werren et al. 2008). It is the only bacterial species known to employ all four strategies of reproductive parasitism, allowing invasion and maintenance in host populations by enhancing the fitness of infected female insects at the expense of males and uninfected females. These strategies are cytoplasmic incompatibility (CI) between infected males and uninfected females, feminization of male offspring, male killing (MK), and parthenogenesis induction (PI) (Stouthamer et al. 1999). Microscopy and inverse correlations between *Wolbachia* and WO abundance indicate that WO likely lyses bacterial cells and thereby influences bacterial titers (Bordenstein et al. 2006). As *Wolbachia* density is the best predictor of CI levels in several divergent insect taxa (Noda et al. 2001; Ikeda et al. 2003), WO may routinely affect the strength of CI, and other density dependent phenotypes, through its effects on symbiont abundance. By reducing the strength of CI, phage WO may limit the invasion potential of both phage and symbiont to new hosts. This may be unavoidable, however, if routine lysis is required for within-host maintenance of WO infections. Of course, if pathogen defense or other *Wolbachia*-mediated beneficial phenotypes are also density-dependent, then WO activity may similarly affect these traits. In vitro experiments suggest that *Wolbachia*-mediated viral resistance, at least, is directly dependent on per-cell *Wolbachia* density (Frentiu et al. 2010). While there is no correlation between the strength of APSE-*H. defensa*-mediated protection and the within-host densities of either partner (Martinez et al. 2014b), APSE's role in limiting *H. defensa*'s fitness costs in aphids indicates that phage effects on symbiont density in both systems are important determinants of reproductive success for the insect hosts and their heritable bacteria.

As noted in **§1.4.4**, phage-bacteria density interactions are dependent on a number of biotic and abiotic factors. Temperature, for example, decreases the abundance of *Wolbachia*

but increases the abundance of WO in the wasp *Nasonia* (Bordenstein and Bordenstein 2011). As heat shock is a common trigger for lytic activity in lysogenic phages, WO may often mediate temperature effects on *Wolbachia*-conferred phenotypes. The protective phenotype conferred by *H. defensa* is lost at higher temperatures (Bensadia et al. 2006), though this has not been associated with changes in either APSE or *H. defensa* abundance. Establishment of equilibria in phage-bacteria density interactions may also be specific to host genotype: introgression of *Nasonia girault*i's genome into the cytoplasm of *N. vitripennis* infected with *Wolbachia* led to a dramatic increase in *Wolbachia* densities and an equally-precipitous decline in WO densities (Chafee et al. 2011). Thus, novel environments, or genotype x genotype x genotype interactions, may routinely result in varying effects of phage on symbiont abundance, and thereby influence the stability of the interaction, the strength of conferred phenotypes, and the likelihood of successful establishment of a horizontally acquired symbiont.

1.4.3 Phage WO may provide effector molecules necessary for the induction of reproductive parasitism. While no association between WO and *Wolbachia*-induced defensive phenotypes has been identified, there is speculation that WO may contribute to manipulative *Wolbachia* phenotypes through more than density interactions. This is unlikely to be universally the case, however, as some WO-free strains of *Wolbachia* are effective manipulators (Gavotte et al. 2007). A proliferation of ankyrin-repeat proteins are encoded in *Wolbachia* genomes, and are often located on, or near, WOs. Given that Ankyrin-repeat motifs are involved in a range of cell functions in eukaryotes, such as protein-protein interactions, and are otherwise rare in bacteria, they have been hypothesized to contribute to *Wolbachia'*s facility for reproductive manipulation (reviewed in: Metcalf and Bordenstein 2012; Kent and Bordenstein 2010).

Alternately, WO-encoded factors may limit the detrimental effects of CI. CI occurs when a male insect (a dead end for *Wolbachia*) harbors a strain of *Wolbachia* not present in his mate, decreasing the fitness of uninfected females relative to infected females, which can mate with

infected or uninfected males (reviewed in: Werren et al. 2008). Often though, multiple strains of *Wolbachia* infect both individuals in a mating pair, resulting in complex compatibility dynamics. It has been hypothesized that WO associated with some *Wolbachia* strains encode "rescue" factors capable of negotiating among strain incompatibilities (Saridaki et al. 2011). In brief, WO prophages encode a DNA methyltransferase, *met2*, found in all CI-rescuing strains of "A" group *Wolbachia,* but absent from strains not capable of rescuing CI. Phage-encoded factors could therefore render these CI-rescuing strains of *Wolbachia* circumstantial reproductive mutualists in particular coinfections. Note that *Met2* is not present in CI-rescuing *Wolbachia* from other phylogenetic groups, indicating that this mechanism, if valid, is not universal. The importance of methylation to CI-induction, however, is unclear: while methylation patterns have been found to underlie some *Wolbachia*-induced feminizations, there is no similar evidence in CI induction (Metcalf and Bordenstein 2012). Unlike the APSE system, no direct comparisons of phenotypes induced by phage-harboring and phage-free bacterial populations have been possible, so only genomic and correlation-based evidence currently inform this aspect of the association.

1.5 Bacteriophage roles in the evolution of heritable symbiont genomes

1.5.1 Bacteriophages contribute to the content and architecture of symbiont genomes.

High rates of nucleotide change are associated with obligate, bacteriocyte-associated symbionts due to relaxations in selection pressure, loss of repair mechanisms, and genetic bottlenecks, but a lack of mobile genetic elements provides them with fairly stable architectures (e.g., Tamas et al. 2002; Degnan et al. 2005; van Ham et al. 2003). In contrast, many facultative symbionts, such as *Wolbachia,* exhibit low rates of sequence divergence but high levels of rearrangement due to the relatively high percentage of mobile genetic elements in their genomes (Wu et al. 2004; Degnan et al. 2010).

Though bacteriophages are absent in obligate associations, most of those are ancient, and phages may have proliferated early in the development of the association. Any evidence of

MGEs, however, has been lost due to genetic drift and a bias toward deletions (Moran and Plague 2004; Kuo et al. 2009). In species which have recently transitioned to a symbiotic lifestyle, such as *Sodalis glossinidus* and *Serratia symbiotica,* there is a proliferation of all classes of mobile genetic elements (MGEs), including phages (Belda et al. 2010; Burke and Moran 2011). Phages can transport other MGEs into the genome, as in the case of the insertion sequence (IS)-carrying WO phage in *Wolbachia*, indicating that MGE proliferation can occur as a self-reinforcing treadmill (Tanaka et al. 2009). Phages provided nearly 18% of the coding sequence in the *S. glossinidus* genome, although more than half were pseudogenized; a major process in the transition to a host-dependent lifestyle (Moya et al. 2008; Belda et al. 2010; Burke and Moran 2011). MGEs, including phages, can inactivate genes, however, in *S. glossinidus,* the role of MGEs in pseudogenization is dwarfed by the role of frameshift and nonsense mutations (Belda et al. 2010). Inactivated genes may also proliferate due to the lack of purifying selection (Burke and Moran 2011). Prior phage infections may also give rise to modern extrachromosomal DNA features: *S. glossinidius*'s plasmid pSOG3 may be producing virions (occasionally spotted in culture, and the ORFs for assembly are present), and appears to be a coalescence of two ancestral phages, one P22-like and one epsilon 15-like (Clark et al. 2007).

1.5.2 Bacteriophages allow for recombination between coinfecting strains and more divergent taxa. Superinfections by multiple heritable symbionts (and different strains of the same symbiont species) are common in many insect groups, including aphids, although heritable symbiont "communities" are usually less complex than gut communities, and coinfecting mutualists may even occupy separate tissues within the host (Engel and Moran 2013; Oliver et al. 2013). Superinfections provide opportunities for the exchange of phage and other MGEs, though there may be substantial barriers. For example, the sister symbionts *H. defensa* and *R. insecticola* are quite distinct, with high nucleotide divergence and only 55% of

genes shared in common. This is despite frequent host and tissue sharing and high percentages of their genomes dedicated to MGEs; two factors that would seem to facilitate genetic exchange (Degnan et al. 2010). On the other hand, comparative *Wolbachia* genomics suggests that significant recombination and mixing occurs between coinfecting *Wolbachia* strains, even those in different supergroups (Klasson et al. 2009). This includes whole-phage transfer and recombination of phage-encoded regions, not only among *Wolbachia* strains but also into other Rickettsiaceae. More dramatically, WO-associated regions have moved into the genomes of some of *Wolbachia's* eukaryotic hosts (reviewed in: Metcalf and Bordenstein 2012).

1.6 Future directions in insect-associated phage research

Given that only two heritable phage-symbiont systems have received much attention, it is unsurprising that there remain many basic questions unanswered about both APSE and WO, and phage-symbiont interactions more broadly. Further investigation is needed both to determine the prevalence of active bacteriophages in insect symbionts, and to understand how their interactions may be similar to or distinct from the behavior of phages in other bacterial communities.

1.6.1 Questions remaining in the APSE-*H. defensa* system. As described in **§1.3.1**, the mere presence of the variable toxin cassette region in APSE led to speculation that APSE-encoded toxin homologs caused wasp mortality (Moran et al. 2005), yet no functional assays confirm this. APSE-associated toxins could be moved into expression vectors and the purified products assayed in vitro for toxicity to cultivable wasp tissues (eggs, larvae and teratocytes) (e.g., Lawrence 1990; Okuda and Kadonookuda 1995; Vinson et al. 1994; Grbic and Strand 1998). Furthermore, it is unknown whether these putative toxins are capable of functioning in isolation or whether they require other factors located on the phage or *H. defensa* chromosomes. The CdtB subunit, for example, is integrated into a holotoxin in other organisms, including *E. coli* and *Campylobacter* spp., comprised of three subunits (Ohara et al. 2004); the CdtA and CdtC

subunits bind to the target cell via an unknown receptor and allow the B subunit to enter the cell (Asakura et al. 2007). The three APSE variants that carry *cdtB*-homologs, however, do not include any ORFs homologous to the A and C subunits, indicating that they use a different delivery system (Degnan and Moran 2008a). Some CdtB-APSEs do contain uncharacterized ORFs in close association with the putative toxin and these possibility perform analogous roles (Degnan and Moran 2008a). Despite their lack of strong homology to known proteins, the genomic placement of these ORFs has led to speculation that they interact with APSEs' toxins. Uncharacterized ORFs are also associated with other APSE toxins, including *ydp* (APSE3) and *stx* (APSE1, 4 and 5). In general, mechanisms of toxin delivery remain unclear across APSE variants and *H. defensa* strains. *H. defensa* has an intact type three secretion system that may allow for APSE toxin secretion, but toxin release into the aphid hemocoel may also be accomplished via lysis of a subset of *H. defensa* cells (Moran et al. 2005; Martinez et al. 2014b).

Facultative symbionts are generally uncultivable in cell-free media, and not easily amenable to transformation or reverse genetics, but some have been cultured in association with insect cells (Pontes and Dale 2006). *H. defensa* has been successfully grown and maintained in culture with a number of dipteran and lepidopteran cell lines (Darby et al. 2005), opening the possibility of in vitro studies aimed at understanding the functional mechanisms underlying host defense as well as key questions about phage lifestyle. As described in **§1.3**, APSE loss is a major determinant of both parasitoid protection and aphid fecundity. However, nothing is known about how or why APSEs are lost, in part because very little is known about how it is normally maintained; the ability to work with APSEs in culture may provide answers.

The range of effects attributable to APSEs will be, at least in part, a function of their diversity, which is poorly characterized. Three of the seven known APSE variants were identified from *H. defensa* strains infecting a single aphid species – the pea aphid *Ac. pisum* - but this likely reflects only depth of sampling (Degnan and Moran 2008a). Furthermore, the pea

aphid has diversified into numerous genetically distinct host races that specialize on cultivated herbaceous legumes (e.g., Ferrari et al. 2007; Frantz et al. 2006; Peccoud et al. 2009; Via et al. 2000), and APSE sampling to date has concentrated on pea aphids found on alfalfa. Historical contingencies, natural enemy identity, and transmission opportunities relating to environmental factors may have each contributed to the development and maintenance of current APSE associations, and we need a better appreciation of APSE diversity to adequately investigate these components.

1.6.2 Determining the prevalence and roles of active phage in other heritable bacteria.

Mobile genetic elements, particularly prophage-like regions, are quite common in heritable facultative symbionts (as discussed in **§1.5.1**). In *H. defensa*, for example, mobile DNA, including a 59kbp plasmid, one active prophage (APSE), and 22 phage-like regions, comprises 21% of the genome (Degnan et al. 2009). However, this says little about the proportion of insect symbionts containing active phages. More genomic surveys, combined with microscopy and bioassays (such as those done for APSEs), are needed before we can assess whether the unique selective pressures on maternally inherited bacteria alter the likelihood of phage acquisition and maintenance.

When phage are present in heritable insect symbionts, then we expect that the general roles - mutualism factor provisioning, horizontal gene transfer, and symbiont population control - identified in the APSE and WO systems will occur, although each system is likely to exhibit its own particular features and dynamics. There are several potential advantages to phage-encoded effector molecules, such as increased genomic copy number of key mutualism factors, allowing for higher transcription rates. This potential benefit has been ascribed to plasmids encoding products for amino acid production, though in the polyploid obligate aphid symbiont *B. aphidicola,* this shift may instead decrease transcription (Plague et al. 2003). It is also possible that encoding factors critical for cooperative functions on mobile elements prevents non-

productive cheating (reviewed in: Rankin et al. 2011). Bacteria often secrete public goods, such as toxins enabling invasion of eukaryotic tissues, which benefit not only the secretor but also other bacteria in the population. Many components of the "cooperative" portion of the bacterial secretome are encoded by mobile genetic elements, including phages. This may simply be a coincidence of secreted goods' inclusion in the general class of secondary metabolites: horizontal transmission of core functions is lethally dangerous, so the only molecules that are consistently exchanged are those that the bacterium could survive without. An alternative and intriguing explanation is that infectious public goods provide cooperators with a neat solution to the control of cheaters: non-cooperators can be transformed into cooperators via infection.

The spread of phage-encoded public goods may be limited by the acquisition of phage resistance by noncooperators; however, resistance in itself may be as costly as the averted lysogenic conversion, and may also be insufficient: some WO strains, for example, are capable of not only infecting cheaters, but also of killing phage-resistant defectors via a toxin-antitoxin addiction system (Kent and Bordenstein 2010; Engelberg-Kulka and Glaser 1999). Of course, populations of infectious public-goods-carrying particles would themselves be susceptible to invasion by incompatible non-cooperators. Heavy purifying selection, enhanced by trans-generational bottlenecks that increase within-host relatedness, may also limit cheaters among heritable symbiotic bacteria, decreasing the utility of correction by infection (Herre et al. 1999). Furthermore, phage in heritable bacterial systems may be more likely to lose their capacity for horizontal transmission relative to those found in free-living bacteria, due to a lack of access to uninfected hosts. Selection may also favor the transfer of phage-encoded mutualism factors to the bacterial genome to avert the costs of infection, and phages may be lost through vertical transmission failure more readily than bacterial symbionts from processes such as competition between infected and uninfected heritable bacterial symbionts (discussed in **§1.4.4**).

Phages associated with heritable symbionts may be less prone to lateral exchange than their free-living relatives. A large majority of prokaryotic viruses are dsDNA tailed bacteriophages in the order Caudovirales; functional and genetic diversity in this group is driven primarily by non-homologous recombination and the promiscuous lateral exchange of whole functional modules (Krupovic et al. 2011). This modularity renders dsDNA phages highly effective carriers of secondary bacterial metabolites. However, while Caudovirales may generally evolve according to the Modular Theory (Botstein 1980), the extreme host-limited lifestyle of symbiont-associated phages may isolate them from common phage gene pools to the extent that modular exchange takes a back seat to other processes, such as non-modular recombination and mutation (reviewed in: Metcalf and Bordenstein 2012).

Finally, the role of phage in density interactions with host bacteria is particularly difficult to predict due to our limited understanding of phage reproductive behavior outside of readily manipulated model systems. Phage-bacteria interactions are dependent on transmission rate, length of survival outside the host, and timing of self-replication, all of which may be limited by phage, bacterial, and environmental factors (Abedon 2008). Phages occurring in closed systems, including insect-restricted bacteria, are at risk of running through their limited host population. This may be prevented by processes such as latent-time mutability (Heilmann et al. 2010), superinfection exclusion or immunity (Susskind 1980; Susskind et al. 1974; McGrath et al. 2002), and mechanical shielding by dead bacteria, where the number of dead reach a density where phages are likely to irreversibly inject DNA into dead cells rather than the remaining uninfected live cells (Rabinovitch et al. 2003). Nutritional limitations can also decrease bacterial receptivity to phage infection (Ptashne 2004), or phage reproductive rate (Ptashne 2004; Lenski 1988). Environmental hostility to free phage particles, such as pH extremes, can increase the rate of lysogenization, and rates of decay by insect host hemolymph factors may be a major unexplored determinant of bacteriophage lifestyle (Pantastico-Caldas et

al. 1992). Of course, environmental stressors severe enough to damage DNA may lead to prophage induction, and abiotic influences may predictably and seasonally influence phage-bacteria-insect relations, as seen in other viral communities: marine phages, for example, produce fewer lysogens in summer months (McDaniel et al. 2002).

1.6.3 Technical challenges of phage discovery. While detecting and characterizing the diversity of uncultivable insect-associated bacteria has become routine through the development of universal 16S rRNA techniques, no simple universal tool for identifying bacteriophages in symbionts is available. Instead they are often found by chance through electron microscopy or bacterial genome sequencing. High throughput sequencing has allowed for the production of genomic data on total organismal and tissue specific viral samples. Sequence data alone, however, is often uninformative due to the high genic richness and sequence divergence associated with viruses: phage metagenomic studies consistently report high proportions of totally novel sequences with no known function (Hatfull 2008). A study of mosquito viromes, for example, was unable to match nearly half of the sequences to any known viral genome (Ng et al. 2011). Furthermore, overall virome samples may miss phage diversity, or fail to pick up on phage presence all together, particularly if eukaryotic viruses are present. For instance, a metavirome sample taken from worker honeybees suffering from an unknown source of malaise was dominated by a small handful of eukaryotic viruses, and only 0.7% of those reads were from known phages (Granberg et al. 2013).

The host ranges of even well-studied phages are seldom known (Flores et al. 2011), which can limit the interpretation of virome results. One possible way to estimate host ranges is to analyze the legacy of bacteriophage infections. Stern has suggested that clustered regularly interspaced short palindromic repeat (or CRISPR) spacers in microbiome samples be viewed as a “database of fragments from phage and plasmid genomes” (Stern et al. 2012). This could allow researchers to associate phages with at least a subset of their potential hosts even in the

absence of direct experimental evidence or integrated prophage. Interestingly, there appears to be variation in the presence of CRISPR systems in two aphid heritable symbionts: *H. defensa* has CRISPR sequences while its sister species, *R. insecticola,* does not (Degnan et al. 2009; Hansen et al. 2012), but the basis of this variation in unknown. More generally, it is unknown why CRISPR/cas systems are absent from 60% of bacterial genomes despite their near-universality in archaea and the ubiquity of bacteriophage predation (Horvath and Barrangou 2010).

1.6.4 Phage roles in the more complex insect-associated bacterial communities. Recently, much attention has been paid to the broader microbiome, and to the nutritional and immune services it provides to its animal host. Studies in humans suggest that a characteristic, somewhat taxonomically-restricted bacterial community may itself host a characteristic, somewhat taxonomically-restricted bacteriophage community (Stern et al. 2012), and that microbiomes and viromes co-vary between individuals (Minot et al. 2011). Though functional studies of insect gut microbiota lag behind those in mammals, it is known that these consortia play significant parts in host development, nutrition, and immune function (Dillon and Dillon 2004; Engel and Moran 2013). The effects of phages in these more complex insect-associated bacterial communities are largely unknown, but may differ from those found in heritable symbionts. Phages may influence community diversity through species-specific predation and community structure by lysing the most abundant species or strains (reviewed in: Weinbauer 2004). Such density-dependent “kill-the-winner” dynamics potentially reduce functions associated with mutualistic bacteria, limit population sizes, and may also drive bacterial strain diversification (Middleboe et al. 2009). Heterogeneity in bacterial groups tends to reduce group productivity, so bacterial diversity per se may lower the efficiency of mutualistic functions provisioned by the microbiota (Mendes-Soares et al. 2014). However, phage-driven diversification can increase a bacterial population’s adaptability to changing environments

(Williams 2013), decreasing the likelihood that environmental perturbations will catastrophically disrupt the microbiome.

Bacteriophage-mediated horizontal gene transfer may also contribute to the development of functional redundancy and allow newly acquired bacteria to rapidly adapt to a shared host. This has been proposed in sponge-associated bacterial communities, with "forcible" adaptation spreading mutualistically-important functions across taxa (Fan et al. 2012), which may limit the risk of function loss via density-dependent strain predation. Microbiome and virome diversity therefore reciprocally influence one another, and phage may contribute to the maintenance of diversity with or without disrupting the mutualistic function of the insect-associated bacterial community. However, the metabolic loads or receptor modifications associated with phage resistance could theoretically decrease the efficiency of a mutualism. Flyg and colleagues (1980) identified a strain of *Serratia marcescens* (an insect pathogen with symbiont relatives), that was more resistant to phage, but more vulnerable to the cercropid immune response and less virulent in drosophilids.

1.7 Concluding remarks

While bacteriophages are the most abundant biological entities on earth, we currently know little about their prevalence in insect-associated bacteria. When they are present, they can profoundly influence not only the ecology and evolution of their bacterial hosts, but also that of the insect in which they both reside. While tripartite mutualisms in insects remain understudied, there is growing interest in their roles, as recent findings indicate that phages can provide key services required for symbiont function and dramatically alter the within-insect population dynamics of symbiotic microorganisms. These within-host effects can in turn be important in the spread and maintenance of heritable symbionts in host populations. Phages also likely play key roles in the horizontal transfer of ecologically relevant traits and in the transition to a host-associated lifestyle, potentially resulting in more dynamic and responsive bacterial symbionts.

Even less is known about tripartite interactions operating under natural conditions. The additional complexity of tripartite interactions potentially renders them less stable when faced with environmental perturbations. Thus, phage infection in heritable symbiosis likely provides advantages and disadvantages for all interacting players.

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CHAPTER 2

PHAGE LOSS AND THE BREAKDOWN OF A DEFENSIVE SYMBIOSIS IN APHIDS[1]

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2.1 Abstract

Terrestrial arthropods are often infected with heritable bacterial symbionts which may themselves be infected by bacteriophages. However, what role, if any, bacteriophages play in the regulation and maintenance of insect-bacteria symbioses is largely unknown. Infection of the aphid *Acyrthosiphon pisum* by the bacterial symbiont *Hamiltonella defensa* confers protection against parasitoid wasps, but only when *H. defensa* is itself infected by the phage APSE. Here we use a controlled genetic background and correlation-based assays to show that loss of APSE is associated with up to seven-fold increases in the intra-aphid abundance of *H. defensa*. APSE loss is also associated with severe deleterious effects on aphid fitness: aphids infected with *H. defensa* lacking APSE have a significantly delayed onset of reproduction, lower weight at adulthood, and half as many total offspring as aphids infected with phage-harbouring *H. defensa,* indicating that phage loss can rapidly lead to the breakdown of the defensive symbiosis. Our results overall indicate that bacteriophages play critical roles in both aphid defence and the maintenance of heritable symbiosis.

2.2 Introduction

Bacteriophages are the most abundant biological entities on earth, and they perform key ecological functions at scales ranging from local to global (Weinbauer 2004). Among free-living bacteria, phages can influence host population dynamics via host cell lysis and other mechanisms, which can affect community structure. Temperate phages often encode functional pathways, such as antibiotic resistance or virulence factors, which enhance bacterial host fitness, and vector these traits within and among bacterial lineages (Clokie et al. 2011). Many bacterial lineages, however, persist only in association with animal cells. Heritable bacterial infections, for example, are widespread among terrestrial arthropods, where many have evolved into beneficial symbionts that provide nutritional or defensive services (Moran et al. 2008; Oliver et al. 2010). Several heritable symbionts also harbour phage infections, yet the prevalence and

roles of phages in heritable symbioses remain poorly understood (Belda et al. 2010; Darby et al. 2010).

Bacteriophages named APSEs (*Acyrthosiphon pisum* secondary endosymbiont) infect *Hamiltonella defensa*, a gammaproteobacterial symbiont of aphids and related insects (van der Wilk et al. 1999; Moran et al. 2005a; Degnan and Moran 2008b, a). APSEs are temperate bacteriophages related to the lambdoid phage P22 (Podoviridae) (van der Wilk et al. 1999; Sandstrom et al. 2001). There are two APSE variants (APSE-2 & 3) commonly found in North American populations of *A. pisum.* Each variant shares a core of conserved genes but also contains a variable region consisting of holin, lysozyme and toxin genes from two protein families: cytolethal distending toxin (CdtB) (APSE-2), and YD-repeat toxin (Ydp) (APSE-3) (Moran et al. 2005a; Degnan and Moran 2008b). Phylogenetic evidence shows that APSEs move these pathways horizontally between *H. defensa* lineages (Degnan and Moran 2008b). Prior studies with the pea aphid, *Acyrthosiphon pisum*, established that *H. defensa* confers protection against an important natural enemy, the parasitic wasp *Aphidius ervi,* by killing wasp offspring that otherwise develop within the aphid hemocoel (Oliver et al. 2005; Oliver et al. 2003). This protective phenotype was further found to depend on whether the bacterial symbiont was infected by APSE, and to differ with phage variant: *H. defensa* strains carrying APSE-3 confer near-complete resistance and those with APSE-2 confer partial resistance (Degnan and Moran 2008a; Oliver et al. 2009).

Given the lytic capabilities of phages, APSEs and other temperate viruses have the potential to influence symbiont abundance in insect hosts. Within-host bacterial abundance can affect conferred phenotypes (e.g., Ikeda et al. 2003; Noda et al. 2001), rates of horizontal transfer and establishment of novel infections (Chafee et al. 2010), and maintenance of tripartite symbioses (Bordenstein and Bordenstein 2011; Jaenike 2009). All stable beneficial heritable symbiont infections must also be coordinated between host and symbiont(s) to strike a balance between sufficient titre to produce the beneficial phenotype and ensure vertical transmission to

progeny, while limiting over-replication that might be detrimental to host fitness (Jaenike 2009). The mechanisms underlying the regulation of heritable symbionts, however, are poorly understood. Hosts may restrict symbionts to particular tissues and the host immune system may regulate symbiont infection (Buchner 1965; Moran et al. 2005b; Login et al. 2011), though some facultative symbionts maintain a pathogen-like capacity for colonization of novel host tissues. Symbionts, in turn, may employ chemical communication (e.g. quorum sensing) to assess titres, but quorum sensing has been characterized in only one heritable insect symbiont, *Sodalis* (Pontes et al. 2008). Temperature has also been shown to affect within-host density of endosymbionts in several insects (Hurst et al. 2000), including wasps in the genus *Nasonia,* where temperature decreases the abundance of *Wolbachia* but increases the abundance of the phage WO (Bordenstein and Bordenstein 2011). We became interested in the role of APSE in regulation of *H. defensa* densities when we anecdotally observed that hemolymph from *A. pisum* infected with *H. defensa* lacking APSE contained higher densities of this symbiont than aphids infected by *H. defensa* with APSE. To elaborate on this observation, we conducted a set of experimental and correlation-based studies to examine whether APSE was responsible for reducing symbiont titres, and if so, whether phage loss and symbiont deregulation affect aphid fitness.

2.3 Materials and methods

2.3.1 Study organisms *Acyrthosiphon pisum* is a cosmopolitan pest of herbaceous legumes, including important forage crops (Van Emden and Harrington 2007). In most temperate regions, *A. pisum* is cyclically parthenogenetic; aphids reproduce asexually and viviparously for most of the growing season, and only in response to a shortening photoperiod in autumn are sexual morphs produced, which lay overwintering eggs. (Brisson and Stern 2006). In the laboratory, clonal lines can be maintained indefinitely by mimicking long day-length conditions. Single parthenogenetic females collected from the field were used to initiate the lines in this study

(Table S2.1). All aphids were reared on *Vicia faba* on a 16L: 8D cycle at temperature of 19 °C +/- 1 °C.

In laboratory–reared pea aphid clones, *H. defensa* is vertically transmitted at rates approaching 100% (Oliver et al. 2010). APSE-3 infections are also transmitted with very high fidelity, but can be spontaneously lost at very low rates (Oliver et al. 2009). We therefore used previously established sublines from the aphid clone 5A that had been inoculated with the APSE-3-harboring *H. defensa* strain A1A ($A1A^{+}\rightarrow5A$), some sublines of which subsequently lost APSE-3 ($A1A^{-}\rightarrow5A$) (Oliver et al. 2009). Lines 82B→5A-1 and 82B→5A-2 were established by a single transfer of *H. defensa* (82B, collected in Cayuga Co, NY 2000) via microinjection into line 5A, but parthenogenetically reproducing lines were maintained separately for at least three years (Oliver et al. 2003). All other aphid clonal lines used in this study contained their natural symbiont infections (Table S2.1).

2.3.2 APSE effects on *H. defensa* titres.

2.3.2.1 To determine if phage loss influences symbiont abundance, we used real time quantitative PCR (qPCR) to compare *H. defensa* titres in aphids that share the same genotype and symbiont strain, but which differed in status of phage infection ($A1A^{+/-}\rightarrow5A$). To create cohorts of equal-aged aphids, between 10 and 15 actively reproducing female *A. pisum* were placed on a single *V. faba* plant. The twenty-four hour-old cohorts were produced within +/- 2 h, all other cohorts (i.e. 48 – 336 h) were produced within +/- 4 h. Aphids were destructively sampled and each time point represents a unique cohort. Quantities of aphid symbiont levels at different time points during development (24-336 h) were then determined by preparing whole aphid DNA extractions in a lysis buffer (10mM Tris-Cl, pH8.2; 1mM EDTA; 25 mM NaCl) with 1% proteinase K (20 mg/mL) scaled by aphid size from 10 µL for a first instar aphid to 100 µL for adults (Gloor et al. 1993). Standard curves for quantification were produced via serial dilutions from 1E2 to 1E9 (Oliver et al. 2006), and efficiencies for all quantification reactions were above 93%. After extraction, aphids from line $A1A^{+}\rightarrow5A$ were first tested using diagnostic

PCR to confirm phage infection (primers and reaction conditions in **Table 2.1**). Unique fragments of the single-copy gene *dnaK* were used to quantify the abundance of *H. defensa* by qPCR (Table 2.1). For all aphid ages except for 336 h, the relative bacterial and phage titres were calibrated using the aphid gene EF1α to account for differences in extraction efficiency and body size. All 10 µL reactions were performed on a Roche LightCycler 480 II using Roche LightCycler 480 SYBR Green I Master chemistry and 0.5 µM of each primer. Preincubation: 95 °C for 5 min; amplification (repeated 45 times): 95 °C for 10 s, 68 °C to 55 °C touchdown with 1 °C steps each at 10, 72 °C for 10s; melting curve: 95 °C for 5 s, 65 °C for 1 min, then ramped to 97 °C; hold at 40 °C.

Table 2.1. Primer sequences used with diagnostic and quantitative procedures.

Target gene (source organism)	5'-Primer sequences-3'	Primer and reaction source
P2 (bacteriophage APSE)	F: GTC CAG GCA TTA TTA GCG C R: TTT TTC TAA GGC AAC CAT	(Moran et al. 2005a)
P28 (bacteriophage APSE)	F: TGA TAA AAG CGG ATA ATG CC R: GCG TTT GTC ATA CTG AAA AGG	(Moran et al. 2005a)
cdtB (variant APSE-2)	F: ATA TTT TTT TTA CCG CCC CG R: CCA GCT TCA TTT CTA CCA CCT C	(Moran et al. 2005a)
Ydp (variant APSE-3)	F: CGC CCA CGC CCT CAA CGA TT R: CTG GCC GGC CTT TGA CCA GG	This publication
dnaK *(Hamiltonella defensa)*	F: GGT TCA GAA AAA AGT GGC AG R: CGA GCG AAA GAG GAG TGA	(Moran et al. 2005a)
ef1α *(Acyrthosiphon pisum)*	F: CTG ATT GTG CCG TGC TTA TTG R: TAT GGT GGT TCA GTA GAG TCC	(Wilson et al. 2006)

2.3.2.2 We also discovered that one sub-line of clone A1A^{+}→5A produced a small percentage of offspring infected with *H. defensa* but without APSE-3. This finding allowed us to examine the differences in symbiont titre between phage negative and phage positive siblings from the same (phage positive) mother. We used qPCR, as described above, to estimate *H. defensa* titres from 24 h +/- 2 h APSE-infected and APSE-free offspring produced by a single mother aphid. Diagnostic PCR was used to determine phage infection status.

2.3.2.3 Diagnostic screening identified additional laboratory-held clonal *A. pisum* lines that were either fixed for or lacked APSE-3 (Table S2.1). Using the same protocols, we conducted qPCR on 72 h +/- 4 h offspring of four clones infected with *H. defensa* plus APSE-3 and three clones without APSE to determine if phage-free lines generally have higher *H. defensa* titres than phage-infected lines.

2.3.2.4 The other common North American phage variant, APSE-2, has never been reported lost from a laboratory-held line, preventing us from directly assessing the effects of APSE-2 loss on *H. defensa* abundance. However, we were able to examine two lines that shared the same aphid clonal background (5A), strain of *H. defensa* (82B), and haplotype of APSE-2, but which had been reared as separate parthenogenetically reproducing colonies for at least three years. Using qPCR as described above, we estimated *H. defensa* and APSE-2 titres (amplifying a unique fragment of APSE gene P28; **Table 2.1**) at two time points in aphid development: 96 hour (third instar) and 144 hour (fourth instar) nymphs.

2.3.3 Effects of phage loss on aphid fitness To assess the effects of phage loss on aphid fitness, we compared three aphid fitness parameters (fecundity, development time, and fresh weight at adulthood) in our experimental lines ($A1A^{+/-}\rightarrow 5A$) that shared the same genotype and symbiont strain, but differed in phage infection status. Fitness assays were conducted as in Oliver et al. 2006 (Oliver et al. 2006). For each replicate (N = 10) of the fecundity assay, a cohort of four similarly aged (+/- 16 h), pre-reproductive, apterous female aphids were placed on a single *V. faba* plant in an isolated cup cage. Offspring were counted and removed every three days after the onset of reproduction. The number of surviving adults from the initial cohort was also noted at each time point until day 26. At this point, most aphids had ceased reproducing and more than a quarter of all cohorts had no surviving adults. Plants were changed occasionally to promote optimal conditions for aphid development. We also examined development time, defined here as time from birth to first reproduction (TFR). To determine TFR, nymphs were moved to new plants after birth (±1.5 h) and, starting at seven days post-

birth, were monitored every three hours during the light cycle until all reproduced. Adult fresh weight of apterous aphids was taken at time of first reproduction.

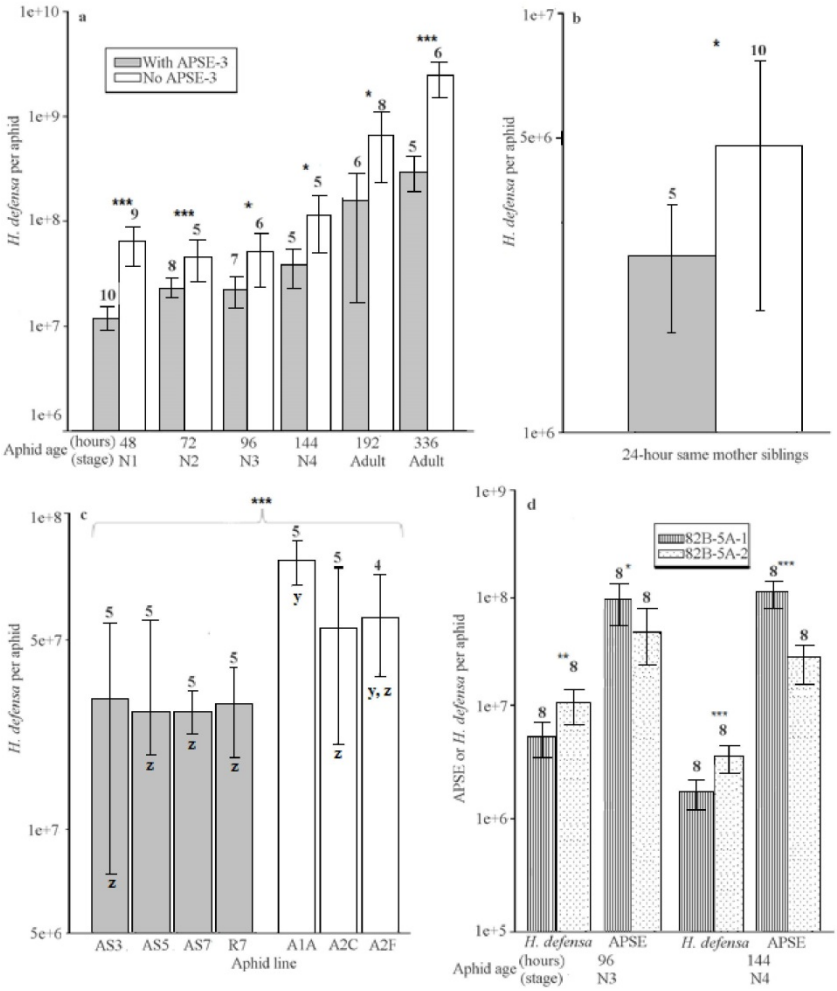

Figure 2.1. Bacteriophage APSE affects *H. defensa* abundance. (N = nymphal instar, t-test, **P*<0.05; ***P*<0.01; ****P*<0.001). Columns represent mean symbiont abundances for APSE-3 infected (dark) and phage-free (open) treatments: bolded numbers indicate number of aphids in the treatment. Bars represent 95% confidence intervals. (**a**) *H. defensa* titres in experimental line A1A^{+}→5A (aphid clone 5A infected with *H. defensa* A1A and with phage APSE-3) vs. line A1A^{-}→5A (aphid clone 5A infected with *H. defensa* A1A but without phage APSE-3). (**b**) *H. defensa* titres in siblings from the experimental line demonstrating changed *H. defensa* titres within a single generation. All aphids, with and without phage, used for the 24 hour time point were the offspring of a single A1A^{+}→5A mother. (**c**) *H. defensa* titres in 72 h/2nd instar clonal lineages with and without APSE-3. ANOVA comparison of summed phage-infected group to summed phage-free group is presented at top; individual lines were compared via post-hoc Tukey-Kramer HSD: shared letters (y or z) indicate levels not significantly different. (**d**) APSE-2 and *H. defensa* in lines 82B→5A-1 and 82B→5A-2.

2.3.4 Fidelity of *H. defensa* and APSE transmission

2.3.4.1 The vertical transmission rate of *H. defensa* is near 100% under standard laboratory conditions (~20°C, 16 light: 8 dark hours) (Oliver et al. 2010), but no comparison of *H. defensa* transmission rates has been reported for lines with and without APSE. To do this, we regularly screened all lines in Table S2.1 for *H. defensa* infection via diagnostic PCR (Table 2.1). Laboratory-based cage experiments show that uninfected aphids spread at the expense of *H. defensa*-infected aphids (Oliver et al. 2008), which would increase the likelihood of detecting any instances of symbiont loss.

2.3.4.2 We also determined the phage variant of each line (Table S2.1) using primers (Table 2.1) that amplify fragments of the *cdtB* and *Ydp* genes, which are found on APSE2 and APSE3, respectively. To examine vertical transmission of APSEs, these lines were regularly screened for phage presence using the *P2* and *P28* primers (Table 2.1).

2.3.5 Data analysis All within-treatment qPCR estimates of symbiont titres, except for the 192 h time point in the experimental line comparisons (section 2.b.i, see below), were normally distributed. Thus, comparisons among treatments were analysed by Analysis of Variance (ANOVA). Confidence intervals presented in Fig. 2.1 were calculated with unpooled variance. Since our phage-infected and phage-uninfected treatments for 192 h exhibited a log-normal distribution, we log transformed these data prior to ANOVA. The resulting means and confidence intervals were then back transformed for presentation in Fig. 2.1a. For cross-line comparisons of symbiont abundances (section 2.b.iii), we conducted an ANOVA to compare all phage-infected and phage-free lines, followed by a post-hoc Tukey-Kramer HSD test to assess which mean differences were significant. For our fitness assays (section 2.c), lifetime fecundity and fresh weight were normally distributed and subjected to t-tests, whereas TFR was non-normally distributed which necessitated our use of a non-parametric Wilcoxon rank-sum test. All statistical analyses were performed using the JMP v. 8.0.2 platform (SAS Institute Inc., Cary, NC, 1989-2007).

2.4 Results

2.4.1 APSE-3 loss is associated with increases in *H. defensa* titre The A1A→5A sublines, identical in aphid genotype and *H. defensa* strain but differing in APSE-3 infection status, allowed us to experimentally investigate the consequences of phage loss. Our qPCR estimates of symbiont abundance revealed that phage-free aphids contained significantly more *H. defensa* than aphids with APSE-3 at all examined time points in aphid development (Fig. 2.1a). *H. defensa* titres rose throughout aphid development, such that older aphids lacking APSE contained much larger numbers of *H. defensa* than adult aphids with phage (Fig. 2.1a).

2.4.2 Phage loss results in immediate increases in *H. defensa* titres In a sub-line of clone A1A^{+}→5A, which infrequently produced APSE-free offspring, we compared symbiont titres of 24 h-old offspring produced by a single APSE-3/*H. defensa* positive mother and found that nymphs lacking APSE-3 carried on average 83% more *H. defensa* than their phage-harbouring sisters (Fig. 2.1b, ANOVA, $F_{1, 14} = 6.0$, $P = 0.03$), indicating that phage loss results in immediate increases in *H. defensa* abundance per aphid.

2.4.3 Phage-free lines generally exhibit higher *H. defensa* titres We screened 72 h old offspring of laboratory-held lines infected with *H. defensa* and APSE-3, and lines infected with phage-free *H. defensa* (Table S2.1) to determine if phage-free lines generally contain higher symbiont titres. We found that clones lacking APSE-3 contained on average more than twice the number of *H. defensa* per aphid than clones with APSE-3 (Fig. 2.1c, ANOVA $F_{6, 33} = 11.85$, $P < 0.0001$).

2.4.4 APSE-2 and *H. defensa* titres have an inverse relationship To determine if APSE-2 also influenced symbiont titres, we assessed the abundance of *H. defensa* and phage in lines sharing the same aphid background (5A) and same *H. defensa* strain and APSE-2 haplotype (from line 82B). We found that aphids from the 82B→5A-1 line contained more APSE-2 than aphids from the 82B→5A-2 line (Fig. 2.1d). Conversely, the abundance of *H. defensa* was

significantly lower in 82B→5A-1 aphids than 82B→5A-2 aphids (Fig. 2.1d), indicating an inverse association between phage and symbiont titre.

Table 2.2. Aphid fitness assays in experimental lines with APSE ($A1A^+$→5A) and without APSE ($A1A^-$→5A). Includes maternal age, in hours, at time of first live offspring produced, maternal mass immediately after first reproduction, and total offspring produced by cohorts of four adult aphids by age 26 d. The statistical test used for each fitness measure is shown in the right column. P-values in each case were also highly significant.

Assay		**+ APSE-3**	**- APSE-3**	**P-value**
Time to first reproduction (hours)	Range **Mean** Aphids	189.5-236.5 **205.87** 19	199.5-236.5 **223.20** 20	0.000006 (Wilcoxon rank-sum)
Fresh weight (mg)	Range **Mean** Aphids	2.74-4.29 **3.79** 19	1.99-4.01 **3.17** 20	0.0002 (t-test)
Offspring per cage by day 26	Range **Mean** Cages	116-335 **237** 10	66-183 **122** 10	<0.0001 (t-test)

2.4.5 APSE loss has severely deleterious effects on measures of aphid fitness The loss of APSE and concomitant rise in the abundance of *H. defensa* could affect aphid fitness. To test this idea we used the $A1A^+$→5A and $A1A^-$→5A aphid sublines, which were genetically identical and contained the same strain of *H. defensa* but differed in whether or not they contained APSE-3. We then measured three fitness parameters: fecundity, development time, and fresh weight at adulthood. In each instance, our results showed that the absence of APSE-3 significantly increased fitness costs to the aphid host (**Table 2.2**). Aphids lacking APSE-3 (line $A1A^-$→5A) reproduced, on average, 18 hours later than $A1A^+$→5A aphids with APSE-3. They also weighed 20% less than their phage-harbouring counterparts at adulthood, and produced roughly half as many offspring (**Table 2.2**).

2.4.6 *H. defensa* is vertically transmitted with high fidelity with and without APSE We have held numerous *H. defensa*-infected lines with and without APSE in continuous culture for many years (Table S2.1) and despite routine screening we have not detected any losses of *H. defensa*. Based on a conservative average of 30 generations per year, we calculated the

number of generations with successful vertical transmission (Moran and Dunbar 2006). We estimate 1470 generations of successful transfer in APSE-3 infected lines and 540 generations in APSE-free *H. defensa* infected lines.

2.4.7 APSE-2 has higher vertical transmission fidelity than APSE-3 We currently maintain sixteen aphid lines bearing APSE-2-*H. defensa*, most of which have been held for at least one year, and despite routine screening we have documented no instances of APSE-2 loss, including in one line held, in multiple subclones, for more than twelve years. In contrast, we have held at least ten lines infected with APSE-3, and most have lost phage within four years (Table S2.1).

2.5 Discussion

By controlling aphid genotype, symbiont genotype, and environmental conditions, such as temperature, the current study shows that APSE reduces within-host densities of *H. defensa.* In lines with identical aphid genotypes and *H. defensa* strains, APSE loss resulted in significant increases in *H. defensa* titre across all examined time points ranging from first instar nymphs to adults (Fig. 2.1a). While it is possible that additional changes (other than APSE loss) that influence *H. defensa* abundance have occurred in our clonal experimental lines, our finding that APSE-free offspring contain fewer *H. defensa* than their APSE-3-harbouring siblings (Fig. 2.1b), strongly suggests that phage loss results in immediate increases in symbiont titres in this line (Fig. 2.1b) and that APSE loss alone is a sufficient explanation for *H. defensa* titre differences we observe in the experimental lines. Furthermore, among genetically diverse *A. pisum* lines, those lacking APSE-3 consistently contain roughly twice the number of *H. defensa* as lines maintaining the phage (Fig. 2.1c). These experimental and correlation-based findings indicate that APSE-3 infection significantly reduces the abundance of *H. defensa* in *A. pisum.* While no APSE-2 loss event has been reported, genetically identical aphid sub-lines with the same strain of *H. defensa* have *H. defensa* titres inversely associated with APSE-2 titre. This inverse

relationship is consistent with the lysis of *H. defensa* by APSE-2, and, along with our APSE-3 results, suggests that APSE-2 also reduces the abundance of *H. defensa* in *A. pisum.*

We also found that the higher *H. defensa* titres associated with phage loss correlated with severe fitness costs to *A. pisum*. In our experimental line sharing *H. defensa* strain and aphid genotype, phage-free *H. defensa*- infected aphids developed more slowly, reached a smaller fresh weight at adulthood, and produced approximately 50% fewer offspring their APSE-3 infected counterparts (**Table 2.2**). The underlying cause of these costs was not investigated, but *H. defensa* is auxotrophic for most essential amino acids and likely relies on the aphid and its obligate nutritional symbiont *Buchnera aphidicola* for growth (Degnan et al. 2009) and increases in *H. defensa* abundance may reduce resources available for aphid growth and reproduction.

Costs associated with phage loss may play an important role in the maintenance of this protective symbiosis. *H. defensa* is found at intermediate frequencies in nature (e.g., (Oliver et al. 2006); (Ferrari et al. 2012)) and most field collected *H. defensa*-infected aphids are also infected by APSE (Oliver et al. 2009; Russell et al.). Population cage studies reveal that aphids infected with *H. defensa* and APSE-3 rapidly spread to near-fixation when parasitism pressure is present, while uninfected aphids are favoured in the absence of parasitism (Oliver et al. 2008). Thus, aphids infected with *H. defensa* plus APSE have a fitness advantage over uninfected aphids when exposed to parasitism pressure due to the resistance traits APSE encodes. In contrast, aphids infected by *H. defensa* alone derive no protection from parasitism and incur higher fitness costs than aphids infected by *H. defensa* plus APSE. Moreover, while individual *H. defensa* cells could benefit from the absence of APSE infection, the within-host reductions in symbiont density APSE causes do not appear to adversely affect transmission fidelity, as symbiont inheritance approaches 100% under standard laboratory conditions whether or not APSE is present. We conclude that APSE is likely essential for maintenance of the *H. defensa*-aphid symbiosis because its loss favours reductions in the prevalence of

symbiont-infected aphids under conditions of both high and low parasitism pressure. APSE is therefore a vital component in the *H. defensa*-aphid symbiosis not only because of the pathways it encodes but also for its ability to regulate symbiont density without compromising transmission fidelity. The loss of this bacteriophage, in contrast, leads to an immediate proliferation of bacterial symbionts, deleterious effects on the animal host, and the rapid breakdown of the heritable symbiosis.

The fitness costs of phage loss to aphids may also explain why aphids harbouring APSE-2 are maintained in natural populations despite being inferior protectors against parasitism. We found that, in the laboratory, APSE-2-*H. defensa* interactions appear more stable than those involving APSE-3, albeit in a limited sample (Table S2.1). The underlying basis for the differential persistence of APSE-2 and 3 is currently unclear. The reduction in *H. defensa* titre that occurs with phage infection suggests both phage variants undergo lytic cycles, but both also persist in *H. defensa* as integrated prophages (Degnan and Moran 2008a). Thus, differences in the timing of lytic and lysogenic activity during the life cycle of the aphid or *H. defensa* may underlie the differential persistence of these APSE variants. Given evidence that higher temperatures reduce the protective benefits of *H. defensa,* abiotic factors may play a role in the within-host dynamics between APSE and *H. defensa* (Bensadia et al. 2006). Studies with *Nasonia* also show that temperature shock reduces the abundance of *Wolbachia* while increasing the abundance of phage WO (Bordenstein and Bordenstein 2011).

In general, phage infections have the potential to exert dynamic and profound influences on animal-bacterial symbioses. In addition to encoding pathways that benefit both the bacterial and animal hosts (Moran et al. 2005a; Degnan and Moran 2008a; Oliver et al. 2009), bacteriophages may alter symbiont abundance within individual hosts and thereby play critical roles in the maintenance of heritable symbiosis within host populations.

2.6 Acknowledgements

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2.7 References and notes

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Table S2.1. Aphid line collection and infection status.

A. pisum line	Current APSE status (or at year discarded)	Original APSE	Variant	Collection
A1A→5A	**-**	+	APSE-3	Utah, USA 2003
A1A	**-**	+	APSE-3	Utah, USA 2003
A2C	**-**	?	? (APSE-3)	Utah, USA 2003
A2F	**-**	+	APSE-3	Utah, USA 2003
A2H	**- (2009)**	+	APSE-3	Utah, USA 2003
A2D	**- (2009)**	+	APSE-3	Utah, USA 2003
AS3	**+**	+	APSE-3	Utah, USA 2007
AS5	**- (2011)**	+	APSE-3	Utah, USA 2007
AS7	**+ (2010)**	+	APSE-3	Utah, USA 2007
R7	**+ (2010)**	+	APSE-3	Utah, USA 2007
82B→5A	**+**	**+**	APSE-2	NY, USA 2000
CJ1-41	**+**	+	APSE-2	Utah, USA 2011
CJ3-14	**+**	+	APSE-2	Utah, USA 2011
NY2-6	**+**	+	APSE-2	NY, USA 2011
PB35	**+**	+	APSE-2	PA, USA 2011
WA4	**+**	+	APSE-2	PA, USA 2010
WI-16	**+**	+	APSE-2	WI, USA 2010
XA6	**+**	+	APSE-2	PA, USA 2010
YA3	**+**	+	APSE-2	PA, USA 2010
YA4	**+**	+	APSE-2	PA, USA 2010
YA12	**+**	+	APSE-2	PA, USA 2010
YA17	**+**	+	APSE-2	PA, USA 2010
ZA11	**+**	+	APSE-2	PA, USA 2010
ZA12	**+**	+	APSE-2	PA, USA 2010
ZA17	**+**	+	APSE-2	PA, USA 2010
ZA33	**+**	+	APSE-2	PA, USA 2010

CHAPTER 3

DIVERSITY, FREQUENCY, AND PHENOTYPES OF PHAGE INFECTIONS IN AN APHID BACTERIAL MUTUALISM[1]

[1]Weldon S.R., A.J. Martinez, E. Smith, J.A. Russell and K.M. Oliver. To be submitted to *Applied and Environmental Microbiology.*

3.1 Abstract

Viruses are the most abundant and genetically diverse segment of the biosphere, and many viruses engage in commensal or even mutualistic relationships with eukaryotes. Viruses can also interact with eukaryotes through proxies: bacteriophage infection is a well-known contributor to virulence in pathogenic bacteria associated with animal hosts, but phage infections may also modulate the interactions of beneficial bacteria with their eukaryotic hosts. Aphids, a model insect host for mutualistic microorganisms, harbor heritable bacteria that mediate diverse ecological interactions. One aphid symbiont, *Hamiltonella defensa*, confers protection against parasitic wasps, but only when the bacterial symbiont is itself infected with bacteriophages called APSEs. All APSEs share a backbone of conserved genes, while also containing a toxin-encoding variable cassette region. Levels of conferred protection differ depending on *H. defensa* strain and associated APSE variant, but little is known about APSE diversity within and among aphid populations. We used phage structural genes and multi-locus sequence typing of *H. defensa* to identify the variety of phage strains present and look for evidence of lateral exchange of coding regions and whole phage. Here we present evidence that only four strains of APSE, three previously identified and one new to this study (APSE8), account for the overwhelming majority of strain diversity in North American pea aphids living on alfalfa. We additionally confirmed that APSE8 provides anti-parasitoid protection, and found evidence of lateral phage exchange between *H. defensa* strains within alfalfa-feeding *A. pisum.*

3.2 Introduction

Bacteriophage are an enormous source of novel functional pathways in bacteria and, through lytic and lysogenic cycles, influential architects of bacterial communities (Calendar and Abedon 2005). Their bacterial hosts may, in turn, provide phenotypic diversity to, and structure the populations of, eukaryotes. The majority of insect species are infected with one of more heritable bacterial endosymbionts, but the overall prevalence, diversity and roles of bacteriophages infecting insect-associated bacteria are largely unknown. While the obligate

nutritional symbionts of insects appear to generally lack phages and other mobile genetic elements, many common facultative symbionts, including *Wolbachia, Arsenophonus, Spiroplasma* and *Hamiltonella,* are associated with active viruses, and the genomes of other secondary symbionts, such as *Sodalis glossinidus* and *Regiella insecticola*, contain the remnants of inactivated prophage (Bordenstein et al. 2006; Darby et al. 2010; Belda et al. 2010). Facultative symbionts often interact with their hosts as reproductive manipulators or defensive mutualists (Duron et al. 2008; Hamilton and Perlman 2013; Oliver and Martinez 2014), and the phages infecting them can confer additional functions, move ecologically-important traits among symbiont lineages, and influence within-host bacterial titers (Saridaki et al. 2011; Oliver et al. 2009; Weldon et al. 2013; Kent and Bordenstein 2010; Degnan and Moran 2008b).

Aphids, a diverse and cosmopolitan group of herbivorous insects, harbor an array of heritable bacterial symbionts, many of which confer protection against natural enemies (Oliver et al. 2009; Oliver et al. 2014). One symbiont of aphids and related hemipterans, *Hamiltonella defensa* (Gammaproteobacteria: Enterobacteriaceae), defends at least three aphid species (*Acyrthosiphon pisum*, *Aphis fabae* and *Ap. craccivora*) against parasitism by some endoparasitic aphidiine braconid wasps (Oliver et al. 2005; Schmid et al. 2012; Asplen et al. 2014). This interaction has been best-studied in *A. pisum*, where *H. defensa* confers protection against *Aphidius ervi*. In *A. pisum* with susceptible genotypes (Martinez et al. 2014a) and without *H. defensa* infections, after the parasitoid *Ap. ervi* oviposits in an aphid nymph the wasp egg will hatch and develop within the living aphid over the course of eight to nine days, until the wasp larva kills the aphid and pupates (Digilio et al. 2000; Oliver et al. 2005). While *Ap. ervi* will readily oviposit in aphids infected with *H. defensa* (Oliver et al. 2012), most wasps fail to complete development in *H. defensa*-infected hosts, and the aphid survives. *H. defensa* also rescues the reproductive capacity of parasitized aphids (Oliver et al. 2008), which are normally castrated by wasp venom (Digilio et al. 2000). While the specific mechanisms driving wasp

mortality and fecundity rescue are not known, it is clear that *H. defensa* cannot provide this defensive effect on its own: an associated temperate bacteriophage, called APSE (*Acyrthosiphon pisum S*econdary Endosymbiont), is required for protection: phage-free *H. defensa* infections fail to provide any anti-wasp protection to their aphid hosts (Oliver et al. 2009). APSE also influences within-host *H. defensa* abundance, and in the absence of APSE *H. defensa* populations multiply to an extent deleterious to the fecundity of their aphid hosts (Weldon et al. 2013).

APSE infection in *H. defensa* likely originated via lateral transfer from the widespread insect endosymbiont *Arsenophonus*, although the effects of APSE infection, if any, on *Arsenophonus* or its insect hosts remain unknown (Duron 2014). APSEs have not been found in *Arsenophonus* relatives that persist as obligate symbionts, nor in the *H. defensa* strains infecting the aphids in the New World *Uroleucon* radiation: these *Hamiltonella* are universally present in their host aphids, and have been hypothesized to be obligate rather than facultative symbionts (Duron 2014; Degnan and Moran 2008b; Wernegreen 2002). When present in *H. defensa,* APSEs are highly conserved in terms of both synteny and nucleotide identity, with the exception of a virulence cassette region (VCR) of variable length, which encodes homologs of eukaryotic toxins putatively underlying the defensive symbiosis. Seven *H. defensa*-infecting APSE strains (called simply APSE1-7), isolated from a variety of aphid species and some allied hemipterans, were characterized on the basis of host identity, phylogeny, and VCR content (Degnan and Moran 2008a). The known APSE VCRs encode one of three different characteristic eukaryotic toxins: cytolethal-distending toxin subunit B (CdtB; APSEs 2 in North American *A. pisum*, 6 in *Chaitophorus* sp & 7 from *B. tabaci* biovar B), homologs of which in pathogenic bacteria disrupt actively dividing eukaryotic cells (Ohara et al. 2004), a Shiga-like toxin (Stx; APSE 1 from European *Ac. pisum*, 4 from *Ap. craccivora* & 5 from *Uroleucon rudbeckiae*), and a putative toxin containing a YD repeat motif (characterized by a tyrosyl-

aspartate dipeptide and associated with carbohydrate binding) (Ydp; APSE3 from North American *A. pisum*) (Moran et al. 2005; Degnan and Moran 2008a; van der Wilk et al. 1999).

Protective phenotypes associated with APSEs have only been investigated for a limited number of host species and strains, but levels of protection conferred differ between APSE variants in *Acyrthosiphon pisum*: *H. defensa* strains harboring APSE1&3 confer 80-90% resistance while those with APSE2 confer only 30-40% resistance - however, the effects of viral and bacterial genotypes generally cannot be separated (Oliver et al. 2005; Oliver et al. 2009; McLean and Godfray 2015). Our inability to culture and manipulate *H. defensa* and APSEs limits the tools available for functional studies, so whether phage toxins, acting alone or in concert with *H. defensa* or aphid-encoded factors, can actually cause harm or mortality to wasps is not yet known. The homologs of *cdtB*, for example, are integrated into a holotoxin in other hosts (such as *E. coli* and *Campylobacter* spp.) comprised of three subunits, cdtA, cdtB and cdtC (Ohara et al. 2004); the cdtA and cdtC subunits bind to the target cell via an unknown receptor and allow the B subunit to enter the cell (Asakura et al. 2007). The APSE strains that encode *cdtB*-homolog do not have any genes homologous to those coding for the A and C subunits, suggesting that APSE cdtB makes use of some other delivery system (Degnan and Moran 2008a). The mechanism of toxin delivery also remains unclear - APSE toxin delivery may be carried out through *H. defensa*'s T3SS, but lysis of *H. defensa* has been additionally proposed as a requirement for toxin release into the aphid hemocoel, and there is evidence of a decrease in *H. defensa* abundance in response to parasitism (Moran et al. 2005; Martinez et al. 2014b).

In addition to differences in protective phenotype, the fidelity with which the APSE-*H. defensa* combination is transmitted from mother to offspring also seems to vary by strain. While transmission of both *H. defensa* and APSE may sometimes approach 100% in pea aphids (Oliver et al. 2010), in some instances mothers infected with phage-harboring *H. defensa* populations will start to produce daughters with phage-free *H. defensa* populations (Oliver et al.

2009). In *A. pisum* lines held long-term in the lab, such losses have been observed for aphids and *H. defensa* harboring APSE3, but never in those with APSE2 (Weldon et al. 2013); the fidelity of other APSE strains is unknown.

The range of mutualistic effects attributable to APSEs will be at least in part a function of their diversity, yet this is poorly characterized. Previous MLST-based sampling of North American APSEs in *A. pisum* has been limited to a handful of samples from New York and Utah (Degnan and Moran 2008a); the APSE *P3* gene has also been included in *H. defensa* strain-typing from a broad North American survey (Russell et al. 2013), but no further attempts to identify *H. defensa*-infecting APSE strains within *A. pisum* have been made. Historical contingencies, natural enemy identity, and transmission opportunities relating to environmental factors may all have played some role in the development and maintenance of current APSE-*H. defensa*-aphid associations, but before we can adequately address the ecological and evolutionary questions posed by a heritable-symbiont-associated bacteriophage, we need to know what those current associations are.

3.3 Methods

3.3.1 Aphid sample collection. *Acyrthosiphon pisum* are cyclical parthenogens in temperate regions: they undergo asexual reproduction through the summer months, and only produce sexual morphs as the photoperiod shortens in fall (Van Emden and Harrington 2007). *A. pisum* is sorted into biotypes on the basis of host plant, and phylogenies of the aphid obligate symbiont *Buchnera* for the most part support the phylogenetic distinctness of these biotypes, with a notable exception: aphids found on *Medicago sativa* and *M. lupulina* (the alfalfa host races) do not form a coherent matriline (Peccoud et al. 2009); *M. sativa* is the host of the vast majority of aphids in our new survey, with exceptions marked in **Table S3.1 ColD**. Individual aphids were collected at least 30 feet apart to limit resampling from clonal lineages. Aphids were either collected in 95% ethanol and DNA extracted (as below), or field-collected nymphs were reared to adulthood in the lab, with processing of first generation offspring for subsequent diagnostics;

all aphid clones used in this study are listed in **Table S3.1**.

3.3.2 APSE and *H. defensa* diagnostics and multi-locus characterization. DNA was obtained from aphid samples via whole-aphid E.Z.N.A Tissue DNA Kit extraction (Omega Bio-Tek). All diagnostic PCRs described below were carried out in 10uL reactions with Lucigen EconoTaq PLUS GREEN 2X Master Mix (Middleton, WI). We identified lines with *H. defensa* using either denaturing gradient gel electrophoresis (DGGE) with universal 16S primers (Russell et al. 2013), *H. defensa*-specific diagnostic PCR using the MLST primers and PCR protocols published in (Degnan and Moran 2008b), or with qPCR using *H. defensa-dnaK* primers (primers and conditions published in Weldon et al. 2013); qPCR was used for all single-aphid ethanol-collected samples and as a supplement where agarose results were unclear. DGGE diagnostics for some populations were previously published in (Russell et al. 2013). Aphids with *H. defensa* were then tested for APSE phage using the primers bAPSE3-13530F 5'-CAGGATACGGGTTCAATTCC-3' and bAPSE3-14275R 5'-GTATTGCGCGGTTTAACGAC-3' cycled at 94^{0}C for 4 min; cycled 25x at 94^{0}C for 1 min, 50^{0}C for 1 min, 72^{0}C for 2 min, then 10 min 72^{0}C; and finally held at 4C indefinitely; or with *P2* qPCR primers (Weldon et al. 2013). Phage false positives in the lab have previously been reported (Degnan and Moran 2008b), and qPCR's sensitivity renders it susceptible to overestimation; we therefore required that phage gene numbers be equal to or greater than *H. defensa dnaK* in concentration for a positive call. Existing primers and PCR protocols were used to amplify five APSE structural genes: *P3* (lysis), *P35* (host cell entry), *P41* (excision/integration), *P45* (DNA synthesis), and *P51* (regulation), and four *H. defensa* genes, *accD, hrpA, murE,* and *recJ* for MLST (Degnan and Moran 2008b). The structural genes were selected for their even distribution across the APSE genome and have a variety of putative functions. *H. defensa* MLST genes are similarly spaced throughout this symbiont's genome. These genes have been used in previous surveys of *H. defensa* diversity (ex: Henry et al. 2013) and perform various housekeeping functions. The pea aphid matrilineal phylogeny was inferred from the amplification of intergenic regions in the obligate symbiont

Buchnera aphidicola, which is strictly maternally inherited, structurally stable, and incapable of recombinatorial repair (van Ham et al. 2003). The regions spanned *groEL-efp* and *cof-metE,* and primers and methods were as per (Peccoud et al. 2009). Amplification was confirmed with agarose gel electrophoresis. Amplicons were purified with an E.Z.N.A CyclePure Kit (Omega Bio-Tek) and sequenced in both directions through Eurofins Operon Sequencing (Pond and Frost 2005). Additionally, the full VCR for APSE8 was sequenced using the primers and conditions in **Table S3.2**.

3.3.3 Variable Cassette Region (VCR) amplification and identification. VCR toxin genes were identified with diagnostic PCR: *Ydp* was amplified with either the pair bAPSE3-F6082 5'-GTCTGAAACCTCTGCCCAAG-3' and bAPSE3-R6843 5'- CCGGTGACGCTAATGGAA-3' or bAPSE3-F6744 5'- GTCGGGTATTAACCCAAGCA-3' and bAPSE3-R7523 5'-GGGAAGCCAATAGACCGTTT-3'; *stx* was amplified using either APSE1_P7P9F 5'-CCGGCGATCATGGTTTCGCCT-3' and APSE1_P7P9R 5'-ACCTCCGCCTAAAACAAGCACT-3' or APSE1_P8P9F 5'-AAAGCACTGATACTTGGAACAAA-3' and APSE1_P8P9R 5'-CCAATCTGGAGGCTCAAACCA-3', using the same cycle described above for APSE detection; *cdtB* was amplified using primers and conditions described in (Martinez et al. 2014b). To distinguish between the *cdtB1* allele published in (Degnan and Moran 2008a) and the *cdtB2* allele published in (Martinez et al. 2014b), we used either Sanger sequencing of the *cdtB* product or subjected the product to TscAI endonuclease degradation, following the FastDigest protocol in a 10uL reaction as per standard specifications from Thermo Scientific (Marietta, OH). Due to concerns of potential APSE DNA contamination, all samples underwent all PCR diagnostics (i.e., samples were not excluded from further testing after a positive hit, and multi-hits then underwent discriminant qPCR screening as described below); in some retests samples were initially pooled in sets of 4 to 12 due to the size of the Wisconsin field samples; subsequent individual screening indicated no loss of sensitivity due to pooling. In the instance of a double toxin hit (<10% of phage-positive samples) qPCR crossing point was used to call

toxin identity; *cdtB* and *YD* primers for qPCR are from (Martinez et al. 2014b), and the *stx* primers were stxF5'-GCGCTAGTGCTTGTTTTAGGCGG-3' and shigatoxr 5'-TGACGCGCTGCCCTTACTGT-3'. Generational bottlenecks and the absence of multiple phage strains observed in long-held lab colonies makes it relatively unlikely that most double hits were the result of genuinely coinfecting phage strains, but our methodology renders this difficult to say with confidence; for the purposes of this study any phage strain not clearly identifiable after discriminant qPCR is therefore marked "unclear."

3.3.4 Sequence editing and phylogenetic analyses. Each pair of sequences was assembled to the closest reference published in NCBI using Geneious 6.1.8's Map to Reference function (Kearse et al. 2012). Ambiguities were resolved by default to the nearest haplotype where possible (Peccoud et al. 2009); where this was not justified by the chromatogram the sequence was excluded from the final alignment. Initial alignments were performed with ClustalW using IUB and then trimmed; published references included in the sequence were used to determine coding frame, and any sequences with early stop codons were excluded at this point (Larkin et al. 2007). Differential Akaike information criteria (δAIC) was calculated with jModelTest 2 prior to tree construction; best fit models are reported in **Table S3.3**, along with alignment lengths, AT%, and dN/dS ratios (Darriba et al. 2012; Guindon and Gascuel 2003). PhyML was used to construct Maximum likelihood trees. Trees for the concatenated 5-gene APSE set (**Fig 3.1)**, for the concatenated 4-gene *H. defensa* set (**Fig 3.2**), and the concatenated intragenic region *B. aphidicola* tree (**Fig 3.3**) were run with 1000 bootstrap iterations. Supplemental trees for individual gene alignments were built with 100 iterations (Kearse et al. 2012). Haplotypes for *H. defensa* were marked on the basis of 100% sequence identity, and unique sequences not taken from other studies were uploaded (waiting on NCBI numbers) and identified using the names of host aphid clones in **Table S3.1**. The subset of unique concatenated phage MLST haplotypes was realigned and underwent DualBrothers recombination analysis**.**

3.3.5 Protective phenotype of *H. defensa* strain with *cdtB2*-encoding bacteriophage.

Aphid lines used for parasitism studies were reared on *Vicia faba* for a 16L:8D cycle at 20 ± 1C to mimic summer conditions. The wasps used for the parasitism studies include the descendants of *Aphidius ervi* collected from aphid mummies in fields in Wisconsin and North Dakota, supplemented by commercially-supplied mummies (sourced with Arbico Organics) held in a single, mixed laboratory colony. Wasps were reared continuously on the same susceptible line used in the parasitism experiment, and adults were provided with constant access to honey and water. The aphid clones used were the highly susceptible AS3-AB, produced by curing line AS3 of its *H. defensa* infection via antibiotics, and the highly resistant native host of the *cdtB2*-encoding *H. defensa* strain ZA17, produced in the same manner (Martinez et al. 2014a). Aphids were reared for several generations and regularly screened via PCR, as described above, to ensure that they were free of *H. defensa* infection. *H. defensa*-strain ZA17 was then transferred into the antibiotically-cured AS3-AB host via hemolymph-to-hemolymph microinjection. Descendent aphids were reared for several generations, and screened periodically to ensure that they maintained the infection, before they were utilized in any parasitism assay. Parasitism assays were carried out as in (Martinez et al. 2014a), and data from that paper on the effects of ZA17 in its native host is partially reproduced for comparative purposes.

3.3.6 Statistics. Statistical comparisons for APSE strain associations with 1) *H. defensa* haplotype, 2) aphid matriline, and 3) collection time and year were done using a Fisher's exact test. For *H. defensa,* sequences from strains in 74 aphids were binned into one of seven haplotypes on the basis of unique concatenated MLST sequence identity (**Table S3.1 ColC**). For the *B. aphidicola* matriline determination, all of our aphids fell clearly into one of two clearly defined sets within the tree partially reproduced from (Peccoud et al. 2009) (**Fig 3.3**); we therefore binned even those aphids with only one amplified intragenic region into one of those two matrilines, marked in (**Table S3.1 ColB**), for a total set of 64. The *H. defensa* and *B.*

aphidicola binned clones overlapped by 44 aphid hosts. The Wisconsin set – the only locality for which we have more than two years of data - consists of all 823 aphids collected in Dane County, WI from 2011 to 2014 (**Table S3.1 ColE,F**). Within-set prevalence of haplotypes, matrilines and phage strains were used to calculate ideal random-assorted expected combinations. Expected, real values, and P-values, and Bonferroni-corrected p-value cut-offs for each set of comparisons are listed in **Supplemental Table S3.4**.

3.4 Results

3.4.1 New phage strains and evidence for phage lateral transfer. While only APSE2 and APSE3 had been reported from *H. defensa* of North American pea aphids, our ML tree of concatenated phage structural genes identified four distinct APSE strains across our sampled aphids. APSE1 was among the two new-to-North America APSE variants (an *stx*-encoding strain previously reported in European *A. pisum*). We also identified a novel *cdtB2*-encoding APSE strain, referred to below as APSE8 (**Fig 3.1**). While our phylogenetic analysis of structural genes clearly indicates that APSE8 forms a distinct clade, the VCR (the region from the terminus of *P5* to the terminus of *holin F*) of this strain is overall 98% identical to that of APSE2, with the majority of variation found in non-coding regions (waiting on NCBI numbers). In general, primary toxin identity (i.e. *Ydp, stx, cdtB1,* and *cdtB2*) acts as a proxy for a coherent and functionally-identical strain designation within our North American pea aphid sample set. The only exception to this rule in our set of 77 fully-sequenced *A. pisum*-sourced APSEs was the strain found in aphid clone YA4, which has unique haplotypes at all structural loci but P35 (**Table S1**), yet encodes *cdtB1*. The YA4 APSE strain also provides the only probable example of intraphage recombination in the new set, with a breakpoint between *P45* and *P51* in DualBrothers. We therefore use primary toxin identity as a proxy for APSE strain to mark our *H. defensa* and *B. aphidicola* phylogenetic trees (**Fig 3.2 and 3.3)**, as well as in our field surveys below.

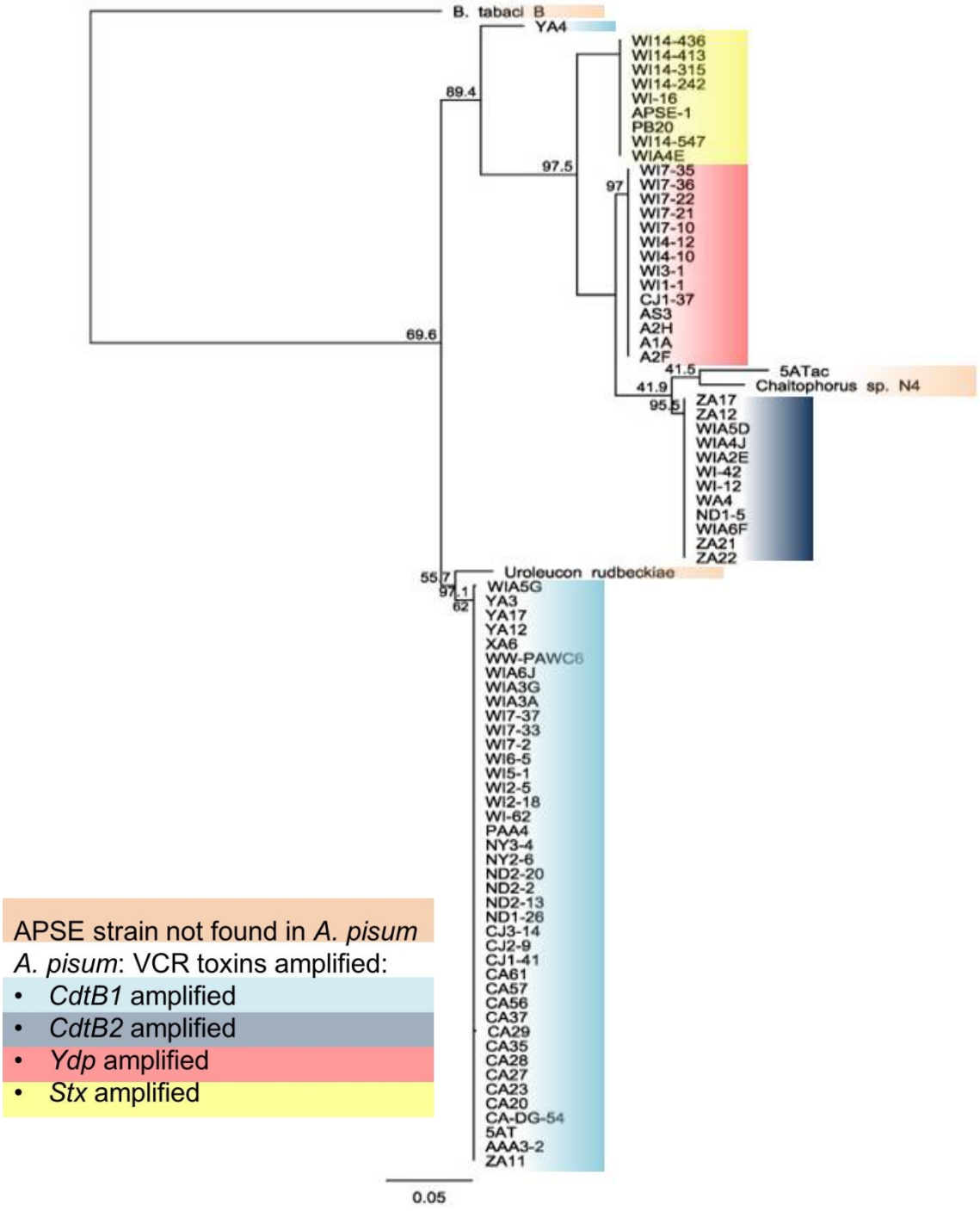

Figure 3.1. Concatenated GTR+I+G ML PhyML tree of phage structural genes *P3, P35, P41, P45,* and *p51.* APSE-7 from *B. tabaci* biovar B is use to root the tree. Branch bootstrap support ≥40 is reported (tree run with 1,000 bootstrap iterations). The bar indicates substitutions/site.

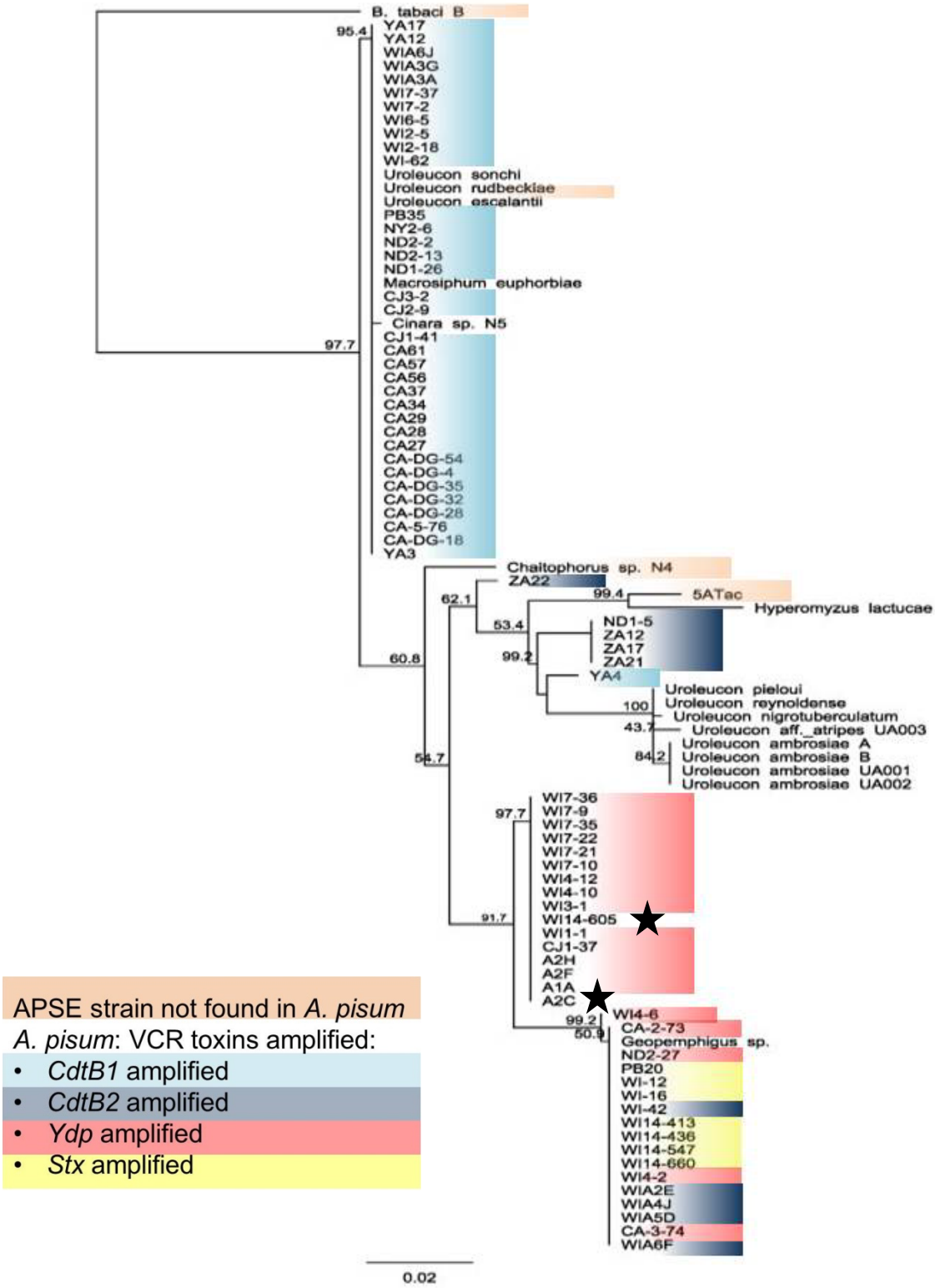

Figure 3.2. TVM +G PhyML tree of the concatenated sequences of *accD, hrpA, accD, gyrB* and *recJ* from *Hamiltonella defensa.* Uncolored species/strain names represent *H. defensa* that do not harbor APSE: the two APSE-free *A. pisum*-infecting *H. defensa* collected from the field are starred. The bar indicates substitutions/site.

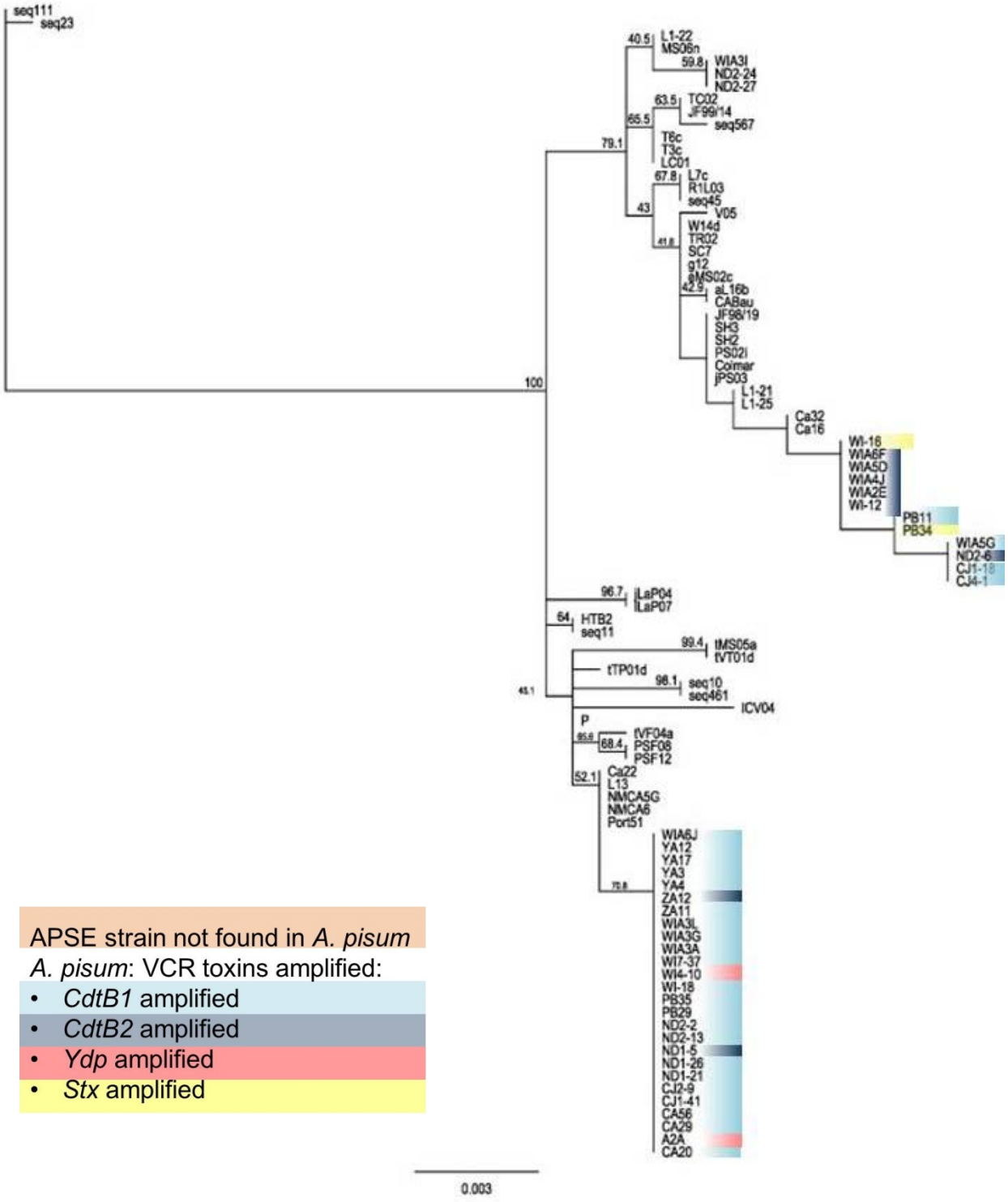

Figure 3.3. HKY PhyML tree of the concatenated sequences of two intragenic regions from *Buchnera aphidicola.* Uncolored species/strain names represent aphids from other studies, where infection status is unknown. The bar indicates substitutions/site.

There is evidence of APSE lateral exchange between *H. defensa* haplotypes (**Figure 3.2)**, though some strains and haplotypes do group together**.** APSE2 is positively associated (p=0.0032) with *H. defensa* haplotype 1, while the other strains are entirely absent from that haplotype. APSE3 has a weaker positive association with haplotype 3 (p=0.0265); neither interaction is significant after the application of Bonferroni correction to the set (p-value cutoff=0.0018, **Table S3.4A**). Associations for APSE8 and APSE1 are less obvious due to their relative rarity. Both of the phage-free *H. defensa* are haplotype 3, and the unusual YA4 APSE has an equally unusual *H. defensa* haplotype – it is the single representative of a haplotype not closely related to others in the pea aphid set (**Table S3.1, Fig 3.2)**. There is no significant association between aphid matriline and phage strain (**Figure 3.3, Table S3.4B**); however, in the overlapping set for which we have both *H. defensa* haplotype and matriline, while there are no statistically significant relationships, every representative of matriline 2 is infected with haplotype 2, which is entirely absent from matriline 1 **(Table S3.4C**). Finally, while our clover sample is limited, there was no evidence of further phage diversity in it (**Table S3.1, Fig 3.1**).

3.4.2 Toxin allele association with defensive phenotype. When *cdtB2* was first identified, it was reported that curing a *cdtB*2-encoding APSE-*H. defensa* infection from its native host resulted in no change in parasitism resistance, leading to speculation that its encoding phage (identified here as APSE8) might be non-protective (Martinez et al. 2014b). However, it was afterwards determined that the aphid background in which the testing had been done had a high aphid-genotypic resistance to parasitism (Martinez et al. 2014a), which may have masked effects provided by *cdtB2*-carrying *H. defensa.* The same strain of *H. defensa* with *cdtB2* bearing APSE8 (ZA17) used in those studies was artificially introduced into an aphid lineage with little to no aphid-genotypic resistance to *Ap. ervi* (AS3-AB) for this study. Our parasitism assays found that this *H. defense*-phage combination significantly increased aphid survival and reduced rates of successful parasitism, to degrees comparable to those of *H. defensa* with *cdtB1*-encoding APSE2 (Oliver et al. 2009) (**Figure 3.1)**. In our assays, the focal *H. defensa*-

APSE8 strain failed to reduce wasp mummification in its native host aphid (clone ZA17), while leading to a significant decrease in aphid survival post-parasitism.

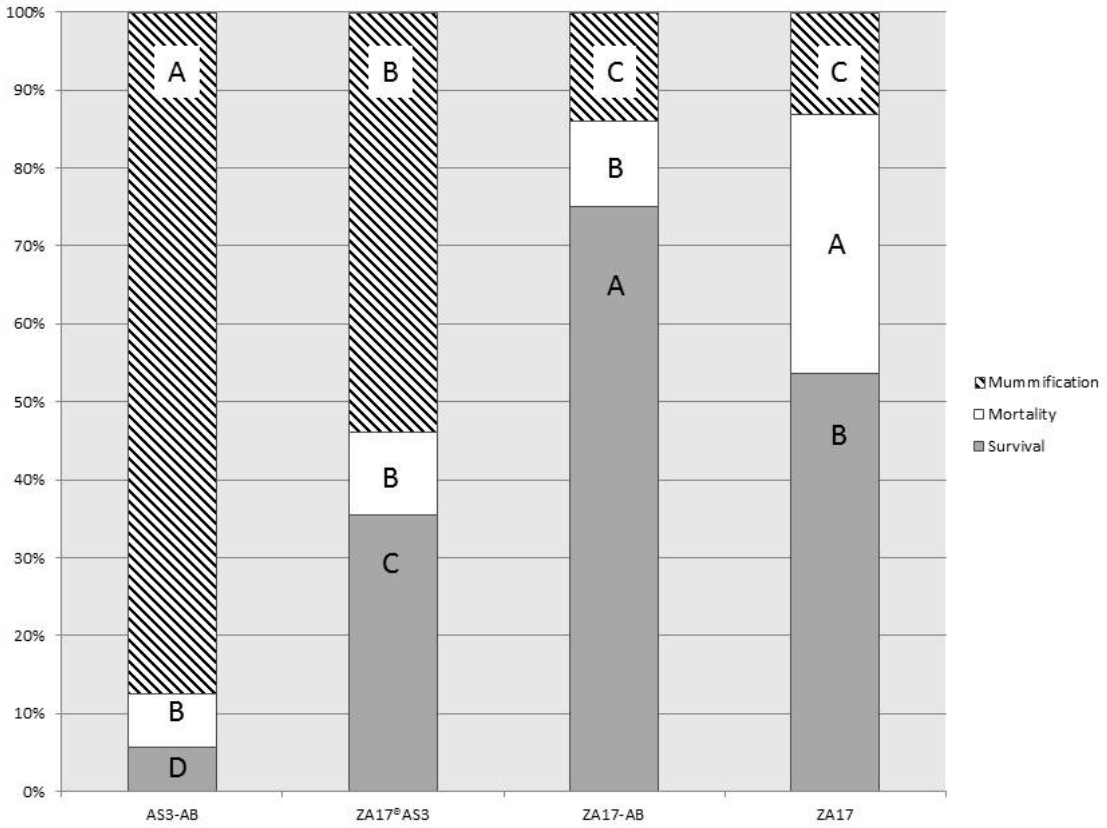

Figure 3.4 Post-parasitism survival, mummification and non-mummification mortality for *H. defensa*-free aphid clones AS3-AB and ZA17-AB (the antibiotically-cured native host of *H. defensa* strain ZA17, see (Martinez et al. 2014b)), and for aphid clone AS3-AB with an artificial ZA17 *H. defensa* infection (ZA17->AS3). *H. defensa* ZA17 drastically increases survival rates in AS3 (groups assigned on basis of arcsin transformed data's Tukey-Kramer HSD comparison, $p<0.0001$ in all instances). Data for ZA17-AB and AS3-AB reproduced from (Martinez et al. 2014a) for comparative purposes.

3.4.3 Prevalence and diversity *H. defensa* and APSE strains in pea aphid populations.

Forty-eight percent (541) of the 1131 aphid clones examined were infected with *H. defensa*; within-population infection frequencies ranged from fifteen to sixty percent. All but one of our new field-collected *H. defensa* clones were infected with APSE, despite the previously-reported spontaneous development of phage-free *H. defensa*-harboring aphid lines in lab populations

(Weldon et al. 2013; Oliver et al. 2009). Diagnostic PCR indicated that all phage-positive samples encoded one of the four known toxins – ambiguous diagnostics were the result of multiple positive toxin amplifications indistinguishable by downstream qPCR, rather than no amplification of a known toxin (Degnan and Moran 2008a; Martinez et al. 2014b).

The most common and geographically widespread phage strain, infecting 40% of sampled *H. defensa,* was APSE2, which encodes *cdtB1* and thus far appears to confer only moderate levels of anti-parasitoid protection against *A. ervi*. APSE8s, which encode the similar *cdtB2* allele while conferring moderate protection in limited assays, were found in 18% of *H. defensa* samples. Thus, the APSE strains currently associated with weaker protection were found in nearly 60% of *H. defensa*. *Ydp*-encoding APSE3 strains, which confer high levels of protection to pea aphids against *A. ervi*, were moderately common (30%) but restricted to mid-western and western populations. APSE3's overall prevalence is likely elevated as an artifact of our in-depth sampling in Wisconsin. Finally, the APSE1 strain was rarest, and found only in Wisconsin and Pennsylvania. In limited assays to date, this phage is associated with high protective levels, much like APSE-3. While all APSE1 strains were found in Wisconsin this may be another side effect of our in-depth sampling in that region. We, thus, find it plausible that this phage may be more geographically dispersed across North America, albeit at low frequency. Within the Wisconsin set, *cdtB1* is sizably, and significantly, overrepresented in collections taken in August ($p=0.0027$); *stx* is underrepresented in 2013 ($p=0.0023$), but recovers to expected values by 2014 (all toxin vs. time comparisons within the Wisconsin set are presented in tables **S3.4F** and **S3.4G**).

Table 3.1. Results of toxin diagnostics performed on pea aphids, *Acyrthosiphon pisum*, clones collected across the US. Results marked unclear when qPCR diagnostics failed to distinguish strain identity.

Collection Location (Table S1 code)	**Collection date**	**Total aphids**	***H. defensa* + aphids (*H+* / total aphids)**	***cdtB1* (*cdtB1*/*H+*)**	***cdtB2* (*cdtB2*/*H+*)**	***YD* (*YD*/*H+*)**	***stx* (*stx*/*H+*)**	**Phage free (*Phage-* / *H+*)**	**unclear (*?*/*H+*)**
Solano, CA (CARUSS)	Aug-13	79	25 (0.32)	21 (0.84)	0 (0.00)	4 (0.16)	0 (0.00)	0 (0.00)	0 (0.00)
Yolo, CA (CAUCD)	Jun-13	45	22 (0.49)	21 (0.95)	0 (0.00)	1 (0.05)	0 (0.00)	0 (0.00)	0 (0.00)
Cache, UT (CJ)	Aug-11	49	25 (0.51)	24 (0.96)	0 (0.00)	1 (0.04)	0 (0.00)	0 (0.00)	0 (0.00)
Cass, ND (ND)	Sep-12	61	19 (0.31)	13 (0.68)	4 (0.21)	2 (0.11)	0 (0.00)	0 (0.00)	0 (0.00)
Tompkins, NY (NY)	Jul-11	10	6 (0.60)	6 (1.00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)
Lansdale, PA (PBALF)	May-11	21	12 (0.57)	9 (0.75)	0 (0.00)	0 (0.00)	2 (0.17)	0 (0.00)	1 (0.08)
Cache, UT (UTAS)	Aug-13	30	10 (0.33)	2 (0.20)	0 (0.00)	7 (0.70)	0 (0.00)	0 (0.00)	1 (0.10)
Cache, UT (UTKMRT)	Jun-13	13	2 (0.15)	2 (1.00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)
Dane, WI (WI11)	Sep-11	38	19 (0.50)	7 (0.37)	10 (0.53)	0 (0.00)	1 (0.05)	0 (0.00)	1 (0.05)
Dane, WI (WIA)	Aug-12	54	20 (0.37)	11 (0.55)	6 (0.30)	1 (0.05)	2 (0.10)	0 (0.00)	0 (0.00)
Dane, WI (wiaaug2013)	Aug-13	76	44 (0.58)	30 (0.68)	0 (0.00)	12 (0.27)	2 (0.05)	0 (0.00)	0 (0.00)
Dane, WI (wioct13)	Oct-13	232	125 (0.54)	25 (0.20)	32 (0.26)	58 (0.46)	1 (0.01)	0 (0.00)	9 (0.07)
Dane, WI (wiaug14)	May-14	252	129 (0.51)	19 (0.15)	29 (0.22)	62 (0.48)	17 (0.13)	0 (0.00)	2 (0.02)
Dane, WI (wioct14)	Oct-14	171	83 (0.49)	28 (0.34)	16 (0.19)	17 (0.20)	20 (0.24)	1 (0.01)	1 (0.01)
	Sums:	1131	541 (0.48)	218 (0.40)	97 (0.18)	165 (0.30)	45 (0.08)	1 (0.00)	15 (0.01)

3.5 Discussion

The pea aphid has emerged as an important model for the study of defensive symbiosis, including *H. defensa*-mediated protection against parasitoids (Oliver et al. 2014). Understanding the diversity, prevalence, and dynamics of defensive symbionts can provide important insights into the range and strength of microbially-mediated protective services available to natural populations. While *H. defensa* is common and widespread in natural pea aphid populations (Ferrari et al. 2012; Russell et al. 2013) much less is known about its strain diversity, or the diversity, prevalence and distributions of APSE bacteriophages that infect *H. defensa.* APSEs are required to produce the protective phenotype, and distinct phage strains correlate with varying levels of protection, though this is confounded by *H. defensa* strain (Oliver et al. 2005; McLean and Godfray 2015; Oliver et al. 2009). Prior to this study, phylogenetic relatedness of *A. pisum*-associated APSEs, as well as characterization of the VCRs which contain the toxins hypothesized to harm wasps had only been carried out for the *H. defensa* of five pea aphid clones: 39.5, 5AT, A1A, A2F, and A2H (Degnan and Moran 2008a; van der Wilk et al. 1999). These studies identified three APSE strains (APSE1-3), but only two (APSE2 and 3) were detected in North America. The addition of 541 toxin-diagnostic samples, including 72 that were sequenced at five additional loci, revealed the presence of the third strain (APSE1) in the United States, strongly suggesting that these three phage, combined with the newly identified APSE8 strain, encompass most of the diversity of APSEs within alfalfa-feeding pea aphids from the United States. Overall, bacteriophage infecting host-associated bacteria may be expected to be more limited in terms of diversity due to generational bottlenecks and limited associations with the broader microbial arena. In this sample set, however, diversity may be further limited due to a recent host-insect genetic bottleneck: pea aphids are indigenous to the Palearctic, and only colonized North America in the 19th century (Brisson and Stern 2006).

Despite limited diversity overall, lateral transfer of *H. defensa* and associated APSEs may introduce novel genotypes and functions into alfalfa *A. pisum*. One candidate for a recent

lateral transfer event is APSE strain YA4, which has an otherwise-unreported haplotype at all structural genes other than *P35* (**Fig 3.1**). The fact that YA4-phage infects a strain of *H. defensa* distinct from others in *A. pisum* is also consistent with recent horizontal transmission of both parties from a currently-unidentified *H. defensa* host. Alternately, other rare strains of *H. defensa* and APSE not captured by our combination of diagnostics and subset sequencing may persist in *A. pisum*: while toxin identity acts as a perfect proxy for strain identity for the vast majority of our samples, YA4's example suggests that a full structural gene panel should be sequenced for any strain of APSE used in lab assays.

The new strain APSE8, identified in this study based on ML phylogeny of concatenated structural loci, which place it closely to APSE3, is nearly identical across its VCR to that of strain APSE2, indicating that the entire VCR likely moved laterally between phage strains. Previous assays show that APSE2-*H. defensa* strains in susceptible aphid genotypes are associated with a 30-40% elevation in post-parasitism survival (Oliver et al. 2005). In this study, our parasitism assays show that APSE8-*H. defensa* confers similar levels of protection to APSE2 (**Fig 3.4**). That similar protective phenotypes occur in strains sharing VCRs but having distinct phage and *H. defensa* genotypes provides more evidence for the hypothesis that the toxin-encoding VCRs are key contributors to the parasitism-resistance phenotype (Oliver et al. 2009). However, while the protective phenotype may be similar between APSE2 and APSE8, other biological attributes, including those influencing APSE-*H. defensa* interactions, may differ. For example, our sequenced structural loci include those involved in the phage lysogenic cycle, and in this respect APSE8 is more closely related to APSE3 than to APSE2 (**Fig 3.1**). Given that laboratory observations reveal that APSE2-*H. defensa* exhibits greater integration rates in the bacterial chromosome and higher vertical transmission fidelity than APSE3 (Weldon et al. 2013), we may find that APSE8 has a protective phenotype similar to APSE2 but interacts with *H. defensa* more like an APSE3. *In vitro* and *in vivo* studies are underway to investigate these patterns and processes.

In our toxin-screen survey of *A. pisum* populations, we found that a moderately-protective strain, APSE2, was most prevalent (**Table 3.1**). If APSE3-bearing *H. defensa* are consistently the most effective defenders—a possibility called into question by recent discoveries on context-dependency of *H. defensa* driven defense—we would expect APSE3s to represent the most common APSE strains. However, we found that only 30% of *H. defensa* carried an APSE3, and given the greater sampling intensity in regions where APSE3 was present, we likely over-estimated their abundance. One potential explanation for the relative paucity of APSE3-*H. defensa* is APSE3 loss, which leads to the breakdown of the defensive symbiosis and could potentially contribute to the rapid removal of phage-free *H. defensa*-infected aphids via purifying selection. Bolstering this hypothesis, despite the frequency of APSE3 loss in lab (Weldon et al. 2013), only one unambiguous case of APSE-free *H. defensa* was detected, and this instance occurred in an *H. defensa* haplotype associated with APSE3 – though this may be due to rarity of loss under field conditions rather than intensity of purifying selection post-phage-loss. We do note that in some WI populations, APSE3s were unusually prevalent (**Table 3.1, Table S3.1)**, and historical contingency or modern parasitism rates may counterbalance the costs of phage loss. APSE8's relative rarity (18%) may be a consequence of protection similar to APSE2's and fidelity in line with APSE3's. No information is currently available about integration rates and stability in the also highly-protective (McLean and Godfray 2015), and extremely rare (8%), APSE1.

Annual sexual reproduction in temperate zones (Moran and Dunbar 2006), "dirty needle" parasitoid transfer (Gehrer and Vorburger 2012), and ingestion (Darby and Douglas 2003) all provide potential mechanisms of horizontal transmission of *H. defensa* by which multiple *H. defensa* strains, associated with multiple APSEs, might share the same host. Phages associated with heritable symbionts may be less prone to lateral exchange than their free-living relatives. APSEs, like a large majority of prokaryotic viruses, are dsDNA tailed bacteriophages in the order Caudovirales; generally evolution in this group is described by the Modular Theory,

where non-homologous recombination of whole functional modules renders these phage effective carriers of secondary bacterial metabolites (Krupovic et al. 2011; Botstein 1980); in the case of APSE, however, the extreme host-limited lifestyle of symbiont-associated phages may isolate them from common phage gene pools, and, based on *H. defensa*-APSE phylogenetic congruence in our experimental set and the lack of both interphage recombination, the whole APSE phage, rather than chunks thereof, acts as the coherent module exchanged between *H. defensa* strains, though this too is relatively rare. In support of that, we found little evidence of a strong correlation between *H. defensa* haplotype and APSE strain within our new set. Outside of alfalfa relationships may differ: in the global set, *H. defensa* strain in *A. pisum* is strongly associated with aphid host plant/biotype, and has been repeatedly reacquired with pea aphid entry into novel ecological niches (Henry et al. 2013); as APSE strain is considered a primary determinant of wasp-resistance phenotype strength (Oliver et al. 2005), but APSEs are not necessarily equally effective against all endoparasitoids (Lukasik et al. 2013), the differential parasitoid pressures on different host plants might drive detectable associations at broader scales. Within our heavily-sampled Dane, Wisconsin fields, we found no indication of temporal shifts in either APSE strain prevalence or total *H. defensa* infection rates (**Table 3.1**). However, these time scales are both crude and unevenly sampled: a recent study found strong temporal modification of *H. defensa* infection rates over the course of a field season related to both parasitoid prevalence and temperature changes (Smith et al. 2015), and a similarly-focused study might be necessary to find temporal frequency variation or modulation by external environmental factors in either *H. defensa* haplotype or APSE strain.

Despite the well-described importance of bacteriophages to bacterial pathogens, little is known about their influence on the many bacterial species that form beneficial symbioses with eukaryotes. APSE is the first known instance of a bacteria-insect mutualism requiring a viral partner, and its association with an already-developed model insect makes it an ideal target for development as an experimental system.

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3.7 References

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Table S3.1 Full list of all aphids used in chapter 3. Phage toxins marked "X" indicate multiple amplifications indistinguishable by qPCR.

Aphid clone	Matriline	*H. defensa* haplotype	Host plant	State	County	Collection date	*H. defensa*	Set name	Phage toxin
CA-1-72			alfalfa	CA	Solano	8/2013	1	CARUSS	cdtB1
CA-2-73		2	alfalfa	CA	Solano	8/2013	1	CARUSS	YD
CA-3-74		2	alfalfa	CA	Solano	8/2013	1	CARUSS	YD
CA-4-75			alfalfa	CA	Solano	8/2013	0	CARUSS	-
CA-5-76		1	alfalfa	CA	Solano	8/2013	1	CARUSS	cdtB1
CA-6-77			alfalfa	CA	Solano	8/2013	1	CARUSS	cdtB1
CA-7-78			alfalfa	CA	Solano	8/2013	1	CARUSS	cdtB1
CA-8-79			alfalfa	CA	Solano	8/2013	0	CARUSS	-
CA-dg-1			alfalfa	CA	Solano	8/2013	0	CARUSS	-
CA-dg-10			alfalfa	CA	Solano	8/2013	0	CARUSS	-
CA-dg-11			alfalfa	CA	Solano	8/2013	0	CARUSS	-

CA-dg-12			alfalfa	CA	Solano	8/2013	0	CARUSS	-
CA-dg-13			alfalfa	CA	Solano	8/2013	0	CARUSS	-
CA-dg-14			alfalfa	CA	Solano	8/2013	0	CARUSS	-
CA-dg-15			alfalfa	CA	Solano	8/2013	0	CARUSS	-
CA-dg-16			alfalfa	CA	Solano	8/2013	0	CARUSS	-
CA-dg-17			alfalfa	CA	Solano	8/2013	0	CARUSS	-
CA-dg-18		1	alfalfa	CA	Solano	8/2013	1	CARUSS	cdtB1
CA-dg-19			alfalfa	CA	Solano	8/2013	0	CARUSS	-
CA-dg-2			alfalfa	CA	Solano	8/2013	0	CARUSS	-
CA-dg-20			alfalfa	CA	Solano	8/2013	0	CARUSS	-
CA-dg-21			alfalfa	CA	Solano	8/2013	1	CARUSS	cdtB1
CA-dg-22			alfalfa	CA	Solano	8/2013	1	CARUSS	cdtB1
CA-dg-23			alfalfa	CA	Solano	8/2013	0	CARUSS	-
CA-dg-24			alfalfa	CA	Solano	8/2013	0	CARUSS	-
CA-dg-25			alfalfa	CA	Solano	8/2013	0	CARUSS	-
CA-dg-26			alfalfa	CA	Solano	8/2013	0	CARUSS	-
CA-dg-27			alfalfa	CA	Solano	8/2013	0	CARUSS	-
CA-dg-28	1	1	alfalfa	CA	Solano	8/2013	1	CARUSS	cdtB1
CA-dg-29			alfalfa	CA	Solano	8/2013	0	CARUSS	-
CA-dg-3			alfalfa	CA	Solano	8/2013	0	CARUSS	-
CA-dg-30			alfalfa	CA	Solano	8/2013	0	CARUSS	-
CA-dg-31			alfalfa	CA	Solano	8/2013	0	CARUSS	-
CA-dg-32		1	alfalfa	CA	Solano	8/2013	1	CARUSS	cdtB1
CA-dg-33			alfalfa	CA	Solano	8/2013	0	CARUSS	-
CA-dg-34			alfalfa	CA	Solano	8/2013	0	CARUSS	-
CA-dg-35		1	alfalfa	CA	Solano	8/2013	1	CARUSS	cdtB1
CA-dg-36			alfalfa	CA	Solano	8/2013	0	CARUSS	-
CA-dg-37			alfalfa	CA	Solano	8/2013	1	CARUSS	YD
CA-dg-38			alfalfa	CA	Solano	8/2013	0	CARUSS	-
CA-dg-39			alfalfa	CA	Solano	8/2013	0	CARUSS	-
CA-dg-4		1	alfalfa	CA	Solano	8/2013	1	CARUSS	cdtB1
CA-dg-40			alfalfa	CA	Solano	8/2013	0	CARUSS	-
CA-dg-41			alfalfa	CA	Solano	8/2013	0	CARUSS	-
CA-dg-42			alfalfa	CA	Solano	8/2013	1	CARUSS	cdtB1
CA-dg-43			alfalfa	CA	Solano	8/2013	0	CARUSS	-
CA-dg-44			alfalfa	CA	Solano	8/2013	0	CARUSS	-
CA-dg-45			alfalfa	CA	Solano	8/2013	0	CARUSS	-
CA-dg-46			alfalfa	CA	Solano	8/2013	0	CARUSS	-
CA-dg-47			alfalfa	CA	Solano	8/2013	0	CARUSS	-
CA-dg-48			alfalfa	CA	Solano	8/2013	1	CARUSS	cdtB1
CA-dg-49			alfalfa	CA	Solano	8/2013	1	CARUSS	cdtB1

CA-dg-5			alfalfa	CA	Solano	8/2013	0	CARUSS	-
CA-dg-50			alfalfa	CA	Solano	8/2013	1	CARUSS	cdtB1
CA-dg-51			alfalfa	CA	Solano	8/2013	1	CARUSS	cdtB1
CA-dg-52			alfalfa	CA	Solano	8/2013	0	CARUSS	-
CA-dg-53			alfalfa	CA	Solano	8/2013	0	CARUSS	-
CA-dg-54		1	alfalfa	CA	Solano	8/2013	1	CARUSS	cdtB1
CA-dg-55			alfalfa	CA	Solano	8/2013	0	CARUSS	-
CA-dg-56			alfalfa	CA	Solano	8/2013	0	CARUSS	-
CA-dg-57			alfalfa	CA	Solano	8/2013	0	CARUSS	-
CA-dg-58			alfalfa	CA	Solano	8/2013	0	CARUSS	-
CA-dg-59			alfalfa	CA	Solano	8/2013	0	CARUSS	-
CA-dg-6			alfalfa	CA	Solano	8/2013	0	CARUSS	-
CA-dg-60			alfalfa	CA	Solano	8/2013	0	CARUSS	-
CA-dg-61			alfalfa	CA	Solano	8/2013	1	CARUSS	YD
CA-dg-62			alfalfa	CA	Solano	8/2013	0	CARUSS	-
CA-dg-63			alfalfa	CA	Solano	8/2013	1	CARUSS	cdtB1
CA-dg-64			alfalfa	CA	Solano	8/2013	0	CARUSS	-
CA-dg-65			alfalfa	CA	Solano	8/2013	0	CARUSS	-
CA-dg-66			alfalfa	CA	Solano	8/2013	0	CARUSS	-
CA-dg-67			alfalfa	CA	Solano	8/2013	0	CARUSS	-
CA-dg-68			alfalfa	CA	Solano	8/2013	1	CARUSS	cdtB1
CA-dg-69			alfalfa	CA	Solano	8/2013	0	CARUSS	-
CA-dg-7			alfalfa	CA	Solano	8/2013	0	CARUSS	-
CA-dg-70			alfalfa	CA	Solano	8/2013	0	CARUSS	-
CA-dg-71			alfalfa	CA	Solano	8/2013	1	CARUSS	cdtB1
CA-dg-8			alfalfa	CA	Solano	8/2013	1	CARUSS	cdtB1
CA-dg-9			alfalfa	CA	Solano	8/2013	0	CARUSS	-
CA18			alfalfa	CA	Yolo	6/2013	1	CAUCD	cdtB1
CA19			alfalfa	CA	Yolo	6/2013	1	CAUCD	cdtB1
CA20	1		alfalfa	CA	Yolo	6/2013	1	CAUCD	cdtB1
CA21			alfalfa	CA	Yolo	6/2013	0	CAUCD	-
CA22			alfalfa	CA	Yolo	6/2013	1	CAUCD	cdtB1
CA23		1	alfalfa	CA	Yolo	6/2013	1	CAUCD	cdtB1
CA24			alfalfa	CA	Yolo	6/2013	1	CAUCD	cdtB1
CA25			alfalfa	CA	Yolo	6/2013	1	CAUCD	cdtB1
CA26			alfalfa	CA	Yolo	6/2013	1	CAUCD	cdtB1
CA27		1	alfalfa	CA	Yolo	6/2013	1	CAUCD	cdtB1
CA28		1	alfalfa	CA	Yolo	6/2013	1	CAUCD	cdtB1
CA29	1		alfalfa	CA	Yolo	6/2013	1	CAUCD	cdtB1
CA30			alfalfa	CA	Yolo	6/2013	0	CAUCD	-
CA31			alfalfa	CA	Yolo	6/2013	0	CAUCD	-

CA32			alfalfa	CA	Yolo	6/2013	0	CAUCD	-
CA33			alfalfa	CA	Yolo	6/2013	0	CAUCD	-
CA34		1	alfalfa	CA	Yolo	6/2013	1	CAUCD	cdtB1
CA35			alfalfa	CA	Yolo	6/2013	1	CAUCD	cdtB1
CA36			alfalfa	CA	Yolo	6/2013	1	CAUCD	cdtB1
CA37	1	1	alfalfa	CA	Yolo	6/2013	1	CAUCD	cdtB1
CA38			alfalfa	CA	Yolo	6/2013	1	CAUCD	cdtB1
CA39			alfalfa	CA	Yolo	6/2013	0	CAUCD	-
CA40			alfalfa	CA	Yolo	6/2013	0	CAUCD	-
CA41			alfalfa	CA	Yolo	6/2013	0	CAUCD	-
CA42			alfalfa	CA	Yolo	6/2013	0	CAUCD	-
CA43			alfalfa	CA	Yolo	6/2013	1	CAUCD	cdtB1
CA44			alfalfa	CA	Yolo	6/2013	0	CAUCD	-
CA45			alfalfa	CA	Yolo	6/2013	0	CAUCD	-
CA46			alfalfa	CA	Yolo	6/2013	0	CAUCD	-
CA47			alfalfa	CA	Yolo	6/2013	0	CAUCD	-
CA48			alfalfa	CA	Yolo	6/2013	0	CAUCD	-
CA49			alfalfa	CA	Yolo	6/2013	0	CAUCD	-
CA50			alfalfa	CA	Yolo	6/2013	0	CAUCD	-
CA51			alfalfa	CA	Yolo	6/2013	0	CAUCD	-
CA52			alfalfa	CA	Yolo	6/2013	1	CAUCD	YD
CA53			alfalfa	CA	Yolo	6/2013	0	CAUCD	-
CA54			alfalfa	CA	Yolo	6/2013	0	CAUCD	-
CA55			alfalfa	CA	Yolo	6/2013	0	CAUCD	-
CA56	1	1	alfalfa	CA	Yolo	6/2013	1	CAUCD	cdtB1
CA57		1	alfalfa	CA	Yolo	6/2013	1	CAUCD	cdtB1
CA58			alfalfa	CA	Yolo	6/2013	0	CAUCD	-
CA59			alfalfa	CA	Yolo	6/2013	0	CAUCD	-
CA60			alfalfa	CA	Yolo	6/2013	0	CAUCD	-
CA61		1	alfalfa	CA	Yolo	6/2013	1	CAUCD	cdtB1
CA62			alfalfa	CA	Yolo	6/2013	1	CAUCD	cdtB1
CJ1-1			alfalfa	UT	Cache	8/2011	1	CJ	cdtB1
CJ1-10			alfalfa	UT	Cache	8/2011	1	CJ	cdtB1
CJ1-13			alfalfa	UT	Cache	8/2011	0	CJ	-
CJ1-14			alfalfa	UT	Cache	8/2011	0	CJ	-
CJ1-15			alfalfa	UT	Cache	8/2011	1	CJ	cdtB1
CJ1-23			alfalfa	UT	Cache	8/2011	0	CJ	-
CJ1-4			alfalfa	UT	Cache	8/2011	1	CJ	cdtB1
CJ4-10			alfalfa	UT	Cache	8/2011	1	CJ	cdtB1
CJ4-2			alfalfa	UT	Cache	8/2011	0	CJ	-
CJ4-3			alfalfa	UT	Cache	8/2011	0	CJ	-

CJ4-7			alfalfa	UT	Cache	8/2011	1	CJ	cdtB1
CJ4-9			alfalfa	UT	Cache	8/2011	0	CJ	-
CJ1-18	2		alfalfa	UT	Cache	8/2011	1	CJ	cdtB1
CJ1-19			alfalfa	UT	Cache	8/2011	1	CJ	cdtB1
CJ1-20			alfalfa	UT	Cache	8/2011	0	CJ	-
CJ1-21			alfalfa	UT	Cache	8/2011	1	CJ	cdtB1
CJ1-22			alfalfa	UT	Cache	8/2011	0	CJ	-
CJ1-24			alfalfa	UT	Cache	8/2011	1	CJ	cdtB1
CJ1-25			alfalfa	UT	Cache	8/2011	1	CJ	cdtB1
CJ1-26			alfalfa	UT	Cache	8/2011	1	CJ	cdtB1
CJ1-28			alfalfa	UT	Cache	8/2011	0	CJ	-
CJ1-30			alfalfa	UT	Cache	8/2011	0	CJ	-
CJ1-31			alfalfa	UT	Cache	8/2011	0	CJ	-
CJ1-32			alfalfa	UT	Cache	8/2011	1	CJ	cdtB1
CJ1-33			alfalfa	UT	Cache	8/2011	1	CJ	cdtB1
CJ1-35			alfalfa	UT	Cache	8/2011	0	CJ	-
CJ1-37	1	3	alfalfa	UT	Cache	8/2011	1	CJ	YD
CJ1-38			alfalfa	UT	Cache	8/2011	1	CJ	cdtB1
CJ1-39			alfalfa	UT	Cache	8/2011	1	CJ	cdtB1
CJ1-40			alfalfa	UT	Cache	8/2011	1	CJ	cdtB1
CJ1-41	1	1	alfalfa	UT	Cache	8/2011	1	CJ	cdtB1
CJ1-43			alfalfa	UT	Cache	8/2011	0	CJ	-
CJ1-45			alfalfa	UT	Cache	8/2011	0	CJ	-
CJ1-46			alfalfa	UT	Cache	8/2011	0	CJ	-
CJ1-47			alfalfa	UT	Cache	8/2011	0	CJ	-
CJ1-48			alfalfa	UT	Cache	8/2011	0	CJ	-
CJ1-50			alfalfa	UT	Cache	8/2011	0	CJ	-
CJ1-51			alfalfa	UT	Cache	8/2011	0	CJ	-
CJ1-53			alfalfa	UT	Cache	8/2011	0	CJ	-
CJ1-54			alfalfa	UT	Cache	8/2011	0	CJ	-
CJ2-5			alfalfa	UT	Cache	8/2011	1	CJ	cdtB1
CJ2-6			alfalfa	UT	Cache	8/2011	1	CJ	cdtB1
CJ2-9	1	1	alfalfa	UT	Cache	8/2011	1	CJ	cdtB1
CJ3-10			alfalfa	UT	Cache	8/2011	0	CJ	-
CJ3-14			alfalfa	UT	Cache	8/2011	1	CJ	cdtB1
CJ3-16			alfalfa	UT	Cache	8/2011	0	CJ	-
CJ3-2	1	1	alfalfa	UT	Cache	8/2011	1	CJ	cdtB1
CJ3-8			alfalfa	UT	Cache	8/2011	0	CJ	-
CJ4-1	2		alfalfa	UT	Cache	8/2011	1	CJ	cdtB1
WCO-PA RC2			red clover	PA		6/2014	0	CLOVER LA	-
WCO-PA			red	PA		6/2014	0	CLOVER	-

RC4			clover					LA	
WCO-PA RC5			red clover	PA		6/2014	0	CLOVER LA	-
WCO-PA WC3			white clover	PA		6/2014	0	CLOVER LA	-
WW-PA RC1			red clover	PA		6/2014	0	CLOVER LA	-
WW-PA WC 11			white clover	PA		6/2014	0	CLOVER LA	-
WW-PA WC 12			white clover	PA		6/2014	0	CLOVER LA	-
WW-PA WC 13			white clover	PA		6/2014	1	CLOVER LA	cdtB1
WW-PA WC 14			white clover	PA		6/2014	1	CLOVER LA	cdtB1
WW-PA WC 15			white clover	PA		6/2014	0	CLOVER LA	-
WW-PA WC 2			white clover	PA		6/2014	0	CLOVER LA	-
WW-PA WC 5			white clover	PA		6/2014	0	CLOVER LA	-
WW-PA WC 6			white clover	PA		6/2014	1	CLOVER LA	cdtB1
WW-PA WC 7			white clover	PA		6/2014	0	CLOVER LA	-
KY-1			alfalfa	KY	Jefferson	10/2011	0	KY	-
kY-2			alfalfa	KY	Harrison	10/2011	0	KY	-
KY-3			alfalfa	KY	Clark	10/2011	0	KY	-
ND1-1			alfalfa	ND	Cass	9/2012	0	ND	-
ND1-10			alfalfa	ND	Cass	9/2012	0	ND	-
ND1-11			alfalfa	ND	Cass	9/2012	0	ND	-
ND1-12			alfalfa	ND	Cass	9/2012	0	ND	-
ND1-13			alfalfa	ND	Cass	9/2012	0	ND	-
ND1-14			alfalfa	ND	Cass	9/2012	0	ND	-
ND1-15			alfalfa	ND	Cass	9/2012	1	ND	cdtB1
ND1-16			alfalfa	ND	Cass	9/2012	0	ND	-
ND1-17			alfalfa	ND	Cass	9/2012	0	ND	-
ND1-18			alfalfa	ND	Cass	9/2012	1	ND	cdtB1
ND1-19			alfalfa	ND	Cass	9/2012	0	ND	-
ND1-2			alfalfa	ND	Cass	9/2012	0	ND	-
ND1-20			alfalfa	ND	Cass	9/2012	0	ND	-
ND1-21	1		alfalfa	ND	Cass	9/2012	1	ND	cdtB1
ND1-22			alfalfa	ND	Cass	9/2012	0	ND	-
ND1-23			alfalfa	ND	Cass	9/2012	0	ND	-
ND1-24			alfalfa	ND	Cass	9/2012	0	ND	-

ND1-25			alfalfa	ND	Cass	9/2012	0	ND	-
ND1-26	1	1	alfalfa	ND	Cass	9/2012	1	ND	cdtB1
ND1-27			alfalfa	ND	Cass	9/2012	0	ND	-
ND1-28			alfalfa	ND	Cass	9/2012	0	ND	-
ND1-29			alfalfa	ND	Cass	9/2012	0	ND	-
ND1-3			alfalfa	ND	Cass	9/2012	0	ND	-
ND1-30			alfalfa	ND	Cass	9/2012	0	ND	-
ND1-4			alfalfa	ND	Cass	9/2012	0	ND	-
ND1-5	1	4	alfalfa	ND	Cass	9/2012	1	ND	cdtB2
ND1-6			alfalfa	ND	Cass	9/2012	0	ND	-
ND1-7			alfalfa	ND	Cass	9/2012	0	ND	-
ND1-8			alfalfa	ND	Cass	9/2012	0	ND	-
ND1-9			alfalfa	ND	Cass	9/2012	0	ND	-
ND2-1			alfalfa	ND	Cass	9/2012	0	ND	-
ND2-10			alfalfa	ND	Cass	9/2012	0	ND	-
ND2-11			alfalfa	ND	Cass	9/2012	0	ND	-
ND2-12			alfalfa	ND	Cass	9/2012	0	ND	-
ND2-13	1	1	alfalfa	ND	Cass	9/2012	1	ND	cdtB1
ND2-14			alfalfa	ND	Cass	9/2012	0	ND	-
ND2-15			alfalfa	ND	Cass	9/2012	0	ND	-
ND2-16			alfalfa	ND	Cass	9/2012	1	ND	cdtB1
ND2-17			alfalfa	ND	Cass	9/2012	0	ND	-
ND2-18			alfalfa	ND	Cass	9/2012	1	ND	cdtB1
ND2-19			alfalfa	ND	Cass	9/2012	0	ND	-
ND2-2	1	1	alfalfa	ND	Cass	9/2012	1	ND	cdtB1
ND2-20			alfalfa	ND	Cass	9/2012	1	ND	cdtB1
ND2-21			alfalfa	ND	Cass	9/2012	0	ND	-
ND2-22			alfalfa	ND	Cass	9/2012	1	ND	cdtB1
ND2-23			alfalfa	ND	Cass	9/2012	0	ND	-
ND2-24	2		alfalfa	ND	Cass	9/2012	1	ND	YD
ND2-25			alfalfa	ND	Cass	9/2012	1	ND	cdtB1
ND2-26			alfalfa	ND	Cass	9/2012	0	ND	-
ND2-27	2	2	alfalfa	ND	Cass	9/2012	1	ND	YD
ND2-28			alfalfa	ND	Cass	9/2012	1	ND	cdtB2
ND2-29			alfalfa	ND	Cass	9/2012	1	ND	cdtB1
ND2-3			alfalfa	ND	Cass	9/2012	1	ND	cdtB1
ND2-30			alfalfa	ND	Cass	9/2012	0	ND	-
ND2-31			alfalfa	ND	Cass	9/2012	0	ND	-
ND2-4			alfalfa	ND	Cass	9/2012	1	ND	cdtB2
ND2-5			alfalfa	ND	Cass	9/2012	0	ND	-
ND2-6	2		alfalfa	ND	Cass	9/2012	1	ND	cdtB2

ND2-7			alfalfa	ND	Cass	9/2012	0	ND	-
ND2-8			alfalfa	ND	Cass	9/2012	0	ND	-
ND2-9			alfalfa	ND	Cass	9/2012	0	ND	-
A1A		3					1	NOPOP	YD
A2A	1						1	NOPOP	YD
A2C		3		UT			1	NOPOP	none
A2F		3					1	NOPOP	YD
A2H		3					1	NOPOP	YD
AAA 3-2			alfalfa	PA	?	?6/2011	1	NOPOP	cdtB1
aaa3-2	1			PA			1	NOPOP	cdtB1
ga10	1			GA			1	NOPOP	cdtB1
NYC 4			clover	NY	?	?6/2011	0	NOPOP	-
PAA 4			alfalfa	PA	?	?6/2011	1	NOPOP	cdtB1
V1			hvetch	PA	?	?6/2011	1	NOPOP	cdtB1
V5			hvetch	PA	?	?6/2011	0	NOPOP	-
wa4	1			PA			1	NOPOP	cdtB2
XA6	1			PA			1	NOPOP	cdtB1
YA12	1	1		PA			1	NOPOP	cdtB1
YA17	1	1		PA			1	NOPOP	cdtB1
YA3	1	1		PA			1	NOPOP	cdtB1
YA4	1	17		PA			1	NOPOP	cdtB1
ZA11	1			PA			1	NOPOP	cdtB1
ZA12	1	4		PA			1	NOPOP	cdtB2
ZA17	1	4		PA			1	NOPOP	cdtB2
ZA21	1	4		PA			1	NOPOP	cdtB2
ZA22	1	9		PA			1	NOPOP	cdtB2
NY2-2			alfalfa	NY	Tompkins	7/2011	0	NY	-
NY2-3			alfalfa	NY	Tompkins	7/2011	0	NY	-
NY2-4			alfalfa	NY	Tompkins	7/2011	1	NY	cdtB1
NY2-5			alfalfa	NY	Tompkins	7/2011	1	NY	cdtB1
NY2-6		1	alfalfa	NY	Tompkins	7/2011	1	NY	cdtB1
NY2-7			alfalfa	NY	Tompkins	7/2011	1	NY	cdtB1
NY3-1			alfalfa	NY	Tompkins	7/2011	1	NY	cdtB1
NY3-2			alfalfa	NY	Tompkins	7/2011	0	NY	-
NY3-3			alfalfa	NY	Tompkins	7/2011	0	NY	-
NY3-4			alfalfa	NY	Tompkins	7/2011	1	NY	cdtB1
PB-11	2		alfalfa	PA	Lansdale	5/2011	1	PBALF	cdtB1
PB-12			alfalfa	PA	Lansdale	5/2011	1	PBALF	cdtB1
PB-17			alfalfa	PA	Lansdale	5/2011	0	PBALF	-
PB-20		2	alfalfa	PA	Lansdale	5/2011	1	PBALF	stx
PB-21			alfalfa	PA	Lansdale	5/2011	1	PBALF	X

PB-23			alfalfa	PA	Lansdale	5/2011	0	PBALF	-
PB-24			alfalfa	PA	Lansdale	5/2011	0	PBALF	-
PB-25			alfalfa	PA	Lansdale	5/2011	0	PBALF	-
PB-28			alfalfa	PA	Lansdale	5/2011	0	PBALF	-
PB-29	1		alfalfa	PA	Lansdale	5/2011	1	PBALF	cdtB1
PB-30			alfalfa	PA	Lansdale	5/2011	1	PBALF	cdtB1
PB-31			alfalfa	PA	Lansdale	5/2011	0	PBALF	-
PB-33			alfalfa	PA	Lansdale	5/2011	0	PBALF	-
PB-34	2		alfalfa	PA	Lansdale	5/2011	1	PBALF	stx
PB-35	1	1	alfalfa	PA	Lansdale	5/2011	1	PBALF	cdtB1
PB-37			alfalfa	PA	Lansdale	5/2011	1	PBALF	cdtB1
PB-38			alfalfa	PA	Lansdale	5/2011	0	PBALF	-
PB-39			alfalfa	PA	Lansdale	5/2011	1	PBALF	cdtB1
PB-41			alfalfa	PA	Lansdale	5/2011	1	PBALF	cdtB1
PB-43			alfalfa	PA	Lansdale	5/2011	1	PBALF	cdtB1
PB-5			alfalfa	PA	Lansdale	5/2011	0	PBALF	-
PB-44			clover	PA	Lansdale	5/2011	0	PBCLOV	-
PB-46			clover	PA	Lansdale	5/2011	1	PBCLOV	cdtB1
PB-47			clover	PA	Lansdale	5/2011	1	PBCLOV	stx
PB-49			clover	PA	Lansdale	5/2011	1	PBCLOV	cdtB1
UTAS1			alfalfa	UT	Cache	8/2013	1	UTAS	YD
UTAS10			alfalfa	UT	Cache	8/2013	1	UTAS	YD
UTAS11			alfalfa	UT	Cache	8/2013	0	UTAS	-
UTAS12			alfalfa	UT	Cache	8/2013	1	UTAS	cdtB1
UTAS13			alfalfa	UT	Cache	8/2013	1	UTAS	cdtB1
UTAS14			alfalfa	UT	Cache	8/2013	0	UTAS	-
UTAS15			alfalfa	UT	Cache	8/2013	0	UTAS	-
UTAS16			alfalfa	UT	Cache	8/2013	0	UTAS	-
UTAS17			alfalfa	UT	Cache	8/2013	0	UTAS	-
UTAS18			alfalfa	UT	Cache	8/2013	0	UTAS	-
UTAS19			alfalfa	UT	Cache	8/2013	0	UTAS	-
UTAS2			alfalfa	UT	Cache	8/2013	1	UTAS	X
UTAS20			alfalfa	UT	Cache	8/2013	0	UTAS	-
UTAS21			alfalfa	UT	Cache	8/2013	0	UTAS	-
UTAS22			alfalfa	UT	Cache	8/2013	0	UTAS	-
UTAS23			alfalfa	UT	Cache	8/2013	1	UTAS	YD
UTAS24			alfalfa	UT	Cache	8/2013	0	UTAS	-
UTAS25			alfalfa	UT	Cache	8/2013	0	UTAS	-
UTAS26			alfalfa	UT	Cache	8/2013	0	UTAS	-
UTAS27			alfalfa	UT	Cache	8/2013	0	UTAS	-
UTAS28			alfalfa	UT	Cache	8/2013	0	UTAS	-

UTAS29			alfalfa	UT	Cache	8/2013	0	UTAS	-
UTAS3			alfalfa	UT	Cache	8/2013	1	UTAS	YD
UTAS30			alfalfa	UT	Cache	8/2013	0	UTAS	-
UTAS4			alfalfa	UT	Cache	8/2013	1	UTAS	YD
UTAS5			alfalfa	UT	Cache	8/2013	0	UTAS	-
UTAS6			alfalfa	UT	Cache	8/2013	0	UTAS	-
UTAS7			alfalfa	UT	Cache	8/2013	0	UTAS	-
UTAS8			alfalfa	UT	Cache	8/2013	1	UTAS	YD
UTAS9			alfalfa	UT	Cache	8/2013	1	UTAS	YD
KM2			alfalfa	UT	Cache	6/2013	0	UTKMRT	-
KM3			alfalfa	UT	Cache	6/2013	0	UTKMRT	-
KM5			alfalfa	UT	Cache	6/2013	1	UTKMRT	cdtB1
KM6			alfalfa	UT	Cache	6/2013	0	UTKMRT	-
R341			alfalfa	UT	Cache	6/2013	0	UTKMRT	-
R348			alfalfa	UT	Cache	6/2013	0	UTKMRT	-
UT18			alfalfa	UT	Cache	6/2013	0	UTKMRT	-
UT19			alfalfa	UT	Cache	6/2013	0	UTKMRT	-
UT20			alfalfa	UT	Cache	6/2013	0	UTKMRT	-
UT21			alfalfa	UT	Cache	6/2013	0	UTKMRT	-
UT22			alfalfa	UT	Cache	6/2013	0	UTKMRT	-
UT23			alfalfa	UT	Cache	6/2013	0	UTKMRT	-
UT24			alfalfa	UT	Cache	6/2013	1	UTKMRT	cdtB1
WI-1			alfalfa	WI	Dane	9/2011	1	WI11	cdtB1
WI-10			alfalfa	WI	Dane	9/2011	0	WI11	-
WI-12	2	2	alfalfa	WI	Dane	9/2011	1	WI11	cdtB2
WI-14			alfalfa	WI	Dane	9/2011	0	WI11	-
WI-16	2	2	alfalfa	WI	Dane	9/2011	1	WI11	stx
WI-18	1		alfalfa	WI	Dane	9/2011	1	WI11	cdtB1
WI-23			alfalfa	WI	Dane	9/2011	0	WI11	-
WI-24			alfalfa	WI	Dane	9/2011	0	WI11	-
WI-26			alfalfa	WI	Dane	9/2011	0	WI11	-
WI-27			alfalfa	WI	Dane	9/2011	0	WI11	-
WI-28			alfalfa	WI	Dane	9/2011	1	WI11	cdtB1
WI-29			alfalfa	WI	Dane	9/2011	1	WI11	cdtB1
WI-31			alfalfa	WI	Dane	9/2011	1	WI11	cdtB2
WI-35			alfalfa	WI	Dane	9/2011	1	WI11	cdtB2
WI-36			alfalfa	WI	Dane	9/2011	0	WI11	-
WI-38			alfalfa	WI	Dane	9/2011	1	WI11	cdtB2
WI-39			alfalfa	WI	Dane	9/2011	1	WI11	cdtB2
WI-4			alfalfa	WI	Dane	9/2011	1	WI11	cdtB1
WI-40			alfalfa	WI	Dane	9/2011	1	WI11	cdtB2

WI-41			alfalfa	WI	Dane	9/2011	0	WI11	-
WI-42		2	alfalfa	WI	Dane	9/2011	1	WI11	cdtB2
WI-43			alfalfa	WI	Dane	9/2011	0	WI11	-
WI-45			alfalfa	WI	Dane	9/2011	1	WI11	X
WI-46			alfalfa	WI	Dane	9/2011	0	WI11	-
WI-47			alfalfa	WI	Dane	9/2011	1	WI11	cdtB2
WI-48			alfalfa	WI	Dane	9/2011	0	WI11	-
WI-49			alfalfa	WI	Dane	9/2011	0	WI11	-
WI-51			alfalfa	WI	Dane	9/2011	1	WI11	cdtB2
WI-52			alfalfa	WI	Dane	9/2011	0	WI11	-
WI-55			alfalfa	WI	Dane	9/2011	1	WI11	cdtB2
WI-56			alfalfa	WI	Dane	9/2011	0	WI11	-
WI-57			alfalfa	WI	Dane	9/2011	0	WI11	-
WI-58			alfalfa	WI	Dane	9/2011	0	WI11	-
WI-6			alfalfa	WI	Dane	9/2011	0	WI11	-
WI-62	1	1	alfalfa	WI	Dane	9/2011	1	WI11	cdtB1
WI-63			alfalfa	WI	Dane	9/2011	0	WI11	-
WI-7			alfalfa	WI	Dane	9/2011	1	WI11	cdtB1
WI-9			alfalfa	WI	Dane	9/2011	0	WI11	-
WIA2A			alfalfa	WI	Dane	8/2012	1	WIA	cdtB1
WIA2B			alfalfa	WI	Dane	8/2012	1	WIA	stx
WIA2C			alfalfa	WI	Dane	8/2012	1	WIA	cdtB1
WIA2D			alfalfa	WI	Dane	8/2012	0	WIA	-
WIA2E	2	2	alfalfa	WI	Dane	8/2012	1	WIA	cdtB2
WIA2F			alfalfa	WI	Dane	8/2012	0	WIA	-
WIA2G			alfalfa	WI	Dane	8/2012	1	WIA	cdtB1
WIA2H			alfalfa	WI	Dane	8/2012	0	WIA	-
WIA2I			alfalfa	WI	Dane	8/2012	0	WIA	-
WIA2J			alfalfa	WI	Dane	8/2012	0	WIA	-
WIA2K			alfalfa	WI	Dane	8/2012	0	WIA	-
WIA2L			alfalfa	WI	Dane	8/2012	0	WIA	-
WIA3A	1	1	alfalfa	WI	Dane	8/2012	1	WIA	cdtB1
WIA3B			alfalfa	WI	Dane	8/2012	0	WIA	-
WIA3C			alfalfa	WI	Dane	8/2012	0	WIA	-
WIA3D			alfalfa	WI	Dane	8/2012	0	WIA	-
WIA3E			alfalfa	WI	Dane	8/2012	0	WIA	-
WIA3F			alfalfa	WI	Dane	8/2012	0	WIA	-
WIA3G	1	1	alfalfa	WI	Dane	8/2012	1	WIA	cdtB1
WIA3H			alfalfa	WI	Dane	8/2012	0	WIA	-
WIA3I	2		alfalfa	WI	Dane	8/2012	1	WIA	YD
WIA3J			alfalfa	WI	Dane	8/2012	1	WIA	cdtB1

WIA3K			alfalfa	WI	Dane	8/2012	0	WIA	-
WIA3L	1		alfalfa	WI	Dane	8/2012	1	WIA	cdtB1
WIA3M			alfalfa	WI	Dane	8/2012	0	WIA	-
WIA3N			alfalfa	WI	Dane	8/2012	0	WIA	-
WIA4A			alfalfa	WI	Dane	8/2012	0	WIA	-
WIA4B			alfalfa	WI	Dane	8/2012	1	WIA	cdtB1
WIA4C			alfalfa	WI	Dane	8/2012	0	WIA	-
WIA4D			alfalfa	WI	Dane	8/2012	0	WIA	-
WIA4E			alfalfa	WI	Dane	8/2012	1	WIA	stx
WIA4F			alfalfa	WI	Dane	8/2012	1	WIA	cdtB2
WIA4G			alfalfa	WI	Dane	8/2012	1	WIA	cdtB2
WIA4H			alfalfa	WI	Dane	8/2012	0	WIA	-
WIA4I			alfalfa	WI	Dane	8/2012	0	WIA	-
WIA4J	2	2	alfalfa	WI	Dane	8/2012	1	WIA	cdtB2
WIA5A			alfalfa	WI	Dane	8/2012	0	WIA	-
WIA5B			alfalfa	WI	Dane	8/2012	0	WIA	-
WIA5C			alfalfa	WI	Dane	8/2012	0	WIA	-
WIA5D	2	2	alfalfa	WI	Dane	8/2012	1	WIA	cdtB2
WIA5E			alfalfa	WI	Dane	8/2012	0	WIA	-
WIA5F			alfalfa	WI	Dane	8/2012	1	WIA	cdtB1
WIA5G	2		alfalfa	WI	Dane	8/2012	1	WIA	cdtB1
WIA6A			alfalfa	WI	Dane	8/2012	0	WIA	-
WIA6B			alfalfa	WI	Dane	8/2012	0	WIA	-
WIA6C			alfalfa	WI	Dane	8/2012	0	WIA	-
WIA6D			alfalfa	WI	Dane	8/2012	0	WIA	-
WIA6E			alfalfa	WI	Dane	8/2012	0	WIA	-
WIA6F	2	2	alfalfa	WI	Dane	8/2012	1	WIA	cdtB2
WIA6G			alfalfa	WI	Dane	8/2012	0	WIA	-
WIA6H			alfalfa	WI	Dane	8/2012	0	WIA	-
WIA6I			alfalfa	WI	Dane	8/2012	0	WIA	-
WIA6J	1	1	alfalfa	WI	Dane	8/2012	1	WIA	cdtB1
WIA6K			alfalfa	WI	Dane	8/2012	0	WIA	-
WI14-1			alfalfa	WI	Dane	05/29/2014	1	wiaug14	X
WI14-100			alfalfa	WI	Dane	05/29/2014	0	wiaug14	-
WI14-101			alfalfa	WI	Dane	05/29/2014	1	wiaug14	cdtB1
WI14-102			alfalfa	WI	Dane	05/29/2014	0	wiaug14	-
WI14-109			alfalfa	WI	Dane	05/29/2014	1	wiaug14	YD
WI14-110			alfalfa	WI	Dane	05/29/2014	1	wiaug14	YD
WI14-111			alfalfa	WI	Dane	05/29/2014	1	wiaug14	YD
WI14-112			alfalfa	WI	Dane	05/29/2014	0	wiaug14	-
WI14-113			alfalfa	WI	Dane	05/29/2014	1	wiaug14	cdtB2

WI14-114			alfalfa	WI	Dane	05/29/2014	1	wiaug14	cdtB2
WI14-121			alfalfa	WI	Dane	05/29/2014	1	wiaug14	cdtB1
WI14-122			alfalfa	WI	Dane	05/29/2014	1	wiaug14	YD
WI14-123			alfalfa	WI	Dane	05/29/2014	1	wiaug14	cdtB2
WI14-124			alfalfa	WI	Dane	05/29/2014	1	wiaug14	cdtB2
WI14-125			alfalfa	WI	Dane	05/29/2014	0	wiaug14	-
WI14-126			alfalfa	WI	Dane	05/29/2014	1	wiaug14	cdtB2
WI14-13			alfalfa	WI	Dane	05/29/2014	0	wiaug14	-
WI14-133			alfalfa	WI	Dane	05/29/2014	1	wiaug14	YD
WI14-134			alfalfa	WI	Dane	05/29/2014	1	wiaug14	cdtB1
WI14-135			alfalfa	WI	Dane	05/29/2014	1	wiaug14	YD
WI14-136			alfalfa	WI	Dane	05/29/2014	0	wiaug14	-
WI14-137			alfalfa	WI	Dane	05/29/2014	0	wiaug14	-
WI14-138			alfalfa	WI	Dane	05/29/2014	1	wiaug14	YD
WI14-14			alfalfa	WI	Dane	05/29/2014	0	wiaug14	-
WI14-145			alfalfa	WI	Dane	05/29/2014	1	wiaug14	YD
WI14-146			alfalfa	WI	Dane	05/29/2014	0	wiaug14	-
WI14-147			alfalfa	WI	Dane	05/29/2014	0	wiaug14	-
WI14-148			alfalfa	WI	Dane	05/29/2014	1	wiaug14	YD
WI14-149			alfalfa	WI	Dane	05/29/2014	0	wiaug14	-
WI14-15			alfalfa	WI	Dane	05/29/2014	0	wiaug14	-
WI14-150			alfalfa	WI	Dane	05/29/2014	1	wiaug14	cdtB2
WI14-157			alfalfa	WI	Dane	05/29/2014	1	wiaug14	cdtB1
WI14-158			alfalfa	WI	Dane	05/29/2014	1	wiaug14	YD
WI14-159			alfalfa	WI	Dane	05/29/2014	0	wiaug14	-
WI14-16			alfalfa	WI	Dane	05/29/2014	0	wiaug14	-
WI14-160			alfalfa	WI	Dane	05/29/2014	1	wiaug14	cdtB2
WI14-161			alfalfa	WI	Dane	05/29/2014	1	wiaug14	cdtB2
WI14-162			alfalfa	WI	Dane	05/29/2014	1	wiaug14	cdtB1
WI14-169			alfalfa	WI	Dane	05/29/2014	1	wiaug14	YD
WI14-17			alfalfa	WI	Dane	05/29/2014	0	wiaug14	-
WI14-170			alfalfa	WI	Dane	05/29/2014	1	wiaug14	YD
WI14-171			alfalfa	WI	Dane	05/29/2014	1	wiaug14	YD
WI14-172			alfalfa	WI	Dane	05/29/2014	0	wiaug14	-
WI14-173			alfalfa	WI	Dane	05/29/2014	1	wiaug14	YD
WI14-174			alfalfa	WI	Dane	05/29/2014	1	wiaug14	YD
WI14-18			alfalfa	WI	Dane	05/29/2014	0	wiaug14	-
WI14-181			alfalfa	WI	Dane	05/29/2014	1	wiaug14	stx
WI14-182			alfalfa	WI	Dane	05/29/2014	1	wiaug14	YD
WI14-183			alfalfa	WI	Dane	05/29/2014	1	wiaug14	cdtB2
WI14-184			alfalfa	WI	Dane	05/29/2014	0	wiaug14	-

WI14-185			alfalfa	WI	Dane	05/29/2014	0	wiaug14	-
WI14-186			alfalfa	WI	Dane	05/29/2014	0	wiaug14	-
WI14-193			alfalfa	WI	Dane	05/29/2014	1	wiaug14	cdtB2
WI14-194			alfalfa	WI	Dane	05/29/2014	1	wiaug14	YD
WI14-195			alfalfa	WI	Dane	05/29/2014	1	wiaug14	cdtB2
WI14-196			alfalfa	WI	Dane	05/29/2014	0	wiaug14	-
WI14-197			alfalfa	WI	Dane	05/29/2014	0	wiaug14	-
WI14-198			alfalfa	WI	Dane	05/29/2014	1	wiaug14	stx
WI14-2			alfalfa	WI	Dane	05/29/2014	0	wiaug14	-
WI14-205			alfalfa	WI	Dane	05/29/2014	0	wiaug14	-
WI14-206			alfalfa	WI	Dane	05/29/2014	1	wiaug14	YD
WI14-207			alfalfa	WI	Dane	05/29/2014	0	wiaug14	-
WI14-208			alfalfa	WI	Dane	05/29/2014	1	wiaug14	YD
WI14-209			alfalfa	WI	Dane	05/29/2014	0	wiaug14	-
WI14-210			alfalfa	WI	Dane	05/29/2014	0	wiaug14	-
WI14-217			alfalfa	WI	Dane	05/29/2014	0	wiaug14	-
WI14-218			alfalfa	WI	Dane	05/29/2014	0	wiaug14	-
WI14-219			alfalfa	WI	Dane	05/29/2014	1	wiaug14	YD
WI14-220			alfalfa	WI	Dane	05/29/2014	0	wiaug14	-
WI14-221			alfalfa	WI	Dane	05/29/2014	1	wiaug14	cdtB1
WI14-222			alfalfa	WI	Dane	05/29/2014	0	wiaug14	-
WI14-229			alfalfa	WI	Dane	05/29/2014	1	wiaug14	cdtB1
WI14-230			alfalfa	WI	Dane	05/29/2014	1	wiaug14	YD
WI14-231			alfalfa	WI	Dane	05/29/2014	0	wiaug14	-
WI14-232			alfalfa	WI	Dane	05/29/2014	0	wiaug14	-
WI14-233			alfalfa	WI	Dane	05/29/2014	0	wiaug14	-
WI14-234			alfalfa	WI	Dane	05/29/2014	1	wiaug14	stx
WI14-241			alfalfa	WI	Dane	05/29/2014	0	wiaug14	-
WI14-242			alfalfa	WI	Dane	05/29/2014	1	wiaug14	stx
WI14-243			alfalfa	WI	Dane	05/29/2014	0	wiaug14	-
WI14-244			alfalfa	WI	Dane	05/29/2014	1	wiaug14	YD
WI14-245			alfalfa	WI	Dane	05/29/2014	0	wiaug14	-
WI14-246			alfalfa	WI	Dane	05/29/2014	1	wiaug14	stx
WI14-25			alfalfa	WI	Dane	05/29/2014	0	wiaug14	-
WI14-253			alfalfa	WI	Dane	05/29/2014	0	wiaug14	-
WI14-254			alfalfa	WI	Dane	05/29/2014	0	wiaug14	-
WI14-255			alfalfa	WI	Dane	05/29/2014	0	wiaug14	-
WI14-256			alfalfa	WI	Dane	05/29/2014	1	wiaug14	stx
WI14-257			alfalfa	WI	Dane	05/29/2014	0	wiaug14	-
WI14-258			alfalfa	WI	Dane	05/29/2014	1	wiaug14	stx
WI14-26			alfalfa	WI	Dane	05/29/2014	0	wiaug14	-

WI14-265			alfalfa	WI	Dane	05/29/2014	1	wiaug14	cdtB2
WI14-266			alfalfa	WI	Dane	05/29/2014	1	wiaug14	cdtB2
WI14-267			alfalfa	WI	Dane	05/29/2014	1	wiaug14	stx
WI14-268			alfalfa	WI	Dane	05/29/2014	1	wiaug14	YD
WI14-269			alfalfa	WI	Dane	05/29/2014	1	wiaug14	cdtB1
WI14-27			alfalfa	WI	Dane	05/29/2014	0	wiaug14	-
WI14-270			alfalfa	WI	Dane	05/29/2014	0	wiaug14	-
WI14-277			alfalfa	WI	Dane	05/29/2014	0	wiaug14	-
WI14-278			alfalfa	WI	Dane	05/29/2014	1	wiaug14	YD
WI14-279			alfalfa	WI	Dane	05/29/2014	1	wiaug14	YD
WI14-28			alfalfa	WI	Dane	05/29/2014	0	wiaug14	-
WI14-280			alfalfa	WI	Dane	05/29/2014	1	wiaug14	YD
WI14-281			alfalfa	WI	Dane	05/29/2014	0	wiaug14	-
WI14-282			alfalfa	WI	Dane	05/29/2014	0	wiaug14	-
WI14-289			alfalfa	WI	Dane	05/29/2014	1	wiaug14	cdtB1
WI14-29			alfalfa	WI	Dane	05/29/2014	0	wiaug14	-
WI14-290			alfalfa	WI	Dane	05/29/2014	1	wiaug14	cdtB1
WI14-291			alfalfa	WI	Dane	05/29/2014	0	wiaug14	-
WI14-292			alfalfa	WI	Dane	05/29/2014	0	wiaug14	-
WI14-293			alfalfa	WI	Dane	05/29/2014	1	wiaug14	YD
WI14-294			alfalfa	WI	Dane	05/29/2014	0	wiaug14	-
WI14-3			alfalfa	WI	Dane	05/29/2014	0	wiaug14	-
WI14-30			alfalfa	WI	Dane	05/29/2014	0	wiaug14	-
WI14-301			alfalfa	WI	Dane	05/29/2014	0	wiaug14	-
WI14-302			alfalfa	WI	Dane	05/29/2014	1	wiaug14	YD
WI14-303			alfalfa	WI	Dane	05/29/2014	1	wiaug14	cdtB2
WI14-304			alfalfa	WI	Dane	05/29/2014	1	wiaug14	YD
WI14-305			alfalfa	WI	Dane	05/29/2014	1	wiaug14	cdtB2
WI14-306			alfalfa	WI	Dane	05/29/2014	1	wiaug14	cdtB2
WI14-313			alfalfa	WI	Dane	05/29/2014	1	wiaug14	YD
WI14-314			alfalfa	WI	Dane	05/29/2014	1	wiaug14	stx
WI14-315			alfalfa	WI	Dane	05/29/2014	1	wiaug14	stx
WI14-316			alfalfa	WI	Dane	05/29/2014	1	wiaug14	cdtB2
WI14-317			alfalfa	WI	Dane	05/29/2014	1	wiaug14	YD
WI14-318			alfalfa	WI	Dane	05/29/2014	1	wiaug14	cdtB2
WI14-325			alfalfa	WI	Dane	05/29/2014	1	wiaug14	stx
WI14-326			alfalfa	WI	Dane	05/29/2014	1	wiaug14	stx
WI14-327			alfalfa	WI	Dane	05/29/2014	0	wiaug14	-
WI14-328			alfalfa	WI	Dane	05/29/2014	1	wiaug14	cdtB2
WI14-329			alfalfa	WI	Dane	05/29/2014	1	wiaug14	stx
WI14-330			alfalfa	WI	Dane	05/29/2014	0	wiaug14	-

WI14-337			alfalfa	WI	Dane	05/29/2014	1	wiaug14	YD
WI14-338			alfalfa	WI	Dane	05/29/2014	1	wiaug14	YD
WI14-339			alfalfa	WI	Dane	05/29/2014	0	wiaug14	-
WI14-340			alfalfa	WI	Dane	05/29/2014	1	wiaug14	YD
WI14-341			alfalfa	WI	Dane	05/29/2014	1	wiaug14	cdtB2
WI14-342			alfalfa	WI	Dane	05/29/2014	1	wiaug14	YD
WI14-349			alfalfa	WI	Dane	05/29/2014	1	wiaug14	cdtB2
WI14-350			alfalfa	WI	Dane	05/29/2014	0	wiaug14	-
WI14-351			alfalfa	WI	Dane	05/29/2014	1	wiaug14	YD
WI14-352			alfalfa	WI	Dane	05/29/2014	0	wiaug14	-
WI14-353			alfalfa	WI	Dane	05/29/2014	1	wiaug14	cdtB2
WI14-354			alfalfa	WI	Dane	05/29/2014	1	wiaug14	YD
WI14-361			alfalfa	WI	Dane	05/29/2014	1	wiaug14	YD
WI14-362			alfalfa	WI	Dane	05/29/2014	0	wiaug14	-
WI14-363			alfalfa	WI	Dane	05/29/2014	1	wiaug14	cdtB2
WI14-364			alfalfa	WI	Dane	05/29/2014	1	wiaug14	YD
WI14-365			alfalfa	WI	Dane	05/29/2014	0	wiaug14	-
WI14-366			alfalfa	WI	Dane	05/29/2014	0	wiaug14	-
WI14-37			alfalfa	WI	Dane	05/29/2014	0	wiaug14	-
WI14-373			alfalfa	WI	Dane	05/29/2014	1	wiaug14	YD
WI14-374			alfalfa	WI	Dane	05/29/2014	0	wiaug14	-
WI14-375			alfalfa	WI	Dane	05/29/2014	1	wiaug14	YD
WI14-376			alfalfa	WI	Dane	05/29/2014	1	wiaug14	YD
WI14-377			alfalfa	WI	Dane	05/29/2014	0	wiaug14	-
WI14-378			alfalfa	WI	Dane	05/29/2014	0	wiaug14	-
WI14-38			alfalfa	WI	Dane	05/29/2014	0	wiaug14	-
WI14-385			alfalfa	WI	Dane	05/29/2014	1	wiaug14	cdtB2
WI14-386			alfalfa	WI	Dane	05/29/2014	1	wiaug14	YD
WI14-387			alfalfa	WI	Dane	05/29/2014	1	wiaug14	YD
WI14-388			alfalfa	WI	Dane	05/29/2014	0	wiaug14	-
WI14-389			alfalfa	WI	Dane	05/29/2014	1	wiaug14	YD
WI14-39			alfalfa	WI	Dane	05/29/2014	0	wiaug14	-
WI14-390			alfalfa	WI	Dane	05/29/2014	0	wiaug14	-
WI14-397			alfalfa	WI	Dane	05/29/2014	1	wiaug14	cdtB1
WI14-398			alfalfa	WI	Dane	05/29/2014	1	wiaug14	cdtB1
WI14-399			alfalfa	WI	Dane	05/29/2014	0	wiaug14	-
WI14-4			alfalfa	WI	Dane	05/29/2014	0	wiaug14	-
WI14-40			alfalfa	WI	Dane	05/29/2014	0	wiaug14	-
WI14-400			alfalfa	WI	Dane	05/29/2014	0	wiaug14	-
WI14-401			alfalfa	WI	Dane	05/29/2014	1	wiaug14	cdtB2
WI14-402			alfalfa	WI	Dane	05/29/2014	0	wiaug14	-

WI14-409			alfalfa	WI	Dane	05/29/2014	1	wiaug14	YD
WI14-41			alfalfa	WI	Dane	05/29/2014	0	wiaug14	-
WI14-410			alfalfa	WI	Dane	05/29/2014	1	wiaug14	cdtB1
WI14-411			alfalfa	WI	Dane	05/29/2014	0	wiaug14	-
WI14-412			alfalfa	WI	Dane	05/29/2014	1	wiaug14	cdtB1
WI14-413		2	alfalfa	WI	Dane	05/29/2014	1	wiaug14	stx
WI14-414			alfalfa	WI	Dane	05/29/2014	1	wiaug14	cdtB2
WI14-42			alfalfa	WI	Dane	05/29/2014	0	wiaug14	-
WI14-421			alfalfa	WI	Dane	05/29/2014	1	wiaug14	YD
WI14-422			alfalfa	WI	Dane	05/29/2014	0	wiaug14	-
WI14-423			alfalfa	WI	Dane	05/29/2014	0	wiaug14	-
WI14-424			alfalfa	WI	Dane	05/29/2014	1	wiaug14	stx
WI14-425			alfalfa	WI	Dane	05/29/2014	1	wiaug14	cdtB2
WI14-426			alfalfa	WI	Dane	05/29/2014	1	wiaug14	cdtB2
WI14-433			alfalfa	WI	Dane	05/29/2014	1	wiaug14	YD
WI14-434			alfalfa	WI	Dane	05/29/2014	1	wiaug14	cdtB2
WI14-435			alfalfa	WI	Dane	05/29/2014	1	wiaug14	stx
WI14-436		2	alfalfa	WI	Dane	05/29/2014	1	wiaug14	stx
WI14-437			alfalfa	WI	Dane	05/29/2014	0	wiaug14	-
WI14-438			alfalfa	WI	Dane	05/29/2014	0	wiaug14	-
WI14-445			alfalfa	WI	Dane	05/29/2014	1	wiaug14	YD
WI14-446			alfalfa	WI	Dane	05/29/2014	1	wiaug14	YD
WI14-447			alfalfa	WI	Dane	05/29/2014	1	wiaug14	cdtB1
WI14-448			alfalfa	WI	Dane	05/29/2014	1	wiaug14	cdtB1
WI14-449			alfalfa	WI	Dane	05/29/2014	0	wiaug14	-
WI14-450			alfalfa	WI	Dane	05/29/2014	0	wiaug14	-
WI14-457			alfalfa	WI	Dane	05/29/2014	1	wiaug14	cdtB1
WI14-458			alfalfa	WI	Dane	05/29/2014	1	wiaug14	X
WI14-459			alfalfa	WI	Dane	05/29/2014	0	wiaug14	-
WI14-460			alfalfa	WI	Dane	05/29/2014	1	wiaug14	cdtB1
WI14-461			alfalfa	WI	Dane	05/29/2014	1	wiaug14	YD
WI14-462			alfalfa	WI	Dane	05/29/2014	0	wiaug14	-
WI14-469			alfalfa	WI	Dane	05/29/2014	1	wiaug14	YD
WI14-470			alfalfa	WI	Dane	05/29/2014	0	wiaug14	-
WI14-471			alfalfa	WI	Dane	05/29/2014	0	wiaug14	-
WI14-472			alfalfa	WI	Dane	05/29/2014	1	wiaug14	YD
WI14-473			alfalfa	WI	Dane	05/29/2014	1	wiaug14	YD
WI14-474			alfalfa	WI	Dane	05/29/2014	1	wiaug14	YD
WI14-481			alfalfa	WI	Dane	05/29/2014	0	wiaug14	-
WI14-482			alfalfa	WI	Dane	05/29/2014	0	wiaug14	-
WI14-483			alfalfa	WI	Dane	05/29/2014	0	wiaug14	-

WI14-484			alfalfa	WI	Dane	05/29/2014	1	wiaug14	YD
WI14-485			alfalfa	WI	Dane	05/29/2014	1	wiaug14	YD
WI14-486			alfalfa	WI	Dane	05/29/2014	1	wiaug14	YD
WI14-49			alfalfa	WI	Dane	05/29/2014	0	wiaug14	-
WI14-493			alfalfa	WI	Dane	05/29/2014	1	wiaug14	YD
WI14-494			alfalfa	WI	Dane	05/29/2014	1	wiaug14	YD
WI14-495			alfalfa	WI	Dane	05/29/2014	0	wiaug14	-
WI14-496			alfalfa	WI	Dane	05/29/2014	0	wiaug14	-
WI14-497			alfalfa	WI	Dane	05/29/2014	0	wiaug14	-
WI14-498			alfalfa	WI	Dane	05/29/2014	0	wiaug14	-
WI14-5			alfalfa	WI	Dane	05/29/2014	0	wiaug14	-
WI14-50			alfalfa	WI	Dane	05/29/2014	0	wiaug14	-
WI14-51			alfalfa	WI	Dane	05/29/2014	0	wiaug14	-
WI14-52			alfalfa	WI	Dane	05/29/2014	0	wiaug14	-
WI14-53			alfalfa	WI	Dane	05/29/2014	0	wiaug14	-
WI14-54			alfalfa	WI	Dane	05/29/2014	0	wiaug14	-
WI14-6			alfalfa	WI	Dane	05/29/2014	0	wiaug14	-
WI14-61			alfalfa	WI	Dane	05/29/2014	0	wiaug14	-
WI14-62			alfalfa	WI	Dane	05/29/2014	0	wiaug14	-
WI14-63			alfalfa	WI	Dane	05/29/2014	0	wiaug14	-
WI14-64			alfalfa	WI	Dane	05/29/2014	0	wiaug14	-
WI14-65			alfalfa	WI	Dane	05/29/2014	0	wiaug14	-
WI14-66			alfalfa	WI	Dane	05/29/2014	0	wiaug14	-
WI14-73			alfalfa	WI	Dane	05/29/2014	0	wiaug14	-
WI14-74			alfalfa	WI	Dane	05/29/2014	0	wiaug14	-
WI14-75			alfalfa	WI	Dane	05/29/2014	0	wiaug14	-
WI14-76			alfalfa	WI	Dane	05/29/2014	0	wiaug14	-
WI14-77			alfalfa	WI	Dane	05/29/2014	0	wiaug14	-
WI14-78			alfalfa	WI	Dane	05/29/2014	0	wiaug14	-
WI14-85			alfalfa	WI	Dane	05/29/2014	0	wiaug14	-
WI14-86			alfalfa	WI	Dane	05/29/2014	0	wiaug14	-
WI14-87			alfalfa	WI	Dane	05/29/2014	0	wiaug14	-
WI14-88			alfalfa	WI	Dane	05/29/2014	0	wiaug14	-
WI14-89			alfalfa	WI	Dane	05/29/2014	0	wiaug14	-
WI14-90			alfalfa	WI	Dane	05/29/2014	0	wiaug14	-
WI14-97			alfalfa	WI	Dane	05/29/2014	1	wiaug14	YD
WI14-98			alfalfa	WI	Dane	05/29/2014	1	wiaug14	YD
WI14-99			alfalfa	WI	Dane	05/29/2014	1	wiaug14	cdtB1
WI 1-1			alfalfa	WI	Dane	08/14/2013	1	wiaug2013	YD
WI 2-1			alfalfa	WI	Dane	08/14/2013	0	wiaug2013	-
WI 2-10			alfalfa	WI	Dane	08/14/2013	1	wiaug2013	cdtB1

WI 2-11			alfalfa	WI	Dane	08/14/2013	1	wiaug2013	cdtB1
WI 2-12			alfalfa	WI	Dane	08/14/2013	0	wiaug2013	-
WI 2-13			alfalfa	WI	Dane	08/14/2013	0	wiaug2013	-
WI 2-14			alfalfa	WI	Dane	08/14/2013	0	wiaug2013	-
WI 2-15			alfalfa	WI	Dane	08/14/2013	0	wiaug2013	-
WI 2-16			alfalfa	WI	Dane	08/14/2013	0	wiaug2013	-
WI 2-17			alfalfa	WI	Dane	08/14/2013	0	wiaug2013	-
WI 2-18	1	1	alfalfa	WI	Dane	08/14/2013	1	wiaug2013	cdtB1
WI 2-2			alfalfa	WI	Dane	08/14/2013	1	wiaug2013	cdtB1
WI 2-3			alfalfa	WI	Dane	08/14/2013	0	wiaug2013	-
WI 2-4			alfalfa	WI	Dane	08/14/2013	0	wiaug2013	-
WI 2-5	1	1	alfalfa	WI	Dane	08/14/2013	1	wiaug2013	cdtB1
WI 2-6			alfalfa	WI	Dane	08/14/2013	0	wiaug2013	-
WI 2-7			alfalfa	WI	Dane	08/14/2013	0	wiaug2013	-
WI 2-8			alfalfa	WI	Dane	08/14/2013	0	wiaug2013	-
WI 3-1		3	alfalfa	WI	Dane	08/14/2013	1	wiaug2013	YD
WI 3-4			alfalfa	WI	Dane	08/14/2013	0	wiaug2013	-
WI 3-6			alfalfa	WI	Dane	08/14/2013	0	wiaug2013	-
WI 3-7			alfalfa	WI	Dane	08/14/2013	0	wiaug2013	-
WI 3-9			alfalfa	WI	Dane	08/14/2013	0	wiaug2013	-
WI 4-1			alfalfa	WI	Dane	08/14/2013	0	wiaug2013	-
WI 4-10	1	3	alfalfa	WI	Dane	08/14/2013	1	wiaug2013	YD
WI 4-12	1	3	alfalfa	WI	Dane	08/14/2013	1	wiaug2013	YD
WI 4-13			alfalfa	WI	Dane	08/14/2013	0	wiaug2013	-
WI 4-14			alfalfa	WI	Dane	08/14/2013	0	wiaug2013	-
WI 4-2		2	alfalfa	WI	Dane	08/14/2013	1	wiaug2013	YD
WI 4-3			alfalfa	WI	Dane	08/14/2013	0	wiaug2013	-
WI 4-6		12	alfalfa	WI	Dane	08/14/2013	1	wiaug2013	YD
WI 4-7			alfalfa	WI	Dane	08/14/2013	0	wiaug2013	-
WI 4-9			alfalfa	WI	Dane	08/14/2013	0	wiaug2013	-
WI 5-1			alfalfa	WI	Dane	08/14/2013	1	wiaug2013	cdtB1
WI 5-2			alfalfa	WI	Dane	08/14/2013	1	wiaug2013	cdtB1
WI 5-3			alfalfa	WI	Dane	08/14/2013	1	wiaug2013	cdtB1
WI 5-4			alfalfa	WI	Dane	08/14/2013	1	wiaug2013	cdtB1
WI 5-5			alfalfa	WI	Dane	08/14/2013	1	wiaug2013	cdtB1
WI 6-5	1	1	alfalfa	WI	Dane	08/14/2013	1	wiaug2013	cdtB1
WI 7-2	1	1	alfalfa	WI	Dane	08/14/2013	1	wiaug2013	cdtB1
WI 7-4			alfalfa	WI	Dane	08/14/2013	0	wiaug2013	-
WI 7-5			alfalfa	WI	Dane	08/14/2013	1	wiaug2013	cdtB1
WI 7-6			alfalfa	WI	Dane	08/14/2013	1	wiaug2013	cdtB1
WI7-10	1	3	alfalfa	WI	Dane	08/14/2013	1	wiaug2013	YD

WI7-11			alfalfa	WI	Dane	08/14/2013	1	wiaug2013	cdtB1
WI7-12			alfalfa	WI	Dane	08/14/2013	1	wiaug2013	cdtB1
WI7-13			alfalfa	WI	Dane	08/14/2013	1	wiaug2013	cdtB1
WI7-16			alfalfa	WI	Dane	08/14/2013	1	wiaug2013	cdtB1
WI7-17			alfalfa	WI	Dane	08/14/2013	1	wiaug2013	cdtB1
WI7-18			alfalfa	WI	Dane	08/14/2013	1	wiaug2013	cdtB1
WI7-21	1	3	alfalfa	WI	Dane	08/14/2013	1	wiaug2013	YD
WI7-22	1	3	alfalfa	WI	Dane	08/14/2013	1	wiaug2013	stx
WI7-23			alfalfa	WI	Dane	08/14/2013	1	wiaug2013	cdtB1
WI7-25			alfalfa	WI	Dane	08/14/2013	1	wiaug2013	cdtB1
WI7-26			alfalfa	WI	Dane	08/14/2013	1	wiaug2013	cdtB1
WI7-27			alfalfa	WI	Dane	08/14/2013	1	wiaug2013	stx
WI7-28			alfalfa	WI	Dane	08/14/2013	1	wiaug2013	cdtB1
WI7-30			alfalfa	WI	Dane	08/14/2013	1	wiaug2013	cdtB1
WI7-31			alfalfa	WI	Dane	08/14/2013	1	wiaug2013	cdtB1
WI7-32			alfalfa	WI	Dane	08/14/2013	1	wiaug2013	cdtB1
WI7-33			alfalfa	WI	Dane	08/14/2013	1	wiaug2013	cdtB1
WI7-34			alfalfa	WI	Dane	08/14/2013	1	wiaug2013	YD
WI7-35	1	3	alfalfa	WI	Dane	08/14/2013	1	wiaug2013	YD
WI7-36	1	3	alfalfa	WI	Dane	08/14/2013	1	wiaug2013	YD
WI7-37	1	1	alfalfa	WI	Dane	08/14/2013	1	wiaug2013	cdtB1
WI7-38			alfalfa	WI	Dane	08/14/2013	0	wiaug2013	-
WI7-39			alfalfa	WI	Dane	08/14/2013	0	wiaug2013	-
WI7-40			alfalfa	WI	Dane	08/14/2013	0	wiaug2013	-
WI7-41			alfalfa	WI	Dane	08/14/2013	0	wiaug2013	-
WI7-42			alfalfa	WI	Dane	08/14/2013	0	wiaug2013	-
WI7-43			alfalfa	WI	Dane	08/14/2013	0	wiaug2013	-
WI7-44			alfalfa	WI	Dane	08/14/2013	0	wiaug2013	-
WI7-45			alfalfa	WI	Dane	08/14/2013	0	wiaug2013	-
WI7-46			alfalfa	WI	Dane	08/14/2013	0	wiaug2013	-
WI7-8			alfalfa	WI	Dane	08/14/2013	1	wiaug2013	cdtB1
WI7-9		3	alfalfa	WI	Dane	08/14/2013	1	wiaug2013	YD
WI 1-1	1	3	alfalfa	WI	Dane	10/14/2013	1	wioct13	cdtB2
WI 1-10			alfalfa	WI	Dane	10/14/2013	1	wioct13	cdtB2
WI 1-11			alfalfa	WI	Dane	10/14/2013	1	wioct13	YD
WI 1-12			alfalfa	WI	Dane	10/14/2013	0	wioct13	-
WI 1-13			alfalfa	WI	Dane	10/14/2013	0	wioct13	-
WI 1-14			alfalfa	WI	Dane	10/14/2013	1	wioct13	YD
WI 1-15			alfalfa	WI	Dane	10/14/2013	1	wioct13	YD
WI 1-16			alfalfa	WI	Dane	10/14/2013	1	wioct13	cdtB2
WI 1-17			alfalfa	WI	Dane	10/14/2013	1	wioct13	YD

WI 1-2			alfalfa	WI	Dane	10/14/2013	1	wioct13	cdtB2
WI 1-3			alfalfa	WI	Dane	10/14/2013	1	wioct13	cdtB2
WI 1-4			alfalfa	WI	Dane	10/14/2013	1	wioct13	YD
WI 1-5			alfalfa	WI	Dane	10/14/2013	1	wioct13	YD
WI 1-6			alfalfa	WI	Dane	10/14/2013	1	wioct13	YD
WI 1-7			alfalfa	WI	Dane	10/14/2013	1	wioct13	X
WI 1-8			alfalfa	WI	Dane	10/14/2013	1	wioct13	cdtB2
WI 1-9			alfalfa	WI	Dane	10/14/2013	1	wioct13	cdtB2
WI 2-1			alfalfa	WI	Dane	10/14/2013	1	wioct13	cdtB2
WI 2-10			alfalfa	WI	Dane	10/14/2013	0	wioct13	-
WI 2-11			alfalfa	WI	Dane	10/14/2013	0	wioct13	-
WI 2-12			alfalfa	WI	Dane	10/14/2013	0	wioct13	-
WI 2-13			alfalfa	WI	Dane	10/14/2013	0	wioct13	-
WI 2-14			alfalfa	WI	Dane	10/14/2013	0	wioct13	-
WI 2-15			alfalfa	WI	Dane	10/14/2013	0	wioct13	-
WI 2-16			alfalfa	WI	Dane	10/14/2013	0	wioct13	-
WI 2-17			alfalfa	WI	Dane	10/14/2013	0	wioct13	-
WI 2-18			alfalfa	WI	Dane	10/14/2013	1	wioct13	YD
WI 2-19			alfalfa	WI	Dane	10/14/2013	0	wioct13	-
WI 2-2			alfalfa	WI	Dane	10/14/2013	1	wioct13	cdtB2
WI 2-20			alfalfa	WI	Dane	10/14/2013	1	wioct13	cdtB1
WI 2-21			alfalfa	WI	Dane	10/14/2013	1	wioct13	YD
WI 2-22			alfalfa	WI	Dane	10/14/2013	0	wioct13	-
WI 2-23			alfalfa	WI	Dane	10/14/2013	1	wioct13	YD
WI 2-24			alfalfa	WI	Dane	10/14/2013	1	wioct13	YD
WI 2-25			alfalfa	WI	Dane	10/14/2013	0	wioct13	-
WI 2-26			alfalfa	WI	Dane	10/14/2013	0	wioct13	-
WI 2-27			alfalfa	WI	Dane	10/14/2013	0	wioct13	-
WI 2-28			alfalfa	WI	Dane	10/14/2013	0	wioct13	-
WI 2-29			alfalfa	WI	Dane	10/14/2013	1	wioct13	cdtB1
WI 2-3			alfalfa	WI	Dane	10/14/2013	0	wioct13	-
WI 2-30			alfalfa	WI	Dane	10/14/2013	1	wioct13	YD
WI 2-31			alfalfa	WI	Dane	10/14/2013	0	wioct13	-
WI 2-32			alfalfa	WI	Dane	10/14/2013	1	wioct13	cdtB1
WI 2-33			alfalfa	WI	Dane	10/14/2013	0	wioct13	-
WI 2-34			alfalfa	WI	Dane	10/14/2013	1	wioct13	cdtB2
WI 2-35			alfalfa	WI	Dane	10/14/2013	1	wioct13	cdtB2
WI 2-36			alfalfa	WI	Dane	10/14/2013	0	wioct13	-
WI 2-37			alfalfa	WI	Dane	10/14/2013	0	wioct13	-
WI 2-38			alfalfa	WI	Dane	10/14/2013	0	wioct13	-
WI 2-39			alfalfa	WI	Dane	10/14/2013	1	wioct13	YD

WI 2-4			alfalfa	WI	Dane	10/14/2013	0	wioct13	-
WI 2-40			alfalfa	WI	Dane	10/14/2013	1	wioct13	cdtB1
WI 2-41			alfalfa	WI	Dane	10/14/2013	1	wioct13	cdtB2
WI 2-42			alfalfa	WI	Dane	10/14/2013	0	wioct13	-
WI 2-43			alfalfa	WI	Dane	10/14/2013	1	wioct13	cdtB2
WI 2-44			alfalfa	WI	Dane	10/14/2013	1	wioct13	cdtB1
WI 2-45			alfalfa	WI	Dane	10/14/2013	0	wioct13	-
WI 2-46			alfalfa	WI	Dane	10/14/2013	1	wioct13	X
WI 2-47			alfalfa	WI	Dane	10/14/2013	1	wioct13	cdtB2
WI 2-48			alfalfa	WI	Dane	10/14/2013	1	wioct13	cdtB1
WI 2-49			alfalfa	WI	Dane	10/14/2013	1	wioct13	cdtB1
WI 2-5			alfalfa	WI	Dane	10/14/2013	1	wioct13	YD
WI 2-50			alfalfa	WI	Dane	10/14/2013	0	wioct13	-
WI 2-51			alfalfa	WI	Dane	10/14/2013	0	wioct13	-
WI 2-52			alfalfa	WI	Dane	10/14/2013	1	wioct13	cdtB1
WI 2-53			alfalfa	WI	Dane	10/14/2013	1	wioct13	cdtB2
WI 2-54			alfalfa	WI	Dane	10/14/2013	1	wioct13	cdtB2
WI 2-55			alfalfa	WI	Dane	10/14/2013	1	wioct13	cdtB2
WI 2-56			alfalfa	WI	Dane	10/14/2013	1	wioct13	cdtB1
WI 2-57			alfalfa	WI	Dane	10/14/2013	1	wioct13	YD
WI 2-58			alfalfa	WI	Dane	10/14/2013	1	wioct13	cdtB1
WI 2-59			alfalfa	WI	Dane	10/14/2013	0	wioct13	-
WI 2-6			alfalfa	WI	Dane	10/14/2013	0	wioct13	-
WI 2-60			alfalfa	WI	Dane	10/14/2013	0	wioct13	-
WI 2-61			alfalfa	WI	Dane	10/14/2013	0	wioct13	-
WI 2-62			alfalfa	WI	Dane	10/14/2013	1	wioct13	cdtB2
WI 2-63			alfalfa	WI	Dane	10/14/2013	1	wioct13	cdtB2
WI 2-64			alfalfa	WI	Dane	10/14/2013	0	wioct13	-
WI 2-65			alfalfa	WI	Dane	10/14/2013	1	wioct13	cdtB2
WI 2-7			alfalfa	WI	Dane	10/14/2013	1	wioct13	YD
WI 2-8			alfalfa	WI	Dane	10/14/2013	1	wioct13	cdtB2
WI 2-9			alfalfa	WI	Dane	10/14/2013	1	wioct13	cdtB2
WI 3-1			alfalfa	WI	Dane	10/14/2013	0	wioct13	-
WI 3-10			alfalfa	WI	Dane	10/14/2013	1	wioct13	cdtB2
WI 3-11			alfalfa	WI	Dane	10/14/2013	1	wioct13	cdtB2
WI 3-12			alfalfa	WI	Dane	10/14/2013	1	wioct13	YD
WI 3-13			alfalfa	WI	Dane	10/14/2013	1	wioct13	YD
WI 3-14			alfalfa	WI	Dane	10/14/2013	1	wioct13	YD
WI 3-2			alfalfa	WI	Dane	10/14/2013	0	wioct13	-
WI 3-3			alfalfa	WI	Dane	10/14/2013	0	wioct13	-
WI 3-4			alfalfa	WI	Dane	10/14/2013	0	wioct13	-

WI 3-5			alfalfa	WI	Dane	10/14/2013	0	wioct13	-
WI 3-6			alfalfa	WI	Dane	10/14/2013	0	wioct13	-
WI 3-7			alfalfa	WI	Dane	10/14/2013	1	wioct13	YD
WI 3-8			alfalfa	WI	Dane	10/14/2013	1	wioct13	cdtB2
WI 3-9			alfalfa	WI	Dane	10/14/2013	1	wioct13	cdtB1
WI 4-46			alfalfa	WI	Dane	10/14/2013	0	wioct13	-
WI 4-47			alfalfa	WI	Dane	10/14/2013	1	wioct13	cdtB1
WI 4-48			alfalfa	WI	Dane	10/14/2013	0	wioct13	-
WI 4-49			alfalfa	WI	Dane	10/14/2013	1	wioct13	YD
WI 4-50			alfalfa	WI	Dane	10/14/2013	1	wioct13	X
WI 4-51			alfalfa	WI	Dane	10/14/2013	0	wioct13	-
WI 4-52			alfalfa	WI	Dane	10/14/2013	1	wioct13	X
WI 4-53			alfalfa	WI	Dane	10/14/2013	1	wioct13	YD
WI 4-54			alfalfa	WI	Dane	10/14/2013	0	wioct13	-
WI 4-55			alfalfa	WI	Dane	10/14/2013	0	wioct13	-
WI 4-56			alfalfa	WI	Dane	10/14/2013	1	wioct13	YD
WI 4-57			alfalfa	WI	Dane	10/14/2013	0	wioct13	-
WI 4-58			alfalfa	WI	Dane	10/14/2013	0	wioct13	-
WI 4-59			alfalfa	WI	Dane	10/14/2013	0	wioct13	-
WI 4-60			alfalfa	WI	Dane	10/14/2013	0	wioct13	-
WI 4-61			alfalfa	WI	Dane	10/14/2013	1	wioct13	cdtB1
WI 4-62			alfalfa	WI	Dane	10/14/2013	0	wioct13	-
WI 4-63			alfalfa	WI	Dane	10/14/2013	0	wioct13	-
WI 4-64			alfalfa	WI	Dane	10/14/2013	0	wioct13	-
WI 4-65			alfalfa	WI	Dane	10/14/2013	1	wioct13	YD
WI 4-66			alfalfa	WI	Dane	10/14/2013	1	wioct13	YD
WI 4-67			alfalfa	WI	Dane	10/14/2013	0	wioct13	-
WI 4-68			alfalfa	WI	Dane	10/14/2013	0	wioct13	-
WI 4-69			alfalfa	WI	Dane	10/14/2013	1	wioct13	cdtB2
WI 4-70			alfalfa	WI	Dane	10/14/2013	0	wioct13	-
WI 4-71			alfalfa	WI	Dane	10/14/2013	0	wioct13	-
WI 4-72			alfalfa	WI	Dane	10/14/2013	1	wioct13	cdtB1
WI 4-73			alfalfa	WI	Dane	10/14/2013	1	wioct13	X
WI 4-74			alfalfa	WI	Dane	10/14/2013	1	wioct13	YD
WI 4-75			alfalfa	WI	Dane	10/14/2013	0	wioct13	-
WI 4-76			alfalfa	WI	Dane	10/14/2013	0	wioct13	-
WI 4-77			alfalfa	WI	Dane	10/14/2013	1	wioct13	cdtB2
WI 4-78			alfalfa	WI	Dane	10/14/2013	0	wioct13	-
WI 4-79			alfalfa	WI	Dane	10/14/2013	1	wioct13	cdtB2
WI 4-80			alfalfa	WI	Dane	10/14/2013	1	wioct13	YD
WI 5-1			alfalfa	WI	Dane	10/14/2013	1	wioct13	cdtB2

WI 5-10			alfalfa	WI	Dane	10/14/2013	0	wioct13	-
WI 5-11			alfalfa	WI	Dane	10/14/2013	0	wioct13	-
WI 5-12			alfalfa	WI	Dane	10/14/2013	1	wioct13	X
WI 5-13			alfalfa	WI	Dane	10/14/2013	0	wioct13	-
WI 5-14			alfalfa	WI	Dane	10/14/2013	0	wioct13	-
WI 5-15			alfalfa	WI	Dane	10/14/2013	1	wioct13	YD
WI 5-16			alfalfa	WI	Dane	10/14/2013	0	wioct13	-
WI 5-17			alfalfa	WI	Dane	10/14/2013	0	wioct13	-
WI 5-18			alfalfa	WI	Dane	10/14/2013	0	wioct13	-
WI 5-19			alfalfa	WI	Dane	10/14/2013	1	wioct13	YD
WI 5-2			alfalfa	WI	Dane	10/14/2013	1	wioct13	cdtB1
WI 5-20			alfalfa	WI	Dane	10/14/2013	0	wioct13	-
WI 5-21			alfalfa	WI	Dane	10/14/2013	1	wioct13	stx
WI 5-22			alfalfa	WI	Dane	10/14/2013	0	wioct13	-
WI 5-3			alfalfa	WI	Dane	10/14/2013	0	wioct13	-
WI 5-4			alfalfa	WI	Dane	10/14/2013	1	wioct13	YD
WI 5-5			alfalfa	WI	Dane	10/14/2013	0	wioct13	-
WI 5-6			alfalfa	WI	Dane	10/14/2013	1	wioct13	YD
WI 5-7			alfalfa	WI	Dane	10/14/2013	1	wioct13	cdtB1
WI 5-8			alfalfa	WI	Dane	10/14/2013	0	wioct13	-
WI 5-9			alfalfa	WI	Dane	10/14/2013	1	wioct13	YD
WI 6-1			alfalfa	WI	Dane	10/14/2013	0	wioct13	-
WI 6-10			alfalfa	WI	Dane	10/14/2013	0	wioct13	-
WI 6-11			alfalfa	WI	Dane	10/14/2013	0	wioct13	-
WI 6-12			alfalfa	WI	Dane	10/14/2013	0	wioct13	-
WI 6-13			alfalfa	WI	Dane	10/14/2013	0	wioct13	-
WI 6-14			alfalfa	WI	Dane	10/14/2013	0	wioct13	-
WI 6-15			alfalfa	WI	Dane	10/14/2013	0	wioct13	-
WI 6-16			alfalfa	WI	Dane	10/14/2013	1	wioct13	YD
WI 6-17			alfalfa	WI	Dane	10/14/2013	1	wioct13	YD
WI 6-18			alfalfa	WI	Dane	10/14/2013	1	wioct13	YD
WI 6-19			alfalfa	WI	Dane	10/14/2013	1	wioct13	YD
WI 6-2			alfalfa	WI	Dane	10/14/2013	0	wioct13	-
WI 6-20			alfalfa	WI	Dane	10/14/2013	0	wioct13	-
WI 6-21			alfalfa	WI	Dane	10/14/2013	0	wioct13	-
WI 6-22			alfalfa	WI	Dane	10/14/2013	0	wioct13	-
WI 6-23			alfalfa	WI	Dane	10/14/2013	1	wioct13	YD
WI 6-24			alfalfa	WI	Dane	10/14/2013	0	wioct13	-
WI 6-25			alfalfa	WI	Dane	10/14/2013	0	wioct13	-
WI 6-26			alfalfa	WI	Dane	10/14/2013	0	wioct13	-
WI 6-27			alfalfa	WI	Dane	10/14/2013	0	wioct13	-

WI 6-28			alfalfa	WI	Dane	10/14/2013	1	wioct13	X
WI 6-29			alfalfa	WI	Dane	10/14/2013	0	wioct13	-
WI 6-3			alfalfa	WI	Dane	10/14/2013	0	wioct13	-
WI 6-30			alfalfa	WI	Dane	10/14/2013	1	wioct13	YD
WI 6-31			alfalfa	WI	Dane	10/14/2013	0	wioct13	-
WI 6-32			alfalfa	WI	Dane	10/14/2013	1	wioct13	cdtB1
WI 6-33			alfalfa	WI	Dane	10/14/2013	1	wioct13	YD
WI 6-34			alfalfa	WI	Dane	10/14/2013	1	wioct13	YD
WI 6-35			alfalfa	WI	Dane	10/14/2013	0	wioct13	-
WI 6-36			alfalfa	WI	Dane	10/14/2013	1	wioct13	YD
WI 6-37			alfalfa	WI	Dane	10/14/2013	0	wioct13	-
WI 6-38			alfalfa	WI	Dane	10/14/2013	1	wioct13	cdtB2
WI 6-39			alfalfa	WI	Dane	10/14/2013	0	wioct13	-
WI 6-4			alfalfa	WI	Dane	10/14/2013	0	wioct13	-
WI 6-40			alfalfa	WI	Dane	10/14/2013	0	wioct13	-
WI 6-41			alfalfa	WI	Dane	10/14/2013	1	wioct13	YD
WI 6-42			alfalfa	WI	Dane	10/14/2013	0	wioct13	-
WI 6-43			alfalfa	WI	Dane	10/14/2013	1	wioct13	YD
WI 6-44			alfalfa	WI	Dane	10/14/2013	0	wioct13	-
WI 6-45			alfalfa	WI	Dane	10/14/2013	1	wioct13	cdtB1
WI 6-46			alfalfa	WI	Dane	10/14/2013	1	wioct13	YD
WI 6-47			alfalfa	WI	Dane	10/14/2013	0	wioct13	-
WI 6-48			alfalfa	WI	Dane	10/14/2013	1	wioct13	cdtB1
WI 6-49			alfalfa	WI	Dane	10/14/2013	0	wioct13	-
WI 6-5			alfalfa	WI	Dane	10/14/2013	1	wioct13	YD
WI 6-50			alfalfa	WI	Dane	10/14/2013	0	wioct13	-
WI 6-51			alfalfa	WI	Dane	10/14/2013	0	wioct13	-
WI 6-52			alfalfa	WI	Dane	10/14/2013	1	wioct13	YD
WI 6-53			alfalfa	WI	Dane	10/14/2013	1	wioct13	YD
WI 6-54			alfalfa	WI	Dane	10/14/2013	1	wioct13	cdtB1
WI 6-55			alfalfa	WI	Dane	10/14/2013	1	wioct13	cdtB1
WI 6-56			alfalfa	WI	Dane	10/14/2013	1	wioct13	cdtB2
WI 6-57			alfalfa	WI	Dane	10/14/2013	1	wioct13	YD
WI 6-58			alfalfa	WI	Dane	10/14/2013	1	wioct13	cdtB1
WI 6-59			alfalfa	WI	Dane	10/14/2013	0	wioct13	-
WI 6-6			alfalfa	WI	Dane	10/14/2013	1	wioct13	X
WI 6-60			alfalfa	WI	Dane	10/14/2013	0	wioct13	-
WI 6-61			alfalfa	WI	Dane	10/14/2013	1	wioct13	YD
WI 6-62			alfalfa	WI	Dane	10/14/2013	1	wioct13	YD
WI 6-63			alfalfa	WI	Dane	10/14/2013	1	wioct13	YD
WI 6-64			alfalfa	WI	Dane	10/14/2013	0	wioct13	-

WI 6-65			alfalfa	WI	Dane	10/14/2013	1	wioct13	YD
WI 6-66			alfalfa	WI	Dane	10/14/2013	0	wioct13	-
WI 6-67			alfalfa	WI	Dane	10/14/2013	1	wioct13	cdtB1
WI 6-68			alfalfa	WI	Dane	10/14/2013	0	wioct13	-
WI 6-69			alfalfa	WI	Dane	10/14/2013	1	wioct13	YD
WI 6-7			alfalfa	WI	Dane	10/14/2013	0	wioct13	-
WI 6-70			alfalfa	WI	Dane	10/14/2013	1	wioct13	YD
WI 6-71			alfalfa	WI	Dane	10/14/2013	1	wioct13	YD
WI 6-72			alfalfa	WI	Dane	10/14/2013	1	wioct13	YD
WI 6-73			alfalfa	WI	Dane	10/14/2013	0	wioct13	-
WI 6-74			alfalfa	WI	Dane	10/14/2013	1	wioct13	YD
WI 6-75			alfalfa	WI	Dane	10/14/2013	1	wioct13	cdtB1
WI 6-76			alfalfa	WI	Dane	10/14/2013	0	wioct13	-
WI 6-77			alfalfa	WI	Dane	10/14/2013	1	wioct13	YD
WI 6-78			alfalfa	WI	Dane	10/14/2013	1	wioct13	cdtB1
WI 6-79			alfalfa	WI	Dane	10/14/2013	1	wioct13	X
WI 6-8			alfalfa	WI	Dane	10/14/2013	1	wioct13	cdtB2
WI 6-9			alfalfa	WI	Dane	10/14/2013	0	wioct13	-
WI14-499			alfalfa	WI	Dane	10/09/2014	0	wioct14	-
WI14-500			alfalfa	WI	Dane	10/09/2014	1	wioct14	YD
WI14-501			alfalfa	WI	Dane	10/09/2014	1	wioct14	cdtB2
WI14-502			alfalfa	WI	Dane	10/09/2014	1	wioct14	cdtB2
WI14-503			alfalfa	WI	Dane	10/09/2014	1	wioct14	YD
WI14-504			alfalfa	WI	Dane	10/09/2014	0	wioct14	-
WI14-505			alfalfa	WI	Dane	10/09/2014	1	wioct14	cdtB2
WI14-506			alfalfa	WI	Dane	10/09/2014	1	wioct14	cdtB2
WI14-507			alfalfa	WI	Dane	10/09/2014	0	wioct14	-
WI14-508			alfalfa	WI	Dane	10/09/2014	1	wioct14	YD
WI14-509			alfalfa	WI	Dane	10/09/2014	1	wioct14	YD
WI14-510			alfalfa	WI	Dane	10/09/2014	1	wioct14	YD
WI14-511			alfalfa	WI	Dane	10/07/2014	0	wioct14	-
WI14-512			alfalfa	WI	Dane	10/07/2014	0	wioct14	-
WI14-513			alfalfa	WI	Dane	10/07/2014	0	wioct14	-
WI14-514			alfalfa	WI	Dane	10/07/2014	1	wioct14	cdtB1
WI14-515			alfalfa	WI	Dane	10/07/2014	0	wioct14	-
WI14-516			alfalfa	WI	Dane	10/07/2014	0	wioct14	-
WI14-517			alfalfa	WI	Dane	10/07/2014	0	wioct14	-
WI14-518			alfalfa	WI	Dane	10/07/2014	1	wioct14	cdtB2
WI14-519			alfalfa	WI	Dane	10/07/2014	1	wioct14	cdtB1
WI14-520			alfalfa	WI	Dane	10/07/2014	0	wioct14	-
WI14-521			alfalfa	WI	Dane	10/07/2014	1	wioct14	cdtB1

WI14-522			alfalfa	WI	Dane	10/07/2014	1	wioct14	cdtB1
WI14-523			alfalfa	WI	Dane	10/07/2014	1	wioct14	cdtB2
WI14-524			alfalfa	WI	Dane	10/07/2014	1	wioct14	cdtB1
WI14-525			alfalfa	WI	Dane	10/07/2014	1	wioct14	cdtB1
WI14-526			alfalfa	WI	Dane	10/07/2014	0	wioct14	-
WI14-527			alfalfa	WI	Dane	10/07/2014	1	wioct14	cdtB2
WI14-528			alfalfa	WI	Dane	10/07/2014	0	wioct14	-
WI14-529			alfalfa	WI	Dane	10/07/2014	1	wioct14	cdtB1
WI14-530			alfalfa	WI	Dane	10/07/2014	1	wioct14	cdtB1
WI14-531			alfalfa	WI	Dane	10/07/2014	0	wioct14	-
WI14-532			alfalfa	WI	Dane	10/07/2014	1	wioct14	cdtB2
WI14-533			alfalfa	WI	Dane	10/07/2014	1	wioct14	cdtB1
WI14-534			alfalfa	WI	Dane	10/07/2014	1	wioct14	YD
WI14-535			alfalfa	WI	Dane	10/07/2014	0	wioct14	-
WI14-536			alfalfa	WI	Dane	10/07/2014	0	wioct14	-
WI14-537			alfalfa	WI	Dane	10/07/2014	0	wioct14	-
WI14-538			alfalfa	WI	Dane	10/07/2014	1	wioct14	cdtB2
WI14-539			alfalfa	WI	Dane	10/07/2014	1	wioct14	stx
WI14-540			alfalfa	WI	Dane	10/07/2014	0	wioct14	-
WI14-541			alfalfa	WI	Dane	10/07/2014	1	wioct14	YD
WI14-542			alfalfa	WI	Dane	10/07/2014	0	wioct14	-
WI14-543			alfalfa	WI	Dane	10/07/2014	1	wioct14	cdtB1
WI14-544			alfalfa	WI	Dane	10/07/2014	1	wioct14	cdtB1
WI14-545			alfalfa	WI	Dane	10/07/2014	0	wioct14	-
WI14-546			alfalfa	WI	Dane	10/07/2014	1	wioct14	YD
WI14-547		2	alfalfa	WI	Dane	10/09/2014	1	wioct14	stx
WI14-548			alfalfa	WI	Dane	10/09/2014	1	wioct14	cdtB1
WI14-549			alfalfa	WI	Dane	10/09/2014	1	wioct14	cdtB1
WI14-550			alfalfa	WI	Dane	10/09/2014	0	wioct14	-
WI14-551			alfalfa	WI	Dane	10/09/2014	0	wioct14	-
WI14-552			alfalfa	WI	Dane	10/09/2014	0	wioct14	-
WI14-553			alfalfa	WI	Dane	10/09/2014	0	wioct14	-
WI14-554			alfalfa	WI	Dane	10/09/2014	1	wioct14	stx
WI14-555			alfalfa	WI	Dane	10/09/2014	0	wioct14	-
WI14-556			alfalfa	WI	Dane	10/09/2014	0	wioct14	-
WI14-557			alfalfa	WI	Dane	10/09/2014	0	wioct14	-
WI14-558			alfalfa	WI	Dane	10/09/2014	1	wioct14	YD
WI14-559			alfalfa	WI	Dane	10/09/2014	0	wioct14	-
WI14-560			alfalfa	WI	Dane	10/09/2014	1	wioct14	cdtB2
WI14-561			alfalfa	WI	Dane	10/09/2014	1	wioct14	stx
WI14-562			alfalfa	WI	Dane	10/09/2014	0	wioct14	-

WI14-563			alfalfa	WI	Dane	10/09/2014	0	wioct14	-
WI14-564			alfalfa	WI	Dane	10/09/2014	0	wioct14	-
WI14-565			alfalfa	WI	Dane	10/09/2014	1	wioct14	stx
WI14-566			alfalfa	WI	Dane	10/09/2014	1	wioct14	cdtB1
WI14-567			alfalfa	WI	Dane	10/09/2014	1	wioct14	stx
WI14-568			alfalfa	WI	Dane	10/09/2014	1	wioct14	stx
WI14-569			alfalfa	WI	Dane	10/09/2014	1	wioct14	cdtB2
WI14-570			alfalfa	WI	Dane	10/09/2014	0	wioct14	-
WI14-571			alfalfa	WI	Dane	10/09/2014	0	wioct14	-
WI14-572			alfalfa	WI	Dane	10/09/2014	1	wioct14	stx
WI14-573			alfalfa	WI	Dane	10/09/2014	0	wioct14	-
WI14-574			alfalfa	WI	Dane	10/09/2014	1	wioct14	stx
WI14-575			alfalfa	WI	Dane	10/09/2014	1	wioct14	cdtB1
WI14-576			alfalfa	WI	Dane	10/09/2014	0	wioct14	-
WI14-577			alfalfa	WI	Dane	10/09/2014	1	wioct14	cdtB1
WI14-578			alfalfa	WI	Dane	10/09/2014	1	wioct14	cdtB1
WI14-579			alfalfa	WI	Dane	10/09/2014	1	wioct14	stx
WI14-580			alfalfa	WI	Dane	10/09/2014	1	wioct14	YD
WI14-581			alfalfa	WI	Dane	10/09/2014	1	wioct14	cdtB1
WI14-582			alfalfa	WI	Dane	10/09/2014	1	wioct14	X
WI14-583			alfalfa	WI	Dane	10/09/2014	1	wioct14	stx
WI14-584			alfalfa	WI	Dane	10/09/2014	0	wioct14	-
WI14-585			alfalfa	WI	Dane	10/07/2014	1	wioct14	cdtB1
WI14-586			alfalfa	WI	Dane	10/07/2014	0	wioct14	-
WI14-587			alfalfa	WI	Dane	10/07/2014	0	wioct14	-
WI14-588			alfalfa	WI	Dane	10/07/2014	0	wioct14	-
WI14-589			alfalfa	WI	Dane	10/07/2014	1	wioct14	cdtB2
WI14-590			alfalfa	WI	Dane	10/07/2014	0	wioct14	-
WI14-591			alfalfa	WI	Dane	10/07/2014	0	wioct14	-
WI14-592			alfalfa	WI	Dane	10/07/2014	0	wioct14	-
WI14-593			alfalfa	WI	Dane	10/07/2014	0	wioct14	-
WI14-594			alfalfa	WI	Dane	10/07/2014	0	wioct14	-
WI14-595			alfalfa	WI	Dane	10/07/2014	1	wioct14	YD
WI14-596			alfalfa	WI	Dane	10/07/2014	0	wioct14	-
WI14-597			alfalfa	WI	Dane	10/07/2014	0	wioct14	-
WI14-598			alfalfa	WI	Dane	10/07/2014	0	wioct14	-
WI14-599			alfalfa	WI	Dane	10/07/2014	0	wioct14	-
WI14-600			alfalfa	WI	Dane	10/07/2014	0	wioct14	-
WI14-601			alfalfa	WI	Dane	10/07/2014	1	wioct14	cdtB1
WI14-602			alfalfa	WI	Dane	10/07/2014	0	wioct14	-
WI14-603			alfalfa	WI	Dane	10/07/2014	0	wioct14	-

WI14-604			alfalfa	WI	Dane	10/07/2014	0	wioct14	-
WI14-605		3	alfalfa	WI	Dane	10/07/2014	1	wioct14	none
WI14-606			alfalfa	WI	Dane	10/07/2014	0	wioct14	-
WI14-607			alfalfa	WI	Dane	10/07/2014	0	wioct14	-
WI14-608			alfalfa	WI	Dane	10/07/2014	0	wioct14	-
WI14-609			alfalfa	WI	Dane	10/07/2014	0	wioct14	-
WI14-610			alfalfa	WI	Dane	10/07/2014	0	wioct14	-
WI14-611			alfalfa	WI	Dane	10/07/2014	0	wioct14	-
WI14-612			alfalfa	WI	Dane	10/07/2014	1	wioct14	YD
WI14-613			alfalfa	WI	Dane	10/07/2014	0	wioct14	-
WI14-614			alfalfa	WI	Dane	10/07/2014	0	wioct14	-
WI14-615			alfalfa	WI	Dane	10/07/2014	0	wioct14	-
WI14-616			alfalfa	WI	Dane	10/07/2014	1	wioct14	cdtB1
WI14-617			alfalfa	WI	Dane	10/07/2014	0	wioct14	-
WI14-618			alfalfa	WI	Dane	10/07/2014	0	wioct14	-
WI14-619			alfalfa	WI	Dane	10/07/2014	1	wioct14	cdtB1
WI14-620			alfalfa	WI	Dane	10/07/2014	0	wioct14	-
WI14-621			alfalfa	WI	Dane	10/07/2014	0	wioct14	-
WI14-622			alfalfa	WI	Dane	10/07/2014	0	wioct14	-
WI14-623			alfalfa	WI	Dane	10/07/2014	0	wioct14	-
WI14-624			alfalfa	WI	Dane	10/07/2014	0	wioct14	-
WI14-625			alfalfa	WI	Dane	10/07/2014	0	wioct14	-
WI14-626			alfalfa	WI	Dane	10/07/2014	0	wioct14	-
WI14-627			alfalfa	WI	Dane	10/07/2014	0	wioct14	-
WI14-628			alfalfa	WI	Dane	10/07/2014	1	wioct14	YD
WI14-629			alfalfa	WI	Dane	10/07/2014	0	wioct14	-
WI14-630			alfalfa	WI	Dane	10/07/2014	0	wioct14	-
WI14-631			alfalfa	WI	Dane	10/07/2014	0	wioct14	-
WI14-632			alfalfa	WI	Dane	10/07/2014	1	wioct14	YD
WI14-633			alfalfa	WI	Dane	10/07/2014	1	wioct14	cdtB1
WI14-634			alfalfa	WI	Dane	10/17/2014	1	wioct14	cdtB1
WI14-635			alfalfa	WI	Dane	10/17/2014	0	wioct14	-
WI14-636			alfalfa	WI	Dane	10/17/2014	0	wioct14	-
WI14-637			alfalfa	WI	Dane	10/17/2014	1	wioct14	YD
WI14-638			alfalfa	WI	Dane	10/17/2014	1	wioct14	stx
WI14-639			alfalfa	WI	Dane	10/17/2014	1	wioct14	stx
WI14-640			alfalfa	WI	Dane	10/17/2014	1	wioct14	stx
WI14-641			alfalfa	WI	Dane	10/17/2014	1	wioct14	YD
WI14-642			alfalfa	WI	Dane	10/17/2014	1	wioct14	stx
WI14-643			alfalfa	WI	Dane	10/17/2014	0	wioct14	-
WI14-644			alfalfa	WI	Dane	10/17/2014	1	wioct14	cdtB1

WI14-645			alfalfa	WI	Dane	10/17/2014	1	wioct14	stx
WI14-646			alfalfa	WI	Dane	10/17/2014	0	wioct14	-
WI14-647			alfalfa	WI	Dane	10/17/2014	1	wioct14	cdtB1
WI14-648			alfalfa	WI	Dane	10/17/2014	0	wioct14	-
WI14-649			alfalfa	WI	Dane	10/17/2014	0	wioct14	-
WI14-650			alfalfa	WI	Dane	10/17/2014	0	wioct14	-
WI14-651			alfalfa	WI	Dane	10/17/2014	1	wioct14	YD
WI14-652			alfalfa	WI	Dane	10/17/2014	1	wioct14	stx
WI14-653			alfalfa	WI	Dane	10/17/2014	1	wioct14	cdtB2
WI14-654			alfalfa	WI	Dane	10/17/2014	1	wioct14	stx
WI14-655			alfalfa	WI	Dane	10/17/2014	0	wioct14	-
WI14-656			alfalfa	WI	Dane	10/17/2014	0	wioct14	-
WI14-657			alfalfa	WI	Dane	10/17/2014	1	wioct14	cdtB1
WI14-658			alfalfa	WI	Dane	10/17/2014	1	wioct14	cdtB1
WI14-659			alfalfa	WI	Dane	10/17/2014	0	wioct14	-
WI14-660		2	alfalfa	WI	Dane	10/17/2014	1	wioct14	stx
WI14-661			alfalfa	WI	Dane	10/17/2014	1	wioct14	stx
WI14-662			alfalfa	WI	Dane	10/17/2014	1	wioct14	cdtB2
WI14-663			alfalfa	WI	Dane	10/17/2014	0	wioct14	-
WI14-664			alfalfa	WI	Dane	10/17/2014	0	wioct14	-
WI14-665			alfalfa	WI	Dane	10/17/2014	1	wioct14	cdtB2
WI14-666			alfalfa	WI	Dane	10/17/2014	1	wioct14	cdtB2
WI14-667			alfalfa	WI	Dane	10/17/2014	0	wioct14	-
WI14-668			alfalfa	WI	Dane	10/17/2014	0	wioct14	-
WI14-669			alfalfa	WI	Dane	10/17/2014	0	wioct14	-

Table S3.2 Primers and conditions. Until bar: 94C for 2 min, then underwent 35 cycles of 94C for 30 s, 58C for 45 s, 72C for 30 s and then a final 5 min extension at 72C before being held at 4C. Below bar: 94C for 4 min, cycled 25 x 94 C for 1 min 50 C for 1 min 72 C for 2 min, then 10 min 72C and finally held at 4C indefinitely; or with P2 qPCR primers.

APSE2/8 Variable Cassette Region primer set			
Primer name	**5'-seq-3'**	**nucleotide (APSE2 ref genome)**	**location**
vrs1f	GCGGTCCGCAAAAAGTTTGGT	4186	P5
vrs1R	AGACCATGCATTAATTTTCGCTCCTGT	4690	inter (P5-A)
vrs2f	CGACAACGGAAAAGGCTCTGCT	4612	inter (P5-A)
vrs2r	GCCATCCCTTTGCCTTCGTCAT	5159	CDS A
vrs3f	CGAAGGCAAAGGGATGGCAATGT	5142	CDS A
vrs3r	TCGCACTCACAACACCACCAC	5803	CDS B
vrs4f	TGGCTGGTAATTCTTATGCGATGCAA	5747	CDS B
vrs4r	GCGTTTGACAATGACTTCTATGCCG	6265	inter (CDS B-cdtB)
vrs5f	GCACTAAGCGATGTTATCGGCATAGAA	6224	inter (CDS B-cdtB)
vrs5r	TGCCAACTATACTCTCTCACTGTTCCA	6730	cdtB
vdr6f	GCTGGAGCGCTTCCTACATCATCG	6576	cdtB
vrs6r	CCCTGATGGCCTACCGGATTAGT	7375	CDS D
vrs7f	TGGTGTATTGGGTGGGTCAGCA	7202	cdtB
vrs7r	CGTATATGCCGTATCCGAAGGCG	7916	CDS D
vrs8f	TCGAGCCAAAAACGGTGGAGGA	7813	CDS D
vrs8r	TGTCGTCACAAGATAAACGTGCCG	8612	inter (CDS D-P11)
vrs9f	AGGGGCTGTGGTCACGAGTT	8427	inter (CDS D-P11)
vrs9r	CCAAGCTTTTTCTTCAACCAACTGCCA	9123	P11
APSE2VR4.1F	CTCGCCGTGCGATTAGACAT	4100	P5
APSE2VR5.1R	TTGCCATCCCTTTGCCTTCG	5100	CDS A
APSE2VR5.0F	TGGAAAACCGCAAACAACCA	5000	CDS A
APSE2VR6.0R	ACACCAGTTAGCAGTGAAGT	6000	CDS B
APSE2VR5.8F	TGCCCTGATGATGGAAATTGT	5800	CDS B
APSE2VR6.9R	GGTCGATTTATATTAGTACGACGTT	6900	cdtB
APSE2VR6.8F	ACGTGATGAGTAACGGAAACCA	6800	cdtB
APSE2VR7.8R	TCTTGTAAAATCCTCCACCGT	7800	CDS D
APSE2VR7.7F	TCAGGTAGACCAGTTAATCATGTG	7700	CDS D
APSE2VR9.0R	GCCACCGAACCGAATAAAGC	9000	P11

Table S3.3 All sequences used in tree construction included, including those from prior studies; detailed references for individual clones in Table S1; sequences from *Arsenophonus* were included in dN/dS calculations for P3 but excluded for *P45* due to frame-shift issues. All *Uroleucon* samples excluded from hrpA *due to frameshift.* jModelTest2 run with default settings apart from number of substitution schemes decreased from 11 to 7. All +I and +G in PhyML set to "estimate" the relevant parameter.

	N	length	best fit tree	AT%	dN/dS
P3	114	792	GTR+G	55	0.17584
P35	113	624	F81	51	0.88678
P41	103	783	TVMef+I+G	52	0.16926
P45	122	504	TVMef+G	50	0.16422
P51	119	774	HKY+I+G	54	0.25825
concat set:			TVM +I +G	n/a	n/a
accD	122	396	TPM1uf	53	5.00E-09
hrpA	122	692	HKY+I	64	0.16752
recJ	117	672	HKY+I	53	0.30703
murE	109	762	TVM+G	56	0.23927
concat set:	92	2617	TVM +I +G	n/a	n/a

Supplemental tables 3.4 Fischer p-values from comparisons of observed and expected values. The single "phageless" clone and the unclear samples are excluded from all final comparisons. Bonferroni corrections were made by dividing 0.05/the total number of comparisons in the subset; significant p values are starred. Samples all represented in **Supplemental table 3.1**.

S3.4A: H. defensa haplotype vs. toxin

H. defensa haplotype	*cdtB1*	*cdtB2*	*stx*	*yd*
1	0.0032	0.0583	0.2449	0.0064
12	1.0000	1.0000	1.0000	1.0000
17	1.0000	1.0000	1.0000	1.0000
2	0.0135	0.2754	0.1161	1.0000
3	0.0135	1.0000	1.0000	0.0265
4	1.0000	0.1199	1.0000	1.0000
9	1.0000	1.0000	1.0000	1.0000

p-value cut-off: 0.0018

S3.4B: Matriline vs. toxin

Matriline	*cdtB1*	*cdtB2*	*stx*	*yd*
1	0.4792	0.7901	1.0000	1.0000
2	0.3638	0.4917	0.4961	1.0000

p-value cut-off: 0.0063

S3.4C: Matriline vs *H. defensa* haplotype

Matriline	1	17	2	3	4	9
1	0.5210	1.0000	0.0554	0.7830	1.0000	1.0000
2	0.2414	1.0000	0.0580	1.0000	1.0000	1.0000

p-value cut-off: 0.0042

S3.4D: Wisconsin set, *H. defensa* infection vs. month

Month	*H. defensa* infection
May	1.0000
August	0.9272
September	1.0000
October	0.9095

p-value cut-off: 0.013

S3.4E: Wisconsin set, *H. defensa* infection vs. year

Collection year	*H. defensa* infection
2011	1.0000
2012	0.3748
2013	0.4963
2014	0.9105

p-value cut-off: 0.013

S3.4F: Wisconsin set, toxin vs. month

Month	*cdtB1*	*cdtB2*	*stx*	*YD*
May	0.0248	1.0000	0.5777	0.1218
August	*0.0027	0.1110	0.7524	0.1664
September	0.7728	0.1759	1.0000	0.0307
October	0.6120	0.9129	1.0000	1.0000

p-value cut-off: 0.0031

S3.4G: Wisconsin set, toxin vs. year

Collection year	*cdtB1*	*cdtB2*	*stx*	*YD*
2011	1.0000	0.1759	1.0000	0.0307
2012	0.2057	0.7524	1.0000	0.0690
2013	1.0000	0.6155	*0.0023	0.3906
2014	0.5281	1.0000	0.0404	0.7891

p-value cut-off: 0.0031

CHAPTER 4

COSTS AND BENFITS OF COINFECTION BY MULTIPLE DEFENSIVE MUTUALISTS[1]

[1]Weldon S.R., and K.M. Oliver. To be submitted to *Journal of Evolutionary Biology*.

4.1 Abstract

Worldwide, many economically important insect species are infected with heritable bacterial symbionts that mediate ecological interactions affecting their pest status. Pea aphids (*Acyrthosiphon pisum*), long a model for symbiosis research, are infected with a diverse assemblage of symbionts affecting heat tolerance, host-plant utilization, and defense against natural enemies, including biocontrol agents. Infection benefits have almost exclusively been studied in aphids infected with a single symbiont, yet field surveys indicate that many individual aphids are infected with two or more symbiont species (i.e. superinfected). Particular co-infections are more or less common than expected by chance, indicating that specific inhibitory or synergistic symbiont-symbiont interactions may affect frequencies. We investigated the costs of superinfection by two prevalent symbionts, *Hamiltonella defensa*, which protects against parasitoids, and the anti-fungal protector *Regiella insecticola*, and found that superinfection benefited anti-fungal defense, and in certain instances aphid fecundity in the absence of natural enemies. We further found that *H. defensa* titers are unaffected by superinfection, while *R. insecticola* titers increase in response thereto. These benefits are surprising in the light of field surveys, which have found that *H. defensa* and *R. insecticola* rarely share the same host. Further investigation into other factors that may limit host-sharing, such as competitive exclusion during transmission, or into the subtleties of strain-specific coinfection prevalence, may be necessary to account for this apparent contradiction.

4.2 Introduction

Symbiotic bacteria are prevalent in terrestrial arthropods and are major sources of phenotypic diversity, providing their arthropod hosts with nutritional, defensive and immune services (Oliver and Martinez 2014). The importance of indwelling bacterial communities to multicellular organisms has become increasingly evident in recent years (e.g., Ezenwa et al. 2012; Flier and Mekalanos 2009), and the relative simplicity of insect microbial consortia renders insect mutualists interesting both in themselves and as simplified models of host-

microbe interactions in more complex systems. Prolonged association with one or more heritable bacterial symbionts is common across insect species (Moran et al. 2008), and in many insect groups, heritable symbionts occur in multi-species communities (Russell et al. 2013; Ferrari et al. 2012; Chiel et al. 2007; Toju and Fukatsu 2011), but most studies examining symbiont function are based on assays isolating the effects of infection with a single symbiont (Oliver et al. 2014). The forces underlying the maintenance of multiple symbionts, as well as the consequences of co-infection, are poorly understood.

Aphids are important models for studying the phenotypic effects of infection with endosymbionts. Their inherited symbionts can be roughly placed into two groups: obligate symbionts, which are generally defined as those necessary for host survival and reproduction, and facultative symbionts, which are not strictly necessary for host survival (Oliver et al. 2010). Aphids live off of plant phloem, which has a limited essential amino acid profile. The intake gap is made up via an obligate partnership with *Buchnera aphidicola*, which is confined to specialized aphids cells called bacteriocytes (Moran et al. 1993). While all individual aphids harbor the obligate nutritional symbiont *B. aphidicola*, individual aphids may also carry one or more facultative symbionts (Oliver et al. 2010). Some facultative symbionts have been experimentally shown to confer ecologically important traits valuable to the host organism, including heat protection, defense against natural enemies and host-plant specification (Montllor et al. 2002; Koga et al. 2003; Scarborough et al. 2005; Ferrari et al. 2007). Though primarily maternally inherited in insects and thus highly dependent on their current host's reproductive success, facultative symbionts may also be occasionally horizontally transferred resulting in the lateral transfer of ecologically relevant traits (Oliver et al. 2010; Henry et al. 2013). Due to their retained pathogen-like capacity for both horizontal transmission and the invasion of novel host tissues facultative symbionts are more likely than obligate symbionts to encounter other bacteria, exposing them to both sources of genomic diversity and competitive pressures that obligate symbionts have left behind (Gottlieb et al. 2008).

The roles of particular facultative symbionts have been best explored in the pea aphid, *Acyrthosiphon pisum,* where all seven commonly occurring heritable symbionts have been implicated in mediating aphid ecological interactions (Oliver et al. 2010). However, population-level surveys of European and North American pea aphids indicate that as many as 25% of individuals carry between 2 and 4 facultative symbiont species, with population averages ranging from ~0.7 to 3.73 distinct symbionts per individual aphid (Russell et al. 2013; Ferrari et al. 2012; Smith et al. 2015). The prevalence of superinfection in the field is perhaps surprising, given the observed lower fidelity in the transmission of double infections (Sandstrom et al. 2001; personal observations) and additional costs to infection accruing in superinfections (Oliver et al. 2006), potentially due to competition between coinfecting mutualists or between host and symbionts. However, superinfections may also exhibit enhanced mutualistic phenotypes, as seen in the additive anti-wasp defense provided by two coinfecting aphid symbionts (Oliver et al. 2006). Few studies, however, have considered how superinfection affects aphid phenotypes in a systematic fashion.

Two of the best-characterized pea aphid defensive symbionts are *Hamiltonella defensa,* which provides protection against parasitoids (Oliver et al. 2003), and *Regiella insecticola,* which confers resistance to the specialized fungal pathogen *Pandora neoaphidis* (Scarborough et al. 2005). In North America these two symbiont species are widely distributed and often occur at relatively high infection frequencies: they also co-occur naturally as superinfections (Russell et al. 2013).

Infection by *H. defensa* protects the pea aphid from parasitism by a prevalent natural enemy, the endoparasitic koinobiont braconid wasp *Aphidius ervi*. *Ap. ervi* will still oviposit in *H. defensa*-infected aphids, but wasps die before completing development. *H. defensa* can save the reproductive capacity of older parasitized aphids, which are normally castrated by parasitoid venoms (Oliver et al. 2008). *H. defensa* cannot, however, provide this defensive effect on its own: an associated temperate bacteriophage, called APSE, is required for it to be at all

protective (Oliver et al. 2009). APSEs also influence within-host *H. defensa* abundance (Weldon et al. 2013), and, despite being primarily vertically transmitted, can be lost during maternal *H. defensa* transmission and laterally transferred across *H. defensa* strains (Degnan and Moran 2008; Weldon et al. 2013).

The closest known relative of *H. defensa, R. insecticola*, is protective against a variety of entomopathogenic fungi in pea aphids, and is found in about 23% of all aphid species (Henry et al. 2015). Specialized entomopathogenic fungi are important natural enemies of aphids, and defensive symbionts are beneficial due to both an increase in aphid survivorship and reproduction and a decrease in spore production from deceased aphids (Lukasik et al. 2013; Parker et al. 2013; Scarborough et al. 2005). *R. insecticola* has also been associated with changes in wing frequency, host plant utilization, and the timing of sexual reproduction (Ferrari et al. 2007; Leonardo and Mondor 2006; Tsuchida et al. 2004). Different strains of *R. insecticola* may provide different defensive phenotypes: localized Australian populations of the peach-potato aphid, *Myzus persicae,* contain a strain that demonstrated high resistance to the aphid parasitoid, *Aphidius colemani;* thus far only anti-fungal defense has been demonstrated in strains found naturally in pea aphids, though cross-species transfer of strains native to *M. persicae* will produce an anti-aphidiine wasp phenotype in *Ap. fabae* (Vorburger et al. 2010).

Lab-based population cage studies show that parasitism pressure can lead to rapid increases in infection frequencies with defensive symbionts within host populations (Oliver et al. 2008), but field studies correlating enemy-driven mortality and infection frequencies of natural infections find a less direct connection: factors apart from natural enemy prevalence are clearly part of the dynamics of symbiont infection rates in the field (Smith et al. 2015). *R. insecticola* and *Hamiltonella defensa* present a particular paradox: they each show at worst mild negative effects on phenotype in the absence of natural enemies, and provide large benefits against common dangers, but remain at only intermediate levels in pea aphid field populations (Scarborough et al. 2005; Oliver et al. 2008; Russell et al. 2013). Furthermore, coinfections

between *R. insecticola* and *H. defensa* are found in the field at less than one sixth the rate that chance alone would predict, despite their overlapping geographic ranges and non-overlapping protective benefits (Russell et al. 2013), and double-infections by *R. insecticola* and *H. defensa* have been observed reducing to single *H. defensa* infections in lab-held lines (Sandstrom et al. 2001). Coinfections between *H. defensa* and *R. insecticola* therefore provide a suitable system for investigating the potential costs and benefits of superinfection.

To better understand superinfection in *A. pisum* we evaluated superinfections between *H. defensa* and *R. insecticola* relative to singly-infected and uninfected aphids of the same clonal aphid genotype in terms of aphid fitness in the presence and absence of natural enemies. We additionally quantified *B. aphidicola*, *R. insecticola*, and *H. defensa* titers, and measured phage APSE integration rates, over the course of aphid development.

4.3 Methods

4.3.1 Aphid subclone collection and maintenance. *Acyrthosiphon pisum* is a cyclical parthenogen across much of its cosmopolitan range, and clonally reproducing aphids can be maintained indefinitely in the lab using summer-like lighting conditions (Brisson and Stern 2006). All aphids in this study were reared at 20C on a 16L:8D lightcycle at a relative humidity of 70% on *Vicia fava* unless otherwise specified. The three aphid genotypes are naturally uninfected by any secondary symbionts (this was thoroughly confirmed via universal primers with denaturing gradient gel electrophoresis, standard and quantitative PCR with specific primers for the known aphid endosymbionts, and microscopy in (Martinez et al. 2014); the same paper determined that these strains were unique clones via microsatellite analysis), and represent a range of innate immunities, with CJ113 highly resistant to *Ap. ervi* and unresistant to *P. neoaphidis*, PB17 highly resistant to *P. neoaphidis* and unresistant to *Ap. ervi*, and WI27 with high resistance to *Ap. ervi* and moderate resistance to *Pandora neoaphidis* (Martinez et al. 2014; Ben Parker, personal communication). We have used hemolymph-to-hemolymph transfer via glass needles to

produce experimental lines (**Table 4.1**) for use in bioassays (technique adapted from Chen and Purcell 1997).

Table 4.1. Experimental lines combine one cell from each of these three columns: thus each aphid genotype will be used to produce six subclones varying in infection status for 18 treatments in all. Subclones will be referred to below by (infection status)-(genotype), ex. A2C-PB17 for *H. defensa* strain A2C in the PB17 genotype aphid, A2C-5AU-PB17 for *H. defensa* strain A2C and *R. insecticola* strain 5AU coinfecting the same PB17 genotype.

Aphid genotype (collected state, year) [genotypic resistance: *Ap. ervi*/*P. neoaphidis*]	**Strain of *Hamiltonella defensa* (aphid host collection state, year)**	**Strain of *Regiella insecticola* (aphid host collection state, year)**
WI27 (WI, 2011) [high/moderate]	None	None
PB17 (PA, 2011) [low/high]	*H. defensa* phage-free strain A2C (Utah, 2003)	*R. insecticola* strain 5AU (NY, 2000)
CJ113 (UT, 2011) [high/low]	*H. defensa* strain AS3 with phage APSE3 (Utah, 2007)	

The A2C *Hamiltonella defensa* line was originally collected in Utah, and, though uninfected by phage APSE the first time they were screened in the lab, collection location and phylogenetic reconstruction has led to the hypothesis that A2C was likely originally host to APSE3s (Degnan and Moran 2008; Oliver et al. 2009). As a phage free strain of *H. defensa,* A2C provides no known mutualistic benefit. *H. defensa* strain AS3, which provides near-total anti-wasp protection, was also collected from Utah (Oliver et al. 2009), and is 1) host to highly-protective phage strain APSE3 and 2) identical to A2C at all sequenced MLST loci (**§3**). The *Regiella insecticola* line 5AU was collected from a pea aphid strain in New York (Oliver et al. 2003). In all coinfected lines, the *R. insecticola* strain 5AU infection was established first, and the *H. defensa* infection then introduced into the 5AU-infected subclone. All experiments included in this paper occurred minimum of ten generations after the establishment of the injected subclone. Experimental lines were screened for infection status prior to major experimental blocks, and at random intervals over the course of the experimental period, to limit the risks of both cross subclone contamination and infection loss.

4.3.2 Aphid fitness in the absence of natural enemies. All aphid fitness in the absence of natural enemies assays below were adapted from (Oliver et al. 2006). All statistical tests were performed within aphid genotypes (the 6 subclones of CJ113 as one set, and so on for PB17 and WI27) using Dunnet's tests, with the uninfected subclone of the same genotype acting as the control.

Fecundity: 7 day-old ± 12 hour-old aphids were placed in cohorts of three on single fava plants. Starting at a maternal age of 11 days all offspring were removed and counted every 3 days until day 26. Mortality among the reproductive adults was also recorded. Total reproductive output by day 26 was summed.

Fresh weight (FW) and time to first reproduction (TFR): 13 ± 1 day-old aphids were allowed to reproduce for four hours: offspring born in this time period were reared to adulthood under standard conditions. Apterous adults were checked every three hours during the light cycle for onset of reproduction; when it occurred, the time was noted and the adult was weighed live.

4.3.3 Natural enemy challenge: parasitoid wasp *Aphidius ervi*. *Aphidius ervi* (Hymenoptera: Braconidae) is a solitary endoparasitic wasp; the wasps in this study were from a mixed colony of commercially produced (Syngenta Bioline Ltd.) and field-collected *Ap. ervi* reared on susceptible aphids. Mated female wasps were allowed to make a single oviposition into 2^{nd}-3^{rd} instar aphids; aphids were then placed on fresh fava plants in groups of 20 and reared under standard conditions. Nine days after parasitism all aphids were scored as living, mummified, or non-mummified deceased (Oliver et al. 2009). A total of ten replicates were produced for each subclone over the course of three time blocks. Arcsin-transformed mummification, survival, and non-mummified mortality proportions were compared within genotypes using Tukey's HSD; the back-transformed data is presented in **Fig 4.1** and significantly different groups (p=0.05) are marked with different letters.

4.3.4 Natural enemy challenge: entomopathogenic fungus *Pandora neoaphidis*. The corpses of aphids killed by *P. neoaphidis* (genotype ARSEF 2588 – obtained ultimately from the USDAARS Collection of Entomopathogenic Fungal Cultures and proximally from N. Gerardo) and stored post-death for fewer than eight weeks at 4C in a sealed contained with a dessicator bag were removed from storage and placed centrally on fresh 1.5% tap water agar plates in sets of two: the plates were then sealed with parafilm and held in the dark at 20C for 14 hours to encourage sporulation. Plates with large visible spore showers around their corpses were then used in the following exposure protocol: twenty apterous 10 $\pm$ 1 day aphids of each subclone were placed in a 35 mm diameter 10 mm deep petri dish and a fungal plate was inverted over them. After fifteen minutes of exposure, fungal plates were rotated within aphid genotype to normalize the number of spores; this was repeated until total exposure time reached an hour and thirty minutes (i.e. every subclone in a single timeblock would be exposed to the same set of sporulating corpses for the same length of time). Aphids were then placed on fresh fava plants in groups of five at 100% humidity (accomplished via an unvented cup lid) but normal temperature and light conditions for 24 hours. The unvented cup was replaced with a standard vented cup and aphids were checked every twenty-four hours for ten days after fungal exposure for survival, mortality, and sporulation. Corpses were left in place unless/until they sporulated, at which point they were removed to avoid secondary fungal infections. Offspring were thinned out at every check-point to minimize crowding, and plants were changed on an as-needed basis. To better mimic natural conditions, no attempt was made to encourage non-sporulating corpses to sporulate. The assay was performed twice, producing 8 replicates of each treatment, with the exception of AS3-PB17, A2C-PB17, and A2C-5AU-PB17, for which only 7 replicates were produced due to a shortage of non-alate adults in the source populations. Exposure methods were modified from (Ferrari et al. 2001; Lukasik et al. 2013; Parker et al. 2014). Total sporulation proportion over the course of the ten day treatment was arcsin transformed and then compared using Tukey's HSD; the back-transformed data is presented in

Fig 4.2a and significantly different groups (p=0.05) are marked with different letters**.** Survival data is presented as a Kaplan-Meier plot with a probable of 50% survival timepoint (a=0.05) calculated after Weibull-fitting the data; all comparisons were performed within genotypes.

4.3.4 Intrahost PCR-based symbiont quantification. Aphid symbiont levels were estimated from whole aphid extractions of adults and their newborn first. Fragments of the single-copy per bacterium *dnaK* genes from *R. insecticola*, *H. defensa* and the obligate nutritional endosymbiont *B. aphidicola* were amplified via RT-qPCR (Moran et al. 2005; Wilson et al. 2006), using standard reaction conditions described in (Weldon et al. 2013); the *Regiella* primers, new to this study, are: forward (CCG ATG CCA GCG GCC CTA AA) and reverse (TGC TGC ACC AGC GGC ATA CG). APSE was amplified with a forward primer located on the phage (GCA CAC CTT TCG CCC AGC CA) and one of two reverse primers: one, to detect phage integrated into the *H. defensa* genome (CGT GAG AGC GAG GAA AGA TGC GG) and one to amplify nonintegrated phage (GCT GCT TAC TAG AGA GGT GTT GAC GC). Standards were run in duplicate and all experimental samples were run without replicates; outliers (determined on the basis of a non-standard melting temperature for amplification – usually indicative of a pipetting error - or a crossing-point >3 cycles from the treatment median with no other samples from the treatment falling in that range) were rerun and the results of the reruns were used in final reporting unless the rerun indicated that the original data range was reasonable, in which case the original data was used to maintain plate block integrity. Aphid elongation factor 1 α quantity was used as a control of extraction efficiency within genotype x time blocks. For all *dnaK* sets the log transformed data was analyzed within genotype x time blocks using Tukey-HSD. For APSE, the ratio of nonintegrated:integrated phage was found and compared within timepoint x genotype via student's t.

4.4 Results

Table 4.2. P-values for time to first reproduction, fresh weight, and total reproductive output calculated using Dunnett's tests with the uninfected same-aphid-genotype sub-clone acting as control. Survivorship data fit to lognormal for estimates of 0.5 survival time, α = 0.05.

Clone and infection status	Time to first reproduction (h) ± SE [p-Value]	Fresh weight (mg) ± SE [N, p-Value]	Offspring by 26d ± SE 10 sets of 3 [p-Value]	½ Survival (day) N=30
CJ113 (control)	238.65 ± 3.58 [34, 1.0000]	2.606 ± 0.037 [34, 1.0000]	183.0 ± 15.0 [1.0000]	23.79
5AU-CJ113	239.55 ± 4.02 [34, 0.9999]	3.127 ± 0.105 [34, 0.0054*]	179.4 ± 16.7 [0.9998]	23.63
AS3-CJ113	256.23 ± 4.12 [30, 0.0036*]	2.500 ± 0.113 [30, 0.9513]	58.2 ± 12.7 [<0.0001*]	14.54
A2C-CJ113	246.05 ± 2.79 [41, 0.3951]	2.840 ± 0.100 [41, 0.3983]	159.3 ± 9.56 [0.5889]	21.23
AS3-5AU-CJ113	244.70 ± 2.61 [30, 0.6576]	2.224 ± 0.109 [30, 0.0800]	89.8 ± 10.6 [<0.0001*]	17.65
A2C-5AU-CJ113	227.75 ± 4.83 [24, 0.1785]	2.810 ± 0.159 [24, 0.6576]	127.3 ± 13.5 [0.0189*]	21.83
PB17 (control)	250.04 ± 2.11 [27, 1.0000]	3.816 ± 0.149 [27, 1.0000]	203.1 ± 25.9 [1.0000]	23.99
5AU-PB17	236.81 ± 3.70 [26, 0.0269*]	3.521 ± 0.141 [26, 0.3866]	173.2 ± 15.7 [0.5724]	21.21
AS3- PB17	253.13 ± 2.89 [39, 0.9202]	2.874 ± 0.093 [39, <0.0001*]	94.7 ± 10.2 [<0.0001*]	18.00
A2C- PB17	250.00 ± 3.25 [46, 1.0000]	3.208 ± 0.107 [46, 0.0021*]	187.7 ± 15.1 [0.9443]	22.24
AS3-5AU- PB17	242.00 ± 2.89 [25, 0.3137]	3.533 ± 0.136 [25, 0.4337]	155.4 ± 12.4 [0.1628]	21.46
A2C-5AU-PB17	241.60 ± 2.39 [42, 0.1795]	3.259 ± 0.114 [42, 0.0068*]	152.4 ± 14.6 [0.1249]	22.38
WI27 (control)	251.90 ± 2.86 [61, 1.0000]	2.401 ± 0.064 [61, 1.0000]	99.6 ± 8.79 [1.0000]	20.96
5AU- WI27	251.30 ± 2.95 [47, 1.0000]	2.737 ± 0.078 [47, 0.0079*]	175.6 ± 16.2 [0.0002*]	31.74
AS3- WI27	266.76 ± 3.01 [29, 0.0050*]	2.049 ± 0.096 [29, 0.0207*]	48.6 ± 7.8 [0.0173*]	16.98
A2C- WI27	242.56 ± 3.21 [48, 0.0679]	2.957 ± 0.085 [48, <0.0001*]	113.2 ± 10.1 [0.8884]	20.89
AS3-5AU- WI27	261.44 ± 3.11 [34, 0.1089]	2.396 ± 0.103 [34, 1.0000]	81.9 ± 14.0 [0.7452]	18.24
A2C-5AU- WI27	260.56 ± 2.44 [27, 0.2360]	2.512 ± 0.099 [27, 0.8461]	84.6 ± 12.8 [0.9984]	19.32

4.4.1 Aphid fitness in the absence of natural enemies. In all three aphid genotypes, single infection with the protective *H. defensa* strain AS3 resulted in drastic reductions in lifetime

reproductive output relative to uninfected controls indicating clear infection costs in the absence of enemies (Table 4.2 Col 4). Costs to infections were not similarly observed with the non-protective A2C *H. defensa*. Most interestingly, and in all cases, coinfection with *R. insecticola* ameliorated the deleterious effects of *H. defensa* AS3, though in background CJ113 total reproductive output was still significantly lower than in the uninfected subclone. Single infection by *R. insecticola* strain 5AU was not associated with infection costs, and even improved components of aphid fitness relative to uninfected controls, especially in aphid genotype WI27. Co-infection with either H. defensa strain, however, removed benefits associated with single *R. insecticola* infections. The high, early mortality induced by AS3 (Table 2 Column 5) is likely the major contributor to lowered reproductive output, though the reproductive curves for AS3-infected sub-lines are lower than those of their clonemates even prior to the onset of significant mortality (**Supp Fig. 4.1**).

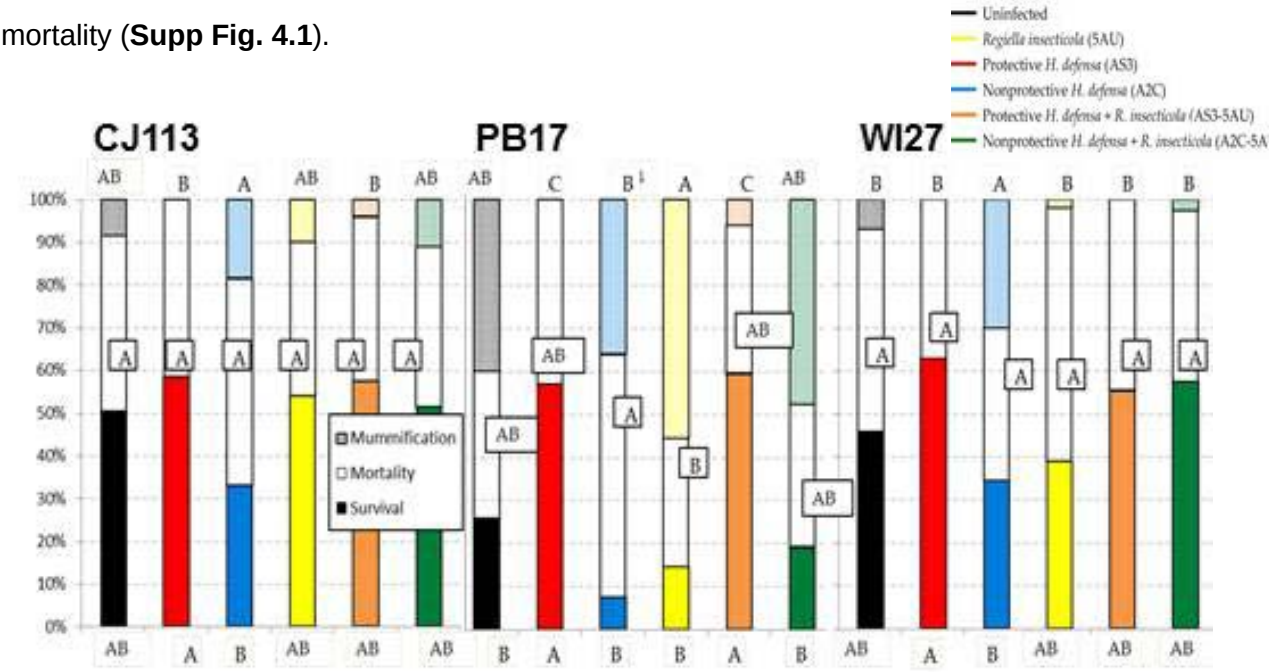

Figure 4.1. Post-wasp parasitism survival, mummification and non-mummification; groups assigned on basis of arcsin transformed data's Tukey-Kramer HSD comparison, $\alpha<0.05$. Each treatment represents 10 replicates of 20 aphids.

4.4.2 Natural enemy challenge: parasitoid wasp *Aphidius ervi*. As previous work in the system has shown, infection by *H. defensa* strain AS3 dropped the proportion of aphids

mummifying (i.e. successful parasitism) to near zero. Coinfection by *R. insecticola* seems to allow for occasional successful mummification in AS3-infected lines, but the effects are not significant. Single infection by *R. insecticola* had no consistent influence on mummification or aphid survival rates. Infection by the non-protective *H. defensa* strain A2C increases mummification rates and decreases survival rates in aphid genotypes that are wasp resistant (**Fig. 4.1**), though this is significant only in WI27. Coinfection by *R. insecticola* ameliorates this effect.

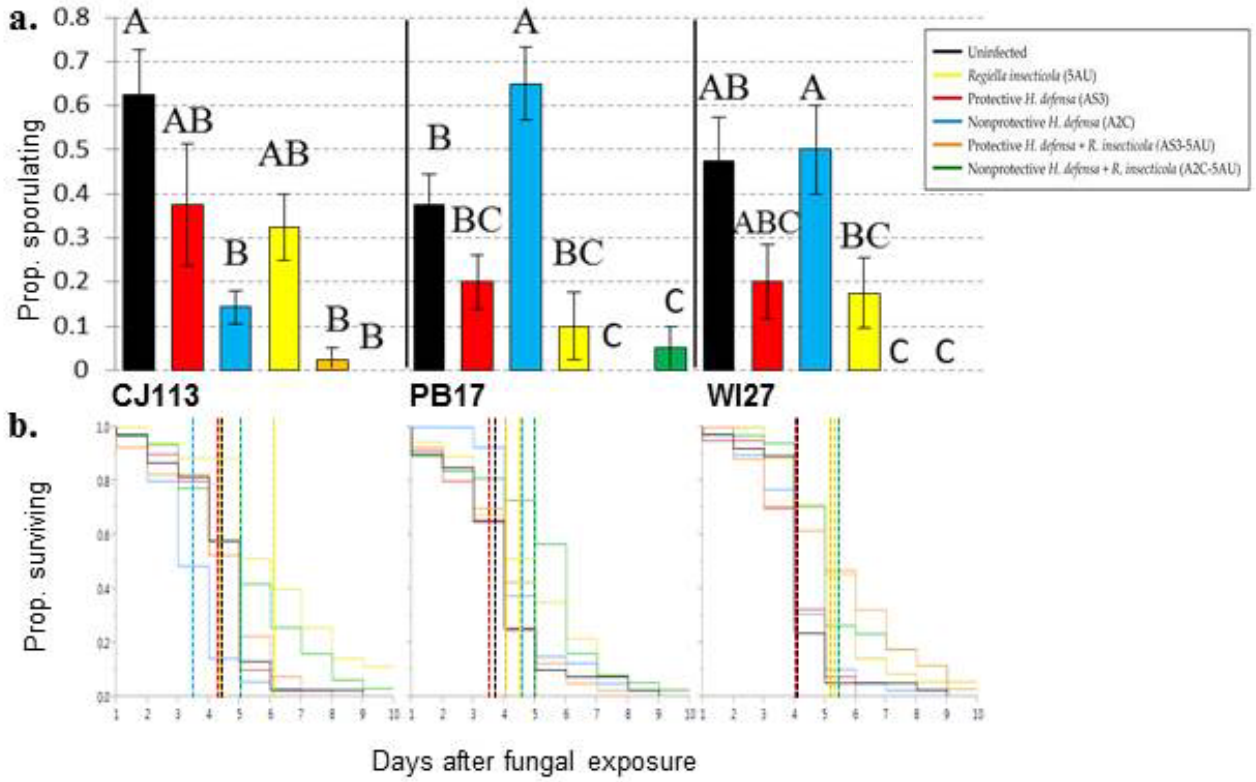

Figure 4.2. a. Proportion of *P. neoaphidis*-exposed aphids that have sporulated by day 10; groups assigned on basis of arcsin transformed data's Tukey-Kramer HSD comparison, $\alpha<0.05$. Bars represent SEM. b. Kaplan-Meier plot of aphid survival: dotted vertical lines represent the predicted 0.5 mortality time point ($\alpha<0.05$) for the Weibull-fitted data.

4.4.3 Natural enemy challenge: entomopathogenic fungus *Pandora neoaphidis*. As previously shown, aphids singly infected with *R. insecticola* exhibited a decrease in total sporulation relative to uninfected, susceptible CJ113, and a non-significant reduction in natively-

resistant lines PB17 and WI27 (**Fig 4.2a**). Coinfection by either A2C or AS3 *H. defensa* consistently enhanced this effect. All sporulation occurred after the previously-reported four day-post-exposure incubation period for *P. neoaphidis* (Parker et al. 2014). Single infection by *H. defensa* A2C appears to decrease sporulation in genotype CJ113, but this is actually due to a sizeable hazard rate prior to the end of the four-day incubation period (**Fig 4.2b, panel 1**). Similar non-significant "benefits" conferred by *H. defensa* strain AS3 in every aphid genotype are also explicable by early post-exposure mortality. From the standpoint of longevity post fungal-exposure, coinfection with 5AU is preferable to single *H. defensa* infection in all genotypes; single infection by 5AU, however, is consistently inferior to coinfection with either *H. defensa* strain in preventing sporulation, and only superior in post-exposure longevity in genotype CJ113.

4.4.4 Intrahost PCR-based symbiont quantification. While *B. aphidicola* genome copy number varied among aphid genotypes, and over time within genotypes, we found no consistent effects of infection with facultative symbionts on *Buchnera* copy number (**Fig 4.3**a). In terms of *H. defensa,* we observed general increases in symbiont titers over time, and differences among genotypes, indicating host x symbiont genotype interactions. In general, estimates of the within-genotype *H. defensa* abundance were similar between the protective and non-protective strains, but when different the AS3-only lines tended to be higher than titers in A2C-only lines. This result is somewhat surprising given our previous work comparing *H. defensa* abundance in phage-infected and phage-free lines (including these two *H. defensa* strains in their native aphid hosts – though the inverse difference observed there was also nonsignificant in the crosswise comparison) (Weldon et al. 2013) (**Fig 4.3b**). Coinfection by *R. insecticola* 5AU had no significant effects on *H. defensa* abundance relative to single infection, however, titers of *H. defensa* in AS3-5AU lines were significantly higher in line CJ113 relative to A2C-5AU at the 2d time point. *Regiella* titers also generally increase over time and vary with respect to aphid genotype. Notably, we observed that co-infection with *H. defensa* often leads to increases in *R.*

insecticola abundance. Some effect of coinfection on *R. insecticola* titers: with the exception of an unusually low mean for 8 day-old AS3-5AU-PB17, all coinfected strains have higher titers of *R. insecticola* than the single-infected subclone in their same genotype, and these difference are significant for some crosswise comparisons in all 2 day-old samples, 4 day-old WI27, and 6 day-old CJ113 (**Fig 4.3c**).

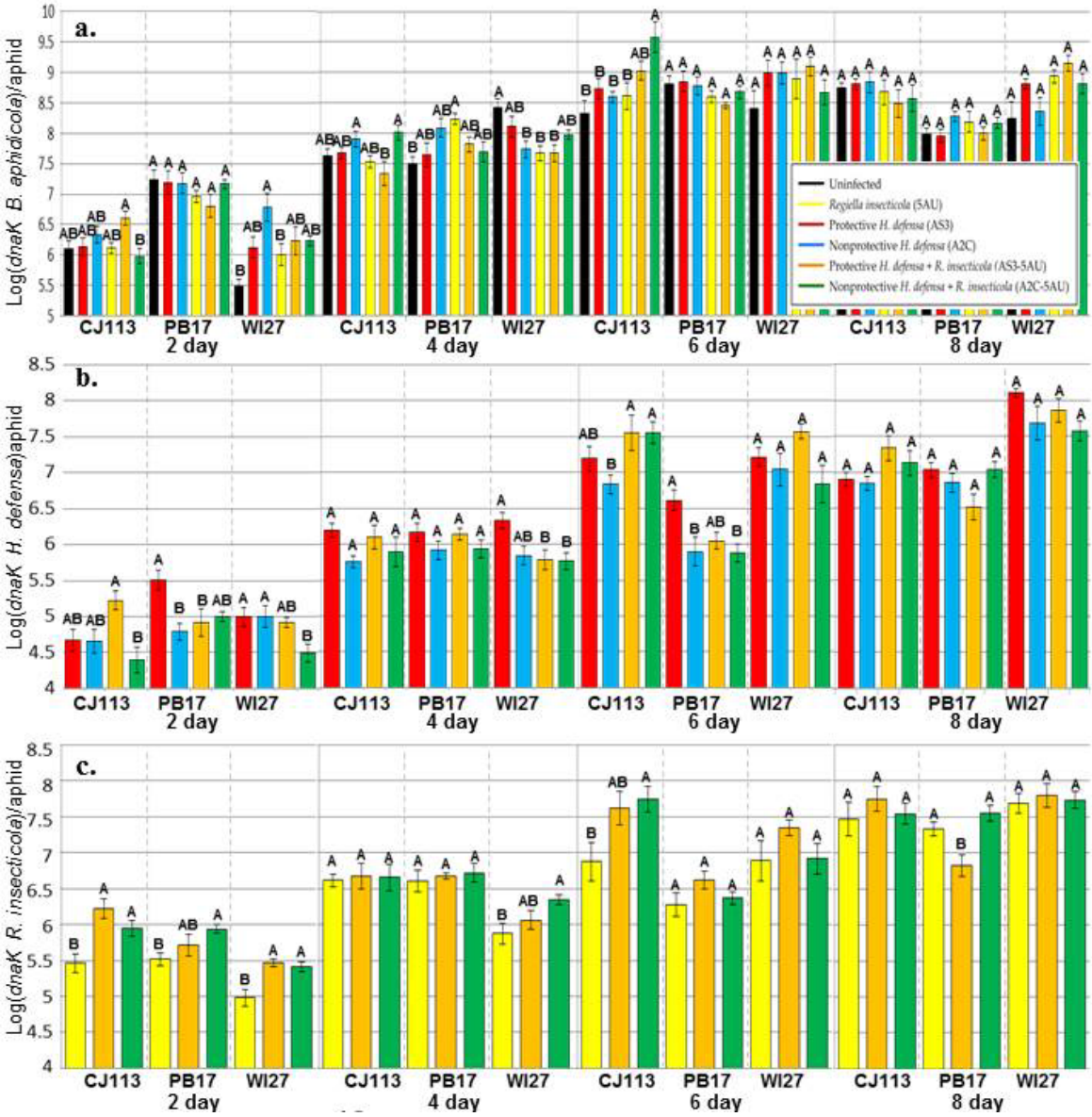

Figure 4.3. RT-qPCR absolute quantification of *B. aphidicola, R. insecticola,* and *H. defensa dnaK*, corrected within genotype x time by aphid ***ef1α***. Within-genotype x time group comparisons made using Tukey-Kramer HSD. Within-group significant differences are marked by letter. In all treatments N=7 except for the 2 day old uninfected CJ113 and A2C-5AU-CJ113, where N=6.

Coinfection had no effect on phage APSE integration rates in *H. defensa*: the only significant effects are non-directional. In fact, with the exception of the uptick in nonintegrated phage at 6 days in PB17, phage integration over time seems strongly responsive to host aphid genotype, behaving similarly over time in the same host regardless of the presence of a coinfecting *R. insecticola* 5AU (**Fig 4.4**). Unfortunately, since qPCR sample aphids were produced/collected with the expectation that they would be analysed genotype x time-based blocks, the effects of production block, age, and aphid genotype can't be disentangled.

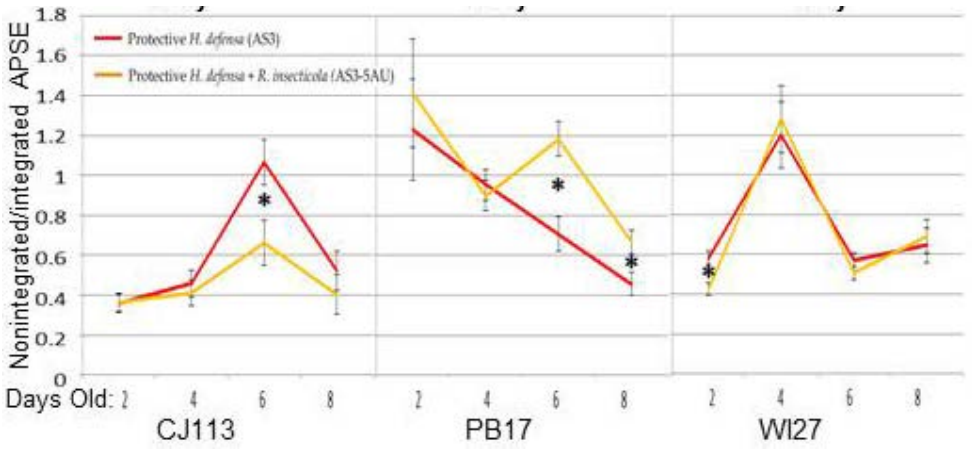

Figure 4.4. The ratio of nonintegrated: integrated phage APSE3 in lines infected with *H. defensa* strain AS3. Cross-subclone comparisons were made at individual time points using student's t, and significance of $\leq$0 .05 is astrixed. In all treatments N=7 except for the 2 day old AS3-5AU-PB17, where N=6.

4.5 Discussion

While isolating single symbiont infections via selective screening or antibiotic curing has been a powerful and invaluable tool for investigating aphid symbioses, it obscures the fact that facultative symbionts are exposed to competitors and, possibly, cooperators, in their hosts: in the field around a quarter of all aphids carry 2-4 different facultative symbionts (Russell et al. 2013), and this snapshot fails to capture other competitive interactions, such as facilitation or exclusion of attempted horizontal transmission (horizontal transmission of aphid facultative symbionts can occur either during sexual reproduction or parasitism (Gehrer and Vorburger 2012; Moran and Dunbar 2006), and personal observations during microinjection suggests that

H. defensa-infected aphids are more refractory to novel infections than are *R. insecticola*-infected aphids, at least when those novel infections are introduced via glass needle), or during vertical transmission (Sandstrom et al. 2001). Superinfections may offer aphids multiple benefits, or perhaps stacked benefits (Oliver et al. 2006), or the additional symbiont taxa may be costly for the aphid host, or both may occur simultaneously. Facultative symbionts may have entered a détente with their aphid hosts, but they still must compete with one another for limited host space, especially during transmission bottlenecks. The benefits they provide may be only a part of that competition, with the rest occurring in a scramble for host resources and direct competition. *H. defensa* and *R. insecticola* were selected on the basis of their mutual reliance on *B. aphidicola* for the same eight essential amino acids (Degnan et al. 2010; Degnan et al. 2009), their status as an unusually rare coinfection partners (Russell et al. 2013), and the observed loss of one partner in lab-held coinfections (Sandstrom et al. 2001) as likely candidates for a costly coinfection: we found, instead, the opposite.

In the absence of natural enemy challenge, it was a single infection that most seriously damaged aphid fitness: *H. defensa* strain AS3's major impact appears to be on longevity, with a serious increased hazard of early mortality in infected aphids that was ameliorated by coinfection with *R. insecticola* – longevity costs to *H. defensa* have previously been identified in the black bean aphid, *Aphis fabae*, where, as here, the presence and severity of decreased lifespan varied by aphid genotype (Vorburger et al. 2009). The lack of a similarly deleterious effect from non-protective *H. defensa* strain A2C is contrary to previous findings about the effects of phageless *H. defensa* on host aphid fitness (Weldon et al. 2013). While A2C (2003) and AS3 (2007) were collected four years apart, they are from the same location and MLSTs suggest that they are quite closely related: the presence/absence of APSE is the major known difference between them. Phage APSE encodes putative eukaryotic toxins (the presumed cause of the anti-parasitoid phenotype); this, or transcriptional changes in *H. defensa* caused by phage infection, may explain the deleterious effects of phage-harboring but not phage-free *H.*

defensa. Contradictory previous findings may be due, like the differences in longevity costs in *Ap. fabae*, to the effect of aphid genotype, or to *H. defensa* strain, as they were performed with a distinct *H. defensa* isolate A1A - though, again, according to published MLSTs this strain is either identical to or very closely related to AS3 and A2C, making aphid genotype the more likely culprit, particularly since *H. defensa* strains A2C and AS3 seem to have established themselves at different relative titers in all genotypes examined here than in their native hosts (**Fig 3b**), where the titer difference goes the other way. One more thing to keep in mind is that A2C, despite its similarity to APSE3-hosting *H. defensa*, was as far as anyone can tell phage-free at its time of collection in the field, though it may have lost its phage very shortly after establishment in the lab instead (all other phage free lines, including those assayed in previous work, are very clearly the result of in-lab phage loss); this is an incredibly rare circumstance (a recent survey of >500 *H. defensa* strains in North American *Ac. pisum* turned up one phagefree strain – Weldon et. al. 2015 in prep) and phage-free *H. defensa* isolates that survive field conditions may do so due to unique phenotypic effects. *R. insecticola* strain 5AU induced no similar costs, and in fact partially ameliorated the deleterious costs of AS3. In genotype WI27, which reproduced at far lower rates than the other genotypes used in the experiment, *R. insecticola* drastically improved reproduction as a single infection but not when it shared a host with either A2C or AS3: this is the only hint from fitness assays performed in the absence of natural enemies of a reason that a single infection might be selected over a coinfection in some genotypes.

Natural enemy challenges produced unexpected fitness results: in terms of fungal resistance, coinfection resulted in a drop in sporulation relative not only to uninfected clones but also to infection by *R. insecticola* alone. It was also found to enhance, in some instances, post-exposure longevity (since adult aphids continue to reproduce after exposure to *P. neoaphidis*, and a sporulating adult aphid's first victims are likely to be its most recent clonal offspring, both of these measures are important to understanding the impact of fungal infection). Coinfection

with *R. insecticola* nonsignificantly decreased the anti-wasp defensive phenotype of *H. defensa* AS3. As with challenges in the absence of natural enemies, the most drastic phenotypic effects were found in single infections by *H. defensa*, in this case strain A2C: while it is already known that phage-free strains do not improve host resistance to wasp parasitism, this is the first report of a negative effect on aphid genotypic resistance; as with AS3's effects on longevity, coinfection by *R. insecticola* strain 5AU ameliorated the negative effects. *R. insecticola*'s general good-neighborliness in response to different costs induced by both of these strains, and the benefits of coinfection to *R. insecticola*'s major protective phenotype, render the fact that *R. insecticola* is unusually rarely found in any coinfection with any other aphid symbiont somewhat mysterious (Russell et al. 2013).

In *Wolbachia*-infected hosts, several studies have found that titers for each individual strain are more dependent on strain identity than the presence/absence of coinfecting strains (Ikeda et al. 2003; Mouton 2003), while others show density suppression during coinfection (Kondo et al. 2005). Work done on *H. defensa* and *S. symbiotica* indicates that one partner may alter abundance in response to coinfection, but not the other, which is what we found here: *R. insecticola* titers increase in coinfected subclones, while *H. defensa* numbers do not (Oliver et al. 2006); the uptick in titer may be why the protective phenotype associated with *R. insecticola* (anti-fungal protection) seems improved in coinfected lines. Coinfection seems irrelevant to phage APSE integration rates, but phage behavior over time seems heavily influenced by host genotype, a finding in line with introgression studies performed in the *Wolbachia*/WO system, though in that case the hosts were different species rather than different clonal genotypes within a species (Chafee et al. 2011). Unfortunately, our qPCR studies were not designed with that question in mind, and detailed investigations of phage x *H. defensa* strain x host genotype over time remain for future authors.

The effects of coinfections are mixed, but on the whole, positive or neutral for the aphid host, with the exception of the nonsignificant decrease in AS3's anti-wasp effect in coinfections

with *R. insecticola*. This contradicts the field-survey picture that inspired this study. However, recent evidence suggests that, despite the fact that *H. defensa* is overall depauperate for *R. insecticola* coinfections and vice-versa (Russell et al. 2013), at least one strain of *H. defensa* is relatively enriched for coinfection with *R. insecticola* (Drew Smith, personal communication). While AS3/A2C's strain of *H. defensa* is underrepresented in current field surveys, preventing any strong statement about its likelihood of association with *R. insecticola,* strain variation in field coinfection rates generally suggests that *H. defensa* AS3/A2C and *R. insecticola*-5AU are unlikely to be representative of all possible *R. insecticola-H. defensa* coinfection dynamics. On the other hand, this may represent the general state of this coinfection, and its rarity may be due to transmission differences and difficulties, or responses to biotic and abiotic factors outside of the major phenotypes provided by both bacteria.

4.6 Acknowledgements

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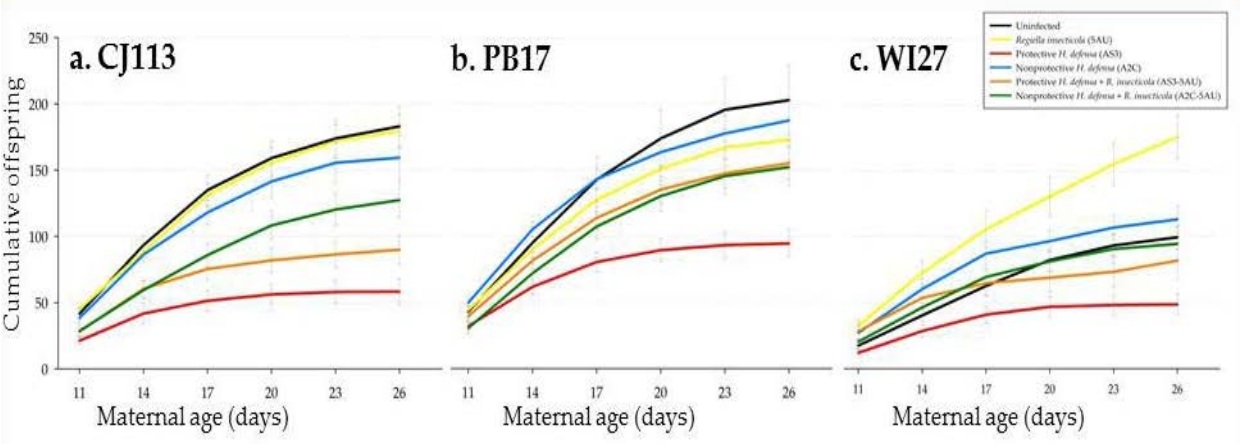

Figure S4.1. Cumulative fecundity by age 26 for cohorts of three aphids, broken down by aphid clone. Bars represent standard error; total fecundity by day 26 that differs significantly from the control is marked in the reproductive output column of **Table 4.2**.

CHAPTER 5

TRANSCRIPTIONAL BASIS OF ANTI-PARASITOID DEFENSE IN APHIDS[1]

[1]Weldon S.R., A.J. Martinez, K.M. Oliver and M.R. Strand. To be submitted to *Insect Molecular Biology.*

5.1 Abstract

The pea aphid, a developing model system for ecological and evolutionary studies, has a reduced set of identifiable immune genes relative to other insects with sequenced genomes, and exhibits little-to-no encapsulation response when challenged by parasitic wasps or other sizeable foreign objects. We used RNA-Seq to investigate the aphid transcriptional response to challenge by the parasitoid wasp, *Aphidius ervi.* We used 1) aphids with high rates of innate resistance to wasp mummification, 2) aphids with low rates of innate resistance to wasp parasitism, and 3) aphids with low innate rates of resistance infected by a heritable facultative mutualist, *Hamiltonella defensa,* which induces wasp resistance in its aphid hosts, and we compared them 30 hours post-parasitism (at the approximate time of *H. defensa* –induced wasp mortality), and found little evidence for the involvement of canonical immune genes in the aphid response to parasitism. The largely uncharacterized transcripts that we did find upregulated in resistant lines may make a solid foundation for future investigations into the aphid anti-parasitoid response.

5.2 Introduction

Acyrthosiphon pisum (Family Aphididae) is a polyphagous pest of legumes and a developing model system for heritable bacterial symbioses and herbivore-enemy interactions. Aphids are attacked by a number of specialized natural enemies, including parasitic wasps and fungal pathogens, which can lead to the evolution and maintenance of diverse resistance traits (Parker et al. 2014; Martinez et al. 2014). Despite a number of major adaptations, including cyclical parthenogenesis and telescoping generations that serve to increase reproductive rates, this aphid also exhibits full-spectrum clonal variation in susceptibility to specialized pathogens and parasitoids (Henter and Via 1995; Ferrari et al. 2001; Parker et al. 2014). With respect to parasitoids, different aphid clones employ multiple resistance strategies, including bacterial symbiont-mediated resistance and innate defenses (Martinez 2015). Eukaryotic toxins are hypothesized to underlie symbiont-based defense, but innate mechanisms of resistance in this

aphid are unclear, especially as there is little encapsulation response to parasitism. It is furthermore unknown whether or not host-based aphid immune responses to parasitism differ when defensive symbionts are present.

The dominant parasitoid of pea aphids in North America is the braconid wasp *Aphidius ervi*, which has the standard solitary koinobiont lifecycle. After an attack by *Aphidius ervi* aphids continue to develop (Digilio et al. 2000), but five to eight days after infection the wasp kills the aphid and the latter's cuticle will harden into a mummy where the wasp spends its pupal stage (Oliver et al. 2005). At oviposition, *Ap. ervi* injects a castrating venom that degrades the aphid's reproductive system, presumably to redirect resources from aphid reproduction to wasp development (Pennacchio et al. 1999). From the wasp's perspective, it must not only avoid aphid or symbiont immune responses, but also create an environment suitable for its larva's stage-specific nutritional needs (Caccia et al. 2005). In addition to venom, teratocytes, which dissociate from the extra-embryonic material approximately three days after deposition, are thought to play important roles in the redirection of host resources, partially through the disruption of already-formed aphid embryos (Falabella et al. 2009).

Pea aphids are infected with a variety of facultative heritable bacterial endosymbionts (Oliver et al. 2010): *Hamiltonella defensa,* a gammaproteobacterial endosymbiont found in about 41% of aphid species (Henry et al. 2015), provides variable, strain-dependent protection from *Ap. ervi* (Oliver et al. 2005). *Ap. ervi* readily oviposits in aphids infected with *H. defensa*, but most wasps fail to complete development, and the aphid survives; furthermore, *H. defensa* can save the reproductive capacity of older parasitized aphids. *H. defensa* strains vary in protection provisioned from between 30% to 90% (Oliver et al. 2005; McLean and Godfray 2015; Moran et al. 2005); the most-aggressively defensive strains (such as the AS3 strain used in our study) lead to the production of small, undersized teratocytes or prevent their production altogether, so everything too developed to be damaged by the initial castrating venom can continue developing (Oliver et al. 2008).

Sequencing of the pea aphid genome revealed the absence of large chunks of the known insect innate immune pathways in the aphid genome (Richards et al. 2010); this, in tandem with the high rates of successful *Ap. ervi* mummification in *H. defensa*-free aphids (Oliver et al. 2005), and the minimal-to-lacking encapsulation response observed after artificial immune challenge (Laughton et al. 2011), led to a broad assumption that variations in pea aphid wasp immunity observed prior to 2005 (ex: Ferrari et al. 2001) were the result of undiagnosed facultative mutualist infections. However, aphids do have five c-type lectin paralogs (utilized in *D. melanogaster* to mark surfaces for encapsulation), serpins that may be involved in Toll-pathway activation, and portions of the JAK/STAT pathway, which, while poorly understood in detailed function, is upregulated in *D. melanogaster* in response to parasitoid challenge (Gerardo et al. 2010), and recent studies have confirmed the presence of innate variation in aphid immune response to wasp parasitism (Martinez et al. 2014). Aphid clone ZA17-AB has rates of post-parasitism survival comparable to aphids infected by the most highly-protective strains of *H. defensa* (Martinez et al. 2014), and these genotypically-resistant aphids retain as much or more of their reproductive potential post-parasitism survival, despite the fact that the timing of wasp mortality occurs later, at some time post-hatching (Martinez 2015).

To better understand the processes underlying *Ap. ervi* resistance in particular and *Ac. pisum* humoral immunity in general, we compared the post-parasitism global transcriptomes of two genotypes of *Ac. pisum*, called AS3-AB and ZA17-AB: the former has no genotypic resistance to wasp parasitism, and the latter has, as was mentioned above, very high resistance. We furthermore included a subclone of the non-resistant line that is infected with a strain of *H. defensa* (also called AS3) which induces high parasitoid protection. To date, transcriptomics have been performed on pea aphids infected with the heat-shock protecting bacterium *Serratia symbiotica* (Burke and Moran 2011): limited upregulation of genes encoding chitin-binding proteins was found in infected aphids, but the majority of the immune-related genes annotated in pea aphids underwent no significant changes in expression level on

infection with *S. symbiotica. H. defensa* is more metabolically constrained than *S. symbiotica* due to a longer adaptation to the symbiotic lifestyle, and may therefore make more demands on its host, though it may also be relatively less virulent (Degnan et al. 2009).

5.3 Methods

5.3.1 Biological sample production *Aphids* *Ac. pisum*'s distribution is currently world-wide, though its historic range was limited to the Palearctic. Pea aphids are cyclical parthenogens: in summer months: all-female generations skip the reductional division of meiosis and clone themselves, allowing for rapid proliferation. These clonal daughters go through embryogenesis in the ovarioles and are born as first instar nymphs. Shortened day-lengths in the fall trigger the production of sexual females and XO males. The autumn's sexual aphids mate and produce all-female eggs that overwinter and hatch into asexual females in the spring. In addition to this annual sexual polyphenism, pea aphids have a stress-related wing polyphenism. Mothers exposed to overcrowding, poor plant quality or predation may produce winged offspring (Brisson and Davis 2008). The pea aphid's parthenogenetic reproduction makes her very well-suited to laboratory studies, as controlled lighting and temperature allows them to be maintained permanently as clonal populations via a long-day lightcycle (Brisson and Stern 2006). All aphids in this study were therefore maintained on *Vicia fava* at 20^0C 16L:8D photoperiod. Three aphid clonal lines with previously-published parasitism-resistance phenotypes, AS3-HD, AS3-AB, and ZA17-AB, were used in this experiment. AS3-AB and ZA17-AB are both free of facultative heritable symbionts, and represent the two opposite poles of aphid genotypic resistance to wasp parasitism: AS3-AB is highly susceptible to mummification, while ZA17-AB is highly resistant (Martinez et al. 2014). As both of these lines were, in the field, infected with secondary symbionts, they are designated –AB, to indicate that they are now symbiont-free due to antibiotic treatment; a treatment which occurred a minimum of ~20 aphid generations prior to the use of these lines in the current study (Martinez et al. 2014). AS3-HD and AS3-AB are the

same aphid clonal genotype, but AS3-HD is infected with a highly protective *H. defensa* (strain also referred to as AS3), which renders it nigh-impervious to mummification (Oliver et al. 2009).

Parasitism assays *Aphidius ervi* (Hymenoptera: Braconidae) is a solitary koinobiont endoparasitic wasp; the wasps in this study were from a mixed colony of commercially produced (Syngenta Bioline Ltd.) and field-collected *Ap. ervi* reared on susceptible aphids. For the parasitized treatments, female wasps were allowed to make a single oviposition into 4±1 day-old (2^{nd}-3^{rd} instar) aphids; aphids were then placed on fresh fava plants in groups of 20 and reared under standard conditions (Oliver et al. 2009). Simultaneously, a matched set of 20 unparasitized aphids from the same line was made for each cup. For each of the six treatments (lines AS3-HD, AS3-AB, and ZA17-AB, parasitized and unparasitized), three biological replicates consisting of ~15 mg of whole live aphids were frozen 30 hours after parasitism.

5.3.2 Sample prep Total RNA was isolated using the standard protocol in the Omega Bio-Tek E.Z.N.A. Mollusc RNA Isolation Kit (Norcross, GA), followed by passage through a Life Technologies (Carlsbad, CA) Turbo DNA-*free* treatment. Samples were then submitted to the Georgia Genomics Facility (Athens, GA) where they were processed as follows: quality and concentration was analyzed with both Qubit 2.0 Fluorometry and an Agilent 2100 Bioanalyzer run; samples were polyA selected and then stranded libraries were constructed using Kapa Biosystems (Wilmington, MA) Stranded RNA-Seq chemistry and multiplexed in a single lane on an Illumina NextSeq 150 cycle Paired End 75 Mid Output Flow Cell.

5.3.3 Analysis pipelines Pea aphid genome assembly version 2 scaffolds NC_011594.1 (Richards et al. 2010) were built into a reference database using Bowtie2 (Langmead et al. 2009); reads where more than 90% of bases were ≥ Q30 were repaired and mapped to the genome database using TopHat (Trapnell et al. 2009), and then passed through the standard Cufflinks/Cuffcompare/Cuffdiff pipe (Trapnell et al. 2014). All figures were produced with cummeRbund (Goff and Trapnell 2011). GO and functional annotations were made via comparisons of the ACYPI OGS v2.1b official gene set with InterproScan and Blast2GO results

(v2.1) available at AphidBase (http://www.aphidbase.com/Downloads) to our transcript ids. Unless otherwise specified, all alpha cutoffs for significance are 0.01. Canonical immune genes come from the Insect Innate Immunity Database (IIID; Bordenstein Lab, NSF DEB-1046149, last accessed Oct 2015); other detailed protein-function descriptions are taken from Uniprot where not otherwise cited (Bateman et al. 2015).

5.4 Results

5.4.1 Overall summary statistics

Table 5.1. Reads assembled to version 2 of the aphid genome, broken down by treatment and biological replicate.

Line	Condition	% total reads mapped to aphid genome	# assembled reads (E6) (replicate 1/2/3)
AS3-HD	Parasitized	88.2-89.5	2.106/1.860/1.953
AS3-HD	Unparasitized	89.2-90.5	2.628/2.022/2.209
AS3-AB	Parasitized	77.9-87.8	2.055/2.074/2.661
AS3-AB	Unparasitized	84.1-90.1	2.068/2.215/2.519
ZA17-AB	Parasitized	54.4-84.4	2.380/1.296/1.699
ZA17-AB	Unparasitized	70.6-83.7	2.041/2.607/2.515

No transcripts were differentially regulated between the unparasitized AS3-AB and unparasitized AS3-HD– that is to say, infection by *H. defensa* per se does not have an effect on the aphid transcriptome: this is in line with a previous study of the aphid transcriptome after infection by another defensive facultative symbiont, *Serratia symbiotica* (Burke and Moran 2011). Furthermore, no transcripts were differentially regulated between the parasitized and unparasitized conditions for non-resistant, *H. defensa*-free line AS3-AB: as post-parasitism survival rates in this line are a bit less than 5% (Martinez et al. 2014), no aphid immune response was expected, and 30 hours is prior to the emergence of the wasp larva (He 2008).

More surprisingly, no transcripts are differentially regulated between the parasitized and unparasitized treatment for the resistant genotype ZA17-AB: wasp mortality in these lines does not occur until after the 30 hour time point we selected for this comparative study, so this may simply be too early to detect any active response. 532 transcripts (mapping to 435 genes in the

OGS) are differentially regulated between the parasitized ZA17-AB (high genotypic protection) and parasitized AS3-AB (no genotypic protection) (**Fig 5.1a**), while a set of 422 transcripts (326 OGS, overlapping with the prior comparison by 215 OGS) are differentially regulated between the unparasitized ZA17-AB and AS3-AB treatments (**Fig 5.1b**): we will primarily focus on those genes solely upregulated in parasitized ZA17, with some reference to similarities/differences in the unparasitized cross comparison set. As to *H. defensa*-induced defensive response, in the cross-comparison between parasitized AS3-HD and AS3-AB, 359 transcripts (255 OGS) are differentially regulated (**Fig 5.1d**); and between parasitized and unparasitized AS3-HD lines, 315 transcripts (224 OGS) (**Fig 5.1c**), with an overlap of 112 OGS between the two comparison sets.

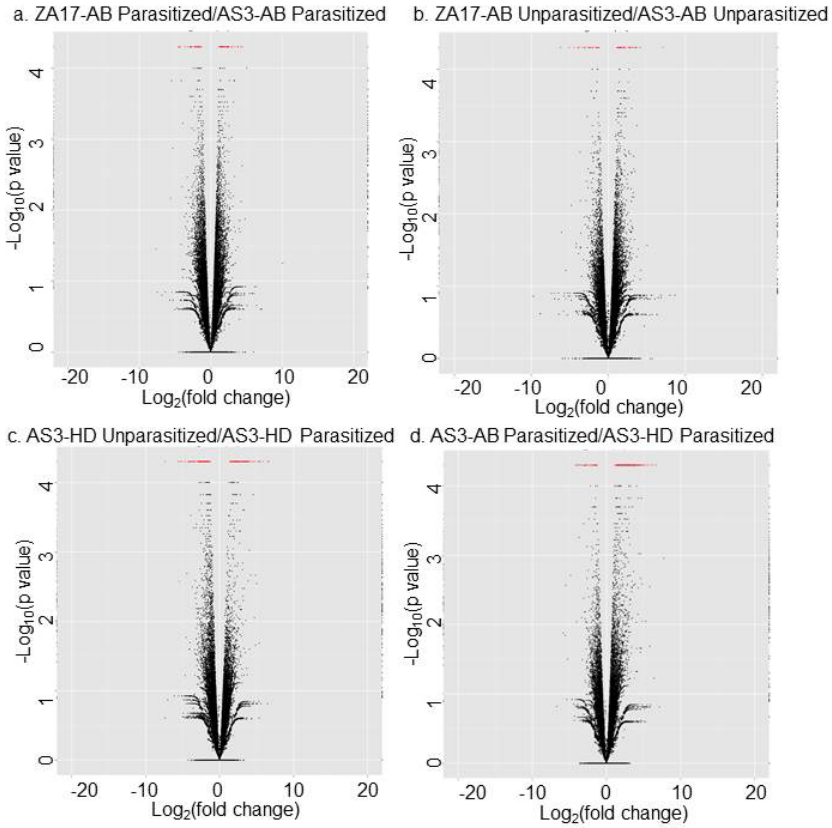

Figure 5.1. Volcano plot of cuffDiff output for those biologically important cross comparisons where some transcripts were significantly differentially expressed: transcripts meeting the alpha=0.01 significance criterion are marked in red.

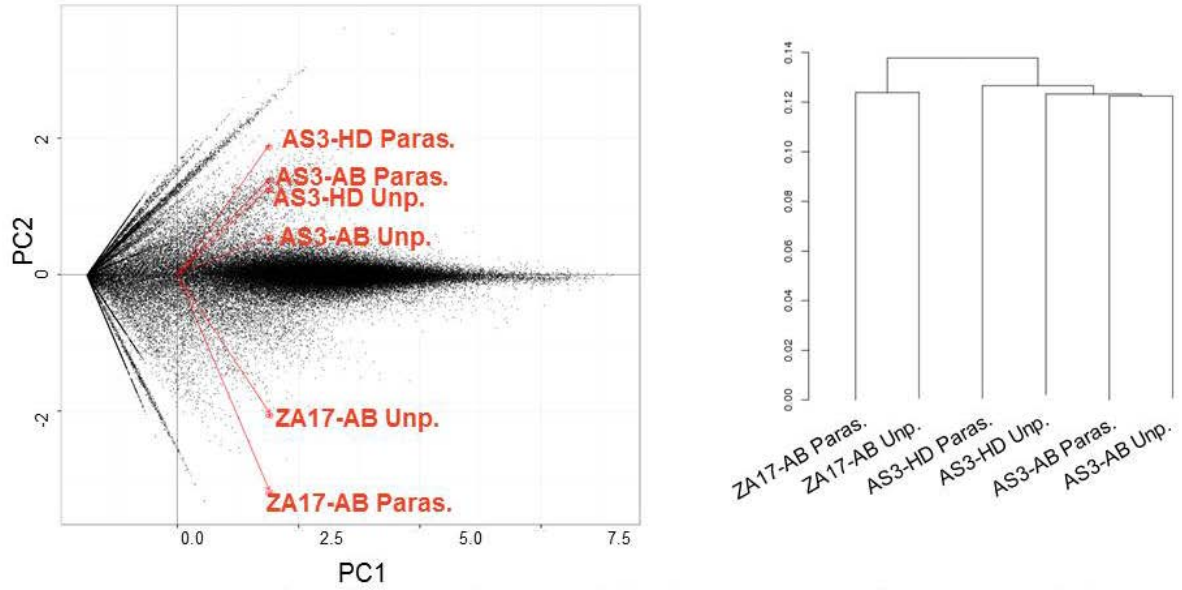

Figure 5.2. Two-factor principal component analysis plot and dendrogram (marked with Jensen-Shannon distances) of all transcripts mapped to the aphid genome.

5.4.2 Aphid-genotypic immunity to parasitism Component analysis and the overall dendrogram suggests a strong aphid-genotypic effect on expression (**Fig 5.2**): therefore, to exclude the effects of aphid genotype and focus on a potential active immune response to parasitism, we isolated the subset of genes (71) upregulated in parasitized ZA17-AB relative to parasitized AS3-AB and *not* upregulated in nonparasitized ZA17-AB relative to nonparasitized AS3-AB. Of those with functional annotations, the majority are involved either in bodyplanning, cellular construction or the mobilization of fat resources, and one is ecdysone: the relative upregulation here may be due to the developmental arrest that occurs in parasitized, non-resistant lines (Digilio et al. 2000). No genes from the canonical aphid immune gene set were upregulated in this crossover, nor were there any in the less-stringent full set of all upregulated parasitized ZA17-AB vs. parasitized AS3-AB. The portion of our upregulated subset inexplicable by instar transition includes one protein of unknown function and probable retroviral origin and a reverse transcriptase (ACYPI53943 and ACYPI002501); as well as two N-end rule pathway–associated proteins (ACYPI000843 and ACYPI003083) and homolog of a *Drosophila* protein that promotes the degradation of RNAs that were derived from transposons or are

otherwise aberrant (ACYPI004857) (Kuan et al. 2009). The substantial number of genes upregulated relative to ZA17-AB in parasitized AS3-AB but not unparasitized AS3-AB (152 in all) all lacked functional annotations.

5.4.3 Aphid contributions to *H. defensa*-induced immunity Most differential regulation between the parasitized AS3-HD and AS3-AB lines were upregulation in AS3-AB (196) – some of this involves mobilization of fatty acids and sugars; outside of that area, many of the identified proteins are cuticular, and four of them are immune: prophenoloxidase 1 and 2 (canonical portions of the *A. pisum* humoral response –directly related to parasitism in stimulating cellular defense and involvement in melanization), spätzle 5, and a homolog of a *Bombyx mori* gene (annotated as "immune related" but otherwise uncharacterized) are all upregulated in the *non*defended parasitized line, as is a serpin (ACYPI000915) of unknown broader function. Note that eight cuticular proteins were downregulated in parasitized AS3-HD relative to both unparasitized AS3-HD and parasitized AS3-AB, as were a chemosensory protein (ACYPI000093) and a takeout-like ACYPI009456, both of which are associated with the regulation of feeding behavior. Chitinase-like protein 7 was upregulated in parasitized AS3-HD relative to both unparasitized AS3-HD and parasitized AS3-AB; relative to unparasitized AS3-HD a Toll-like gene (ACYPI004287) was also upregulated, and relative to parasitized AS3-AB, a member of the JAK/STAT pathway (ACYPI005292 – Tep III-2) was upregulated. Other than chitinase-like protein 7 and an alaserpin (ACYPI005016), the 45 genes upregulated in parasitized AS3-HD relative to both unparasitized AS3-HD and parasitized AS3-AB (and therefore the best candidates for an active, *H. defensa*-related parasitism response) lack for the most part obvious associations with any discrete biological process: the upregulation of dusky cg9355-pa (whose *D. melanogaster* homolog is involved in wing morphogenesis cite) might suggest that looking at rates of alate morph production in parasitism-surviving aphids would be worthwhile, and, as was the case with ZA17-AB, two genes of retroviral origin (integrase ACYPI000314 and ACYPI082654, which has a conserved zinc-finger like domain) were

upregulated in response to parasitism. Finally, nine genes were upregulated in both parasitized ZA17-AB and AS3-HD relative to parasitized AS3-AB, but none have any functional annotations.

5.5 Discussion

Here we examined the global expression patterns of aphid genomes following enemy challenge by the parasitoid *Aphidius ervi*. We used three lines that varied in susceptibility to parasitism, including one line that was resistant due infection with *H. defensa*, one line innately resistant and lacking symbionts, and a third innately susceptible and without symbiont infection. Despite assaying these responses thirty hours post-parasitism, after the parasite has exited its rupture chorion and is exposed, within the hemocoel, to the aphid immune system (He 2008), we found few significant differences among pathways involved in innate immunity in enemy-challenged vs control aphids. Of course, examining additional time points, including shortly after the oviposition of eggs and other maternal factors, as well as further along in wasp development, may produce different, and possibly more intuitive, responses.

When considering innately-defended aphids, we primarily detected changes in genes associated with developmental timing that presumably result from wasp-induced developmental delay and ultimate arrest (Digilio et al. 2000), though a few of our characterized genes did not fall into that category. Since the mechanism by which suppressor of sable identifies and targets a wide variety of mutant and exogenous RNAs for exosome degradation is unclear (Kuan et al. 2009), it may not be too farfetched to postulate that the identification of exogenous eukaryotic RNA, such as those produced by a developing endoparasitic wasp, might be within its capabilities, but this is highly speculative, and there are no other clear potential targets for immune-function investigation. As the timing of wasp mortality is later than thirty hours in resistant lines, this may simply be a timepoint selection issue (Martinez 2015). Due to the relatively limited damage done to lifetime fecundity in wasp-parasitized genotypically-protected lines, future studies may want to deemphasize humoral immunity in genotypically resistant

aphids and instead focus on the effects of *Ap. ervi*'s castrating venoms in these clones: failure to effectively hijack sufficient host nutritional resources may play a part in inhibiting full larval development.

Aphid immune response to infection by *H. defensa* was even lower than that observed in response to infection by *S. symbiotica* (Burke and Moran 2011). This demonstrates that immune permissiveness is likely a general strategy employed by pea aphids with facultative bacterial symbionts: as has been previously hypothesized, the overall limited aphid immune response may be due to a combination of protection of endosymbionts from the immune system and farming out of immune functions to endosymbionts (Laughton et al. 2011). One oddity in the AS3-HD line is a downregulation of two genes associated with feeding behavior thirty hours after wasp parasitism, which may also suggest a useful target future behavioral studies.

The current data set is by no means exhausted: it will be rescreened for differential expression at a summed transcript/total gene output level, with more relaxed determinants of significance and a greater exploration of differential splicing and gene sets under similar regulatory schemes. Currently-unassigned high quality reads will be blasted against 1) the obligate intracellular endosymbiont *Buchnera aphidicola* genome (the heavy AT bias in obligate mutualists renders it likely that some bacterial transcripts survived polyA selection), and 2) the *H. defensa* genome where appropriate. Afterwards, remaining transcripts in the parasitized sets will be built de novo in the hopes of recovering some *Aphidius ervi* expression profiles.

The development of the aphid model system will require a combination of "big data" projects to identify sets of interest and serious wetwork to make up for the limited homologies some aphid protein coding genes have to known sets. The sizeable store of unannotated differentially regulated genes produced by this project will be best used as a comparison and target set for future transcriptional and proteomic studies.

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CHAPTER 6

CONCLUSIONS

Insects, which are easily the most diverse and abundant group of animals, have varied and dynamic interactions with microbes. The majority of insect species are infected with heritable bacterial symbionts, which may be obligate (that is, required for insect survival and reproduction) or facultative (that is, not required for insect survival and reproduction). These facultative bacterial associates are an extra-genomic source of heritable variation in defense from natural enemies, nutrient acquisition, and reproduction. Bacteriophages are present as prophage in 62% of all sequenced bacterial genomes: in bacteria living in complex communities, phage are important drivers of diversity via density-dependent kill-the-winner interactions, and they can also drastically alter bacterial phenotypes via lysogenic conversion, famously enhancing virulence in the case of the Shiga toxin-encoding phages of *Escherichia coli*. Despite key roles mediated by viruses in free-living bacteria, little is known, about how phages interact with the diverse symbiotic bacteria infecting insects.

The pea aphid, *Acyrthosiphon pisum*, has become a model organism for insect-bacteria mutualisms. Aphids tap plant phloem for sustenance: this substrate is extremely limited in terms of essential amino acids, and the intake gap is made up via a symbiotic relationship with the bacterium *Buchnera aphidicola*. The majority of pea aphids are additionally infected with one or more of seven known heritable facultative symbiotic bacteria. These secondary symbionts produce aphid phenotypes that utilize host plants differently, are less sensitive to thermal stress, and are better defended from some natural enemies. Resistance to an important natural enemy of aphids, aphidiine braconid wasps, is enhanced in aphids infected with the widespread gammaproteobacterial aphid symbiont *Hamiltonella defensa. H. defensa* represents the only

known case where a bacteriophage engages in a tripartite mutualistic interaction via lysogenic conversion: *H. defensa* strains in *A. pisum* uninfected by a bacteriophage called APSE (*Acyrthosiphon pisum* Secondary Endosymbiont) provide no protective phenotype.

APSE-infected *H. defensa* kill the endoparasitic wasps developing in pea aphids before they can pupate and kill the aphid host. The provisional hypothesis is that putative eukaryotic toxins encoded by the virus are the cause of wasp mortality and therefore the source of aphid defense. My first research project found that APSE performs an additional key role in intra-host bacterial population control, and in the case of its loss, the host insect's reproductive output is severely depressed. APSE loss therefore leads to the conversion of a heritable mutualist to an over-replicating heritable pathogen and thus plays key roles in the population-level maintenance of the defensive symbiosis. Secondarily, I established the range of APSE diversity in *Ac. pisum* collected from alfalfa in the US, identifying a new strain of APSE and characterizing the defensive phenotype in the process. Thirdly, I investigated the interactions between another secondary symbiont, *Regiella insecticola,* and phage-infected and phage-free *H. defensa* infecting the same host, and determined that infection by *R. insecticola* ameliorated harms done by both *H. defensa* strains to aphid fitness. More generally, I found that aphids co-infected with these two symbionts often outperformed singly-infected and uninfected aphids sharing the same genotype, which may explain why superinfections are common in natural populations. Finally, I analyzed the transcriptomes of *H. defensa*+APSE infected aphids, and genotypically wasp-resistant and non-resistant aphids, and found little evidence for the upregulation of canonical insect innate immune genes in any aphid at the approximate time of *H. defensa*-induced wasp mortality.

The work presented here furthers the development of the pea aphid as a model system for insect-bacteria mutualisms, and establishes it as the first to have a functionally-obligate viral partner of a bacterial mutualist.