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THE ROLE OF THE BACTERIAL ENDOSYMBIONT, *ARSENOPHONUS,* IN THE SOYBEAN APHID, *APHIS GLYCINES*

DISSERTATION

A dissertation submitted in partial fulfillment of the
requirements for the degree of Doctor of Philosophy in the
College of Agriculture, Food and Environment
at the University of Kentucky

By

Jason A. Wulff

Lexington, Kentucky

Director: Dr. Jennifer A. White, Professor of Entomology

Lexington, Kentucky

2014

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ABSTRACT OF DISSERTATION

THE ROLE OF THE BACTERIAL ENDOSYMBIONT, *ARSENOPHONUS,*
IN THE SOYBEAN APHID, *APHIS GLYCINES*

Bacterial endosymbionts can have profound impacts on their host's ecology. Notably, endosymbionts can protect their hosts against natural enemies and influence host plant interactions. The endosymbiont Candidatus *Arsenophonus* infects a wide taxonomic range of arthropod hosts, and is suspected of an uncharacterized mutualistic role in hemipterous insects. In the soybean aphid, *Aphis glycines*, an introduced pest of soybeans in the United States, *Arsenophonus* is the sole facultative endosymbiont. The focus of this dissertation is to characterize the role of *Arsenophonus* in the aphid, with an overall emphasis on its impact on aphid management strategies.

I first used diagnostic PCR to determine *Arsenophonus* infection frequency and strain diversity for native and introduced soybean aphids. I found that *Arsenophonus* infection is a uniform strain that is highly prevalent in soybean aphid. I then determined if *Arsenophonus* was a defense symbiont by curing two genotypes of soybean aphid of their natural *Arsenophonus* infection, resulting in infected and uninfected isolines within the same genetic background. I subjected these isolines to assays with three parasitoid species and a common aphid fungal pathogen, *Pandora neoaphidis.* I did not find differences in parasitism or fungal infections within the treatments. These results indicate that, although *Arsenophonus* is widespread, the symbiont should not interfere with biological control efforts.

I next examined the influence of *Arsenophonus* on the ability of soybean aphid "biotypes" to colonize resistant *Rag* plants. I cured three additional soybean aphid biotypes. All isolines were subjected to growth rate assays on resistant *Rag* versus

susceptible soybean. My results indicate that *Arsenophonus* infected soybean aphids have an increased population growth compared to uninfected aphids regardless of soybean plant type

Finally, I induced soybean plants with jasmonic acid (JA) or salicylic acid (SA) to determine the effective plant defense against soybean aphid feeding. I also used *Arsenophonus* infected and uninfected aphids to determine any interaction between *Arsenophonus* and plant defense. I found SA treatment decreased soybean aphid population growth for one experiment, but had no effect when replicated. JA treatment had no effect, and there were no interactions between *Arsenophonus* infection and plant treatments.

Key words: plant resistance, plant defense, aphid biotypes, *Rag*, *Arsenophonus*

Jason A. Wulff

03/21/2013

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Chapter 1

Introduction

1.1 Overview

Soybean aphid, *Aphis glycines*, represents a critical challenge for soybean growers in the United States. The aphid's heavy economic impact, due to yield loss and pesticide input, has mobilized significant research effort into control alternatives to pesticides, including classical biological control and plant resistance. However, these efforts have proceeded without evaluating the role of *Arsenophonus,* a widespread vertically transmitted endosymbiont in soybean aphid. Aphid bacterial endosymbionts have profound influences on their host ecologies, including protection against natural enemies and influencing performance on host plants. The presence of an endosymbiont is often variable in an aphid population, which makes these bacteria a potential gene reservoir for rapid adaptation to selection from biological control introductions. Prior to the research presented in this dissertation, fundamental questions concerning infection frequency, diversity, and function of *Arsenophonus* had not been examined; it was unknown if the symbiont presented an obstacle to controlling this introduced pest.

1.2 Soybean Aphid

Soybean aphid, *Aphis glycines,* is a small aphid that has both parthenogenetic and sexual portions to its lifecycle (holocyclic), which take place on different host plants (heteroecious). The aphid is only 1-2 mm long (McCornack et al. 2004) and ranges in morphology from yellowish green to small "white dwarf" aphids. It has diagnostic black tipped cornicles, and three distinct hairs on its cauda that are used to distinguish it from

another nearly identical *Aphis* species, *Aphis gossypii* (Tilmon et al. 2011). The aphid has approximately 15 generations per year, late spring to early fall. As the day length and temperature decrease in fall, soybean aphids undergo their sexual phase, and migrate from their secondary soybean host to their primary buckthorn host, *Rhamnus* spp., where they overwinter as eggs.

Soybean aphid was accidentally introduced into the north central United States, where it has become the most destructive pest of soybeans. It was first identified in Wisconsin in 2000, thought to have originated from Japan, and remained undetected for several years, being misidentified as *Aphis gossipii* (Venette and Ragsdale 2004, Tilmon et al. 2011). Since 2003, soybean aphid has been detected in several states, including Delaware, Georgia, Illinois, Indiana, Iowa, Kansas, Kentucky, Michigan, Minnesota, Mississippi, Missouri, Nebraska, New York, North Dakota, Ohio, Pennsylvania, South Dakota, Virginia, West Virginia, and Wisconsin (Venette and Ragsdale 2004). Kim et al. (2008) estimated that the economic impact of soybean aphid could result in a cost of $3.6 to $4.9 billion annually. And, despite a decade of research into a variety of control methods, this economic figure still looms over the soybean industry.

Besides damaging crops directly though feeding, soybean aphids also vector a variety of plant viruses (Clark and Perry 2002, Davis et al. 2005, Wang et al. 2006, Davis and Radcliffe 2008). Of particular concern in the United States, Soybean mosaic virus (SMV) and Alfalfa mosaic virus (AMV) have been identified, but no major outbreaks have yet occurred (Wang and Ghabrial 2002, Domier et al. 2003, Ragsdale et al. 2011). One of the most destructive soybean viruses, Soybean dwarf virus (SbDV), can cause significant yield loss in Japan and Indonesia (Tamada 1970, Iwaki et al. 1980). However,

soybean aphid is a very poor vector for the strains of SbDV that are present in the United States (Wang et al. 2006, Damsteegt et al. 2011). This is likely why, although the virus has been detected in soybean fields within the United States, it has not had much of an impact.

The soybean aphid has a vast Asian host range, and occasionally becomes a soybean pest in China, Indonesia, Japan, Korea, Malaysia, the Philippines and Thailand. Controlling the aphid in the native range, are over 55 species of natural enemy, including Coccinellid predators: *Propylaea japonica*, *Harmonia axyridis*, and *H. arcuate*, several species of syrphids, lacewings, several species of parasitoids and some fungal pathogens (Wu et al. 2004, Rutledge et al. 2004). However, within the introduced range, soybean aphid natural enemies are mostly comprised of generalist predators, including coccinellids, *H. axyridis* and *Coccinella septempunctata*, and the anthocorid *Orius insidiosus*. Parasitism of soybean aphid is very low within the introduced range (Fox et al. 2004, 2005, Rutledge and O'Neil 2005, Costamagna and Landis 2006, Mignault et al. 2006, Costamagna et al. 2007).

The limited natural enemies in the introduced range encouraged exploration of parasitoid mediated classical biological control. The first agent, *Binodoxys communis,* was approved for release in 2007 in MN, and subsequently it was released in additional states, but failed to establish (Tilmon et al. 2011). This failure could have resulted from some unknown life history aspect of the wasp, especially diapause. While *B. communis* does parasitize egg-laying (oviparous) soybean aphids on buckthorn, it is unclear if *B. communis* can overwinter/ diapause in these aphids (Heimpel et al. 2010, Asplen et al. 2011).

Coinciding with the introduction of *B. communis,* another parasitoid native to Asia, *Aphelinus certus,* was detected parasitizing soybean aphids within the introduced range from Ontario, Canada to Kentucky, United States (Frewin et al. 2010, Wulff et al. 2013). While the parasitoid is well established in the introduced range, it is unclear whether it will make an appreciable impact on aphid densities (Frewin et al. 2010, Heimpel and Asplen 2011).

A second option for soybean aphid control, developed from the discovery of soybean genes that confer "Resistance to *Aphis glycines*" (*Rag*). To date five *Rag* genes have been identified (Hill et al. 2012). These genes have been backcrossed into commercial varieties of soybean, and in 2012, there were more than 18 resistant varieties of soybean, 17 with *Rag1* and one with a R*ag1*+*Rag2* gene pyramid (Hesler et al. 2013). However, soon after the discovery of these resistance genes, virulent aphid biotypes able to colonize these plants were also identified. And, while *Rag1* plants are cheaper than the cost of foliar insecticides and there is no yield penalty, soybean aphids can still develop past the EIL (economic injury level) on resistant plants, casting doubts on the effectiveness of these resistance genes (Hesler et al. 2013).

The mechanisms underlying soybean (*Rag*) resistance and aphid biotype determination are unknown. In tomato, the interaction is suspected of being gene-for-gene, in which a single plant gene, *Mi*-1.2, conveys resistance against aphid feeding. The *Mi*-1.2 gene is in the CC-NBS-LRR (coiled coil-nucelotide binding site-leucine rich repeat) gene family, and it is thought to recognize an aphid elicitor protein, encoded by a single aphid gene, which triggers a defensive response in the plant (Elzinga and Jander 2013). In the soybean aphid/soy system, there is some suggestive evidence that some *Rag*

genes are CC-NBS-LRR type genes (Kim et al. 2010), but there is no evidence that soybean aphid biotype virulence results from a gene-for-gene interaction. This indicates biotype determination is not novel genetic variation, but could be the product of phenotypic variation or endosymbiont infection (Wenger and Michel 2013).

1.3 Bacterial Endosymbionts

It is well established that microbes make considerable contributions to their host's ecology, acting as accessory genomes that are necessary for the holobiont's survival or are beneficial within narrow contexts (reviewed in Hussa and Goodrich-Blair 2013). These microbes have a spectrum of benefits and dependence on their hosts. At one end are common environmental microbes that are likely beneficial in the host gut, e.g. *lactobacillus* spp. in Hymenoptera (McFrederick et al. 2013). At the other end of the spectrum lie endosymbiotic bacteria, which mostly are vertically transmitted from mother to offspring, often have reduced genomes making them unable to survive outside the host, and are under selection to increase the proportion of infected hosts (Buchner 1965, Douglas 1998, Sandstrom et al. 2001, Duron et al. 2008, Hilgenboecker et al. 2008, Gehrer and Vorburger 2012, McCutcheon and Moran 2012).

From the host's perspective, endosymbiont bacteria are either obligate or facultative. Obligate endosymbionts are common, especially for blood and phloem feeding insects. These bacteria are confined to specialized host organelles and provide necessary amino acids and nutrients required for the host survival and reproduction. Obligate endosymbionts have extremely reduced genomes, the smallest known bacterial genome belonging to a mealybug obligate endosymbiont (McCutchion and Moran 2012).

Regarding aphids, the vast majority of species are infected with the obligate symbiont, *Buchnera aphidicola* (Buchner 1965, Douglas 1998).

In contrast to obligate symbionts, facultative endosymbionts often have larger genomes, are not required for host survival, and must either reduce their infection cost, increase their benefits, or manipulate host reproduction to increase the prevalence of infected female lineages (Werren et al. 2008, Oliver et al. 2010). While a great deal is known about reproductive manipulators (Werren et al. 2008), the focus for this dissertation is facultative endosymbionts that benefit their hosts within certain ecological contexts (Hedges et al. 2008, Oliver 2010).

Defense against natural enemies is a common role for facultative endosymbionts (Hedges et al. 2008, Jaenike et al. 2010). In aphids, three species of bacteria, from distinct genera, provide defense against parasitoid wasps (Oliver et al. 2003, Oliver et al. 2005, Vorburger et al. 2009). The best characterized of these interactions is the protection provided by *Hamiltonella defensa* to the pea aphid *Acyrthosiphon pisum* against the parasitoid wasp *Aphidius ervi*. After the wasp deposits an egg in the host, pea aphids infected with *H. defensa* have up to an 80% greater survivorship over uninfected pea aphids of the same genotype (Oliver et al. 2006). The mechanism for this resistance involves the phage APSE, (*Acyrthosiphon pisum* Secondary Endosymbiont), which is inserted within the bacterial genome (Oliver et al. 2009). Rates of protection against the parasitoid were found to vary based on the APSE phage genotype and loss of protection occurred with phage loss (Oliver et al. 2009). Several additional aphid endosymbionts defend against natural enemies and abiotic factors. Strains of *Serratia symbiotica* and *Regiella insecticola* can also protect their aphid host against parasitoid wasps (Oliver et

al. 2003, Vorburger et al. 2009). Bacterial strains in the genera *Rickettsia*, *Rickettsiella*, *Spiroplasma*, and *Regiella* protect against the common aphid fungal pathogen, *Pandora neoaphidis* (Scarborough et al. 2005, Lukasik et al. 2013). In addition to biotic factors, a strain of *S. symbiotica* also protects aphids against heat shock (Montllor et al. 2002, Russell and Moran 2006). The advent of this defensive role within several different genera suggests that selection favors a defensive, evolutionary trajectory for vertically transmitted endosymbionts. Also, this prevalence makes it critical to assess the defensive potential of an endosymbiont within an aphid targeted for biological control.

Besides defense, endosymbionts also may mediate interactions between their hosts and the plants they feed on. Aphids feed on plant sap and many, including soybean aphid, have evolved very specialized relationships with their hosts, feeding on a limited range of plant species. Plants and aphids co-evolve in response to one another: as plants evolve defenses against their pests, pests evolve to overcome plant defenses (Kamphuis et al. 2013). Aphid endosymbionts are likely a third player in this evolutionary interaction, but it can be difficult to tease apart each player's role. This three-way interaction has been examined in detail within only one aphid, *Acythrosiphum pisum*, the pea aphid, yielding contradictory results. In this polyphagous aphid, strong correlations between plant host species and endosymbiont infection led to a series of studies aimed at untangling the relationships. For example, natural populations of pea aphid on white clover are associated with the symbiont *R. insecticola* (e.g., Tsuchida et al. 2002, Leonardo and Muiru 2003, Frantz et al. 2009), and some authors have shown a causal relationship wherein *R. insecticola* improved aphid performance on white clover (Tsuchida et al. 2004). However, other studies found that *R. insecticola* decreased aphid

performance on clover (Ferrari et al. 2007), had no effect on aphid performance (Leonardo 2004), or improved performance for some aphid × bacterial genotype combinations on some host plant species (Ferrari et al. 2007). McLean et al. (2011) found that endosymbiont removal from naturally infected hosts had an overall reduction in fecundity regardless of the plant type, indicating a general benefit from natural endosymbiont infection. In the case of pea aphid, it doesn't appear that symbionts facilitate host plant specialization (Hansen and Moran 2013). However, there is evidence that symbionts influence interaction with plants in other ways, e.g., endosymbiont proteins are associated with aphid biotypes exploiting aphid resistant plants (Francis et al. 2010) and a leaf mining insect symbiont is associated with a "green island" phenomenon, where leaf senescence is halted in active feeding sites (Kaiser et al. 2010). Finally, there are likely interactions that have been missed, as the majority of aphid symbiont studies have focused on the pea aphid and associated symbionts, overlooking other unique aphid/ symbiont/ host plant systems (Oliver et al. 2010). Notably, pea aphid has not been found to harbor *Arsenophonus*, which is probably why it had not previously been studied in aphids.

1.4 Arsenophonus

Arsenophonus represents a large endosymbiont clade estimated to infect ~ 5% of arthropods (Duron et al 2008; Novakova et al. 2009), and its possible roles are diverse. This symbiont was first identified as infecting the parasitoid wasp *Nasonia vitripennis*, where it functions as a male killing reproductive parasite, and infection is horizontally transmitted (Werren et al. 1986, Wilkes et al. 2010). In contrast, *Arsenophonus* is thought to be a strictly vertically transmitted obligate symbiont in triatomine bugs, hippoboscid

and streblid flies and some lice (Hypsa 1993, Trowbridge et al. 2006, Perotti et al. 2007). The bacteria has also been found to be phytopathogenic in two plant species (Zreik et al. 1998; Semetey et al. 2007, Bressan et al. 2012). In the order Hemiptera, the endosymbiont is suspected of an uncharacterized facultative relationship with its host. In a geographic survey of *Arsenophonus* infection and parasitism in the lerp psyllid, *Glycaspis brimblecombei,* Hansen et al. (2007) found a positive correlation between infection and parasitism, indicating that individuals with the endosymbiont have a selective advantage over uninfected individuals under increasing parasitism pressure, which suggests a defensive role. This type of correlation was also established for the defensive symbiont *H. defensa* in a population cage study (Oliver et al. 2009).

Soybean aphid is widely infected with *Arsenophonus* (Enterobacteriaceae), in its introduced range and much of its vast native range (Willie and Hartman 2008, Wulff et al. 2013, Bansal et al. 2013). The function of this bacteria is unknown in aphids, despite it being found in 7% of all aphid species, and 30% of species within the genus *Aphis*, including several pest species (Jousselin et al. 2012). This dissertation represents the first effort to understand the role of this bacteria in aphid ecology.

Considering how little is known about the function of *Arsenophonus* in aphids generally, and in soybean aphid specifically, it is critical to evaluate the symbiont's role in the aphid's ecology to better predict the efficacy of a biological control strategy.

The presence of *Arsenophonus* in soybean aphid could determine the outcome of control attempts; especially when considering how common defensive endosymbionts are in aphids. More broadly, assessing the function of *Arsenophonus* in soybean aphid adds insight into its function in other aphids, including additional pest species.

Objectives

The main goal of my dissertation was to determine the function of *Arsenophonus* in soybean aphid, particularly within the context of biological control and host plant defense. A secondary goal was to evaluate the efficacy of plant defense induction to reduce aphid feeding.

My specific objectives were to:

1. Determine the infection frequency of *Arsenophonus* in soybean aphid within the introduced and native range.
2. Manipulate infection in both naturally infected and uninfected individuals through either antibiotic microinjection or hemolymph transfer.
3. Investigate the defensive potential of *Arsenophonus* against natural enemies, including three species of parasitoid wasps and a common fungal pathogen.
4. Assay population growth of differentially infected aphids on resistant and susceptible plant types, to determine if the endosymbiont aids virulent biotypes in colonizing resistant plants.
5. Determine if there is an additional cost to *Arsenophonus* infection for avirulent biotypes on resistant soybean plants.
6. Evaluate the interaction between *Arsenophonus* infection and plant defense.
7. Assess the role of jasmonic acid and salicylic acid in soybean defense against soybean aphid feeding.

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Chapter 2

The endosymbiont *Arsenophonus* is widespread in soybean aphid, *Aphis glycines*, but does not provide protection from parasitoids or a fungal pathogen

Introduction

Maternally inherited bacterial endosymbionts are common in arthropods (Buchner 1965, Douglas 1998, Sandstrom et al. 2001, Duron et al. 2008, Hilgenboecker et al. 2008). Many insects are infected with obligate nutritional endosymbionts that are required for survival, e.g. *Buchnera aphidicola* in aphids (Buchner 1965, Douglas 1998, Akman et al. 2002). In contrast, facultative endosymbionts are not strictly required for insect survival, but can provide a selective advantage in certain ecological contexts (Oliver et al. 2010). For example, facultative endosymbionts have been shown to provide their hosts with heat shock resistance (Russell and Moran 2006), modify host color (Tsuchida et al. 2010), and potentially facilitate host plant colonization (Ferrari et al. 2007). A subset of these facultative endosymbionts can also defend their insect hosts against natural enemies such as parasitoids, entomopathogenic fungi, viruses, and nematodes (Oliver et al. 2003, Scarborough et al. 2005, Hedges et al. 2008, Jaenike et al. 2010).

Bacterial symbionts in the genus *Arsenophonus* are estimated to infect approximately 5% of arthropods (Duron et al. 2008, Novakova et al. 2009). In the

parasitoid wasp *Nasonia vitripennis, Arsenophonus nasoniae* acts as a male killing reproductive parasite (Huger et al. 1985, Werren et al. 1986, Gherna et al. 1991, Duron et al. 2010). Other strains are thought to be obligate symbionts of triatomine bugs, hippoboscid and streblid flies, and lice (Hypsa 1993, Trowbridge et al. 2006, Perotti et al. 2007), and yet others are plant pathogens (Zreik et al. 1998, Bressan et al. 2009, Bressan et al. 2012). *Arsenophonus* is also found in multiple whitefly, psyllid, and aphid species (Subandiyah et al. 2000, Thao et al. 2000, Russell et al. 2003, Thao et al. 2004), but its function among these hosts remains uncharacterized. However, there have been suggestions that *Arsenophonus* may play a defensive role. In a geographic survey of the lerp psyllid, *Glycaspis brimblecombei,* Hansen et al. (2007) found a positive correlation between parasitism and the frequency of *Arsenophonus* infection, potentially indicating that *Arsenophonus* provides the psyllid with a selective advantage in populations under heavy parasitism pressure (Hansen et al. 2007).

If *Arsenophonus* provides defense against natural enemies, then it could be an important consideration in biological control programs against *Arsenophonus*-bearing pests. For example, a defensive symbiont that is present at low prevalence within a population could become common under selective pressure provided by a newly released classical biological control agent, thus undercutting the efficacy of the agent (Clay et al. 2005, Oliver et al. 2008). Alternatively, laboratory populations, which experience vastly different selective environments and frequent population bottlenecks (Heimpel and Lundgren 2000), might be expected to have a different frequency of symbiont infection than field populations. In such a case, conclusions about natural enemy efficacy drawn

from laboratory studies may have little bearing on natural enemy performance in the field.

Multiple important pest species are infected with *Arsenophonus*, including the lerp psyllid, the cotton aphid, *Aphis gossypii,* the sweet potato whitefly, *Bemisia tabaci,* and the soybean aphid, *Aphis glycines* (Thao and Baumann 2004, Hansen et al. 2007, Carletto et al. 2008, Willie and Hartman 2009). Soybean aphid is a serious invasive pest of soybeans in North Central United States, causing extensive yield loss and requiring intensive pesticide applications to a crop that required little pesticide input prior to the introduction of the soybean aphid (Ragsdale et al. 2011). Early parasitism surveys in North America found that soybean aphids were infrequently parasitized (Costamagna et al. 2008, Kaiser et al. 2007, Noma and Brewer 2008) leading to ongoing biological control investigations that incorporate augmentation of ambient fungal pathogens and introduction of parasitoids from the aphid's native range (Heimpel et al. 2004, Wyckhuys et al. 2009, Nielsen and Hajek 2005). The function and prevalence of *Arsenophonus* in field populations of soybean aphid has the potential to affect these pest management tactics.

The goals of this study were 1) to document the frequency and diversity of *Arsenophonus* infection in field-collected soybean aphids from the aphids' native and introduced range and 2) to investigate whether *Arsenophonus* protects soybean aphid against parasitoid wasps or entomopathogenic fungi by assessing natural enemy efficacy against infected versus experimentally cured aphid isolines. For the first goal, we performed *Arsenophonus* diagnostic PCR on six native and seven introduced populations of soybean aphid, followed by multi-locus strain typing (MLST) of *Arsenophonus* using

3 bacterial genes (Jousselin et al. 2013, Wilkes et al. 2011). For the latter goal, we assayed three species of parasitoid wasp and one species of fungal pathogen. The first parasitoid species assayed was *Binodoxys communis*, which currently is the only exotic parasitoid to have been intentionally released in the United States to control the soybean aphid as part of a classical biological control program (Wyckhuys et al. 2009). The second wasp, *Aphelinus certus*, has been identified from parasitized North American soybean aphids, although estimates of parasitism rates are still forthcoming. This parasitoid is native to China, was potentially co-introduced with soybean aphid, and is of interest as a biological control agent (Heimpel et al. 2010). The third wasp, *Aphidius colemani,* is a commercially-available generalist parasitoid of aphids that is known to be susceptible to a defensive symbiont in pea aphid (Vorburger et al. 2009). The aphid fungal pathogen, *Pandora neoaphidis*, is also known to be susceptible to defensive symbionts in pea aphid, and is being investigated for augmentative biological control of the soybean aphid (Scarborough et al. 2005, Lukasik et al. 2013b, Koch and Ragsdale 2011).

Materials and Methods

Geographic survey

To evaluate the prevalence of *Arsenophonus*, soybean aphids were collected from the Asian native range and North American invasive range. Collections were made either at university agricultural stations or on private lands with landowner permission (Table 1). For each population, 30 adult aphids were collected from plants at least 1 meter apart to minimize sampling of siblings, and immediately placed in 95% ethanol. Five aphids

were selected at random from each introduced range population and ten aphids were selected from each native range population for molecular analysis. We extracted DNA by homogenizing individual aphids in 100 µl of 10% w/ v Chelex (Sigma-Aldrich, St Louis, MO, USA) in PCR-grade purified water. We added 6 µl of proteinase K to each sample, vortexed, incubated overnight at 56°C, and then incubated samples at 96°C for ten minutes. We screened for the presence of *Arsenophonus* using a diagnostic PCR protocol modified from Thao and Baumann (2004), which uses *Arsenophonus* specific primers to amplify the intervening region between 16S and 23S rDNA: Ars23S-1 (5'-CGTTTGATG AATTCATAGTCAAA-3') and Ars23S-2 (5'-GGTCCTCCAGTTAGTGTTACCCAAC - 3'). Reactions totaled 10 µl, containing: 2.0 µl of DNA template, 1.0 µl of 25 mM $MgCl_2$, 1.0 µl of 10 mM dNTP mixture, 1.0 µl of Invitrogen 10X buffer ($MgCl_2$ free), 0.8 µl of 5.0 pmole $µl^{-1}$ of each primer, 0.1 µl of 5U/µl Invitrogen Taq polymerase, and ddH_2O to 10 µl. PCR conditions were: initial denature at 95°C for 5 min; followed by 30 cycles of (95°C, 30 s; 55°C, 30 s; 72°C, 45 s); and final elongation at 70°C for 10 min. All PCRs included negative and positive controls. Product from multiple samples was sequenced to confirm *Arsenophonus*. All sequences were identical and the shared sequence was submitted to Genbank (Accession number KC019882). As a further control of extraction quality, we ran samples with the primers CAIF (5'-GCCTGATGCAGCCATGCCGCGTGTATG-3') and CAIR (5'-GTCATCCCCACCTTCC-3') with the same PCR conditions as previously listed. These primers were developed by Dale et al. (Dale et al. 2006) to target *Arsenophonus* 16S sequence in the hippoboscid fly, *Pseudolynchia canariensis*. However, they reliably detected 16S sequence from the obligate symbiont *Buchnera aphidicola* in soybean

aphid, as confirmed by sequencing results (Accession number KC019881). Because this obligate symbiont should be present in all extractions, any samples that failed to amplify *B. aphidicola* were considered to be of poor quality and discarded. To compare *Arsenophonus* infection prevalence between the native and introduced ranges, we used logistic regression (Arc v. 1.06). To avoid overrepresentation of heavily sampled geographic regions, aphids collected from within the same county were considered to come from a single population, and pooled prior to statistical analysis.

MLST

We investigated potential genetic diversity in *Arsenophonus* using an MLST approach. We randomly selected a single extraction from each native and introduced population (Table 1), as well as from our two experimental colonies (KY and MN). We amplified DNA from each sample with the following primer sets: fbaAf (5'-GCYGCYAAAGTTCRTTCCC-3') and fbaAr2 (5'-GGCAAATTAAATTTCTGCGCAACG-3'), ftsKf (5'-GTTGTYATGGTYGATGAATTTGC-3') and ftsKr (5'-GCTCTTCATCACYTCAWAACC-3'), yaeTf (5'-GCATACGGTTCAGACGGGTTTG-3') and yaeTr (5'-GCCGAAACGCCTTCAGA AAAG-3'). PCR reaction recipe followed above protocol and PCR conditions were: initial denature at 93°C for 3 min; 30 cycles of (93°C, 30 s; 52°C, 30 s; 72°C, 1min); and final elongation at 72°C for 5 min (Jousselin et al. 2013, Wilkes et al. 2011). Because sequences generated from each population were identical for each of the genes, *fbaA*, *ftsK*, and *yaeT*, a single sequence per gene was submitted to Genbank (KC701199, KC701198, KC701197).

Arsenophonus curing and colony maintenance

We used two soybean aphid clones for experimental manipulations. These clones were collected independently of the geographic survey specimens. One aphid clone, "KY", was initially collected in Fayette County, KY in 2009.The second clone, "MN", was originally collected in Ramsey County, MN and was maintained in culture at the University of Minnesota prior to transfer to Kentucky in 2010 (USDA Permit # P526P-10-00818). In addition to *Arsenophonus*, each aphid clone was screened diagnostically for other known bacterial symbionts of aphids (Russell et al. 2003), and examined for total bacterial diversity using denaturing gradient gel electrophoresis of bacterial 16S sequences (Russell et al. 2013). The only bacterial endosymbionts detected were *Arsenophonus* and *Buchnera* (J. Wulff, unpublished data).

We cured these aphid clones of *Arsenophonus* infection using antibiotic microinjection, following a protocol modified from Oliver et al. (2003). Individual aphids from each clone were immobilized on a screen-covered pipette tip attached to vacuum, under a stereo microscope. Antibiotic was fed into a borosilicate microinjection needle attached to a syringe via tubing. Fourth-instar aphids were injected with 1.0 mg ml^{-1} ampicillin solution (Ruan et al. 2006). *Arsenophonus* is susceptible to ampicillin, but the aphid's primary symbiont, *Buchnera aphidicola*, is not (Griffiths and Beck 1974). After the initial injection, aphids were individually placed on excised soybean leaves maintained on 1% w/v agar, monitored for survivors, and a subset of offspring were checked for *Arsenophonus* via diagnostic PCR. This procedure was repeated for two subsequent generations using offspring of survivors from the previous bout of injections. Cured and infected isoline colonies were kept at 25± 1°C and 16L: 8D on

Asgrow® AG4303 variety commercial soybeans in 10 cm pots. Plants were individually caged in 3.78 liter plastic jars that had panels of mesh to allow ventilation while preventing aphid escape. Aphids were transferred to new plants as needed, approximately twice per month, to avoid overcrowding and prevent alate production. All aphid isolines were maintained in this manner for at least 3 months prior to experiments. Five individuals from each soybean aphid isoline were screened with diagnostic PCR at least every 2 months to assure that the isoline retained the expected infection status. The cured aphid isolines never tested positive for *Arsenophonus*.

Parasitism assays

We evaluated the influence of *Arsenophonus* in soybean aphid on parasitism success by three parasitoid wasp species. The classical biological control agent *Binodoxys communis* was initially collected in August 2002 near Harbin, in the Chinese province of Heilongjaing, and was maintained in quarantine in St. Paul, Minnesota prior to initiation of our colony in Kentucky (USDA-APHIS permit P526P-10-01532) (Wyckhuys et al. 2008). *Aphelinus certus* was collected locally in Lexington, KY in August 2010 from parasitized soybean aphids. *Aphidius colemani* is a commercially available biological control agent of aphids (APHIPAR, Koppert Biological Systems, The Netherlands). Each species of parasitoid was maintained in culture with *Arsenophonus*-cured soybean aphids and soybean plants at 25 ± 1°C and 16L: 8D in the previously described culture jars with supplemental honey and water for at least two generations prior to use in parasitism assays.

Cage parasitism assays

We conducted cage parasitism assays using methodology adapted from Oliver et al. (2003). For each *Arsenophonus* infected/cured isoline pair, we assayed parasitism success by each of the three parasitoid species in separate experiments (6 assays total). For each assay, 12 vegetative stage 2 (V2) soybean plants were infested with *Arsenophonus*-infected aphids and 12 V2 soybean plants were infested with *Arsenophonus*-cured aphids. We transferred a leaf with >100 juvenile aphids to each experimental plant. Experimental plants were covered with cup cages, constructed from 947 ml translucent plastic containers, organza screening material, and weather stripping to provide a tight seal between cage and pot. After allowing 24 h for aphid establishment, we culled the aphids to either 30 aphids (*A. certus* assays), or 50 aphids (*B. communis* and *A. colemani* assays). *B. communis* and *A. certus* assays were conducted primarily with 2nd and 3rd instar aphids, whereas *A. colemani* assays were conducted primarily with 3rd and 4th instar aphids (Wyckhuys et al. 2008, Lin and Ives 2003). A single mated female wasp was introduced to each cup cage and removed after 24 h. If the wasp was dead or missing after this interval, the replicate was discarded. After 10 d, parasitized aphids (mummies) were counted, and proportion parasitism was calculated by dividing the number of mummies observed by the initial aphid number for that replicate. For each assay, the effect of aphid infection status on proportion parasitism was assessed using a t-test (IBM SPSS v20). Proportion data required an arcsine square-root transformation to satisfy the assumptions of the model.

Observation assays

Six observation assays were conducted in parallel to the cage assays, using the same three parasitoid species and two aphid genotypes. For each experiment, soybean leaves infested with either *Arsenophonus*-infected or cured aphids of all instars were embedded, adaxial side, in 1% agar in 100×15 mm petri dishes. Five to ten wasps of the same species were aspirated onto the embedded leaf. Wasps were allowed to settle and then culled to four actively parasitizing wasps. Wasps were observed continuously under a dissecting microscope. When oviposition was observed, each stung aphid was moved to a 35 mm leaf disk embedded in 1% agar, until a total of 10-15 aphids were parasitized, constituting a replicate. This procedure was repeated with fresh wasps until 10 replicates were generated per treatment per assay.

We regularly removed aphid progeny from leaf disks to avoid confusing progeny with the original stung aphids. Wasp mummies typically formed within 5-7 days, regardless of the parasitoid species. On day 10, we calculated proportion parasitism by dividing the number of mummies by the number of aphids that had survived until just prior to mummy formation. Aphids that died prior to day 5 were excluded from the data. Proportions were arcsine square-root transformed and analyzed using a t-test for each assay.

Fungal assays

To assess the effect of *Arsenophonus* infection status on soybean aphid susceptibility to the entomophthoralean fungus *P. neoaphidis*, we conducted bioassays of *Arsenophonus*-infected versus cured aphids using the same two aphid genotypes as the parasitism assays. For each replicate, we transferred 25, 3rd - 4th instar alatoid nymphs to

a 100×15 mm, sterile, polystyrene petri dish containing moistened filter paper and an excised soybean leaflet (variety S19R5; NK, Golden Valley, MN). The petiole of each leaflet was placed in moist florist foam to prevent leaflet desiccation. To measure aphid exposure to fungal conidia, a glass cover slip was attached to each leaflet to allow for enumeration of conidia after aphid exposure to cultures.

We initiated a total of 20 replicates for each aphid isoline pair, 10 each from the infected and cured isolines. We used actively sporulating *P. neoaphidis* cultures to inoculate aphids. Subcultures used in the assays had been established 30-40 days prior to use and were only used after sporulation became evident (i.e., when conidia became visible on culture lids). All fungal cultures originated from the same *P. neoaphidis* isolate, which had been initially isolated from an infected, field-collected pea aphid (*Acyrthosiphon pisum*). The field collected isolate was used to infect soybean aphids in the laboratory, after which, the fungus was recovered from a single infected soybean aphid. The resulting isolate was periodically passed through and recovered from single soybean aphid individuals prior to use in the assays. Such periodic infection and recovery was necessary to maintain culture pathogenicity. Cultures used to infect soybean aphids in these assays originated from a single culture recovered from an infected soybean aphid immediately prior to assay initiation. The *P. neoaphidis* isolate has been deposited in the USDA, Agricultural Research Service's Collection of Entomopathogenic Fungal Cultures (ARSEF 11663).

Fungal cultures were inverted over each soybean aphid replicate. After 2 h, the fungal cultures and coverslip were removed from each replicate, and the dishes were sealed with parafilm to maintain the humidity required for fungal disease initiation. Each

cover slip was stained with aceto-orcein stain, and examined at 200x magnification. Spores had been deposited on all, indicating that all replicates were exposed to fungal conidia. We then counted spores in 10 randomly chosen fields of view per replicate, and calculated mean spore number per field as an estimate of fungal exposure.

We examined the aphids once per day over the next 5 days. Dead or apparently infected aphids were removed from the experimental dish and transferred to a 50 mm tissue culture dish containing 1% water agar to induce sporulation. If sporulation occurred, the aphid was considered to be infected. We confirmed fungal species identity for two aphids exhibiting successful sporulation on each of the 5 days that assays were monitored. Conidia were stained with aceto-orcein stain and species identity was confirmed via spore morphology at 200x magnification (Samson et al. 1988).

We calculated the proportion of aphids infected per replicate, and used Pearson's correlation coefficient to determine whether this value was significantly associated with fungal exposure per replicate. We observed substantial variation in both variables, but they were not strongly correlated ($R = 0.067$, $P = 0.72$), so we proceeded to compare fungal infection between treatments without including fungal exposure as a covariate. We arcsine square-root transformed the proportion of aphids infected by *P. neoaphidis*, and performed t-tests (IBM SPSS v20) to determine whether this proportion differed as a function of *Arsenophonus* presence/absence in either aphid isoline.

Results

Geographic survey

When the prevalence of *Arsenophonus* in native and introduced populations of the soybean aphid was surveyed, we found that the symbiont was very common in all examined populations (Table 1). In the introduced North American range, a mean (±S.E.) of 98 ± 1% of aphids were infected, which was slightly, but significantly, higher than the 85 ± 6% infection found in the native Asian range (Wald = 2.128, df =11, P = 0.0334).

Arsenophonus MLST

Arsenophonus fbaA, ftsK, yaeT genes were sequenced from one aphid from each of our surveyed populations (Jousselin et al. 2013, Wilkes et al. 2011). We did not detect any genetic variation among sequences from the native and introduced populations, giving no evidence for multiple strains of *Arsenophonus* within soybean aphid.

Parasitism assays

The influence of *Arsenophonus* on soybean aphid susceptibility to parasitism was assessed using three different parasitoids. Parasitism by the introduced biological control agent *B. communis* did not differ significantly between *Arsenophonus*-infected and experimentally cured aphids of a Kentucky (KY) origin isoline within either a cage assay (t =0.88, df =18, P =0.39), or an observation assay (t =0.22, df =22, P =0.83; Figure 1A). Parasitism of a Minnesota (MN) origin isoline of aphids was substantially lower than the KY isoline, but again did not differ between *Arsenophonus*-infected and experimentally cured aphids in either the cage assay (t =0.86, df =22, P =0.40), or the observation assay (t =0.12, df =22, P =0.90).

There were no differences in *A. certus* parasitism of the KY isoline in the cage assay (t =0.38, df =22, P =0.71) or the observation assay (t =0.52, df =20, P =0.61), nor of the MN isoline in the cage assay (t =0.02, df =19, P =0.98) or the observation assay (t =0.99, df =18, P =0.33; Figure 1B). *A. certus* had the greatest disparity in performance between the two assays, with very low rates of parasitism for cage assays compared to the observation assays.

There were also no differences in proportion parasitism by *A. colemani* between infected and experimentally cured soybean aphid for either isoline or parasitism assay (KY cage assay: t =0.33, df =20, P =0.75; KY observation assay: t =0.29, df =24, P =0.77; MN cage assay: t =0.97, df =20, P =0.34; MN observation assay:, (t =1.87, df =18, P =0.07; Figure 1C).

Fungal assays

In a challenge using the entomopathogenic fungus *P. neoaphidis*, observed proportions of infection were highly variable, ranging from 0 to 0.76 per replicate. Mean (± SE) proportion *P. neoaphidis* infection in the *Arsenophonus* infected and uninfected aphids in the KY isoline were 0.15 ± 0.05 and 0.12 ± 0.06 respectively, and arcsine squareroot transformed values did not differ significantly from one another (t = 0.58, df = 18, P = 0.57). Likewise, *Arsenophonus* infected and uninfected aphids in the MN isoline had 0.22 ± 0.07 and 0.13 ± 0.05 proportion infected, and again did not differ significantly from one another (t = 1.46, df = 18, P = 0.16).

Discussion

Our primary goal was to assess whether *Arsenophonus* defends soybean aphid against natural enemies. Using three parasitoid wasp species, we found no evidence that *Arsenophonus* provides this defense in either of two genotypes of soybean aphid. All three species of parasitoids were able to successfully attack soybean aphid, and there were no significant differences in successful parasitism of *Arsenophonus*-infected versus cured aphids in either cage or observation assays. Likewise, we found no difference in aphid mortality from the fungus *P. neoaphidis* based on *Arsenophonus* infection.

Our aggregated results indicate that *Arsenophonus* is likely not a defensive symbiont in soybean aphid, but some caveats should be considered. First, we used only two genotypes of aphids, which were infected with the same strain type of *Arsenophonus*, based on identical *Arsenophonus* ribosomal and MLST sequences. It is possible that other *Arsenophonus* strains may provide protection to other genotypes of soybean aphid host. For example, different strains of the bacterial endosymbiont *Hamiltonella defensa* provide differential protection against parasitism to pea aphid based on the presence or absence and type of APSE phage (Oliver et al. 2009). Additionally, a strain of *Regiella insecticola* was recently shown to protect its aphid host against parasitism, a trait not previously associated with the symbiont (Vorburger et al. 2009), indicating that bacterial strains can vary in their defensive properties. However, in soybean aphid, our MLST survey of *Arsenophonus* did not identify any additional bacterial strains in either the native or introduced range, indicating that hypothetical alternate strain types are rare, if they exist at all. Furthermore, soybean aphid is a recent introduction to North America, and is notably lacking in genetic diversity (Michel et al. 2009); consequently, it seems

unlikely that additional sampling of aphid/symbiont genotypes in the invaded range would yield different results.

We limited our parasitism assays to wasp species relevant to the North American introduced range of soybean aphid. *B. communis* and *A. certus* are both of interest for biological control and represent two different families of parasitoids (Braconidae and Aphelinidae, respectively), the latter being a more generalized parasitoid species (Heimpel et al. 2010, Desneux et al. 2009). However, there is growing evidence that defensive symbiont-mediated selection can favor parasitoid genotypes that are insensitive to the symbiont (Vorburger et al. 2009). The high prevalence of *Arsenophonus* infection in the field makes it likely that field-collected parasitoids of soybean aphid have encountered and potentially adapted to the symbiont. *A. colemani,* the third wasp we assayed, was commercially cultured on other aphid species and presumably naïve to soybean aphid, yet it was also unaffected by *Arsenophonus.*

Although our results indicate that *Arsenophonus* does not defend its host against these natural enemies, it does have a very high infection rate in both the introduced and native populations. Several possible explanations could underlie this widespread infection. First, *Arsenophonus* could manipulate host reproduction. Reproductive manipulation is a common means by which endosymbionts promote their own infection, and has recently been documented in the sexual generation of pea aphid by the endosymbiont *Spiroplasma* (Engelstadter and Hurst 2009, Simon et al. 2011). Second, *Arsenophonus* could be providing other context-specific benefits to soybean aphid, e.g., heat tolerance, defense against other pathogens (Russell and Moran 2006, Jaenike et al. 2010), or general fecundity or longevity effects (Himler et al. 2011). Third,

Arsenophonus may be transmitted horizontally, either directly between aphids or indirectly through the plant (Moran and Dunbar 2006, Caspi-Fluger et al. 2012). Finally, high fidelity vertical transmission, coupled with a very low metabolic cost to the host, could permit *Arsenophonus* to persist in a population without any benefit to the host (Hoffmann et al. 1998). However, other endosymbionts that had been considered previously to be neutral passengers were subsequently found to be extremely beneficial to their hosts under certain circumstances (Hedges et al. 2008, Brownlie et al. 2009). Given the very high prevalence of *Arsenophonus* in soybean aphid, it is therefore reasonable to presume that *Arsenophonus*, too, provides soybean aphid with a context-specific benefit that remains to be elucidated.

Table 1. Soybean aphid, *Aphis glycines,* collection locations, year collected, collector, and *Arsenophonus* prevalence

Locality	*Year*	*Collector*	*Arsenophonus positive/Aphids screened*
Native			
Hebei Province, China	2008	Wu Kongming	8/8
Shangdong Province, China	2008	Wu Kongming	9/10
Guangxi Province, China	2008	Wu Kongming	10/10
Hangzou District, China	2008	Wu Kongming	7/10
Yangling District, China	2008	Wu Kongming	9/10
Harbin Province, China	2008	Wu Kongming	5/8
Introduced			
Whitley Co., Indiana, USA	2008	Marc Rhainds	23/25
Tippecanoe Co., Indiana, USA	2008	Marc Rhainds	10/10
Wabash Co., Indiana, USA	2008	Marc Rhainds	5/5
Huntington Co., Indiana, USA	2008	Marc Rhainds	5/5
Olmsted Co., Minnesota, USA	2008	Fritz Breitenbach	5/5
Waseca Co., Minnesota, USA	2008	George Heimpel	5/5
Fayette Co., Kentucky, USA	2011	Jason Wulff	27/28

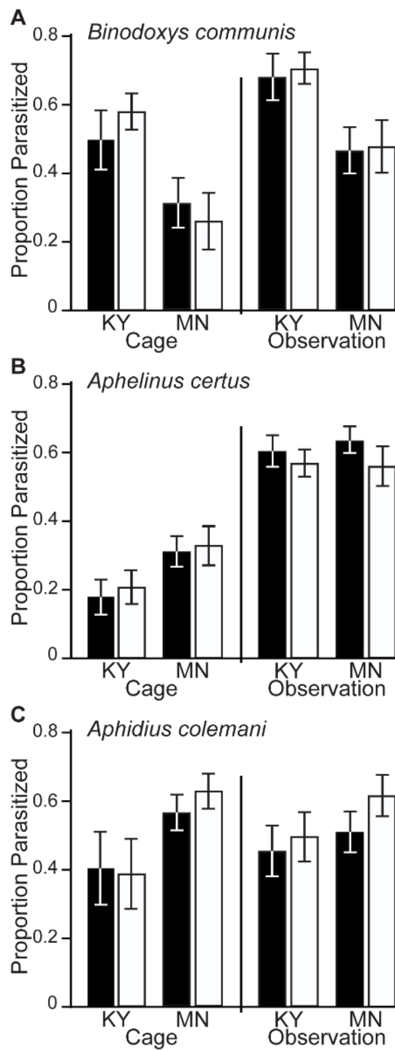

Figure 1. Mean (± SE) proportion of soybean aphids parasitized by *Binodoxys communis* (A), *Aphelinus certus* (B), and *Aphidius colemani* (C). Black bars represent naturally *Arsenophonus*-infected soybean aphids and white bars represent experimentally cured isolines with the same genetic background. Two isoline pairs (KY and MN) were each evaluated in two experiments (cage and observation assays) for each parasitoid species. No significant differences were detected in any assay.

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Chapter 3

The endosymbiont *Arsenophonus* provides a general benefit to its host soybean aphid, *Aphis glycines*, regardless of host plant resistance (*Rag*).

Introduction

Soybean aphid is the primary pest of soybean in North America, causing substantial economic cost from both yield loss and chemical treatment (Ostlie 2001, Johnson et al. 2009). Multiple avenues of research have been developed in an effort to control soybean aphid, including biological control (Heimpel et al. 2004) and traditional breeding for resistant plants. Discovery of soybean genotypes resistant to soybean aphid (e.g. Hill et al. 2004, Mensah et al. 2005, Zhang et al. 2010) has led to breeding efforts to incorporate resistance genes (*Rag1, Rag2,* etc.) into commercial soybean varieties. As of 2012, there were 18 varieties, 17 *Rag1* and one *Rag1* + *Rag2* pyramid, commercially available (McCarville et al. 2012, Hesler et al. 2013). However, the discovery of soybean aphid "biotypes" that are unaffected by these resistance genes (Kim et al. 2008b, Hill et al. 2010), has cast some doubt on the durability of soy resistance (Hill et al. 2010). The mechanism for soybean aphid virulence on resistant soy is currently unknown.

The biotype designation within soybean aphid is, to date, based purely on differential performance on resistant soybean varieties, without reference to underlying aphid genotypes. Overall, soybean aphid has limited genetic diversity within North America, as would be predicted based on founder effects (Michel et al 2009). Wenger and Michel (2013) found that the genetic variation that does exist in soybean aphid has

no clear linkage between biotype and genotype, indicating aphid virulence is not a single gene trait. They suggest that broader mechanisms, such as gene complexes, non-genetic environmental cues or endosymbionts may determine aphid virulence biotypes rather than the single gene for gene model indicated in other systems (Hogenhout and Bos 2011). In the present paper we explore the potential role of a bacterial endosymbiont, *Arsenophonus,* in soybean aphid biotype determination.

Endosymbiotic bacteria that infect arthropods can provide a range of benefits to their hosts, including nutrient provisioning, increased fecundity, and defense against biotic and abiotic factors (Moran et al. 2008, Brumin et al. 2011, Himler et al. 2011, Jaenike and Brekke 2011). Aphids, in particular, have a wide array of maternally-inherited bacterial endosymbionts, including an obligate nutritional symbiont, *Buchnera aphidicola,* which is found in virtually every aphid species (Vogel and Moran 2013). Some aphids are also infected with one or more strains of "facultative" endosymbionts, which have been shown to defend their host against natural enemies (Oliver et al. 2010, Lukasik et al. 2013b) as well as potentially influencing interactions between aphids and their host plants (Frago et al. 2012, Biere and Tack 2013). Endosymbionts may also aid in aphid biotypes' virulence; a *Rickettsia*-like symbiont in the potato aphid is suspected of facilitating the aphid's ability to colonize tomato plants bearing a resistance gene against aphid feeding (Hebert et al. 2007, Francis et al. 2010).

Most soybean aphids are infected with the facultative endosymbiont *Arsenophonus* (Willie et al. 2009, Chapter 2, Wenger and Michel 2013, Bansal et al. 2014). *Arsenophonus* infects many arthropods, but has an unknown role in hemipterous

insects (Duron et al. 2008, Nováková et al. 2009). Among aphids, Jousselin et al. (2013) found that *Arsenophonus* infected 7% of species, with more than 31% of species in the genus *Aphis* infected. However, within an infected species, they found that *Arsenophonus* was present at low to intermediate frequencies. In contrast, soybean aphid has a very high rate of infection, both in its native Asian range, and introduced North American range (Chapter 2, Bansal et al. 2014). This high prevalence of infection suggests the symbiont could be playing a critical role in soybean aphid life history, potentially mediating interactions with its soybean host.

On the surface, *Arsenophonus* does not appear to be a likely candidate for determining soybean aphid virulence on *Rag* plants, but it has never been directly investigated. *Arsenophonus* has been found in all soybean aphid biotypes examined for the symbiont (Bansal et al. 2014, Wenger and Michel 2013, Chapter 2); therefore it is not the simple presence or absence of *Arsenophonus* that determines biotypes. Additionally, while strain variation within a bacterial species can cause profoundly different host phenotypes (Hansen et al. 2012), Wulff et al. (2013) did not find evidence of multiple *Arsenophonus* strains in soybean aphid; however, it is possible that the four gene regions assessed (Duron et al. 2010, Wilkes et al. 2011, Jousselin et al. 2013) overlooked subtle but relevant differences. Despite the apparent uniformity of *Arsenophonus* in both distribution and strain type, there are other examples of identical or very similar strains of endosymbiont infections that can produce different phenotypes within the same host species (Brumin et al. 2011, Caspi-Fluger et al. 2011). Mobile genetic elements such as bacteriophages, can be the critical determinant of phenotype rather than the

endosymbiont strain (Oliver et al. 2009). The same endosymbiont strain in different host genetic backgrounds can also profoundly influence phenotype (Jaenike 2007, Chafee et al. 2011, Raychoudhury et al. 2011, Veneti et al. 2012). Thus it remains possible that *Arsenophonus* may be a critical element in determining virulence biotypes. *Arsenophonus* could also play a more general role in interactions between the aphid and its plant host, independent of soybean resistance.

The overall goal of this study was to investigate how *Arsenophonus* influences host/plant interactions between soybean aphid and soybean. Our first objective was to determine if *Arsenophonus* influences soybean aphid virulence on soybean containing one of two resistance genes, *Rag1* or *Rag2.* The presence of *Arsenophonus* could be necessary for virulent biotypes to overcome resistance genes, either directly through bacterial gene products, or through interactions among bacterial, plant, and aphid genes. Our second objective was to more generally explore the cost and benefits of *Arsenophonus* infection in soybean aphid, comparing the fitness of infected versus cured aphids.

Materials and Methods

Arsenophonus curing and colony maintenance

We manipulated the *Arsenophonus* infection of five soybean aphid clones. Our first two clones, KY and MN, were collected in Fayette County, KY and Ramsey County, MN, respectively (Wulff et al. 2013). Three additional clones were provided by Andy

Michel at The Ohio State University. Biotype 1 was originally collected in Urbana, Illinois in 2000. It is avirulent on *Rag1* and *Rag2* plants, and has been used in several studies (Hill et al. 2004a, 2004b, Li et al. 2004, Hill et al. 2006a, 2006b). Biotype 2 is an Ohio isolate collected and established in the summer of 2005 at the Ohio Agricultural Research and Development Center (OARDC), Wooster, OH. It is virulent on *Rag1* and avirulent on *Rag2* plants (Kim et al. 2008b, Hill et al. 2009). Biotype 3 was originally collected in 2007, from an overwintering host of soybean aphid, *Rhamnus frangula,* in Springfield Fen, IN. It is avirulent on *Rag1* and virulent on *Rag2* plants (Hill et al. 2010). Preliminary studies with our KY and MN lines established KY as avirulent on *Rag1* and *Rag2* (similar to Biotype 1) whereas MN was virulent on *Rag1* and avirulent on *Rag2* (similar to Biotype 2). All clones except Biotype 1 were naturally infected with *Arsenophonus*. We cured naturally infected lines through use of antibiotic microinjection following the protocol described in Chapter 2. We transferred *Arsenophonus* to Biotype 1 from the MN clone through hemolymph microinjection using the same microinjection apparatus. Treated aphids were monitored, and descendants were checked for *Arsenophonus* status using diagnostic PCR, and maintained in colony for at least two months prior to experiments, following protocols from Wulff et al. (2013). At least every two months, five randomly chosen aphids from each cured and infected clone (ten isolines) were screened for *Arsenophonus* status as a check against contamination or spontaneous symbiont loss under culturing conditions. All ten isolines retained the expected infection status throughout the experiment timeframe.

DNA extraction and Diagnostic PCR

To test for the presence of *Arsenophonus* we homogenized individual aphids in 50 µl of 10% w/v Chelex (Sigma-Aldrich, St Louis, MO, USA). We added 3 ml of proteinase K to each sample, vortexed, incubated for 3 hours at 50°C, and then ten minutes at 96°C. We routinely used the following two primer sets to screen for the presence of *Arsenophonus*. With Ars23S-1 (5'-CGTTTGATG ATTCATAGTCAAA-3') and Ars23S-2 (5'- GGTCCTCCAGTTAGTGTTACCCAAC -3') we used 95°C for 5 min for initial denaturation, followed by 30 cycles (95°C, 30s; 55°C, 30s; 72°C, 45s); and a final elongation at 70 °C for 10 min (Thao and Baumann 2004). With yaeTf (5'-GCATACGGTTCAGACGGGTTTG-3') and yaeTr (5'-GCCGAAACGCCTTCAGA AAAG-3'), we used 93°C for 3 min for the initial denaturation, followed by 30 cycles of (93°C, 30 s; 52°C, 30 s; 72°C, 1 min), and a final elongation at 72°C for 5 min (Duron et al. 2010, Wilkes et al. 2012, Jousselin et al. 2013). For both primer sets, reactions totaled 10 µl, containing 2.0 µl of DNA template, 1.0 µl of 25mM $MgCl_2$, 1.0 µl of 10 mM dNTP mixture, 1.0 µl of Invitrogen 10X buffer ($MgCl_2$ free), 0.8 µl of 5.0 pmole μl^{-1} of each primer, 0.1 µl of 5 U/ µl Invitrogen Taq polymerase and ddH_2O to 10 µl.

Population Growth Assays

Two weeks prior to experiments, we established at least three new colonies per clone on early vegetative state, V1-V2, soybean plants at low density. This minimized aphid stress and partially synchronized aphid development amongst the different clones

and between paired infected/cured isolines. Source colonies that produced substantial alates (winged morphs) or “white dwarfs” were considered stressed and discarded. Soybean aphid colonies were maintained on Asgrow® AG4303 variety commercial soybeans. Experimental soybean genotypes, provided by Brian Diers, University of Illinois, were Dwight (non-aphid resistant), LD06-16721 (*Rag1* in the background of Dwight), LD02-4485 (non-aphid resistant), and LD08-12427a (*Rag2* in the background of LD02-4485). All plants were grown in ten cm pots at 25± 1°C and 16L:8D. Aphid colonies were caged in 3.78 L plastic jars that had panels of mesh, allowing ventilation.

In total we performed five experiments to assess aphid performance as a function of *Arsenophonus* infection and plant resistance. We used the same basic experimental set up for all five experiments, although we used different combinations of aphid clones and/or plant types. In each experiment, an experimental unit was a single soybean plant, at the V1-V2 developmental stage that we initially infested with ten 4th instar aphids. To reduce maternal effects from using a single cohort, we choose aphids randomly from at least three source colonies for each of the isolines used on each experimental date, and transferred the aphids to the experimental plant using a fine tipped paint brush. Each experimental plant was caged and maintained at 25± 1°C and 16L: 8D. Aphids were counted for establishment at ~24 h, and counted again at the end of each experiment on day 11. DNA was extracted from at least ten randomly selected aphids per treatment per experiment, and diagnostic PCR performed to verify expected infection.

The first three experiments used paired control and resistant (*Rag1* or *Rag2*) plants to test the hypothesis that *Arsenophonus* provides an advantage to virulent biotypes

on resistant plants. We set up **Experiment 1** on *Rag1* plants, paired with the susceptible counterparts, using three soybean aphid clones: KY, MN, and Biotype 2. This experiment had a fully factorial design with two plant types and two infection types per each of the three clones for a total of 12 treatments. We set up two replicates per treatment per date for a total of 24 aphid populations per date, and we ran the experiment across four dates, giving us a total sample size of 96 aphid populations. **Experiment 2** also used *Rag1* with paired susceptible plants, and added another avirulent biotype, Biotype 1, for a total of 4 aphid clones. This experiment was conducted on a single date, but increased replication to three experimental units for each of the four aphid clones, for a total sample size of 48 aphid populations. **Experiment 3** switched to a different aphid resistance gene, *Rag2*. Experimental design was parallel to Experiment 2, except we added a fifth aphid clone, Biotype 3, which is virulent on *Rag2*. The other four aphid clones were all avirulent on *Rag2*. There were two replicates per treatment for a total sample size of 40 aphid populations.

The last two experiments focused only on susceptible aphid biotypes on resistant plants, to increase experimental power for detecting potentially subtle *Arsenophonus* effects for poorly performing avirulent aphids on resistant plants. **Experiment 4** was a single date experiment using only *Rag1* plants and three avirulent clones: KY, Biotype 1, and Biotype 3. There were eight replicates for each *Arsenophonus* infected and uninfected isolines for a total sample size of 48 populations. **Experiment 5** used *Rag2* plants and three avirulent clones: KY, MN, and Biotype 2. It was repeated on two dates

with six replicates for each clone on each date for a total sample size of 72 aphid populations.

For each of the five population experiments, we calculated population growth by dividing the total day 11 aphid count by the day 1 count. To better fit assumptions of normality and equal variance, we log (x+1) transformed population growth. We analyzed these experiments as fully factorial general linear models in SAS v9.3 statistical software package (SAS Institute, Cary, NC). Single date experiments include aphid clone, plant type, and *Arsenophonus* as fixed factors. Multi-date experiments also included date as a random factor.

Additionally, we were interested in determining if there was an overall *Arsenophonus* main effect when all five population growth experiments were analyzed in aggregate. For each date, we calculated the mean population growth for each treatment (aphid clone × infection status × plant type) and paired values that were the same in all factors except infection status. This effectively created pairs blocked by date, plant type and aphid clone, resulting in 51 *Arsenophonus* plus/minus pairs across all experiments. We compared the log (x+1) transformed population growth between infected and uninfected aphids using a non-parametric Wilcoxon Signed Rank test for paired data (JMP v.10 statistical software package (SAS Institute, Cary, NC)).

Individual fitness experiment

In **Experiment 6,** we assayed individual fecundity for all five aphid clones of our *Arsenophonus* pairs, ten total isolines. We first placed ~fifteen 4th instar soybean aphids from each isoline on at least four soybean leaf disks embedded in 1% w/v agar in a petri dish. These aphids matured to adulthood and immediately started producing offspring. Of which, twenty individual aphids from each isoline, born within a 24-h period, were transferred individually to fresh soybean leaf disks, for a total of 200 aphids across the ten aphid isolines. These aphids were individually reared to adulthood; any aphids that developed wings (alates) were excluded from further study. Final sample size ranged from 10 to 19 aphids per treatment, and there were no differences in alate production between *Arsenophonus* infection categories (χ^2=25.568, d.f. = 1, p= .062). For the remaining apterous aphids, progeny were counted and removed every three days until the aphid died. Total progeny per aphid was square root transformed and analyzed using ANOVA with clone and *Arsenophonus* as fixed factors.

Results

In **Experiment 1**, there were significant interactions between date and all of the experimental factors (Table 2, Figure 2A), making interpretation of other main and interactive effects difficult. There was substantial variability in clone performance across dates, particularly on the control (non-*Rag*) plants. The virulent lines (MN and Biotype 2) performed well on resistant *Rag1* plants, whether *Arsenophonus* was present or not, and the avirulent line (KY) performed more poorly on *Rag1* plants than on control plants,

regardless of infection status. Overall, *Arsenophonus* infected lines performed slightly better than their uninfected counterparts, but with exceptions for some clones on some dates.

In **Experiment 2**, which was conducted on a single date and included an additional susceptible aphid clone, we found a significant effect for clone ($F_{3,32}$= 34.88, p <0.001), for plant type ($F_{1,32}$= 123.34, p <0.001), and between aphid clone and plant type ($F_{3,32}$= 41.01, p <0.001; Table 3, Figure 2B). We also found a significant *Arsenophonus* main effect, in which infected aphids performed better than their uninfected counterparts regardless of plant type ($F_{1,32}$= 4.13, p= 0.050). We found no significant higher order interactions with *Arsenophonus*. In **Experiment 3**, which compared performance of all 5 clones on *Rag2* plants, we found a similar pattern to Experiment 1 and 2, with a significant aphid clone by plant type interaction ($F_{4,20}$= 19.91, p <0.001; Table 4, Figure 2C), but no significant main or interactive effects of *Arsenophonus*, indicating that *Arsenophonus* does not affect aphid virulence on *Rag* plants.

For the last two experiments, we specifically considered whether *Arsenophonus* might impact avirulent clones on resistant *Rag1* or *Rag2* plants. In **Experiment 4**, on *Rag1* plants, we found significant differences in performance among clones ($F_{2,42}$= 16.57, p <0.001; Table 5, Figure 3A), but did not find a significant main or interactive *Arsenophonus* effect ($F_{1,42}$= 0.01, p= 0.950; $F_{2,42}$= 0.45, p= 0.640). In **Experiment 5**, on *Rag2* plants, we found a significant difference between clone performance ($F_{2,60}$= 4.95, p= 0.010; Table 6, fig 3B). We also again found a significant date effect ($F_{1,60}$= 4.12, p=

0.047). We did not find a main *Arsenophonus* effect nor did we find any two or three way interactions.

Despite inconsistent *Arsenophonus* main effects in the individual population growth experiments, when considered in aggregate, we did find that Ars+ clones performed significantly better than their paired Ars- counterparts, when aphid clone, plant type, and experimental date were all taken into account (d.f.= 50, WS= 247, p= 0.02). Per population cage, infected populations averaged 39 ±16 more aphids after 10 d than their corresponding uninfected populations.

When we evaluated progeny production for individual aphids, **Experiment 6**, we found a significant interaction between *Arsenophonus* infection and clone ($F_{4,161}$= 12.68, p= 0.022; Table 7, Figure 4), but not an *Arsenophonus* main effect ($F_{1,161}$= 0.24, p= 0.625). The *Arsenophonus* infected isolines sometimes outperformed their uninfected counterpart (e.g. the MN clone), but not always (e.g., Biotype 1).

Discussion

We explored whether *Arsenophonus*, the facultative endosymbiont of soybean aphid, contributes to biotype virulence on resistant, *Rag*, soybean plants. Our results indicate that *Arsenophonus* does not aid virulent biotypes in colonizing *Rag1* or *Rag2* plants. The three virulent soybean aphid clones exhibited no reduction in performance on resistant plants when cured of their natural *Arsenophonus* infection. Also, transmission of the symbiont from a virulent biotype to an avirulent biotype did not enable the recipient

to better exploit resistant plants. This result is perhaps unsurprising given the widespread prevalence of *Arsenophonus* infection in both avirulent and virulent biotypes (Chapter 2, Wulff et al. 2013, Wenger and Michel 2013). However, given that endosymbiont effects on their host can vary based on endosymbiont genotype (Oliver et al. 2009, Hansen et al. 2012, Weldon et al. 2013) and host genotype (Vorburger and Gouskov 2011) it remained possible that *Arsenophonus* played a role in determining biotype virulence. Overall, it remains unknown how virulent soybean aphid biotypes exploit resistant (*Rag*) soybean plants. In other similar aphid/plant interactions, resistance and biotype formation is determined by a plant gene by insect gene interaction (Elzinga and Jander 2013). It is unlikely that this model underlies soybean aphid biotypes (Wenger and Michel 2013). Our results further reduce the possible mechanisms underlying biotype differentiation, indicating that biotype formation in soybean aphid is independent of *Arsenophonus*.

Secondly, our results indicate that *Arsenophonus* infection does not impose a further cost to avirulent aphids on *Rag* plants. The overall poor performance of avirulent lineages on resistant plants was not improved by curing *Arsenophonus.* Endosymbionts can impart a range of costs on their hosts, which can become more evident when feeding on suboptimal hosts (Chandler et al. 2008). If an additional cost were incurred by *Arsenophonus* infection in conjunction with this stress, we would have expected that introduction of *Rag* resistant plants in the field might select for a shift in *Arsenophonus* infection dynamics, reducing the field prevalence of *Arsenophonus*. In total, we did not find an *Arsenophonus* interaction between either virulent or avirulent biotypes and *Rag1* or *Rag2* plants.

However, when we considered all experiments in aggregate, we found a slight significant benefit to *Arsenophonus* infection. We determined this *Arsenophonus* main effect by incorporating all other experimental variables, such as date, plant type, and aphid clone, into a blocking factor, and conducted a simple comparison of population growth for our paired *Arsenophonus* infected and uninfected treatments. This *Arsenophonus* main effect was usually not significant within individual population experiments due to limited replication and power per experiment. Similarly, we did not find an overall *Arsenophonus* effect in the individual fecundity experiment, but we did observe a clone by *Arsenophonus* interaction.

Prior studies on the fitness impacts of endosymbiont infection have also produced mixed results from individual experiments (Russell et al. 2006, Oliver et al. 2008, Lukasik et al. 2013a). Despite uniform experimental conditions, throughout our experiments there was significant variation in performance among clones and between isolines across dates. In particular, for Experiment 1, consisting of four experimental blocks on different dates, all the fixed factors had significant interactions with date. It is difficult to isolate the source of this date to date variation as all the controllable experimental elements were kept as static as possible. However, aphids have extensive polyphenism in response to subtle environmental cues and rapid generation time that can exaggerate small initial reproductive differences (Chen et al. 2000, Richardson et al. 2011). This variation introduces statistical noise that can shroud subtle symbiont effects and host genotype by endosymbiont interactions. In the present study, extensive replication over dates overcame this variation to reveal that *Arsenophonus* infected

soybean aphids generally performed slightly better than uninfected aphids. That this pattern was not evident for every clone on every date, however, emphasizes the potential pitfalls of drawing sweeping conclusions from single date experiments.

By itself, the slight fitness advantage of *Arsenophonus* infection might contribute to its high field infection frequency in soybean aphid, suggesting a general competitive advantage of infected individuals over uninfected individuals. The overall average aphid totals from across all plant treatments was 176 aphids per plant. The observed fitness benefit of 39±16 *Arsenophonus* infected aphids over uninfected aphids after 10 days, might be amplified in field populations over a longer timeframe, given that soybean aphid populations can double in about seven days (Ragsdale et al. 2007).

We still do not know the underlying mechanism for the *Arsenophonus* benefit. It could provide a slight nutritional advantage. It could also have additional more profound benefits under field conditions; while *Arsenophonus* does not appear to be defensive against parasitoids or fungal pathogens (Chapter 2) the symbiont could mediate interactions with other abiotic or biotic factors. Alternatively, *Arsenophonus* could influence soybean aphid performance on its primary host, buckthorn (*Rhamnus* spp). However, the observed general fitness benefit on soybean may alone be sufficient to explain the near fixation of *Arsenophonus* infection in soybean aphid populations. In summary, our results indicate that *Arsenophonus* increases soybean aphid population growth and may contribute to the pest status of this aphid, but should not impact attempts to control soybean aphid though host plant resistance.

Table 2. ANOVA of log (x+1) transformed aphid population growth (Experiment 1)

Factor (s)	d.f.	ss	ms	*F*	*P*
Arsenophonus (Ars)	1	0.111	0.111	5.22	0.027
Plant type (Pt)	1	0.446	0.446	20.98	<0.001
Clone	2	2.917	1.458	68.63	<0.001
Date	3	1.104	0.368	17.32	<0.001
Ars x Pt	1	0.001	0.001	0.05	0.817
Ars x Clone	2	0.040	0.020	0.94	0.399
Ars x Date	3	0.251	0.084	3.93	0.014
Pt x Clone	2	1.795	0.898	42.24	<0.001
Pt x Date	3	0.214	0.071	3.36	0.026
Clone x Date	6	1.107	0.185	8.69	<0.001
Ars x Pt x Clone	2	0.058	0.029	1.37	0.264
Ars x Pt x Date	3	0.007	0.002	0.11	0.953
Ars x Clone x Date	6	0.717	0.120	5.62	<0.001
Pt x Clone x Date	6	0.078	0.013	0.61	0.720
Ars x Clone x Pt x Date	6	0.617	0.103	4.84	<0.001
Error	48	1.020	0.021		

Population growth was log (x+1) transformed to better fit assumptions of normality and analyzed as fully factorial general linear models

Table 3. ANOVA of the effect of *Arsenophonus* and *Rag1* on clone performance on single date (Experiment 2)

Factor (s)	d.f.	ss	ms	*F*	*P*
Arsenophonus (Ars)	1	0.187	0.187	4.13	0.050
Plant type (Pt)	1	5.594	5.594	123.34	<0.001
Clone	3	4.746	1.582	34.88	<0.001
Ars x Pt	1	0.081	0.081	1.79	0.190
Ars x Clone	3	0.127	0.042	0.94	0.435
Pt x Clone	3	5.580	1.860	41.01	<0.001
Ars x Clone x Pt	3	0.171	0.057	1.25	0.307
Error	32	1.451	0.045		

Population growth was log (x+1) transformed to better fit assumptions of normality and analyzed as fully factorial general linear models

Table 4. ANOVA of the effect of *Arsenophonus* and *Rag2* on clone performance (Experiment 3)

Factor (s)	d.f.	ss	ms	*F*	*P*
Arsenophonus (Ars)	1	0.010	0.010	0.36	0.555
Plant type (Pt)	1	5.548	5.548	200.70	<0.001
Clone	4	0.920	0.230	8.32	<0.001
Ars x Pt	1	0.000	0.000	0.02	0.897
Ars x Clone	4	0.293	0.073	2.65	0.063
Pt x Clone	4	2.201	0.550	19.91	<0.001
Ars x Clone x Pt	4	0.176	0.044	1.59	0.215
Error	20	0.553	0.028		

Population growth was log (x+1) transformed to better fit assumptions of normality and analyzed as fully factorial general linear models

Table 5. ANOVA of the effect of *Arsenophonus* on avirulent soybean aphid performance on resistant (*Rag1)* plants (Experiment 4)

Factor (s)	d.f.	ss	ms	*F*	*P*
Arsenophonus (Ars)	1	0.000	0.000	0.01	0.950
Clone	2	1.622	0.811	16.57	<0.001
Ars x Clone	2	0.044	0.022	0.45	0.640
Error	42	2.056	0.049		

Population growth was log (x+1) transformed to better fit assumptions of normality and analyzed as fully factorial general linear models

Table 6. ANOVA of the effect of *Arsenophonus* on avirulent soybean aphid performance on resistant (*Rag2)* plants over two dates (Experiment 5)

Factor (s)	d.f.	ss	ms	*F*	*P*
Arsenophonus (Ars)	1	0.110	0.110	1.40	0.241
Clone	2	0.777	0.389	4.95	0.010
Date	1	0.323	0.323	4.12	0.047
Ars x Clone	2	0.115	0.058	0.73	0.485
Ars x Date	1	0.208	0.208	2.64	0.109
Clone x Date	2	0.168	0.084	1.07	0.350
Ars x Clone x Date	2	0.054	0.027	0.34	0.711
Error	60	4.711	0.079		

Population growth was log (x+1) transformed to better fit assumptions of normality and analyzed as fully factorial general linear models

Table 7. ANOVA of the effect of *Arsenophonus* on individual soybean aphid fecundity for five clones (Experiment 6).

Factor (s)	d.f.	ss	ms	*F*	*P*
Arsenophonus	1	0.259	0.259	0.24	0.625
Clone	4	8.731	2.183	2.02	0.094
Arsenophonus x Clone	4	12.679	3.170	2.94	0.022
Error	161	173.903	1.080		

Total progeny was square root transformed to better fit assumptions of normality and analyzed as fully factorial general linear models

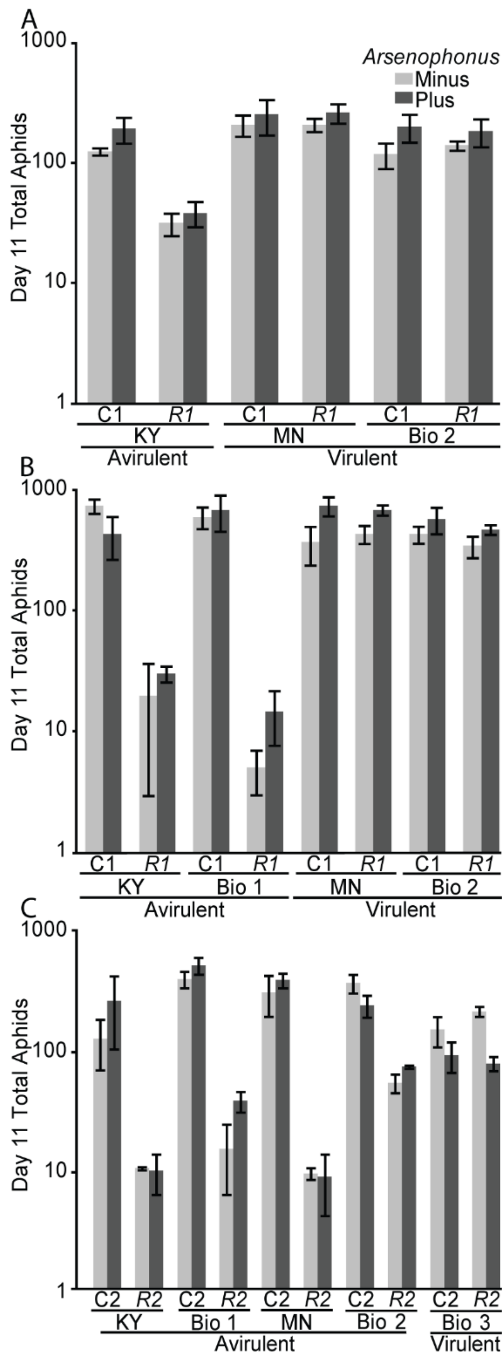

Figure 2 Mean (± SE) populations of *Arsenophonus* infected and uninfected soybean aphid on resistant and susceptible soybean plants. Data presented on a log scale for [A] Experiment 1, [B] Experiment 2, and [C] Experiment 3. [A] Experiment 1 assessed the performance of one avirulent clone, KY, and two virulent clones, MN and Biotype 2 (Bio 2), on *Rag1 (R1)* and corresponding control (C1) soybean plants over 4 dates. [B] Experiment 2 assessed the performance of two avirulent clones, KY and Biotype 1 (Bio 1), and two virulent clones, MN and Bio 2, on *R*1 and C1. [C] Experiment 3 assessed the performance of four avirulent clones, KY, Bio1, MN, Bio2 and one virulent clone, Biotype 3 (Bio3), *Rag2* (*R2*) *and* corresponding control plants (C2).

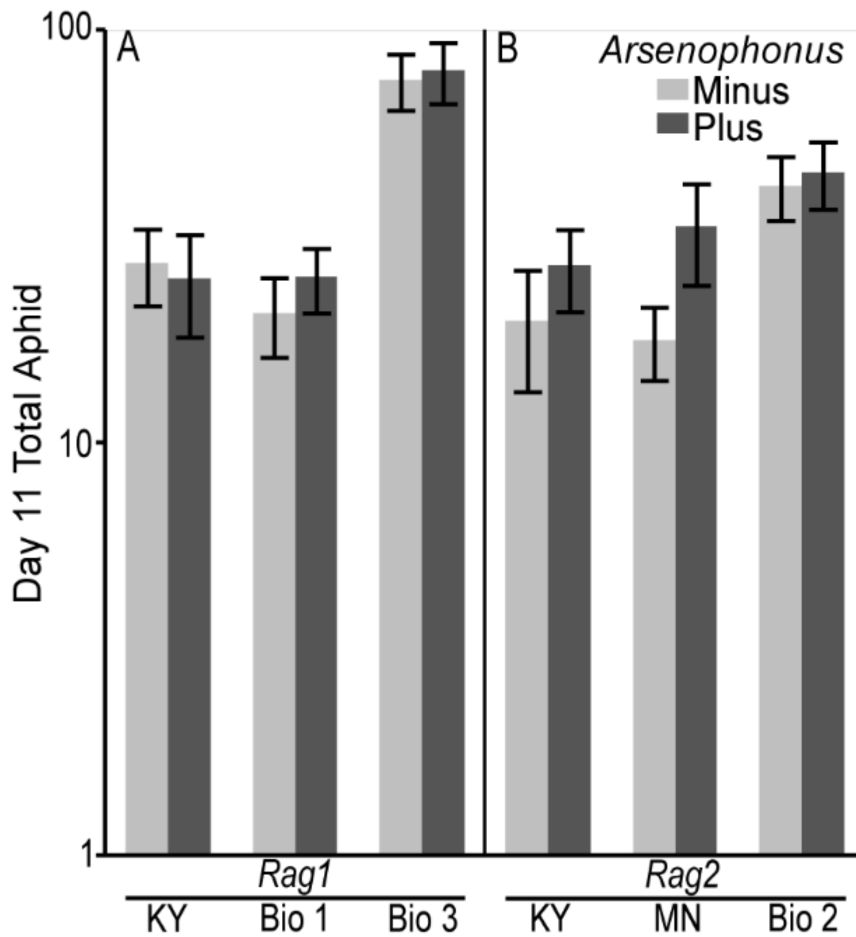

Figure 3. Mean (± SE) populations of *Arsenophonus* infected and uninfected soybean aphid on resistant and susceptible plants. Data presented on a log scale for [A] Experiment 4, and [B] Experiment 5. [A] Experiment 4 assessed three avirulent clones, KY, Biotype 1 (Bio1), and Biotype 3 (Bio3) on resistant (*Rag1)* plants. [B] Experiment 5 assessed three avirulent clones, KY, MN, and Biotype 2 (Bio2) on resistant (*Rag2*) plants across two dates.

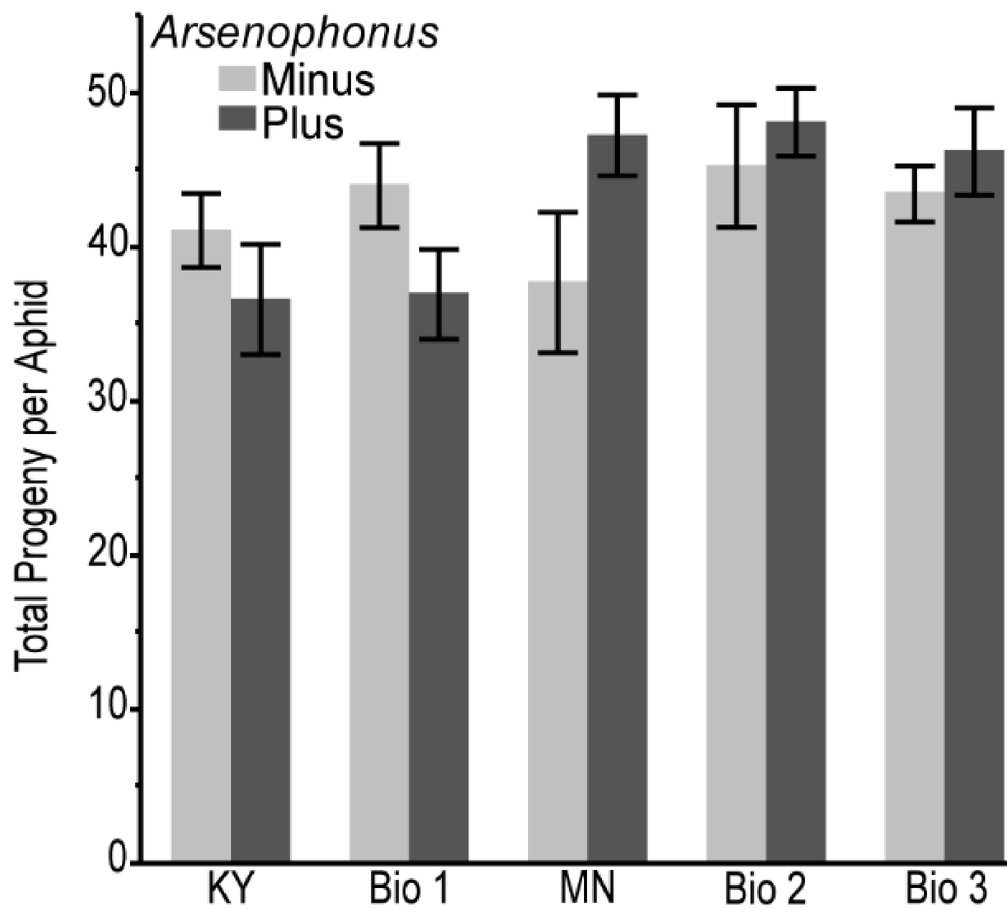

Figure 4. Mean (± SE) lifetime progeny produced by individual soybean aphids, either *Arsenophonus* infected or uninfected for five clones: KY, Biotype 1 (Bio 1), MN, Biotype 2 (Bio 2), and Biotype 3 (Bio 3). There was a significant interaction between clone and *Arsenophonus* status.

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Chapter 4
Plant defense against soybean aphid, *Aphis glycines,* and the influence of the endosymbiont *Arsenophonus*

Introduction

Plants defend themselves against natural enemies by undergoing complex changes to their physiology that make them less susceptible to attack. A paradigm of plant defense is that herbivores and pathogens trigger expression of plant hormones, jasmonic acid (JA) and salicylic acid (SA), which induce a cascade of gene expression tailored to defend against a particular natural enemy guild. Typically, genes in the JA pathway are up-regulated in response to tissue damage by rasping or chewing insects such as thrips and caterpillars. This produces a variety of proteins including those involved in wound response and secondary metabolites that inhibit protein metabolization (e.g. tannins), or are toxic (e.g. glucosinolates). The salicylic acid (SA) pathway is induced by fungal, viral, and bacterial pathogens. It also induces a complex array of genes, which can increase reactive oxygen species, pathogen-related (PR) and structural proteins, and triggers the hypersensitive response, i.e. rapid cell death. (Reviewed in Glazebrook 2005, Loake and Grant 2007, Walling 2009). It is often not clear which of these defenses are mobilized in response to the order Hemiptera, especially to the family Aphididae.

Gene expression studies reveal that aphid feeding triggers a mixture of defensive and metabolic pathways (Morkunas et al 2011, Kamphuis et al. 2013). This is partially because, unlike chewing herbivores, aphids have a much subtler form of herbivory involving intercellular stylet penetration and phloem feeding, which reduces overall tissue damage and induction of a dominant plant defense. Additionally, some aphids can

suppress effective defenses by inducing an ineffective, decoy defense that exploits the antagonistic cross-linkage between plant defensive pathways, such as JA and SA (Glazebrook 2005, Thaler et al. 2012). The clearest example of this comes from a whitefly, *Bemisia tabaci,* which strongly induces the SA pathway when feeding. This pathway does not directly defend against the whitefly; instead, it indirectly benefits the whitefly by suppressing the effective JA defense response (Zarate et al. 2007, Walling 2008). Experimental evidence from chemical plant hormone induction and, when available, plants with mutations in gene pathways, has been used to disentangle effective and ineffective plant responses to aphid herbivory. Supporting JA as the effective defense against aphids, *Arabadopsis* mutants that are insensitive to JA are more susceptible to green peach aphids, while those plants that over express JA are less susceptible (Ellis et al. 2002a, Mewis et al. 2005). Additionally, exogenous application of JA reduces aphid feeding in a variety of plants (Bruce et al. 2003, Zhu-Salzman et al. 2004, Cooper and Goggins 2005, Goa et al. 2007). For SA, chemical induction of *Arabidopsis* found no effect (Moran and Thompson 2001), but exogenous applications of SA decreased aphid colonization of wheat in the field (Pettersson et al. 1994) and negatively affected performance in assays on tomato (Cooper et al. 2004, Li et al. 2006, Thaler et al. 2010). Protein analysis also indicates the SA pathway is the dominant plant defense in tomato against potato aphid (Coppola et al. 2013).

In the present study, our first goal was to determine if chemical induction of either the SA pathway or JA pathway reduced soybean aphid population growth. Whether either defense is effective against soybean aphid is unclear, as the aphid induces both JA and SA expression (Li et al. 2008; Studham and MacIntosh 2013). Additionally, Studham and

MacIntosh (2013) found that soybean aphid causes a greater induction of genes in the abscisic acid (ABA) and ethylene (ET) pathway, which are typically not defensive and, in this case, are thought to function as a decoy defense to suppress the effective JA or SA plant defense. The authors also tested SA as the effective herbivory defense through applying exogenous SA treatments to resistant (*Rag1*) and susceptible soybean (see Chapter 3). They found reduced aphid population growth only on aphid resistant *Rag1* plants, which already had greatly reduced aphid performance. They found no effect of SA on susceptible soybean plants. They did not test JA chemical induction.

Resolving which pathway is defensive against soybean aphid will help predict indirect interactions between the aphid and the different feeding guilds and pathogens that attack soybean plants. For example, both soybean cyst nematode (*Heterodera glycines*) and a pathogenic fungus, *Cadophora gregata,* decrease soybean aphid population (McCarville et al. 2012). Both of these likely induce SA as a defense, and the reduction in soybean aphid might be due to heightened SA induction (Walling 2009, Lin et al. 2013). Alternatively, the reduction in soybean aphid population could be due to nutrient limitations in plants exploited by three natural enemies.

Insect-associated microbes may add another level of complexity to these plant/insect interactions. Many insects have long evolutionary associations with symbiotic bacteria (Moran et al. 2008), and multiple studies have started to uncover a layer of prokaryotic influence in plant/insect interactions. For example, Kaiser et al. (2010) identified *Wolbachia* as inducing photosynthetic “green-islands” in otherwise senescent plant leaves, which likely promotes the survival of the leaf miner host. In the pine beetle *Dendroctonus ponderosa,* gut microbes have genes that breakdown plant

defensive terpenes (Adams et al. 2013). Rotation resistant corn rootworm (*Diabrotica virgifera*) also have gut bacteria that allow them to tolerate soybean cysteine protease inhibitors and feed on soybean (Chu et al. 2013). In another example, a gut symbiont of Colorado potato beetle (*Leptinotarsa decemlineata*) induces a SA decoy plant defense, interfering with the effective JA plant defense and improving the beetles' performance (Chung et al. 2013). However, these interactions can be complex and bacterial roles potentially misinterpreted. In *Diabrotica virgifera,* the bacteria *Wolbachia* was initially thought to interfere with corn plant defense (Barr et al. 2010) but subsequent work determined *Wolbachia* not to be involved and suggested that there was another cause, potentially an additional unknown bacterial actor (Robert et al. 2013).

While the above examples are mostly limited to gut bacteria, studies from a few model bacteria have identified microbial effector proteins that could make it possible for non-gut associated endosymbionts to manipulate the host plant. Plants typically induce an SA defense in response to Pathogen Associated Microbial Patterns (PAMPs). Bacterial PAMPs include flagellin, elongation factor TU, cold-shock proteins, certain lipopolysaccharides (LPS), and peptidoglycans (PGN) (Gust et al. 2007, Nürnburger and Kemmerling 2009). However, some plant pathogens, *Pseudomona syringae* and *Xanthomonas* spp., secrete effector proteins that induce potentially ineffective plant defenses (Block and Alfano 2011, Deslandes and Rivas 2012, Feng and Zhou 2012). In one example, *P. syringae* produces the bacterial effector AvrPtoB, which induce the plant hormone, ABA. This decoy defense is thought to then suppress the effective SA defense (de Torres-Zabala *et al.* 2007). Similar molecules could be produced by endosymbionts,

which could be secreted into the plant while feeding. Aphid symbiont proteins have been detected in aphid honeydew indicating that they pass into the gut (Sabri et al. 2013).

Soybean aphid is widely infected with a bacterial endosymbiont, *Arsenophonus,* which has an unknown role in its host. We have determined that this symbiont does not defend its host against some natural enemies (Chapter 2, Wulff et al 2013) nor does it aid its host in exploiting resistant soybean (Chapter 3). *Arsenophonus* infection does provide a slight fitness benefit to its host (Chapter 3), but it remains unknown how *Arsenophonus* provides this benefit and if it aids in fundamental interactions between the aphid and its host plant.

The second goal of this project was to determine if *Arsenophonus* interferes with plant defense against soybean aphid. The symbiont could modify plant defense in several ways. Some *Arsenophonus* species invade plant tissues, causing disease and likely directly interacting with pattern-recognition receptors (PRRs) plant genes (Bressan et al. 2009, Bressan 2014). *Arsenophonus* cell wall proteins (LPS or PGN fragments) could pass through the aphid salivary glands and trigger a decoy response (Erbs and Newman 2012). Alternatively, *Arsenophonus* effector proteins could underlie the induction of the ABA pathway by soybean aphid. Finally, *Arsenophonus* could protect its aphid host against downstream soybean protein products originating from SA induction such as glyceollins, a type of soybean phytoalexin (Graham et al. 1990, Landini et al. 2003). Phytoalexins in *Arabidopsis* reduced population growth of the cabbage aphid (*Brevicoryne brassicae*), but their effect is unknown on soybean aphid (Kusnierczyk et al. 2008).

Our experiments have two main goals: 1) To clarifying if SA or JA is the effective defense against soybean aphid, and 2) to determine if *Arsenophonus* interferes with plant defense. To address these goals, we assayed the performance of *Arsenophonus* infected and cured soybean aphids on soybean plants with either the salicylic acid or jasmonic acid pathways induced.

Materials and Methods

Arsenophonus curing and colony maintenance

We cured four soybean aphid clones, KY, MN, Biotype 2, and Biotype 3, of their natural *Arsenophonus* infection through Ampicillin microinjection and established an infection in one clone, Biotype 1, using hemolymph injection from the infected MN clone. The details of clone origins and microinjection protocol are described in Chapters 2 and 3. This resulted in five total clones, in each of which we had paired *Arsenophonus* infected and uninfected isolines.

DNA extraction and diagnostic PCR

We monitored aphid infection status through diagnostic PCR by, briefly, homogenizing individual aphids in 3ml of proteinase K on parafilm (Pechiney Plastics) and adding the homogenate to 50 µl of 10% w/v Chelex solution. These were then vortexed and incubated at 50°C for 3 hours, after which samples were heat shocked for 10 min at 96°C. *Arsenophonus* status was determined through use of either of two primer sets: for the first set, yaeTf (5'-GCATACGGTTCAGACGGGTTTG-3') and yaeTr (5'-GCCGAAACGCCTTCAGA AAAG-3'), we used an initial denature at 93°C for 3 min;

30 cycles of (93°C, 30 s; 52°C, 30 s; 72°C, 1 min); and final elongation at 72°C for 5 min (Wilkes et al. 2012). The second primer set, adapted from Thao and Baumann (2004), was Ars23S-1 (5'-CGTTTGATGATTCATAGTCAAA-3') and Ars23S-2 (5'-GGTCCTCCAGTTAGTGTTACCCAAC-3'), with an initial denature at 95°C for 5 min; followed by 30 cycles of (95°C, 30s; 55°C, 30s; 72°C, 45s); and final elongation at 70 °C for 10 min. For both primer sets, reactions totaled 10 µl, containing 2.0 µl of DNA template, 1.0 µl of 25mM $MgCl_2$, 1.0 µl of 10 mM dNTP mixture, 1.0 µl of Invitrogen 10X buffer ($MgCl_2$ free), 0.8 µl of 5.0 pmole μl^{-1} of each primer, 0.1 µl of 5 U/ µl Invitrogen Taq polymerase and ddH_2O to 10 µl.

Soybean Plants

Laboratory colonies of soybean aphids were reared on Asgrow® AG4303 variety commercial soybeans. Experiments were conducted on either Dwight, or LDO6-16721 (Aphid resistant *Rag1* gene backcrossed into Dwight). The latter variety was only used in conjunction with virulent aphid clones that are not affected by the *Rag1* gene (Chapter 3). Experimental plants were grown in ten cm pots at 25± 1°C and 16L:8D in a Percival incubator (Percival Scientific).

Experimental setup

To chemically induce the jasmonic acid pathway, we adapted a protocol from Hamm et al. (2010) and Accamando and Cronin (2012). We prepared a 1-mM jasmonic acid solution by dissolving 31.5 mg of jasmonic acid (Sigma-Aldrich, St. Louis, MO) in 1 ml of 95% ethanol and diluting the solution in distilled water to 150 ml. We then used a Preval aerosol sprayer (CA Acquisitions, Coal City, IL) and sprayed all leaves until run off with either the jasmonic acid solution or a control solution (1 ml of 95% ethanol in

149 ml of distilled water). This procedure was done first for the control in a fume hood, and then the experimental plants were sprayed within a fume hood, allowed to dry and kept separate from the controls throughout the experiment to avoid induction of control plants through JA volatiles.

We chemically induced the salicylic pathway by using a protocol modified from Thaler et al. (2010), which used BTH (Benzothiadiaozole) to induce salicylate expression and subsequent downstream genes. We used Actigard™ 50W (Syngenta), which is 50% BTH with additional proprietary ingredients presumably for application purposes (Inbar et al. 1999). We dissolved 0.504g of Actigard in 1 L distilled water for a total concentration of 1.2 mM BTH. We followed the same application methodology as with JA, except that the control was only distilled water.

Population growth experiments

Our set up followed a protocol modified from Chapter 3. Two weeks prior to each experiment, we set up at least three new source colonies per experimental isoline. From these source colonies we used a fine tipped paint brush to randomly remove sets of 10 4th instar soybean aphids and added them to each experimental plant. After aphids were added, experimental plants were caged using modified 3.78 liter plastic jars with mesh panels for ventilation. After allowing aphids to settle for ~24 hours, we did our first count. We counted experimental aphids again on day 11. Following this count, we randomly choose at least 10 aphids from each isoline per treatment per experiment and performed diagnostic PCR to verify expected infection status.

Experiments

We performed three experiments. **Experiment 1** explored the impact of JA induction on population growth of one soybean aphid clone, KY, with paired *Arsenophonus* infected and uninfected isolines. We hypothesized that JA would decrease aphid performance overall, but *Arsenophonus* would buffer the impact in infected aphids. We repeated this experiment on two dates, each with factorial combinations of 2 aphid types (*Arsenophonus*-infected or uninfected) and 2 plant treatments (JA treated or control). Total sample size was 32 on the first experimental date and 60 on the second date.

Experiment 2 tested the impact of SA pathway induction on soybean aphid performance. We designed a 4-way factorial experiment to compare population growth among factorial combinations of two aphid clones (MN or Biotype 2), with two different infection statuses (*Arsenophonus* infected or uninfected) on two plant types (*Rag1* or susceptible) with one of two plant induction treatments (treated with BTH, inducing the SA pathway, or untreated). For the MN clone, we had a total of 16 experimental units, representing 2 replicates per treatment combination. For the Biotype 2 clone, we had 24 total experimental units, representing 3 replicates per treatment combination. We included both *Rag1* plants and susceptible plants because the only previously published effect of SA on soybean aphid performance was shown for *Rag1* plants, suggesting an interplay between the SA pathway and the resistance gene (Studham and MacIntosh 2013). **Experiment** 3 also used a BTH treatment to test SA induction, but had a simplified design, with only susceptible plant types. This experiment also added three

additional clones, KY, Biotype 1, and Biotype 3, to explore potential interactions between *Arsenophonus* and aphid clone.

For all three experiments, population growth was calculated by dividing the total day 11 aphid count by the day 1 count. We log (x +1) transformed population growth to better adhere to assumptions of normality and homoscedacity, and analyzed the data using fully factorial general linear models in IBM SPSS v20. For experiment 1, we analyzed each date separately with plant treatment and *Arsenophonus* infection as fixed factors. Experiment 2 had plant treatment, plant type, *Arsenophonus,* and aphid clone as fixed factors. Experiment 3 had plant treatment, *Arsenophonus,* and aphid clone as fixed factors.

Results

JA Treatment

For **Experiment 1,** we found no effect of JA treatment on soybean aphid population growth for the first date ($F_{1,32}= 0.372$, $p= 0.546$; Table 8, Figure 5A) or the second date ($F_{1,56} = 0.007$, $p= 0.935$; Table 9, Figure 5B) indicating JA is not the effective defense against soybean aphid. We also did not find an *Arsenophonus* main effect or an interaction between *Arsenophonus* infection and plant treatment.

SA treatment

In **Experiment 2,** we found a significant plant treatment main effect in which SA induction decreased aphid population growth ($F_{1,24}= 14.907$, $p= 0.001$; Table 10, Figure 6), but there was no interaction between plant treatment and plant type, *Rag1* or control ($F_{1,24}= 1.251$, $p= 0.274$).

We found a significant *Arsenophonus* main effect ($F_{1,24}$= 5.174, p= 0.032), and an interaction between *Arsenophonus* infection and clone ($F_{1,24}$= 7.517, p= 0.011). This interaction was likely driven by the poor performance of the uninfected relative to infected Biotype 2, whereas uninfected and infected MN clone performed more equivalently. There were no interactions between *Arsenophonus* and plant type nor between *Arsenophonus* and plant treatment. The former is as expected (Chapter 3) and the latter indicates *Arsenophonus* infection does not provide an advantage to its infected host on SA induced plants.

Experiment 3 contradicted our first SA experiment, and we found no significant main effects or interactions for plant treatment, *Arsenophonus* status, or clone (Table 11; Figure 7).

Discussion

For our first objective we determined if chemical induction of either JA or SA pathway reduced aphid population growth. Previous soybean transcriptome results and chemical induction assays had not clarified which, if any, pathway provides effective defense against soybean aphid (Li et al. 2008, Studham and MacIntosh 2013). Our results indicate mixed support for SA, but do not support JA as defensive against soybean aphid. We found no difference in aphid performance on JA treated plants compared to controls. For our SA experiments, the BTH treatment in the first experiment significantly reduced soybean aphids on average by 37% on susceptible plants and 54% on Rag plants. However, in the second experiment BTH treatment did not show any significant effect on aphid population growth. Studham and MacIntosh (2013) chemically induced plants with SA and found reduced avirulent aphid populations on treated resistant (*Rag1*) plants, but

no effect on susceptible plants. Their experiment indicates the SA effect is embedded within a broader incompatible-like response to soybean aphid feeding. The effect we found was on both types of plants, suggesting that SA pathway induction is a general plant defense against soybean aphid. Supporting the first SA results, Thaler et al. (2010), chemically induced tomato plant with BTH causing a 23% reduction in aphids on treated plants versus untreated plants. However, our second BTH experiment, which only used susceptible soybeans, found no SA effect, making it uncertain if SA induction affects soybean aphid.

Overall, the effective plant response to soybean aphid feeding is still not resolved. It was previously thought that JA was induced in response to chewing herbivores and SA was induced against phloem feeding insects such as aphids (Moran and Thompson 2001, Rodriguez-Saona et al. 2005, Thompson and Goggin 2006, Thaler et al. 2010). However, these defense induction pathways might not be strictly dichotomized and JA, alongside SA, has also been implicated as having a role in defense against aphid feeding, with SA still having the large effect (Thaler et al. 2001, Thaler et al. 2002, Cooper et al. 2004, Copper and Goggins 2005, Li et al. 2006).

In general, these experiments illustrate limitations in chemical defense induction. Plants with mutations in defense pathways, e.g. *Arabidopsis* knockout plants with either overexpression or insensitivity, offer a binary way to test the defense against aphid feeding. Chemical induction likely has more variation in gene expression and is less reliable. However, for most plants including soybean, these mutants are either not available or not widely available. Lacking these mutants requires a reliance on chemical induction, and its subsequent lack of resolution.

Concurrently with our defense induction study, we also tested whether *Arsenophonus* infection provides an advantage to its soybean aphid host in response to inducible plant defenses. We found no interaction between symbiont infection and either chemically induced jasmonic acid or salicylic acid pathways. Soybean aphid feeding causes similar plant defense gene induction as the bacterial pathogen *Pseudomonas syringae* (Zou et al. 2005, Li et al. 2008), which induces both the SA and JA pathways (Thaler et al. 2004). Also similar to soybean aphid, *P. syringae* induces biosynthesis of abscisic acid (ABA), likely as a decoy defense (de Torres-Zabala et al. 2007, Studham and MacIntosh 2013). While *P. syringae* induces the decoy defense through bacterial effectors, soybean aphid induces ABA through an unknown mechanism. Our hypothesis was that *Arsenophonus* might have a role in this decoy defense. Our secondary hypothesis was that JA was the effective defense, which would be suppressed by a SA decoy defense induced by aphid salivary excretion of *Arsenophonus*-origin microbe-associated molecular patterns (MAMPs) such as peptidoglycan fragments. In both cases we expected that *Arsenophonus* infected populations would out-perform uninfected ones on treated plants. Our results indicate that it is unlikely that *Arsenophonus* interferes with soybean defense against aphid feeding.

We did find a clone-dependent significant benefit from *Arsenophonus* in our first BTH experiment, which was independent of plant type and hormone treatment. However, it was not consistent across all experiments. Our previous population growth assays also found a great deal of statistical noise within and among individual experiment (Chapter 3), which is likely an inherent feature of these multi-trophic aphid studies.

Overall our conclusions are somewhat conjectural due to the limitation of chemical induction. While we did not find an *Arsenophonus* interaction or an effect of the JA defensive pathway on aphid population growth, we did find a SA dependent effect for one experiment. In total, we conclude that SA is likely the effective defense against soybean aphid, but subsequent studies using soybean plants with defensive pathway mutations would allow more definitive conclusions.

Table 8. ANOVA of the effect of *Arsenophonus* (Ars) and JA (Ptr) on clone (KY) performance (Experiment 1)

Factor (s)	d.f.	ss	ms	*F*	*P*
Intercept	1	53.664	53.664	1111.589	<0.000
Arsenophonus (Ars)	1	0.058	0.058	1.208	0.280
Plant treatment (Ptr)	1	0.018	0.018	0.372	0.546
Ars x Ptr	1	0.161	0.161	3.326	0.078
Error	32	1.545	0.048		

Population growth was log (x+1) transformed to better fit assumptions of normality and analyzed as fully factorial general linear models

Table 9. ANOVA of the effect of *Arsenophonus* (Ars) and JA (Ptr) on clone (KY) performance (Experiment 2)

Factor (s)	d.f.	ss	ms	*F*	*P*
Intercept	1	146.819	146.819	1572.433	<0.000
Arsenophonus (Ars)	1	0.128	0.128	1.372	0.246
Plant treatment (Ptr)	1	0.001	0.001	0.007	0.935
Ars x Ptr	1	0.012	0.012	0.127	0.722
Error	56	5.229	0.093		

Population growth was log (x+1) transformed to better fit assumptions of normality and analyzed as fully factorial general linear models

Table 10. ANOVA of the effect of *Arsenophonus* (Ars) and SA (Ptr) on performance of two clones (Experiment 3)

Factor (s)	d.f.	ss	ms	*F*	*P*
Intercept	1	107.254	107.254	2246.430	0.008
Arsenophonus (Ars)	1	0.230	0.230	4.819	0.038
Plant type (Pt)	1	0.001	0.001	0.017	0.898
Clone	1	0.247	0.247	5.174	0.032
Plant treatment (Ptr)	1	0.712	0.712	14.907	0.001
Ars x Pt	1	0.007	0.007	0.141	0.711
Ars x Clone	1	0.359	0.359	7.517	0.011
Ars x Ptr	1	0.069	0.069	1.454	0.240
Pt x Ptr	1	0.060	0.060	1.251	0.274
Pt x Clone	1	0.086	0.086	1.803	0.192
Ptr x Clone	1	0.019	0.019	0.396	0.535
Ars x Pt x Clone	1	0.016	0.016	0.344	0.563
Ars x Pt x Ptr	1	0.128	0.128	2.677	0.115
Ars x Ptr x Clone	1	0.002	0.002	0.032	0.859
Pt x Ptr x Clone	1	0.045	0.045	0.934	0.988
Ars x Pt x Ptr x Clone	1	0.000	0.000	0.008	0.928
Error	24	1.146	0.048		

Population growth was log (x+1) transformed to better fit assumptions of normality and analyzed as fully factorial general linear models

Table 11. ANOVA of the effect of *Arsenophonus* (Ars) and SA (Ptr) treatment on performance of five clones (Experiment 4)

Factor (s)	d.f.	ss	ms	*F*	*P*
Intercept	1	120.450	120.450	1948.574	<0.000
Arsenophonus (Ars)	1	0.009	0.009	0.171	0.683
Plant treatment (Ptr)	1	0.001	0.001	0.029	0.866
Clone	4	0.322	0.081	1.565	0.211
Ars x Ptr	1	0.002	0.002	0.043	0.838
Ars x Clone	4	0.317	0.079	1.540	0.218
Ptr x Clone	4	0.477	0.112	2.170	0.098
Ars x Clone x Ptr	4	0.192	0.048	0.932	0.460
Error	28	1.442	0.051		

Population growth was log (x+1) transformed to better fit assumptions of normality and analyzed as fully factorial general linear models

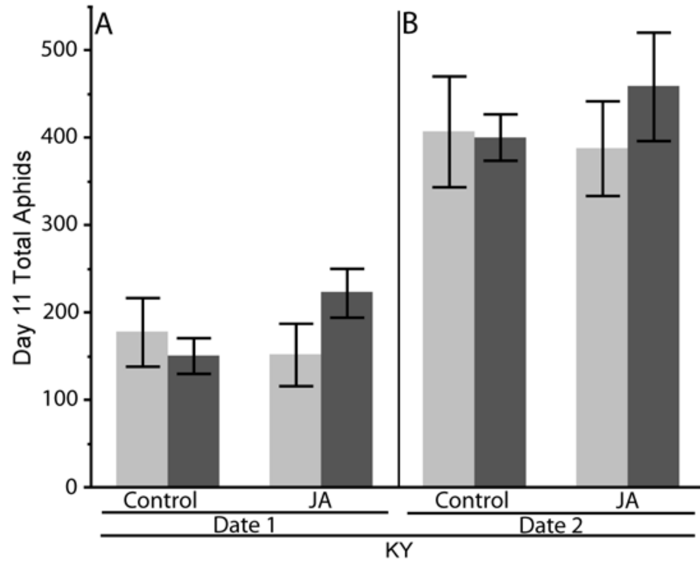

Figure 5 Experiment 1: Jasmonic Acid (JA) effect on soybean aphid population growth. Mean (± SE) populations of *Arsenophonus* infected and uninfected soybean aphid after 10 d on jasmonic acid (JA) treated and untreated (Control) soybean plants on two separate dates, [A] first date and [B] second experimental date.

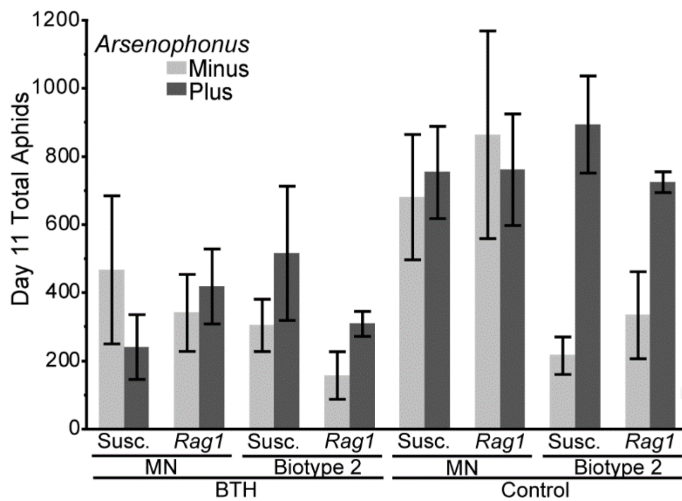

Figure 6. Experiment 2: Salicylic Acid (SA) effect on soybean aphid population growth. Mean (± SE) populations of *Arsenophonus* infected and uninfected soybean aphid after 10d on (*Rag1*) resistant or (Susc.) susceptible plants that were either (BTH) salicylic acid induced or (Control) untreated. Two virulent clones, Biotype 2 and MN were used. There was a significant reduction in aphid population growth on the BTH treated plants regardless of plant type.

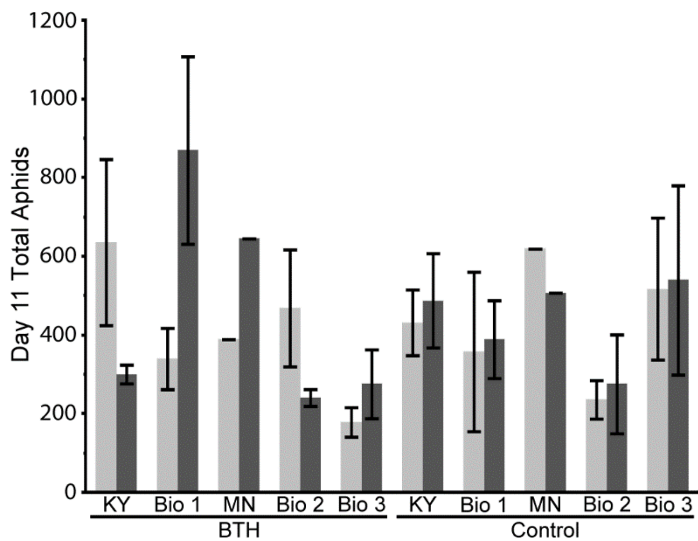

Figure 7. Experiment 3: Salicylic Acid (SA) effect on soybean aphid population growth. Mean (± SE) populations of *Arsenophonus* infected or uninfected soybean aphid on (BTH) salicylic acid induced or (Control) untreated susceptible soybean plants for five clones: KY, Biotype 1 (Bio 1), MN, Biotype 2 (Bio 2), and Biotype 3 (Bio 3).

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Conclusions

Soybean aphid is an introduced pest of soybean in the United States, requiring extensive pesticide input to curb severe economic loss from feeding damage and vectored viruses. The advent of this aphid motivated considerable research into its biology and ecology, aimed at developing control alternatives to pesticides. This produced two major control strategies, parasitoid mediated classical biological control and development of soybean varieties resistant to aphid feeding. However, despite nearly a decade of intense soybean aphid research, the facultative endosymbiont of soybean aphid, *Arsenophonus*, remained unexplored until the research presented in this dissertation. Facultative endosymbionts are known to protect their hosts against natural enemies and influence host plant interactions, making it possible that *Arsenophonus* could interfere with attempts to control the soybean aphid. More broadly, this research is the first to focus on the role of *Arsenophonus* in any of the 7% of aphid species infected with the symbiont, increasing the scope and application for these findings.

The frequency of an endosymbiont in an aphid population provides a clue to the dynamics of the infection. My first objective determined that *Arsenophonus* infection is widespread in soybean aphid populations in both the native range and the introduced range. This could suggest that *Arsenophonus* protects against a common pathogen or parasitoid, interacts with a fundamental aspect of the insect / plant interaction or provides a general benefit to infected individuals. The infection pattern made it unlikely that the symbiont would have a narrow beneficial context or a high general costs. My work established that under relatively permissive laboratory conditions, *Arsenophonus* does not induce a cost to soybean aphid, but significantly increases population growth. This fitness

benefit could underlie the high infection rate in soybean aphid. Further field study follow ups will be needed to determine if this benefit occurs in a more natural setting.

My second goal was to determine if *Arsenophonus* was defensive against intimately associated enemies, such as parasitoid wasps and fungal pathogens. Research on the pea aphid, *Acyrthosiphon pisum,* a model for endosymbiont studies, indicates that these defensive roles are common for endosymbionts. Additionally, a geographic survey of *Arsenophonus* infection in lerp psyllid found a positive correlation between parasitism and infection, suggesting a defensive role for *Arsenophonus.* However, my work indicates that *Arsenophonus* is not defensive against three wasp species or a common fungal pathogen. Prior to this finding, the extensive vetting of an importation biological control agent against soybean aphid did not consider the symbiont infection. It was unknown if *Arsenophonus* was widespread in soybean aphid populations or defensive against parasitoid wasps. Considering that defensive symbionts in pea aphid are often sporadically dispersed in the field and, in laboratory population cage studies, increase in frequency in response to parasitism pressure, *Arsenophonus* infected aphids could have been overlooked during testing, but could become more prevalent in response to the field release of a biological control agent, protecting the aphid and contributing to the biocontrol failure. However, my work suggests that *Arsenophonus* does not have a defensive role and does not need to be a consideration in these programs.

Rag soybean plants, resistant to soybean aphid, are another control strategy actively being researched and commercially available. However, the identification of aphid biotypes able to exploit these plants questions the field durability of the resistance. The mechanism behind biotype differentiation is unknown, and it was suggested that

Arsenophonus contributed to virulent biotype performance. My work has eliminated *Arsenophonus* as a factor in biotype determination. Additionally, my work indicates the presence of the symbiont is not further deleterious to avirulent aphids on resistant plants.

Finally, my work suggests that salicylic acid induced plant defenses are effective against soybean aphid. However, this result was not obtained when replicated on a second date, casting doubt on my conclusion. I found no impact from jasmonic acid induced soybean plants on aphid population growth on two separate experiments. My results also indicate that the fitness advantage from *Arsenophonus* infection is not the result of symbiont interference with plant defense.

Overall, this work required substantial replication of population growth experiments across several dates, which, when dates are considered individually, illustrates the variability and statistical noise inherent in aphid studies. The variability is presumably driven by the extensive polyphenism and fast population growth exhibited by aphids, which exaggerates the effects from small differences in plant quality, maternal and grand-maternal effects, and microenvironment. This contributes to significant differences between and within each date for clones and differentially infected isolines. Additionally, when experimental dates are considered individually, a significant aphid genotype x *Arsenophonus* interaction is sometimes recovered. Several symbiont studies in the literature have also found an aphid genotype x symbiont interaction. However, when this dissertation's experimental dates are combined, the aphid genotype x symbiont interactions are ephemeral. For fitness studies, extensive replication is likely necessary to determine subtle symbiont benefits or costs, and illuminate actual aphid genotype x symbiont interactions, which may not be reliably detectable in individual experiments.

While I did find an overall benefit of increased population growth for *Arsenophonus* infected populations, it is possible that I did not have the appropriate ecological context to resolve the underlying function of *Arsenophonus* in soybean aphid. Along these lines, the most promising avenue for future *Arsenophonus* research is testing its influence on aphid performance on the primary host, buckthorn, *Rhamnus* spp. I did some limited experiments exploring this interaction. I found that cured soybean aphid clones still produce males, gynoparous and oviparous aphids, and eggs, all of which can establish on buckthorn despite still lacking the symbiont. However, it would be interesting to test if the loss of *Arsenophonus* significantly alters aphid performance on the primary host.

In general for these types of studies, deciphering the role of an endosymbiont requires the appropriate ecological context to isolate its advantage. Many endosymbiont studies start with an observation of some variable aspect within an aphid population, such as extreme differences in susceptibility to parasitism, which can be correlated to symbiont infection. These observations are then followed by experiments on differentially cured, same genotype, isolines to fully attribute the effect to the symbiont and not host genotype. Blindly testing and eliminating the various known roles of symbiont infections can lead to a scientific cul-de-sac in which the symbiont function remains elusive. However, negative results still have value, especially when the host is an important pest species and the symbiont could be interfering with control efforts. This dissertation aids soybean aphid research by ruling out several *Arsenophonus* functions that could impede control efforts. It also lays the groundwork for studying this common bacterial endosymbiont in other aphid species, including additional pest species.

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VITA

EDUCATION

University of Kentucky	Entomology	Ph.D., expected	2014
University of Wisconsin	Biology	Non-degree	2008
University of Minnesota	English	B.A.	2002

RELEVANT EXPERIENCE

- Graduate Research Assistant, Symbiont Ecology Laboratory (Dr. JA White)
  University of Kentucky, Dept. of Entomology (09/08- present)
- Research Assistant, Plant Systematics Laboratory (Dr. SB Hoot)
  University of Wisconsin/Milwaukee, Dept. of Biol. Sci.(5/06-6/08)
- Teacher Training/Agricultural Extension, Peace Corps, Nepal
  (February 2004 – October 2004, evacuated)

PUBLICATIONS:

Brady, C.M., M.K. Asplen, G.E. Heimpel, K.R. Hopper, C.R. Linnen, K.M. Oliver, **J.A. Wulff**, J. A. White (2013). “Worldwide populations of *Aphis craccivora* have diverse facultative bacterial symbionts.” Accepted in Microbial Ecology.

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White, J. A., C. Hurak, **J.A. Wulff**, M. S. Hunter, S. E. Kelly (2011). Parasitoid bacterial symbionts as markers of within-host competitive outcomes: superparasitoid advantage and sex ratio bias. Ecological Entomology **36**(6): 786-789.

MANUSCRIPTS IN PREPARATION:

Wulff, **J.A**., J. A. White (2014). The endosymbiont *Arsenophonus* provides a general benefit to its host soybean aphid, *Aphis glycines*, regardless of host plant resistance (*Rag*). Environmental Entomology (In Prep).

INVITED PRESENTATIONS:

- (Oral) Wulff, J.A., and J.A. White. Effects of an aphid bacterial endosymbiont, *Arsenophonus,* on natural enemies and host plant use in soybean aphid on resistant and susceptible plants. University of Kentucky Ecolunch, Lexington, KY 2012.

- (Oral) Wulff, J.A., and J.A. White. The defensive potential of the bacterial endosymbiont *Arsenophonus* in the soybean aphid. International Symposium on the Ecology of Aphidophaga 11, Perugia, Italy, 2010.

PRESENTATIONS:

- (Oral) Wulff, J.A., and J.A. White. The endosymbiont, *Arsenophonus,* influences soybean aphid, *Aphis glycines,* performance on soybean. National Meeting of the Entomological Society of America, Austin, TX 2013.

- (Oral) Wulff, J.A., and J.A. White. The role of the bacterial endosymbiont, *Arsenophonus*, in soybean aphid, *Aphis glycines*. University of Kentucky PhD Exit Seminar, Lexington, KY 2013.

- (Oral) Wulff, J.A., and J.A. White. The influence of the endosymbiont *Arsenophonus* on soybean aphid, *Aphis glycines*, performance on resistant and susceptible plants. National Meeting of the Entomological Society of America, Knoxville, TN 2012.

- (Poster) Wulff, J.A., and J.A. White. Interactions of *Arsenophonus* and host-plant resistance on soybean aphid performance. Seventh International *Wolbachia* conference, Ile d'Oléron, France 2012.

- (Oral) Wulff, J.A., and J.A. White. The influence of the endosymbiont *Arsenophonus,* on soybean aphid performance on resistant and susceptible plants. Annual meeting of the University of Kentucky Center for Ecology, Evolution, and Behavior, Lexington, KY 2012.

- (Oral) Wulff, J.A., and J.A. White. The defensive potential of the bacterial endosymbiont *Arsenophonus* in the soybean aphid. North Central Branch Meeting of the Entomological Society of America, Minneapolis, MN 2011.

- (Oral) Wulff, J.A., and J.A. White. The defensive potential of the bacterial endosymbiont *Arsenophonus* in the soybean aphid. National Meeting of the Entomological Society of America, San Diego, CA 2010.

- (Poster) Wulff, J.A., and J.A. White. Does *Arsenophonus* defend the soybean aphid against parasitism? Sixth International *Wolbachia* conference, Asilomar, CA 2010.

- (Oral) Wulff, J.A., and J.A. White. *Arsenophonus* a defensive symbiont of soybean aphid. Annual meeting of the University of Kentucky Center for Ecology, Evolution, and Behavior, Lexington, KY 2010.

- (Oral) Wulff, J.A., and J.A. White. Do facultative symbionts influence the outcome of superparasitism in a solitary endoparasitoid? National meeting of the Entomological Society of America, Indianapolis, IN 2009.

TEACHING/OUTREACH

- Teaching assistant, Conservation and Environmental Science, University of Wisconsin, Milwaukee, WI, September 2006 - May 2007
- Primary Public School, Peace Corps, Nepal, February 2004 – October 2004; evacuated

Invited lecturer

- Wulff, J.A., Classical Biological Control. Agroecology,
  University of Kentucky, Spring, 2012
- Wulff, J.A., Weighing Costs and Benefits of Implementing Parasitoid mediated Classical
  Biological Control. Agroecology, University of Kentucky, Spring, 2011
- Wulff, J.A., Parasitoids and Biological Control. Agroecology,
  University of Kentucky, Spring, 2010
- Wulff, J.A., Introduction to Entomology. Invertebrate Function and Evolution,
  University of Wisconsin, Milwaukee, Fall, 2007

Outreach Events

- "Night Insect Walk" at Raven Run Nature Sanctuary; 2009-2013
- "120 years of Entomology" at University of Kentucky; 2011
- "Hope in the City" health services event targeted to underserved communities; 2010-2013

GRANTS AND AWARDS:

- University of Kentucky, Graduate School Professional Meeting Travel
  Scholarship to attend the Entomological Society of America meeting, Austin, TX
  2013 ($400)

- University of Kentucky, Department of Entomology Publication Scholarship 2012 ($500)
- University of Kentucky, Graduate School Professional Meeting Travel Scholarship to attend the Seventh International *Wolbachia* conference, Oleron, France 2012 ($800)
- Travel Award to attend the North Central Branch Meeting of the Entomological Society of America, Minneapolis, MN 2011 ($250)
- Student Paper Competition (Ph.D.), North Central Branch Meeting of the Entomological Society of America, Minneapolis, MN 2011 ($200)
- University of Kentucky, Graduate School Professional Meeting Travel Scholarship to attend the Sixth International *Wolbachia* conference, Asilomar, CA 2010 ($400)

EDITORIAL

Reviewer for:

Biological Control (1)

International Society for Microbial Ecology (1)