Actual and potential host range of *Arsenophonus nasoniae* in an ecological guild of filth flies and their parasitic wasps

by

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Supervisory Committee

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Abstract

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The gammaproteobacterium *Arsenophonus nasoniae* infects *Nasonia vitripennis* (Hymenoptera: Pteromalidae), a parasitic wasp that attacks filth flies. This bacterium kills virtually all male offspring of infected females. Female wasps transmit A. *nasoniae* both vertically (from mother to offspring) and horizontally (to unrelated *Nasonia* developing in the same fly). This latter mode may enable the bacterium to colonize novel species and spread throughout a filth fly-parasitoid guild. This spread may be important for maintenance of the bacterium. The ecology of novel hosts may be significantly impacted by infection.

The actual and potential host range of *A. nasoniae* was assessed. I used *Arsenophonus*-specific primers to screen a large sample of filth flies and their parasitoids. The bacterium infects a wide range of wasp species in the environment. The potential host range was determined by inoculating three wasp and one fly species with an isolate of *A. nasoniae* from Lethbridge, AB. The bacterium successfully infected all insects and was transmitted by two wasp species. It reduced host longevity, but did not kill males, in *Trichomalopsis sarcophagae*. It also caused pupal mortality in *Musca domestica*.

Table of Contents

List of Tables

List of Figures

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Chapter 1: An introduction to inherited bacterial symbionts of insects, with a focus on male-killers

Symbiosis, or intimate associations between organisms, is one of the most ubiquitous and profound forces in the evolution of life. The acquisition and evolution of mitochondria and chloroplasts are perhaps the most striking examples of endosymbiotic relationships. Symbiotic interactions can range from mutualistic, where both members benefit from the association, to parasitic, where one organism prospers to the detriment of the other. Arthropods harbour an enormous diversity of symbionts that have shaped their evolution and ecology (MORAN *et al.* 2008), and recently there has been an explosion of research in this area. In particular, arthropods are commonly infected with symbionts that are transmitted primarily from mothers to their offspring, often in the egg cytoplasm (WERREN 1987; GOTTLIEB *et al.* 2002; KELLNER 2002). As a result, the fitness of these symbionts is intimately tied to that of their hosts.

Inherited symbionts are divided into two major categories: primary symbionts, and secondary symbionts (MORAN *et al.* 2008). Primary symbionts are strictly required for host survival and/or reproduction, and provide essential functions, most commonly supplementing host nutrition. Thus far, all insects that feed exclusively on plant sap or animal blood have been shown to harbour obligate primary bacterial symbionts (BAUMANN *et al.* 1995; AKMAN *et al.* 2002; ALLEN *et al.* 2007; DOUGLAS 2009). The best studied nutritional primary symbiont is *Buchnera aphidicola*, a bacterium that provides its aphid host with essential amino acids that are lacking from its plant sap diet (BAUMANN *et al.* 1995). The strictly obligate nature of the association between primary symbionts and their hosts has led to extensive cospeciation between the two lineages. In

addition, primary symbionts are often housed in specialized host cells called bacteriocytes that permit the host to regulate its microbiota (DOUGLAS 1989; MORAN and BAUMANN 1994; AKSOY 1995; ZCHORI-FEIN *et al.* 1998; CHEN *et al.* 1999; GOTTLIEB *et al.* 2008). Cospeciation and bottlenecks associated with longterm vertical transmission also cause a characteristic reduction in the genome of primary symbionts, with many non-essential genes being lost (e.g., AKMAN *et al.* 2002; MORAN *et al.* 2005).

Secondary symbionts, alternatively, are not strictly required for host survival and reproduction (i.e., they are facultative). As such, they are often found at lower frequencies in hosts populations. In contrast to the high levels of cospeciation exhibited by primary symbionts, secondary symbionts are often characterized by repeated colonization of novel host species over evolutionary timescales, despite being transmitted maternally over ecological timescales (SCHILTHUIZEN and STOUTHAMER 1997; VAVRE *et al.* 1999; ZCHORI-FEIN and PERLMAN 2004; NOVAKOVA *et al.* 2009). They are also not restricted to specialized cells and can invade multiple tissues within infected hosts (HUGER *et al.* 1985; GOTO *et al.* 2006). After invading a novel host, the maintenance of the symbiont is moderated by both its vertical transmission efficiency and its effect on host fitness (HEATH *et al.* 1999; JAENIKE *et al.* 2007). This transmission efficiency, and the phenotype expressed in the host, may even change depending on the host background (BORDENSTEIN and WERREN 1998; HUIGENS *et al.* 2004; SASAKI *et al.* 2005; TINSLEY and MAJERUS 2007). This results in a wide variety of phenotypes exhibited by secondary symbionts.

Facultative secondary symbionts are inherently detrimental to the host because they utilize host resources. There are two ways that secondary symbionts ensure there

transmission and maintenance in the population: by augmenting host fitness and acting as mutualists, or by manipulating the reproductive behaviours of the host to increase the prevalence of the symbiont in the next generation. The benefits conferred by facultative symbionts are often conditional, and may increase heat tolerance, protection from parasitism and pathogens, and alteration of food specialization (MONTLLOR *et al.* 2002; OLIVER *et al.* 2003; FERRARI *et al.* 2004; TSUCHIDA *et al.* 2004; HEDGES *et al.* 2008). These benefits are not mutually exclusive, and increasing the host's resilience allows the symbiont to be maintained. However, the symbiont may be lost from the population if the selective agent is subsequently removed (DALE and MORAN 2006; OLIVER *et al.* 2008; OLIVER *et al.* 2009).

Instead of serving as a mutualist, a secondary symbiont can also be maintained if it exploits the host's reproductive behaviours to the symbiont's advantage, and this is termed 'reproductive manipulation'. These reproductive manipulators increase the prevalence of infected female hosts in the subsequent generation. This is achieved by increasing either the quality or absolute number of females produced by an infected host, or by limiting the ability of uninfected hosts to reproduce in the population. These phenotypic modifications will be discussed in more detail subsequently.

Major secondary symbiont lineages infecting arthropods

Several major lineages of microbes infect arthropods as vertically transmitted secondary symbionts, although the effects they have on most of their hosts remain largely unknown. *Wolbachia*, a gram-negative member of the class Alphaproteobacteria, is the most common arthropod secondary symbiont and is thought to infect ~66% of all insect

species (WERREN *et al.* 1995; HILGENBOECKER *et al.* 2008). It is also the best-studied secondary endosymbiont to date, likely as a result of both its ubiquity and the remarkable diversity of phenotypes that it can induce in its hosts. *Wolbachia* is the only secondary symbiont lineage that can induce every known form of host reproductive manipulation (feminization, parthenogenesis induction, cytoplasmic incompatibility, and male-killing) (ROUSSET *et al.* 1992; BREEUWER and WERREN 1993; STOUTHAMER *et al.* 1993; HURST *et al.* 1999). Additionally, other strains of *Wolbachia* provide direct benefits to their host, including defending their hosts against RNA viruses (HEDGES *et al.* 2008). Distantly related lineages of *Wolbachia* serve as primary symbionts in both filarial nematodes and bedbugs (BANDI *et al.* 2001; HOSOKAWA *et al.* 2009).

Cardinium is a recently discovered lineage of secondary symbionts that demonstrates an arthropod host range almost as broad as *Wolbachia*; infections are known from five orders of arthropods (i.e., ticks and mites, wasps, true bugs, spiders, and flies; ZCHORI-FEIN and PERLMAN 2004; GOTOH *et al.* 2007; DURON *et al.* 2008). *Cardinium* induces three of the four known types of reproductive manipulation (WEEKS *et al.* 2001; ZCHORI-FEIN *et al.* 2001; HUNTER *et al.* 2003).

Spiroplasma, a member of the Mollicutes, possesses a peculiar cell morphology; it is a gram-positive bacterium that lacks a cell wall. Another characteristic that sets this lineage apart is its highly diverse host range, infecting plants as well as arthropods. *Spiroplasma* cause a more limited set of phenotypes than *Wolbachia* and *Cardinium*, and are generally regarded as parasites. For example, some strains of *Spiroplasma* cause disease in plants, and other strains kill male offspring of insects (LEE *et al.* 1998; VENETI

et al. 2005). In one remarkable example, a beneficial *Spiroplasma* protects its *Drosophila* host against nematode infections (JAENIKE *et al.* 2010).

A particularly notable lineage is the recently discovered gram-negative bacterium, *Arsenophonus*. This bacterium infects a broad range of arthropods as well as plants, based on 16S rDNA screening surveys (HYPSA and DALE 1997; THAO and BAUMANN 2004; DALE *et al.* 2006; HANSEN *et al.* 2007; NOVAKOVA *et al.* 2009). The effects of infection in nearly all hosts are unknown. One strain of *Arsenophonus* appears to cause disease in strawberries (ZREIK *et al.* 1998). One lineage appears to serve as a primary nutritional symbiont of lice where it supplements the blood diet of the host (ALLEN *et al.* 2007). Perhaps the most intriguing host phenotype is caused by *Arsenophonus nasoniae,* which kills most of the male offspring produced by its wasp host (SKINNER 1985). This strain is also transmitted extra-cellularly and can be cultured in cell-free media, both of which are exceptional features for vertically inherited symbionts.

Modes of reproductive manipulation

Some symbionts are able to increase their fitness by manipulating the host's reproductive abilities to their own advantage. The prevalence of the symbiont in the host population is increased if its host produces more daughters, or more competitive daughters, than uninfected hosts. This hijacking by the symbiont can be enacted through different modifications of the host, and thus far four types of manipulation are known. The symbiont will spread if it increases the absolute number of female offspring the host produces, which can be done through two mechanisms: feminization and parthenogenesis-induction. Feminizing symbionts convert genetic males into phenotypic

females (ROUSSET *et al.* 1992; TERRY *et al.* 1997; STOUTHAMER *et al.* 1999; KAGEYAMA *et al.* 2002). In parthenogenesis-induction, symbionts convert genetic males into genetic females (STOUTHAMER *et al.* 1999; ZCHORI-FEIN *et al.* 2001). This latter manipulation is common in wasps, which have haplodiploid sex determination. The symbionts convert unfertilized haploid eggs, which would normally develop as males, into diploid (i.e., female) individuals (STOUTHAMER *et al.* 1993). A third sex ratio distorting strategy is male-killing, whereby the sons of infected females die early in development. This will be discussed in more detail below.

Finally, some symbionts cause mating incompatibilities between infected males and uninfected females; i.e., cytoplasmic incompatibility. As a result, the fitness of uninfected females is reduced relative to infected females. As the prevalence of infection increases in a population, so does the advantage for being infected. As a result, incompatibility-inducing symbionts often reach very high frequencies in host populations (TURELLI and HOFFMANN 1991). Cytoplasmic incompatibility is the most common phenotype induced by *Wolbachia,* and occurs in a wide variety of hosts. It is also induced by *Cardinium* in wasps and mites (WERREN 1997; HUNTER *et al.* 2003; GOTOH *et al.* 2007).

Male-killing

One of the most common reproductive manipulations in arthropods is male-killing, where symbionts kill the sons (but not daughters) of infected females. This strategy has evolved independently at least seven times by microorganisms infecting at least seven orders of arthropods (HURST 1991; HURST and JIGGINS 2000). These

endosymbionts usually kill the males early in development (i.e., as embryos; HURST and JIGGINS 2000) through unknown mechanisms (but see DOSAGE COMP AND FERREE). Male-killers can cause significant effects on the host population. If male-killers reach high frequencies within a host population they can induce an extremely female-biased sex-ratio, potentially resulting in extirpation of the host species. A male-killer is therefore expected to have less than perfect inheritance if it is to remain stable in a population (HURST 1997). In the butterfly *Acraea encedon* however, a male-killing *Wolbachia* has near-perfect transmission, and this bacterium has reached a stable prevalence of 95% in some populations (JIGGINS *et al.* 2000a). This causes the peculiar situation where males are the limiting sex, and results in a reversal of traditional sex-roles where females now compete for males (JIGGINS *et al.* 2000b). Vertically inherited male-killers may also be lost from the population if their transmission is too low. Low inheritance of the endosymbiont will reduce its prevalence and its ability to be maintained, as all females from the population will produce uninfected offspring (HURST 1991).

Although the short-term benefit for male-killing symbionts is not controversial (i.e., there is no selection for symbiont function in males), one of the major unresolved issues in male-killing is how they are maintained in host populations. This is because under exclusively vertical transmission, uninfected lineages are predicted to replace infected ones if there is no fitness benefit to infection. Male-killing has been modeled extensively, and thus far, three major types of fitness benefits resulting from male-killing have been proposed (SKINNER 1985). However, empirical support for these theorized benefits is slim (IKEDA 1970; HURST *et al.* 1994; BALAS *et al.* 1996). First, infected

females may increase their fitness by directly feeding on their dead brothers. Second, male-killing may reduce competition for resources. Third, male-killing might serve to reduce inbreeding. However, high levels of inbreeding are considered rare in most field populations, causing the reduction of inbreeding to likely be irrelevant (HURST *et al.* 1996). Perhaps the best understood cases of male-killing occur in ladybird beetles. These insects appear to be particularly susceptible to male-killing (MAJERUS and HURST 1997; MAJERUS *et al.* 2000). Ladybird beetles lay their eggs in clutches; both early larval mortality and cannibalism are common. The latter provides a clear benefit to the beetle (OSAWA 1992). A fitness benefit to male-killing was also recently demonstrated in the viviparous pseudoscorpion *Cordylochernes scorpioides*, where it has been shown that females infected with male-killing *Wolbachia* produce more numerous, and higher quality, daughters (KOOP *et al.* 2009). It is also formally possible that the symbiont provides an unrelated fitness benefit to its pseudoscorpion host, although this has not yet been demonstrated.

Alternatively, a male-killing symbiont may be maintained in the population via horizontal transmission. This uncouples the symbiont's fitness from that of its host (UYENOYAMA and FELDMAN 1978) and allows the development of alternative phenotypes. For example, some microsporidia kill male mosquitoes late in larval development, resulting in the release of high densities of spores that are then horizontally transmitted to new hosts (ANDREADIS 1985; HURST 1991). This form of male-killing differs from most by killing males as larvae rather than eggs, an approach thought to maximize horizontal transmission potential. In typical male-killing symbionts, horizontal transmission may assist the symbiont during the initial invasion of a host population, but

may play only a supporting role in transmission once the symbiont is established (LIPSITCH *et al.* 1995). An important differentiation to make is horizontal transmission on an evolutionary scale versus that on an ecological scale. Most male-killers, such as *Wolbachia* and *Spiroplasma,* demonstrate rare transmission events between host lineages over evolutionary time. Others, such as microsporidia infecting mosquitoes, demonstrate ecological transmission that may be imperative for their maintenance.

Male-killing *Arsenophonus* in *Nasonia* wasps

An excellent model system for studying the dynamics of a male-killer is the bacterium *Arsenophonus nasoniae* (Gammaproteobacteria: Enterobacteriaceae) infecting the wasp *Nasonia vitripennis* (Hymenoptera: Pteromalidae), a pupal parasitoid of filth flies. Across N. America roughly 4% of *N. vitripennis* are infected by this bacterium (BALAS *et al.* 1996) which kills 80% of the sons of infected females as embryos; 95% of the daughters and the surviving males inherit the bacterium (SKINNER 1985). Mechanistically, *A. nasoniae* kills male *Nasonia* by inhibiting the formation of the maternal centrosome within the embryo using currently unknown factors (FERREE *et al.* 2008). Hymenoptera have haplodiploid sex determination, whereby unfertilized haploid eggs develop as males, and fertilized diploid eggs develop as females. Unfertilized (male) embryos use a maternally derived centrosome to control the nuclear division of cells, while fertilized (female) eggs use a centrosome derived from the sperm (CALLAINI *et al.* 1999). It has been proposed that *A. nasoniae* produces a toxin that inhibits maternal centrosome formation, resulting in the developmental arrest and death of haploid embryos (FERREE *et al.* 2008).

Arsenophonus nasoniae is not inherited via the egg, as in most insect symbionts. Instead, female *Nasonia* deposit an inoculum of bacteria into the fly host during oviposition. This inoculum survives within the fly puparium until it is ingested by the developing wasp larvae, where it infects the larva through the midgut (HUGER *et al.* 1985; SKINNER 1985). This infectious stage of *A. nasoniae* within the fly creates a potential avenue for horizontal transmission of the bacterium. Multiple *Nasonia* females can parasitize a single fly pupa (GRILLENBERGER *et al.* 2008), and under these conditions all of the wasps developing within the host are equally likely to become infected, regardless of parental lineage (SKINNER 1985). A single fly can also be coparasitized by different wasp species (WYLIE 1972), and these interactions may allow *A. nasoniae* to be transferred into novel wasp lineages. While coparasitism occurs at low levels in the field (FLOATE *et al.* 2000), these transfers may have significant ecological impacts if *A. nasoniae* is vertically transmitted in the naïve lineage. *Nasonia longicornis* is a sympatric and closely related species of *N. vitripennis,* and both species are found coparasitizing flies in pupal clutches (DARLING and WERREN 1990). Field-collected individuals from both *N. vitripennis* and *N. longicornis* have been found infected with *A. nasoniae* (BALAS *et al.* 1996). No other hosts of *A. nasoniae* are known. However, with the wide overlapping host ranges of parasitoid wasps, interactions between wasp species and *A. nasoniae* may occur in complex webs, moving *A. nasoniae* throughout the ecological guild.

The successful invasion of other wasp species by *A. nasoniae* may be limited by differences in the genetic backgrounds of the wasps. The effect a symbiont will have on host fitness may vary between host species (CHANG and WADE 1994; JAENIKE *et al.*

2007; TINSLEY and MAJERUS 2007). For example, the strain of *Wolbachia* that causes cytoplasmic incompatibility in the almond moth *Cadra cautella* causes male-killing when introduced into the flour moth *Ephestia kuehniella* (SASAKI *et al.* 2005). Vertical transmission of the symbiont may also be affected by the genotype of the wasp; for example, a male-killing *Spiroplasma* shows reduced vertical transmission when experimentally transferred to genetically dissimilar hosts (TINSLEY and MAJERUS 2007); other systems show a similar loss of transmission in novel hosts (CHANG and WADE 1994; HEATH *et al.* 1999).

As for most male-killing systems, definitive fitness benefits for infection by *A. nasoniae* have remained elusive. Infected females are not larger than uninfected females (BALAS *et al.* 1996); body size is a major fitness correlate in these wasps (WYLIE 1966). Additionally, *Nasonia* does not conform to many of the life history traits that are thought important in maintaining a male-killer. For example, the death of male *N. vitripennis* would not directly increase the nutrition of larvae, as they do not feed upon conspecific eggs (WYLIE 1972). These larvae are also not typically resource-limited in the fly host, and a reduction in competition would be irrelevant. Although inbreeding suppression may potentially provide a benefit to *N. vitripennis* (BALAS *et al.* 1996), *Nasonia* inbreed to such a large degree that its ecological relevance is unknown (GRANT *et al.* 1980; MOLBO and PARKER 1996; LUNA and HAWKINS 2004). Additionally, limited numbers of males are still produced within *A. nasoniae* infected clutches. Males can mate multiple times (GRANT *et al.* 1980), and a single male could fertilize a large number of sisters (WERREN 1980). These traits suggest host mating dynamics may be relatively unaffected

by moderate levels of male-killing. Horizontal transmission of the bacterium may thus be integral for the maintenance of *A. nasoniae* in the host population (BALAS *et al.* 1996).

Arsenophonus nasoniae is an excellent candidate system for studying a male-killer bacterium in both an ecological and lab setting. The ability to culture the bacterium in vitro (GHERNA *et al.* 1991) opens avenues for experimentation that are simply unavailable using obligate symbionts. Microinjection studies using pure cultures of *A. nasoniae* mimic the natural transmission of the bacterium, and allow for an ecologically relevant way to introduce the bacterium into new host lineages. Additionally, the nonfastidious nature of *A. nasoniae* may represent an evolutionary transition between a free-living bacterium and an obligate endosymbiont. The genome of *A. nasoniae* reflects this notion, demonstrating early degradation of metabolic genes and overall genome stream-lining (DARBY *et al.* 2010; WILKES *et al.* 2010). This bacterium is also an excellent candidate for studying symbiont spread in an ecosystem.

Current Study

In this thesis, I use two complementary approaches to investigate the host range of *A. nasoniae*. To estimate the extent of the symbiont's host range, I screened a large sample of filth flies and their parasitoids. I characterized *Arsenophonus*-positive samples by sequencing three variable loci. I then examined the potential host range of *A. nasoniae* using microinjection techniques. Four ecologically related hosts were inoculated with the bacterium: the fly *Musca domestica,* and the wasps *Trichomalopsis sarcophagae, Urolepis rufipes,* and *Muscidifurax raptorellus*. Transmission efficiency was investigated in each species, and symbiont effects on host longevity, fecundity, and

offspring sex-ratios were determined in *T. sarcophagae*. Mortality and offspring sex-ratios were also examined in the fly.

Practical applications of this research for biocontrol programs

Filth flies, e.g., house flies (*Musca domestica*), stable flies (*Stomoxys calcitrans*), and horn flies (*Haematobia irritans*), are major pests of livestock and cause significant economic losses; these flies are also known vectors of many diseases (MCLINTOCK and DEPNER 1954; MALIK *et al.* 2007). Infestations have traditionally been managed using chemical pesticides, but alternative control methods are being investigated to minimize both pesticide resistance and non-target effects (MALIK *et al.* 2007). 'Integrated pest management' (IPM) employs multiple approaches in concert, including physical, chemical and biological, to control pests. Parasitic wasps play an important role in many IPMs and several species are commercially available as biocontrol agents, e.g., *Muscidifurax* spp., *Spalangia* spp., and *Nasonia vitripennis* (MORGAN 1986; CRANSHAW *et al.* 1996). These wasps, along with *Trichomalopsis* spp., are found sympatrically in North America and all target filth flies with high specificity (FLOATE *et al.* 1999).

Recently, the potential of insect symbionts to augment IPMs has been investigated; for example, CI-inducing strains of *Wolbachia* may reduce host numbers through incompatible matings, or allow desirable traits to be introduced into a pest population, e.g., genes that reduce either host survival or disease transmission (DOBSON *et al.* 2002; RASGON *et al.* 2003). Male-killing bacteria may reduce pest populations by lowering mating opportunities (HURST and JIGGINS 2000). The unusual ability of *A. nasoniae* to be cultured should allow for transformation studies to be performed, i.e., the

introduction of novel genes into the bacterium, such as pesticide resistance traits. While the generality of male-killing by *A. nasoniae* remains to be determined, horizontal transmission of the bacterium may vector these genes across an entire fly-parasitoid guild. As wasps are susceptible to chemical and biological agents (AXTELL and ARENDS 1990; RUIU *et al.* 2007), using this process may increase wasp fitness under IPMs. Additionally, Son-killer may also play a role in traditional IPMs by reducing male wasp production and thus lowering the cost of wasp mass-rearing programs.

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Chapter 2: The wide host range of the male-killing symbiont *Arsenophonus nasoniae* in a guild of filth flies and their parasitic wasps

Insects harbour a great diversity of microbial symbionts. In particular, they are commonly infected with bacterial endosymbionts that are primarily maternally transmitted, often within the egg. This mode of transmission causes their fitness to be intimately linked to that of their host (KELLNER 2002). Because these microbes utilize resources within the host, and are thus inherently detrimental, selection will act against infections and cause symbionts to be lost from the host population (HURST 1991). To counter-act this, many symbionts provide a direct benefit to their host, and some are absolutely required for host survival. For example, virtually all aphids harbour an obligate symbiont, *Buchnera aphidicola,* which produces essential amino acids that are lacking in aphid diets (BAUMANN *et al.* 1995). Alternatively, many symbionts are facultatively beneficial, and only provide a fitness benefit under certain conditions. Examples include symbionts that defend their hosts against natural enemies, and others that buffer hosts against environmental stresses (MONTLLOR *et al.* 2002; MORAN *et al.* 2005).

Instead of directly increasing the survival of the host, many symbionts manipulate their host's reproduction to increase their own fitness; these are termed 'reproductive manipulators' (MORAN *et al.* 2008). Symbionts commonly do this by increasing the relative number of females produced by an infected host, allowing the symbiont to spread through the host population. As these symbionts are transmitted through the egg, male insects represent an evolutionary dead-end to the symbiont. The symbiont increases its

prevalence in the host population by skewing host reproduction towards females. This sex distortion occurs in either the primary sex ratio, through feminization or induction of parthenogenesis in the host, or in the secondary sex ratio, through killing of males during early embryogenesis (STOUTHAMER *et al.* 1993; HURST and JIGGINS 2000; WEEKS *et al.* 2001).

This male-killing strategy has evolved independently in bacteria at least five times as well as at least once in each of the microsporidia and viruses, and affects a diverse array of arthropods with vastly different sex-determination mechanisms (ANDREADIS 1985; HURST 1991; HURST and JIGGINS 2000; NAKANISHI *et al.* 2008). Almost all of these agents kill male hosts during the embryonic stage, and this is thought to maximize benefits for (infected) female siblings. For example, in a viviparous pseudoscorpion (*Cordylochernes scorpioides*), females infected by a male-killing strain of *Wolbachia* produce daughters of higher quality (determined by female size), as well as an increased number of daughters, than uninfected females (KOOP *et al.* 2009). A number of fitness benefits conferred by male-killers have been proposed, including reduced inbreeding, reduced competition by male siblings for resources, and increased resources through consumption of dead male siblings (HURST and JIGGINS 2000).

In oviparous insects, however, empirical support for the benefits of male-killing is lacking and how these male-killers are maintained in host populations remains a mystery. Despite this ambiguity, male-killing agents can reach an extremely high prevalence within host populations. For example, the male-killing *Wolbachia* infects 95% of females in populations of the butterfly *Acraea encedon* (JIGGINS *et al.* 2000). This symbiont appears to be at equilibrium in the population (JIGGINS *et al.* 2000) suggesting it

may provide cryptic benefits to the host (HURST and JIGGINS 2000). Horizontal transmission could also account for this high infection frequency. A parasitic microsporidium in mosquitoes, for example, kills males as larvae rather than as embryos (ANDREADIS 1985). Upon male death the bacterium is released to the environment where it infects additional hosts (ANDREADIS 1985). This horizontal transmission is thought to play a crucial role in the maintenance of this parasite (ANDREADIS 1985).

Although most early male-killers are transmitted in a strictly maternal fashion, horizontal transmission may assist maintenance of the male-killing bacterium *Arsenophonus nasoniae* (Gammaproteobacteria: Enterobacteriaceae) in its host *Nasonia vitripennis* (Hymenoptera: Pteromalidae), a filth fly parasitoid wasp. Known as Son-killer, *A. nasoniae* causes 80% of infected males to die as embryos (SKINNER 1985). It does this by targeting the maternal centrosome, which is required for proper development of male wasps (BALAS *et al.* 1996; FERREE *et al.* 2008). The bacterium is found systemically in the body of infected females and surviving males without apparent detrimental effects to the host (HUGER *et al.* 1985; BALAS *et al.* 1996). However, there is also no clear fitness benefit conferred by infection (BALAS *et al.* 1996). Wasps infected with *A. nasoniae* are found across the continental United States between 4% and 10% prevalence (SKINNER 1985; BALAS *et al.* 1996) and how it is maintained in these populations is unknown.

Within infected lineages, Son-killer is transmitted with high efficiency (95%) from the female to her offspring (SKINNER 1985). This transmission occurs not through the egg, as in most insect inherited symbionts including all other known strains of *Arsenophonus* (NOVAKOVA et al. 2009) but via the tissue of the wasp's fly host (HUGER

et al. 1985). *Nasonia* wasps parasitize the pupal stage of filth flies. During this parasitism, an inoculum of bacteria is mechanically transferred alongside the egg into the fly pupa. Son-killer is then acquired by the wasp larvae extra-orally and infects them through the midgut (HUGER *et al.* 1985; SKINNER 1985). Interestingly, and perhaps related to this mode of transmission, *A. nasoniae* is unusual for insect symbionts by being easily cultured outside of the host. This generalist nature of the bacterium may permit Son-killer to infect a wide range of hosts.

In the field, multiple female *Nasonia* wasps can parasitize the same individual filth fly; i.e., 'superparasitism' (GRILLENBERGER *et al.* 2008). During episodes of superparasitism, all wasp larvae within the fly will interact with the bacteria deposited by an infected female. About 95% of the *Nasonia* emerging from an inoculated host are infected, regardless of their mother's infection status (SKINNER 1985). This element of horizontal transmission may play an important role in maintaining the bacterium in *Nasonia* populations, as well as potentially spreading the bacterium across wasp species boundaries. Many closely related parasitoid wasp species share overlapping host ranges and can co-parasitize a single host (FLOATE *et al.* 1999; FLOATE 2002). A sympatric congener, *Nasonia longicornis*, is also infected with *A. nasoniae* (BALAS *et al.* 1996). However, thus far, *A. nasoniae* is not known to infect any insects other than *Nasonia*.

In this study I investigated the actual host range of *A. nasoniae* in a guild of filth flies and their parasitic wasps. This was done by conducting diagnostic PCR using 16S rDNA, as well as a protein encoding gene, infB, that has previously been used to resolve the Enterobacteriaceae at a species level (HEDEGAARD *et al.* 1999). InfB encodes translation initiation factor 2 (IF2), an essential protein for initiating protein synthesis

within prokaryotes (HEDEGAARD *et al.* 1999). I also screened *Arsenophonus*-positive insects for a phage gene that is associated with a number of insect symbionts. This gene potentially represents a rapidly evolving marker capable of distinguishing closely related *Arsenophonus* strains.

Methods

Sample collection and DNA extraction

Filth flies act as hosts for a wide range of parasitic wasps and represent a common link between different wasp species in the community. While previous research has examined *Arsenophonus nasoniae* as an endosymbiont specific to *Nasonia* wasps (BALAS *et al.* 1996), the overlapping host range of different wasp species may allow for horizontal transfer of the bacterium. The actual host range of *A. nasoniae* was therefore determined by screening members of the filth fly-parasitoid guild.

The specimens examined encompass 29 species of Hymenoptera (432 total individuals) and 12 species of Diptera (18 total individuals). The majority of these samples were accumulated by Kevin Floate and his lab (Agriculture Canada, Lethbridge AB) and used in a previous screening survey for *Wolbachia* (FLOATE *et al.* 2006), and represent individuals collected from both laboratory colonies and the field (Table 2.1). The DNA of these samples was previously extracted by washing the insect (1 minute with 95% EtOH followed by three rinses of sterile dH2O for 1 minute each) then processing the insect using either the Qiagen Blood and Tissue kit (following the instructions of the manufacturer), or by the STE method, where the insect is ground using a pipette tip in 25 uL of STE buffer [10mM Tris buffer (pH 8.0), 1mM EDTA (pH 8.0),

100mM NaCl] and 5 uL of proteinase K [20 mg/mL], and incubated at 37°C for 1 hour, followed by 3 minutes at 96°C.

I obtained additional insects by collecting mountain bluebird nest material from nest boxes near Lethbridge, AB, on two separate occasions in July, 2008, and placing the material within a culture cage incubated at 26°C (16:8 hour light:dark cycle). Emergent insects were killed in 95% EtOH and were identified using a light microscope. DNA from *Nasonia vitripennis* (n = 75), *Eupelmus vesicularis* (n = 8), and *Protocalliphora sialia* (n = 3) collected in this manner was extracted using the STE method.

Screening for *Arsenophonus*

The presence of *A. nasoniae* in each sample was determined using diagnostic polymerase chain reaction (PCR). I first screened samples using established primers designed to amplify 23 rDNA from *Arsenophonus*-like bacteria (THAO and BAUMANN 2004). As these primers also amplify related bacteria (most commonly *Proteus*), I then screened the 23S rDNA positive samples with conserved *Arsenophonus*-specific 16S rDNA primers (DURON *et al.* 2008b). I confirmed that these bands were *Arsenophonus* by sequencing bands from at least two samples per insect population, where possible (see below).

PCRs were carried out in a 25 uL reaction volume using either Invitrogen Taq Polymerase or TaKaRa LA Taq following the recommended reagent concentrations of the manufacturer. PCRs were run on an Eppendorf Mastercycler Gradient or a Bio-Rad DNA Engine Dyad Peltier Thermal Cycler. A 700bp fragment of the 23S rDNA was amplified using Ars23SF and Ars23R (THAO and BAUMANN 2004) at 95°C for 5 mins,

followed by 34 cycles of 30s at 94°C, 30s at 54°C, 45s at 72°C, and a final elongation for 10 min at 72°C. For those positive by Ars23SF/R, a 600bp fragment of the 16S rDNA was amplified using ArsF and ArsR3 (DURON *et al.* 2008b) at 95°C for 2 mins, followed by 35 cycles of 30s at 94°C, 30s at 54°C, 1 min at 72°C, and a final elongation for 5 min at 72°C. Both a positive control (DNA extracted from *A. nasoniae* from the American Type Culture Collection [ATCC strain 49151]) and negative water blank were used in all reactions.

Because 16S rDNA evolves so slowly and is considered undesirable for phylogenetic studies of highly similar sequences (CILIA *et al.* 1996), I attempted to design primers that would amplify a more variable region of the *Arsenophonus* genome. The protein encoding gene, infB, was amplified from *Arsenophonus* positive samples. This single-copy gene allows for better discrimination between bacterial strains than 16S rDNA; it encodes an initiation factor for protein synthesis, and is thought to undergo higher evolutionary rates of change than 16S rDNA (STEFFENSEN *et al.* 1997). It has previously been used to differentiate *Escherichia coli* strains and to resolve the phylogeny of the Enterobacteriaceae at the species level (STEFFENSEN *et al.* 1997; HEDEGAARD *et al.* 1999). Using infB sequence of the *A. nasoniae* type strain (ATCC 49151) initially obtained using general Enterobacteriaceae primers for the infB locus (1186F: 5'-ATYATGGGHCAYGTHGAYCAYGGHAARAC-3', 1833R: 5'-TATCCGACGCCGAACTCCGRTTNCGCATNGCNCGNAYNCGNCC-3') (HEDEGAARD *et al.* 1999), I designed primers using Primer3 (ROZEN and SKALETSKY 2000) to maximize the mismatches between *A. nasoniae* and closely related bacteria. The primers A-InfBF (5'-GATCCGGCCATACTCAAAAC-3') and A-InfBR (5'-

GACCACGGCAAAACTTCATT-3') amplify 618bp from *A. nasoniae* under the following PCR conditions: 95°C for 3 mins, followed by 34 cycles of 60s at 94°C, 60s at 53°C, 90s at 72°C, and a final elongation for 10 min at 72°C. All reactions were run alongside a positive control and negative water blank.

Finally, to ensure the DNA extractions were successful, samples testing negative in all reactions were amplified using primers (H3AF/H3AR (COLGAN *et al.* 1998)) designed to amplify 400bp of the single copy histone H3 gene. The PCR conditions for histone amplification were 94°C for 3 mins, followed by 40 cycles of 45s at 94°C, 30s at 65°C, 1 min at 72°C, and a final elongation for 6 min at 72°C. Both a positive DNA control and negative water blank were used in all reactions.

All PCR products were subjected to electrophoresis using a 1% agarose gel, stained using an ethidium bromide solution, and imaged using an UVP Bio Doc-it Imaging System. The amplified PCR products were purified for sequencing through either gel extraction using the Qiagen Gel Extraction kit or by the Qiagen PCR Purification Kit, following the instructions of the manufacturer.

Presence of phage sequences in *Arsenophonus*

During the course of this project, I identified a sequence in *A. nasoniae* highly similar to a lysogenic bacteriophage found in *Hamiltonella defensa*, a facultative symbiont infecting pea aphids (DEGNAN and MORAN 2008a; DEGNAN and MORAN 2008b). In aphids, this phage produces a toxin that protect its insect host from parasitic wasps (OLIVER *et al.* 2009), and a related phage has been recently identified in a strain of *Arsenophonus* infecting a psyllid (HANSEN *et al.* 2007). These closely related phages

have been designated APSE. The presence and variation of this APSE viral sequence was investigated for the insect samples infected with *A. nasoniae*. As phage sequences are often hotspots for recombination and tend to evolve rapidly (JUHALA *et al.* 2000), the viral locus is an excellent marker to use for analysis of closely related strains of bacteria.

DNA extracts were amplified by PCR using the primers APSE30.1 and APSE31.1 (DEGNAN and MORAN 2008a). These primers specifically amplify 1000bp of the viral P45 gene which encodes a DNA polymerase. The reaction conditions were as follows: 94°C for 2 mins, followed by 36 cycles of 30s at 94°C, 50s at 53°C, 90s at 72°C, and a final elongation for 5 min at 72°C. Samples were directly sequenced for identification and phylogenetic analysis.

Screening for potential hosts of APSE in addition to *Arsenophonus*

It is formally possible that the isolated APSE sequences occur in bacteria other than *Arsenophonus*. I therefore wanted to screen for the presence of symbionts other than *Arsenophonus* within each infected sample. In order to do this, I performed a preliminary study using SuPER PCR. This method, which stands for 'suicide polymerase endonuclease restriction' removes target DNA (in this case *Arsenophonus*) from a sample using restriction digestion (GREEN and MINZ 2005). Subsequently, universal primers are used to amplify the DNA of the remaining species which is then cloned and sequenced for identification. SuPER PCR can be performed on conventional PCR equipment and provides a cursory exploration of the bacterial flora present in a sample; future studies using denaturing gradient gel electrophoresis (DGGE) techniques would allow for a more thorough analysis.

An individual *Spalangia cameroni* wasp from Russia (a sample that was positive for both *Arsenophonus* and APSE) was analyzed using SuPER PCR to remove *Arsenophonus nasoniae,* and the remaining flora was amplified using universal 16S rDNA primers. Kill primers, specific for the 16S rDNA of *A. nasoniae,* were designed using Primer3 (ROZEN and SKALETSKY 2000), Ars315F: 5'-TCAGTCGTGAGGAAGGTGTTAAGG-3', Ars718R: 5'-TGACCACAACCTCCAAATCGACA-3', and a reaction mixture containing 1.5 U of Taq DNA polymerase, 10ug of BSA, 0.86x PCR buffer, 5.6mM MgCl, and 0.56uM of each primer in a 29 uL final reaction volume was incubated at 95°C for 3 mins. The heat was then reduced to 70°C, and deoxynucleoside triphosphate (0.172 mM final concentration in the reaction mixture) and 5U of the restriction enzyme Tsp509I (New England Biolabs, Beverly, MA) along with 1.38x NEBuffer 1, preheated to 70°C, was added to the sample. This thermostable restriction enzyme removes the annealed sequences from subsequent reactions. The reaction mixture was incubated at 60°C for 60min, 95°C for 30 min, then held at 4°C, at which time proteinase K (20 mg/mL) was added to a final concentration of 3.3ug/uL. The mixture was incubated at 58°C for 30 mins, 95°C for 10 min, then cooled and stored at 4°C for a subsequent PCR reaction.

2 uL of the reaction mixture was used directly in a PCR using universal 16S rDNA primers to survey the remaining bacterial species in the sample. In a 26 uL reaction volume, 0.172 uM of the primers 63F and 907R (MARCHESI *et al.* 1998; SCHABEREITER-GURTNER *et al.* 2003), 10ug BSA, 0.96x PCR buffer, 1.92 mM MgCl, 0.192 mM dNTP mixture, and 1.5U of Taq, were subjected to PCR at 94°C for 4 mins,

followed by 38 cycles of 1min at 95°C, 1min at 50°C, 1min at 72°C, and a final elongation for 5 min at 72°C.

This 16S rDNA amplified product was cleaned using a Qiagen PCR Purification kit and a clone library was developed using the Stratogene Cloning Kit, following the manufacturer's instructions. 13 clones were prepared using the Qiagen Miniprep Kit, according to the manufacturer's instructions, and sequenced using M13F (5'-GTAAAACGACGGCCAGT-3') and M13R (5'-GCGGATAACAATTTCACACAGG-3') primers.

Sequencing and analysis

Products were sequenced by Macrogen, South Korea, in both directions using the same primers as were used for the initial amplification unless otherwise noted. Sequences and chromatograms were analyzed, and vector sequences removed if necessary, using Biomatters' Geneious software. Sequences were identified through BLAST using the top hit by identity.

Phylogenetic analyses were conducted on the 16S rDNA (not shown), infB, and viral P45 genes. The alignment datasets were curated using Gblocks v0.91b (TALAVERA and CASTRESANA 2007) under the default settings. Sequences from related bacteria were retrieved from GenBank. The infB dataset contains sequences from known insect symbionts, while sequences in the P45 dataset were selected from GenBank solely on nucleotide similarity to those collected from the wasp samples. I also successfully amplified and sequenced *Arsenophonus* 16S rDNA, infB, and APSE from genomic DNA from the common greenhouse whitefly *Trialeurodes vaporariorum* (Taylor *et al.*

unpublished); these represent the only infB and APSE sequence from another *Arsenophonus* lineage and therefore serve as useful comparisons. The accession numbers for all sequences used in the phylogenetic analysis are shown in Figure 2.1. *Escherichia coli* shows moderate levels of divergence from *Arsenophonus* and was used as the outgroup for the infB phylogeny; this bacterium has previously been used to root 16S rDNA trees of *Arsenophonus* (THAO *et al.* 2000; DALE *et al.* 2006; TROWBRIDGE *et al.* 2006). As the most ancestral viral sequence is unknown, phylogenetic trees for the APSE gene were visualized using midpoint rooting. For each gene the nucleotide DNA sequences were aligned using MUSCLE (EDGAR 2004) and output as a PHYLIP file. These alignments were trimmed and confirmed using MacClade v.4.08, and the best model was found using Modeltest 3.7 (POSADA and CRANDALL 1998) in PAUP* 4.0b10 (SWOFFORD 2003). Maximum likelihood phylogenies were constructed through http://www.phylogeny.fr (DEREEPER *et al.* 2008) using PHYML (GUINDON and GASCUEL 2003; GUINDON *et al.* 2005) with the optimum parameters as suggested by Modeltest. Node support was determined using 100 bootstrap replicates.

Results

Presence of *A. nasoniae* infection amongst different host species

Four of the 29 tested species of wasps were infected with *A. nasoniae* (Table 2.1); i.e., *Nasonia vitripennis, Spalangia endius, Spalangia cameroni* (Pteromalidae), *Eupelmus vesicularis* (Eupelmidae). Infected individuals were collected from field populations from widespread locales: i.e., Canada, United States, Romania, Turkey, Peru. In the populations represented by multiple individuals (*E. vesicularis, N. vitripennis, S.*

endius, S. cameroni) not all of the individuals in a population were infected. Only representatives from field collections, rather than culture collections, tested positive for infection in the survey. Additionally, a large proportion of *N. vitripennis* collected from Lethbridge, AB, were infected with *A. nasoniae*. None of the flies tested were infected with *A. nasoniae*.

These initial *Arsenophonus* identifications were made using 16S rDNA sequence. Sequences at this locus from all samples were identical over 470 nucleotide positions, and matched the *A. nasoniae* type strain in Genbank (AY264674.1) with 99% identity. As 16S rDNA is highly conserved, other *Arsenophonus* strains are nearly identical at this locus. The wasp samples also matched *Arsenophonus* from *T. vaporariorum* with 99% identity.

The infB phylogeny for *Arsenophonus* was reconstructed with 426 nucleotide characters of the infB locus using maximum likelihood (Fig. 2.1). Three of the infected samples (*N. vitripennis* from Canada, *E. vesicularis* from Canada, *S. cameroni* from Russia) contain identical infB sequences to the type strain of *A. nasoniae*, while the other sample (*E. vesicularis* from Romania) differed in one nucleotide position. Thus, all sequences obtained from insects in the filth fly/parasitoid wasp guild clustered tightly together into a single clade (ML bootstrap 86%). The infB sequences from these wasps differed at 29 of the 501 nucleotides from the *Arsenophonus* symbiont of *T. vaporariorum* (94% similarity). Sequence for the infB gene of *S. endius* samples was not obtained.

The infB phylogeny was performed with a GTR+G+I model, with the following transition/transversion rates: AC:0.5310, AG:2.7576, AT:0.7580, GC:0.4391, CT:2.7576,

GT:1.0000. There was a 0.5612 proportion of invariable sites, and a gamma distribution of 2.3964. The base frequencies were estimated from the data (A:0.246705, C:0.232561, G:0.249850, T:0.270884), and the final tree had a score of 2966.02.

Prevalence of a viral marker sequence among individuals infected with *A. nasoniae*

I identified P45 viral sequence from 3 of the 4 *Arsenophonus nasoniae* positive wasp species; i.e., *E. vesicularis, N. vitripennis, S. cameroni* (Table 2.1). The remaining species, *S. endius,* exhibited the expected band but was not confirmed by sequencing. APSE infections also appear polymorphic within species. *E. vesicularis* collected from both Canada and Romania contained the sequence, while it was not amplified from the individual sample from Turkey. *S. cameroni* samples from both Russia and Peru contained the viral sequence. All APSE-like sequences were highly similar from the three wasp species, differing at only 39 nucleotide positions over 702 total bases (94% identical).

The maximum likelihood analysis for the APSE sequence (Fig. 2.2) was performed using 702 nucleotide bases under a GTR+G model with the following transition/transversion rates: AC:2.1017, AG:6.8375, AT:0.9115, GC:1.9309, CT:6.8375, GT:1.0000. A gamma distribution of 0.2319 was used, and with estimated base frequencies (A: 0.264381, C: 0.240636, G: 0.257898, T: 0.237085) the produced tree had a score of 2070.32.

The APSE-like sequence from *A. nasoniae* is highly similar to the APSE sequence found in *H. defensa* (89% nucleotide similarity), and all P45 sequences found in the filth fly parasitic wasps clustered tightly together with high bootstrap support (ML

bootstrap 99.9%). A viral sequence found in the whitefly *T. vaporariorum* is allied with the *A. nasoniae* clade (ML bootstrap 96.4%); this whitefly host is also infected with *Arsenophonus* and the viral nucleotide sequence is 89% identical to the *A. nasoniae* type strain.

Arsenophonus nasoniae **is the most likely host of APSE in filth fly parasitic wasps**

All 13 clones sequenced from the SuPER PCR assay were highly similar to one another (99% nucleotide similarity). They showed the highest similarity (>99%) in BLAST searches to *Sodalis*, a gammaproteobacterial symbiont that infects diverse insects, including the weevil *Curculio sikkimensis* and tsetse flies (DALE and MAUDLIN 1999; TOJU *et al.* 2010).

To determine whether *Sodalis* was present in other APSE positive insects, I designed *Sodalis*-specific 16S rDNA primers using Primer3 (ROZEN and SKALETSKY 2000). The samples were run with the designed primer set (Sod320F: 5'-ATATTGCACAATGGGGGAAA-3', Sod801R: 5'-CAAGGCCACAACCTTCAAAT-3'; this study) at 95°C for 5 min, 30 cycles at 94°C, 56°C, 72°C for 1 min each, and a final elongation step at 72°C for 10 min, along with a positive control for *Sodalis*, and three negative controls: DNA extracted from *E.coli*, DNA extracted from the *A. nasoniae* type strain, and a water blank. Only the single *S. cameroni* sample from Russia tested positive for *Sodalis*.

Discussion

Diverse *Arsenophonus* strains infect arthropods from diverse taxa across the globe (DURON *et al.* 2008a; NOVAKOVA *et al.* 2009). Within these hosts, infection frequencies appear highly variable but almost never approaching 100%, i.e., at or near fixation (BALAS *et al.* 1996; HYPSA and DALE 1997; THAO *et al.* 2000). The effects of most *Arsenophonus* infections on their hosts are not yet known. Here I report *Arsenophonus* infection in a parasitic wasp guild where all infected hosts appear to share a single *Arsenophonus* species. *Arsenophonus* sequences from the infected wasps exhibit extremely high similarity across the loci examined, including the viral locus P45. The highest level of divergence between *Arsenophonus* strains at this locus (*S. cameroni* (RUS) and *N. vitripennis* (AB); 97.2% nucleotide similarity) is similar to the divergence between known APSE strains infecting the bacterial host *H. defensa* (APSE-5 and APSE-3; 97.2% nucleotide similarity). It is concluded that the *Arsenophonus* strains within the wasp guild are composed of a single species, *Arsenophonus nasoniae*.

It is formally possible that samples testing positive for *Arsenophonus nasoniae* do not represent true infections within the insect. The bacterium may instead be restricted to the body surface or inside the gut of the insect. These possibilities are unlikely for two reasons. The adult wasps used are unlikely to have gut contaminants because larvae purge gut contents prior to pupation (TORMOS *et al.* 2009). Additionally, insect samples were washed prior to DNA extraction to reduce surface contaminants. The present study thus strongly suggests *Arsenophonus nasoniae* can infect multiple species. Hence, this bacterium should be considered a generalist rather than a specialist of *N. vitripennis*. This ability to infect different hosts may be permitted by the mode of transmission of *A.*

nasoniae through fly tissue under conditions of superparasitism. However, this postulation will need to be directly tested. The transmission outside of the host also suggests that this bacterium has a broader set of metabolic capabilities than most insect symbionts. This supposition is corroborated by the recently sequenced *A. nasoniae* genome, which contains diverse genes for carbohydrate metabolism (DARBY *et al.* 2010). No flies were infected with *A. nasoniae.* This result is not surprising, however, because flies typically die during interactions with wasps. For a bacterial transfer to occur, the fly would need to a) be parasitized by an infected wasp, b) survive envenomation and parasitism by the wasp, c) maintain the bacterium during fly development through to emergence, and d) transmit the bacterium to its offspring.

The number of species infected by *A. nasoniae* suggests both horizontal and vertical transmission are possible in this system, and suggests horizontal transmission may play an integral role in bacterial maintenance. To determine if these alternate hosts act as ecologically relevant reservoirs for the bacterium, the vertical transmission and effects of the bacterium must be evaluated. If *A. nasoniae* is a generalist and is maintained in these alternate hosts, male-killing may simply be incidental. Horizontal transmission could also fundamentally alter host systems; symbionts exchange genetic material within a shared host (BORDENSTEIN and WERNEGREEN 2004), and a generalist could vector genetic components between maternally-inherited symbionts. This would increase genetic diversity in otherwise isolated microbes.

Previous studies have found that *A. nasoniae* infects *Nasonia vitripennis* at low frequencies, with ~5-10% wasps infected across North America (BALAS *et al.* 1996). It is therefore surprising that I identified infected individuals in my study given these expected

frequencies, especially since some of the populations I sampled had very small numbers. For example, some infected individuals are the sole representative of their population (i.e., *E. vesicularis* from Turkey, *S. cameroni* from Russia) and this suggests that *A. nasoniae* may occur in some species at much higher frequencies than previous reports. Infection frequencies in my field-caught *N. vitripennis* collected in 2008 from Lethbridge, AB, were also unexpectedly high (51%). While previous studies have shown some populations with similarly high prevalence, there is a high degree of variation in the prevalence of this symbiont (BALAS *et al.* 1996).

How can one reconcile these differences in infection frequencies? One possibility is that *A. nasoniae* does not function as a male-killer in this system, but exerts other fitness effects that might result in higher population frequencies, such as a cryptic fitness benefit. Supporting this, *A. nasoniae* was only found in field-collected individuals and not from laboratory maintained cultures. This suggests infection is only beneficial under certain conditions and is lost from a lab culture in the absence of environmental factors. Balas *et al.* have proposed that inbreeding depression maintains the bacterium (1996). This phenomenon may contribute mildly to host fitness (LUNA and HAWKINS 2004), but its effect in field populations is unclear (MOLBO and PARKER 1996).

The existence of non-male-killing strains has also been hypothesized (BALAS *et al.* 1996). In these cases, perhaps *A. nasoniae* helps protect its insect hosts against natural enemies. Defensive symbionts are often lost from populations, including lab cultures, which are no longer exposed to the enemy (OLIVER *et al.* 2008). The phage sequences identified in *A. nasoniae* are very similar to those found in two other defensive symbionts – a strain of *Hamiltonella defensa* that protects pea aphids against parasitic

wasps (OLIVER *et al.* 2009) and a strain of *Arsenophonus* that infects the psyllid *Glycaspis brimblecombei,* and whose prevalence is correlated with parasitism pressure (HANSEN *et al.* 2007). In *H. defensa* this dsDNA phage expresses toxins during lysogeny, and is able to induce lysis in the host (VAN DER WILK *et al.* 1999; MORAN *et al.* 2005)

While the phage sequence in *A. nasoniae* may have potential ecological implications, it can also be exploited as a useful marker for phylogenetic studies. Because viral sequences often undergo extremely high rates of evolutionary change (JUHALA *et al.* 2000) the P45 gene of APSE provides an excellent candidate for examining divergence between closely related bacterial strains. While some phages have broad host ranges (JENSEN *et al.* 1998), most are restricted to closely-related hosts (WELKOS *et al.* 1974); two phages related to APSE infect single hosts within the Enterobacteriaceae (VANDER BYL and KROPINSKI 2000; CLARK *et al.* 2001). While it is formally possible that the phage sequences identified in this study are found in a symbiont other than *A. nasoniae*, a number of lines of evidence suggest otherwise. First, there was a very strong correlation between *A. nasoniae* infection and P45 sequence. While SuPER PCR identified *Sodalis* in *S. cameroni*, *Sodalis* was not found in any other *A. nasoniae* positive samples. Most significantly, two independent isolates of *A. nasoniae* contain P45 sequence that is nearly identical to that of the infected wasps. This demonstrates that *A. nasoniae* is able to host the virus, and the high similarity between P45 sequences from wasps and the isolated *A. nasoniae* suggest it is the same viral species in both. The phages found in the mixed samples are thus likely to be infecting the same host.

Phage was not amplified from all *A. nasoniae* samples, possibly as a result of sequence divergence at the primer binding locations, or secondary loss of the phage (DEGNAN and MORAN 2008a), and it is unclear what effects viral infection may have on the wasp host. All sequences from the viral marker in this study are highly similar to APSE strains in pea aphids and psyllids; these are two hosts where the phage provides a protective phenotype to the insect. It is important to determine if the phage in *A. nasoniae* is functional and expresses proteins within the host, as its high relatedness suggests the phage in *A. nasoniae* may have similar phenotypic effects for infection. Because toxins produced by APSE in the pea aphid prevent wasps from developing in the host (OLIVER *et al.* 2009), it is tantalizing to hypothesize that the mechanisms for phage-mediated defense and male-killing may be similar.

No fitness benefit has been found for *A. nasoniae* infection in *N. vitripennis*, but the bacterium infects multiple wasps in the environment. This broad host range suggests horizontal transmission may play an integral role in bacterial maintenance. Its importance remains to be directly tested, and the bacterium may have significant impacts on novel hosts in the environment - particularly given the lysogenic phage present. Perplexingly, the Son-killer bacterium is defined by its male-killing phenotype, but the importance of this reproductive manipulation for its maintenance remains completely unknown.

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Table 2.1: *Arsenophonus* screening results for a sample of filth flies and their parasitic wasps. Numbers represent infected individuals out of the total number tested for *A. nasoniae*, P45, and *Sodalis* infections. Only *A. nasoniae* infected samples were examined for P45 and *Sodalis*.

Insect θ	**Family**	**Location collected**	**Number screened**	**Sex$^{\phi}$**	***A.nasoniae***	**P45**	***Sodalis***	**Collection information† (f = field, c = lab culture)**
Flies (Diptera)								
Calliphorid sp.	Calliphoridae	Lethbridge, AB	1	♀	-	n.t‡	n.t	F
Haematobia irritans	Muscidae	Lethbridge, AB	2	♀	-	n.t	n.t	F
Lucilla cuprina	Calliphoridae	Bali, Indonesia	1	?	-	n.t	n.t	F
Musca domestica	Muscidae	Lethbridge, AB	1	♀	-	n.t	n.t	C
Neobellieria	Sarchophagidae	AAFC	1	?	-	n.t	n.t	C
Phormia sp.	Calliphoridae	San Antonio, TX	1	?	-	n.t	n.t	C
Pollenia rudius	Calliphoridae	Lethbridge, AB	3	?	-	n.t	n.t	F
Protocalliphora sialia	Calliphoridae	Lethbridge, AB	3	♀	-	n.t	n.t	F
Protophormia terraenovae	Calliphoridae	Lethbridge, AB	1	?	-	n.t	n.t	F
Ravinia querula	Sarcophagidae	Lethbridge, AB	2	?	-	n.t	n.t	F
Sarcophaga bullata	Sarcophagidae	Lethbridge, AB	1	?	-	n.t	n.t	F
Sepsis sp.	Sepsidae	Lethbridge, AB	1	?	-	n.t	n.t	F
Wasps (Hymenoptera)								
Aphaereta pallipes	Braconidae	Ottawa, ON	1	♀	-	n.t	n.t	F
Brachymeria podagrica	Chalcididae	Buenos Aires, ARG	1	♀	-	n.t	n.t	F
Diapria conica	Diapriidae	Lethbridge, AB	1	♀	-	n.t	n.t	F

Insect	Family	Location collected	Number screened	Sex	*A.nasoniae*	P45	*Sodalis*	Collection information (f = field, c = lab culture)
Dirhinus himalayanus	Chalcididae	Morocco	1	♀	-	n.t	n.t	F
Eupelmus vesicularis	Eupelmidae	Agassiz, BC	8	♀	**1/8**	**1/1**	**0/1**	F
		Lethbridge, AB	8	♀	-	n.t	n.t	F
		Romania	4	♀	**2/4**	**1/2**	**0/2**	F
		Turkey	1	♀	**1/1**	**0/1**	n.t	F
Kleidotoma sp.	Eucoilidae	Lethbridge, AB	1	♀	-	n.t	n.t	F
Muscidifurax raptor	Pteromalidae	Demmark	1	♀	-	n.t	n.t	F
		Lethbridge, AB	8	♀	-	n.t	n.t	C
		Ottawa, ON	1	♀	-	n.t	n.t	F
		PNE	2	♀	-	n.t	n.t	C
Muscidifurax raptorellus	Pteromalidae	AAFC	1	♀	-	n.t	n.t	C
		PNE	1	♀	-	n.t	n.t	C
Muscidifurax uniraptor	Pteromalidae	MLIRL	1	♀	-	n.t	n.t	C
Muscidifurax zaraptor	Pteromalidae	KSU	1	♀	-	n.t	n.t	C
		Lethbridge, AB	2	♀	-	n.t	n.t	C
		PNE	1	♀	-	n.t	n.t	C
Nasonia vitripennis	Pteromalidae	Buenos Aires, ARG	1	♀	-	n.t	n.t	F
		Lethbridge, AB	81	♀	**38/81***	**10/11****	**0/5**	F
		Ottawa, ON	1	♀	-	n.t	n.t	F
		Lethbridge, AB	39	♀	-	n.t	n.t	C
		Utah	17	♀	-	n.t	n.t	F
		Indiana	31	♀	-	n.t	n.t	F
Pachycrepoideus vindemiae	Pteromalidae	Denmark	1	♀	-	n.t	n.t	F
		AAFC	4	♀	-	n.t	n.t	C

Insect	Family	Location collected	Number screened	Sex	*A.nasoniae*	P45	*Sodalis*	Collection information (f = field, c = lab culture)
Phygadeuon sp.	Ichneumonidae	Denmark	1	♀	-	n.t	n.t	F
Phygadeuon fumator	Ichneumonidae	Denmark	3	♀	-	n.t	n.t	F
		Ottawa, ON	1	♀	-	n.t	n.t	F
Pteromalus venustus	Pteromalidae	Lethbridge, AB	5	4 ♀ 1 ♂	-	n.t	n.t	F
Spalangia cameroni	Pteromalidae	Kazakstan	1	♀	-	n.t	n.t	F
		Russia	1	♀	**1/1**	**1/1**	**1/1**	F
		Ottawa, ON	1	♀	-	n.t	n.t	F
		AAFC	39	♀	-	n.t	n.t	C
		Florida	7	♀	-	n.t	n.t	F
		Denmark	6	♀	-	n.t	n.t	F
		Peru	2	♀	**2/2**	**2/2**	**0/2**	F
		France	1	♀	-	n.t	n.t	F
		Mead, Nebraska	1	♀	-	n.t	n.t	F
		Arkansas	1	♀	-	n.t	n.t	F
		Minnesota	1	♀	-	n.t	n.t	F
Spalangia endius	Pteromalidae	Ottawa, ON	1	♀	-	n.t	n.t	F
		Florida	10	♀	-	n.t	n.t	F
		Peru	1	♀	-	n.t	n.t	F
		Mead, Nebraska	7	♀	-	n.t	n.t	F
		Kazakstan	8	♀	-	n.t	n.t	F
		Russia	6	♀	-	n.t	n.t	F
		Arkansas	2	♀	-	n.t	n.t	F
		Minnesota	7	♀	**7/7***	**3/4****	n.t	F
		PNE	2	♀	-	n.t	n.t	C
		N Illinois University	1	♀	-	n.t	n.t	F
		Maine	1	♀	-	n.t	n.t	F

Insect	Family	Location collected	Number screened	Sex	*A.nasoniae*	P45	*Sodalis*	Collection information (f = field, c = lab culture)
Spalangia gemina	Pteromalidae	Brazil	4	♀	-	n.t	n.t	F
		PNE	2	♀	-	n.t	n.t	C
Spalangia nigra	Pteromalidae	Ottawa, ON	2	♀	-	n.t	n.t	F
		UMN	1	♀	-	n.t	n.t	C
		Mead, Nebraska	1	♀	-	n.t	n.t	F
Spalangia nigroaenea	Pteromalidae	Ottawa, ON	1	♀	-	n.t	n.t	F
		Russia	2	♀	-	n.t	n.t	F
		Kazakstan	2	♀	-	n.t	n.t	F
		Florida	1	♀	-	n.t	n.t	F
		UMN	1	♀	-	n.t	n.t	C
		Mead, Nebraska	11	♀	-	n.t	n.t	F
Tachinophaegus zealandicus	Encyrtidae	Buenos Aires, ARG	1	♀	-	n.t	n.t	F
		Brazil	1	♀	-	n.t	n.t	F
Trichomalopsis sarcophagae	Pteromalidae	Lethbridge, AB	20	♀	-	n.t	n.t	F
		Lethbridge, AB	20	♀	-	n.t	n.t	C
Trichomalopsis viridiscens	Pteromalidae	Ottawa, ON	1	♀	-	n.t	n.t	F
Trichopria nigra	Diapriidae	Russia	2	♀	-	n.t	n.t	F
		Kazakstan	2	♀	-	n.t	n.t	F
Urolepis rufipes	Pteromalidae	Lethbridge, AB	20	♀	-	n.t	n.t	C
		Denmark	2	♀	-	n.t	n.t	F
		Ottawa, ON	2	♀	-	n.t	n.t	F
		PNE	2	♀	-	n.t	n.t	C

Θ Most insect samples were previously used in a screening survey for *Wolbachia*; see FLOATE *et al.* 2006 for further information regarding insect host range and food sources. Eupelmidae were not included in the aforementioned study; *Eupelmus vesicularis* is a generalist parasitoid and hyper-parasitoid (GIBSON 1990), and has been rarely collected from filth fly pupae (FLOATE *et al.* 2000; PETERS and ABRAHAM 2010).

ϕ If no number is given, sex listed is for all tested samples. If present, the number gives individuals of each sex tested. ? signifies insects were not sexed prior to DNA extraction.

†Location collected lists geographic location where samples were originally collected; if collection location is unknown the supplier is listed: PNE = PNE, Inc., UMN = University of Minnesota, KSU = Kentucky State University, AAFC = Agriculture and Agrifoods Canada, MLIRL = Midwest Livestock Insects Research Laboratory.

‡n.t represents samples not tested

* At least 2 individuals were confirmed infected by direct sequencing of 16S rDNA. The remaining specimens were identified as infected by presence of the expected band.

** Only a subset of *Arsenophonus* samples were screened for P45 sequence; 11 and 4 infected samples of *N. vitripennis* and *S. endius,* respectively. Products from 2 *N. vitripennis* and none from *S. endius* were confirmed by direct sequencing.

Note: *Sodalis* infection in *N. vitripennis* was examined in only a subset of *Arsenophonus*-infected samples

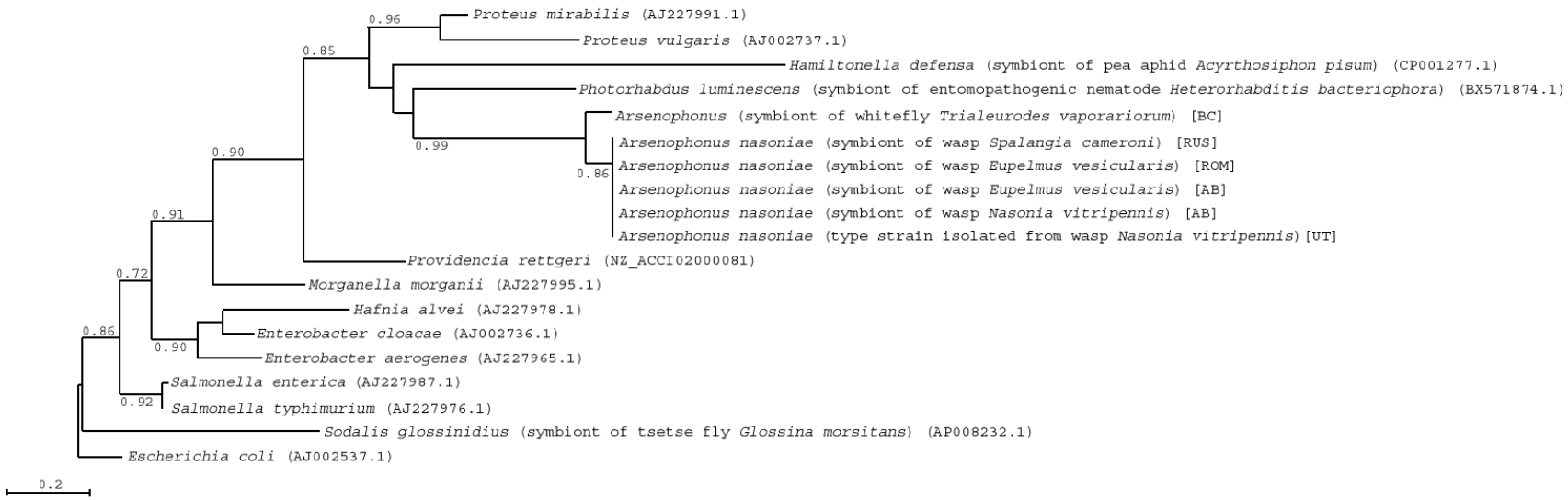

Figure 2.1: Maximum likelihood phylogeny of *Arsenophonus nasoniae* using the infB protein encoding gene. Numbers indicate bootstrap percentage at each node (out of 100 bootstraps). Labels in brackets are NCBI accession numbers or location collected if newly reported in this study.

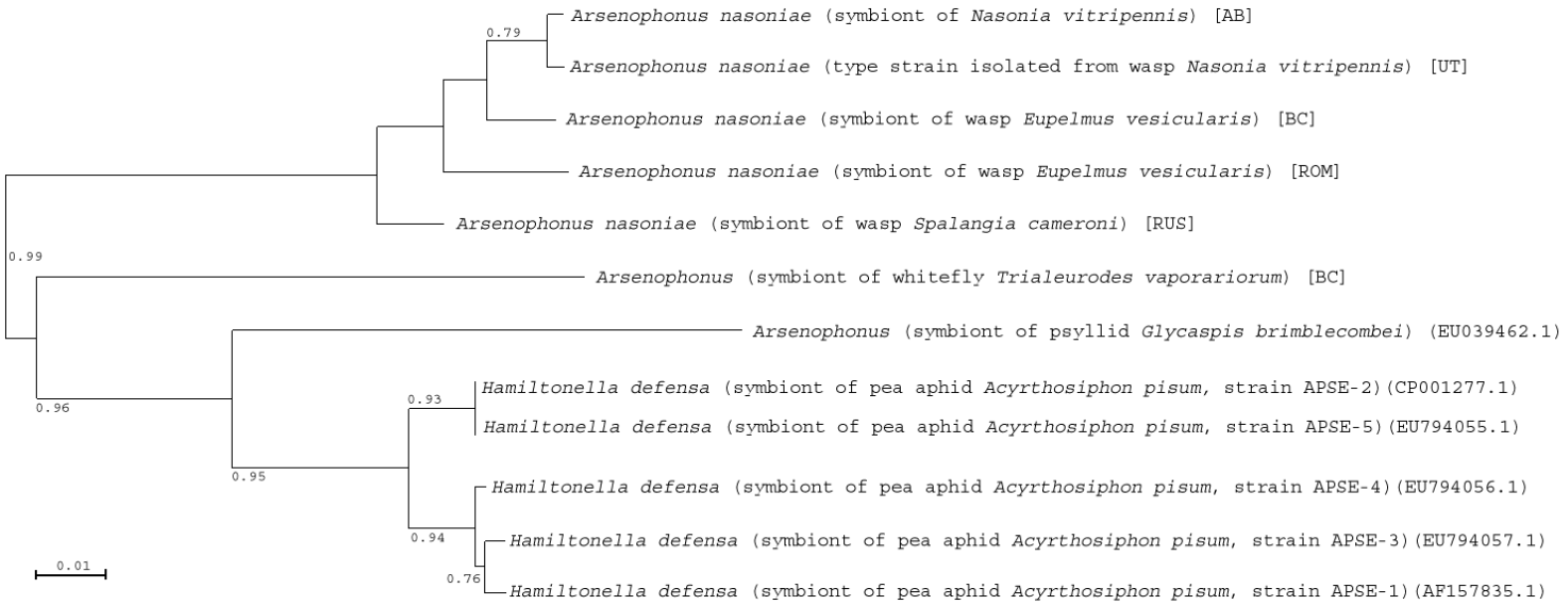

Figure 2.2: Maximum likelihood phylogeny of the P45 viral protein encoding gene in a clade of insect symbionts. Numbers indicate bootstrap percentage (100 bootstrap replicates) for each node. Labels in brackets are NCBI accession numbers or location collected if newly reported in this study.

Chapter 3: Potential host range of *Arsenophonus nasoniae*, a symbiont of the filth fly parasitoid *Nasonia vitripennis*

Symbiotic interactions between microorganisms and arthropods are extremely diverse, and many have shaped the ecology and evolution of their hosts (MORAN *et al.* 2008). The nature of these interactions can vary on a spectrum from beneficial to detrimental for the host. Endosymbionts living within an arthropod host can increase the host's fitness relative to uninfected hosts (mutualism), provide a benefit equivalent to the cost of infection (commensalism), or reduce a host's fitness relative to uninfected hosts (parasitism). Commonly, endosymbionts are transmitted strictly maternally, often within the egg cytoplasm, and as a result of this mode of transmission their fitness is intimately linked to that of their host.

There are two major types of inherited endosymbionts: primary and secondary symbionts. Primary symbionts are beneficial symbionts obligately required for host survival, and infect all known arthropods that feed on nutrient deficient food sources such as blood and plant sap (BAUMANN *et al.* 1995; AKMAN *et al.* 2002; ALLEN *et al.* 2007; DOUGLAS 2009). The primary symbiont of aphids, *Buchnera aphidicola*, is the best-studied example and augments the host's diet with essential amino acids (BAUMANN *et al.* 1995). As they are required for host survival, primary symbionts are found within all hosts within the population (i.e., at fixation). Associations between primary symbionts and their hosts are often ancient and exhibit patterns of strict cospeciation between host and symbiont. Many primary symbionts are housed in specialized tissues or organs of the host (AKSOY 1995; BAUMANN *et al.* 1995; SANDSTROM *et al.* 2001; PEROTTI *et al.* 2007).

Secondary symbionts are not obligately required for host survival, and are often found at lower prevalence in hosts. Contrary to the long-term host fidelity displayed by primary symbionts, secondary symbionts demonstrate horizontal transfer between species on an evolutionary timescale, despite being predominately maternally transmitted (SCHILTHUIZEN and STOUTHAMER 1997; VAVRE *et al.* 1999; ZCHORI-FEIN and PERLMAN 2004; NOVAKOVA *et al.* 2009). Symbionts utilize host resources and are thus inherently detrimental to the host. Selection will act against infections in the host population. Secondary symbionts counteract this selection by either providing a benefit to the host, or by manipulating the host to avoid these pressures. Benefits provided by facultative symbionts are often only exhibited under specific conditions. For example, these symbionts may protect the host against natural enemies, facilitate the colonization of new host plants, or protect the host against environmental stresses (MONTLLOR *et al.* 2002; OLIVER *et al.* 2003; TSUCHIDA *et al.* 2004).

Other secondary symbionts have developed the ability to exploit the host's reproductive behaviours, and are called 'reproductive manipulators'. These symbionts have evolved diverse strategies to increase the frequency of infected females in the population. Thus far, four types of manipulation are known. Some symbionts induce mating incompatibilities between infected males and uninfected females; this is termed 'cytoplasmic incompatibility'. Others increase their prevalence by distorting the host sex-ratio in one of three known ways; inducing parthenogenesis, feminization, or by killing males. Male-killing is the most commonly derived strategy of reproductive manipulators, and has independently evolved at least five times in bacteria and at least once in both the microsporida and viruses (HURST and JIGGINS 2000; NAKANISHI *et al.*

2008). Some male-killers have been shown to reach very high frequencies, with drastic effects on host mating systems and population genetic structure. For example, a male-killing strain of *Wolbachia* is found at a 95% equilibrium frequency in the butterfly *Acraea encedana*, causing the reversal of sex-roles of the host (JIGGINS *et al.* 2000a; JIGGINS *et al.* 2000b).

Male hosts represent an evolutionary 'dead-end' for symbionts as they do not transmit maternally inherited symbionts. Male-killing symbionts typically kill these hosts during the embryonic phase (HURST and JIGGINS 2000). However, one major unresolved issue is how male-killers are maintained in populations over the long-term. This is because unless male-killing symbionts increase host fitness, uninfected lineages are expected to replace infected ones over evolutionary time. Although a number of fitness benefits have been suggested, such as reduced inbreeding and reduced competition (HURST 1991; HURST and JIGGINS 2000), empirical support has been elusive (but see KOOP *et al.* 2009). Alternatively, other factors may maintain male-killers, such as high rates of horizontal transmission.

An interesting system to study male-killer maintenance is the 'Son-Killer' bacterium *Arsenophonus nasoniae* that infects the wasp *Nasonia vitripennis*, a pupal parasitoid of filth flies. Like all wasps, *N. vitripennis* has haplodiploid sex determination, whereby fertilized diploid eggs develop as females and unfertilized haploid eggs develop as males. Throughout its range in North America, 4-10% of these wasps are infected with *A. nasoniae* (SKINNER 1985; BALAS *et al.* 1996). Because no benefits for infection have been identified (BALAS *et al.* 1996), how this maternally inherited symbiont is maintained in the population is unknown. Balas *et al.* proposed an 'incremental gain

hypothesis', suggesting the male-killing bacterium supplanted a non-male-killing strain already established within the wasp population (1996). Providing a benefit to the host by reducing inbreeding is a possible explanation for this hypothetical spread, however the relevance of inbreeding reduction remains to be tested in this system (BALAS *et al.* 1996; LUNA and HAWKINS 2004). *Arsenophonus nasoniae* inhibits cell division in males and prevents 80% of unfertilized eggs from developing (SKINNER 1985; BALAS *et al.* 1996; FERREE *et al.* 2008). The bacterium is found systemically in infected females, including the reproductive tissues where maternally inherited symbionts are expected to be located (HUGER *et al.* 1985; O'NEILL *et al.* 1993).

Son-killer has two unusual traits compared to other secondary symbionts. It can be easily cultured on laboratory media. This is likely directly related to its unusual mode of transmission. Instead of being transmitted in the egg, the bacterium is inoculated by an infected wasp during oviposition into fly pupae (WERREN *et al.* 1986; GHERNA *et al.* 1991). Wasp larvae acquire the bacterium per-orally from the fly's tissue and are infected by the bacterium through the midgut (HUGER *et al.* 1985). About 95% of *Nasonia* wasp larvae within an infected fly acquire the Son-Killer infection (HUGER *et al.* 1985; SKINNER 1985).

This mode of transmission via the fly also allows the symbiont to be horizontally transmitted between wasp lineages. A single fly pupa can be parasitized by multiple *Nasonia* wasps (GRILLENBERGER *et al.* 2008). When this occurs, and one of the parasitizing females is infected with *A. nasoniae*, 95% of all emerging wasps (regardless of their parentage) will be infected (SKINNER 1985). *N. longicornis*, a sympatric sister species of *N. vitripennis*, is also infected with *A. nasoniae*, suggesting that transfers may

occur between species (BALAS *et al.* 1996). Although screening surveys have demonstrated that *A. nasoniae* infects multiple wasp species in nature (Chapter 2; BALAS *et al.* 1996; DURON *et al.* 2008; NOVAKOVA *et al.* 2009), the transmission efficiency and fitness effects of this symbiont on non-*Nasonia* hosts are not known. It is crucial to determine how *A. nasoniae* affects these additional hosts to understand the symbiont's ecology. If *A. nasoniae* is indeed a generalist, horizontal transmission across the guild may significantly alter the life histories of a wide range of insects. Horizontal transmission may also account for the symbiont's maintenance within the host population.

In this study I investigate the infection success and transmission efficiency of *A. nasoniae* in three common filth fly parasitoid species, as well as fitness effects in one of these species. All wasps are sympatric and have overlapping host ranges with *Nasonia vitripennis*: *Trichomalopsis sarcophagae, Urolepis rufipes,* and *Muscidifurax raptorellus* (Hymenoptera: Pteromalidae) (FLOATE *et al.* 1999). I also tested whether the house fly, *Musca domestica* (Diptera: Muscidae), could also serve as a suitable host for *A. nasoniae*. Filth flies, including house flies, are major economic pests (MCLINTOCK and DEPNER 1954; MALIK *et al.* 2007) and parasitic wasps have been used for biological control of these flies (MORGAN 1986; CRANSHAW *et al.* 1996; KAUFMAN *et al.* 2001). Biological control releases consist of either the introduction or the augmentation of wasp species, and the horizontal transmission of *A. nasoniae* may impact current programs.

Methods

Establishment of a strain of *Arsenophonus nasoniae* from wild-caught *Nasonia vitripennis* wasps

Nasonia vitripennis wasps were collected from Lethbridge, AB, to isolate a strain of *A. nasoniae* and to establish an *A. nasoniae*-infected culture. Mountain bluebird (*Sialia currucoides*) nest material was collected from nest boxes set on posts at Hubbard Farm in West Lethbridge, Alberta, in July, 2008. The material was placed in a culture cage and incubated at 26°C (16:8 hour light: dark cycle). Emergent *N. vitripennis* (P generation) were allowed to mate en-masse for 48 hours with males from an *Arsenophonus*-free lab culture. Females were then placed individually in vials containing either 10 live or freeze-killed *M. domestica* pupae and allowed to oviposit for 72 hours before being placed in 95% EtOH. These females were washed and DNA was extracted using the STE method (see Chapter 2). The parasitized pupae were incubated at 26°C, and emergent F_1 females were examined for sex-ratio and number of wasps produced. We were not able to induce unmated wasps to oviposit; this would have been a more straightforward assay for male-killing as unmated wasps are able to produce only haploid (male) offspring.

To establish an isolate of *A. nasoniae*, 26 wasps from the P generation were killed in 95% EtOH and washed as above, then decapitated using a razor blade. DNA was extracted from the head of the wasp using the STE extraction method and the infection status of the host was assessed by diagnostic PCR. The body was ground in 50 uL of TSB media, and 30 uL of the homogenate was spread onto GC plates and incubated at 26°C for 96 hours (GHERNA *et al.* 1991). GC plates were prepared using GC Medium

Base (DIFCO) and Kellogg's supplement B (KELLOGG *et al.* 1963). The growth plates generated from *A. nasoniae* infected wasps were examined for *A. nasoniae* colonies based on morphological characteristics (GHERNA *et al.* 1991). Colonies that were mucoid, grey-white, round, and convex with entire edges were subcultured to obtain a monoculture of the bacterium. Monocultures were confirmed as *A. nasoniae* by directly sequencing the amplicon from diagnostic PCR. Two monocultures were combined and designated as TSB58.

Experimental injection of *A. nasoniae* into novel host species

To determine the effects of *A. nasoniae* on novel wasp hosts, the bacterium was injected into fly pupae that were already parasitized by wasps using a procedure similar to that of WERREN *et al.* (1986). Bacteria were also injected into unparasitized house flies, to determine whether these might serve as potential hosts. Three wasp species were examined: *U. rufipes*, *M. raptorellus*, and two strains of *T. sarcophagae* that differed in the presence of *Wolbachia*. The infected line of *T. sarcophagae* (TPOS) is fixed for a strain of *Wolbachia* that causes cytoplasmic incompatibility (Kevin Floate, personal communication) and the uninfected line of *T. sarcophagae* (TNEG) was established from the TPOS line using tetracycline. This bacteriostatic antibiotic inhibits protein synthesis in both gram-negative and gram-positive bacteria (CHOPRA *et al.* 1992). TNEG has remained *Wolbachia*-free for at least 50 generations without subsequent application of antibiotics. These wasps were maintained in Kevin Floate's lab at Agriculture and Agri-Food Canada, Lethbridge. Unfortunately, we were not able to establish infection in the native host *N. vitripennis* as the *N. vitripennis* laboratory culture died during the

experiment. This species is not presented in the statistical analyses, but did produce six females from the injection treatment.

Microinjections were conducted using a borosilicate pipette connected to a 10cc syringe with either air-filled or oil-filled (Dow Corning 710) capillary tubing. The pipette was pulled from Sutter Instruments Borosilicate filament capillary tubing (1.0 x 0.5 x 100mm) stock using a DKI 700C vertical pipette puller with a Nichrome filament and tips were sharpened by puncturing two layers of Kimwipe. The pipettes were filled with bacterial culture via aspiration, and were replaced between each treatment and wasp culture to minimize contamination. Injections contained approximately 600 cells. This quantity was estimated using a colony-forming unit assay with GC plates.

Wasps:

Injections were performed at two locations: at Agriculture and Agri-Food Canada in Lethbridge, AB, each wasp culture was injected with TSB58 in two replicates; and at the University of Victoria in Victoria, BC, *T. sarcophagae* (TNEG) was injected with TSB58 and TSB58RI (reisolated from F_1 *T. sarcophagae* (TPOS) wasps, see below) in three replicates. In Victoria the type strain of *A. nasoniae*, SKI4 (ATCC strain 49151) was also injected into *T. sarcophagae* (TNEG) in four replicates.

Tryptic soy broth (TSB) media was inoculated from a plate culture of the bacterium and incubated at 26°C for 96 hours, vortexing halfway through the incubation. The broth cultures were vortexed again after 96 hours and an aliquot was put into separate 1.5mL tubes to be used for each set of injections. 15 uL of the broth was spread on a GC plate and incubated for 96 hours to confirm a viable monoculture was used for

injection; the most common contaminants are *Salmonella* and *Proteus*, both of which exhibit a colony morphology distinct from *A. nasoniae* (GHERNA *et al.* 1991). Contaminated plates occurred infrequently, and both plates and corresponding tubes were discarded. The liquid bacterial culture was injected into house fly (*M. domestica*) puparia that had been previously parasitized by one of the wasp species under study. Each wasp species parasitized a separate set of fly pupae. These pupae were parasitized in Lethbridge by placing a mesh bag containing approximately 1000 fly pupae (7 days post-hatch) in the wasp culture cage for 24 hours and maintained at 25°C. In Victoria these pupae were parasitized by placing ~100 female wasps from the lab colony in three culture tubes each containing ~200 pupae for 24 hours and maintained at 25°C; these pupae were combined prior to injection.

The first experimental replicate conducted in Lethbridge consisted of three treatment groups for each wasp species. Sterile TSB was injected into 96 puparia as a control, and 192 puparia were injected with *A. nasoniae* culture. The viability of wasp cultures was confirmed using 192 parasitized pupae with no additional treatment. The second replicate in Lethbridge consisted of only untreated (n = 96) and *A. nasoniae* injected puparia (n = 192). Although parasitic wasps are able to develop successfully on both live and dead hosts (FLOATE 2002), it was unknown if *A. nasoniae* is transmitted when infected wasps oviposit in freeze-killed flies. Live fly pupae were thus used as wasp hosts. Fly viability was assessed by incubating 192 unparasitized pupae at 25°C (16:8 light cycle) for one week; 99% of these flies successfully emerged. *Arsenophonus nasoniae* was successfully transmitted when wasps oviposit in freeze-killed fly pupae (this study), and freeze-killed hosts were used in subsequent experiments.

In Victoria, 70 parasitized puparia were injected for each treatment per replicate. These treatments consisted of sterile TSB and either TSB58 and TSB58RI, or SKI4 *A. nasoniae* injections. The parasitized puparia were individually inoculated according to the different treatment groups under study.

In both locations, 0.75 uL of the bacterial broth culture or sterile TSB was injected into the fly puparia. The pupa was then placed on a Kimwipe to remove excess inoculum and either placed individually into the wells of a 96 well plate covered with foam and parafilm, or placed into a plastic culture tube stopped with rayon. Treated pupae were incubated at 25°C (16:8 light cycle) for 4 weeks.

Flies:

The injection of house flies was performed in Lethbridge similarly to wasps, except that they were not parasitized prior to injection. *M. domestica* were injected using the same apparatus and preparation as above; fly pupae were subjected to one of four treatments 7 days after hatching (no manipulation, mechanical penetration of the puparium without injection, injection of 0.75 uL sterile TSB, injection of 0.75 uL of TSB58) then placed on a Kimwipe to dry before being placed into 96 well plates and incubated at 25°C (16:8 light cycle). These were examined for emergence to assess fly mortality in each treatment. A portion of injected pupae were placed in open salad cups (Dixie brand) in culture cages, and similarly incubated until emergence. These plastic salad cups measured 10 cm (diameter) by 5 cm (height).

Assaying *A. nasoniae* infection in emergent flies and wasps

Wasps:

Although the wasp species used can be conclusively identified to species under a microscope, precautions were taken to prevent cross-contamination of wasp species. All wasp species were managed separately.

Emergent wasps were pooled in salad cups for 72 hours for mating; these cups contained a paper towel moistened with a sugar solution (10g sugar/100mL water) and 25 wildtype males. After this mating period, female wasps were placed individually into glass vials containing 10 thawed freeze-killed *M. domestica* pupae. After allowing the females to oviposit for 48 hours the females were placed in 95% EtOH and males were discarded. All female wasps emerging from injection treatments were set up in this manner, and the number of wasp lineages established is listed as generation P in Table 3.1. Females from the sterile TSB treatment were set up similarly in this manner as controls for sex-ratio and total offspring.

Infection status of female wasps was determined by diagnostic PCR. Wasp DNA was individually extracted using the STE DNA extraction method (Chapter 2). Each batch of extractions contained 18 females to be assessed and 2 wasps from the sterile TSB injections as controls; no cross-contamination of *A. nasoniae* from the treated samples was observed. PCR was conducted on the DNA extracts using the diagnostic primers Ars23S4F (5'-AAATCAACCGAGATTCCCCTA-3') and Ars23S516R (5'-AGTCACCCCATCCTCAAATG-3') designed to specifically amplify a 516bp region of the *A. nasoniae* 23S rDNA gene (this study). The reaction conditions for the PCR were as follows: 95°C for 5 mins, followed by 34 cycles of 30s at 94°C, 30s at 54°C, 45s at

72°C, and a final elongation for 10 min at 72°C. All reactions were run with a positive control (DNA extracted from SKI4) and negative water blank. The PCR products were subjected to electrophoresis using a 1% agarose gel, stained using an ethidium bromide solution, and imaged using an UVP Bio Doc-it Imaging System. Treated wasp lineages not infected with *A. nasoniae* were discarded. The success of DNA extractions was confirmed using primers designed to amplify 400bp of the single copy histone H3 gene (Chapter 2).

Arsenophonus nasoniae was isolated from *Wolbachia*-infected T. *sarcophagae* one generation after injection. Seven F_1 wasps from *T. sarcophagae* (TPOS) were washed and half of the wasp tissue was ground in 50 uL of TSB medium prior to DNA extraction. This entire mixture was spread plated on GC plates and incubated at 26°C for 5 days. DNA was extracted from the remaining half using STE and screened similar to the previous wasp injections. Bacterial monocultures were established from colonies matching the morphology of *A. nasoniae* and confirmed using diagnostic PCR. This isolate was designated TSB58RI.

To confirm diagnostic PCR results, 23 of the 16S rDNA amplicons were randomly selected from wasp samples, along with an additional 7 amplicons from TSB58RI colonies, and directly sequenced. Products were sequenced by Macrogen, South Korea, in both directions using the same primers as the initial amplification. Chromatogram analysis and sequence alignments were performed using Biomatters' Geneious software. All 30 samples were confirmed as *A. nasoniae*; no contamination was identified.

Flies:

Culture cages containing treated flies were supplied with 10% sugar water in a beaker with a paper towel wick. This sugar solution and wick were changed every 3 days. Four days after fly emergence a salad cup containing paper towel soaked in a milk solution (1:1 condensed milk to water) was added to the cage to help egg maturation in the flies. All female flies (n = 16) were assumed to be mated at 5 days post-emergence, and were removed to individual salad cups. These contained paper towel soaked with milk solution for oviposition. The paper towel was replaced every 24 hours and examined for eggs under a field scope at 10x magnification. Females were placed in 95% EtOH upon death, DNA extracted using a modified STE method (200 uL STE, 16 uL proteinase K) and *A. nasoniae* infection was determined by diagnostic PCR screening. Additional flies (14 males and 14 unsexed) were collected from the TSB58 culture cage and washed as above with 95% EtOH and water, and extracted using STE. Bacterial growth plates were also generated from male flies by taking a portion of tissue prior to DNA extraction, grinding it in TSB, and streaking the mixture on GC plates; these plates were incubated for one week at 25°C and examined for *A. nasoniae* colonies by morphological characteristics. DNA extractions and prospective *A. nasoniae* colonies were tested with diagnostic PCR. The sex-ratio of offspring from infected females was examined for evidence of male-killing.

Transmission efficiency of *A. nasoniae* in novel wasp and fly hosts

Wasps:

The offspring from *A. nasoniae*-infected wasps were individually mated to *A. nasoniae*-free culture males and placed in a glass vial containing 10 freeze-killed fly pupae. Infected lineages were maintained in the lab, and female offspring were screened each generation after mating and oviposition. All uninfected treatment lineages were discarded.

Flies:

Fly egg masses were collected after oviposition and washed in 5mL of distilled water to confirm the viability of the eggs (viable eggs will sink in dH2O). All egg masses contained viable eggs. The eggs were poured into a 1cm deep trench in a portion of fly rearing media (2L wheat bran, 0.5L wheat bran, 0.4L hammered alfalfa pellets, 0.6L dried distiller's grain, 2 tablespoons brewer's yeast, 2L warm tap water) in individual salad cups and incubated at 26°C (16:8 light cycle), stirred once every 24 hours. 10 larvae and 10 pupae from each lineage were selected during the rearing process and placed in 95% EtOH. All adults were also placed in 95% EtOH for future analysis.

The infection status of both female flies, and the offspring of infected females, was assessed. Infection in the offspring was examined at four different host life stages, i.e., on the surface and interior of the egg, larva, pupa, and adult. DNA from the larval and adult flies was extracted using a modified STE method (200 uL of STE, 16 uL of proteinase K). Female flies from a wildtype laboratory culture were extracted alongside the treated flies as negative controls. One egg mass from each female fly was also

selected for analysis. Eggs were collected and then placed in 500 uL of autoclaved distilled water and vortexed. DNA was extracted from 100 uL of this 'egg wash' using a modified STE extraction (400 uL of STE buffer, 40 uL of proteinase K). Surface contaminants were removed from the eggs by washing in 1mL of 10% Javex bleach and vortexing the tube every two minutes for 10 minutes, then decanting the bleach and rinsing the eggs thrice in sterile water. DNA was extracted from the eggs using the STE method. Two egg masses collected from a wildtype fly culture were similarly extracted as negative controls. All extracted DNA samples were examined by diagnostic PCR.

Effects of *A. nasoniae* on *T. sarcophagae*

Infected *T. sarcophagae* (TNEG) females were examined for male-killing induced by three isolates of *A. nasoniae;* TSB58, TSB58RI, and the type strain SKI4. Sex-ratios of these species in the wild are normally highly female-biased when wasps oviposit in isolation (HAMILTON 1967; FLOATE *et al.* 1999, SYKES *et al.* 2007). This inherently low male production makes it difficult to observe reductions caused by male death. Initially we attempted to compare the offspring produced by a female pre-mating (only males are produced) and post-mating (predominately female production). However, treated wasps often died before producing a post-mating brood or failed to be fertilized, and this approach was not further pursued. Instead, male-killing in these wasps was assayed by comparing the sex-ratio and total number of offspring between infected and control females from sterile TSB injections. A number of females did not produce any daughters and are therefore assumed to be virgins. Although these females were not included in

this analysis, they are very informative with respect to male-killing. Table 3 shows the number of males produced by virgin infected females.

Preliminary studies suggested *A. nasoniae* may cause premature death of wasp hosts (G. Taylor, unpublished). The day that each wasp died was therefore recorded and lifespan compared between infected and control wasps.

Statistical analysis

All statistical analysis was performed in the R software package (R DEVELOPMENT CORE TEAM 2009) using the 'stats' (R DEVELOPMENT CORE TEAM 2009) and 'survival' (THERNEAU AND LUMLEY 2009) libraries. Statistical significance was determined using $\alpha = 0.05$.

Differences in infection frequency and transmission efficiency between the four wasp cultures (*M. raptorellus, U. rufipes,* and two strains of *T. sarcophagae*) were analyzed using Pearson's χ^2 test or Fisher's Exact Test. Replicates and generations were analyzed independently.

Distortions in both the sex-ratio and total number of offspring produced by female wasps were analyzed using a generalized linear model with a binomial (sex-ratio) or poisson (total offspring) distribution. The distribution was modified (quasibinomial or quasipoisson) if the data was overdispersed. The dispersion parameter is reported for these and an F test was used instead of χ^2. Model simplification was performed in a stepwise fashion from the saturated model. Because quasibinomial and quasipoisson distributions cannot be compared using AIC, models were compared by testing for deviance with a χ^2. Sex-ratio and total offspring produced were used as main effects, and

treatment, generation, and replicate were analyzed as fixed effects. P-values of the final models were calculated with deviance using a χ^2 test.

Longevity results were analyzed using a Cox-proportional hazards model without censoring. Model simplification was similarly performed in a stepwise fashion from the saturated model with longevity as the main effect, and treatment, generation, and replicate as fixed effects. P-values of the final models were calculated using Likelihood ratio tests.

The emergence success of flies from the four treatments was compared using Pearson's χ^2 tests. Contrasts were made between *A. nasoniae* and sterile TSB treatments, as well as between all injection treatments and no injection.

Results

Isolation of a strain of *Arsenophonus nasoniae* from field-caught *Nasonia vitripennis*

Approximately 300 *N. vitripennis* adult females emerged from the mountain bluebird nest material. A culture of *A. nasoniae*, designated TSB58, was established from extracts from 26 females. Iso-female wasp lines were established using 11 females; four of these 11 were infected with *A. nasoniae.* The sex-ratio of *N. vitripennis* clutches was highly female-biased as expected (WERREN 1980), and there was no difference in the proportion of daughters between infected and uninfected females (infected females: 0.865, uninfected females: 0.874; analysis of deviance with χ^2, $p = 0.985$). Nor was there any difference in offspring number between infected and uninfected females (mean-offspring, infected: 5.5 ± 1.9, uninfected: 12.4 ± 2.8; F, dispersion = 3.767, $p = 0.091$).

Three of the four infected females each produced one male offspring; the 4th produced only two daughters. Unfortunately, *A. nasoniae* infected lines of *N. vitripennis* collapsed after one generation, so whether this strain kills male *Nasonia* is unknown. This collapse was unrelated to the present experiment, and may have occurred through contamination with a pathogen.

Novel wasp species experimentally acquire *A. nasoniae* infection

Arsenophonus nasoniae successfully colonized all three novel wasp species. All four of the wasp cultures investigated (*M. raptorellus*, *U. rufipes*, and both strains of *T. sarcophagae*) acquired infection from the injected fly (Table 3.1). Two of these species, *U. rufipes* and *T. sarcophagae*, successfully transmitted the bacterium to their offspring. Both the *Wolbachia*-infected (TPOS) and *Wolbachia*-free (TNEG) cultures of *T. sarcophagae* acquired and transmitted *A. nasoniae*.

The prevalence of infection of *A. nasoniae* varied between novel wasp species (Table 3.1). Infection in the parental generation significantly differed between wasps in both replicates (replicate 1: $\chi^2 = 16.4995$, $P < 0.001$; replicate 2: $\chi^2 = 53.4377$, $P < 0.001$). *Urolepis rufipes* consistently had high infection levels, and in the first replicate both strains of *T. sarcophagae* show a lower level of acquisition than *M. raptorellus*, while in the second replicate they both show much higher levels than *M. raptorellus*. However, the statistical significance of these results is not explicitly tested.

Infected females of both *U. rufipes* and *T. sarcophagae* successfully transmitted the bacterium to their offspring. While infection in *M. raptorellus* was lost in the F_1 generation, only a single individual was tested. The F_1 offspring from *M. raptorellus* is

not included in further statistical tests. The prevalence of infection of the F_1 generation did not significantly differ between the two wasp species that successfully transmitted the infection (replicate 1: Fisher's, P = 0.072; replicate 2: Fisher's, P = 1.000).

When examining the different *A. nasoniae* isolates (TSB58, TSB58RI, SKI4) in the common host background of *T. sarcophagae* (TNEG), all three demonstrated vertical transmission (Table 3.2). However, injections with SKI4 were performed on a separate occasion than TSB58 and TSB58RI and results could not be directly compared between all three isolates.

Because the laboratory culture of *N. vitripennis* collapsed during the experiment, we were not able to evaluate the effects of infection in the native host. However, six female *N. vitripennis* did emerge from injection treatments with TSB58; four were positively infected with *A. nasoniae.* Only one of these females produced offspring; this infected female produced 11 males and zero females. This suggests that while the *A. nasoniae* isolate is able to infect hosts, it may not be virulent and express male-killing. However, this is a single sample and conclusions cannot be made.

Effects of *A. nasoniae* on fitness of the novel host *Trichomalopsis sarcophagae*: reduced adult female survival but no male-killing

Infected *Trichomalopsis sarcophagae* were examined for distortion in the sex-ratio and number of offspring produced (Table 3.2), as well as effects on adult longevity (Fig. 3.1 and Fig. 3.2). Results are detailed below. There was no evidence that *A. nasoniae* kills males in this wasp host; infected wasps did not produce significantly distorted sex-ratios, did not produce fewer total wasps, and several infected virgins did

produce males. The TSB58RI isolate significantly reduced the longevity of *T. sarcophagae* wasps. Lifespan was consistently lower in wasps infected with the SKI4 type strain than controls. However, this difference was not statistically significant.

A. nasoniae isolates from Lethbridge, AB:

The model for the sex-ratio of wasps infected by TSB58 or TSB58RI was not affected by either generation ($F_{62,61}$ = 1.009, P = 0.463) or replicate ($F_{59,61}$ = 0.969, P = 0.415) and these factors were removed during model simplification. The resulting model contained only the main effect and treatment as a fixed effect. The sex-ratios of infected wasps did not significantly differ from the controls ($F_{1,2}$ = 0.992, dispersion = 10.432, P = 0.798) (Table 3.2).

The model for total offspring produced by TSB58 and TSB58RI infected wasps was affected by generation ($F_{62,61}$= 1.087, P = 0.013) but not by replicate ($F_{61,59}$ = 1.031, P = 0.356), and the latter factor was removed. The final model included both treatment and generation as fixed effects. Treatment did not significantly affect the total number of offspring ($F_{1,2}$= 0.920, dispersion = 4.725, P = 0.106), but wasps in the parental generation did produce significantly fewer offspring than the F_1 generation ($F_{1,2}$ = 0.920, dispersion = 4.725, P = 0.016) (Table 3.2). Because host pupae are damaged during the injection procedure this reduction is expected.

The longevity of *T. sarcophagae* was significantly reduced by infection with Lethbridge isolates of *A. nasoniae* (Fig. 3.1). Survival did not vary across replicates for TSB58 and TSB58RI (χ^2 = 3.467, df = 2, P = 0.415) while generation did have a significant effect on the model (χ^2 = 11.924, df = 2, P < 0.001). The final model used

included treatment and generation as fixed effects, and showed *A. nasoniae* infection significantly reduced wasp survival ($\chi^2 = 11.8$, df = 2, P = 0.009). Longevity was consistently lower in wasps infected with either bacterial isolate than control wasps in all generations.

A. nasoniae type strain:

The SKI4 isolate did not cause a significant bias in sex-ratio compared to controls in *T. sarcophagae*. The model was not significantly affected by replicate ($F_{17,18}$= 0.889, P = 0.232) and the factor was removed; the simplified model was used treatment as a fixed effect. Infected wasps did not produce significantly different sex-ratios from controls (analysis of deviance with χ^2, P = 0.073). Unfortunately, I was not able to determine the effect of SKI4 on the native host *Nasonia vitripennis*.

Replicate also did not affect the model for total offspring ($F_{17,18}$= 0.889, P = 0.162), and the simplified model with only treatment as a fixed effect was used. SKI4-infected and control treatments of *T. sarcophagae* (TNEG) produced similar numbers of offspring ($F_{18.19}$= 0.976, dispersion = 4.7258, P = 0.517).

Wasps infected with SKI4 did not die significantly sooner than controls (Fig. 3.2; $\chi^2 = 5.700$, df = 3, P = 0.126). The longevity of wasps injected with SKI4 varied across replicates ($\chi^2 = 152.530$, df = 5, P < 0.001) but not across generations ($\chi^2 = 6.1816$, df = 4, P = 0.186), and blocking by replicate is included in the final model, with treatment and replicate as fixed effects. When the generations are analyzed individually, infected wasps in the parental generation of the second replicate died significantly sooner than controls

($\chi^2 = 8.210$, df = 1, P = 0.003). None of the other generations showed significant differences.

Although low sample numbers reduced the power to identify male-killing, a total of 20 *A. nasoniae*-infected females from both TPOS and TNEG lines of *T. sarcophagae* produced only male offspring (Table 3.3). At least one virgin female infected with each bacterial isolate produced males. This male production corroborates the sex-ratio data, suggesting the bacterium does not kill males.

A. nasoniae can infect _M. domestica_ flies: increased pupal mortality but no evidence of vertical transmission or male-killing

After injection with *A. nasoniae*, emergent flies were collected from the culture cage to assess the bacterium's persistence in the host. Of these flies, 10 of 14 unsexed, 6 of 14 males, and 1 of 16 females were infected with the bacterium. However, while the infection was retained post-emergence, it was not vertically transmitted in the single female tested. This female's offspring were not infected with *A. nasoniae* at any tested life stage (i.e., on the egg, in the egg, larva, pupa, or adult). *A. nasoniae* infection also did not distort the sex-ratio produced by the infected female; she produced 25 males and 23 females. House flies normally produce equal numbers of males and females.

Injection of flies with *A. nasoniae* significantly increased pupal mortality (Table 3.4) compared to both sterile TSB injected pupae ($\chi^2 = 4.3858$, P = 0.036), and all injection treatments (i.e., sterile TSB, bacterial cultures, mechanical penetration) significantly decreased fly emergence ($\chi^2 = 62.7461$, P < 0.001).

Discussion

Multiple wasp species can be experimentally infected by *Arsenophonus nasoniae*

Field surveys have shown that *Arsenophonus nasoniae* infects three parasitic wasps in addition to *Nasonia* (Chapter 2). These *A. nasoniae* strains are highly similar based on three genetic loci (Chapter 2). It is thus very likely that horizontal transmission of the bacterium has occurred between these wasp species (Chapter 2). However, it is unclear how common these transmission events are and whether infections are subsequently vertically transmitted by the host. Here we show that three additional pteromalid wasps can acquire novel *A. nasoniae* infections. At least two of these species also transmit the infection vertically to the next generation. How these infections are acquired and transmitted by these novel hosts is unknown.

The three wasp species (*M. raptorellus, U. rufipes,* and *T. sarcophagae*) all acquired novel infections from injected fly hosts. The injection protocol used here mimics how the bacterium is ecologically acquired by the native host, and suggests that these wasps will become infected if they parasitize *A. nasoniae*-infected flies. Given the overlapping geographic and host ranges of these wasps with known hosts of *A. nasoniae,* it is feasible the bacterium may infect these species in nature.

How frequently these hosts are infected may vary between species. Studies of a male-killing *Spiroplasma* in coccinellid beetles suggest susceptibility to infection is related to the phylogenetic relatedness between the naive and native hosts (TINSLEY and MAJERUS 2007). Here, *A. nasoniae* infects hosts with high variability. *Urolepis rufipes* was consistently highly susceptible and *T. sarcophagae* was moderately so. The limited number of *M. raptorellus* made it difficult to assess infection and transmission success.

When compared to a wasp phylogeny constructed with the gene *pten*, a gene that encodes a lipid phosphatase (BAUDRY *et al.* 2006), these infection frequencies loosely correlate with phylogenetic distance between the naive wasp host and *N. vitripennis.* In this study, *T. sarcophagae* is the most similar to *N. vitripennis* when examining substitutions at the *pten* locus, followed closely by *U. rufipes*, while *M. raptorellus* represents a more distant relative (BAUDRY *et al.* 2006).

This notion of susceptibility being affected by host genetics is also reflected in the frequency of vertical transmission of *A. nasoniae.* Both *U. rufipes* and *T. sarcophagae* successfully transmitted the bacterium to their offspring. *Muscidifurax raptorellus* did not transmit infection, although only a single female was examined and conclusions cannot be drawn. Transmission efficiency in the novel hosts was lower than the 95% observed in the original host (SKINNER 1985). This is consistent with previous research on symbiont transfer into novel hosts (CHANG and WADE 1994; HEATH *et al.* 1999; VAN MEER and STOUTHAMER 1999; HUIGENS *et al.* 2004; TINSLEY and MAJERUS 2007). The F_1 generation in several of these wasps exhibited a lower infection prevalence than the initial generation. This is anticipated for two reasons. First, the injection protocol used may introduce a larger inoculum of bacteria than an infected wasp deposits. Hence, the transmission to the F_1 generation may be a more accurate indication of transmission efficiency. Second, transmission success may be a function of bacterial density. If so, transmission efficiency would be reduced in successive generations if the symbiont is maladapted to the host (HEATH *et al.* 1999). The growth of the symbiont may improve by passage through successive generations of the novel host (MCMENIMAN *et al.* 2008).

However, because bacterial infection experimentally reduces the longevity of the host, *A. nasoniae* may be lost before sufficient adaptation can occur.

Arsenophonus nasoniae isolate does not kill males in a novel wasp species

While screening surveys can identify hosts, they do not provide information about the effects of infection on the host; phenotypic expression can vary dramatically between host species. For example, a *Wolbachia* strain induces cytoplasmic incompatibility in one moth species, but kills males when transferred into another moth species (SASAKI *et al.* 2005). The infection phenotype may also be affected by minute changes between highly similar strains of a symbiont (e.g., OLIVER *et al.* 2005). Here, *A. nasoniae* obtained from Lethbridge, AB, is identical to the Son-killer type strain at three loci (Chapter 2) but does not appear to kill males in either *N. vitripennis* or *T. sarcophagae*. The SKI4 type strain of *A. nasoniae* also does not kill males in experimentally infected *T. sarcophagae.* However, this lack of male-killing may be an issue with the bacterial strain used, rather than the different genetic backgrounds of the hosts. The effects of SKI4 in *N. vitripennis* need to be confirmed for comparisons to be made.

A. nasoniae is thought to kill males through a general mechanism that affects all parasitic wasps. Wasp death is caused by the inhibition of the maternal centrosome by the bacterium (FERREE *et al.* 2008). As males exclusively use the maternal centrosome for cell division they are not able to develop. In contrast, females preferentially use a paternally derived centrosome and are therefore unaffected by *A. nasoniae* (CALLAINI *et al.* 1999). There are two potential explanations for why the Lethbridge isolate of *A. nasoniae* may not kill males in both *N. vitripennis* and *T. sarcophagae*. 1) The

bacterium may not grow sufficiently in the wasp. Phenotype penetrance is thought to be a function of bacterial density (ANBUTSU and FUKATSU 2003), and this isolate may not reach the minimum bacterial density in the host required for male-killing. 2) The effector molecules cannot inhibit centrosome formation. This may be from a failure to reach or affect the target, or the effector may not be expressed. Quantitative approaches using real-time PCR (qPCR) can address if bacterial density differs between isolates to determine its significance. Additionally, once the effector for male-killing by *A. nasoniae* is identified, the lack of male-killing in the host can be directly assessed.

Potential ecological implications of *A. nasoniae* infecting the filth fly *M. domestica*

Although flies typically shed their bacterial flora upon emergence (ROCHON *et al.* 2005 and references therein), injected flies acquired *A. nasoniae* and retained the infection into adulthood. This retention suggests the bacterium colonizes host tissues and, as the injection used here is analogous to how *A. nasoniae* is naturally deposited, flies may be infected under natural conditions. The bacterium can also be reisolated from the adult fly suggesting it is able to evade the host's immune system and remain viable.

Unfortunately, only a single infected female produced offspring, preventing conclusions to be drawn on both bacterial transmission and effect on sex-ratio. This female did not transmit the bacterium to her offspring. *A. nasoniae* also did not kill males in this house fly. Male-killing would not be expected to occur, because the sex-determination systems in Diptera and Hymenoptera differ. Male wasps exclusively use a maternally-derived centrosome, whereas sex-determination in flies is much more diverse

(CALLAINI *et al.* 1999). Conceivably both male and female flies could be affected by inhibition of the centrosome resulting in general death of offspring.

Infection by *A. nasoniae* significantly increased pupal mortality. This death may be caused directly by the bacterium utilizing host resources, or potentially by the bacterium expressing toxins within the fly. The former possibility is intriguing, as the genome of *A. nasoniae* contains an unexpected number of chitinases (DARBY *et al.* 2010). Darby *et al.* postulate that *A. nasoniae* may be utilizing host resources and be metabolically active in the fly (DARBY *et al.* 2010). This potential growth of *A. nasoniae* while in the fly may play an important role in successful bacterial acquisition by wasps, and may be addressed using quantitative approaches in the future.

Despite flies being susceptible to infection, *A. nasoniae* is likely ecologically irrelevant to filth flies in the field. To become infected the fly needs to survive: a) envenomation by the wasp, b) acute effects of *A. nasoniae* infection (e.g., avoiding mortality), and c) parasitism by the developing wasp larvae (RIVERS and DENLINGER 1994; RIVERS *et al.* 2002). Host defences may allow this survival (VINSON 1990), and a distinct strain of *Arsenophonus* was identified in the blowfly *Protocalliphora sialia* by a recent screen (DURON *et al.* 2008). However, if the bacterium cannot be vertically transmitted it will be quickly lost from the host population.

Importance of *A. nasoniae* in biological control programs

Arsenophonus nasoniae is a generalist that infects multiple insects in the field (Chapter 2) and in the lab (this chapter), potentially allowing infection to spread across naive populations in the field. *Nasonia vitripennis* collected from Lethbridge, AB, were

infected with *A. nasoniae* at a higher prevalence than previous studies (Chapter 2) and may not kill males. The extensive host range and horizontal transmission of *A. nasoniae* suggest mechanisms other than male-killing may account for its maintenance. Establishing bacterial reservoirs in wasp species in the environment (e.g., sympatric wasp species) could play a substantial role in the maintenance of the symbiont. Alternatively, to account for its maintenance *A. nasoniae* may provide an unidentified benefit to its host.

Currently, as *A. nasoniae* does not significantly affect the reproductive output of wasp hosts, it is unlikely to be useful in biological control of filth flies. However, if a cryptic benefit is conferred the bacterium may increase wasp yields during mass-rearing. Additionally, the potential horizontal transmission and wide host range of the bacterium may be exploited in the future. These traits may allow desirable genes to be vectored throughout an ecological guild. *A. nasoniae* also causes mortality in fly pupae and could potentially be applied directly to pest populations, similarly to *Bacillus thuringiensis* (LACEY *et al.* 2001).

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Table 3.1: Infection prevalence and transmission efficiency of the TSB58 *A. nasoniae* isolate in four different wasp hosts. Infection of wasps was examined in both the parent (P) and offspring (F_1) generations (Gen). Offspring from virgin females is not included in the analysis. Prevalence of infection across all individuals screened is in brackets. Comparisons are made between wasps within each generation, and replicates were analyzed independently. DNA quality of all negative samples was checked by amplifying insect DNA. Infection of wasp species significantly differ in the P generation in both replicates (1: $\chi^2 = 16.4995$, $P < 0.001$; 2: $\chi^2 = 53.4377$, $P < 0.001$). Infection status of wasps from the F_1 generation did not significantly differ in either replicate (1: Fisher's, $P = 0.072$; 2: Fisher's, $P = 1.000$).

		M. raptorellus		*U. rufipes*		*T. sarcophagae* (TPOS)		*T. sarcophagae* (TNEG)	
Gen	Replicate	**1**	**2**	**1**	**2**	**1**	**2**	**1**	**2**
P	# infected ♀	8/14 (0.571)	1/48 (0.021)	11/12 (0.917)	8/11 (0.727)	5/17 (0.294)	42/86 (0.488)	7/26 (0.269)	29/39 (0.744)
F_1	# of P ♀ that produced F_1 daughters	2/8	0/1	11/11	8/8	1/5	1/42	5/7	7/29
	# of P ♀ that produced infected F_1 daughters	0/1	-	3/8	1/4	1/1	1/1	4/4	3/6
	Total # F_1 daughters screened (proportion infected)	1	-	16 (0.312)	6 (0.167)	9 (0.778)	15 (0.133)	23 (0.565)	28 (0.143)

Table 3.2: Offspring sex-ratio and total number of offspring produced by female *T. sarcophagae* (TNEG) infected with one of three isolates of *A. nasoniae* (i.e., TSB58, TSB58RI, SKI4). Offspring from virgin females was not included in the analysis. Control wasps are from lineages initially injected with sterile TSB. Comparisons are only made between treatments from the same Block (Block 1: *T. sarcophagae* (TNEG) TSB58 / TSB58RI / control, Block 2: *T. sarcophagae* (TNEG) SKI4 / control). Bacterial infection did not significantly affect mean sex-ratio (Block 1: $F_{1,2} = 0.992$, dispersion = 10.432, P = 0.798, Block 2: analysis of deviance with χ^2, P = 0.073) or total offspring produced (Block 1: $F_{1,2} = 0.920$, dispersion = 4.725, P = 0.106, Block 2: $F_{18.19} = 0.976$, dispersion = 4.7258, P = 0.517).

Gen		Block 1			Block 2	
		TSB58	**TSB58RI**	**TSB Control**	**SKI4**	**TSB Control**
P	Mean sex-Ratio	0.895	0.833	0.770	-	-
	Mean total offspring/♀ (with SEM)	13.5 ±5.5	31.75±4.1	30.95±2.5	-	-
	# broods	2	4	22		
F_1	Mean sex-ratio	0.655	0.782	0.751	0.871	0.812
	Mean total offspring/♀ (with SEM)	30.5±21.5	37.67±5.2	38.44±2.3	27.3±5.8	23.2 ±2.6
	# broods	2	2	34	4	16

Table 3.3: Number of virgin females experimentally infected with *Arsenophonus nasoniae* that produced offspring (i.e., male offspring because wasps are haplodiploid). Males would not be expected if male-killing occurred.

		TSB58		**TSB58RI**		**SKI4**	
Wasp strain	Gen	Total females	Mean # of ♂ produced	Total females	Mean # of ♂ produced	Total females	Mean # of ♂ produced
T.sarcophagae (TNEG) (from injections performed in Victoria, BC)	P	1	4	0	0	0	0
	F_1	1	36	1	34	2	21±6
T.sarcophagae (TPOS) (from injections performed in Lethbridge, AB)	P	14	7.71±1.6	-	-	-	-
	F_1	1	11	-	-	-	-

Table 3.4: Successful emergence of *Musca domestica* after injection of either *A. nasoniae* or sterile TSB media into the pupal fly. Significance categories are based $\alpha < 0.05$ using χ^2 test of independence (no treatment vs injections: $\chi^2 = 62.7461$, $P < 0.001$, TSB58 vs sterile TSB: $\chi^2 = 4.3858$, $P = 0.036$, sterile TSB vs. mechanical: $\chi^2 = 0.9553$, $P = 0.328$).

	No treatment	Mechanical penetration	Sterile TSB injection	TSB58 injection
% emergence (total # of pupae)	0.97 (96)	0.43 (96)	0.31 (96)	0.17 (192)
Significance category	a	b	B	c

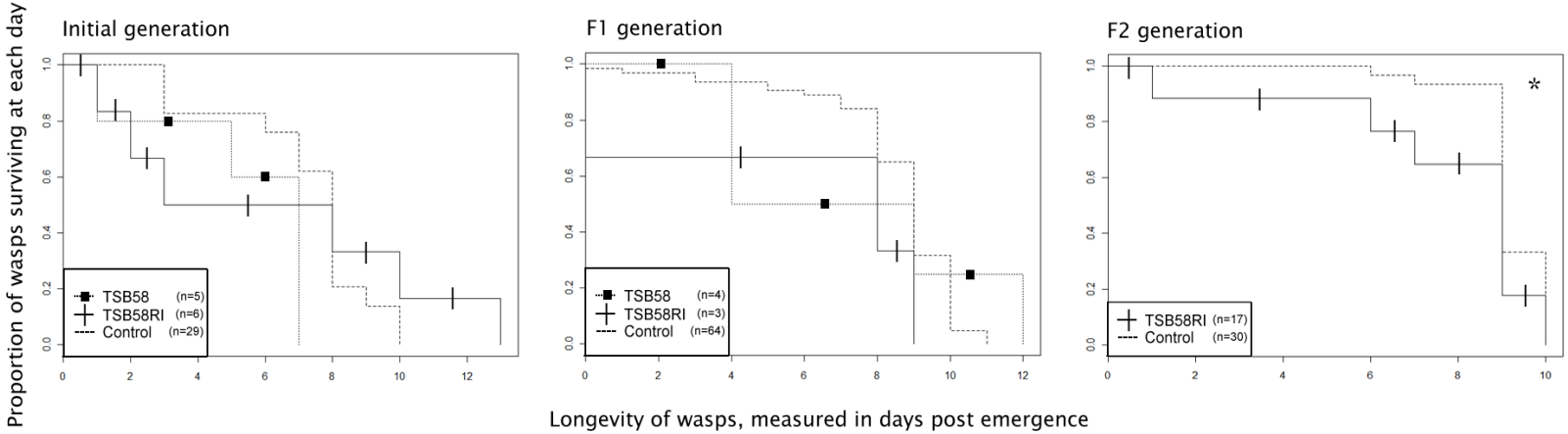

Figure 3.1: Longevity of female wasps (in days) from three generations of *T. sarcophagae* (TNEG) wasps infected with one of two *A. nasoniae* isolates (TSB58 and TSB58RI). Treated wasps are compared to controls established by injection with sterile TSB. Wasp longevity was significantly reduced by *A. nasoniae* ($\chi^2 = 11.8$, df = 2, P = 0.009).

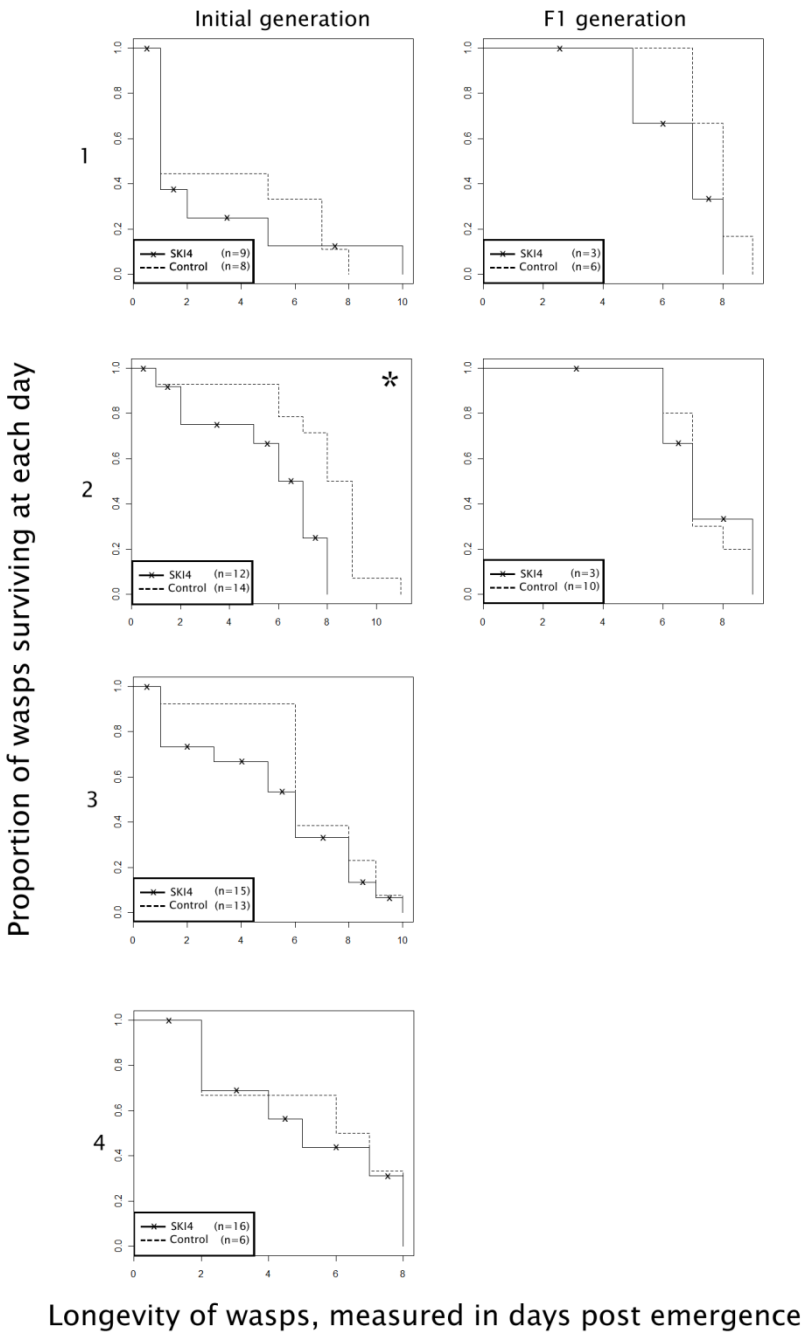

Figure 3.2: Longevity of female wasps (in days) from two generations of *T. sarcophagae* (TNEG) wasps infected with the *A. nasoniae* type strain SKI4. Treated wasps are compared to controls initially exposed to sterile TSB. Longevity differed between the 4 replicates performed, but did not differ across generations. Longevity was significantly reduced by *A. nasoniae* infection in the P generation of replicate 2 ($\chi^2 = 8.210$, df = 1, P = 0.003).