Studies of the Spread and Diversity of the Insect Symbiont *Arsenophonus nasoniae*

Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of Doctor of Philosophy

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Abstract:

Heritable bacterial endosymbionts are a diverse group of microbes, widespread across insect taxa. They have evolved numerous phenotypes that promote their own persistence through host generations, ranging from beneficial mutualisms to manipulations of their host's reproduction. These phenotypes are often highly diverse within closely related groups of symbionts and can have profound effects upon their host's biology.

However, the impact of their phenotype on host populations is dependent upon their prevalence, a trait that is highly variable between symbiont strains and the causative factors of which remain enigmatic. In this thesis I address the factors affecting spread and persistence of the male-killing endosymbiont *Arsenophonus nasoniae* in populations of its host *Nasonia vitripennis*. I present a model of *A. nasoniae* dynamics in which I incorporate the capacity to infectiously transmit as well as direct costs of infection – factors often ignored in treaties on symbiont dynamics. I show that infectious transmission may play a vital role in the epidemiology of otherwise heritable microbes and allows costly symbionts to invade host populations. I then support these conclusions empirically by showing that: a) *A. nasoniae* exerts a tangible cost to female *N. vitripennis* it infects, b) it only invades, spreads and persists in populations that allow for both infectious and heritable transmission. I also show that, when allowed to reach high prevalence, male-killers can have terminal effects upon their host population. Secondly, I examine the phenotypic and genetic differences of a novel strain of *Arsenophonus* that has recently diverged from the male-killer following a host-shift event. I show that interspecific transmission can lead to rapid changes in symbiont biology, shifting away from reproductive parasitism and reliance upon mixed transmission towards mutualism, pure heritability and host-specialisation. I also show that these transitions are underpinned by specific genomic diversification. These findings have important implications for the way in which we view symbiont dynamics in nature and predict their outcome in terms of virulence and phenotype evolution. Further, it highlights the rapid directed selection pressures that symbionts are regularly exposed to following a host-shift and how this may be responsible for the diversity seen in nature.

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Abbreviations:

P-endosymbiont: A primary symbiont, essential for host survival.

S-endosymbiont: A heritable organism that is not-essential for host survival

RP - Reproductive parasitism.

SRD – Sex ratio distorting.

MK – Male-killing.

PI – Parthenogenesis Inducing.

CI – Cytoplasmic incompatibility.

ORF – Open Reading Frame (Genome sequence).

T3SS - Type 3 secretion system

T6SS – Type 6 secretion system

A+ - Individual or treatment infected with *Arsenophonus nasoniae.*

A- Individual or treatment uninfected with *Arsenophonus nasoniae.*

Glossary of Terms:

Co-parasitism: Two or more female parasitoid wasps sharing the same host fly individual.

Horizontal transmission/infectious transmission: Transmission from an infected individual to an uninfected individual through a route other than direct descent.

Vertical transmission/heritable transmission: Symbiont transmission from parent to offspring.

Symbiotic phenotype: The phenotypic consequence of symbiont infection upon host biology.

Drive: The force with which a symbiont spreads through a population of hosts. An emergent property of the symbiotic phenotype and transmission efficiency in balance with segregational loss.

Titre: The density of bacterial cells in a host's body or tissues.

Chapter 1

General Introduction

1.1 Symbiosis

The term 'symbiosis' was coined by Heinrich Anton de Bary in 1879 to describe "The living together of unlike organisms". Our modern definition of the term encompasses the whole continuum of intimate, long-term interactions between individuals belonging to disparate species – typically exhibiting close physical contact. The phenotypic manifestation of these interactions ranges from outright parasitism, through ecologically contingent mutualism to obligate co-dependence (Ewald, 1987). By encompassing such a broad array of life styles, the majority of organisms on earth enter into some form of 'symbiosis', as either host or symbiont.

One notable feature of symbiotic interactions is that they involve very disparate parties, commonly from members of different biological kingdoms. These associations occur at all levels of biological complexity, from those widely considered part of the genome (e.g. symbiotically derived organelles such as plastids and mitochondria), parasitic or mutualistic microbes, mycorrhizal root fungi and social parasites. Symbiotic interactions range from transient, present in only part of a host generation, to, in the case of heritable symbioses, persistent between them. Transient interactions arise through density or frequency dependant contact between host individuals. Chronic 'infections' are passaged through multiple generations of their host, effectively becoming a heritable component of the hologenome (Rosenberg and Zilber-Rosenberg, 2011).

Given the ubiquity of symbiosis in nature, the diversity of organisms that engage in symbiotic relationships and the plethora of ecological effects that they produce, it is reasonable to argue that the phenomena is one of the major evolutionary driving forces on earth. Perhaps this is most eloquently put by Angela E. Douglas in her book *The Symbiotic Habit*: "*Symbiosis is… a first-order process in the evolutionary diversification of living organisms….and a major determinant of the structure of ecological communities*."

1.2 Heritable Symbiosis in insects

1.2.1 Discovery and Background

Many of the recent advances in symbiosis research have been driven by the discovery of numerous, heritable microbial endosymbionts of insects. Insect-bacteria associations are represented at all points on the 'mutualism-parasitism' and 'heritable-acquired' continua. They involve interactions between the most numerous life forms on earth (bacteria) as well as the most diverse eukaryote taxa (insects).

The first discovery of a heritable insect symbiont was a vertically transmitted nutritional mutualist infecting olive flies (Petri, 1909), later named *candidatus Erwinia dacicola.* However, much of our modern understanding is founded on the work of Paul Buchner (Buchner, 1965). Buchner painstakingly documented the obligate, nutritional association between bacteria and sap-feeding insects such as aphids, cicadas, scale insects and, later, blood feeders such as bed bugs, mosquitos, lice, ticks and fleas (Sapp, 2002 and references therein). He found that the microbes were harboured in specific host cells and tissues – mycetocytes and mycetosomes, and that the bacteria were transmitted from parent to offspring, commonly through the cytoplasm of the ova. He also noted that closely related species tended to be infected with bacteria sharing a specific morphology, evidence that the infections were derived from an ancient common ancestor. He postulated that these microbe-insect associations were rooted deep in evolutionary time and had been vertically transmitted from parent to offspring, resulting in stable, persistent, obligate infections.

The second major step in our understanding of heritable insect symbionts came with the discovery of microbes that manipulated host reproduction. In the first half of the 20th century several researchers published on the phenomena of single-sex broods in arthropods and unexplained mating incompatibilities between populations of insects. A maternally heritable trait for female-only broods was reported in natural populations of butterflies (Simmonds, 1926), woodlice (Vandel, 1941) and ladybirds (Lus, 1947). Later, symbiont-induced mating incompatibilities were identified between populations of the mosquito *Culex pipiens* (Laven, 1951). These factors were shown to be inherited through the female line of their host. Later, a diverse set of bacteria were found to be the causative agents of the manipulation observed. Reproductive manipulation is now known to be caused by several major classes of insect endosymbionts, including:

Arsenophonus, Cardinium, Rickettsia, Spiroplasma and *Wolbachia* (Duron *et al.*, 2008) and is manifested as several discreet phenotypes which will be reviewed in detail in section 1.2.4.

Finally, at the turn of the millennium, a third class of insect symbiosis termed 'ecologically contingent mutualists' was described. These microbes convey a benefit to their host under specific ecological conditions, most commonly by providing resistance to natural enemies (Reviewed in Haine., 2008 & Brownlie and Johnson., 2009) but also fulfilling non-essential nutritional roles (Brownlie *et al.*, 2009), or boosting host fitness through as-yet undefined mechanisms (Himler *et al.*, 2011). The ubiquity of ecologically contingent mutualists is still being explored and is now believed to be far more commonplace than once thought (Duron and Hurst, 2013).

The work of Buchner had taken the first steps towards understanding the nature of primary - symbiosis, characterised by mutually obligate associations. Together, reproductive manipulators and ecologically contingent mutualists form the secondary-symbionts: non-obligate, yet vertically transmitted, partners. Both mutualistic and parasitic symbionts form a heritable component of their host's biology and thus represent part of the genetic variation within the host that is subject to natural selection. Research has also focused on the impacts of a symbiotic lifestyle upon the microbes themselves; how they invade hosts, produce their diverse phenotypes and the consequences of prolonged co-evolution.

1.2.2 Primary Endosymbionts

The obligate, vertically transmitted symbioses first discovered by Buchner are now referred to as primary endosymbionts, or P-endosymbionts. Douglas (1998) identified three defining characteristics of P-symbioses (Douglas, 1998):

a. The infection is confined to specialised insect cells (mycetocytes).
b. The infection is heritable and exhibits perfect vertical transmission.
c. The association is obligate for the survival of both parties.

Molecular phylogenetic analyses have demonstrated that the association between these microbes and their hosts are ancient. The high degree of co-cladogenesis between symbiont lineages and their hosts indicates that when host lineages become isolated and diversify, their resident symbiont communities follow suit. This is expected only

when horizontal transmission of the symbiont, which produces host switches, is absent (Baumann, 2005) (See table 1.1 for examples).

Prolonged co-dependency leads to fundamental evolutionary change in the bacteria from its free-living ancestor. The genomes of P-symbionts are typically reduced in size, gene dense, AT rich and relatively stable (Hosokawa *et al.*, 2006; Moran *et al.*, 2008). Indeed the smallest genome of any cellular organism recorded to date is that of a P-endosymbiont of Psyllids; *Carsonella ruddii,* which has been estimated at under 160 kbp (Nakabachi *et al.*, 2006). Genomic decay of P-endosymbionts may come about through two, non-mutually exclusive processes. First, the microbes' effective population size is limited to the number of cells that will fit into an ovum during vertical transmission. This results in repeated population bottlenecks, and deleterious mutations are accumulated and fixed via the phenomena of Muller's ratchet (Rispe and Moran, 2000; Moran, 2007). Secondly, the resource rich environment of a host cell negates the need for many of the factors required for free-living. Thus, a process of adaptive gene-loss occurs in order to shed the costly genetic equipment rendered redundant by host-dependency. This theory of reduced genome evolution through co-dependency, is termed the 'Black Queen Hypothesis' (Morris *et al.*, 2012). The widely accepted endosymbiotic theory of organelle evolution is often cited as the extreme outcome of P-endosymbiosis (Sagan, 1967).

Table 1.1: Known examples of P-endosymbionts that exhibit co-cladogenesis with their host lineages (Adapted from Moran *et al.*, 2008).

Symbiont	Host	Age of association	Ref
Buchnera	*Aphidoidea*	180Mya	(Montllor *et al.*, 2002)
Portiera	*Aleyrodidae*	180 Mya	(Baumann, 2005)
Carsonella	*Psyllidae*	120 Mya	(Baumann, 2005)
Wigglesworthia	*Glossina*	>40 Mya	(Chen *et al.*, 1999)
Blochmannia	*Camponotus*	50 Mya	(Sauer *et al.*, 2000)
Baumannia	*Proconiini*	100 Mya	(Takiya *et al.*, 2006)
Ishikawaella	*Pentatomidae*	Unknown	(Hosokawa *et al.*, 2006)
Nardonella	*Curculionoidea*	125 Mya	(Lefevre *et al.*, 2004)
Tremblaya	*Pseudococcidae*	40 Mya	(Baumann, 2005)
Blattabacterium	*Blattodea*	150 Mya	(Lo *et al.*, 2003)
Uzinuria	*Diaspididae*	100 Mya	(Gruwell *et al.*, 2007)
Sulcia	*Auchenorrhyncha*	>270 Mya	(Moran *et al.*, 2005)

1.2.3 Secondary Symbiosis

Secondary endosymbionts consist of all heritable microorganisms that are not required for survival of the host. Prior to the turn of the century this class was predominantly considered to consist of the reproductive parasites. However, recent research has identified numerous microbes with transient associations with host lineages that confer ecologically contingent benefits such as host protection (Reviewed in Haine, 2008; Brownlie and Johnson, 2009) and stress tolerance (Chen *et al.*, 2000; Montllor *et al.*, 2002; Russell and Moran, 2006). The last two years have seen a growing appreciation that symbionts can express multiple phenotypes within a single host. For example, a *Rickettsia* that recently spread through natural populations of white fly (*Bemisia tabaci*) was driven by both a mutualistic phenotype (increasing longevity and fecundity) and a sex ratio distortion (Himler *et al.*, 2011). In addition, a strain of *Wolbachia* (wMelPop) caused cytoplasmic incompatibility, but also prevented onward transmission of Dengue virus when artificially introduced to *A. aegypti* mosquitoes from its native *Drosophila* host (Walker *et al.*, 2011). It has been hypothesised that a

strategy of mixed symbiotic phenotypes may be beneficial or even necessary for the establishment of novel symbioses (Fenton *et al.*, 2011).

1.2.4 Reproductive Parasitism

Reproductive parasitism (RP) is a widespread phenomenon in arthropod S-symbiosis, stemming from the inherent conflict over sex allocation arising from the asymmetry in inheritance between nuclear and cytoplasmically bound elements (Cosmides and Tooby, 1981). Because heritable microbes are transmitted to progeny through the cytoplasm of the ovum or at the point of oviposition, their fitness is intrinsically linked to that of the female hosts (for an exception, *D. melanogaster* sigma virus (Carpenter *et al.*, 2007; Longdon and Jiggins, 2012). Therefore, microbes have evolved a number of strategies that increase either the fitness or number of female hosts in order to maximize their own vertical transmission (Engelstadter and Hurst, 2009).

Reproductive manipulation phenotypes fall into two broad categories:

a) Incompatibility inducers (CIs)
b) Sex ratio distorters (SRDs)

Cytoplasmic incompatibility

Arguably the best studied of all reproductive manipulation phenotypes is cytoplasmic incompatability (CI). CI was first discovered in the mosquito *C. pipie*ns where a maternally inherited element prevented some strains from successfully breeding (Laven, 1951). In some instances the incompatibility was uni-directional, i.e. males from population 'A' were unable to successfully mate females from population 'B', whilst the reciprocal cross remained viable. Other crosses exhibited bi-directional incompatibility, where both reciprocal crosses were unviable. Two decades after Laven's discovery, the causative agent of the incompatibility was described as the 'Rickettsia-like' microorganism, previously identified as *Wolbachia pipientis* (Hertig and Wolbach, 1924; Yen and Barr, 1971) which is transmitted vertically in the ooplasm.

CI is now known to occur widely across many insect taxa (Bourtzis, 2003; Saridaki and Bourtzis, 2010) and is commonly induced by both *Wolbachia* (Binnington and Hoffmann, 1989; Bourtzis *et al.*, 1996) and *Cardiniuum* (Hunter *et al.*, 2003; Perlman *et al.*, 2006; Wu and Hoy, 2012; Zhang *et al.*, 2012). That CI is caused by such distantly related microbes strongly suggests that the phenotype has evolved multiple times. It is

thought to operate through an effector/rescue system whereby debilitating modifications made to a sperm by bacteria in the testes are rescued by bacteria of the same strain in an infected egg (Werren, 1997; Presgraves, 2000; Zabalou *et al.*, 2008). If no rescue is present (e.g. when an uninfected female mates with an infected male) then either zygotic death ensues, or only males are produced (if the host is haplodiploid) (Vavre *et al.*, 2002).

CI drives the symbiont through populations because it selectively eliminates uninfected individuals. This drive increases in strength with the frequency of the symbiont – a rare CI symbiont has little impact. This creates a 'threshold' prevalence that the symbiont must reach before it can spread deterministically (Hoffmann and Turelli 1997).

Sex ratio distortion (SRD)

SRD covers a range of phenotypes that increase the proportion of females in the host population. These phenotypes have evolved independently a number of times in a diverse group of microbial taxa, including members of the α-proteobacteria, γ-proteobacteria, mollicutes and bacteroidetes.

a) Feminization

Feminizing bacteria convert genetic males into functional females. This has been described in *Wolbachia* infections of the butterfly *Eurema hecabe* (Hiroki *et al.*, 2002), as well as in non-insect arthropod/symbiont systems: The crustacea *Armadillium vulagre* (*Wolbachia*) (Rousset *et al.*, 1992; Rigaud and Juchault, 1995) and *Gammarus duebeni* (Microsporidia)(Terry *et al.,* 1998), and the spider mite *Brevipalpus phoenicis* (*Cardinium*) (Weeks *et al.*, 2001). The feminizing *Wolbachia* in the woodlouse *Armadillium vulgare* operates by disrupting the androgenic hormone, forcing the embryo to revert to the 'default' female phenotype (whilst remaining genetically male) (Rigaud *et al.*, 1997). The feminization of males in the butterfly *Eurema hecabe* has been shown to be an on-going process throughout the organism's development. Experimentally removing the *Wolbachia* at different developmental stages with antibiotic treatment results in intermediate degrees of masculine traits in the adult butterfly (Narita *et al.*, 2007).

b) Induced Parthenogenesis

Induced parthenogenesis (PI) has been recorded in several groups of hymenopteran insects as well as a number of *Bryobia* mite species (Huigens *et al.*, 2000; Weeks and

Breeuwer, 2001). It causes infected individuals to produce daughters without mating (thelytoky) but interestingly fertilization blocks this effect, resulting in the production of both male and female offspring. PI effects have been shown in *Rickettsia, Wolbachia* and *Cardinium* infections. Arthropod species affected by PI are haplodiploid and therefore have a sex-determination system dependent on ploidy. Manipulation is achieved by physically altering the ploidy of embryos destined to become male, switching them to females in a process called diploidization.

c) Male-killing

Male-killing (MK) is a common form of sex ratio distortion employed by a diverse set of microbes (Hurst, 1991; Hurst and Jiggins, 2000; Duron *et al.*, 2008). The causative agents are subdivided into those that cause the death of male embryos (early male-killers) and those that cause death of male larvae (late male-killers). The latter phenomena has only been recorded in microsporidian infections in mosquitoes (Hurst, 1991 and references therein), and viral infection of the tea tortrix moth (Nakanishi *et al.*, 2008). Early male-killers are known in numerous insect groups including Coleoptera, Lepidoptera, Hymenoptera and Diptera.

To date we only have limited data on two mechanisms of male-killing: *Spiroplasma poulsonii* infection in *Drosophila melanogaster* induces apoptosis through a functional dosage compensation complex (Veneti *et al.*, 2005). A similar infection in *Drosophila nebulosa* caused widespread apoptosis in male embryos, resulting in a failure to form segments and inducing embryo death (Bentley *et al.*, 2007). The male-killing action of *Arsenophonus nasoniae* in *Nasonia vitripennis* is induced by blocking maternal centrosome formation (the only centrosome present in the haploid embryo), resulting in uneven mitosis and embryo death. In females, the paternal centrosome rescues the embryo and so they develop normally (Ferree *et al.*, 2008). As the causative agents of male-killing are diverse, it is reasonable to assume that the mechanisms are highly varied.

In all cases of sex ratio distortion, symbiont spread occurs because infected females leave more surviving daughters than uninfected females. The evolutionary drive of feminization and PI are relatively intuitive. Both phenotypes exchange would-be males embryos for functional females. Thus, they have the capacity to double the number of the infectious sex, if the uninfected sex ratio is 1:1. The adaptive advantage of male-killing on the other hand is somewhat more enigmatic. Male-killing phenotypes do not

increase the absolute number of female progeny. Superficially this appears spiteful as it harms the host fitness without increasing bacterial transmission. However, male-killing can promote bacterial transmission if the death of males benefits their female siblings in some way. Specifically this can be achieved through two forms of kin-selection:

First, the death of infected males may release resources to their infected sisters, either through cannibalism or the alleviation of sib-sib competition. Siblings of dead males will then be fitter and more fecund and the male-killing element will drive towards fixation (Hurst, 1991). This is termed the 'resource reallocation' hypothesis and has been inferred in a number of species (Hurst and Majerus, 1993; Hedges *et al.*, 2008; Walker *et al.*, 2011), although only demonstrated in one (Koop *et al.*, 2009). It is presumed to operate for species that lay clutches in discreet patches, where larvae are confined to a finite resource for which they are in direct competition (Jaenike *et al.*, 2003; Baldo *et al.*, 2006; Mouton *et al.*, 2012).

Secondly, the death of males may promote outcrossing and thus diminishes the deleterious effects of inbreeding depression. Werren (1987) modeled this theory and found that inbreeding avoidance could promote the spread of a male-killing element in populations even if fitness gains were very marginal. However this was highly sensitive to the transmission efficiency of the symbiont.

Both theories only apply to species where sib-sib contact/competition occur. Majerus and Majerus (2012) demonstrated that this condition holds true when comparing male-killer infection across brooding and non-brooding beetles.

1.2.5 Ecologically contingent mutualists

Whilst P-endosymbionts persist in host populations through obligate mutualism and reproductive parasites achieve drive through manipulating their hosts reproduction, ecologically contingent mutualists persist through providing an adaptive benefit only under specific conditions. This phenomena is relatively newly discovered, the majority of evidence coming post-2000. The forms of benefit provided are also varied and recent evidence suggests that contingent mutualism and reproductive parasitism may not be mutually exclusive.

Some ecologically contingent mutualists offer defensive roles against predators and parasitic symbionts, thus the contingency is the presence of such enemies. Both pea aphids (*Acyrthosphion pisum*) and black bean aphids (*Aphis fabae*) show increased resistance to parasitoid wasp attack when infected with *Hamiltonella defensa* or *Serratia symbiotica* (Oliver *et al.,* 2003; Ferrari *et al.,* 2005; Vorburger *et al*., 2009). Interestingly, the source of the resistance in the *H. defensa* system has been identified as a bacteriophage (APSE) associated with the symbiont, rather than the symbiont itself (Oliver *et* al., 2009; Wheldon *et al*, 2012). Symbiont mediated parasitoid defense is not just limited to Aphids: *Drosophila hydeii* display enhanced resistance to *Leptopilina heterotoma* when infected with a *Spiroplasma* (Xie *et al*, 2010). *Drosophila* and *Culex sp* can also derive a protective benefit from their native *Wolbachia* infections. Several researchers have demonstrated that resistance to a number of RNA viruses in *D. melanogaster* is strongly linked to presence of the symbiont under laboratory conditions (Hedges 2008, Teixeira 2008) and that naturally occuing Wolbachia infections reduce west nile virus pathology in both *D. melanogaster* and *Culex quinquefasciatus* (Glaser and Meola., 2010). Indeed, *Wolbachia* presence has been cited as a major component dictating the epedimiology of these viruses in nature (Johnson & Brownlie, 2009). Another secondary symbiont, *Regiella insecticola*, has been linked with increased resistance to the fungal pathogen *Pandora (Erynia) neoaphidis* in the its aphid host (Scarborough *et al* 2005). Here the protection not only reduces individual mortality, but also reduces spore production from fungus-infected individuals up to ten-fold, and so conveys a degree of group-level immunity. Whether this latter effect is an adaptation on the part of the symbiont is not clear however. In the presence of the natural enemy against which they protect, these symbionts are highly beneficial to their hosts and so should be under strong selection for maintenance. However, in the absence of the enemy the symbiont shave been shown to be highly costly (Vorburger *et* al., 2013) and in the case of *Hamiltonella defensa*, just the absence of the defense phenotype-linked APSE phage greatly increases the cost of the symbiont to the host (Weldon *et al.,* 2012). Therefore whilst these microbes form in intriguing part of the host resistance complex, they themselves are subject to strong negative selection under certain conditions.

In addition to these defensive microbes, other secondary-endosymbionts convey a benefit to their host under particular environmental conditions. For example, when *Drosophila melanogaster* are reared on iron-depleted or overloaded food, *Wolbachia* infected lines have greater fecundity than uninfected controls (Brownlie *et al*., 2009).

In this case, this ecologically contingent benefit is postulated to be an additional contributor to the symbionts drive on top of the weak CI phenotype also invoked by *Wolbachia* in *D. melanogaster*.

Ultimately many secondary symbionts may posses both beneficial and parasitic traits. For example a Rickettsia has shown to both skew sex ratio and increase fitness in its whitefly host (Himler *et al* 2011). Potentially the phenotype expressed by the symbiont may be plastic, and contingent upon the condition and environment in which it finds its host.

1.2.6 Dynamics of secondary symbionts

The non-obligacy of S-symbionts has led them to exhibit more transient dynamics than the P-symbionts. Their persistence in host lineages is driven either by the action of their reproductive manipulation or by selection upon the beneficial trait they confer, or a synergy of the two.

Reproductive manipulation is a double-edged sword. RP phenotypes have evolved to drive bacterial transmission, however they shift host life-history traits away from their adaptive optima and so are costly to the host. Hosts are therefore under selection to purge reproductive manipulators, limiting the persistence of these elements within any particular host species over evolutionary time or to evolve tolerance to the manipulation (Vala *et al* 2003). Evidence for this instability comes from the discord in cocladogenesis between *Wolbachia* and its various host species and evidence of its regular loss and re-infection in host lineages (Werren *et al.*, 1995; Van Meer *et al.*, 1999; Reuter *et al.*, 2004; Koehncke *et al.*, 2009).

The life-span of ecologically contingent mutualisms are also limited in evolutionary time. The drive of symbionts that protect against natural enemies is dependent upon mortality/morbidity from the predator or parasite against which they defend (Vavre *et al.*, 1999; Sandström *et al.*, 2001; Russell *et al.*, 2003; Wernegreen *et al.*, 2009; Raychoudhury *et al.*, 2009; Kwiatkowski and Vorburger, 2012; Watanabe *et al.*, 2012). Experimental analysis indicates that in the absence of this antagonist, the costs of maintaining the symbiont lead to the loss of the symbiont from host populations. This transient selection pressure has been cited as the reason that protective symbionts are typically only found in a subset of their host population, not at fixation (Tsuchida *et al.*,

2002; Simon *et al.*, 2003; Oliver *et al.*, 2006; Vorburger *et al.*, 2009). For example, *Hamiltonella defensa* carrying the APSE bacteriophage increases survival of its aphid host (*Lysiphlebus fabarum*) under parasitoid attack (Oliver *et al.*, 2003; Ferrari *et al.*, 2004; Vorburger *et al.*, 2009), but is detrimental to host longevity and fitness when parasitoids are absent (Vorburger *et al.*, 2013).

The inherent instability of S-symbioses results in regular loss, host shift and invasion events. As a consequence, S-symbionts display broader phenotypic diversity between closely related strains than P-endosymbionts because selection has differentially acted on incipient strains following a host-switch. *Wolbachia*, for example, has been shown to exhibit broad phenotypic diversity, acting parasitically as a male-killer (Dyson *et al.*, 2002), incompatibility inducer (Bordenstein and Werren, 2007) and feminizer (Rigaud and Juchault, 1995), but also beneficially as a protective partner against natural enemies (Hedges *et al.*, 2008; Walker *et al.*, 2011) and a nutritional mutualist in *D. melanogaster* (Brownlie *et al.*, 2009). The mobility of S-symbionts also increases the chance of heterogeneous infections occurring within single host individuals, thus creating opportunity for horizontal transfer of genetic material (Baldo *et al.*, 2006; Mouton *et al.*, 2012, Ros *et al.,* 2012).

S-symbionts, are therefore a far more diverse collection of organisms, with the potential to impact greatly on their hosts evolutionary trajectory in dynamic and diverse ways, whilst remaining relatively transient in any given population.

1.3 Infectious Transmission in symbiont biology

Although inherently associated with vertical transmission (VT), heritable symbiotic associations can additionally show infectious (horizontal) transmission (HT). Horizontal transmission is currently viewed as a rare, but ecologically important, event through which heritable symbionts host-switch (Engelstadter and Hurst, 2006). This leads to related symbiont lines occurring in non-monophyletic clusters of host species (Moran *et al.*, 2005).

Evidence for this comes from the incongruence between host and symbiont phylogenies (Vavre *et al.*, 1999; Sandström *et al.*, 2001; Russell *et al.*, 2003; Wernegreen *et al.*, 2009; Raychoudhury *et al.*, 2009; Watanabe *et al.*, 2012) and genetic recombination between disparate lines of symbionts (Baldo *et al.*, 2006; Raychoudhury *et al.*, 2009; Ros *et al* 2012). There is also molecular evidence that symbionts that horizontal transmit become associated with hosts that share particular habitats or niches, rather than descent (Stahlhut *et al.*, 2010). This suggests an ecologically mediated rate of horizontal transfer.

Laboratory experiments have also shown that novel heritable symbioses may emerge following horizontal transfer in both facultative beneficial microbes (Russell and Moran, 2005; Weiss *et al.*, 2006; Moran and Dunbar, 2006; Gehrer and Vorburger, 2012) and those that manipulate host reproduction (Grenier *et al.*, 1998; Huigens *et al.*, 2000 & 2004; Duron *et al.*, 2010). These studies show that horizontal transmission occurs following ecological interactions in which host fluids come into contact, such as reproduction (Moran and Dunbar, 2006), shared nutrition source (Sintupachee *et al.*, 2006) or predation (Gehrer and Vorburger, 2012).

What is less clear is how symbionts and hosts evolve following introduction to a novel host. It is known that new interactions may be pathogenic and that selection acts to reduce pathogenicity on quite short evolutionary timescales (McGraw *et al.*, 2002; Weeks *et al.*, 2007; Carrington *et al.*, 2010). However, the conditions that create a descent into primary symbiosis, rather than the retention of the capacity to undergo host shifts remain unclear. Further, whilst the role of horizontal transmission in establishing new symbiont-host interactions is well researched and uncontentious, its impact upon the epidemiology of heritable symbionts remains relatively unexplored.

This is despite several documented cases where heritable symbionts employ mixed strategies of both horizontal and vertical transmission.

1.3.1 Horizontal transmission on ecological timescales

Infectious transmission is normally excluded from models of heritable symbiont population biology as rates are considered to be too low to alter dynamics. Notwithstanding this, it is likely to be important in a number of cases, namely those where the symbiont's mode of transmission routinely exposes it to potential host individuals that are unrelated to its current one. It is also worth recognizing that pure vertical transmission is an evolved state. Ancestors of purely vertically transmitted symbionts will have combined vertical and horizontal transmission, and secondarily lost infectious ability. Further, there are a variety of conditions under which selection will favour maintenance of infectious transmission on ecological timescales.

Symbionts can be selected to promote heritable transmission when the relationship is either mutualistic or parasitic. However, the relative adaptive values of infectious and vertical transmission are likely dependent upon host ecology. It is likely that host density plays a major role in determining the balance between horizontal and vertical transmission. Under low host density (or low vector density if the symbiont is reliant upon a vector for part of its transmission cycle) there is limited opportunity for infected hosts to come into contact with uninfected hosts. Thus, symbionts that rely heavily on horizontal transmission will be under selection to switch to vertical transmission if they are in a small or growing population, i.e. one that has been recently founded or experienced a crash. Ironside *et al* (2011) mathematically demonstrated that feminizing reproductive parasites should only completely lose the capacity for horizontal transmission if their hosts are at low density or has a male-biased primary sex ratio. In contrast, if the host population is at carrying capacity then transmission through reproduction is also limited. Here, infectious transmission should be under positive selection.

The role of host density in determining symbiont transmission strategy has been empirically investigated in *Paramecium* symbionts of the protest *Holospora unulate.* Researchers successfully selected for increased efficiency of vertical transmission by maintaining host populations under constant growth, and select for infectious transmission and virulence in host populations under enforced carrying capacity (Kaltz and Koella, 2003). Furthermore, Microsporidian infections in mosquitos have been

shown to invest more into vertical transmission if their host larvae have a rich diet, as this is an indicator of low host density. Conversely, the symbionts resort to horizontal transmission when ecological indicators imply population crowding (Agnew and Koella, 1999).

As an 'exception that proves the rule', symbionts may evolve to favour one form of transmission over the other in spite of the density of their host. For example, LBvF viral symbionts of the parasitoid *Leptopilina boulardi* has evolved to manipulate host oviposition behavior to encourage host-host contact and therefore boost horizontal transmission (Varaldi *et al.*, 2006). In this case the symbiont has effectively evolved to artificially increase the population density of its host in order to enhance its infectious transmission.

Most symbioses do not establish in a system with stable host densities, but rather with seasonal or developmental cycles where the capacity to transmit infectiously fluctuates. These cycles result in vertical and horizontal transmission having differing adaptive values at different times. For example, when host-host contact is low, due to a sedentary stage of the hosts life-cycle or seasonally-enforced diapause, infectious transmission is of little value. In these scenarios we would expect selection to have maintained the capacity for both forms of transmission. LaCrosse virus in the mosquito *Aedes triseriatus* exhibits such a mixed transmission strategy. In the summer, infectious transmission through intermediate mammalian hosts maintains viral loads in the mosquito populations. However, mammal hosts become rarer in winter and so the virus is maintained in the mosquito population through vertical transmission from adult to offspring, which overwinter as infected eggs (Watts *et al.*, 1973). Whilst the vertical transmission has little impact on the epidemiology of the virus during the summer, it is vital for the year-on-year fitness of the symbiont.

Given the above evidence it is likely that a) selection on the symbiont to evolve vertical transmission will be dependent upon the density of host, and b) retaining the ability to horizontally transmit may be beneficial for symbionts in hosts with variable population sizes or seasonal declines. This then represents a paradox for the symbiont, as vertical and horizontal transmission should select for differing levels of virulence and thus resistance evolution in their host.

Alternatively, maintaining the capacity for infectious transmission may represent a long-term evolutionary strategy for the symbionts, particularly those that have transient or parasitic associations with their host such as secondary-endosymbionts.

Under pure vertical transmission, these symbiont will live and die with their host lineage and are at the mercy of their hosts resistance. Being able to infectiously transmit allows these symbionts to 'jump ship' if their host lineage is at risk or to continuously move between disparate, non-resistant host populations. Arguably, this is why we see evidence of horizontal transmission in the phylogenies of secondary end-symbiont of insects (e.g. Baldo *et al* 2006; Mouton *et al* 2012). However, as with all arguments based upon higher-order selection it is necessary to consider at what point selection is acting. Potentially, Whilst confined to a single host lineage, selection should act to promote vertical transmission, selection for infectious transmission only operates when the host lineage is under threat or in decline. Complete loss of infectious transmission is likely to be an evolutionarily slow process and thus it may be that selection is not acting to maintain horizontal transmission, rather that selection to remove it is easily disrupted and horizontal transmission is retained.

1.3.2 Consequences of horizontal transmission on ecological timescales

Infectious transmission, as discussed above, can contribute to the dynamics of heritable symbionts, providing it occurs at high enough rates. One obvious impact is that symbiont transmission theoretically becomes dependent on host density – a factor to which purely vertically transmitted symbionts are largely unaffected. The dynamics of symbionts showing both infectious and vertical transmission are thus likely affected by a wide array of ecological feedbacks that impact host density, as well as environmental drivers of abundance.

Beyond this, infectious transmission may alter the relationship between host and symbiont. In particular, it may reduce the likelihood of the symbiont being benign or beneficial to the host. This is for three reasons: First, the correlation between symbiont and host fitness is high under vertical transmission, because host fitness represents symbiont transmission (Fine, 1975; Ewald, 1987). This selects for minimization of virulence, and indeed contribution to host function. Second, the act of horizontal transmission will commonly require symbiont replication and release, which may be inherently pathogenic (Frank, 1996b). Third, infectious transmission creates the possibility of different strains of a given symbiont mixing within a host. Combined with infectious transmission, this creates the conditions under which symbionts may be selected to compete for transmission.

1.3.3 Summary: Horizontal transmission on ecological timescales

The recent focus on symbionts whose population biology is dominated by vertical transmission has made symbionts with dual transmission modes the forgotten group. This lack of study belies the importance of symbionts with dual transmission as an essential 'stepping stone' for the evolution of symbionts with vertical transmission alone, and despite the fact that a number of symbionts are known to transmit through both routes.

1.4 Overview of the field and directions of study

Until the mid-2000s research on heritable symbionts was focused on their diversity and abundance throughout arthropod populations, the mechanism of their phenotype and the evolutionary drive behind the diverse phenotypes seen in nature. Since this time there has been a shift towards understanding of the complexity inherent within symbiotic interactions. The future of symbiosis research likely lies in a) understanding how novel symbioses establish, evolve and diversify, particularly with reference to host-shifts, and b) reconciling current models of symbiont prevalence and evolutionary impact with the new-found complexity of phenotype and infectious transmission seen across many symbiont groups. These fields will overlap with current research on the evolution of parasitism and virulence, maintenance of diversity in nature and the emergent field of ecological genomics. From an applied perspective heritable insect symbiosis has long been touted as a route to control of pests in agriculture and key disease vectors. Understanding the variability and duplicity of individual symbiont infections and how these react to host-shifts will be vital if effective widespread symbiont-mediated control measures are to be employed.

The aim of this thesis is to explore both of these issues in the *Arsenophonus* – chalcid parasitoid wasp interactions. I present two systems of study.

a) The interaction between *Arsenophonus nasoniae* and *Nasonia vitripennis*, in which a reproductive parasite (male-killer) undergoes both vertical and infectious transmission. Here the aim is to investigate the impact of infectious transmission on the dynamics of the symbiont.

b) The interaction between *Arsenophonus nasoniae* and *Pachycrepoideus vindemmiae*. My thesis is that this interaction is one established through a host shift, and in which loss of capacity for infectious transmission has recently occurred.

1.5 The *Arsenophonus*/Parasitoid System.

1.5.1 Arsenophonus nasoniae

In the early 1980s Sam Skinner and Prof. Jack Werren at the University of Rochester discovered a sex-ratio distorting factor in a strain (HEB-3) of the parasitic wasp, *Nasonia vitripennis* caught in Utah, USA. This factor skewed the sex ratio of the wasp's brood to between 85-94% female bias (Skinner, 1985). They termed this factor 'son-killer' and later identified the causative agent to be a heritable bacterium (Werren *et al.*, 1986). This bacterium was named *Arsenophonus nasoniae* and identified as a member of the gamma-proteobacteria, the group that also includes *Escherichia, Pseudomonas* and *Proteus,* by DNA sequence identity and fatty acid profiling (Gherna *et al.*, 1991; Bressan *et al.*, 2011) (NCBI Taxon ID: 1121018). The bacterium is described as being *"non-motile, non-spore forming, long rods, occasionally filamentous young culture".*

In *N. vitripennis*, *A. nasoniae* acts as an early male-killer, halting the development of male embryos as eggs. Male mortality occurs after oviposition and females do not alter their sex ratio to compensate for the loss of fecundity (Skinner, 1985), thus infected females ultimately produce fewer offspring than their uninfected counterparts. The male-killing itself is known to be caused by the breakdown of the maternal centrosome, an effect that is rescued by the paternal centrosome in diploid females but results in fatal incomplete chromosome division in haploids (Ferree *et al.*, 2008).

Arsenophonus nasoniae is transmitted between wasps at the point of oviposition. When the female wasp stings and immobilizes her dipteran host she also inoculates it with *A. nasoniae* in the venom. The symbiont then re-infects her progeny per-orally through the gut wall as they feed on the infected body fluids of the fly. The transmission efficiency of this pseudo-vertical infection is very high (95%) (Skinner, 1985), but importantly, is not perfect. In the same study Skinner also noted that the bacterium readily transmitted horizontally between infected (HEB-3) and uninfected (ScDr) *N. vitripennis* that oviposited in the same fly pupae. Duron *et al* (2010) expanded on this when they demonstrated that *A. nasoniae* could successfully undergo horizontal transmission between closely related wasp species that co-parasitised the same pupae. Duron and colleagues demonstrated that the efficacy of this horizontal transmission, and the efficiency of the male-killing phenotype was correlated with genetic distance between native and novel hosts. In the same study, and further expanded upon by (Taylor *et al.*, 2011), they established that several species of chalcid wasp that

parasitised members of the filth-fly community had natural infections closely related to *A. nasoniae* (see table 1.3). These hosts included the solitary parasitoid, *Pachycrepoideus vindemmiae*. Subsequently, *A. nasoniae* infection has been found throughout members of the gall-wasp guild as well (Wilkes *et al* unpublished data).
What is unclear, and yet to be explored, is the role that the fly pupae plays in the transmission and epidemiology of *A. nasoniae*. The bacterium spends a significant portion of its life-cycle inhabiting the body of the fly pupae before it is ingested by the parasitising wasp pupae. The degree of antimicrobial resistance encountered and efficiency with which the microbe is able to live in this environment will almost certainly vary between host species and so may affect the efficiency of transmission to the intended wasp host. Many of *A. nasonia's* hosts are generalist parasitoids (See following sections) and so it is likely that the bacterium will readily encounter differing fly environments.

As with many male-killers, prevalence in natural populations is relatively low. Estimates from field surveys range between 8-15% (Balas *et al.*, 1996; Duron *et al.*, 2008). Previous study has found no costly effect of *A. nasoniae* infection on *N. vitripennis* in terms of body size or fecundity in natural populations (Balas *et al.*, 1996).

Unusually for an endosymbiont, *A. nasoniae* is readily culturable in cell free media (Werren *et al.*, 1986). This is experimentally useful, allowing for dose-controlled artificial inoculation, gene transformation and growth assays. It is hypothesized that this ability for free-living is due to the obligate re-infection stage of the bacteria's life cycle (Darby *et al.,* 2010). In 2010, Darby *et al* completed a draft sequence of the *N. vitripennis* derived *A. nasoniae* (Darby *et al.*, 2010), from which the genetic underpinnings of virulence and symbiosis factors have been explored (Wilkes *et al.*, 2010).

The genus *Arsenophonus* also has a broad distribution across several arthropod taxa and has secondarily evolved to be a plant pathogen multiple times (Bressan *et al.*, 2011). Duron *et al* (Duron *et al.*, 2008) estimated that approximately 5% of insect species harbor *Arsenophonus*. The nature of these symbioses is highly diverse, ranging from the reproductive parasitism discussed above, to obligate P-endosymbiosis (See table 1.2). It is striking that the majority of *Arsenophonus* hosts feed on either phloem or blood (vertebrate or invertebrate). The few exceptions are the Diptera and plants, both of which are regularly parasitised by known *Arsenophonus* hosts. The majority of these other *Arsenophonus* strains have yet to be phenotypically characterized, although

most are implicated in either primary or secondary mutualism. They are all more fastidious than *A. nasoniae* and have yet to be cultured in cell-free media, although *candidatus Arsenophonus arthropodicus*, an infection in Hippoboscid flies, has been cultured in dipteran cell lines (Dale *et al*., 2006). Several of these *Arsenophonus* members are currently undergoing genome sequencing, functional annotation and comparative analysis (Darby *pers comms*). Given that members of the *Arsenophonus* clade occupy almost all positions on the symbiotic spectrum, it is hoped that comparative analysis of these genomes will elucidate the molecular processes that underpin the evolution of heritable symbiosis.

1.5.2 Nasonia vitripennis

Nasonia vitripennis (Walker 1836) is a gregarious wasp that acts as a pupal ectoparasitoid of several dipteran species from the families Sarcophagidae and Calliphoridae (Whiting, 1967). *N. vitripennis* belongs to the Chalcidoidae superfamily (family *Pteromalidae*) and has been variously referred to as *Pteromalus vitripennis, Pteromalus adnormis, Mormoniella* and *Nasonia brevicornis* in the literature, but is now universally recognized as *Nasonia* (=Mormoniella) *vitripennis* (Whiting, 1967) (NCBI Taxon ID: 7425).

Upon encountering a potential host, the adult female will drill through the puparium cuticle to assess the quality of the host and to inject venom into its body. The venom manipulates the host to create an optimal environment for her offspring by arresting development (Rivers and Denlinger, 1994), reallocating resources from growth to lipid production (Rivers and Denlinger, 1995) and manipulating immune responses (Rivers *et al.*, 2002). The female lays a number of eggs in the airspace between the host and the puparial shell, the quantity and sex ratio of which are determined by host species, pupal size and quality (Rivers and Denlinger, 2011) and whether or not the host has been parasitized previously (Shuker and West, 2004; Ivens *et al.*, 2009). Upon hatching, the larvae use their mandibles to attach to the disabled fly and feed on its body fluids. After four larval instars and pupation, adult males eclose a few hours prior to females and chew through the puparial case to escape. Males then wait by the exit hole in the puparium and mate the females (usually their sisters) as they emerge. Female then disperse to parasitise fresh patches of fly pupae, males are flightless and so do not disperse from their natal patch.

As with all Hymenoptera, *N. vitirpennis* has haplodiploid sex determination; fertilized eggs develop into diploid females whilst unfertilized eggs become haploid males. Mothers are thus able to manipulate the sex ratio of their broods by controlling the

fertilization of their eggs and do so to conform to the predictions of the Local Mate Competition theory of sex ratio evolution (Hamilton, 1967). When parasitizing in isolation females produce *c.*80% daughters, but this shifts towards parity when more than one female utilizes the same host – co-parasitism (Werren, 1980 & 1983). The degree of co-parasitism that occurs in nature is dependent upon host density, parasitoid density and the spatial distribution of both, i.e. if they are dispersed or highly aggregated. Surveys of genetic structure in natural *N. vitripennis* populations have identified that *c.*40% of host pupae support broods from 2-4 wasps (Grillenberger *et al.*, 2008). Thus co-parasitism appears to be commonplace.

Nasonia vitripennis enjoys a widespread distribution across the northern hemisphere and anecdotally elsewhere globally. Whiting (1967) claimed that it had been found "wherever it has been sought". *N. vitripennis* is part of a complex of closely related, sympatric species that are naturally isolated by their bi-directional CI inducing *Wolbachia* (Breeuwer and Werren, 1995; Bordenstein *et al.*, 2000; Bordenstein and Werren, 2007; Raychoudhury *et al.*, 2010). Unlike *N. vitripennis,* other *Nasonia* species have only been reported in north America: *N. longicornis, N. giraulti* (Darling and Werren, 1990) and *N. oneida* (Raychoudhury *et al.*, 2010).

Nasonia vitripennis is an ideal experimental model system. Its short generation time, ease of rearing, discrete-stage life cycle and a number of characterized genetic markers make it easily tractable for large scale experimentation. Furthermore, the haplodiploid sex determination makes *N. vitripennis* particularly resistant to inbreeding depression (Werren and Loehlin, 2009). *N. vitripennis* has long been used as a model system with which to investigate sex ratio evolution, LMC and reproductive game-theory (Werren, 1980; 1983; Shuker and West, 2004; Sykes *et al.*, 2007) and to investigate the evolution of nucleic sex ratio distorters (Werren *et al.*, 1981; Skinner, 1982; 1985). It has more recently been employed as a genetic model for analysis of complex traits (Rutten *et al.*, 2004) and the basis of incipient speciation (Bordenstein *et al.*, 2001). To this end, the system benefits from a well-established molecular toolkit that includes specifically designed microarrays and RNAi techniques (Lynch and Desplan, 2006). In 2010 the genomes of *N. vitripennis, N. girualti* and *N. longicornis* were sequenced, opening up a wealth of genetic tools for an already highly tractable system (Werren *et al.*, 2010). This resource has already allowed for huge advances in *Nasonia* research, including the identification of functional QTL and their associated regulatory regions (Pannebakker *et al.*, 2010), evidence of DNA methylation (Werren *et al.*, 2010) and identity of key

factors associated with venom constituents (de Graaf *et al.*, 2010) and immune factors (Tian *et al.*, 2010).

1.5.3 Pachycrepoideus vindemmiae

The wasp *Pachycrepoideus vindemmiae* (Rondai 1875) is a solitary ectoparasitoid of a number of dipteran species including fruit flies, radish flies and houseflies (Crandell, 1939; Nostvik, 1954; Phillips, 1993; Goubault *et al.*, 2003; Wang and Messing, 2004). Like *N. vitirpennis* it belongs to the chalcid superfamily (family Pteromalidae). It is also been referred to in the literature as *Anisopteromalia crassinervis*, *Pachycrepoideus dubius, Pachycrepoideus elongate, Pterosmoidea drosophilae, Tuxeumella dissimilis* and *Toxeumella nigra*. In particular the name *P. dubius* (Ashmead 1904) is still used in much of the literature, but by all accounts refers to the same species (NCBI taxon ID: 632107).

Pachycrepoideus vindemmiae's life history differs to that of *N. vitripennis* in that it only produces 1-3 offspring per host (Crandell, 1939; Nostvik, 1954). This solitary, or near-solitary, parasitism is enforced by the larvae themselves who, for the first few hours post-hatching, roam around their host destroying any competitors. Herbert Crandell described the brutality of this process; *"An encounter between two larvae invariably results in only one survivor – there is no compromise"* (Crandell, 1939). Incidences of females producing more than one offspring per host only occur in larger host species, where the roaming larvae fail to come into contact with their competitors. The sex ratio of *P. vindemmiae* broods is typically close to 50:50 (personal observation). As with many parasitoids, *P. vindemmiae* alters its sex ratio in response to perceived competition, resource quality, size and previous parasitism (Goubault *et al.*, 2011). Females will also readily reject pupae they deem to be of substandard quality (Goubault *et al.*, 2004b).

Like *N. vitripennis*, *P. vindemmiae* is a globally widespread parasitoid, preferring warmer, sub-tropical and temperate climates (Nostvik, 1954). However, its natural range is somewhat difficult to ascertain, as it has been repeatedly introduced to novel localities as a pest control measure for nearly a century (Crandell, 1939). Furthermore, its use of *Drosophila melanogaster* as a host, an insect whose global distribution has well documented anthropological causes, implies that humans may have inadvertently transported it around the globe.

Pachycrepoideus vindemmiae has been used as a model for the study of optimal foraging theory in relation to superparasitism and host discrimination (Goubault *et al.*, 2003;

Goubault *et al.*, 2004b; Goubault *et al.*, 2004a; Plantegenest *et al.*, 2004; Goubault, 2005; Goubault *et al.*, 2011) and natural variation in *Wolbachia* infections (Vavre *et al.*, 2002). It has also gained interest as a biological control agent for dipteran pests (Wang and Messing, 2004). Aside from these topics, and the largely ecological and whole organisms studies that have contributed to them, relatively little is known about *P. vindemmiae*. Indeed, when compared to the vast molecular resources available for *N. vitripennis, P. vindemmiae* remains an enigma.

Duron *et al* (2010) demonstrated *A. nasoniae* infections to exist in *Pachycrepoideus vindemmiae.* The solitary nature of the wasp make male-killing an unlikely phenotype for the bacterium, and suggests the *A. nasoniae* - *P. vindemmiae* interaction may have diversified following transfer into/out of this species.

Table 1.2: Diversity of recorded *Arsenophonus* symbionts across arthropods and plants. X = unnamed strain.

Host	**Strain**	**Known phenotype**	**References**	**Notes**
White fly (*Bemisia tabaci*)	X	Unknown	(Baumann, 2005; Skaljac *et al.*, 2010)	Bound to bacteriocytes (Gottlieb *et al.*, 2008). Diverse within whiteflies, potentially with two different ancestral strains followed by recombination (Duron *et al.*, 2010; Taylor *et al.*, 2011; Mouton *et al.*, 2012).
Aphids (multiple)	X	Unknown	(Jousselin *et al.*, 2013; Wulff *et al.*, 2013)	
Ticks (*Ixodes ricinus*)	X	Unknown	(Mediannikov *et al.*, 2012)	
Nasonia vitripennis	*Arsenophonus nasoniae* (ArN)	male-killing	(Werren *et al.*, 1986; Gherna *et al.*, 1991)	Type strain, sequenced
Pachycrepoideus vindemmiae	*Arsenophonus nasoniae* (ArPv)	No evidence of RP.	(Duron *et al.*, 2010) This thesis	
Nycteribiid Bat flies	*candidatus Aschnera chinzeii*	Nutritional mutualist	(Novakova *et al.*, 2009; Hosokawa *et al.*, 2011)	P-symbiont, bacteriocyte bound, evidence of cocladogenesis.
Lice (*Pediculus humanis capitis, Pediculus humanis humanus, Pthirus pubis*)	*Riesia spp*	B-vitamin mutualist	(Sasaki-Fukatsu *et al.*, 2006; Allen *et al.*, 2007; Perotti *et al.*, 2007; Kirkness *et al.*, 2010)	Different lineages in Human body and hair lice, human pubic lice, and chimpanzee lice allude to multiple acquisition.
Strawberry & sygar beet	*Candidatus Arsenophonus phytopathogenicus*	Causative agent of SBR disease in strawberry and sugar beet.	(Salar *et al.*, 2009; Bressan *et al.*, 2011)	Arthropod- plant transmission with pathological lifestyle - vectored by planthopper *Pentastiridius leporinus* & *Cixius wagneri*. Arisen independently of *P. fragariae*
Strawberrys and sugar beet	*candidatus Phlomobacter fragariae*	Causative agent of marginal chlorosis in W.France and Japan	(Nourrisseau *et al.*, 1993)	
Hippoboscid flies	*Arsenophonus arthropodicus*	Nutritional mutualist	(Dale *et al.*, 2006; Duron *et al.*, 2008)	

Eupelmus vesicularis	*A. nasoniae*	Unknown	(Taylor *et al.*, 2011)	
Spalangia cameroni	*A. nasoniae*	Unknown	(Duron *et al.*, 2010; Taylor *et al.*, 2011)	
Spalangia endius	*A. nasoniae*	Unknown	(Taylor *et al.*, 2011)	
Triatoma spp	*candidatus Arsenophonus triatominarum*	Unknown, presumed beneficial	(Hypša and Dale, 1997; Šorfová *et al.*, 2008)	Culturable in cell lines
Apis mellifera	X	Unknown	(Novakova *et al.*, 2009)	
Ticks (Genus: *Dermacentor*)	X	Likely secondary, negatively correlates with pathogenic bacteria infection.	(Grindle *et al.*, 2003)	*D. andersoni* and *D. variabilis* (Canada) show different infection 16S sequence to *D. variabilis* (USA) and *Amblyomma americanum*.
Psyllids (*Glycaspis brimblecombeil*)	X	Implied secondary parasitoid protection	(Hansen *et al.*, 2007)	
Scale insect (*Ericerus pela*)	X	Not a sex-ratio distorter	(Liu *et al.*, 2012)	
Muscidifurax raptor	*A. nasoniae*	Unknown	(Duron *et al.*, 2010)	
Protocaliphora fly (*Protocalliphora azurea*)	*A. nasoniae*	Unknown	(Duron *et al.*, 2010)	
Spider (*Araneus diadematus*)	X	Unknown	(Duron *et al.*, 2008)	
Cockroach *(Loboptera decipiens)*	X	Unknown	(Duron *et al.*, 2008)	
Fire bug (*Pyrrhocoris apterus)*	X	Unknown	(Duron *et al.*, 2008)	
Vespid wasps (*Polistes nimpha*)	X	Unknown	(Duron *et al.*, 2008)	

1.6 Thesis outline

In this thesis I will address the significance of infectious transmission in the male-killing bacterium *Arsenophonus nasoniae*. I will determine the importance of horizontal transfer in the epidemiology of *A. nasoniae* in its native host *Nasonia vitripennis* both theoretically and empirically. I will address the fitness costs of infection upon females and attempt to reconcile this with standing theory on the evolution and adaptive benefit of male-killing. I will also investigate how horizontal transmission between species generates phenotypic divergence between closely related symbionts and to what extent this is underpinned by molecular divergence and evidence of selection at a genomic scale.

Chapter 2 presents a simple model of the spread of a male-killer through a host population, under joint vertical and horizontal transmission. I investigate the importance of segregational loss of the parasite, adaptive benefit of sex ratio distortion and the rate of horizontal transmission upon infection prevalence. I also explore the impact of direct cost of infection upon transmission dynamics. Ultimately I demonstrate that horizontal transmission alone is sufficient to maintain a male-killer under certain conditions, and that there are reasons to believe that horizontal transmission is necessary for *A. nasoniae* maintenance.

Chapter 3 presents evidence that *A. nasoniae* infection in *N. vitripennis* confers substantial costs to the host in terms of implied fecundity and potential dispersal ability. I discuss the importance of this in the light of standing theories on the adaptive advantage of male-killers and the conclusions drawn from Chapter 1.

Chapter 4 describes a series of experiments that manipulate the density of laboratory populations of *N. vitripennis* in order to test some of the predictions made in Chapter 2. Furthermore I present experimental evidence that *A. nasoniae* is able to rapidly invade susceptible populations of its host when horizontal transmission is permitted, and that under resource stress this can lead to host population extinction.

Chapter 5 demonstrates the rapid evolutionary change in a symbiont following a host shift event. I attempt to discern the symbiotic phenotype of a novel *A. nasoniae* infection in its native parasitoid host *Pachycrepoideus vindemmiae* through a series of experiments. I demonstrate its inability to infect *N. vitripennis* and thus its ongoing transition toward pure vertical transmission and host specificity. Finally, I show that

the phenotypic differences between this *A. nasoniae* strain and the male-killer associated with *N. vitripennis* are underpinned by virulence specific genetic differentiation.

Chapter 2

Incorporating Infectious Transmission into a Model of Male-killer Dynamics

Abstract:

The invasion, spread and persistence of secondary symbionts in populations of their host is dependent upon a balance between the efficiency of transmission, costs of infection and the drive of the symbiotic phenotype. Previous models have established dynamics under joint vertical and infectious transmission, but without reproductive parasitism or dynamics under vertical transmission and reproductive parasitism, but without infectious transmission. Here, I incorporate both vertical and horizontal transmission into a model of the epidemiology of the male-killer *Arsenophonus nasoniae*. I demonstrate that infectious horizontal transmission can drive the spread of the male-killer that would otherwise be unable to invade a population.

2.1 Introduction

The impact of secondary symbionts on populations of their eukaryotic host is unquestionable (Feldhaar, 2011). They drive the evolution of numerous key traits such as disease resistance, predator defense and sex determination (Oliver *et al.*, 2003; Hedges *et al.*, 2008), as well as influencing reproductive behavior (Rousset *et al.*, 1992; Charlat *et al.*, 2007). They facilitate niche expansion (Joy, 2013) and promote diversity and reproductive isolation (Bordenstein *et al.*, 2001).

Observational data indicate that the frequency of these microbes can be dynamic in ecological time, with rapid spread through populations being commonly observed, alongside rapid loss (Turelli, 1994; Hornett *et al.*, 2010; Jaenike *et al.*, 2010; Himler *et al.*, 2011). This causes them to be found at varying prevalence within their host populations (Majerus *et al.*, 1998; Charlat *et al.*, 2005; Duron *et al.*, 2008). Over evolutionary time hosts may suffer loss/gain events that are also rapid, such that sibling species rarely share identical symbionts through descent (Reuter *et al.*, 2004; Ros *et al.*, 2012). It is therefore necessary to understand the factors that allow symbionts to invade host populations and what determines their prevalence at equilibrium. One such factor, which is regularly recognised as important but rarely incorporated into models of symbiont spread is infectious transmission. In this chapter I mathematically model the spread of the male-killer *Arsenophonus nasoniae* through a population of its host *Nasonia vitripennis*. I incorporate *A. nasoniae's* ability to readily infectiously transmit, as well as potential benefits of its reproductive manipulation phenotype and direct physiological costs of infection, into its epidemiology.

2.1.1 Symbiont Dynamics

The first comprehensive attention given to symbiont population biology is that of Paul Fine (1975). He noted that the efficacy of vertical (maternal) transmission was dependent upon an interplay between vertical transmission fidelity, the strength of drive conveyed by the 'symbiotic phenotype', and the potential for compensatory horizontal or paternal transmission (Fine, 1975). This can perhaps be more succinctly boiled down to two core components: segregational loss (as a product of the net inefficiencies of transmission and direct costs of infection) and drive (undefined in his model, but may be construed as a net value of adaptive benefit or reproductive manipulation of the symbiotic phenotype and the efficiencies of transmission). The

emergent property of this interplay is in effect the basic rate of increase (*BRI*) of a symbiont within a host population, a term introduced by Randerson *et al* (2000) in their theoretical treatment of competing male-killer strains.

Segregational loss

It is rarely the case that 100% of the progeny of an infected female acquire the infection. Because of these imperfections in transmission, any otherwise neutral infection will be lost from host populations over time. Furthermore, even where transmission is perfect, simple models would predict that this would not be sufficient for the symbiont to invade the host population, but rather remain at initial prevalence unless increased or decreased through some form of stochastic or selective process (e.g. Fine 1975). Therefore we would expect commensalism to be rare if not non-existent amongst heritable symbionts. Rather, they should possess some form of additional drive mechanism that is either mutualisitic or parasitic in nature.

Inefficiencies in vertical transmission may arise through host-evolved resistance, a phenomena more likely to occur for parasitic symbioses such as reproductive manipulators. However, evidence for resistance to heritable symbionts is rare in nature (Jiggins *et al.*, 2000; Jaenike and Dyer, 2008) and targeted studies have failed to find immune activation in the face of symbiont infection (Hutchence *et al.*, 2011). This is despite evidence that symbionts can be susceptible to the immune effectors of their hosts if they are ectopically activated (Hurst *et al.*, 2003). There is evidence that suppression of the specific deleterious effects of reproductive parasites can occur (Charlat *et al.*, 2005), although this will reduce symbiont prevalence through degrading the effects of drive, rather than increasing segregational loss.

More likely, segregational loss occurs through mistakes or inefficiencies during the process of infection itself. For example, during trans-ovarial infection, cytoplasmically bound symbionts must be present in the portion of the embryo that will form the germ line. Whilst many symbionts have evolved mechanisms by which to maximize transmission (e.g. *Wolbachia* aggregates at the posterior of oocytes, in which poll cells are formed, to maximize transmission (Ferree *et al.*, 2005)) there remains the chance of loss. The risk of segregational loss is even higher for symbionts that exhibit non-ovarial or pseudo-vertical transmission. In these instances microbes are deposited on or near the offspring at the point of oviposition and obligatorily re-acquired through the gut (e.g. Huger *et al.*, 1985) or through the membrane of the developing embryo following

egg smearing (Stammer, 1929). Here, there is a greater risk of the offspring of an infected individual failing to become re-infected with the symbiont. Furthermore, there is the additional risk that once a symbiont re-infects the offspring it will be unable to migrate through its body to the necessary tissues to facilitate onwards transmission. For example, *A. nasoniae* invades developing larvae of its host *Nasonia vitripennis* through the gut. The bacterium must then make its way to the calyx gland of adult females in order to be vertically transmitted during oviposition. In this case transmission can be blocked though failure to be ingested by host larvae, failure to successfully penetrate the gut wall and failure to reach the correct adult tissue for transmission.

Where symbionts are obligate for host survival, segregational loss is not an issue. In this scenario the lack of a symbiont in any individual is analogous to a lethal mutation and so all adult hosts must possess the infection. This leads to selection on the host to transmit the symbiont with perfect, or near perfect, vertical transmission. Ultimately, segregational loss and its negative impact upon symbiont spread will be compounded by any direct cost of infection and must be compensated for by the microbe's symbiotic phenotype in order to achieve positive drive.

Cost of infection

Empirically demonstrated costs of symbiont carriage are relatively rare in the literature, with many targeted studies finding no detrimental effect of infection (Balas *et al.*, 1996; Hoffmann *et al.*, 1996; Poinsot and Mercot, 1997; Montenegro *et al.*, 2006). Notwithstanding this, there have been documented cases of direct costs. For example, *Pseudonocardia* infection in ants has been shown to increase the basal metabolic rate of their host by 10%, potentially due to an increased rate of foraging to support the symbiont population (Poulsen *et al.*, 2003). Ladybirds (*Adalia bipunctata*) carrying the reproductive manipulator *Rickettsia* suffer reduced fecundity and longevity (Hurst *et al.*, 1994). Furthermore, parthenogenesis inducing *Wolbachia* reduces competitive ability of its *Trichogramma* host (Huigens *et al.*, 2004).

Where costs are not observed it could be due to the masking effect of the symbiont's drive phenotype. This is possibly best illustrated in the case of ecologically defensive symbionts. In the presence of a parasitoid, individuals carrying symbionts such as *Hamiltonella* and *Spiroplasma* are at an adaptive advantage (Oliver *et al.*, 2003; 2008; Vorburger *et al.*, 2009). However, when the external selection pressure is absent, the

cost of maintaining a population of symbiont cells within their tissues puts them at a selective disadvantage. For example, black bean aphids (*Aphis fabae*) infected with *Hamiltonella defensa* suffer reduced longevity and lifetime fecundity in the absence of parasitoids (Vorburger *et al.*, 2013).

Costs may also originate from either pathogenicity of the microbe itself or autoimmune effects of a response against an invading symbiont (Armitage *et al.*, 2003). However, evidence for the latter is scarce, and indeed studies have failed to detect an immune response in the face of symbiont infection (Hutchence *et al.*, 2011). The former is possible, although heritable symbionts should be under selection to minimize virulence as their fitness is positively correlated with that of heir host. Studies have shown that virulence is mostly likely to manifest in novel symbioses and then be quickly removed by selection (McGraw *et al.*, 2002; Weeks *et al.*, 2007; Carrington *et al.*, 2010). Ultimately, under a framework of pure vertical transmission, the strength of the drive phenotype must be such that it is able to offset the compounded effects of segregational loss and direct costs of infection.

Drive

Drive is the force with which a symbiont spreads through the host population. Symbionts have evolved to increase their drive with phenotypes by which they overcome segregational loss and direct costs of infection. I will refer to these as 'symbiotic phenotypes'. The 'aim' of all symbiotic phenotypes possessed by maternally transmitted symbionts is to increase the fitness of infected females in the population, or to increase the rate at which they produce infected daughters. This can be achieved by either conveying some form of benefit, or by manipulating host reproduction in favor of the transmitting sex.

Secondary symbionts (non-obligate infections) achieve drive through either beneficial traits or reproductive manipulation, but are subject to costs and loss. Ecologically-contingent mutualists provide a fitness benefit in the presence of an external selection pressure, such as enemy attack, disease risk or environmental stress. For example, *Hamiltonella* reduces parasitoid induced mortality in several aphid species (Oliver *et al.*, 2003; Vorburger *et al.*, 2009); *Spiroplasma* has shown a similar defensive roll in *Drosophila,* protecting against both parasitoids (Xie *et al.*, 2010) and sterilising nematode infections (Jaenike *et al.*, 2010); and *Wolbachia* can convey resistance to viral parasites in *Drosophila* (Hedges *et al.*, 2008; Osborne *et al.*, 2012). These mechanisms

increase the fitness of infected hosts in the presence of the selection pressure they offset. Thus, models of their dynamics should incorporate a frequency dependent term addressing symbiont benefit as a function of external pressure intensity (Kwiatkowski and Vorburger, 2012). Oliver *et al* elegantly demonstrated that when symbiont transmission efficiency neared 100%, the rate of loss of a defensive microbe in the absence of selection by natural enemies was dictated by the cost of the infection alone (Oliver *et al.*, 2008). This delicate balance between cost of symbiont maintenance and the adaptive benefit of protection is hypothesized to be the reason why such symbionts are yet to be found at fixation.

Reproductive manipulation can act to increase the total number of daughters produced by an infected female. This can occur directly through feminization or induced parthenogenesis, or indirectly through male-killing and reallocation of resources from dead males to their surviving sisters. Alternatively, symbionts may increase the fitness of infected females relative to uninfected conspecifics through cytoplasmic incompatibility. The adaptive benefit of reproductive manipulation is based on the conclusion that a maternally heritable element has no 'evolutionary interest' in male hosts (Cosmides and Tooby, 1981). As discussed briefly in Chapter 1, these phenotypes are varied and may not be mutually exclusive. A single infection may posses multiple phenotypes with which it achieves drive (e.g. Hornett *et al.*, 2008; Himler *et al.*, 2011). Drive phenotypes may have additive effects on symbiont spread or may be of varying benefit depending upon the prevalence of the symbiont within the population (Hornett *et al.*, 2010).

The importance of drive, loss and cost in determining symbiont prevalence depends on the strength of each component, and the nature of the symbiosis. Drive phenotypes of secondary symbionts can be subdivided into strong and weak. A strong drive is one which has a large impact on the host individual where it is present, and occurs commonly. Feminization is a strong drive, as is natural enemy resistance where the natural enemy is common. Male-killing, being associated with resource transfer from dead male to female siblings, is likely to be a weak drive. Ultimately, the role of segregational loss and induced cost are likely to be the major determinants of symbiont prevalence where drive is weak. When drive is strong, equilibrium prevalence is less sensitive to rates of segregational loss (Jaenike, 2009).

2.1.2 Modes of horizontal transmission

In his early treaty of symbiont transmission dynamics, Fine (1975) addressed horizontal transmission as a potential route through which any deficit in vertical transmission could be offset. Later, Hurst (1993) further discussed its potential importance in the epidemiology of symbionts that killed male larvae (Hurst and Majerus, 1993). However, infectious transmission of maternally inherited symbionts is rarely incorporated into models of symbiont dynamics within a population. It is more routinely discussed in detail when assessing the spread of symbionts across disparate host species as a rare but ecologically important source of host-shift events (Engelstadter and Hurst, 2006). Ironside *et al* (2011) noted that the capacity for infectious transmission should rarely be lost in a feminizing symbiont over evolutionary time, and that its epidemiological importance will be directly related to the demographic of host populations. Studies from outside insect symbioses have demonstrated that the balance between vertical and horizontal transmission is dependent upon host growth conditions, the latter being favored in populations nearing carrying capacity or with limited growth (Kaltz and Koella, 2003).

Incidences of horizontal transmission occurring alongside vertical transmission have been documented in nature. For example, *Wolbachia* infections in parasitoid *Trichogramma* wasps exhibits horizontal transmission when two host females lay eggs in the same patch (*Lepidoptera* eggs in this case) (Grenier *et al.*, 1998; Huigens *et al.*, 2004). A similar route has been demonstrated by LbFV virus in *Leptopilina* (Patot *et al.*, 2009). Here, the wasp is usually solitary, but viral infection manipulates it into co-parasitising hosts already attacked by other females, thus increasing the rate of horizontal transmission. This indicates the importance of infectious transmission to the epidemiology of these symbionts. Therefore it is necessary to consider the importance of horizontal transmission in theoretical models of the spread of these symbionts. In spite of this evidence and Fine's initial postulations as to its importance, infectious transmission has received very little attention in models of symbiont dynamics, and models have not combined infectious transmission with mal-killing phenotypes.

2.1.3 Male-Killer dynamics

Models of male-killing drive have been based upon the principle that the death of infected males will in some way increase the fitness of their infected siblings. In this way the microbe's fitness (as transmission) is increased through a form of kin selection.

This is broadly termed fitness compensation and can be subdivided into two non-mutually exclusive theories: resource reallocation (Hurst, 1991), and inbreeding avoidance (Werren, 1987). The former will increase the fitness of the sisters of killed males, whilst the later should manifest as a fitness increase for the daughters of the sisters of killed males.

Resource reallocation is predicted to convey relatively marginal benefits (Hurst and Majerus, 1993), although a maximum of 26% increase in fecundity has been demonstrated in *Wolbachia* infected pseudoscorpions (Koop *et al.*, 2009). An analysis of Werren's (1987) model of inbreeding avoidance estimated that as little as a 5.5-5.7% fitness increase was necessary to provide sufficient drive of male-killing *A. nasoniae* in *N. vitripennis* (Balas *et al.*, 1996). However, this model presumed no direct cost of carrying a symbiont. Furthermore, whilst male-killing is perfect in some systems e.g. *Wolbachia* in *Hypolimnas bolina* (Dyson *et al.*, 2002), it is incomplete in others, such as *A*rsenophonus *nasonia* infecting *Nasonia vitripennis* (Skinner, 1985). Given the weakness of the drive, we expect male-killers that persist to exert little cost and have high vertical transmission fidelity. There is a paucity of data concerning cost, and existing studies show conflicting data (Hurst *et al.*, 1994; Jiggins *et al.*, 2002; Montenegro *et al.*, 2006). Male-killers do typically exhibit high vertical transmission efficiency (Huger *et al.*, 1985; Jiggins *et al.*, 2002), although these estimates are typically mad eunde roptimal laboratory conditons. Yet their prevalence in natural populations is highly variable, between 5-100% (Balas *et al.*, 1996; Majerus *et al.*, 1998; Jiggins *et al.*, 2002; Taylor *et al.*, 2011). This suggests that in some systems the balance of infection is highly sensitive to the interplay between cost and drive.

2.1.4 The Nasonia/Arsenophonus system

The transmission efficiency of *A. nasoniae* in its host *N. vitripennis* is high, but sub-perfect with estimates at c. 95% under ideal laboratory conditions (Huger *et al.*, 1985 and this thesis Chapter 4), and so a drive strength >5% associated with male-killing is necesary in order to achieve spread. The adaptive advantage of male-killing in this system has been postulated to lie within both the resource reallocation and inbreeding avoidance frameworks (Werren, 1987; Hurst, 1991; Hurst and Majerus, 1993; Balas *et al.*, 1996). However, studies assessing wild caught, infected females have failed to find a significant increase in fitness compared to uninfected individuals (using head width as an indicator of fecundity) (Balas *et al.*, 1996). In addition, the negative effects of

inbreeding in *N. vitripennis* have only been demonstrated after several generations of sib-sib matings (Luna and Hawkins, 2004), rather than the direct competitive benefit required by Werren's 1987 model. Therefore, the exact processes dictating *A. nasoniae* prevalence in the wild remain somewhat enigmatic.

The unusual transmission biology of *A. nasoniae*, where the bacterium infects each host generation orally (See Chapter 1.5), allows for horizontal transmission when multiple females parasitise the same host patch (Huger *et al.*, 1985). This phenomenon has been demonstrated to facilitate host-shifts of *A. nasoniae* between parasitoid species (Duron *et al.*, 2010). However, it has not been explored as an essential component of the symbiont's epidemiology within a species or population. Surprisingly, despite the observation of infectious transmission following co-parasitism being known at the time, it was omitted from Werren's (1987) initial analysis of the dynamics of the system. Balas *et al* (1996) later presented a very brief verbal model that incorporated horizontal transmission termed the 'incremental gains hypothesis'. However this model stated that infectious transmission was only adaptive to a male-killing bacterium when in competition with individuals carrying a non male-killing bacterium. Here, the beneficial drive is still reliant upon a benefit of male-killing, but infectious transmission allows it to displace a competitor infection. It does not address the more common scenario of a bacterium invading an uninfected population.

Given that estimates of co-parasitism rates in the field suggest 40% of fly pupae in birds nests are subject to co-parasitism (Grillenberger *et al.*, 2008 & 2009), there is a pressing need to examine how infectious transmission alters dynamics in this system. The aim of this chapter is to incorporate varying degrees of horizontal transmission alongside vertical transmission in simple models of *A .nasoniae* spread in populations of *N. vitripennis*. I will also explore both the potential benefit of male-killing, within a resource release framework, and the direct costs of infection to determine which factors may be predominantly affecting *A. nasoniae* prevalence.

2.2 The Model:

2.2.1 Incorporating infectious transmission

I model the dynamics of *A. nasoniae* infection over discrete generations with non-overlapping cohorts. A fraction *P* of female wasps in any generation carry *A. nasoniae* infection. Each generation, wasps are allowed to oviposit in fly pupae. A fraction a of these pupae are utilized by two female wasps that parasitise sequentially and a fraction $(1-a)$ are utilized by a single female wasp. Host pupae utilization is random with respect to *A. nasoniae* infection status, and six oviposition combinations are possible (Table 2.1).

Table 2.1 Frequency with which each oviposition scenario will occur, dictated by infection prevalence and co-parasitism rate.

#	Oviposition Scenario	Frequency
1	Single A^+	$P(1-a)$
2	Single A^-	$(1-P)(1-a)$
3	Co-lay A^+ & A^+	P^2a
4	Co-lay A^- & A^-	$(1-P)^2a$
5	Co-lay A^+ & A^-	$P(1-P)a$
6	Co-lay A^- & A^+	$(1-P)Pa$

The first *N. vitripennis* female to encounter a host pupa lays multiple offspring with a natural sex ratio bias of c. 80% female (Whiting, 1967). Females ovipositing in an already parasitized host (co-parasitism) alter their sex ratio towards parity and sometimes male bias (Ivens *et al.*, 2009) and will lay fewer offspring in the now compromised resource. Therefore, the clutch size of the first female is denoted n_1 with a sex ratio (proportion male) of r_1. The second female to oviposit produces n_2 eggs, which is a fraction of n_1, with a sex ratio of r_2.

For simplicity, the model presented here will initially assume that:

n_1 = 1st mother clutch size = 20

n_2 = 2nd mother clutch size $= 0.2n_1$

r_1 = Proportion male for 1st clutch = 0.2

r_2 = Proportion male for 2nd clutch = 1

Thus, the second female to parasitise a host will lay 4 eggs in total, all males.

Transmission of *A. nasoniae* proceeds both vertically from infected females to her daughters, and infectiously to any wasps within the pupa not infected vertically. Vertical transmission efficiency is denoted by $(1-u)$, where u is the fraction of female progeny that do not inherit the infection, and horizontal transmission efficiency is $(1-y)$. For simplicity we will assume this to be additive, so that when two infected foundresses co-parasitise a pupa (scenario 3 above), those offspring that escape infection through inefficient vertical transmission from their mother are exposed to horizontal transmission from the other foundress. Infection is considered to be chronic throughout an individual's lifetime with no loss once infected. All female wasp individuals are assumed to be susceptible to infection, which accords with the absence of evidence for resistance to male-killing in the *N. vitripennis/A. nasoniae* system. This produces flows of infection pictured in Figure 2.1.

The presence of *A. nasoniae* induced male-killing is determined by maternal infection status, and this combines with the number of foundress wasps, and their order of oviposition, to determine the total number of larvae in a host (Table 2.2). It is a logical assumption that the number of larvae in a host impacts on the fitness of those larvae, with larvae subject to crowding having reduced fitness and those released from crowding through male death having higher fitness. Where two A- females co-parasitise, the hosts contain (n_1+n_2) wasp larvae which for our clutch sizes above results in a 20% increase in within-pupa density. Therefore, costs of crowding are denoted by $(1-S)$, where S is confined to a maximum of 0.2 in models presented here. Male-killing is considered to be perfect in our model, so that in scenario *1* and *3,* where male are killed by the bacterium and not replaced by the sons of uninfected females, there is a benefit of alleviated crowding below n_1 denoted by $(1+k)$. Biologically speaking, it is unlikely that costs and benefits exactly offset one another. The fitness cost of crowding is likely to be density dependent. That is, fitness loss through crowding above an optimum threshold is likely to be greater than the fitness gained by killing males and lowering density below this threshold. Therefore, we set the constraint that $S>k$ (For the special condition where $S=k$ see appendix). Assumptions of the model are summarized in Table 2.3, and parameters (and their likely values) summarized in Table 2.4.

Table 2.2: Proportion of offspring contributed by each oviposition scenario, weighted by fitness. Terms in the column Cost/Benefit, assuming $n_2 = 0.2n_1$, $r_1 = 0.2$ and $r_2 = 1$ are incorporated into the model.

#	Scenario	1st Clutch	2nd Clutch	Total Offspring	Cost/Benefit
1	A^+ Single	$n_1(1-r_1)$	$-$	$n_1(1-r_1)$	$(1+k)$
2	A^- Single	n_1	$-$	n_1	1
3	A^+A^- Co-lay	$n_1(1-r_1)$	n_2	$n_1(1-r_1)+n_2$	1
4	A^-A^+ Co-lay	n_1	$n_2(1-r_2)$	$n_1+n_2(1-r_2)$	1
5	A^+A^+ Co-lay	$n_1(1-r_1)$	$n_2(1-r_2)$	$(n_1(1-r_1))+(n_2(1-r_2))$	$(1+k)$
6	A^-A^- Co-lay	n_1	n_2	n_1+n_2	$(1-S)$

Table 2.3: Biological assumptions of the model and their ecological validity

Assumption	Validity
No host resistance to infection.	No direct study but resistance to male-killers is exceptionally rare (e.g. Jaenike and Dyer 2008)
A. nasoniae infection status does not affect chance of wasp parasitizing a host, nor whether it chooses to single/co-parasitise.	Experiments show no obvious impact of *A. nasoniae* on parasitism choice (unpublished data).
Males are ubiquitous and therefore all females are mated.	Fair when there are many pupae within a nest and prevalence is low. May be violated when *A. nasoniae* prevalence is high.
2nd host female lays only males.	Incorporated for simplicity in initial models. Expanded upon later.
Male-killing complete.	Sex ratio produced by infected mothers is 0-5% male. Variables explored later.
Wasp population size and probability of co-parasitism independent of *A. nasoniae* prevalence in population.	Because *A. nasoniae* affects male host viability, approximately true.

Table 2.4 Parameters and variables used in the above model.

Parameter:	Description:	Notes:
P	Proportion of females infected with *A. nasoniae*	$0 \leq P \leq 1$
a	Rate at which *N. vitripennis* co-parasitise host pupae.	$0 \leq a \leq 1$
$(1-u)$	Vertical (maternal) transmission efficiency of *A.nasoniae*	0.95
$(1-y)$	Horizontal (infectious) transmission efficiency of *A. nasoniae*	0.95 $(unless\ otherwise\ stated)$
S	Cost of crowding when co-parasitism occurs.	$k < S \leq 0.2$
k	Benefit of male-killing through resource reallocation.	$0 \leq k < S$
j	Direct costs of infection incurred by all *A.nasoniae* infected females	$0 \leq j \leq 1$
n_1	Clutch size produced by first female *N. vitripennis* parasitizing a host pupae.	20
n_2	Clutch size produced by second female *N. vitripennis* parasitizing a host pupae.	4
r_1	Sex ratio (proportion male) of first clutch laid in a pupae,	0.2
r_2	Sex ratio (proportion male) of the second clutch laid in a pupae.	1

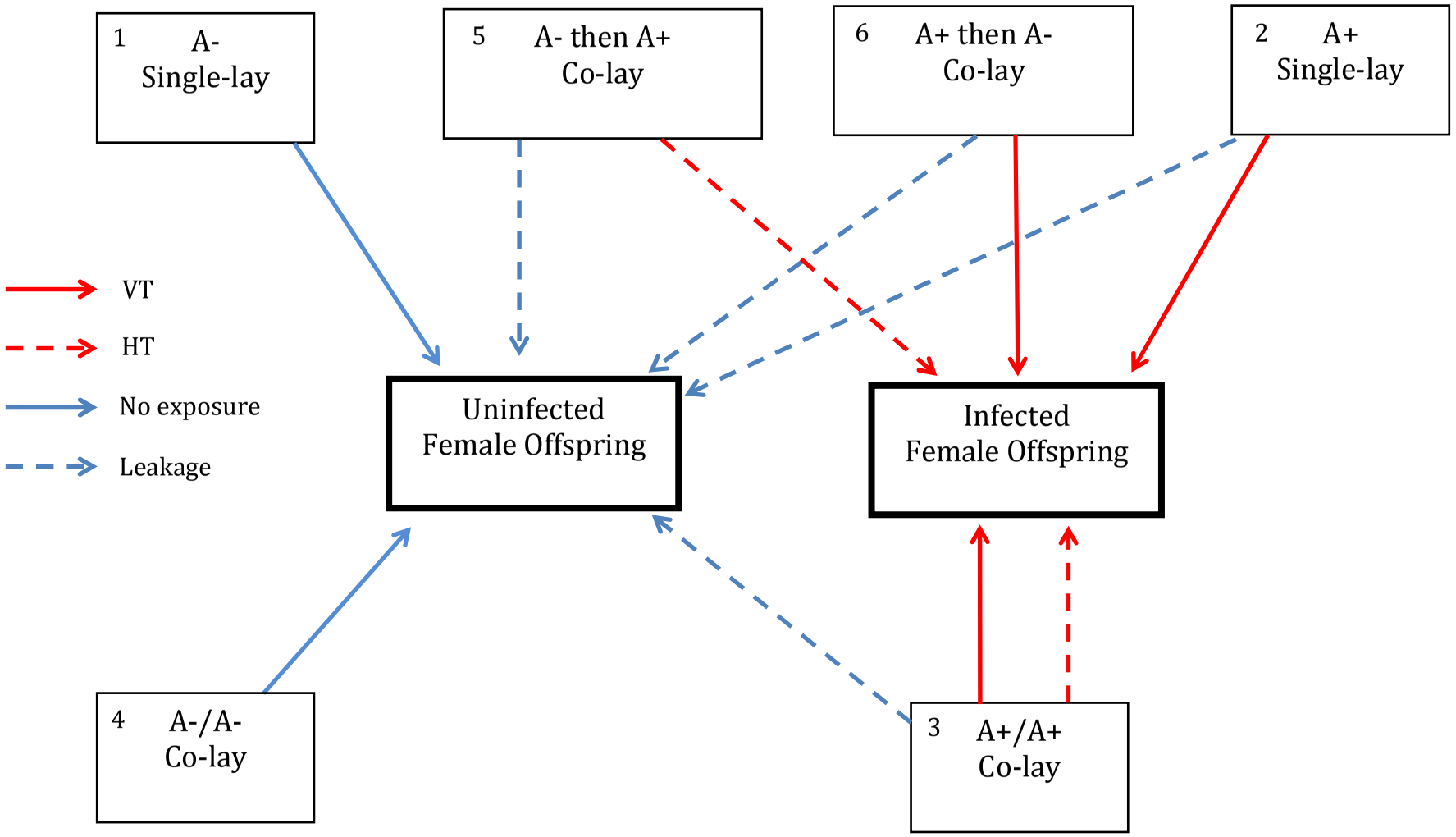


Figure 2.1: Potential contributions of each of the six oviposition scenarios to the number of infected and uninfected females in the next generation. Note that in the scenarios described here, the second female lays only male offspring.

For each scenario we combine the effects of the parameters for co-parasitism rate (a), transmission efficiency $(1-u)$ & $(1-y)$, benefits $(1+k)$ and costs $(1-S)$ with the variable P for proportion of individuals infected to generate the equations given in Table 2.5.

Table 2.5 Each scenario's contribution towards infected and uninfected individuals in the next generation.

#	Scenario	A+ Daughter Production	A- Daughter Production
1	A^+ Single	$P(1-a)(1-u)(1+k)$	$Pu(1-a)(1+k)$
2	A^- Single	-	$(1-P)(1-a)$
3	A^+A^- Co-lay	$P(1-P)a(1-y)$	$P(1-P)ay$
4	A^-A^+ Co-lay	$P(1-P)a(1-u)$	$P(1-P)au$
5	A^+A^+ Co-lay	$P^2a(1-uy)(1+k)$	$P^2auy(1+k)$
6	A^-A^- Co-lay	-	$(1-P)^2a(1-S)$

These equations combine to give the normalized recursion equation for the flux of infection between generations:

$$P' = \frac{P + Pk - Pu - Puk + Pa - Pak + Pauk + P^2ak - P^2uya - P^2uyak - P^2a + P^2au - Pay + P^2ay}{Pk + 1 - Pak + P^2ak - aS + 2PSa - P^2Sa}$$

Which can be solved for $\Delta P = 0$ to give the equilibrium condition:

$$\hat{P} = \frac{a + k - 2ak + 2aS - au - a - ay + auy + akuy - \sqrt{4a(k-S)\big(k - u - ku + a(1 + S + k(-1+u) - y)\big) + \Big(k + a\big(2S + (-1+u)(-1+y) + k(-2+uy)\big)\Big)^2}}{2a(k-S)}$$

Where $a = 0$ (i.e. there is no co-parasitism, and only vertical transmission occurs) the simpler recursion equation below applies:

$$P' = \frac{P + Pk - Pu - Puk}{Pk + 1}$$

And equilibrium prevalence is given by:

$$\hat{P} = \frac{-(u - k + ku)}{k}$$

2.2.2 Incorporating direct cost of infection to offspring number

The model presented thus far only incorporates the cost of crowding (due to co-parasitism) and the benefit of male-killing in alleviating any crowding and resource re-allocation. As argued previously, symbionts may additionally have direct costs, either as an indirect consequence of growth and transmission or an adaptive pathology. Now we incorporate the term $(1 - j)$ where *j* is the direct physiological cost of carrying a symbiont. Thus this term is applied to all equations denoting the proportion of infected individuals produced by a given scenario. The value of *j* must be between 0 (cost free) and 1 (produces complete clutch death/sterility). This alters daughter production according to the schedule in Table 2.6.

Table 2.6. Each scenario's contribution towards infected and uninfected individuals in the next generation including the cost term *j* to denote the effect of direct physiological cost of infection

#	Scenario	A+ Daughter Production	A- Daughter Production
1	A^+ Single	$P(1-a)(1-u)(1+k)(1-j)$	$Pu(1-a)(1+k)$
2	A^- Single	-	$(1-P)(1-a)$
3	A^+A^- Co-lay	$P(1-P)a(1-y)(1-j)$	$P(1-P)ay$
4	A^-A^+ Co-lay	$P(1-P)a(1-u)(1-j)$	$P(1-P)au$
5	A^+A^+ Co-lay	$P^2a(1-uy)(1+k)(1-j)$	$P^2auy(1+k)$
6	A^-A^- Co-lay	-	$(1-P)^2a(1-S)$

These equations give the normalized recursion equation:

$$P' = \frac{-(P(j-1)(a+k-u-Pa-ak-ay-ku+Pak+Pau+Pay+aku-Pauy-Pakuy+1)}{Pk-Pj-Sa+2PSa-Paj-Pak-Pjk+Pju-P^2Sa+P^2aj+P^2ak-P^2ajk-P^2aju-P^2ajy+Pajk+Pajy+Pjku-Pajku+P^2ajuy+P^2ajkuy+1}$$

Equilibria under these conditions are given in the Appendix.

Where there is no co-parasitism, the recursion equation is simplified to:

$$P' = \frac{P-Pj+Pk-Pu-Pjk+Pju-Pku+Pjku}{Pk-Pj-Pjk+Pju+Pjku+1}$$

And equilibrium prevalence is given by:

$$\hat{P} = \frac{-(j-k+u+jk-ju+ku-jku)}{(k-j-jk+ju+jku)}$$

2.3 Results

I first analyse the scenario in which *N. vitripennis* only parasitizes hosts in isolation (single-parasitism). The spread of *A. nasoniae* is sensitive to variation in both the strength of drive (as a resource reallocation benefit of male-killing: k) and the degree of direct costs associated with infection (j) (Figure 2.2). Under single parasitism, when male-killer benefit is at an ecologically realistic maximum (15%), a costly bacterium is unable to invade when the direct cost of infection exceeds 8%.

When co-laying is allowed without the possibility of infectious transmission, the conditions for invasion are broadened, and equilibrium infection prevalence increased (Figure 2.3). This is associated with male-killer infected individuals avoiding the cost of crowding in co-parasitised hosts. Nevertheless, the conditions for invasion remain restrictive. Under the 'best field estimate' of multiparasitism rates (40%, (Grillenberger *et al.*, 2008)), invasion is not possible if the direct cost of infection exceeds 3.55%. This represents the 'best scenario' for *A. nasoniae* invasion, as benefits of male-killing from reduced crowding are on the upper end of those that are biologically plausible (k=0.08).

When horizontal transmission of *A. nasoniae* is permitted and co-parasitism is common, highly costly infections can spread (Figure 2.4). The strength of horizontal transmission is such that it is able to drive invasion even in the absence of any male-killing derived drive (Appendix Figure 2.2). Further, equilibrium prevalence is very high even when co-parasitism is relatively rare ($a = 0.2$). Infectious transmission during co-parasitism also allows the symbiont to invade populations from very low starting prevalence (Figure 2.5, *c, d, e*). When there is no route for infectious transmission, *A. nasoniae* is unable to invade from any starting prevalence (Figure 2.5, *a*).

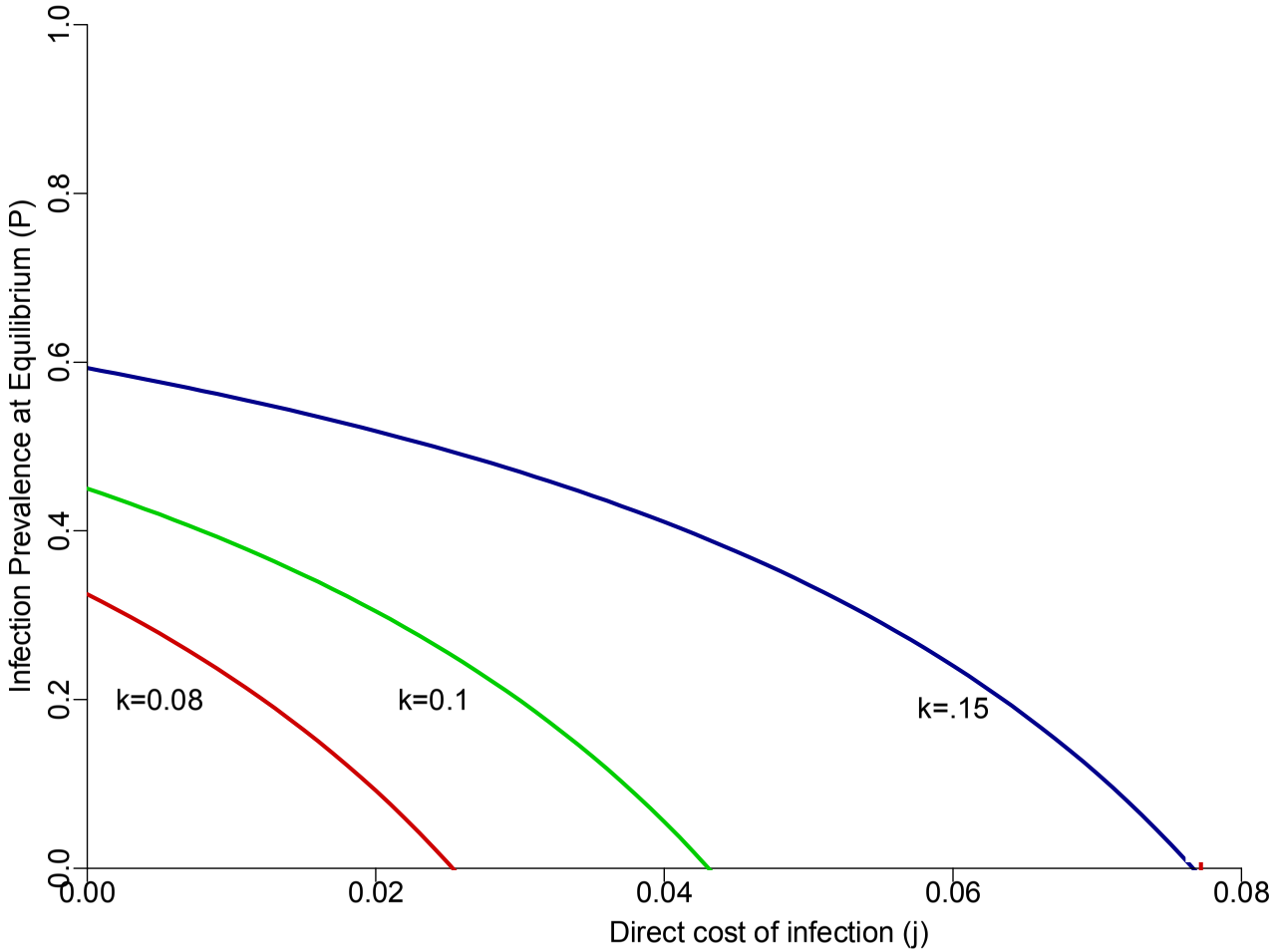


Figure 2.2: Infection equilibrium of *A. nasoniae* in populations of *N. vitripennis* with no co-parasitism ($a = 0$) and varying degrees of male-killer derived benefit and symbiont induced cost. Segregational loss is set at $u = 0.05$. The lines represent scenarios with differing degrees of male-killer benefit through resource reallocation.

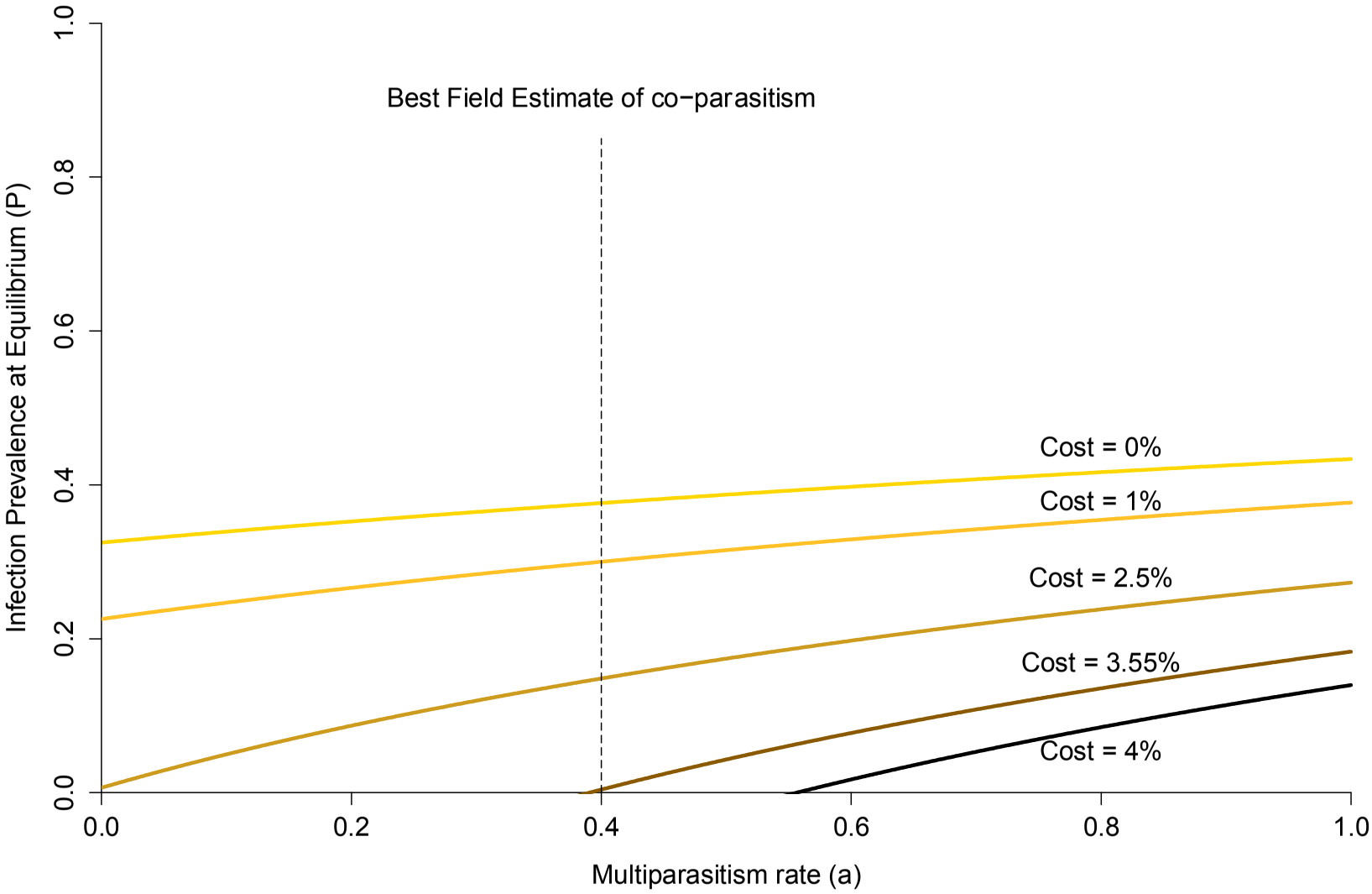


Figure 2.3 Equilibrium prevalence of *A. nasoniae* in populations where co-laying occurs, but only vertical transmission is permitted ($(1-y)=0$) under different levels of multiparasitism. The coloured lines represent infections with symbiont elicits different direct costs. In all scenarios the cost of crowding S is set to 10%, whilst the benefit of male-killing through alleviation of crowding (k) is 8%. *A. nasoniae* can invade populations to a maximum of c40% prevalence when it exerts no direct cost to the host.

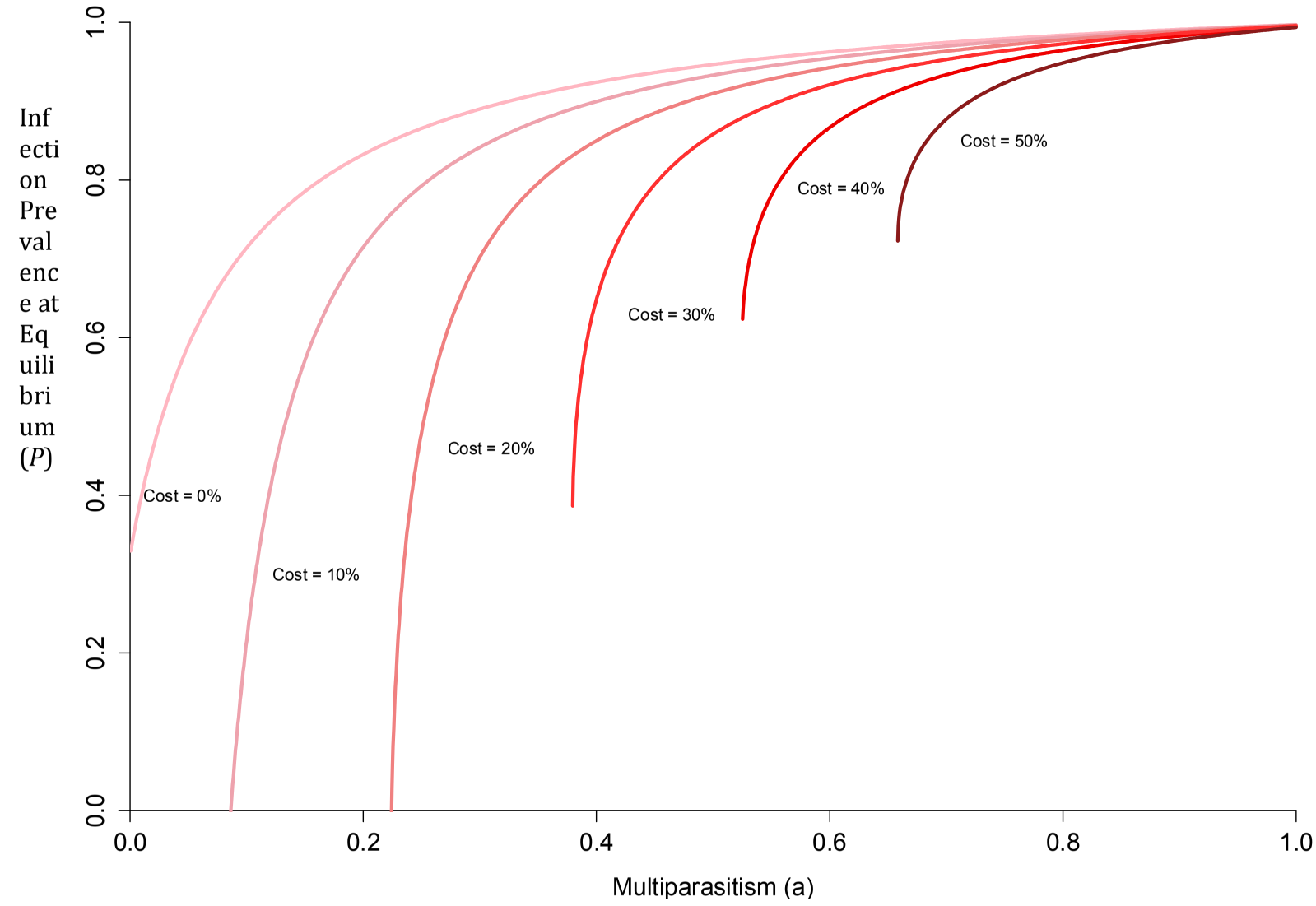


Figure 2.4: Equilibrium prevalence of *Arsenophonus* with horizontal transmission efficiency during co-parasitism set at the ecologically valid value of 95%. Coloured lines represent symbiont strains of different direct costs. Note that even highly costly strains (50%) can be driven to high prevalence when infectious transmission is permitted (darkest line). All scenarios here are subject to a cost of crowding of 10% (S = 0.1) and a benefit of crowding alleviation of 8% (k = 0.08). Transmission efficiencies of both vertical and horizontal transmission are 95%.

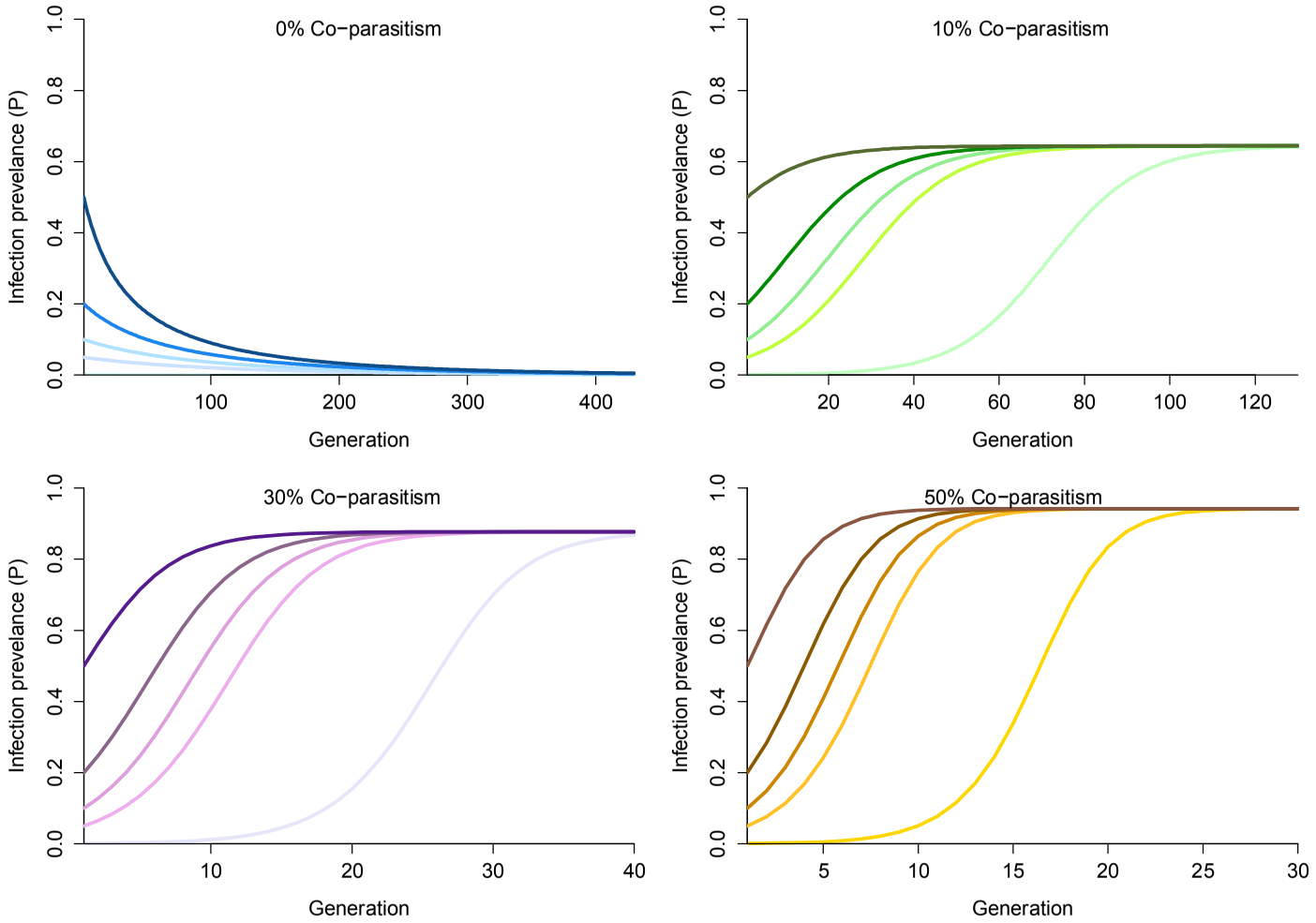


Figure 2.5: Model simulations of symbiont invasion in population of *N. vitripennis* starting from varying degrees of prevalence (coloured lines, light – dark: 0.005%, 5%, 10%, 20% and 50% starting prevalence) and varying degrees of population level co-parasitism and therefore infectious transmission (Blue = 0%, Green = 10%, Purple= 30%, Gold = 50% co-parasitism). Note the difference in x-axis scale. Here, the direct cost of infection is set at -5%, benefit of male-killing at 8% and cost of crowding at 10%. When horizontal transmission is permitted *A.nasoniae* can invade from very rare, the final prevalence being dictated by the level of co-parasitism (See figure 2.4). The intensity of co-parasitism also dictates the speed at which the infection reaches equilibrium. Infection is lost when co-parasitism is absent.

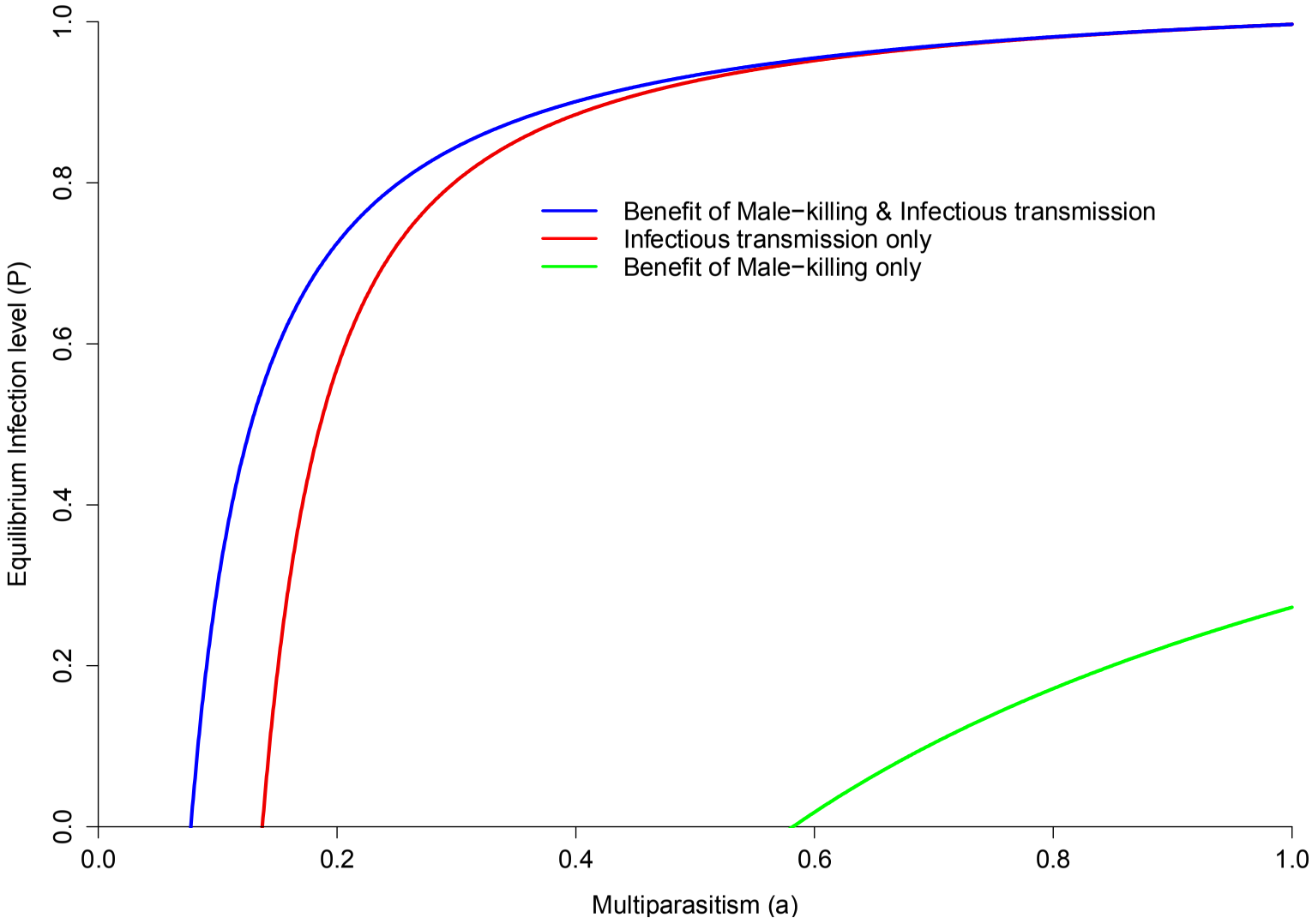


Figure 2.6: Equilibrium prevalence of three *Arsenophonus* strains at varying levels of host multiparasitism. Note that infectious transmission alone has a far greater positive influence on symbiont prevalence at equilibrium than the benefit of male killing. However the combination of the two factors allows symbiont invasion across a larger range of parameters. In these scenarios the direct cost of infection (j) is set at 10%

2.4 Discussion:

Existing models of male-killer spread are based on fitness compensation mechanisms acting through either resource reallocation or inbreeding avoidance. The biased primary sex ratio produced by *N. vitripennis* under single parasitism, 80% female, provides an upper limit to the fitness compensation benefits that could occur through male-killing. Death of males can increases resources available to females by a maximum of 25% per individual female *N. vitripennis* larva. Given resource reallocation is not equivalent to fitness (doubling resource does not double fitness), the fitness benefit of male-killing is likely to be much lower than a 25% gain, which would require absolute correspondence of resource to fitness. Benefits from inbreeding avoidance are also likely to be limited, primarily because, as a haplodiploid species, deleterious recessives are exposed and purged in male individuals. Laboratory measurement suggests that costs of inbreeding depression are only measureable after several generations of inbreeding (Luna and Hawkins, 2004), and so do not manifest between differentially infected broods as is required by Werren's (1987) model.

Given observed segregational loss and estimates of infection prevalence in the field, Balas *et al* (1996) concluded that a benefit from male-killing of 5.5-5.7% was required to drive *A. nasoniae* into a population according to Werren's (1987) model. This estimate required the infection to be otherwise cost-free. In the presence of a cost, the model developed in this chapter suggests invasion under single parasitism will occur rarely. For 8% fitness compensation (a high level), invasion requires a direct cost of <2.5% (Figure 2.2 [red line]).

The model presented here suggests that the addition of co-parasitism, and associated infectious transmission, broadens both the conditions for invasion of *A. nasoniae*, and the equilibrium prevalence achieved. Male-killing is commonly not sufficient for *A. nasoniae* persistence, and the dynamics of *A. nasoniae* is dominated by infectious transfer following co-parasitism. Under this model, male-killing may still be beneficial and adaptive, but is simply not the primary contributor to the drive of *A. nasoniae* dynamics.

One feature of the model is that for the co-parasitism rates recorded in past studies (40% - Grillenberger *et al.*, 2008), equilibrium prevalence of *A. nasoniae* lies between

50-90%. This prevalence is not observed in natural populations. In Europe, *A. nasoniae* prevalence appears to be around 15-20% (C. Frost, *Pers comms*). Thus, the model does not perform well in predicting *A. nasoniae* frequency in the field. One possibility is that infectious transmission rates measured in the laboratory are not achieved in the field. Laboratory estimates of transmission efficiency only focus on specific fly-hosts, usually *Sarcophagia spp* (e.g. Skinner 1985; Balas *et al*, 1996). In nature, *Nasonia* is a generalist parasitoid, and so the species of fly-host may well impact upon transmission as the bacterium must survive and replicate in the fly pupae before orally infecting the wasp. Alternatively, co-parasitism rates may vary over the landscape. This will be high in the birds' nests studied by Grillenberger, where *N. vitripennis* is the dominant parasitic wasp, but lower on filth fly hosts in agricultural landscapes, where it is a minority species (Darling and Werren, 1990; Klunker, 1994; Carvalho *et al.*, 2005). Thus the prevalences reported in exisiting literature may well be highly biased by the sampled soure and wasp movement across this patchy landscape produces prevalence values higher than expected in agricultural landscapes and lower than expected in birds' nests.

A second possibility is that there are additional costs to carrying *A. nasoniae* that prevent the symbiont reaching the equilibrium prevalence predicted by this model. One such cost may be from virginity. Given that *N. vitripennis* females typically mate with their brood-mates upon emerging as adults, it is necessary to factor in female virginity in infected clutches as an additional fitness parameter. Where all females that lay in a pupa are infected with *A. nasoniae*, there may be no males available for local mating. In the six oviposition scenarios detailed previously (table 2.1) the uninfected females will provide the necessary males to offset any virginity cost in scenarios *2*, *3*, *4* & *6*. Therefore it is only necessary to add virginity terms to scenarios *1* and *5*; oviposition by a single infected female and co-parasitism by two infected females. The cost to *A. nasoniae* from female host virginity will depend on male-killing efficiency, and the possibility that females that are unmated locally may acquire a mate derived from other pupal hosts. Male-killing efficiency by *A. nasoniae* in *N. vitripennis* is difficult to accurately estimate as uninfected clutches contain so few males (Whiting, 1967). However, estimates of 80-95% have been postulated in the literature (Skinner, 1985) and it is certainly the case that *A. nasoniae* infected females commonly lay all female broods (pers. obsn). The probability of any given male from an infected brood escaping male-killing can be stated as the Poisson term d^m, where d= individual male survival, m= the number of males in a brood. So, for a brood founded by a single mother the probability of at least one male surviving is $d^{(r1n1)}$, and a co-lay brood is $d^{(r1n1)+(r2n2)}$.

Here, this is fixed at $d^{r1n1} = 0.8^4$ and $d^{(r1n1)+(r2n2)} = 0.8^8$. There is also the chance that females not mated by their brood mates will be mated by males migrating from other broods. Because *N. vitripennis* males are flightless, males will need to derive from other broods within the patch. Birds' nest environments vary in the number of foundresses, and the number of pupae parasitized. There is also unknown synchronicity of wasp emergence from different pupae. Modeling this process is difficult. What is clear is the number of males in a patch will be dependent upon the prevalence of global infection (*P*). As prevalence rises, so the chance of being mated by the son of another female declines. This will create a frequency dependent cost, which would limit the spread of *A. nasoniae.*

Increasing virginity with increasing prevalence also suggests an interesting ecological dynamic. The act of male-killing imposes a demographic stress onto the host population through skewing the operant sex ratio towards males. In extreme cases of fixed, perfect male-killing this will lead to global virginity through high sex ratio skew and ultimately host extinction (Hamilton, 1967; Hatcher *et al.*, 1999; Groenenboom and Hogeweg, 2002; Price *et al.*, 2010).

Further modeling work should place the ecological dynamics outlined here in an evolutionary framework. In the model presented, parameters such as the cost of infection are static. Whilst uniparental inheritance prevents mixing of *A. nasoniae* strains and any competition, infectious transmission increases the frequency with which mixed *A. nasoniae* infections exist. As this occurs, there may be selection for *A. nasoniae* to prevail under within-host competition, which may select for strains with higher cost. Thus the ecological dynamics of *A. nasoniae* select on cost, which then feeds back into ecological dynamics. The development of models that combine evolutionary and ecological dynamics, as provided in the adaptive dynamics framework, would be appropriate.

The model presented here represents an extremely simplistic view of the system. Most notably I have worked on assumptions relating to clutch size and sex ratio which are not necessarily ecologically valid. For example I have assumed that 2nd females to parasitise a pupae lay only males and that male-killing is complete. The purpose of these assumptions was to simplify the mathematics needed to explain the costs of crowding. In effect removing the cost of crowding when co-parasitism occurred between A- and A+ mothers (Oviposition scenarios 3 & 4). A more realistic approach

would be to add an intermediate cost to these scenarios or to make cost (*S*) directly dependent upon the number of developing wasps in a fly pupae rather than the fixed values used here. These enhancements should be incorporated into future work on this model, however their impact upon the adaptive benefit of male-killing are likely to be very marginal and certainly not strong enough to overcome the overriding effect of infectious transmission (See Figure 2.6). Furthermore, I have constructed this model on the assumption that male-killing efficiency is 100%, when estimates from the field and laboratory put it at 80-95% (Skinner 1985; Chapter 3 of this thesis). However, given *Nasonia's* highly female biased sex ratio under single parasitism, these rates of male-killing can regularly produce all-female broods from a single clutch (personal observation). Furthermore, the purpose of this model is to demonstrate that infectious transmission is a more important determinant of symbiont prevalence than the benefit of male-killing. Thus setting male-killing efficiency at 100% actually overestimates its importance and thus only serves to further demonstrate its inadequacy in the absence of infectious transmission.

Chapter 3

Fitness consequences of *Arsenophonus nasoniae* infection in *Nasonia vitripennis*

Abstract:

Maternally inherited microbes are selected to minimize the costs they incur upon their hosts in order to maximize their vertical transmission. Here I compare aspects of *Nasonia vitripennis* development, size, fecundity and dispersal ability in the presence or absence of *Arsenophonus nasoniae*. I conclude that male-killing *A. nasoniae* causes multifaceted costs on its host in terms of fecundity, size and development. These costs overwhelm any benefit from resource release and make vertical transmission likely to be ineffective as a sole means of propagation of the symbiont. They may also impact upon the normal population biology of the host in nature by limiting dispersal.

3.1 Introduction

The fitness of maternally inherited symbionts is dependent upon the longevity and fecundity of infected females in their host population. To this end symbionts should evolve to minimize any costs they may impose by infecting their host's tissues. Indeed, selection acts quickly to reduce symbiont-induced costs in newly formed heritable symbioses (McGraw *et al.*, 2002; Carrington *et al.*, 2009; 2010). However, some unavoidable baseline costs will remain even after selection. These can be direct, associated with the metabolic demands of a resident symbiont population on host resources, or indirect, through the evolutionary consequences of the symbiotic phenotype (See Chapter 1, section 1.3 for examples relating to reproductive parasites). Where unavoidable costs of symbiont infection occur, compensating drive mechanisms are required for the symbiont to invade. Indeed, the established paradigm is that heritable symbiont prevalence is dictated by a delicate balance between; segregational loss, direct costs of symbiont carriage outside of manipulation phenotypes, and the efficiency of the drive phenotype. Randerson *et al* (2000) define the interaction between these factors as producing the Basic Rate of Increase (*BRI*) of the symbiont.

Here I discuss the relative costs imposed by the male-killer *Arsenophonus nasoniae* on its host *Nasonia vitripennis,* and how these costs may be offset through the adaptive benefit of male-killing. I empirically show that male-killing *A. nasoniae* inflicts a multi-faceted cost on *N. vitripennis* and discuss how this demands that we re-evaluate the adaptive significance of male-killing by *A. nasoniae*.

3.1.1 Drive of male-killers

The evolutionary basis for male-killing as a drive phenotype has been explained in terms of fitness compensation, a form of kin selection where the death of infected male embryos benefits the bacterium infecting their surviving sisters (Hurst and Majerus, 1993). Fitness compensation is thought to operate in one of two ways: resource reallocation (Hurst, 1991) and inbreeding avoidance (Werren, 1987). Resource reallocation occurs when sibling competition is high. Here the death of males can release vital resources to their infected sisters either through direct cannibalism or lowered competition for food (Hurst, 1991). Evidence for resource release in nature comes from studies of brood cannibalism across Coccinellidae beetles (Hurst and Majerus, 1993; Majerus and Majerus, 2012) and embryonic competition in live-bearing

pseudoscorpions (Koop *et al.*, 2009). The magnitude of benefit conferred to infected females is dependent upon the number of males being killed and the proportion of freed resources that are then assimilated by the females. Therefore, the fitness gains in real terms are likely to be relatively marginal. Alternatively, Werren (1987) proposed that the fitness compensation of male-killing may manifest by reducing inbreeding (Werren, 1987). If infected mothers only produce females, then their offspring are forced to outcross and so should produce F2 females with a lower chance of suffering from inbreeding depression. However, empirical evidence for this is lacking. Both theories of male-killing should only provide weak drive (See Chapter 2.1) and so are highly sensitive to symbiont induced costs/benefits.

3.1.2 Direct costs/benefits of symbiont carriage

The presence of symbionts may have a direct positive or negative effect on female performance. Infections may exert a metabolic cost to the host, as even well co-evolved symbionts will have to sequester nutrients from the host environment. They may also induce pathogenic reaction (particularly in novel host-symbiont interactions), arising from the microbe's interaction with host tissues or through the mode of its transmission. If these costs are greater than the benefit of male-killing, then the microbe should not spread. Further, it is possible that symbionts directly contribute to female host fitness. Recently, a sex ratio distorter in *Bemisia tabaci* whitefly was observed to increase host performance, aiding its spread across the Western US (Himler *et al.*, 2011). Thus it is important to consider symbiont induced costs and benefits, aside those arising from its manipulative phenotype, when addressing symbiont spread.

Only two studies have investigated the direct costs of male-killer infection. Montenegro *et al* (2006) looked for, and failed to find, any direct cost or benefit in terms of fecundity or longevity of male-killing *Spiroplasma* in its *D. melanogaster* host (Montenegro *et al.*, 2006). Conversely, male-killing *Rickettsia* infection in *Adalia bipunctata* was shown to decrease fecundity and longevity, although not larval survival (Hurst *et al.*, 1994). Evidence for the cost of carrying non-male-killing reproductive parasites varies between studies. No negative consequences of CI-inducing *Wolbachia* infections in *D. simulans* were found in targeted experimental studies (Hoffmann *et al.*, 1996; Poinsot and Mercot, 1997). However, in other symbiont-host complexes physiological costs have been found (Hoffmann *et al.*, 1990; Fleury *et al.*, 2000; Champion de Crespigny and

Wedell, 2006; White *et al.*, 2010). In all of these cases the strong CI phenotype is enough to enable persistence despite the cost imposed.

Direct physiological costs of symbiont infection are most overt after a host shift event, where the symbiont is in contact with a host with which it has not co-evolved (Clancy and Hoffmann, 1997; Russell and Moran, 2005; Kageyama *et al.*, 2006b). Long-term studies of novel symbionts have shown evidence that strict titre control is the key to attenuating these costs by selection. Popcorn *Wolbachia* (*w*MELPop) over-replicates in its native host *D. melanogaster*, shortening adult lifespan (Min and Benzer, 1997) and reduces longevity and fecundity when artificially trans-infected into *D. simulans*. However, following trans-infection, fecundity costs were attenuated after twenty generations (McGraw *et al.*, 2002) and longevity effects were reduced after two hundred generations (Carrington *et al.*, 2009). A similar evolution of pathogenicity attenuation has been observed in natural *Wolbachia*/*D. simulans* associations where fecundity effects were shifted from a 20% deficit to a 10% increase over 20 years of co-evolution (Weeks *et al.*, 2007). This demonstrates a capacity for symbionts to evolve from a directly costly phenotype to a mutualistic one. Although these examples do not come from incidences of male-killers they highlight that whilst direct physiological costs do exist, they are relatively rare and under negative selection.

3.1.3 Arsenophonus /Nasonia interaction

As argued previously, both resource reallocation and inbreeding avoidance represent possible benefits to male-killing in *Nasonia*, but both are limited in their potential strength of drive. Any direct cost/benefit of infection would therefore be an important contributor to symbiont dynamics. The single paper to address the issue of cost empirically examined the size of *N. vitripennis* females taken from the wild. The authors measured head size as an indicator of body size which has been demonstrated to correlate positively with fecundity (O'Neill and Skinner, 1990; Balas *et al*., 1996). No significant difference between the size of infected and uninfected individuals was observed. However, taking only a single measurement of fitness from natural populations is subject to severe variability and bias and thus is not sufficient evidence to discount the hypothesis that *A. nasoniae* infection will affect fitness.

There are reasons to believe that *A. nasoniae* may have direct physiological costs. First, the obligate per-oral infection route at each generation involves transit across the gut

wall. This is likely to incur a greater cost than the more common vertical transmission mechanisms where the bacterium passes through the egg. Costs may manifest due to disruptions in the resident gut microbiota of the insect, a trait strongly linked to viability in *N. vitripennis* (Brucker and Bordenstein, 2013), or by actively penetrating the gut wall to gain access to tissues and allowing opportunistic pathogens access to the host. Secondly, this unusual route of transmission is more likely to generate competing co-infections of different *A. nasoniae* strains in the same host. This would potentially establish competing infections within a wasp host that may select for higher replication, titre and thus virulence.

In this chapter, I measure the key life-history traits of time to eclosion, clutch size, clutch sex ratio and adult body size to test the hypothesis that *N. vitripennis* infected with *A. nasoniae* suffer direct costs of infection. I also score wing deformities in female offspring from infected and uninfected mothers as a metric for dispersal ability and development quality, a key component of fitness.

3.2 Methods

3.2.1 Establishing Arsenophonus nasoniae infected Nasonia vitripennis lines

Infected wasp lines were established by inoculating a male-killing *Arsenophonus nasoniae* strain isolated from wild female *Nasonia vitripennis* caught in Lethbridge, Alberta, Canada (Taylor *et al.*, 2011) into laboratory isofemale lines of *N. vitripennis*. To this end, single bacterial clones were grown up from glycerol stocks maintained at the University of Liverpool and plated as lawns using GC base medium (Difco) supplemented with 3ml/L IsoVitalex (Applied Biosystems). These clone lawns were washed from media plates, suspended in PBS and diluted by a factor of 10 (resulting in c300 CFU/ml).

Recipient fly pupae were then surface sterilized with 70% EtOH before being inoculated with 2µl of the suspension between the 3rd and 4th pupal segment using pulled glass micro lances. Pupae were kept in a sterile Petri dish for 15 minutes post-injection to allow coagulation at the wound site. Single, uninfected *N. vitripennis* females were then allowed to oviposit into these pupae. Upon emergence, F1 offspring were allowed to mate within their broods (or had additional uninfected males introduced if none were present) before individual females were given a host pupae in which to oviposit. F2 offspring from these lays were then assessed for both sex ratio and infection status by PCR analysis, and '*A+*' infected stocks established from the siblings of any individuals that screened positive for *Arsenophonus*. Screening for infection was left until the F2 generation to account for any failures to transmit through the wasp line and to ensure that the male-killing phenotype was present. Parallel uninfected lines were established with sham-injection controls (sterile PBS inoculum).

3.2.2 DNA extraction and infection screening method by PCR

Other than clutch sex ratio there are no consistent phenotypic indicators that an individual *Nasonia vitripennis* is infected with *A. nasoniae*. Thus infection status was determined through diagnostic PCR for *Arsenophonus* specific genes.

DNA extraction on *N. vitripennis* was performed using Chelex 100 resin beads with an overnight incubation at 30^{o}C (Walsh *et al.*, 1991). Whole individual wasps were added to 49ul of Chelex suspended in ddH_2O and 1ul of 100g/l^{-1} protinase-K suspended in 1M Tris-HCl (pH 7.5) in a 1.5ml centrifuge tube and homogenized with a sterile pestle until

insect tissues were ruptured. Samples were then centrifuged for 10s at 600rpm to aggregate the contents to the bottom of the tube and placed in a water bath set at 30°C overnight. The following day samples were exposed to 95°C+ for ten minutes in a waterbath or heat block to denature the proteinase-K before being centrifuged as described above. If samples were not used for PCR directly, they were stored at -80°C.

The quality of DNA extractions was verified for each sample by amplifying a portion of the insect cytochrome oxidase mitochondrial gene (CO1), (Primers: **LCO**. 5' GGT CAA CAA ATC ATA AAG ATA TTG G 3, **HCO**. 5' TAA ACT TCA GGG TGA CCA AAA AAT CA 3'; 35 reaction cycles, 52°C annealing temperature, 30s extension time, (Folmer *et al.*, 1994)), or the insect 18S *r*RNA gene (**NSF4/18:** CTG GTT GAT YCT GCC AGT, **NSR399/19**: TCTCAGGCTCCYTCTCCGG, under the same conditions as HCO/LCO, (Hendriks *et al.*, 1991)). Any samples that failed to amplify a product for these primers to visible standards through gel electrophoresis were deemed to be of poor DNA quality and discarded from analysis.

Diagnostic PCR for *Arsenophonus nasoniae* infection was initially performed using primers designed to amplify a strain specific 846bp portion of the 16S ribosomal *r*RNA gene (primers: **Arse16S**–F: GGG TTG TAA AGT ACT TTC AGT CGT/**Arse16S-R**: CGC AGG CTC GCC TCT CTC, 30 reaction cycles, annealing temp: 59°C, 30 second extension (Duron *et al.*, 2008)). These primers are susceptible to giving type type II errors, which are evident from weak band visualization under gel electrophoresis or incorrect amplicon size. Where this was suspected, screening for the more specific, but type I error prone metallaprotease-1 (zapA - GenBank accession: CBA72251.1, Wilkes *et al.*, 2010) gene was performed (primers **M1-F**: GGGTCACATACCTATTTT, **M1-R**: GTAGTCGCCTGGGTGGG, 35 reaction cycles, annealing temperature: 55°C, 30s extension time). Only samples with a positive consensus between these two diagnostics were included as 'infected' in the data.

PCR reaction volumes were 15µl consisting of 7.5µl GoTAQ GREEN or HotStart GoTAQ GREEN (Promega™), 5.5µl dH_2O, 1µl primer mix and 1µl DNA template. These were run in either a Applied Biosystem® Veriti® or Bio-Rad® T100™ 96-well thermo cycler.
In all cases a positive control, consisting of DNA from pooled infected wasps that had been diluted to a minimum amplifiable concentration, and a negative control of 1µl dH_2O were run alongside samples.

PCR products were run through a 1% agarose gel stained with 2% (w/v) Ethidium bromide. Gels were then visualized under UV-B light and presence/absence of amplicon scored.

3.2.3 Maintaining Nasonia vitripennis stocks

The lines of *N. vitripennis* used in this experiment originate from individual females collected from the Netherlands by Leo Beukeboom. These lines were clear of *Arsenophonus* upon collection but have their native *Wolbachia* infection. Wasp lines were maintained in mass culture at 25^{o}C, 12h:12h day:night cycle. Preliminary studies had indicated the value of enforced co-parasitism in maintaining high infection prevalence and so *A+ N. vitripennis* lines were host limited to 2 female wasps per fly pupa during maintenance. Cultures were kept in this way for 5-6 generations post-artificial *Arsenophonus* infection before individuals were used in experimental procedures.

3.2.4 Sarcophaga fly hosts

Sarcophaga bullata were used as hosts for *N. vitripennis* during the experiment. These were obtained from Fisherman Tackle (Birkenhead, UK) as final instar larvae. They were allowed to pupate at 20^{o}C, and were used for experiments within 2 days of pupation or kept in stasis at 4^{o}C for less than 7 days prior to use.

3.2.5 Experimental procedures

Daughter number, sex ratio and development time:

Females from both *A+* and *A-* stocks were isolated into individual glass vials and allowed to oviposit into a single *S. bullata* pupa for 24 hours. After this time the females were re-claimed from the vials and screened for. At this point any clutches originating from supposedly *A+* mothers that lacked the infection were discarded from the experiment (4 of 58 clutches were removed).

F1 progeny from the remaining replicates were allowed to develop through the larval instars at 25^{o}C until day 12, at which point any non-diapausing offspring will have pupated. The fly pupae were then cracked open to collect the pupated wasps and the fly remains were discarded. The wasp pupae in each clutch were counted and sexed and then left in their glass vials to develop. Maintenance temperature was dropped to 19^{o}C to make recording more precise by elongating development time. Clutches were

then observed at 12 hour intervals to score the number of each sex that had eclosed until all individuals had matured. In some cases the pupae died, so in these cases clutches were considered fully eclosed once 72 hours had passed since the last adult emerged. The uneclosed individuals were omitted from development time analysis but not from clutch size analysis as resource competition occurs before pupation.

Twenty-four hours post full eclosion the females were collected and preserved in 100% EtOH for wing size analysis. *Nasonia* typically lay upwards of 15 offspring in a single pupae and as high as 50 in *S. bullata*, I therefore omitted five clutches that contained <10 individuals from all analysis on the grounds that these extremely low numbers were likely a product of poor host quality (n=3; 4, 7 & 9 individuals respectively). All-male clutches would also have been removed from the experiment, as this would have been indicative of female virginity. However, none were produced.

Wing Size and quality

Wing length was used as an indicator of body size with which to infer symbiont-induced effects on fecundity. Body size is regularly used as an indicator of reproductive fitness in insects including *Nasonia vitripennis* (O.Neill and Skinner 1990) and wing size is used as a proxy for body size (e.g. Reeve *et al.*, 2000). Previous studies have used head width as a proxy for body size in *Nasonia* (e.g. Balas *et al*., 1996). However the ethanol storage used in this study to preserve sample DNA may potentially desiccate larger wasp tissues, leading to biased estimates of head size. Between 10-20 F1 females from each clutch were recorded. The left forewing was carefully removed from the body and photographed with a digital SLR camera attached to a dissecting microscope. Photographs were then randomized and measured using the metrics detailed in Figure 3.1 using ImageJ™ (Rasband, 1997). Wings were also scored for quality; those wings displaying any creasing and folding scoring as 'defomred' and those with typical flat, uncreased morphology scoring 'good' (see Figure 3.1).

The above procedures were carried out in three experimental replicates. The first two contributed data to all measures of fitness whilst the third only contributed to clutch size and sex ratio. This was because a CT room failure during development necessitated the removal of replicate #3 from analyses of development time and wing deformity. To account for this, all statistical analyses include 'replicate' as a random factor. The sample sizes for each metric of fitness were as follows: clutch size and sex ratio: *A*+=progeny from 48 hosts, *A*- =58 hosts. Emergence time and wing quality:

A^+=progeny from 25 hosts, A^- =30 hosts. Wing size: A^+= 247 female wasps, A^-=353 female wasps.

3.2.6 Analysis

All statistical analyses were conducted in R™ using the 'lme4', 'fBasic' and 'survival' packages CRAN repository). In all cases the experimental replicate was incorporated into statistical models as a random factor. The minimum adequate model was deduced by pairwise model simplifications and statistical comparisons using AIC values and χ^2/F-tests where appropriate. If required, overdispersion of data was accounted for by fitting a unique individual marker as a random factor.

Daughter number and sex ratio

The number of daughters produced by each focal mother were recorded upon cracking open the fly pupae. Daughter number was modeled using generalized linear mixed effect models (GLMERs) with infection status and sex ratio as fixed effects. Poisson errors were designated as this is count data. Sex ratio was calculated as the proportion of males in each clutch and analysed with GLMERs assuming binomial errors.

Wing size

Data for wing length was negatively skewed (-1.094 skewness) and so was transformed with a reflected inverse $\left(\frac{1}{x}\right)$ transformation to give a more normal distribution (-0.417 skewness). This transformation satisfied both the Shapiro and Bartlett tests of variance and normality (P>0.05). These data were then analyzed with mixed effects linear models (LMERs) with treatment and clutch size as fixed effects and cohort and replicate as random effects, the latter to account for relatedness between females from the same clutch.

Wing quality

Wing quality was determined to be binary within replicates (0 for good, 1 for deformed) and so was expressed as a 'proportion deformed' for each replicate. These proportions were then analysed using GLMERs assuming binomial errors with treatment level and clutch size as fixed effects and replicate as a random effect.

Development time

Three metrics of development time were analysed using parametric survival analyses based on Weibald distributed errors. These were: a) time to first eclosing female, b) time to 50% female eclosion and c) time to total eclosion. Furthermore, the 'eclosion duration' was calculated as the time elapsed between first eclosion to time at final eclosion. These data were strongly positively skewed (skewness=1.49) and so were transformed using the natural log to satisfy assumptions of normality (skewness= 0.234). This was analysed as a standard linear mixed effect model (LMER).

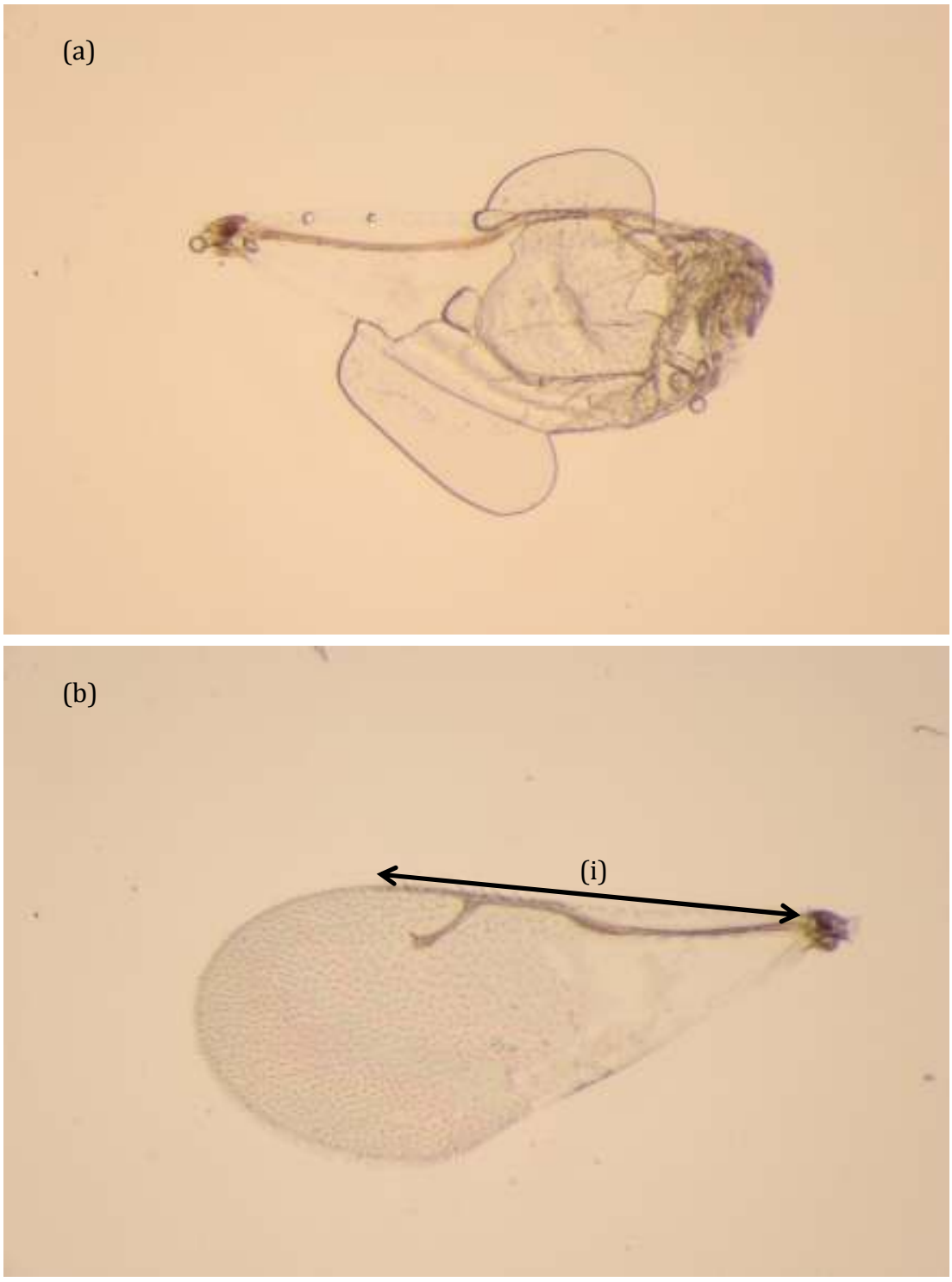


Figure 3.1: Examples of dissected female *N.vitripennis* forewings. (a) Example of damage or warping that qualified wings as 'deformed'. (b) An example of a normal wing that would be classed as 'good'. Note, wing length was measured as the length of the major wing vessel, annotated here as line (i) between the connective tissue that attaches the wing to the body and the base of the furthest most major hair structure on the anterior of the main wing vessel. Wings such as (a) or those that were damaged during dissection were omitted from analysis for length.

3.3 Results:

3.3.1 Daughter number and sex ratio

Clutches produced by infected females contained significantly fewer adult females (22.08 ± 1.08) than those produced by uninfected females (23.03 ± 0.82) (Comparison of LMERS, χ^2 = 8.71, df= 1, *P*=0.003)(Figure 3.2 = [a]). There was also a negative correlation between clutch size and sex ratio (Stepwise simplification of linear mixed models, χ^2 = 23.45, df = 1, *P*<0.001)(Figure 3.2-[b]). There was, however, no significant interaction between infection status and sex ratio in their effect on clutch size (Comparison of LMERs, χ^2= 1.77, df = 1, *P*=0.183). The minimal adequate model was:
Female number ~ Infection status + Sex ratio + (1|replicate).

Infected females produced clutches with a significantly greater proportion of daughters (mean proportion daughters: A^-= 0.84, A^+= 0.97, Figure 3.3)(Pairwise comparisons of GLMERs with binomial errors, χ^2= 109.06, df = 1, *P*<0.001) and sex ratio was significantly negatively correlated with clutch size (Pairwise comparison of GLMERs with binomial errors, χ^2= 4.4641, df = 1, *P*=0.035, See Figure A3.1 in appendix). However, there was no significant interaction between clutch size and infection status on sex ratio (Pairwise simplification of GLMERs with binomial errors, χ^2= 2.36, df = 1, *P*=0.12421). The minimum adequate model was:
Sex ratio ~ Infection status + Clutch size + (1|cohort), errors=binomial.

Wasp mortality during development

Female pupal mortality showed no significant difference between treatments. 3 of 600 female pupae died across A^+ clutches, and 2 of 749 females died across A^- clutches (Two-sample test of equality of proportions χ^2= 0.062, df = 1, *P*= 0.8034). Male pupal mortality was 0 of 32 in A^+ clutches and 2 of 133 across A^- clutches, a non-significant difference (2-way test of equality of proportions, χ^2= 0.0145, df = 1, *P* = 0.904).

3.3.2 Wing length (as proxy for body size)

Wing length was significantly reduced in the daughters of infected females (mean length in mm: A^- = 1.322 ± 0.015, A^+=1.287 ± 0.018, Figure 3.4 [b]) (Comparison of LMERs χ^2 =16.395, df= 1, *P*<0.001). Wing length was also significantly negatively correlated with clutch size in both treatments (Comparison of LMERs, χ^2 =10.715, df= 1,

P=0.0011) (Figure 3.4 [a]). There was no significant interaction between treatment and clutch size on wing length (Comparison of LMER, χ^2 = 0.0191, df= 1, *P*=0.8901). The minimum adequate model was achieved by dropping the interaction between infections status and clutch size:

Wing length ~Infection status + Clutch Size + (1|Replicate) + (1|Clutch ID).

3.3.3 Wing quality

Females infected with *A. nasoniae* produced clutches with a significantly higher proportion of deformed wings (A^- = 0.018, A^+= 0.364) (Pairwise comparisons of LMERs, χ^2= 60.815, df=1, *P*<0.001). This effect was independent of clutch size (Comparisons of LMERs, χ^2=1.199, df=1, *P*=0.274) (Figure 3.5). The minimum adequate model:

Mean Proportion of deformed wings in a clutch ~ Infection status + (1|Replicate) + (1|Clutch), error=binomial.

3.3.4 Development time:

Female wasps infected with *A. nasoniae* had significantly longer development times than uninfected wasps of the same strain. Three different metrics of emergence time were analysed: time to first eclosion (Figure 3.6=[c]), time to 50% eclosion (Figure 3.6=[b]) and time to full eclosion (Figure 3.6=[a]). All three metrics were significantly increased in infected clutches and two were significantly positively correlated with clutch size (Appendix Figure 3.7). In all cases the effect of infection was far greater than the effect of clutch size. There were no significant interactions between these descriptive factors (See table 3.1 for statistical outputs for each metric). Finally, the emergence duration was calculated as time to complete eclosion minus time to first eclosion. Eclosion duration was significantly longer for infected clutches despite these being smaller (Pairwise simplification comparisons of LMERs, χ^2=13.427, df=1, *P*<0.001) (Figure 3.6 =[d]) and was significantly positively correlated with clutch size (Pairwise simplification comparisons of LMERs, χ^2=11.653, df=1, *P*<0.001) (Figure 3.7=[d]). There was no significant interaction between these factors (Pairwise simplification of LMERs, χ^2=0.243, df=1, *P*=0.622).

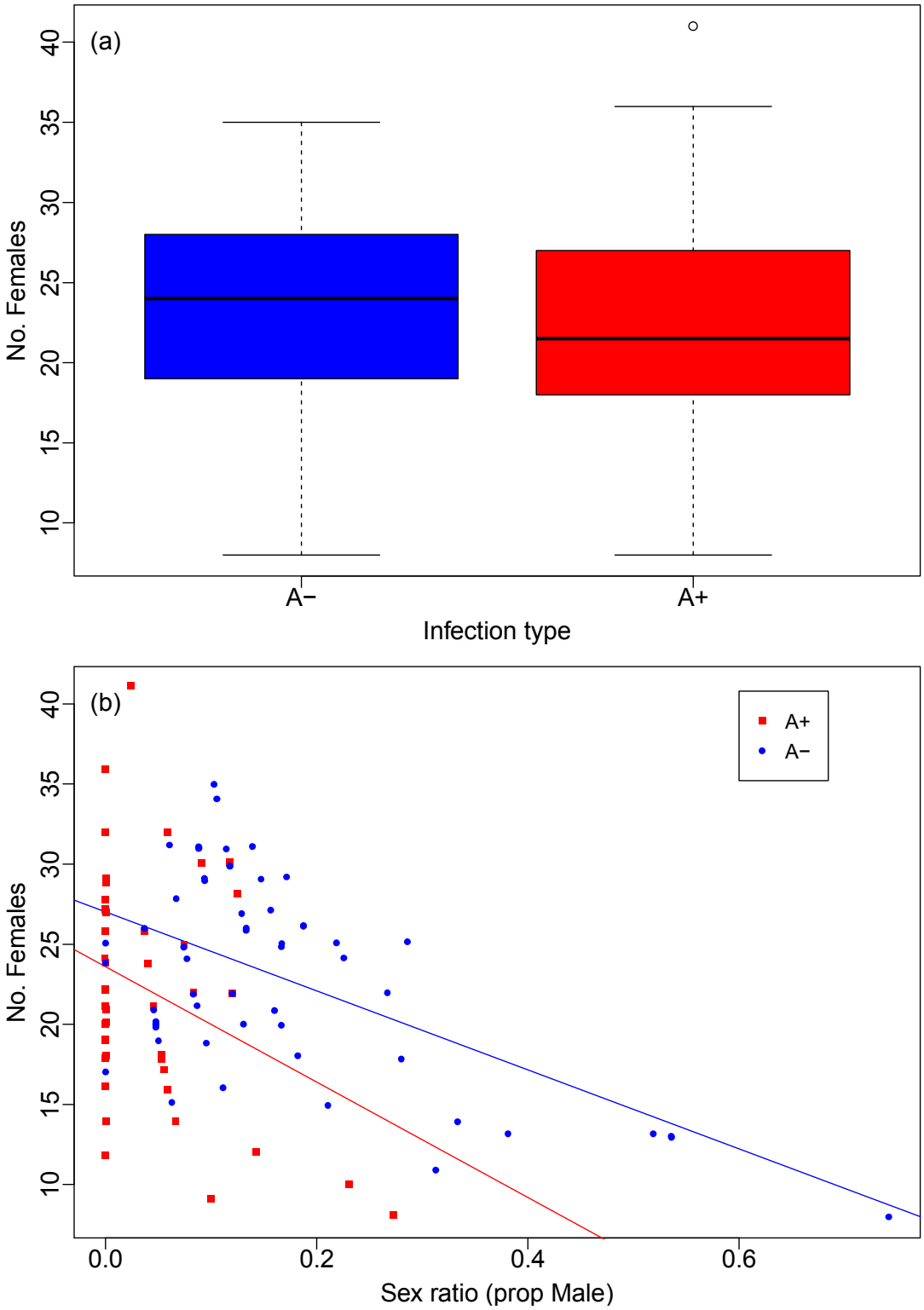


Figure 3.2: (a) Boxplot of the number of daughters produced infected (A+) and uninfected (A-) mothers (Solid line: Median, Box ends: interquartile range, Whiskers: range). (b) Correlation between clutch sex ratio, expressed as proportion of males, and the absolute number of female offspring within that clutch. Red and Blue dots represent clutches from infected and uninfected mothers respectively with fitted linear models as trend lines.

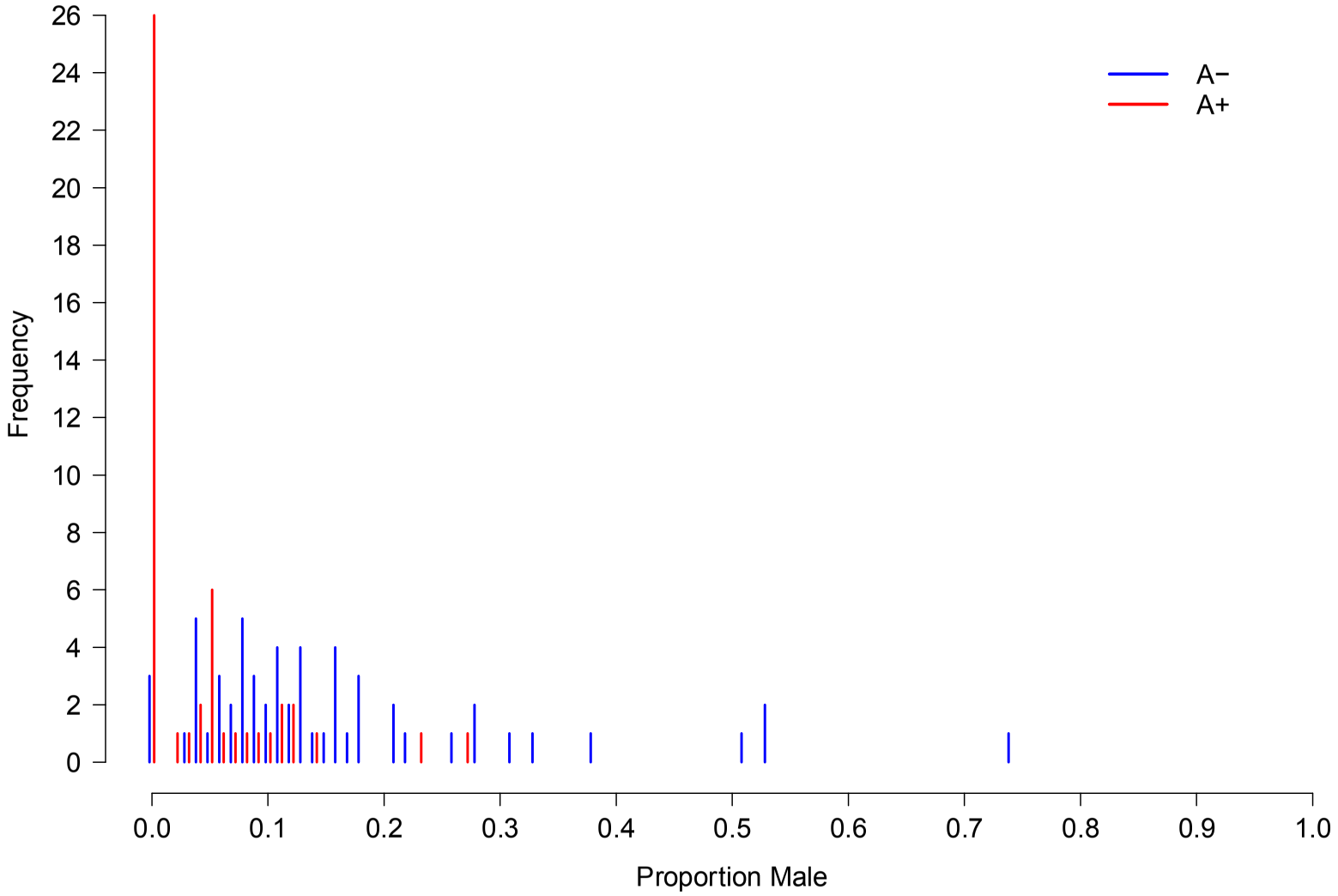


Figure 3.3: Frequency distributions of clutch sex ratios from infected and uninfected mothers. Sex ratio displayed here as the proportion of males in a clutch (to 2 decimal places for clarity).

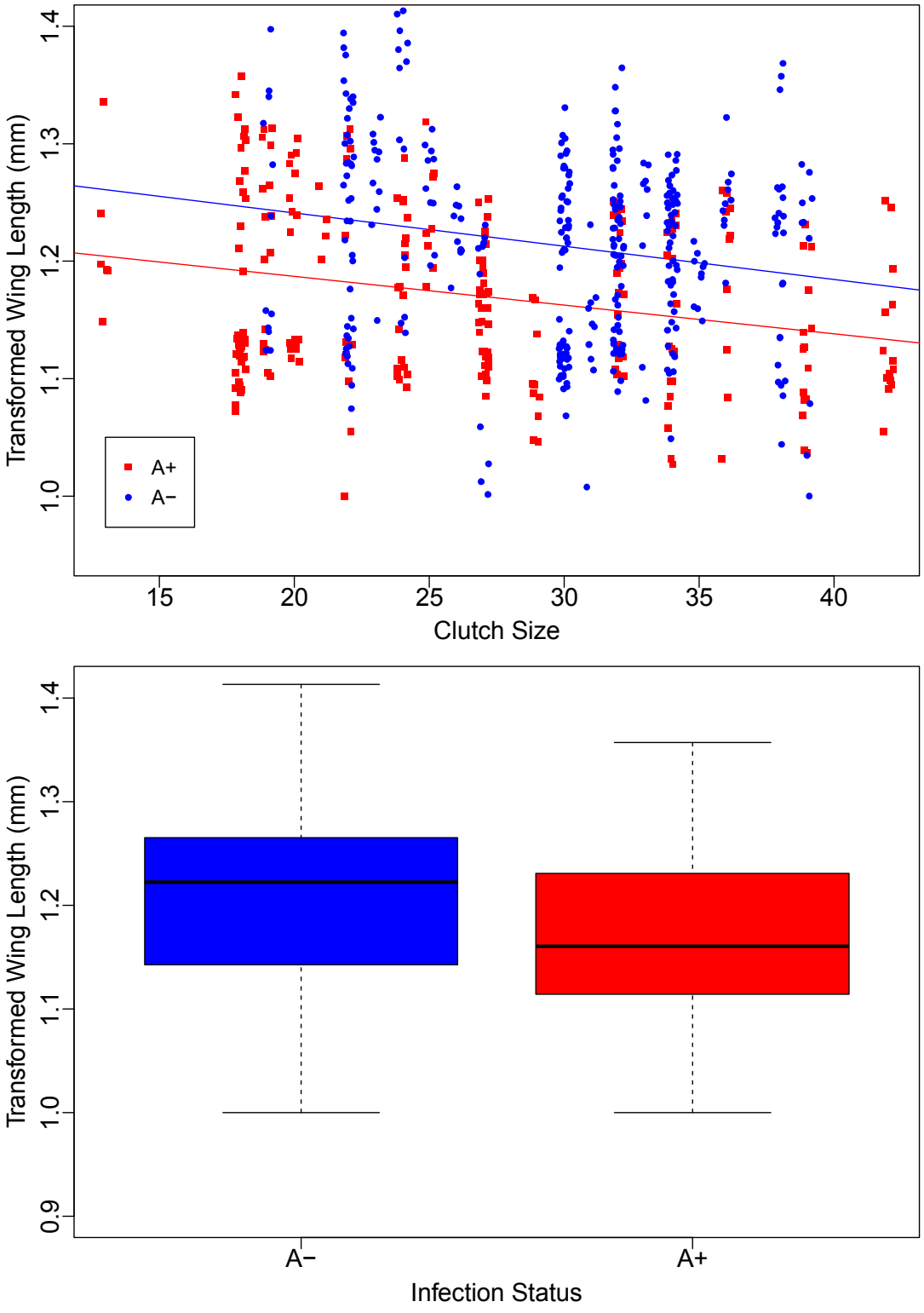


Figure 3.4: Wing length (in mm, reflected inverse (1/x) transformed) of offspring from infected (A+) and uninfected (A-) females. (a) Wing length against clutch size (#females), red and blue points represent infected and uninfected clutches respectively, lines fit with linear regression of the data. (b) Boxplot of transformed wing length by treatment.

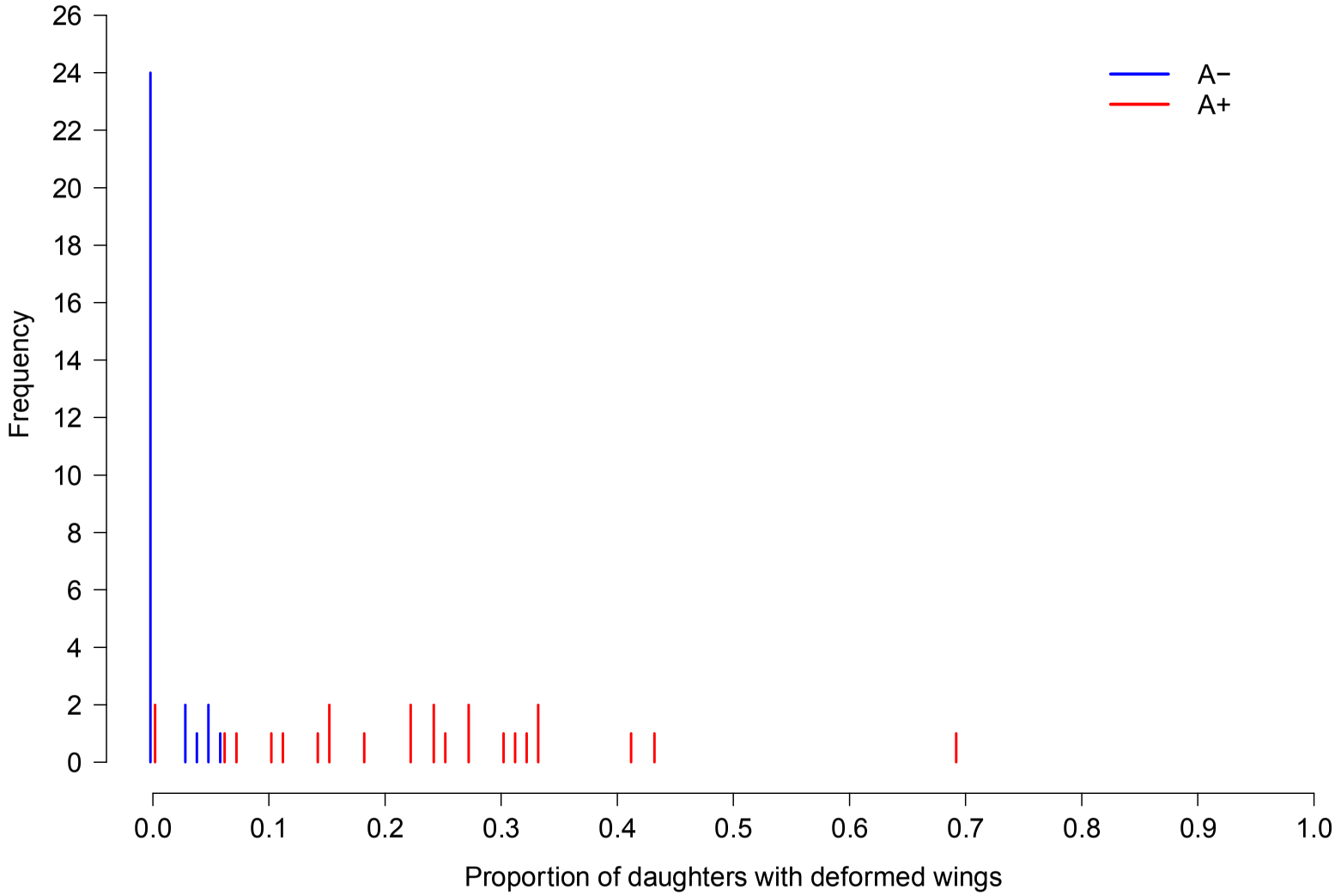


Figure 3.5: Frequency distribution of the proportion of daughters with deformed wings in clutches from infected (A+) and uninfected (A-) mothers. Proportions calculated here to 2 decimal places for clarity.

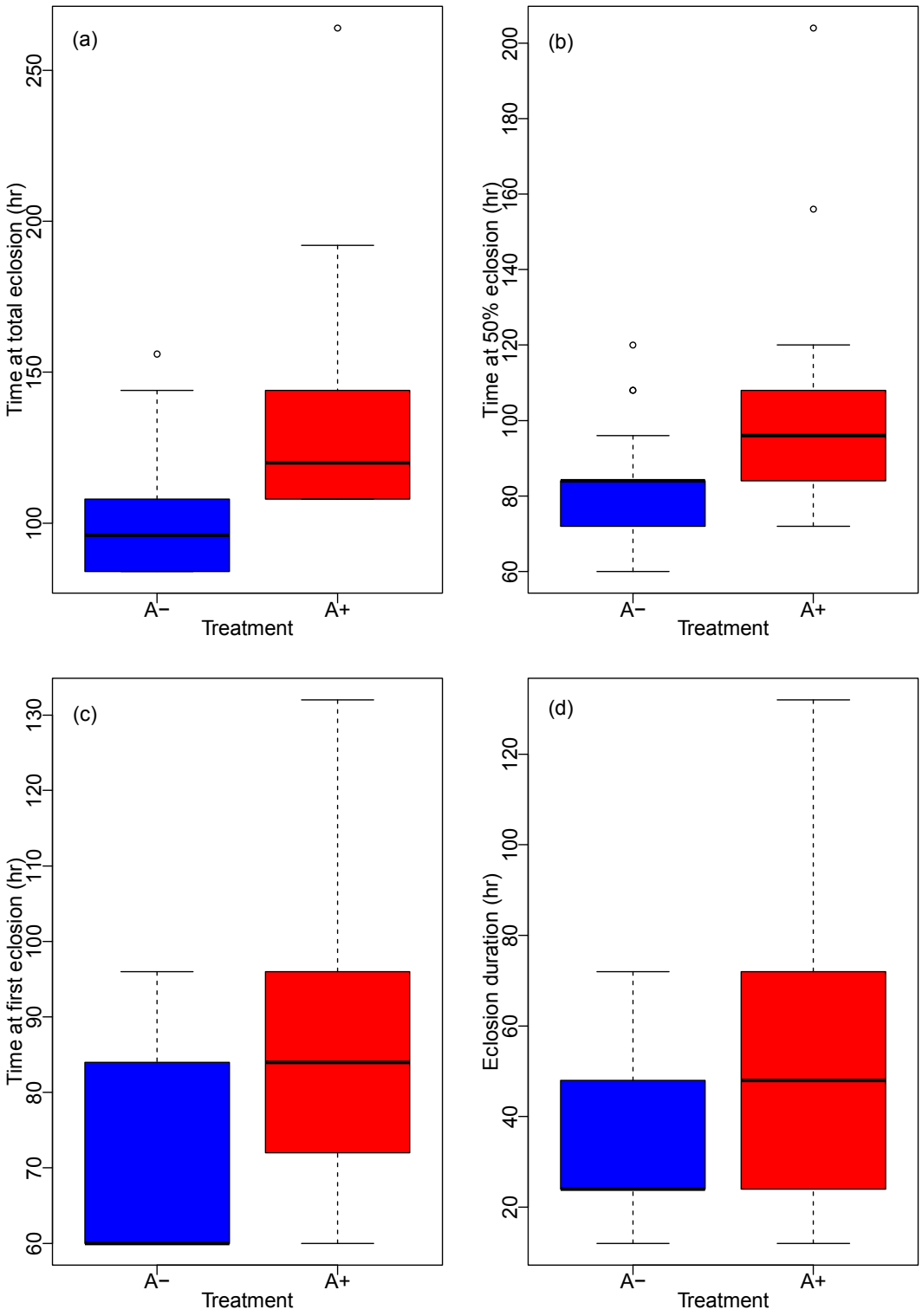


Figure 3.6: The median eclosion metrics for development time of clutches from infected (A+) and uninfected (A-) females. (a) The time taken for all F1 females in a clutch to eclose. (b) The time taken for 50% of F1 females to eclose. (c) The time taken for the first female to eclose. (d) The total time taken between first and last eclosion in a clutch

Table 3.1: Statistical output from step-wise model simplification of parametric survival analyses. All models assume a Weibald distribution of errors. Note that in all cases the infection status had a greater impact upon emergence than clutch size. There were no significant interactions between infections and clutch size. Stated degrees of freedom are based on the number of models being compared, for sample sizes see methods section.

Metric	Independent variable	X^2	df	*P*	
Time to 1st eclosion	Infection	19.15	1	<0.001	***
	Clutch size	0.01	1	0.94	NS
Time to 50% eclosion	Infection	22.31	1	<0.001	***
	Clutch size	7.39	1	0.007	**
Time to complete eclosion	Infection	23.62	1	<0.001	***
	Clutch size	6.49	1	0.011	*

3.4 Discussion:

The results presented here clearly demonstrate direct costs imposed upon *N. vitripennis* females and their offspring when infected with *A. nasoniae*. These costs manifest as a reduction in the number of daughters produced by the parent, delayed development of the progeny with reduced body size and impaired wing development. Effects on sex ratio are consistent with existing literature on the male-killing phenotype of *A. nasoniae* and demonstrate that the strain used here is indeed a reproductive parasite that kills males. As discussed elsewhere this cost should be offset through fitness compensation, translating to fitter female siblings of dead males.

The reduction in daughter number is an overt cost to both host and bacterium as the fitness of both parties is realized through the production of females. The reduction in wing size of females from infected clutches is difficult to interpret as a direct cost from the sample presented, however it is indicative of a reduced body size as a result of infection. Previous studies have demonstrated that female egg number is positively correlated with body size (for which wing length is used as a proxy). However it is not clear how fitness is constrained by the size of the egg complement. Indeed, female *N. vitripennis* may produce dozens of eggs in their lifetime, yet other factors, most likely host availability, will be the limiting factor to reproductive output. Ultimately this proxy serves to demonstrate a limit to female reproductive potential rather than realized fitness.

The increased formation of females with deformed wings represents a real cost. Dispersal ability will be an important fitness component in this species, as female wasps emerge, mate and then commonly need to disperse to find new hosts in which to oviposit. Females with malformed wings will be unlikely to find hosts, and are likely to have near zero fitness in the field.

3.4.1 Implications for fitness compensation theory of male-killing:

3.4.1.1 Resource release

The best-evidenced theory of male-killing states that the daughters of an infected female should incur a fitness benefit from the death of infected male siblings (Hurst, 1991; Hurst and Majerus, 1993; Majerus and Majerus, 2012). This benefit will translate

into greater fecundity and thus an increased bacterial transmission. However, this benefit is highly susceptible to the costs shown here since any detrimental physiological effects of infection erode the marginal benefits conveyed by male death and may translate to a net negative impact of infection. Previous studies have demonstrated that the reproductive potential of *N. vitripennis* is positively correlated to body size (O'Neill and skinner, 1990) and that wing size is a good proxy for body size in insects (Reeve *et al.*, 2000). Therefore the infection-induced reduction in wing size may truncate the fitness of infected females and subsequently harm bacterial transmission. Previous studies have found no significant effect of *A. nasoniae* on *N. vitripennis* body size (Balas *et al.*, 1996). However, these data are from highly variable field collections and have no control for wasp strain. Furthermore, this study shows that infected females produce significantly fewer female offspring than uninfected females, again demonstrating that the presence of infection is deleterious to its own vector.

The data presented here also implies a developmental cost of *A. nasoniae* infection, with infected females eclosing significantly later than symbiont-free counterparts, and a significant proportion of F1 females exhibit wing damage. Delayed development may translate into a real-time cost because it will reduce the number of pristine host patches available to infected females. Assuming that no population is homogeneous for infection (Balas *et al.*, 1996; Taylor *et al.*, 2011) and that adult *N. vitripennis* emerge in waves or groups (discussed as an ecologically valid assumption in Grillenberger *et al.*, 2009a) then infected females will always arrive second at available host patches. If this is the case then infected females are more likely to suffer fitness costs associated with co-parasitism and, crucially for the bacterium, will skew their sex ratio away from female bias thus reducing the viable transmission routes for their infection.

3.4.1.2 Inbreeding avoidance:

This study only permits the transfer of resources associated with male-killing, but prevents any benefit accruing from inbreeding avoidance. Rather, the experiment controlled for genetic background, comparing isofemale lines of *N. vitripennis* transinfected with *A. nasoniae* with lines that were uninfected (sham transinfection controls). Thus, there remains the possibility that the direct costs (and absence of benefit from resource reallocation) observed could be compensated for, in natural populations, by a reduction in the rates of inbreeding.

Despite *Nasonia* being robust to the deleterious effects of inbreeding due to its haplodiploid sex determination system (Werren, 1993), evidence suggests that outbred wasps are fitter (Luna and Hawkins, 2004). However, this study reported that repeatedly inbred wasps (15 generations) were less fit than crosses between these inbred lines, and that this effect was marginal. In nature, mark-recapture experiments demonstrate that *N. vitripennis* females have a range of 2km, and will forgo patches near to their own natal origin in favour of patches further away. The authors of this study postulate that this is in order to promote outcrossing (Grillenberger *et al.*, 2009a). Thus, comparison of deeply inbred lines to outbred lines is not appropriate. Rather, comparison between sib-mated and outcrossed females represents a more natural scenario for establishing the fitness benefits of inbreeding avoidance. Combining the effect of haplodiploidy on inbreeding depression with the failure to measure differences in fitness between sib-mated and outcrossed females, any benefit of male-killing from inbreeding avoidance is likely to be small in magnitude and unlikely to compensate for the costs measured in this study. An analysis of Werren's (1987) model concludes that 5.5-5.7% benefit in the presence of inbreeding depression is sufficient to allow invasion of *A. nasoniae* (Balas., 1996). This conclusion assumes there are no direct costs associated with infection, and so the costs demonstrated here will negate any such marginal benefit of inbreeding avoidance.

The high cost measured in this study, combined with absence of evidence for substantial benefits from resource release, likely low benefits from inbreeding avoidance, and possible costs of virginity to *A. nasoniae* positive females makes it unlikely that this bacterium will spread through vertical transmission alone.

3.4.2 Mechanisms for cost

The experiments described have quantified the effects of *A. nasoniae* on some key life history traits, but have not determined the mechanistic cause of these induced costs. The source of the costs can be considered in terms of proximate mechanism (how the impact occurs), or ultimate (why a microbe would damage its host).

First, it is quite likely that some observed costs are simply the result of metabolic demands of the bacterium on the host's tissues. Typically, direct effects on the host are most common following transfer into a new species, and can be reduced, along with negative effects on fitness through selection (Carrington *et al.*, 2010). Maladaptive

virulence is possible in this system, given the commonness of inter-specific transfer of the bacterium (Duron *et al.*, 2010). Second, virulence may also be a proximate outcome of the bacterium's unusual route of transmission. *A. nasoniae* must infect larval hosts through the gut wall after residing in the dipteran host. This may reduce the quality of the dipteran host, resulting in poor nutrition for *N. vitripennis* larvae and reduced fitness. Further, per-oral transmission may damage the insects' gut, opening a passage for opportunistic pathogens and eliciting self-harming immune responses (Armitage *et al.*, 2003). In addition, *A. nasoniae* may disrupt the resident gut flora of the insect. In *Nasonia*, disruption to gut biota composition has been linked with hybrid inviability (Brucker and Bordenstein, 2013), and more broadly evidence suggests that gut microbes form an intrinsic part of insect fitness (reviewed for honeybee gut microbes in (Hamdi *et al.*, 2011). Thus, if the presence of *A. nasoniae* is disrupting the microbiome, this may be indirectly causing the costs observed here. Other mechanisms that may be resulting in the pathogenicity observed here include autoimmunity and bacterial impacts upon the dipteran host. The wasps may be mounting an immune response against *A. nasoniae* infection which itself imposes costs in terms of resource sinking and self-harm (Moret, 2000; Siva-Jothy *et al.*, 2005; Sadd and Siva-Jothy, 2006). Alternately, the infection of the *S. bullata* host with *A. nasoniae* through the mothers' calyx fluid may be degrading its nutritional value to the wasp larvae.

Under this view, virulence may be adaptive for the bacterium. It is widely assumed that virulence and pathogenicity are in a trade-off with infectious transmission (Anderson and May, 1979) and in Chapter 2 of this thesis I demonstrated that infectious transmission may play a central role in the spread of *A. nasoniae*. Therefore, potentially the pathogenicity detailed here is traded off against the bacterium's ability to horizontally transmit. If this is the case then the basic rate of increase (*BRI*) of *A. nasoniae* is not only dependent upon segregational loss and cost-drive coefficients, but also cost/infectious transmission trade-offs as well. In addition, infectious transmission allows mixing of symbiont infections, which may select for higher titre (and thus cost).

3.4.2 Implications for host population biology:

The costs detailed here may also affect the population biology of *N. vitripennis* at a localized level by increasing density and reducing gene flow. The detrimental effect of infection on wing development may well inhibit the female's ability to disperse effectively, causing her to rely on terrestrial locomotion to find new host patches. As a

result, *Arsenophonus*-infected females will become more localized, as their dispersal ability is limited compared to uninfected individuals. Therefore, there will be greater density of related females on patches within walking distance of the natal patches. This may have implications for inbreeding in the F2 generation and also increase the rate of co-parasitism as more females are sharing localized resources. If this conjecture is true, then *A. nasoniae* has the capacity to increase local density. Furthermore, by limiting female dispersal, *A. nasoniae* is limiting between-patch gene flow and potentially creating a more genetically structured population than is normal for the typically homogenous *N. vitripennis* (Grillenberger *et al.*, 2008).

A similar effect has been demonstrated in *Rickettsia* infected money spiders (*Erigoneatra*) (Goodacre *et al.*, 2009). Here, infection is negatively correlated with long distance dispersal in natural populations and empirically demonstrated to alter the arachnid's dispersal behavior in the laboratory. The authors conclude that this limited dispersal may ultimately affect population structure of the host. However, they were unable to offer an adaptive reason for *Rickettsia* driven dispersal limitation. In the study presented here, limited dispersal may be adaptive for the bacterium as it will increase relatedness at a local level and increase the rate of co-parasitism (and thus horizontal transmission potential), as it effectively boosts host density.

However, ultimately reduced dispersal is likely to be maladaptive for the bacterium. If dispersal is reduced and subsequently infection levels increase through horizontal transmission, then the populations become at risk from local virginity through male-killing. Further, infected wasps will be less able to make use of new fly pupal resource patches. This will then reduce the fitness of the bacterium along with that of its host.

3.4 Conclusion:

The data presented here clearly demonstrates that *A. nasoniae* infection conveys a dramatic cost to its host *N. vitripennis*. Infected individuals develop slower, produce fewer offspring, are smaller, and may have deformed wings. These costs will not only override any adaptive benefit of male-killing (a weak drive phenotype at best estimate), but may also have long term detrimental effects on both host and symbiont biology if infection moves towards high prevalence in localized populations. One caveat to this conclusion is that the data investigates a single *A. nasoniae*/wasp combination, and should be repeated for other combinations to test the generality of the conclusions drawn.

Chapter 4:

Experimentally evaluating vertical and infectious transmission in the spread of *Arsenophonus nasoniae*

Abstract:

In Chapter 3 I presented data showing overt fitness consequence of *A. nasoniae* infection in *N. vitripennis*. This evidence challenges existing theories of male-killer dynamics that presume fitness compensation benefits are sufficient to drive the symbiont into the population. In Chapter 2, however, I demonstrated that co-parasitism and horizontal transfer could play a key role in *A. nasoniae* epidemiology. In this chapter I report the result of experiments to determine the dynamics of *A. nasoniae* under different regimes of co-parasitism. I demonstrate that infectious transmission is necessary for the spread and maintenance of *A. nasoniae* in *N. vitripennis* and note how this is associated with host density. I also demonstrate that resource-limited wasp populations may be driven extinct by *A. nasoniae*.

4.1 Introduction

The reproductive manipulation phenotype of male-killing has evolved numerous times in a broad array of microbes and has been reported from numerous disparate host species (Hurst, 1991; Duron *et al.*, 2008). However, male-killer prevalence within host populations is highly variable, with prevalence ranging from 5-100% (Hurst and Majerus, 1993; Hurst *et al.*, 1997; Majerus *et al.*, 1998), and commonly show both spatial (Majerus *et al.*, 1998) and temporal (Hornett *et al.*, 2009) heterogeneity within species. This therefore begs the question; what explains the different prevalence male-killers achieve in natural populations? As previously discussed in Chapters 2 & 3, the spread of symbionts is a product of their basic rate of increase (*BRI*) (Randerson, *et al.*, 2000b), a trait derived from the combination of rates of segregational loss, direct costs, and varying strength of drive. Chapter 3 presented data demonstrating the costly nature of *Arsenophonus nasoniae* infections in *Nasoniae vitripennis* and discusses how these costs almost certainly undermine any advantage of male-killing through resource reallocation. Here, I empirically test the hypothesis established in Chapter 2, that infectious transmission may be a suitable compensatory transmission strategy to facilitate drive in this system.

4.1.1 Factors affecting the drive of secondary symbionts

Questions regarding the drive of male-killers through their host population are typically addressed within a framework of pure vertical transmission. This is because the act of male-killing is presumed to have evolved to maximize fitness through maternal inheritance and thus this will be the sole or primary route of infection. The potential mechanisms through which male-killing confers an adaptive benefit were outlined in Chapter 1 of this thesis. In brief: the classical evolutionary basis for male-killer drive is through kin-selection based fitness compensation. This can manifest as either reallocation of resources from dead males to infected sisters, or the alleviation of inbreeding depression by enforced outcrossing. Empirical evidence is relatively rare, but mostly concerns resource reallocation e.g. (Hurst *et al.*, 1994; Jaenike *et al.*, 2003; Majerus and Majerus, 2012), whilst inbreeding avoidance has a basis in theory alone (Werren, 1987). Ultimately, fitness compensation is a relatively weak drive phenotype. This is because the adaptive benefit to females is indirect and dependent upon the proportion of males in a brood, the efficiency of their death, the standing risk of inbreeding depression and/or the efficiency of resource reallocation. This is in contrast

to other reproductive manipulations employed by symbionts. For example, feminization and pathogenesis induction directly increase the number of females in a population by either converting genetic males into function females or redirecting parental investment from son to daughter production. These mechanism therefore make a strong contribution towards the drive of the causative symbiont by increasing the potential for maternal transmission. It has been suggested that the drive of male-killing may be supplemented through other mechanisms in order for the bacterium to persist (Hurst and Majerus, 1993). These mechanisms can either contribute towards vertical transmission by increasing the number or fitness of infected offspring, or, they may boost the R_0 of the symbiont through infectious or paternal transmission.

Vertical transmission may be augmented with multiple drive phenotypes. Some sex-ratio distorting bacteria have been shown to possess secondary symbiotic phenotypes that may have an additive impact upon drive. *Wolbachia* (*w*Bol1) infecting populations of the butterfly *Hypolimnas bolina* induces both male-killing and cytoplasmic incompatibility (Hornett *et al.*, 2008). The latter phenotype is masked by the former unless resistance to male-killing spreads. However, it has been theorized that a synergy between the two allows the *w*Bol1 to reach and maintain high prevalence (Hornett *et al.*, 2010). Himler and colleagues demonstrated that a *Rickettsia's* rapid spread from 1% to 97% prevalence in US populations of its whitefly host, *Bemisia tabaci,* was achieved by simultaneously increasing fitness and skewing sex ratio (Himler *et al.*, 2011). However, the relative contributions of each phenotype to drive are yet to be determined. *Wolbachia* infections in *D. melanogaster* have been shown to impart both weak reproductive manipulation and viral resistance on their hosts (Hedges *et al.*, 2008). Here, the authors reason that the protective phenotype and reproductive manipulations may be synergistically driving the global dynamics of *Wolbachia* infection in *D. melanogaster* (e.g. Riegler *et al.*, 2005).

Vertical transmission may also be promoted through utilization of both paternal and maternal routes. Some viral symbionts employ a mixture of both maternal and paternal transmission in order to maximize fitness and overcome the inherent cost of infection. This is particularly well studied in sigma viruses that naturally infect *Drosophila* spp. (reviewed in Longdon and Jiggins., 2012). However, the utility of paternal transmission for bacterial endosymbionts is inhibited by the lack of space in sperm cytoplasm to accommodate symbiont cells. That said, potential paternal leakage of *Wolbachia* has been suggested as an explanation for infection across multiple mitochondrial

haplotypes in *D. willistoni* (Müller *et al.*, 2012). However, a more parsimonious explanation for this phenomenon is that the symbiont has been horizontally vectored between unrelated individuals. Indeed, infectious or horizontal transmission itself is another method by which symbionts may increase their *BRI*.

4.1.2 Horizontal transmission in heritable sex ratio distorters

Horizontal or infectious transmission is typically associated with outright parasites and pathogens. The standing assumption is that parasites trade off virulence with infectivity, so that their fitness is determined by host-host contact rather than host reproduction (a function of longevity). Symbionts that utilize maternal inheritance are thus assumed to minimize their horizontal transmission in order to reduce associated pathology that will limit their prospects for vertical transmission.

Notwithstanding this, the capacity to transmit infectiously has not been completely lost by all heritable symbionts. Horizontal transmission in maternally inherited microbes is traditionally considered to be a rare but ecologically profound occurrence that leads to host shift events (Russell *et al.*, 2003; Russell and Moran, 2005; Duron *et al.*, 2010) (Although see the unusual case of late male-killing microsporidia where it commonly occurs (Agnew and Koella, 1999)). As such, its treatment in the literature has primarily concerned diversity of symbionts across disparate host taxa (Engelstadter and Hurst, 2006) and the non-cocladogenesis between symbionts and host phylogenies (Russell *et al.*, 2003; Baldo *et al.*, 2006; Mouton *et al.*, 2012). However, there are incidences where horizontal transmission has been shown to play a regular part of the microbe's transmission strategy. Furthermore, theory has demonstrated that the capacity for horizontal transmission should rarely be lost in secondary symbionts, including those that manipulate reproduction (Ironside *et al.*, 2011).

In the case of sex ratio distorting symbionts, Ironside and colleagues (Ironside *et al.*, 2011) theoretically demonstrated that the capacity for horizontal transmission would only be completely lost under very specific host conditions, namely extremely low density. Indeed, host density is a major determinant of the evolution of transmission strategy because it determines whether a microbe is more likely to come into contact with unrelated hosts or the offspring of its current host. In dense populations, unrelated individuals are in regular contact and so infectious transmission is optimum (assuming that there is a physiological bridge between individuals). Alternatively, if

hosts are sparse, then vertical transmission will be favored because a) host-host contact will be rare, and b) population growth will be greater and so fitness maximized by hijacking host reproduction. Evidence for mixed infectious/vertical transmission strategies are common. Experimental evolution studies of the protist *Holospora unulate* and its *Paramecium* symbiont have demonstrated increased vertical transmission in growing populations, whilst virulence and infectious transmission evolve in response to host populations at carrying capacity (Kaltz and Koella, 2003). Similarly, microsporidian late male-killers are more likely to utilize vertical transmission if their host larvae have a rich diet, an indicator of low density, and resort to horizontal modes when ecological indicators imply population crowding (Agnew and Koella, 1999). Furthermore, NPV virus infecting African armyworms (*Spodoptera exempta*) alters its rates of VT and HT depending on the density of its host (Vilaplana *et al.*, 2008).

A mixed strategy of HT and VT can also evolve when host conditions are not constant through time. For example, seasonally reduced population size or diapause overwintering will be unfavorable to infectious transmission. In these scenarios we would also expect selection to promote plastic switching of epidemiological strategy in response to changes in the host demographic, or to simply maintain both routes of transmission. Transmission of LaCrosse virus in the mosquito *Aedes triseriatus* exhibits such a mixed strategy. In the summer months, infectious transmission through intermediate mammalian hosts maintains viral loads in the mosquito populations. However, during host hibernation, the base-line load of virus is maintained in eggs that have acquired the infection vertically though transovarial transmission from their parents (Watts *et al.*, 1973). Whilst the vertical transmission has little impact on the epidemiology of the virus during the summer, it is vital for the year-on-year fitness of the symbiont. Similar links between vertical transfer and overwintering have been implied in other mosquito vectored diseases (Goddard *et al.*, 2003), and this is considered a key facet of their long-term epidemiology.

Therefore, we may expect horizontal transmission to evolve or be retained in male-killers if their hosts are at high density, if their hosts experience seasonal cycles where VT and HT will be differentially beneficial, or if pure vertical transmission is inefficient to the point where infectious transmission becomes adaptive.

4.1.3: Horizontal Transmission in A. nasoniae/N. vitripennis

The vertical transmission efficiency of *A. nasoniae* in *N. vitripennis* has been estimated at 95% (Skinner, 1985) and so generates a relatively minor rate of segregational loss. Therefore, male-killing derived fitness compensation, either through resource reallocation or inbreeding avoidance, need only increase mean fitness of infected females by >5% in order to generate drive. However, surveys of *A. nasoniae* in natural populations of *N. vitripennis* have found low infection prevalence, 5-16% (Balas *et al.*, 1996; Taylor *et al.*, 2011), and several populations have failed to show any infection (Taylor *et al.*, 2011; C.Frost *pers comms*). It is reasonable to assume that these data are at, or near, equilibrium given that the surveys were conducted 15 years apart. This then indicates that *A. nasoniae* naturally occurs at low prevalence and that there are barriers preventing its spread to certain populations. Furthermore, fitness compensation in this system is likely to be very weak because a) males are a small fraction of progeny b) the male-killing is only *c.*80% efficient (Skinner, 1985) and c) *N. vitripennis* is robust to the deleterious effects of inbreeding by virtue of its haplodiploid sex determination (but see Luna and Hawkins, 2004). This begs the question as to the factors, aside from the adaptive benefit of male-killing, that mediate the epidemiology of *A. nasoniae*? Given the overt direct costs of infection shown in Chapter 3, and the mathematically demonstrated principle that infectious transmission can maintain prevalence in Chapter 2, I hypothesise that *A. nasoniae* is employing infectious transmission as a central component of its transmission dynamic.

Horizontal transmission of *A. nasoniae* is readily achievable in laboratory cultures of *N. vitripennis* by forcing an infected female to co-parasitise the same dipteran host as an uninfected female. Due to the per-oral transmission of the bacterium from maternal calyx fluid to offspring gut, all larvae present in the pupae can acquire the infection (Skinner, 1985; Huger *et al.*, 1985). Co-parasitism is commonly observed in natural populations of *N. vitripennis*. Their dipteran hosts are typically aggregated around bird's nests and animal corpses and so encourage high densities of wasps to congregate (Grillenberger *et al.*, 2008). Grillenberger and colleagues assessed the parentage of offspring emerging from naturally parasitized fly pupae and found that 40% of broods were founded by 2-4 mothers and, in a later study, demonstrated that up to 9 mothers can contribute offspring to a single pupae (Grillenberger *et al.*, 2009b). Therefore, there exists a natural scenario in which *A. nasoniae* will be able to infectiously transmit.

This capacity for horizontal transfer was explored by Olivier Duron and colleagues who demonstrated that the bacterium would readily transmit interspecifically to other parasitoids that co-parasitised with infected *N. vitripennis* (Duron *et al.*, 2010). These authors also found that the transmission efficiency and strength of male-killing reduced with genetic distance between host species. Furthermore, they surveyed natural populations of several chalcid species and found numerous cases of *A. nasoniae* infection, which they propose to be recent acquisitions through infectious transmission. However, despite *A. nasoniae's* ability to horizontally transmit being known for almost 30 years, and its importance in host shifting being explored, there is a lack of empirical attention given to HT in the context of symbiont drive within *N. vitripennis*. It is ignored in the initial models of the system (Werren, 1987) and only very briefly discussed in reviews of male-killer spread (e.g. Hurst and Majerus, 1993). One verbal model postulated that infectious transmission may be important in mixed infections of MK and non-MK bacterium within a population but without theoretical or rigor or ecological grounding (Balas *et al.*, 1996).

In this chapter I present a series of experiments where I manipulate the density of laboratory population of *N. vitripennis* in order to inhibit or encourage horizontal transmission. I test the overarching hypothesis that infectious transmission is necessary for symbiont invasion and spread in populations of its host.

4.2 Methods

4.2.1: Establishing and maintaining Arsenophonus infected N. vitripennis

In the following experiments, *A. nasoniae* infected lines were established and maintained on *S. bullata* hosts as described in Chapter 3.

4.2.3 Sexing N. vitripennis

Nasonia vitripennis exhibits a strong sexual dimorphism (Whiting, 1967), with males having significantly reduced wings compared to females, yellow antennae and a green/gold metallic iridescence compared to the female's darker colouring.

4.2.4 Screening for infection

Infection status of individual Nasonia vitripennis wasps was determined with diagnostic PCR as detailed in Chapter 3 (3.2.2).

4.2.5: Experiment 1: Assessing the role of Horizontal transmission in Arsenophonus spread

Aim: to test the hypothesis that infectious transmission is required for *A. nasoniae* spread in populations of *N. vitripennis*.

The experiment followed a 2X2 factorial design. The first factor investigated was wasp density (as a proxy for co-parasitism) with two levels: single female wasps per patch (single parasitism) and four female wasps per patch (co-parasitism possible). The second factor was 'patch size' or resource availability. This consisted of either a single host pupa per patch (low resource) or four host pupae (high resource) (See Figure 4.1). The choice of co-parasitism intensity and host density was based on field estimates in (Grillenberger *et al.*, 2008). Here it was found that in rare cases (8<10%) of wild co-parasitisms, four foundresses contribute offspring to a single host pupae, and so the treatment levels used here represent the natural extreme of possible co-parasitism.

Each treatment consisted of populations of 80 wasp females subdivided into either 80 or 20 discreet patches depending upon treatment. The parental wasps used to establish populations were 50:50, *A+*:*A-*, with infected females distributed evenly across all patches within a population. Females were allowed to oviposit individually or in groups of four depending upon their respective treatments. The groups were

treated as analogous to discreet patches within the population with no mating between them. As such, the wasps in each patch were allowed to develop and then hatch and mate amongst themselves for 2-3 days post eclosion but not with the progeny of neighbouring patches.

After within-patch eclosion and mating, all wasps within a population were immobilised with CO_2, pooled, mixed and the population sex ratio estimated by sexing between 100-150 individuals selected at random. Where possible 80 females were then chosen to propagate the next generation whilst a further 20 females were isolated in 90% EtOH for later PCR screening for infection (See Figure 4.2 for a schematic of the procedure). If population size had reduced below the 100 females required for this then the female wasps used to propagate the next generation were reclaimed after 3 days of laying and PCR analysis performed on these individuals. Alongside these treatment populations, corresponding control populations were set up under the same demographic conditions, but with no *A. nasoniae* infection present in order to directly compare sex ratio.

The above treatments (4 experimental treatments, 4 controls) were replicated six times, making 48 populations in total at the outset of the experiment. Propagation of the populations continued for 8 generations, save for infected populations that completely lost *A. nasoniae* infection during the course of the experiment (discontinued at the point of losing infection) or went extinct through lack of males. All control populations were maintained until their corresponding experimental populations were removed or the experiment ceased. Due to the size of the populations and manpower required to maintain them and turn them over this experiment was run in two blocks, each containing three replicates of each treatment and control.

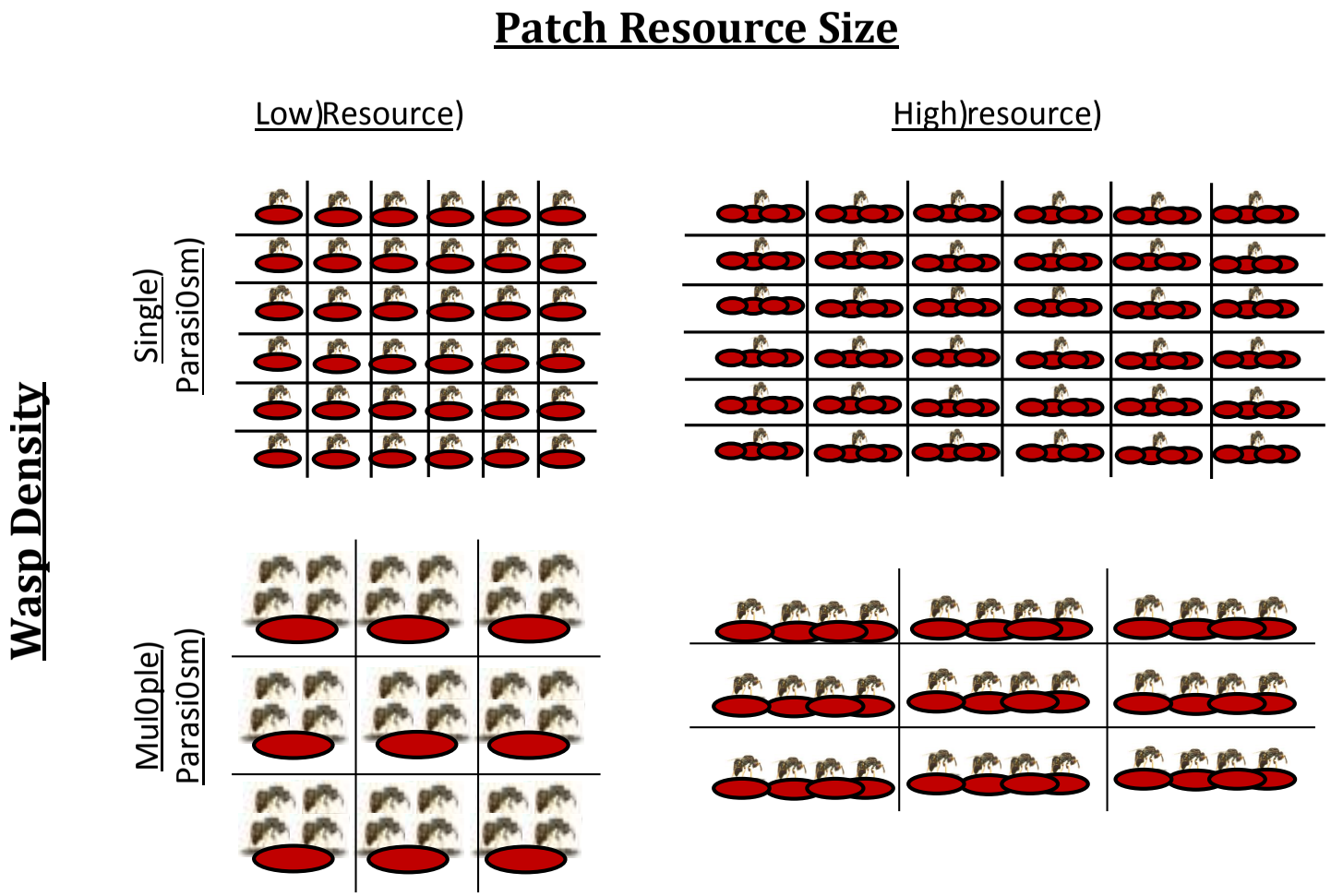


Figure 4.1: Schematic of population set-ups for *Experiment 1*. Note that for clarity fewer wasps are shown per population here than were used in the experiment (80). Wasps oviposit in isolated patches within populations. The number of wasps varies from one to four, as does the number of host pupae offered according to treatment. Mating strictly occurs within-patch. Sex ratio and infection prevalence are scored from offspring emerging from all patches within a population. The experimental unit is therefore the population, which is replicated six times. Control populations in which there is no *A. nasoniae* were set-up in parallel (n=6).

4.2.6 Experiment 2: Can horizontal transmission allow A. nasonaie to invade from rare?

Aim: to test the hypothesis that drive through horizontal transmission will enable *A. nasoniae* to spread into populations from initially low prevalence.

The experimental treatment was the proportion of founding mothers in each population that were infected with *A. nasoniae*. Three treatments of 5%, 25% and 50% starting infection prevalence were set up within populations of 40 reproductive female *N. vitripennis* (Figure 4.2). As with *Experiment 1*, populations were subdivided into 'patches' in which all oviposition and mating took place. All populations for this experiment consisted of 'high density, high resource' patches, with four female wasps and four dipteran pupae per patch. For the parental generation, infected wasps were distributed evenly across patches wherever possible. As each population consisted of 40 females distributed across ten patches the 50% and 25% treatments contained two and one infected wasp per patch respectively. The 5% treatment had only two infected females across the whole population. These were specifically allocated to separate patches in each replicate population.

As in *Experiment 1*, females were allowed access to the hosts until they died. Their offspring were then left to develop, emerge and mate within their patch before being pooled from all patches and mixed under CO_2 immobilization. Forty females were then randomly allocated to fresh patches and a further twenty females were preserved in 90% EtOH at -80^{o}C for later PCR screening for infection prevalence. Populations were propagated in this way for four generations (See Figure 4.4). Each treatment population was replicated six times, to make 24 populations in total. As for Experiment 1, replicates in this experiment were split into two cohorts, staggered by one week.

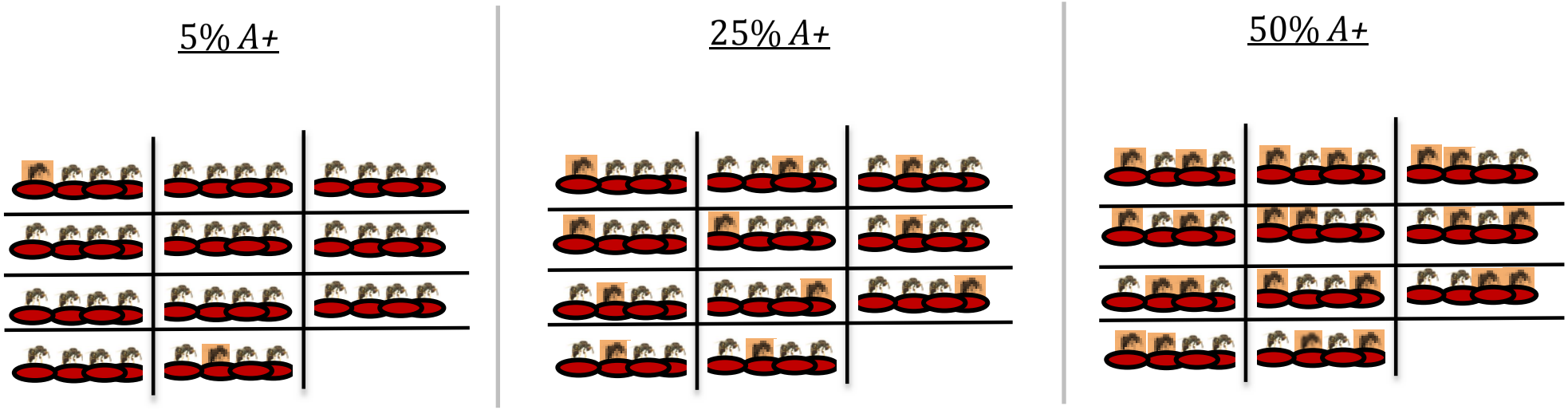


Figure 4.2: Schematic of until population set-ups for Experiment 2. All populations are made-up of ten patches containing four wasps and four fly pupae, thus allowing horizontal transmission. The treatment is initial infection prevalence, indicated here by highlighted individuals. An additional control population was also established with no infection present. This was in order to directly compare the fluctuations in sex ratio of the above treatments with wasps in the same conditions (data in appendix).

4.2.7 Experiment 3: Is A. nasoniae invasion dependent upon a threshold level of horizontal transmission?

Aim: Experiments 1 & *2* examined infection dynamics through populations that were homogeneous in terms of density and horizontal transmission potential – either 100% or 0% of females are co-parasitising. This does not reflect the heterogeneity of patch size and wasp density seen in nature (Grillenberger *et al.*, 2008). Here, I test the degree to which *A. nasoniae* spread depends on the level of co-parasitism, and whether there is a threshold level of co-parasitism required for spread.

Experimental populations of forty females were established with a starting infection prevalence of 50%. As in previous experiments females were restricted to oviposit into discrete patches within their population. The imposed treatment was the proportion of these patches that allowed wasps to co-lay. Levels of co-laying (proportion of female wasps that were placed with three other wasps during the oviposition phase) were 0%, 10%, 20%, 30%, 50% & 100% (Figure 4.3). Populations were turned over for four generations as described in *Experiments 1* & *2*, with the exception that the offspring from all patches were collected under CO_2 sedation and put into a single glass vial for 24 hours of mass-mating prior to exposure to new hosts (See Figure 4.4). Prior to mass mating, 20 females were preserved in 95% EtOH and stored at -80^{o}C for later screening for infection prevalence. Each experimental population was replicated in triplicate to make a total of 18 populations.

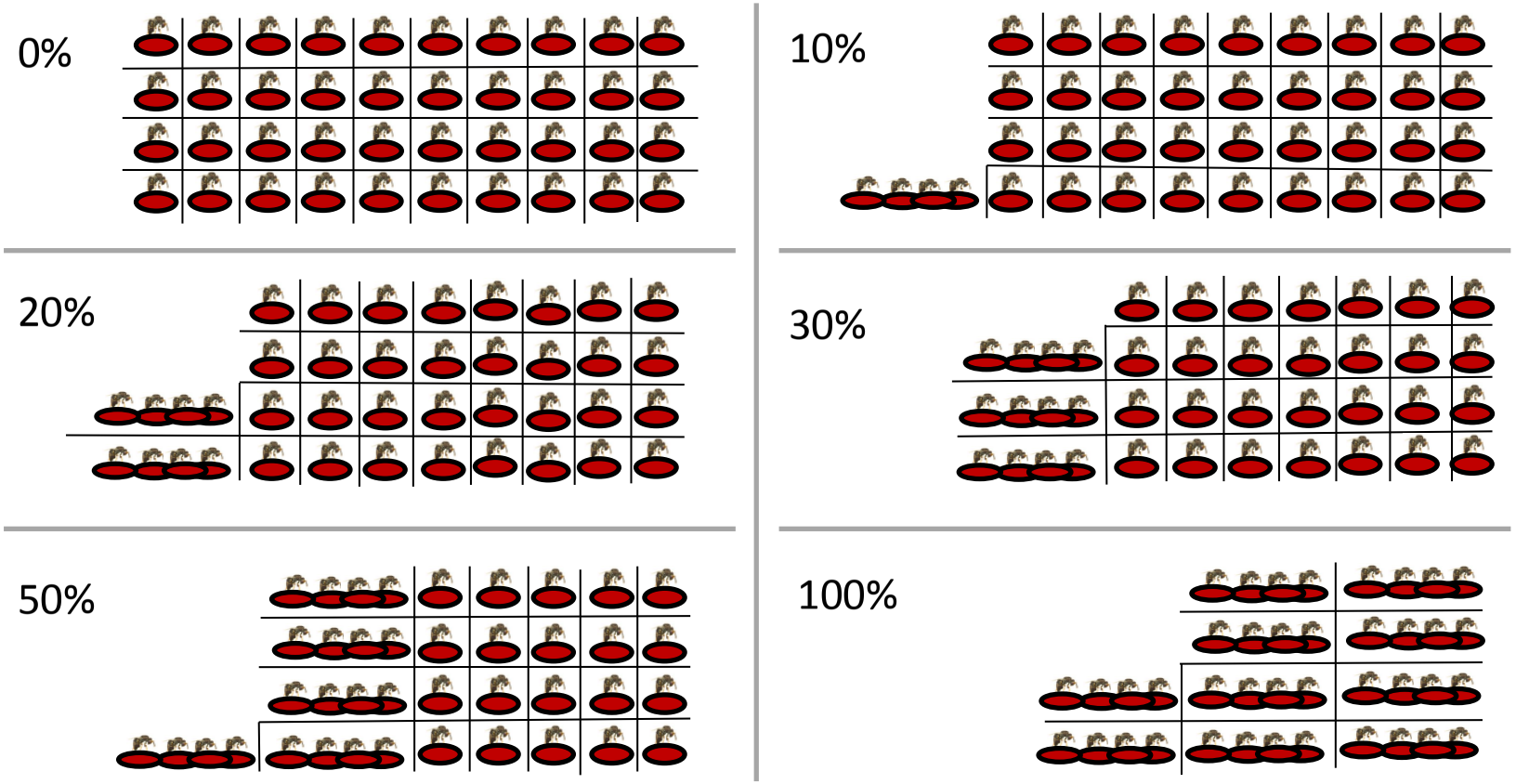


Figure 4.3: Schematic of populations set up for *Experiment 3*. Treatment levels were the varying opportunities for co-parasitism within populations. Wasps were assigned at random to single-lay or co-lay patches at each generation. Initial infection prevalence was set at 50%, with half of female wasps coming from *A+* stocks and randomly distributed across patches in each population.

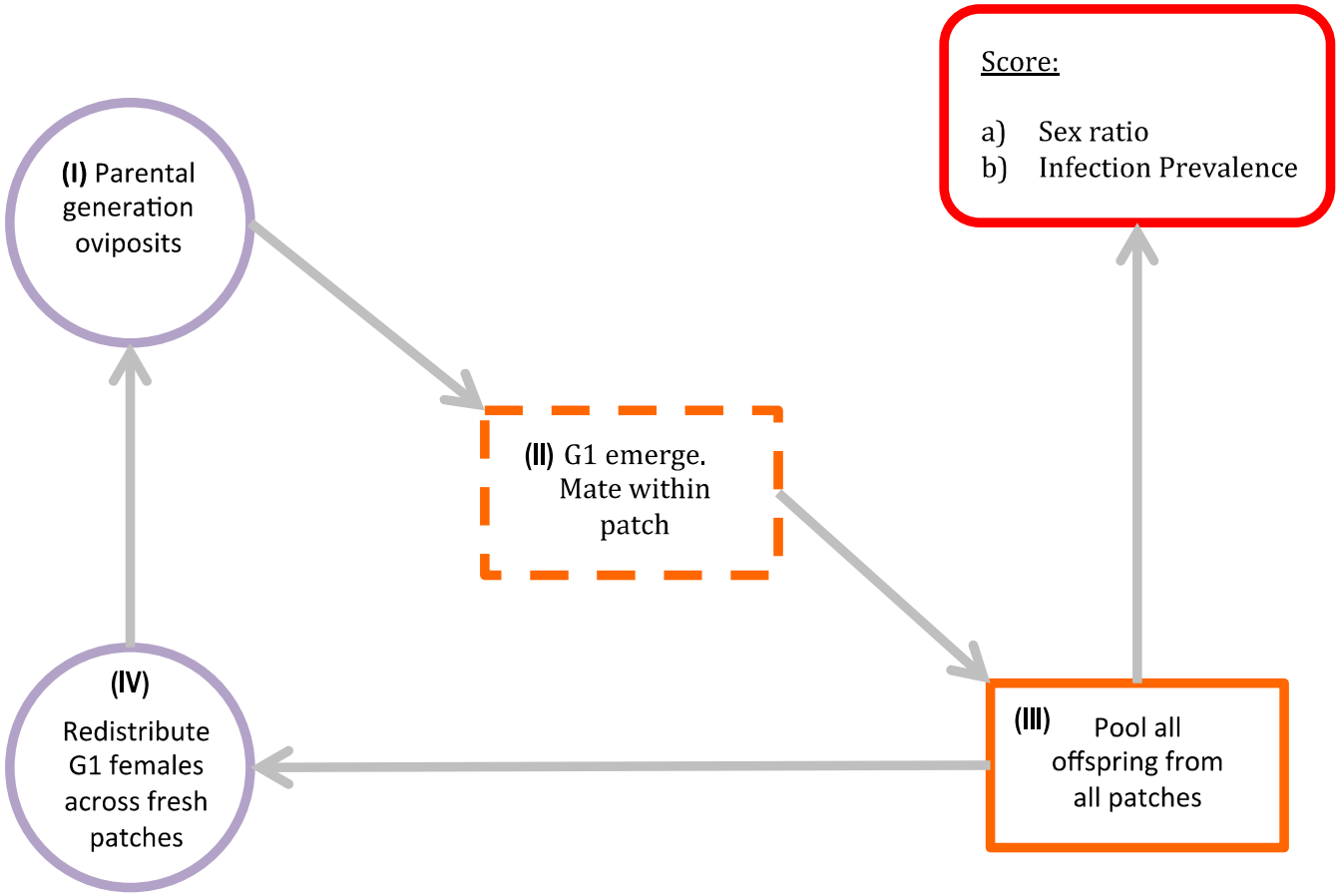


Figure 4.4: Schematic of experimental procedure for *Experiments 1, 2 & 3*. Each replicate population for all treatments goes through the same procedure. Within-patch mating (**II**) is enforced for *Experiment 1* and *2*. In *Experiment 3* pooled offspring are allowed mate on mass at step **(III)**. Sex ratio is only scored for *Experiment 1 & 2.* Procedure is repeated for eight generations in *Experiment 1*, and for four generations in *Experiments 2 & 3*.

4.2.8 Experiment 4: Is transmission efficiency linearly dependent upon host density?

Aim: to test the hypothesis that infection prevalence of G1 progeny from a given patch will be determined by the intensity of co-laying in that patch.

Five treatments of differing parasitism intensities were established with female densities of 8, 4, 2 and 1 individual(s) exposed to four host pupae. All co-laying treatments were established with 50% of foundresses infected with *A. nasoniae*, in order to allow infection to either increase or decrease. The single foundress treatment had 100% infection prevalence to establish vertical transmission efficiency. 50% infection was verified by reclaiming females after 48 hours of oviposition and screening them for infection through PCR. G1 progeny were left to develop at 25°C and females collected after emergence preserved in 95% EtOH and stored at -20°C for later PCR screening for infection prevalence. Each treatment was replicated 12 times, although this was reduced after removing replicates without the correct starting infection frequency. Infection prevalence within a brood was estimated by screening 10 G1 females by PCR. This accounts for an average of 20% of all individuals in each brood.

4.2.9 Statistical analyses

All statistical analyses were conducted in R using the 'lme4', 'fBasic' and 'binom' packages (CRAN repository). Where measured, sex ratio was recorded as the proportion of males in a clutch/population. Analyses of proportional data such as sex ratio and prevalence of *Arsenophonus* were carried out using general linear models with assumption for binomial errors. Where necessary, the experimental replicate was incorporated into statistical models as a random factor (*Experiments 1* & *2*). If models showed evidence of oversdispersion then this was accounted for by fitting an observation level random effect to account for high residual variation. All models were simplified to the minimum adequate form through pairwise tests (F-tests for fixed effect models, χ^2 tests for mixed effect models) and by selecting models with the lowest AIC score when a significant difference was detected. Statistics stated describe the variation in model fit when focal factors are removed.

4.3 Results

4.3.1 Results: Experiment 1: Assessing the role of Horizontal transmission in Arsenophonus spread

When female wasps oviposited in fly hosts in the absence of any opportunity for co-parasitism, *A. nasoniae* was lost from the population through poor transmission efficiency (<five generations in all replicates), irrespective of patch size (Figure 4.5). In contrast, *A. nasoniae* spread rapidly and was maintained (70-95% infected) in experimental populations in which horizontal transfer via co-parasitism was permitted. For simplicity, the infection prevalence was analysed at G3. At this time point, infection prevalence was significantly higher under co-parasitism (Stepwise simplification GLMER, χ^2 = 458.87, df = 2, P<0.001) but was not significantly affected by the level of host resource (Stepwise simplification of GLMER, χ^2 = 2.45, df = 1, P=0.118). The minimum adequate model was: Prop.Infected~Wasp.Density+(1|Cohort)+(1|Popn.ID), errors=Binomial.

High infection prevalence under co-parasitism led to demographic instability in populations. Initially, the spread of *A. nasoniae* reduces male frequency, causing increased female virginity. This is then often followed by a surge in male frequency as the virgins from the previous generation only produced males (Figure 4.6 [c & d]). The resultant inter-generation fluctuations are detailed in Table 4.1. The instability led to rapid extinction in populations with high wasp density but low host resources, in which male mates can only be supplied from the single pupa (Figure 4.7). Extinction was observed after only three generations and all populations were extinct by the 8th generation. Examining the state of the populations at G8, density (number of females laying in a patch) was associated with increased extinction risk (Fisher's exact test, P=0.037). Within high-density lays, low host resource (one host pupa not four) was associated with heightened risk (Fisher's exact test P=0.015). Note that populations that purged *A. nasoniae* are considered extant on the basis that no uninfected control population went extinct.

Table 4.1: Differences in mean sex ratio (proportion male) between infected populations and corresponding controls for treatments where co-parasitism was permitted. Note that the difference in sex ratio fluctuates from highly significant to non-significant. This is a product of demographic instability caused by male-killing and the production of all male broods by virgin females. All statistics are pairwise model comparisons of GLMERs.

	(A) High wasp density, Low Resource.			
Generation	Mean A+ Sex ratio	Mean Control Sex Ratio	Confidence interval	Notes:
G1	0.30	0.28	NS	
G2	0.08	0.23	***	
G3	0.05	0.20	***	Two populations extinct
G4	0.17	0.24	NS	One population extinct, one purged.
G5	0.18	0.18	NS	
G6	0.00	0.21	-	Very small, all-female broods.
G7	-	0.22	-	All populations extinct

	(B) High wasp density, High resource			
Generation	Mean A+ Sex ratio	Mean Control sex ratio	Confidence interval	Notes:
G1	0.20	0.25	NS	
G2	0.07	0.17	***	
G3	0.13	0.17	NS	
G4	0.16	0.20	NS	
G5	0.08	0.22	***	
G6	0.14	0.23	***	
G7	0.07	0.16	***	
G8	0.10	0.23	***	

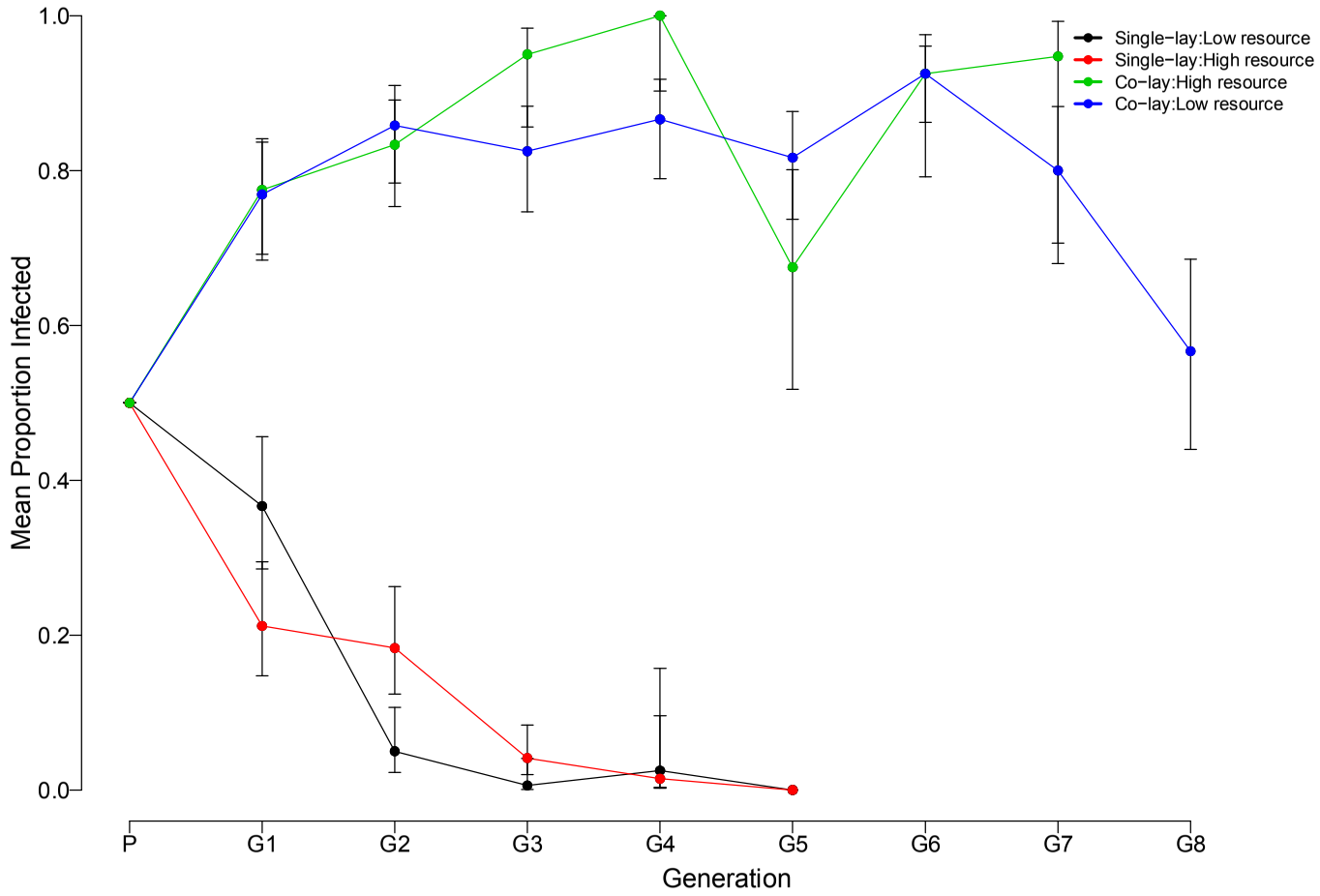


Figure 4.5: Mean infection prevalence over time for populations under four different oviposition treatments. Treatments with females ovipositing in isolation rapidly lose infection (red and black lines). Treatments with enforced co-parasitism saw rapid increases in infection prevalence (blue and green lines). Populations were removed from analysis once complete purging of the infection had occurred (F5 in all replicate populations where density = single). Error bars are 95% confidence intervals calculated for binomial data using the logit link function

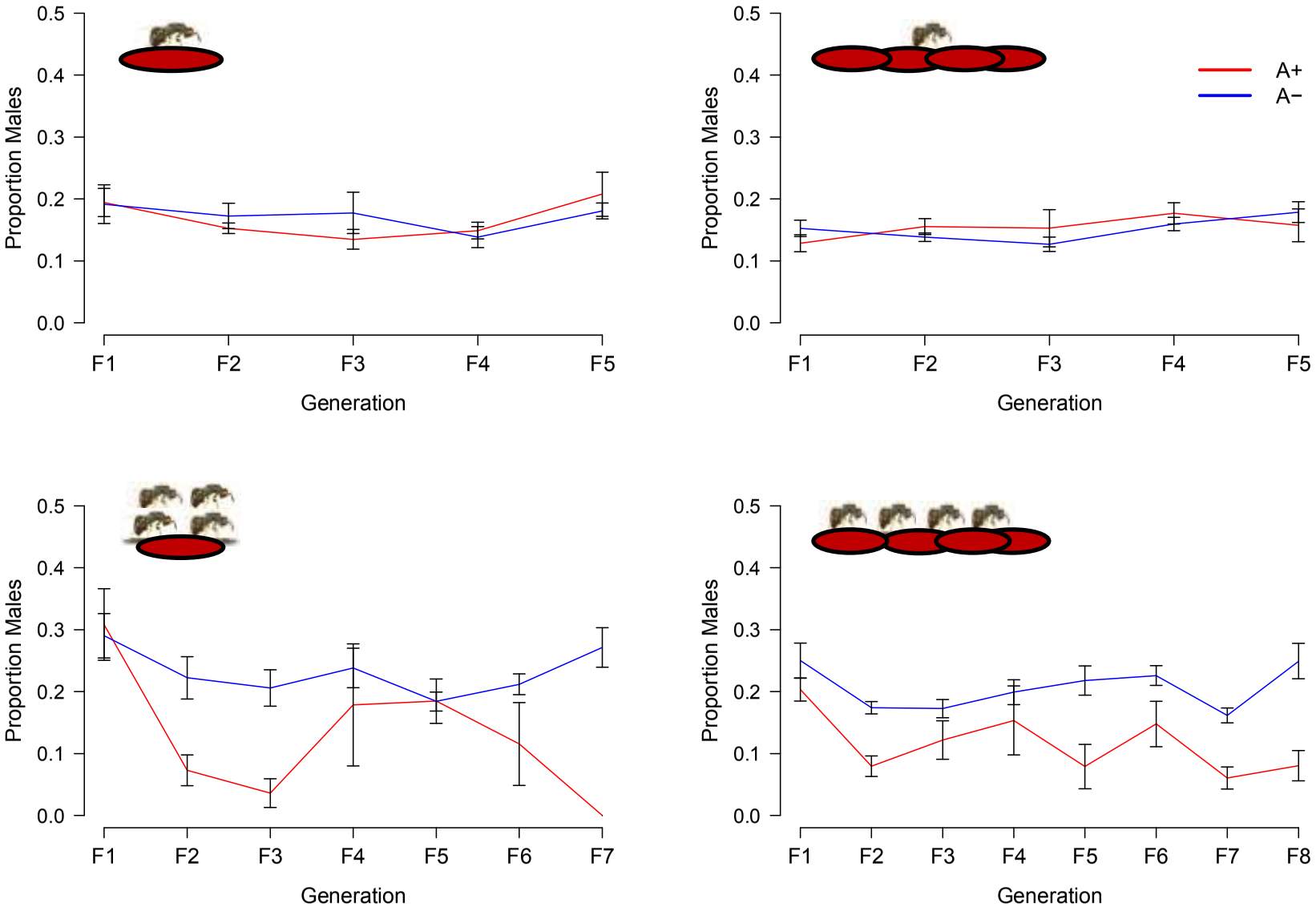


Figure 4.6: Mean sex ratio of control and infected populations (as proportion males). Error bars are 95% confidence intervals calculated for binomial data using the logit link function.

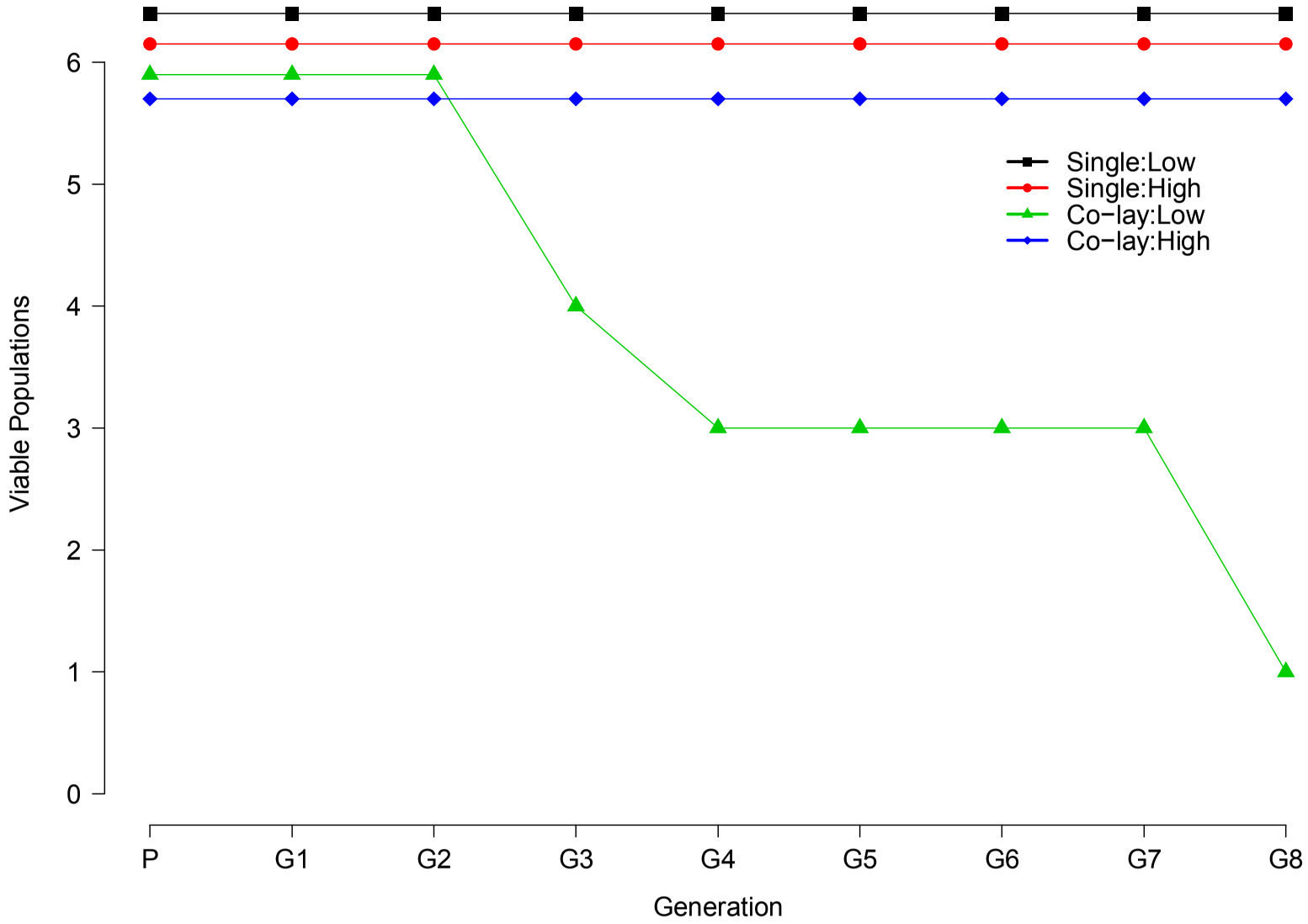


Figure 4.7: The number of replicate populations viable at each generation during the experiment. Any populations that purged the infection were considered viable because no control populations (infection free from the outset) ever went extinct.

4.3.2 Results: Experiment 2: Can horizontal transmission allow A. nasoniae to invade from rare?

Infection can drive into the population quickly and invasion occurs from low prevalence (Figure 4.8). The rate of drive is difficult to determine from these data due to the non-independence of infection prevalence for each treatment at each generation. However, at G4, infection prevalence of populations started with 50% and 5% infection are not significantly different from each other (GLMER with binomial errors, z = 1.286, df = 1, P=0.19). Furthermore, there is a significant increase in the infection prevalence of populations initiated at 5% infection prevalence (only two *A+* individuals of 40) between the G1 and G4 generations from 30% to 70% (stepwise model simplification of GLMER with binomial errors, χ^2 =2.321, df = 1, P<0.001). Regardless of non-independence of the generations this difference is stark and true for all six replicate populations.

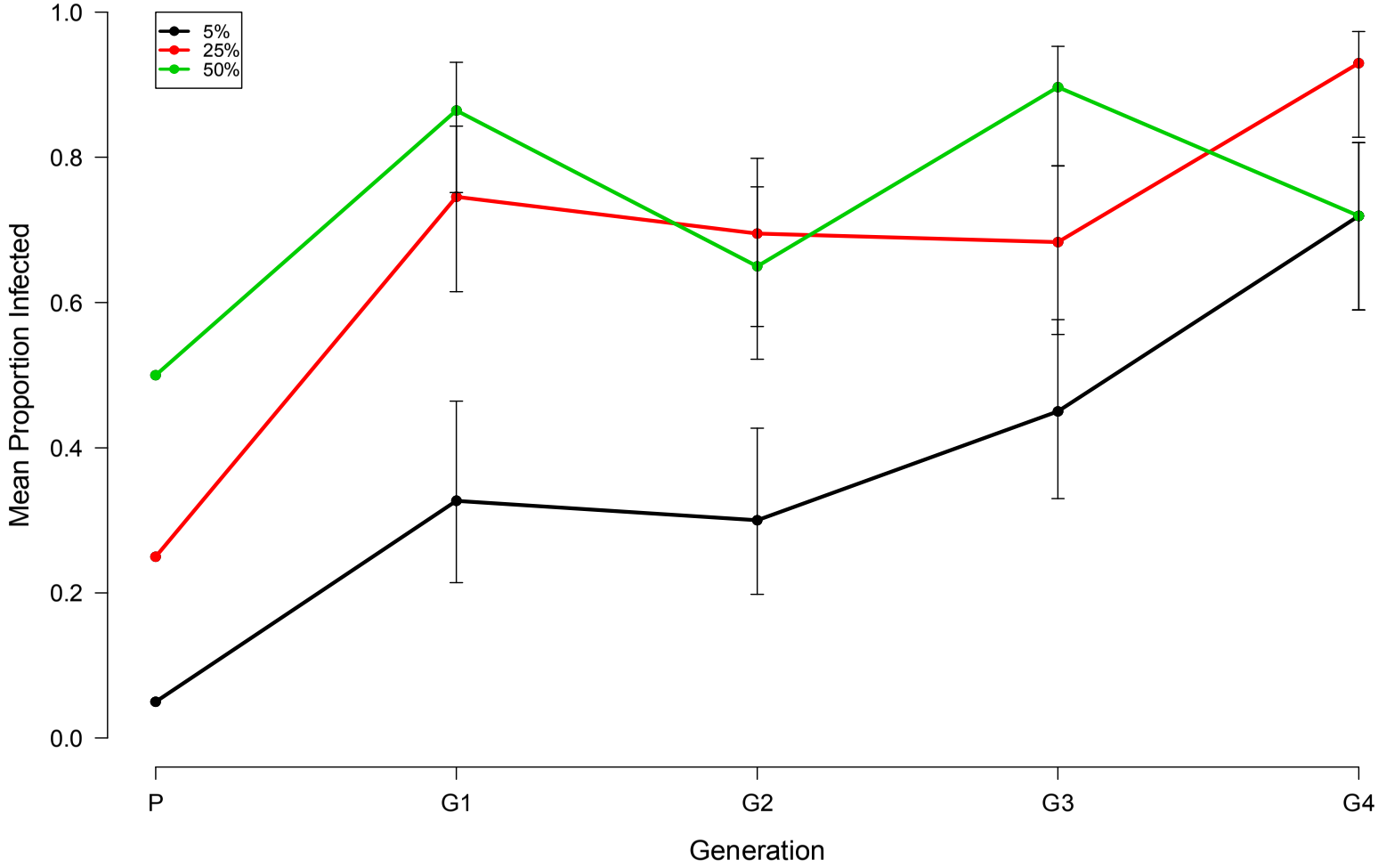


Figure 4.8: Change in *A. nasoniae* infection prevalence of co-laying populations starting from 5%, 25% and 50% initial infection prevalence. Points are the mean proportion infected from 6 replicate populations for each treatment. Error bars denote 95% confidence intervals calculated for binomial data using the logit link function. All populations here were under high density/high resource conditions (See Figure 4.2).

4.3.3 Results: Experiment 3: Is A. nasoniae invasion dependent upon a threshold level of horizontal transmission?

Infection prevalence in populations at G4 was positively correlated with the proportion of individuals in the population that are exposed to co-parasitism (Pairwise simplification of GLMER with binomial errors, χ^2 = 30.154, df= 1, *P*<0.001)(Figure 4.9). Individual binomial tests were used to determine if the combined infection prevalence of each treatment significantly deviated from the starting prevalence of 50%. Results of these tests are given in Table 4.2

Table 4.2 Summary of infection prevalence of populations in experiment 3 and their statistical deviation from the starting infection prevalence of 50%.

Treatment (%) multiparasitising.	Mean probability of being infected	Significance of deviation from 50% *(P)*
100	95%	<0.001
50	73%	<0.001
30	27%	<0.001
20	20%	<0.001
10	12%	<0.001
0	10%	<0.001

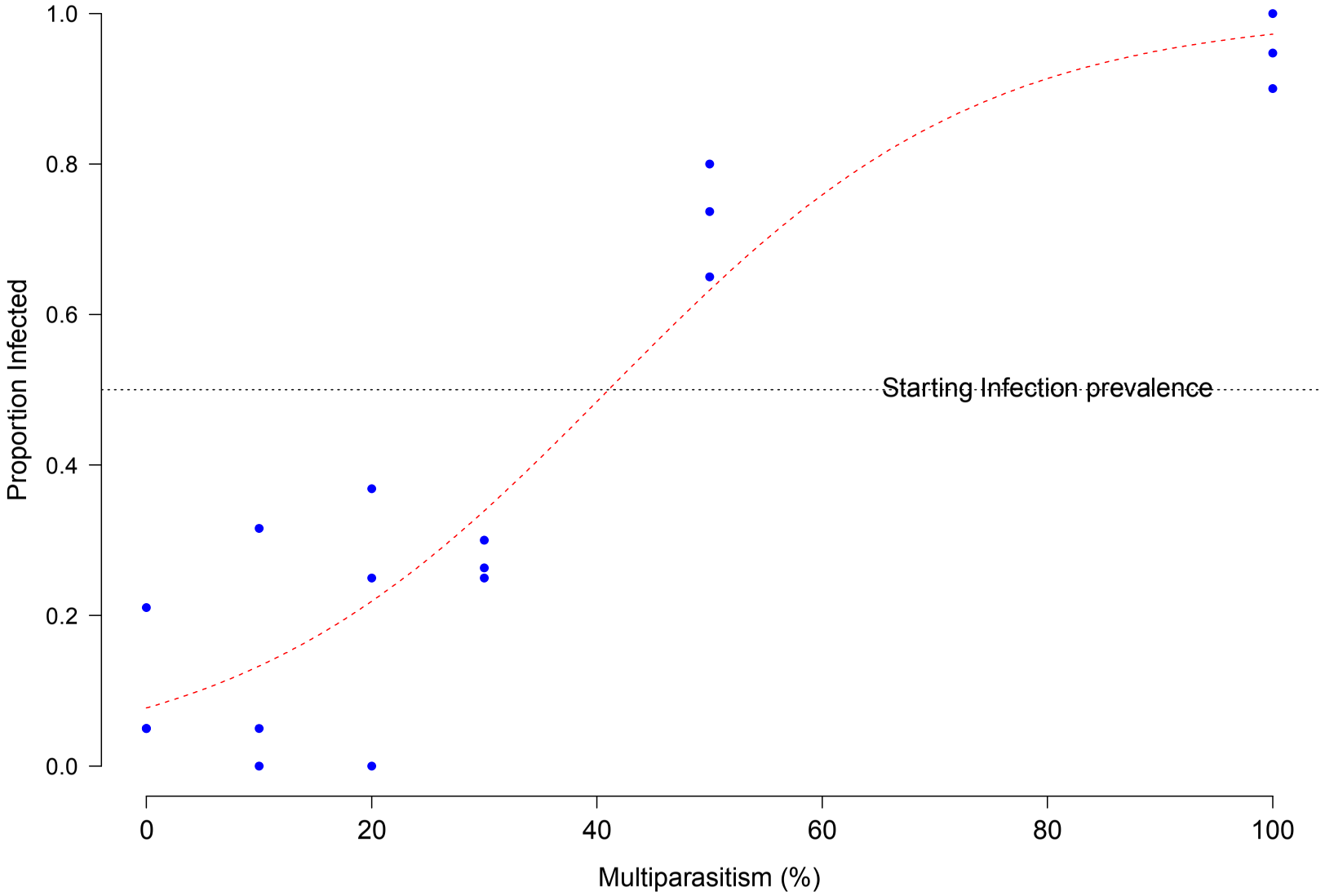


Figure 4.9: Proportion of individuals infected with *A. nasoniae* after 4 generations with different opportunity for multiparasitism within the population. Predicted line (dashed red) generated by GLM with binomial errors (Infection ~ multiparasitism, errors=binomial).

4.3.4 Results: Experiment 4: Is transmission efficiency linearly dependent upon host density?

Both vertical and horizontal transmission are operating at high efficiency (Figure 4.7). There is no significant difference in offspring infection from wasps kept at increasing densities (Pairwise simplification of GLMERs with binomial errors, χ^2=3.89, df=3, *P*=0.9). However, infection through pure vertical transmission is sub-perfect, with a mean of 94% of F1 females being infected. Although this is not statistically deviant from other treatments, it is biologically important.

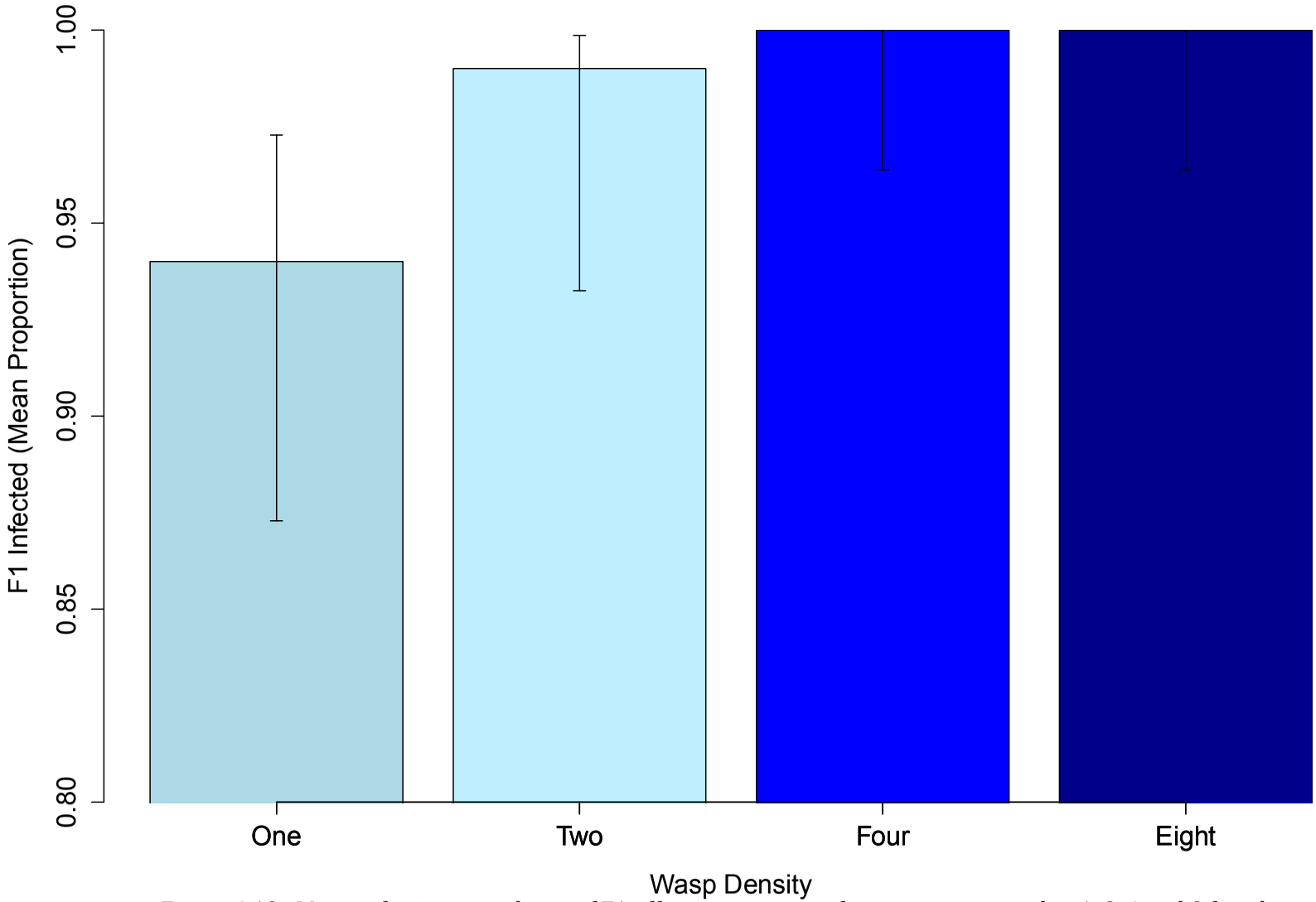


Figure 4.10: Mean infection prevalence of F1 offspring emerging from pupae exposed to 1, 2, 4 and 8 female *N. vitripennis*. In all multi-parasitism treatment s (2, 4, 8) 50% of mothers were infected. There is no significant difference in infection prevalence between treatments, however the sub 100% infection under single parasitism illustrates the imperfect vertical transmission of *A. nasoniae*. Errors are 95% confidence intervals calculated for binomial data with logit link function.

4.4 Discussion:

Arsenophonus nasoniae has traditionally been regarded as a male-killing parasite dependent upon resource reallocation or reduction of inbreeding load to spread. Indeed, whilst infectious transmission was known when *A. nasoniae* was discovered in 1985, early models of its epidemiological dynamics did not incorporate co-infection. In this chapter I demonstrate that *A. nasoniae* is reliant on host co-parasitism for its invasion and maintenance. *A. nasoniae* was lost rapidly from populations where females were forced to oviposit alone, but maintained at high prevalence when females always oviposited alongside con-specifics. Further, when the opportunity to co-parasitise was manipulated more precisely, the extent to which *A. nasoniae* can penetrate a susceptible population was directly dependent upon the level of co-parasitism occurring in that population. A final observation of these population biology experiments is that *A. nasoniae* can rapidly invade co-parasitising populations from rare (5% infection) and move to high prevalence (60% infection in four generations). From these experiments, we can infer that horizontal transmission of *A. nasoniae* from infected mothers to the offspring of uninfected mothers is necessary for the maintenance of infection in natural populations. Conversely, the rapid loss of infection when only a single female parasitizes a host is indicative that pure vertical transmission is not sufficient to maintain the bacterium in natural populations.

It was notable in these experiments that infection prevalence dropped very rapidly in the absence of co-parasitism. The rate of loss (*c*50% per generation) is considerably greater than would be expected from the segregational loss demonstrated under vertical transmission estimated in *Experiment 4*, which suggests that vertical transmission efficiency is c95%, a figure consistent with previous studies (Skinner, 1985). Thus, the inter-generational loss seen in *Experiment 1* implies that *A. nasoniae* is not solely being lost through segregation, but is also being actively selected against under vertical transmission. This is consistent with the evidence of high direct costs of infection observed in Chapter 3, for example the high incidence of wing deformity seen in the daughters of infected mothers. In essence, the main force reducing *A. nasoniae* presence is the low relative fecundity of infected females compared to uninfected, and only when there is a strong infectious force is *A. nasoniae* maintained.

Whilst this study was initially designed to provide insight into the factors affecting *A. nasoniae* population biology, the data also gives us an important insight into the potential impacts upon host population biology. Under particular circumstances (high density of wasps and low resource) male-killer infections can drive their host populations to extinction by causing global virginity (*Experiment 1*). Extinction was not observed under high density of wasps and high resource, indicating that low resource is necessary for extinction in the timescale under study. This result is probably explained by the chance of a single male deriving from a patch. Where there is high resource, there is a greater chance of a male offspring being produced, either from an uninfected female or through surviving male-killing. Low resource limits the opportunity for males to be produced, and thus increases the chance that no males emerge, the condition that creates virginity and loss of either infection or, when played out across multiple host pupae, the population.

4.4.1 The role of horizontal transmission in symbiont spread:

Models of heritable symbiosis predict that symbionts' ability to invade and persist is dependent upon their drive phenotype offsetting any segregational loss (Werren, 1987; Hurst, 1991; Randerson *et al.*, 2000b). Wasp density is an important component of classic male-killer theory. The adaptive benefit of male-killing is based upon density dependent competition between infected siblings, with the death of males relieving this competition, resulting in increased female fitness. This benefit should break down if the competition is between unrelated individuals, as the death of males will benefit both infected and uninfected individuals equally. This phenomena was empirically demonstrated by Jaenike *et al* (2003) when they showed that male-killing *Wolbachia* spread in experimental populations of *Drosophila innubila* under sib-sib competition, but was lost when mixed competition was permitted (Jaenike *et al.*, 2003). The dynamics of *A. nasoniae* presents a marked contrast to this dogma. Invasion occurs only when wasps are permitted to lay at high density, and infectious transmission occurs between individuals from unrelated lineages.

Overall, infectious transmission is likely to be making a greater contribution to the drive of *A. nasoniae* than the adaptive benefit of male-killing. However, if infectious transmission is high within pupae, so that the majority of offspring will emerge infected regardless of parentage, then the resource reallocation hypothesis can still stand. The death of sons from infected mothers will free resources to all F1 females in the brood,

all of whom carry *A. nasoniae* by virtue of infectious transmission. In this scenario all potential *A. nasoniae* carriers enjoy a fitness increase irrespective of lineage. Furthermore, this effect may actually be stronger than resource reallocation under vertical transmission (females ovipositing alone). Costs of crowding will be greater when a pupae is multi-parasitised as there will be more individuals present and so resources are stretched thinner (Sykes *et al.*, 2007). Further, horizontal transmission is most effective when the infected female lays second – here she lays nearly completely male broods. Thus the death of males releases resources that are of greater value to females than when only one clutch is present. This effect is an inherent assumption of the model presented in Chapter 2.

An alternative explanation for the strong drive observed under multiparasitism is that when multiple infected females inoculate a host during co-parasitism they increase bacterial titre in the resource patch and thus boost per-oral infection rate. However, the ability of the infection to invade from rare in *Experiment 2* suggests this is not a good explanation for the results. Infection increased in frequency from initial prevalence levels of 5% and 25% - conditions under which only a single infected female could initially be present on a patch. In this initial scenario the uninfected females co-parasitising with a single infected individual do not contribute to the bacterial titre, but infection nevertheless increased dramatically between the Parental and G1 generations in both treatments (Figure 4.8). This implies that the drive of infection associated with co-parasitism is independent of bacterial titre. Furthermore, direct estimates of transmission parameters (*Experiment 4*) showed there to be no significant difference in G1 infection prevalence between broods produced from one, two, four and eight females. Therefore, vertical transmission is high (the data from *Experiment 4* estimate 95% efficient), as is horizontal transmission. The loss of infection seen in the absence of co-parasitism must be associated with costs of infection rather than just segregational loss through inefficient transmission.

Potentially, there is an effect of multiple stinging (with infected venom or not) on *A. nasoniae* transmission. As the bacterium is exposed to the dipteran host before being eaten by the wasp larvae it is at risk from the immune effectors of the fly. *N. vitripennis* has been shown to modulate immune function in their dipteran hosts (Rivers *et al.*, 2002), so it is reasonable to assume that symbiont survival (and thus transmission efficiency) may be positively correlated with venom dosage. If this were true then the uninfected mothers would be facilitating the infection of their own offspring by stinging

co-parasitised hosts. *Experiment 4* goes someway to disproving this theory, as it demonstrates that the high infection rate driven by horizontal transmission is independent of the intensity of co-parasitism. Indeed, as stated above, vertical transmission is high (95% efficient) so segregational loss alone is unlikely to produce the rapid decline of infection seen in Figure 4.5. It is impossible to completely rule out a venom-mediated effect on transmission from this data, as its margin of effect will be small given *A. nasoniae's* already high transmission efficiency.

Ultimately, the drive associated with co-parasitism is strong because it allows the offspring of uninfected individuals to change class and become infected individuals. Conversely, under only imperfect vertical transmission, only infected individuals can change class and become uninfected. This may operate in conjunction with the adaptive benefit of male-killing through resource release, particularly in the crowded multiparasitised pupae. It is therefore possible to argue that infectious transmission is necessary for the drive of *A. nasoniae*, but likely not solely sufficient. The male-killing phenotype releases resources to all carriers of *A. nasoniae* irrespective of lineage and so still conveys an adaptive advantage to infected females over uninfected females from similarly crowded pupae. Importantly, both vertical and infectious transmission occur solely through female hosts. This may explain why a bacterium that readily horizontally transmits has retained a phenotype associated with pure vertical transmission.

4.4.2 Horizontal transmission and the evolution of virulence

Ultimately, high horizontal transmission is a paradox for heritable symbionts because vertical and infectious transmission success commonly have differing virulence optima. Horizontal transmission should select for a greater level of virulence as microbe strains may be placed in competition with each other, such that microbe fitness is a product not simply of host fitness (as under vertical transmission) but also their ability to gain representation in the face of competition. This may select for increased growth rates and titre within the host (Frank, 1996a). Further, it is easy to imagine that the efficiency of infectious transmission is positively correlated with parasite growth, which itself may cause pathogenicity in the host.

The adaptive trade-off between transmission mode and virulence has been demonstrated using experimental evolution of Barley Stripe Mosaic Virus (*Hordeum vulgare)*. Here the authors found that after just four generations of horizontal transfer,

virulence increased 300%. Following a subsequent three generation of vertical transmission, virulence decreased by 40%. The virus also lost vertical transmission efficiency under regimes of enforced infectious transmission and *vice versa* (Stewart *et al.*, 2005). However, male-killers are an unusual case. The virulence of male-killers is sex specific and indirectly boosts vertical transmission by increasing female fitness (but see Chapter 3). Furthermore, in the specific case of *N. vitripennis*, horizontal and vertical transmission occurs through the same pathway: oral acquisition of the bacterium by feeding on an infected host. Therefore, whilst increasing titre may increase the rate of infection within a brood, this effect should be equal under both transmission routes. Even if increasing titre does result in a virulence increase, this will impact upon both vertical and horizontal transmission routes equally.

Ultimately, the data in Chapter 3 demonstrates that *A. nasoniae* does induce a cost upon its host, which can be considered virulence. Here I have demonstrated that horizontal transmission is a necessary component of *A. nasoniae's* epidemiology. In this case it appears that *A. nasoniae* may be trading off infectious transmission with virulence.

4.4.3 Invasive capacity of Arsenophonus nasoniae

The second experiment demonstrates that very few A+ individuals are required to seed successful infection of *A. nasoniae* in a population if co-parasitism is common. The ability to invade from rare is an important trait for male-killers as well as other parasites, as it is extremely unlikely that 50% of the population will become infected simultaneously (the initial conditions in *Experiment 1*). It is also important to note how rapidly the infection was driven to >50% prevalence (4 generations) and therefore how strong potential sweep of *A. nasoniae* would be in dense natural population of *N. vitripennis*. Documentation of rapid symbiont sweeps are increasing in the literature, from both observed evidence (Himler *et al.*, 2011) and inferred molecular data (Riegler *et al.*, 2005; Lack *et al.*, 2011). High prevalence of symbionts in host populations can have profound effects on host biology, and so it is important that we understand the factors that allow them to invade and sweep.

In *Experiment 3,* heterogeneous levels of single and co-parasitism were imposed on populations to determine how invasion potential will be mediated by population structure. Many models of male-killer drive predict that there should be a threshold drive level within a population above which the symbiont can invade (Werren, 1987;

Hurst, 1991), although these are modeled for fitness compensation and not infectious transmission. The data presented here indicate a linear relationship between the level of co-parasitism in a population and ultimate infection prevalence. The extent of co-parasitism in a population directly dictates the prevalence of *A. nasoniae* infection. One caveat in these experiments is that it is not known whether the populations are at equilibrium for *A. nasoniae*. Whilst co-parasitism exposure >50% clearly drives infection up in frequency, it is not clear whether the low prevalence infections under lower rates of co-parasitism represent equilibria, or infections that are being lost from the population. It is notable that loss of infection was observed where co-parasitism rates were <20%, suggesting this may represent a threshold rate required for persistence.

4.4.4 Symbiont driven extinction

The high extinction risk under co-parasitism with limited resource seen in *Experiment 1* has important consequences for the study of disease-driven host ecology, epidemiology and conservation. These results demonstrate that when male-killer prevalence becomes high enough it causes demographic instability in populations, with many females going unmated and remaining virgins. When host resources are low, i.e. there is only a single host pupae per patch from which a male can be derived, this virginity can become global and the populations suffer extinction. Similar levels of demographic instability are seen when resources are high (four host pupae per patch) but the virginity effect remains weak. I hypothesised that the high mating capacity of a single *N. vitripennis* male means that only one of the four pupae in a patch must produce a single male to keep the population viable. Therefore the extinction effect demonstrated here is likely to occur in dense, resource limited populations.

Bill Hamilton (1967) acknowledged that a sex ratio distorting element may be able to cause host extinction by reducing the effective population size of its host to zero through suppression of either sex (Hamilton, 1967). This phenomenon has been empirically demonstrated with SR meiotic drive chromosomes in laboratory populations of *Drosophila pseudoobscura* (Price *et al.*, 2010). Hatcher *et al* (1999) expanded upon this and acknowledged that sex ratio distorting symbionts could also have the same effect if they were at fixation (Hatcher *et al.*, 1999). Later work explicitly implicates male-killers when spatial population structure is considered (Groenenboom and Hogeweg, 2002). Previous theory for male-killers suggested that they would only

drive extinction where vertical transmission was near 100%, as otherwise segregational loss provides a source of males. This conclusion is reflected in the very extreme sex ratio seen in wild *Hypolimnas bolina* populations (where segregational loss has not been observed (Dyson *et al.*, 2002; Charlat *et al.*, 2005) and in laboratory emulation by Jaenike *et al* 2003, where male-killing *Wolbachia* in *Drosophila innubila* drove experimental populations to extinction through virginity when allowed to drive to high prevalence (Jaenike *et al.*, 2003). The study presented here indicates that the requirement for perfect vertical transmission for population extinction is relaxed when infectious transmission occurs.

A key question is whether extinction is likely to occur in this particular system. The results of *Experiment 1* suggest it is only likely when co-parasitism is very common and host pupae very rare. Field estimates of co-parasitism (c40% - Grillenberger *et al.*, 2008) are not sufficient to produce the extinction dynamic. However, local extinction remains possible in regions of high co-parasitism. It is widely assumed that any sex ratio distorting element is inherently unstable and it is hypothesised that the majority of novel biasing elements that arise quickly drive either themselves or their hosts extinct and are not noticed by researchers (Carvalho and Vaz, 1999). The well studied sex ratio distorters are those that are kept at some level of equilibrium through either compensatory mechanisms (Price *et al.*, 2010), through their own inefficient transmission (this study), or through host response in the form of suppression of sex ratio distortion (Hornett *et al.*, 2006). Therefore it may be that *A. nasoniae* driven extinction occurs rapidly at very localised level, and surveyed prevalence are from incidences where host density does not allow fixation or extinction.

Theory predicts that the spread of a parasite should not result in host extinction by virtue of the dependence of infectious transmission upon host density. The core assumption is that a lethal or castrating parasite should not reach fixation in a population because its own virulence reduces R_0 below 1. Because of this, parasites can reduce their host's population size and limit diversity thus making populations more susceptible to extinction, but their virulence will not be the causative agent. However, there are also predictions that parasites that spread in a frequency dependent fashion, e.g. those that are dependent upon events such as reproduction or foraging, may be able to rapidly reach fixation and thus globally affect their host population and cause extinction (Best *et al.*, 2011). Furthermore, it has been argued that within spatially structured host populations, frequency dependent parasite spread is more likely to

cause local extinctions, which may then be 'rescued' globally by migration from uninfected patches (Boots and Sasaki, 2002).

Realistically, density and frequency dependent transmission are not discrete, and disease spread is often an additive effect of both. In the *Nasonia* system infectious transmission occurs with oviposition, which is frequency dependent, but the rate of co-parasitism is most likely a function of host density. *Arsenophonus nasoniae* spreads because the unusual the sex specific virulence allows the infection to reach high prevalence in the female population. There is no negative feedback from virulence until global male availability becomes low to the point of causing virginity. It is at this point that population level costs are felt and, given external stressors, extinction becomes a realistic risk. Therefore the infection is behaving similarly to a frequency dependent parasite, but the mechanism is unique.

4.5 Conclusion

The data presented here allow us to understand *A. nasoniae* spread in the context of its host's ecology. I demonstrate that the adaptive benefit of male-killing through resource allocation is insufficient to drive or maintain infection in populations where only maternal transmission is permitted. However, allowing individual wasps to regularly co-parasitise offers a route of infectious transmission to the bacterium and results in increased infection levels. Infectious transmission is sufficiently strong to allow the infection to invade from just a few founding females within a population when co-parasitism is high, and the extent to which the infection will penetrate a population is dependent upon the level of co-parasitism. Finally, the male-killing phenotype, coupled with high drive through infectious transmission can leave host population vulnerable to extinction, particularly if patch resources are limited.

Chapter 5:

Assessing the phenotypic and genomic divergence of a novel *Arsenophonus nasoniae* isolate following a recent host shift

Abstract:

Related symbionts are commonly found in evolutionarily disparate hosts. The symbiotic phenotype observed varies between hosts across the mutualist-parasite continuum. It is generally considered that infectious host-shift events, rather than co-speciation is the major driver of secondary symbiont presence, and so microbes that regularly employ horizontal transmission in their within-host species epidemiology are most likely to spread quickly across species. Upon infecting a new host, symbiont infections are typically maladapted, but undergo rapid selection for optimum symbiotic phenotype, often driving innovation of novel traits and reinforcing disparity between closely related symbiont strains. Here I investigate a novel *Arsenophonus* strain that naturally infects a solitary parasitoid *Pachycrepoideus vindemmiae*. I test the hypothesis that this strain has recently diverged from the male-killing strain described previously and is on an evolutionary trajectory towards a specialized mutualism with its new host, at the expense of previously described reproductive manipulation. I investigate the transmission route, fitness effects and manipulative potential of this novel strain. I demonstrate its host-specialization through interspecific transinfection experimentation and finally examine how the observed disparities in symbiotic phenotype may be underpinned by genomic change.

5.1 Introduction:

In Chapters 2, 3 & 4, I addressed the role of horizontal transmission in the within-host species dynamics of *Arsenophonus nasoniae* in *Nasonia vitripennis*. However, the literature on horizontal transmission of symbionts is more widely concerned with their distribution across species and how selection acts to generate diversity across closely related symbiont strains. Previous work on the host range of *A. nasoniae* has found it to be widespread amongst the chalcid parasitoids, but with little genetic variation at conserved MLST markers, implying high relatedness between strains (Duron *et al.*, 2010; Taylor *et al.*, 2011; Wilkes *et al* in prep). However, casual observations have alluded to highly disparate behavior of the infections in some of these host species under laboratory conditions. In this chapter I test the overarching hypothesis that following a natural host-shift event, *A. nasoniae* has evolved towards a more mutualistic symbiotic phenotype. I empirically quantify key symbiotic traits such as transmission route and efficiency, evidence for reproductive manipulation and effects on fitness. I also test for the evolution of host specialization by attempting to artificially transinfect this novel *A. nasoniae* into *N. vitripennis*. Finally, I briefly compare the genome of a novel, non-male-killing strain of *A. nasoniae* to that of a previously published male-killer strain (Darby *et al.*, 2010; Wilkes *et al.*, 2010) with a focus on putative virulence/symbiosis factors.

5.1.1: Symbiont diversity following host shifts:

Insect secondary endosymbionts are widespread, and any given symbiont strain is often found in disparate host species, genera, orders and even classes (Duron *et al.*, 2008). The most readily cited example of this is the distribution of *Wolbachia*. This group of symbionts has been found in most orders of insects, as well as in isopods crustaceans, *Aranea* and even filarial nematodes. Whilst the diversity and phylogeny of *Wolbachia* itself is complex and diverse, it does not reflect the genetic distance between its hosts (Lo *et al.*, 2007). Indeed, secondary symbionts rarely exhibit co-cladogenesis with the lineage of their host species (Raychoudhury *et al.*, 2009; Burke *et al.*, 2009; Jousselin *et al.*, 2013), although evidence does suggest that they can become specialized on particular groups of host species within which they horizontally transmit (Russell *et al.*, 2009). Furthermore, genetic analyses of secondary symbionts often shows evidence of recombination between strains, an indicator that horizontal transmission or hybrid introgression forms co-infections where exchange of genetic material is

possible (Jiggins *et al.*, 2001; Baldo *et al.*, 2006; Mouton *et al.*, 2012). It has therefore been hypothesized that interspecific transmission is key to explaining the distribution of symbionts across host species, rather than co-divergence through evolutionary time as is the case with obligate primary symbionts (Lo *et al.*, 2003; Baumann, 2005; Takiya *et al.*, 2006).

Once enigmatic, routes for interspecific infection are becoming well documented from laboratory experiments. These may be direct, where the symbionts regular transmission mode predisposes it to host-shifts, e.g. the pseudo-vertical transmission of *A. nasoniae* (Duron *et al.*, 2010). Alternately, transmission may be indirect, where infection occurs when host species share an ecological bridge. For example, *Hamiltonella defensa* and *Regiella insecticola* have both shown evidence of interspecific transmission between aphids, vectored by the hosts' hymenopteran parasitoids (Gehrer and Vorburger, 2012). However, the success of a symbiont in a novel host is ultimately dependent upon its ability to persist and exert drive (through manipulation or conveyed benefit).

The relative merits of specific drive mechanisms will vary from host to host, as will the extent to which the symbiont is adapted to the novel host environment. Indeed, host-shift are often followed by decreased transmission efficiency (Kellner, 2002; Jaenike *et al.*, 2007; Duron *et al.*, 2010; Hutchence *et al.*, 2012) and increased virulence (Calvitti *et al.*, 2010; Chafee *et al.*, 2011) or both (Clancy and Hoffmann, 1997; Russell and Moran, 2005; Kageyama *et al.*, 2006a; Tinsley and Majerus, 2007). Therefore, we expect selection to act upon symbionts to maximize, and possibly change, the nature of drive, and to adapt to a potentially hostile novel host environment.

This process of host-shift driven adaptation may explain the diversity of symbiont phenotypes within a given group of microbes. For example, *Wolbachia* has been associated with reproductive manipulation (Stouthamer *et al.*, 1999), mutualistic viral resistance (Hedges *et al.*, 2008), stress tolerance (Brownlie *et al.*, 2009), a key component of its host's virulence (Ferri *et al* 2011) and even obligacy for host oogenesis (Dedeine *et al.*, 2001). Some experimental work has shown that phenotypes may persist in novel hosts following a host shift (Hoffmann *et al.*, 2011), however in other cases the phenotype expressed in the original host is reduced or lost, possibly as a positive function of genetic distances between hosts (Duron *et al.*, 2010). We would therefore expect to see a signature of selection, or rapid differentiation in genes

associated with host interaction between closely related symbiont in disparate host species. These differences may be extreme because a host shift can represent a tidal change in the evolutionary trajectory of the symbiont, even a transition from parasitism to mutualism (Weeks *et al.*, 2007).

5.1.2: Evolution towards mutualism

It is hypothesized that the majority of current mutualistic symbioses have evolved from parasitic associations (Douglas, 2011) in the same way that parasites evolve from free-living organisms. These transitions can come about through a host-shift event, where a parasitic symbiont finds itself in a host where its manipulative phenotype is sub-optimal but there is for the possibility of a mutualistic association. For maternally inherited elements, selection will promote any trait that contributes to the production or survival of female hosts.

Broadly, this evolutionary trajectory towards mutualism is expected to be characterized by three core changes:

a) As the microbe becomes beneficial for the host there should be selection for increased VT efficiency. This can come about through selection on either party and should lead to specialization of the symbiont on that particular host through reciprocal co-evolution.

b) By its very nature, a mutualist should maximize the fitness of its host and minimize the detrimental impact of its own presence. Therefore, it should evolve to decrease direct costs of infection and convey a benefit. At a phenotypic level we should see minimal to no cost associated with symbiont infection and ultimately a benefit that may or may not be ecologically contingent. At a genomic level this should manifest as a loss of factors associated with virulent phenotypes.

c) The evolution of mutualism and associated vertical transmission will be accompanied by genomic decay. This may occur through a process of accumulated deletion mutations acquired through systematic population bottlenecks at transmission (Rispe and Moran, 2000; Moran *et al.*, 2008; Kaltenpoth *et al.*, 2009), or loss of genes that have been rendered redundant due to intimate associations with a host. The former process is a consequence of Muller's ratchet (Moran *et al.*, 2008), whilst the later has been termed the 'Black Queen Hypothesis' (Morris *et al.*, 2012).

Comparative analyses between distantly related genomes of primary-mutualists and their nearest free-living ancestor have verified this that genomic change can be massive following millions of years of vertical transmission. Furthermore, even on a relatively

short time scale, genomic decay was shown to have occurred in the genome of the defensive secondary-mutualist of aphids, *Serratia symbiotica*, compared to its free-living ancestor (Burke and Moran, 2011). However, there has only recently been a focus on genomic transitions within a symbiont group itself. There have been no in-depth studies of genomic evolution following a host shift event married with quantification of a novel symbiotic phenotype.

5.1.3 ArPv, a novel strain of symbiont found in Pachycrepoideus vindemmiae

Surveys of *A. nasoniae* distribution within species of chalcid wasps have found several natural host-symbiont associations (Duron *et al.*, 2010; Taylor *et al.*, 2011). The majority of these wasps share the ecological niche of parasitising dipterans, predominantly of the flesh fly or filth fly guilds. This has been postulated as the route through which the infection has spread between hosts species. Duron and colleagues (2010) demonstrated that *A. nasoniae* could indeed host shift in this way, but that the effectiveness of its male-killing phenotype and vertical transmission efficiency diminished with genetic distance between hosts. Therefore, *A. nasoniae* should regularly arrive in disparate wasp species and will be under strong selection for alternative drive mechanisms and adaptation to the novel host background. They also found high levels of relatedness between *A. nasoniae* strains naturally infecting different wasp species. So we would hypothesis that genetic comparison between *Arsenophonus* strains will reveal high relatedness in conserved genes, contrasted with adaptive variation in virulence/symbiosis associated factors.

One of the *A. nasoniae* strains identified by Duron *et al* (2010) was naturally infecting a pseudo-solitary parasitoid of several fly species, *P. vindemmiae*. This strain will be referred to as *ArPv* hereafter. Casual observations of *ArPv* infection in *P. vindemmiae* showed no evidence of overt reproductive manipulation (personal observation). Furthermore, the infection was kept in populations of its wasp host without selection at both the University of Lyon, France and the University of Liverpool, UK. MLST comparisons by Duron *et al* found no significant sequence differentiation between *ArPv* and the male-killing type strain of *A. nasoniae (ArN* hereafter). This leads to the hypothesis that *ArPv* represents a strain of *A. nasoniae* that is currently in an evolutionary transition between reproductive manipulator and mutualistic association with its host. At around the same time that *ArPv* was discovered, the genome of the male-killing *ArN* strain associated with *Nasonia vitripennis* was sequenced and putative virulence/symbiosis factors identified (Wilkes *et al.,* 2010; Darby *et al*., 2010).

Therefore, this provided a unique opportunity to compare and contrast these infections both phenotypically and genetically in order to understand the evolutionary adaptation to novel host species following host shift events.

In this chapter I briefly characterize the symbiosis between *ArPv* and *Pachycrepoideus vindemmiae*. I test for evidence of reproductive parasitism, induced costs or mutualistic benefits and transmission route and efficiency. I also test the hypothesis that *ArPv* is on an evolutionary trajectory towards specialism with *P. vindemmiae* by attempting to artificially introduce it into laboratory populations of *N. vitripennis.* Finally, I briefly compare the genome of *ArPv* with that of the male-killing *ArN* with a specific focus on putative virulence factors.

5.2 Methods:

5.2.1 Culturing A. nasoniae strains

For the purposes of microinjection and high yield DNA extraction for genome sequencing, it was necessary to culture single clones of *A.nasoniae,* both strain *ArPv* and the *ArN*. In order to do this the bacterium was isolated either from host wasp pupae or glycerol stocks. Isolation from pupae was achieved by extracting wasp pupae from their fly hosts, surface sterilizing them with 70% EtOH, washing with ddH_2O and homogenizing in sterile PBS. This method removes any other microorganisms from the surface of the wasp and also reduces the impact of contamination with gut flora due to the fact that parasitoids eject their gut contents before eclosion. The homogenate was then spread onto cell-free media as described in (Darby *et al.*, 2010) and allowed to grow at 25^{0}C for 4-6 days until single colonies were visible. A single clone was then picked into 100µl of sterile PBS, 20µl aliquots of which were spread onto fresh GC agar plates using sterile resin beads to form bacterial lawns after a second bout of incubation and growth. Once lawns were formed the bacteria were removed into centrifuge tubes by washing the plates with PBS up to a total volume of 400µl. These steps were carried out under sterile conditions in a laminar flow hood with surfaces and equipment sterilised with 70% EtOH and TriGene disinfectant where necessary.

5.2.2 PCR screening for A. nasoniae in P. vindemmiae.

In the following experiments diagnostic PCR screening was routinely used to detect *Arsenophonus* and *Wolbachia* infections.

Preparation of DNA samples from *P. vindemmiae* for PCR screening was conducted using the Promega™ Wizard DNA extraction kit, following standard protocols but with ¼ volume of reagents to account for the small size of individual wasps. Whole, adult wasps were used for extractions. In all cases extracted samples were stored at -20^{o}C for periods of up to two weeks and at -80^{o}C if longer.

DNA extraction of *N. vitripennis* was done according to the protocols detailed in Chapter 3 of this thesis.

5.2.3 Establishing and maintaining differentially infected stocks of P. vindemmiae.

All lines of *P. vindemmiae* used in these experiments were derived from a single *A. nasoniae* infected female caught near Pierrefeu, south East France by F. Vavre *et al,* University of Lyon, France. These lines were also infected with their native strain of *Wolbachia* (Duron *et al.*, 2010).

Pachycrepoideus vindemmiae were maintained on *Drosophila melanogaster* hosts at 25°C, 12h:12h day:night. At this temperature female *P. vindemmiae* has an egg-adult life cycle duration of c. 26 days. When not being used in experiments, stocks were dropped to 20°C to effectively double development time and ease maintenance burden. Stock turn-over involved taking four adult female wasps and placing them in fly vials containing 100+, freshly pupated *D. melanogaster* (Canton S). Where appropriate, wasp stocks were periodically screened for *ArPv* infection, which was maintained without selection.

In order to directly compare the effects of *ArPv* on *P. vindemmiae* it was necessary to segregate the infection within the same host background and also to segregate it from the native *Wolbachia* infection of *P. vindemmiae*. To this end A+/W+ *P. vindemmiae* were offered *D. melanogaster* (Canton S) hosts reared on ASG fly food containing 0.2% (w/v) rifampicin for two generations (Vavre *et al.*, 2000). After this, forty mated females were isolated into separate isofemale lines for a further three generations on hosts that were not exposed to the antibiotic. At G5, females were reclaimed from their vials of hosts after 72 hours of oviposition and screened for infection with *Wolbachia* and *Arsenophonus* specific primers. The lines of wasps detailed in Table 5.1 were established from the differentially infected females identified by this screening process. Experiments here only use wasps from A^- and A+ lines

Table 5.1: Differentially infected strains of *P.vindemmiae* produced through antibiotic treatment (A^+W^+)

Ancestral strain	*Wolbachia*	*Arsenophonus*	Strain ID hereafter
'Pierrefeu'	-	-	A^-
'Pierrefeu'	-	+	A^+
'Pierrefeu'	+	+	A^+W^+
'Pierrefeu'	+	-	W^+

5.2.4 Experiment 1: Testing for Vertical transmission efficiency and horizontal transmission of ArPv

Aim: In previous chapters I have demonstrated that *A. nasoniae* native to *N. vitripennis* requires horizontal transmission to successfully persist. Here, I test the hypothesis that *ArPv* has lost the utility of horizontal transmission.

Approach: measure vertical transmission efficiency, and presence of infection transfer during co-parasitism.

Method: Infected female wasps were set up in vials with ad-libitum *D. melanogaster* hosts in which to oviposit. These females were collected as pupae from stock vials, allowed to eclose and mate for 24 hours with 3 males from their natal line. Females were then given *ad libitum* (100+) *D. melanogaster* (Canton S) pupae in which to oviposit for 48 hours. After this time females were collected and their infection verified by PCR screening as described in 5.2.2. Females lacking infection were discarded from further analysis. The progeny of these females were collected after 30 days, allowing for full development and eclosion. Fifteen female progeny of each mother were then selected at random for PCR screening for infection to generate a transmission efficiency value per mother. Initially, twenty replicates were established, although this was reduced to nineteen due to lack of infection in one mother.

In parallel, group-lays were established to test for horizontal transmission of *ArPv*. The same procedure as before was followed, but four females were given access to the same hosts at a 2:2, *A+*:*A-* ratio. Once females were grouped it was no longer possible to distinguish *A+* from *A-*. Therefore, following ovipositon for 48 hours, all mothers were collected and screened for infection (80 total). Where expected infection was absent in mothers the replicate was discarded. Three replicates had to be dropped from analysis due to a lack of infection in this treatment, thus n=17. These data were analysed as comparisons to the null hypothesis that there is no horizontal transmission. Thus we expect near 100% infection in clutches from a single mother, and 50% infection in offspring emerging from a vial with four foundresses.

5.2.5 Experiment 2: Testing for paternal transmission and evidence of reproductive manipulation by ArPv in P. vindemmiae

Aim: To test the hypothesis that *ArPv* generates drive by manipulating the reproductive biology of *P. vindemmiae*. I also test for paternal transmission routes.

Approach: An experimental design consisting of a 2 X 2 set of factorial crosses of differentially infected males and females was set up according to the scheme in Table 5.2. These crosses allow us to ascertain:

i) Paternal transmission efficiency of *ArPv* (from progeny of A^- female x *A+* male crosses).

ii) Any sex ratio distortion (from sex ratio produced by *A+* females compared to A^-)

iii) Any CI, which would be typified by the production of either no, or all male, progeny from A^- mothers crosses to *A+* fathers

Method: Males and females for the crosses were collected within 24 hours of eclosion and kept in mating groups of 3 males and 3 females according to treatment, with access to honey water for nutrition. Mating in groups was used to reduce the impact any infertile or unresponsive males may have on virginity in the experiment. After mating, females were removed and placed into individual 'host vials' with ten freshly pupated *D. melanogaster* (CS) (11 days old @ 25^{o}C). Initially these 'host vials' were constructed by dislodging fly pupae from stock vials and adhering them to the inner surface of fresh vials with sugar water. However, this resulted in high fly and wasp mortality across treatments. The experiment was therefore repeated by allowing flies to pupate on removable plastic sticks embedded in their culture vials. Sticks were removed and pupae knocked off until only ten remained. These sticks were then offered to female wasps for 48 hours. Infection status of the parents was confirmed *post hoc* by PCR screening as described in 5.2.2. If females were not of their expected infection status then their clutch was discarded from further analysis. If any of the three males in a mating group were not of the correct infection status then the clutches of all females he came into contact with were removed. The experiment was started with twenty replicates per treatment, but this was eroded by lack of infection and female mortality during the 48 hour laying period (see Figure 5.1 for final sample sizes). The number and sex ratio of the F1 offspring were scored for each clutch as they emerged. Where possible, three live F1 female offspring from each clutch were taken for PCR screening for *A. nasoniae*. This was to determine whether transmission of the infection was maternal or paternal.

Table 5.2 Experimental design for Experiment 1. Numbers indicate final sample size once replicates were removed due to incorrect infection of the parents or female death during oviposition. If CI operates we expect clutches from A+ mother and C father to either be un viable or male biased.

		Mother Status	
		A-	*A+*
Father status	*A-*	8	15
	A+	14	16

5.2.6 Experiment 3: Quantifying effects of ArPv on P. vindemmiae fecundity

Aim: to test the hypothesis that *ArPv* has evolved a mutualistic association with *P. vindemmiae,* or that infection results in a cost to host fitness.

Approach: comparison of the fitness of *A+* and A^- females, as established from fecundity and longevity.

Method: Fitness of *A+* and *C* females was determined by two metrics. First, the number of offspring produced by a single female over both 5 days of offering *ad-libitum* hosts and as offspring number per/day over their lifespan (or up to 14 days). Although *P. vindemmiae* can survive as adults for longer than 5 days (Crandell, 1939) early life represents a valid ecological window for realized fitness. Second, longevity of differentially infected females was recorded up to a maximum of 14 days as an adult.

Focal female *Pachycrepoideus* from A^+ and *C* stocks were allowed to eclose, feed and mate for 48 hours before being exposed to thirty, freshly eclosed *D. melanogaster* (Dahommey) pupae every 24 hours for up to 14 days. If females died during this time, their day of death was recorded and they were preserved in 90% EtOH immediately for later screening for infection. On day 14 post-eclosion, any surviving females were collected into 90% EtOH and PCR screened to ensure infection status (n=1). Females were not given access to food other than their hosts. The number and sex of offspring

produced by each female on each day was then scored as they emerged. Focal females were PCR screened to ensure they were of the correct infection type and discarded form analysis if not (n=3).

Large quantities of *D. melanogaster* hosts were required on a daily basis for this experiment. To this end, flies were reared en-mass in cages containing ASG fly food in which were multiple plastic sticks for them to pupate. Fresh cages were set-up every day starting ten days prior to the first *P. vindemmiae* females being available. This created a continuous supply of freshly pupated flies to offer to the female wasps each day. Each day, the plastic 'pupation sticks' were removed from the rearing cages and either excess pupae removed or multiple sticks placed in the same fresh vial to form the requisite 30 host pupae for the wasps. Thirty hosts were offered because previous studies have demonstrated that this is well in excess of a single females daily reproductive limit (Crandell, 1939; Vavre *et al.*, 2002).

Two metrics of offspring production were obtained. First, the total offspring produced per female over the experiment/days present in the experiment (thus allowing for death) (sample sizes: *A+* = 14, *A-* = 17). Secondly, the total number of offspring produced for the first five days of the experiment (sample sizes: *A+* = 11, *A-* = 15). Female death is controlled for in the former by fitting 'days present in the experiment' as a factor to analyses. In the later, only females that lived for five days or more are included in the analyses. The sex ratio or offspring produced over five days was also analysed as a GLM with binomial errors.

A parametric survival analysis with a Wieblad distribution was used to quantify female longevity. This was right censored to account for females that survived until the end of the experiment ($n = 1$). All analyses and data management were conducted in R (CRAN project) and Microsoft Excel™.

5.2.7 Experiment 4: Interspecific transmission of ArPv to Nasonia vitripennis.

Aim: to test the hypothesis that *ArPv* has undergone selection for specialization on *P. vindemmiae* and lost the ability to successfully interspecifically transfer.

Approach: Introduce cultured *ArPv* into *N. vitripennis*, and compare transmission to *ArN* controls.

Method: ArPv was artificially transinfected to *N. vitripennis* to test its ability to establish and vertically transmit in a novel host species. This was done in comparison with two strains native to *N. vitripennis*: *ArN* (PERL), which has been used in the rest of this thesis, and the type strain obtained from the American Type Culture Collection *ArN* (ATCC) (Table 5.2). *ArPv* was isolated from *P. vindemmiae* pupae, whilst both *ArN* strains were taken from glycerol stocks kept at the University of Liverpool.

Single clones of each strain were grown as a bacterial lawn as described in section 5.2.1. The lawn was washed from the growth media and diluted by a factor of 10 before 2µl of this dilution were injected into *Sarcophagia* pupae with pulled glass needles and a mouth pipette. Previous preliminary injection experiments had shown this dosage to successfully infect *N. vitripennis* with native *A. nasoniae*. *Sarcophaga* pupae were surface sterilized with 70% EtOH before inoculation and left in a sterile Petri dish for 15 minutes post-injection to coagulate the wound. A single, mated, uninfected female *N. vitripennis* was then allowed to oviposit in the inoculated pupae for 24 hours. The success rate of these artificial inoculations was scored as pupae that produced viable wasp clutches. Infection prevalence in G1 offspring was not scored, as this can be a measure of topical or gut infection rather than tissue infection and transmission. Instead, G1 offspring were allowed to mate within their sibships (males were provided from the ancestral line if necessary) and then four females were randomly selected to propagate their line by co-parasitising four fresh host pupae (co-parasitism facilitates the spread of *A. nasoniae* in *N. vitripennis*, see Chapter 3). Nine G2 females were taken from each line upon eclosion and pooled into three groups of three for infection screening by PCR. In this way, transmission efficiency is not assessed *per se,* but rather the ability of the bacterium to be transmitted through calyx fluid, into a new host and to the gut of a wasp larva at least at an 11% success rate. Injection lines that were positive for *A. nasoniae* were maintained for a further five generations under co-parasitism conditions (two female wasps to every fly pupae) and screened for infection again at G7, thus testing for the inter-generational stability of the infections under conditions known to be propitious for the maintenance of *ArN*.

Table 5.3 Strains of *A. nasoniae* used in *Experiment 3*.

Strain	*Native Host*	*Source*	*Reference*
ArPv	*P.vindemmiae*	*Univ of Lyon, FRA (F.vavre)*	(Duron *et al.*, 2010)
ArN (ATCC)	*N.vitripennis*	*ATTC*	(Huger *et al.*, 1985)
ArN (PERL)	*N.vitripennis*	*Univ of Victoria, CA (S.Perlman)*	(Taylor *et al.*, 2011)

5.2.8 Comparative genomics of A. nasoniae strains

Aim: to examine in more detail any genetic differentiation between *ArPv* and the male-killing *ArN* native to *Nasonia vitripennis* (Darby *et al.*, 2010; Wilkes *et al.*, 2010).

Approach: Next generation pyrosequencing was used to generate a draft scaffold whole genome sequence of *ArPv*. This was then annotated through the joint genome institute's (JGI) IMG/ER annotation pipeline to generate conservative gene-calls for open reading frames (ORFs) and genome wide-statistics. Comparative analysis between this sequence and that of *ArN* was carried out with a focus on core relatedness (through conserved house keeping genes) and factors identified by Wilkes *et al* as key to virulence/symbiosis.

5.2.8.1 DNA extraction and quality checks

For next generation sequencing, DNA was extracted from a single clone of *ArPv*. This clone was obtained through the process described above (Section 5.2.4).

Genomic bacterial DNA was obtained from the *ArPv* using the Qiagen miniprep DNA extraction kit following standard protocols for gram-negative bacteria. In order to ensure a great enough yield of DNA for sequencing, the process was replicated six times (with clonal bacterial strains) and two elutes were taken from the final step in each preparation, as recommended in the supplier's instructions. To verify that no contamination was present before submitting samples for whole genome sequencing, a 1µl sample of each of the six DNA extractions was subject to PCR analysis using general primers for bacterial 16S ribosomal RNA gene (Primers: **27f:** 5' AGA GTT TGG ATC MTG GCT CAG 3', **1415r:**. 5' CGG TTA CCT TGT TAC GAC TT 3'). These amplicons were then size checked using gel electrophoresis and sequence checked using short read capillary

sequencing (ABi PRISM 3100 sequencing analyzer) with BigDye 3.1 modified dNTPs. If any contaminants were present we would have expected to see polymorphisms/unreadable sequencing output. However, no such pattern was observed and so all extracted DNA was assumed to be pure *A. nasoniae*.

5.2.8.2 Sequencing analysis, annotation and comparative genome analysis:

Genomic DNA preparation and pyrosequencing was performed by generating a standard fragment and 8Kb paired-end single-stranded template DNA library using the GS DNA Library Preparation Kits (Roche Applied Sciences, Indianapolis, IN, USA) that were then amplified by emPCR and sequenced on a GS-FLX454 platform at the centre for genomic research (CGR) at the University of Liverpool). The 454 reads were assembled with Newbler (v1.1.03.24) using default assembly parameters. Following assembly, concatenated contigs were submitted to the joint genome institutes IMG/ER annotation pipeline according to their specifications (Markowitz *et al.*, 2009). This pipeline generates metadata for the genome as well as conservative gene-calls through automated and manual annotation.

5.2.8.3 ArPv/ArN Comparison:

Reciprocal BLAST searches of ORFs of interest were used to identify homologues between strains. Homology was quantified as % identity between sequences and the presence, size and number of gaps (a product of insertion/deletion mutations). BLAST searches were carried out on either the IMG/ER's in-house server (for specifically blasting against the *ArPv* genome) or on the standard NCBI hosted BLAST tool. Initially, BLASTn was used to search for homologues at the nucleotide level as past MLST data suggested differentiation was minor. Where genetic differentiation was found, sequence alignments between *ArN* and *ArPv* ORFs were performed at both nucleotide and amino acid level using MEGA5™ and Geneious™ bioinformatics software. Resultant hits were scored for identity and bit score, and where necessary were aligned through ClustalW or Geneious™ alignment algorithms in the Geneious™ software package to discern differences. Gene topology was assessed for evidence of pseudogenisation (as STOP codon appearance through indel mutations and frame shifts) by direct comparison of genome structures, ORF dispersal and stop codons using the Artemis Comparison Tool (ACT).

i) Assessing core relatedness between ArPv and ArN:

Studies of *Arsenophonus* distribution and diversity across host species have used

sequence polymorphism across a number of conserved loci in order to determine phylogenetic relationships. This method is termed Multi Loci Sequence Typing (MLST) (Duron *et al.*, 2010). Here, the sequence of these genes, along with 50 other single copy loci associated with ribosome synthesis, are compared between strains as an indicator of relatedness.

ii) Assessing divergence in virulence associated factors:

Wilkes *et al* (2010) identified a number of factors in the genome of *ArN* that were putatively associated with symbiosis or virulence. These are factors that showed homology to proteins known to be involved in prokaryote-eukaryote cell interaction. They are sub-divided into Secretion systems, secretion system associated effectors and other toxins. Specifically, *ArN* has two complete type three secretion systems (T3SS) and a third with putative functionality. These systems are used for interaction with host cells and the delivery of toxin effectors. Thus, they and the toxins associated with which they are associated are a key part of virulence and symbiosis. *ArN* also posses several other toxins associated with either active transport out of their cell or other secretion systems. To this end, sequences of ORFs associated with T3SS systems and other toxins identified by Wilkes *et al* were BLASTed against the *ArPv* genome in order to determine presence of the ORF, its size and evidence for pseudogenistation.

5.3 Results:

5.3.1 Experiment 1: Testing for Vertical transmission efficiency and evidence of horizontal transmission of Arsenophonus nasoniae (ArPv) in Pachycrepoideus vindemmiae

ArPv appears to exhibit high vertical transmission efficiency and no evidence of significant levels of horizontal transmission (Figure 5.2). When only a single infected female produces offspring vertical transmission efficiency of *ArPv* is estimated at 98.3%. This is a significant deviation from perfect transmission (Exact binomial test, $P<0.001$). When four disparately infected females share a patch of hosts, the mean infection prevalence of offspring emerging from that patch is 53.3%. This is not a significant deviation from the expected 50:50 infected/uninfected ratio (1-sample test of proportions, $\chi^2=1.33$, df=1, $P=0.2482$) and thus implies that infected females are not at a competitive advantage or disadvantage and that horizontal transmission is unlikely to be occurring.

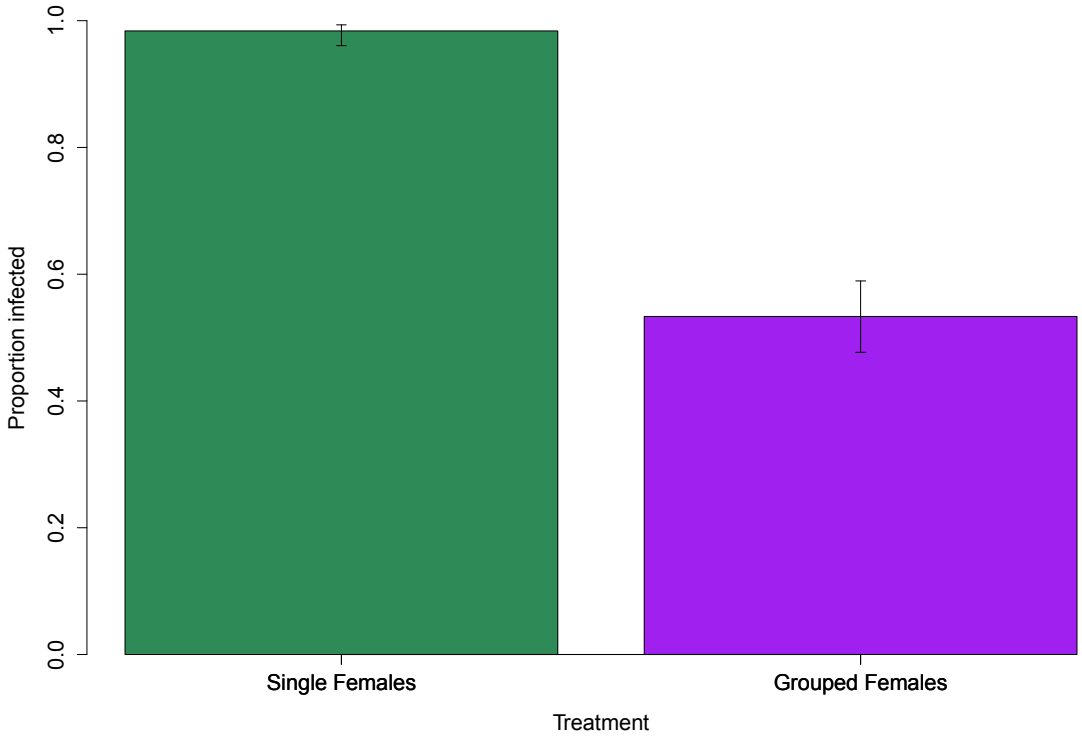


Figure 5.1 Prevalence of *ArPv* in clutches produced by single infected females or groups of two *A+* and two *A-* females.

5.3.2 Experiment 2: Testing for paternal transmission and evidence of reproductive manipulation by ArPv in P. vindemmiae.

Paternal transmission of A. nasoniae (ArPv):

Where the father was infected and the mother was not, none of 29 offspring (at least two from each of 14 clutches) carried the infection (95% Confidence intervals: lower = 0.0, upper = 0.119).

Evidence of reproductive manipulation:

There was no evidence of strong reproductive manipulation by *ArPv* in *P. vindemmiae* (Figure 5.3 [a]). For simplicity, treatments are referred to by the mother's infection status, followed by the father's infection status.

CI has been shown to cause both complete inviability and male-biased clutches in haplodiploid species (Vavre *et al.*, 2001). There was no significant difference in clutch sex ratio between the uninfected control treatment (*A-/A-*) and the crosses where just one parent was infected (*A+/A-* & *A-/A+*) (GLMER, z=-0.243, *P*= 0.8080). CI should manifest when mothers carry the infection but fathers do not, thus this indicates there to be no CI. A marginally significant difference in sex ratio was observed between the bi-parentally infected treatment (*A+/A+*) and all other treatments (GLMER: *z*= -2.068, *P*= 0.0386). However this is unlikely to be the result of CI and is most likely an artifact of the small sample size used.

There is also no significant difference in clutch viability (scored as the proportion of pupae successfully parasitised) between treatments (Test for equality of proportions, P>0.05) (Figure 5.3 [b]). Furthermore, crosses where both parents were infected (*A+/A+*) actually gives the lowest estimate of clutch viability, and so this is not indicative of *ArPv* induced CI.

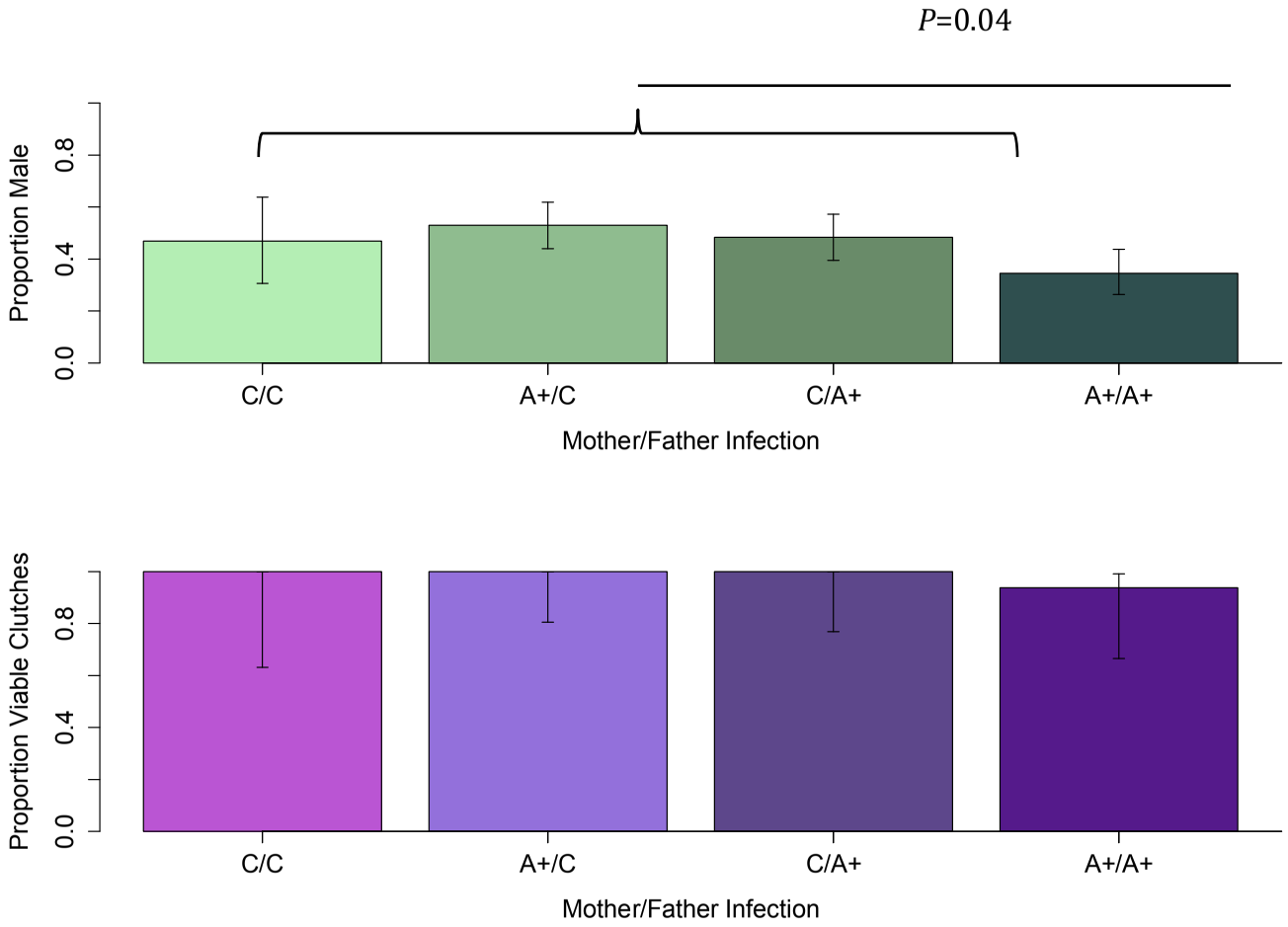


Figure 5.2 (a) Mean sex ratio (as proportion males) of clutches produced by differentially mated pairs in *Experiment 1*. There is a weakly significant reduction in males when both parents are infected. However, this is not a treatment in which we would expect to see CI. (b) Proportion of mothers in each treatment that produced at least one viable offspring. Here there is no negative effect of differentially infected parents upon clutch viability.

5.3.3 Experiment 3: Quantifying effects of ArPv on P. vindemmiae fecundity

There was no evidence for significant costs or benefit of *ArPv* infection in *P. vindemmiae* relative to uninfected controls. Female wasps infected with *ArPv* produced the same mean number of offspring per day (pairwise comparison of linear models: F= 1.3847, df=1, *P*=0.2489) and in total over five days (Welch two-sample t-test; t = 1.0125, df = 22.921, *P* = 0.3219) compared to uninfected wasps (Figure 5.4 [a] & [b]).

There was also no significant difference in the sex ratio of offspring produced over five-days between infected and uninfected mothers (Pairwise comparisons of GLMERs: χ^2, df=1, *P*=0.733) (Figure 5.4 [c]). The minimum adequate model being the null: (sex ratio~1+(1|female.ID), family=binomial). This is in support of the findings in experiment 1 (Figure 5.2).

Finally, there was no significant difference in survival between infected and uninfected females (Pairwise comparison of survival analyses: *P*=0.585).

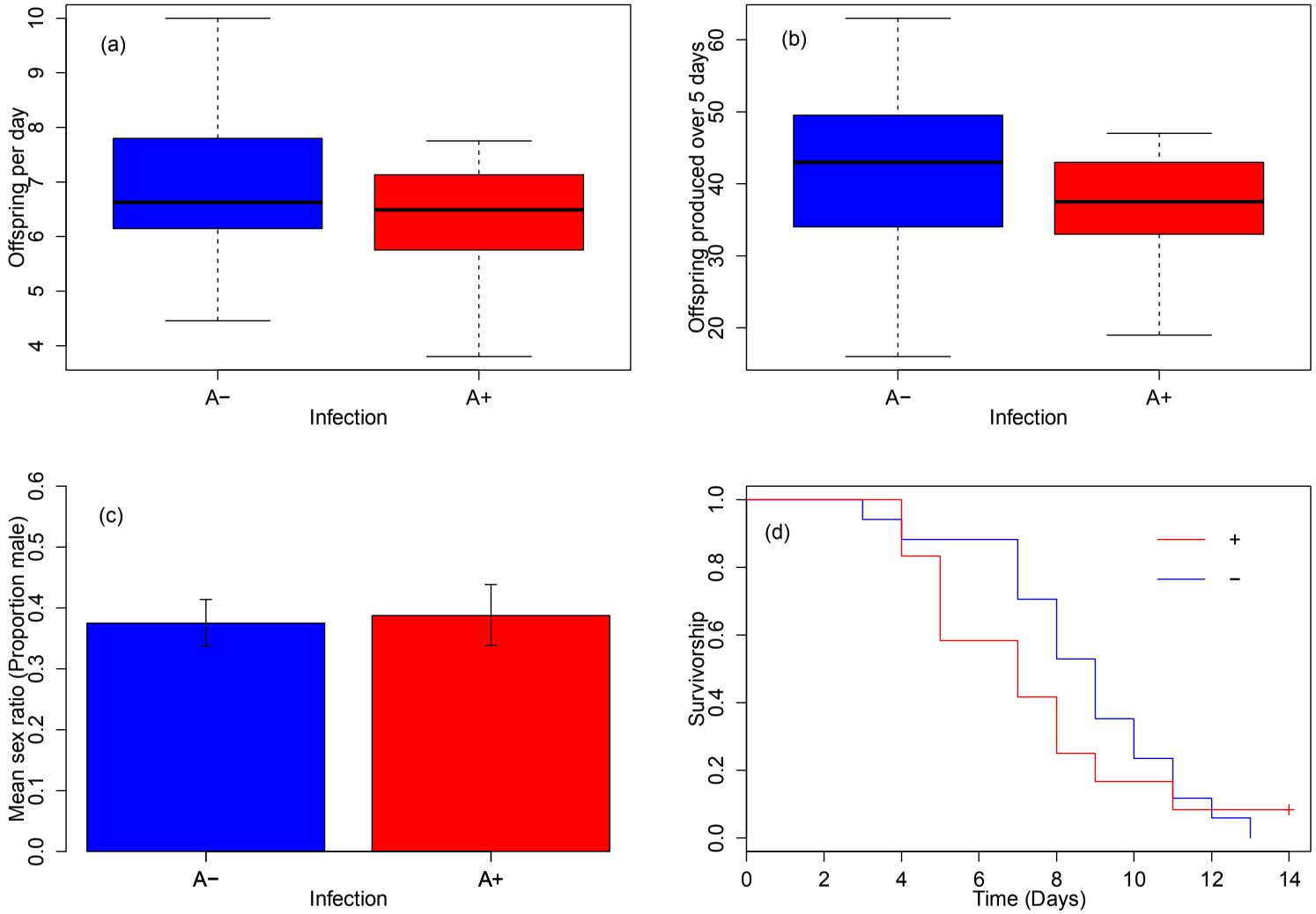


Figure 5.3: Measures of fitness for *P.vindemmiae* with and without *ArPv* infection. (a) Mean number of offspring produced per day over lifetime. (b) Total number of offspring produced over first five days of oviposition, (c) Mean sex ratio (proportion male) of offspring produced over five days of oviposition, (d) Survivorship curve of differentially infected *P.vindemmiae* females.

5.3.3 Experiment 4: Interspecific transmission of ArPv to Nasonia vitripennis

Both native and *P. vindemmiae* derived strains of *A. nasoniae* successfully infected *N. vitripennis* following artificial transinfection and persisted for two generations. However, both native *ArN* strains, *ATCC* and *PERL*, were significantly more successful, appearing in 80% and 100% of clutches respectively, compared to just 12.5% success for *ArPv* (3-sample test for equality of proportions, χ^2=22.43, df=2, *P*<0.001). After seven generations (G7) native *A. nasoniae* strains persisted at the same levels as at G2. The non-native *ArPv* however, was lost (Figure 5.5).

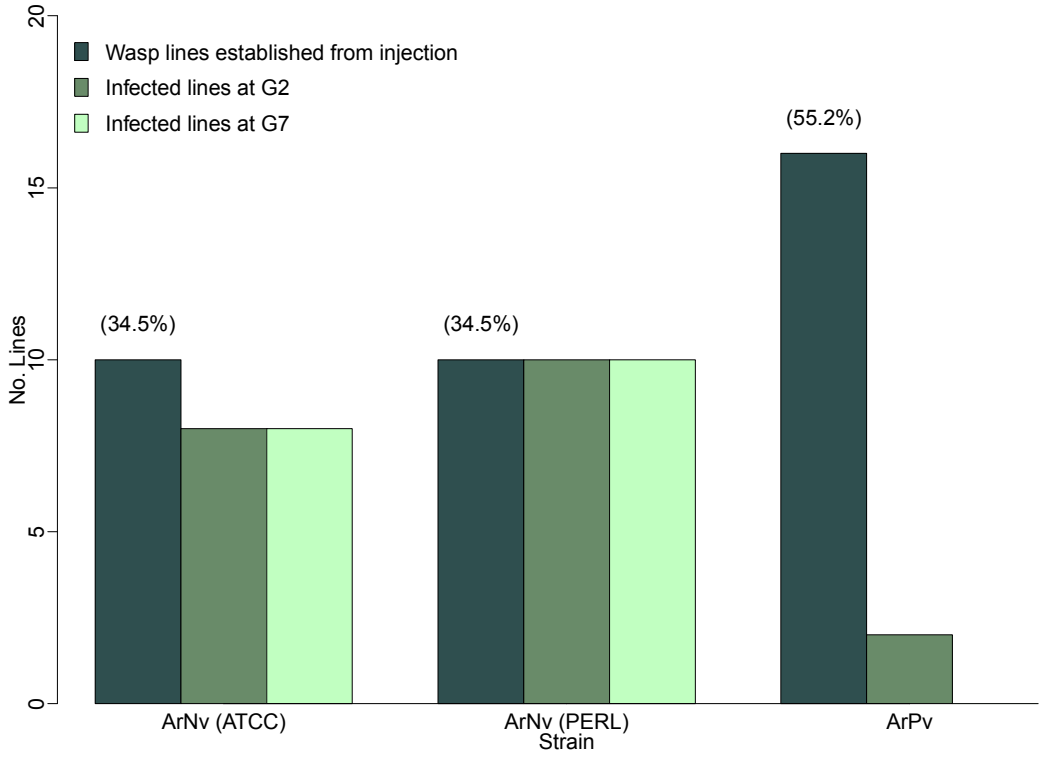


Figure 5.4: Efficiency of infection and subsequent inter-generational transmission of *Arsenophonus* strains in *Nasonia vitripennis*. Numbers in ( ) denote the percentage of inoculated *Sarcophaga* pupae that were successfully parasitised and yielded G1 wasps.

5.3.5 Comparative genomics of ArPv and ArNv

5.3.5.1 Genome metadata

Newbler assembly of 454 reads of the *ArPv* genome yielded 4Mb of DNA assembled into 191 contigs over 65 scaffolds. Annotation through the IMG/ER pipeline estimated that 78.8 % of the genome was coding and distributed across 3851 coding open reading frames (ORFs). Of these, 97.74 % were protein coding, the remainder coding for rRNA and tRNA. The genome was A-T rich at 62%. The IMG/ER's gene calling algorithms assigned putative function to 72.55% of coding ORFs, with the remainder assigned "protein of unknown function". This is considerably larger than the genome of the male-killing *ArN* published by Wilkes *et al* (2010), which also had a smaller coding gene complement. A comparison of these statistics is presented in table 5.3.

Table 5.3 Comparative meta data of *ArPv* sequenced here and the Male-killing *ArN* strain sequenced and annotated by Darby *et al* (2010)

	ArPv	*ArN* (Male-killer)
Size	4029609	3.575339
GC content (%)	38	35.74
Predicted coding ORFs	3764 (78.8%)	3476 (80.07%)
Library type	8kb mate-pair	2kb mate-pair
Scaffolds	65	143
Contigs	191	665

5.3.5.2 Core relatedness of ArPv and ArN.

All loci used in previous studies to construct phylogenetic relationships between *A. nasoniae* isolates demonstrated sequence identity of 98% or greater with the exception of zapA (Table 5.5). Topographical analysis of zapA revealed that it is truncated in *ArPv* due to a 1000bp deletion. ZapA is the only coding region of those tested affected by a deletion. Furthermore, analysis of ORFs annotated as 'ribosomal protein' had 99% or greater identity between strains at both nucleotide and amino acid level (Appendix Table 5.6).

Table 5.4 MLST loci used for phylogenetic analysis of *Arsenophonus* strains, for ARN references see Darby *et al* 2010.

Gene	*ArN* accession	*ArPv* ID (%)	*ArN* ORF length (bp)	Gaps	Function	Comparison notes
zapA	ARN_09690	99	1527	0	Metalloprotease	Identical for 1098 bp. Missing the first 429 bp.
aprA	ARN_04690	100	570	0	Alkaline metalloprotease precursor	Identical.
yaeT	ARN_26640	99	2406	0	Outer membrane protein	Identical.
fbaA	ARN_34300	100	1113	0	Fructose-biphosphate aldolase	Identical.
ftsK	ARN_16600	99	3093	0	Cell division protein	Identical.
spoT	ARN_31680	100	2118	0	Pentaphosphate guanosine - 3' - pyrophosphorylase	Identical.

5.3.5.3 Comparison of virulence associated factors between ArPv and ArN

i) Type 3 Secretion System Effectors

Of twelve putative TTSS effector proteins identified by Wilkes *et al*, ten were present in the *ArPv* genome (Table 5.7). YopJ, a toxin associated with interruption of the innate immune system in *Yersinia pestis* was absent from the assembled genome. In the *ArN* genome this ORF is flanked by traI and traM. Both of these flanking regions are present in the *ArPv* assembly, implying that yopJ may have been lost. There was also no individual hit for one of three SopA ORFs (ARN_26090) and so this appears to be absent from *ArPv*.

Of the remaining ten toxins identified by Wilkes *et al*, exoY and sopB (ARN_24950, ARN_35620 respectively) showed signs of pseudogenistation under ACT comparison, with multiple additional stop codons in comparison to their counterpart *ArN* ORFs. The remaining ORFs found hits in *ArPv* with at least 99% identity.

Table 5.5 Type III secretion system effector ORFs identified in the genome of male-killing *ArN* by Wilkes *et al* 2010 alongside homologues in *ArPv*.

Gene	*ArN* accession	*ArPv* nucleotide ID(%)	*ArN* length (bp)	Gaps	Function identified by Wilkes *et al* 2010	Comparison Notes
YopJ	ARN_36660	ABSENT	-	-	Affecting innate immune signalling and cytokine production. Antiapoptotic.	Absent from *ArPv* assembly.. Three *ArPv* ORFs in a row have partial identity (50%)
YopH	ARN_23010	99	2181	2	Tyrosine phosphatase activity provides resistance to phagocytosis.	Identical.
Shewenella effector	ARN_10280	99	3636	18	Not known. Effector hypothesis is raised by position in TTSS and chaperone binding site.	18bp deletion.
SopA	ARN_35130	99	1239	0	HECT-3 like ubiqutin ligase enzyme, alters ubiquitination status of proteins.	Identical.
SopA	ARN_02810	99	1662	0	HECT-3 like ubiqutin ligase enzyme, alters ubiquitination status of proteins.	Identical.
SopA	ARN_26090	84	1432	26	HECT-3 like ubiqutin ligase enzyme, alters ubiquitination status of proteins.	Absent, partial hit to other two SopA copies.
SopB/IpgB	ARN_35620	99	4746	0	Inositol phosphate phosphatase enzyme.	Fragmented over three ORFs in *ArPv*.
PipA	ARN_23150	99	675	0	Type III secreted protein of unknown function, save important in virulence.	Identical.

ExoY	ARN_24950	99	800	1	Adenylate cyclase activity, creates 100 fold increase in intracellular cAMP, interferes with signaling pathways.	Evidence of pseudogenisation. Stop codons and reading frame shift in *ArPv*.
IpaD	ARN_14250	100	1131	0	Surface antigen, required for bacterial entry into epithelial cells and introduction of late effectors.	Identical.
SipB	ARN_14270	99	2001	24	Important for cell entry and translocation of late effectors. Pro-apoptotic through binding to caspase-1.	Identical.
OspG	ARN_23940	99	594	0	Alters phosphorylation of ikB, interfering with activation of NF-kB pathway and innate immune signaling.	Identical.

ii) Non T3SS associated toxins:

Non-TTSS associated toxins were also variable between strains (Table 5.8). Most notably *ArN* has four copies of the gene with sequence similarity to apoptosis inducing gene *aip56*. Only one copy is present in the assembled *ArPv* genome, and has a different sequence to the *ArN* associated counterpart (See Figure 5.7).

Furthermore, ORFs encoding colicin 1b and cnf1 (cytotoxin necrolizing factor related) were both absent from the assembled *ArPv* genome, although a highly distant match for cnf1 was found at the amino acid level. ORFs with sequence similarity to the remaining three remaining toxin related ORFs were found at >99% identity at the nucleotide level.

Table 5.6: Non-T3SS associated toxins identified by Wilkes *et al* (2010) and the identity score of their closest homologue in *ArPv*.

Gene	*ArN* accession	*ArPv* nucleotide ID(%)	*ArN* length (bp)	Gaps	Function identified by Wilkes *et al* 2010	Comparison Notes
Aip56 (1)	ARN_10220	89	1534	26	Pro-apoptotic	86.2% identity translates to differing amino acid residues and differing predicted structure.
Aip56 (2)	ARN_23450	ABSENT	205	1	Pro-apoptotic	ABSENT. Closest hit is Aip56 (1)
Aip56 (3)	ARN_07720	ABSENT	371	8	Pro-apoptotic	ABSENT. Closest hit is Aip56 (1)
Aip56 (4)	ARN_33080	ABSENT	592	7	Pro-apoptotic	ABSENT. Closest hit is Aip56 (1)
cnf1	ARN_36850	69	3360	8	Alters host cell signaling	*ArN* ORF spans a sequence gap. Low identity at both aa and nucleotide level. ArN spans sequence gap. Low nucleotide ID. V.low amino acid ID
colicin V	ARN_35480	99	456	0	Bactericidal	Identical.
colicin 1b	ARN_22290	ABSENT	-	-	Bactericidal	ABSENT.
serralysin	ARN_31950	99	1161	0	Insecticidal	Identical but interrupted by sequencing gap in *ArPv*.
ast	ARN_28400	99	489	0	Enterotoxin	Identical.

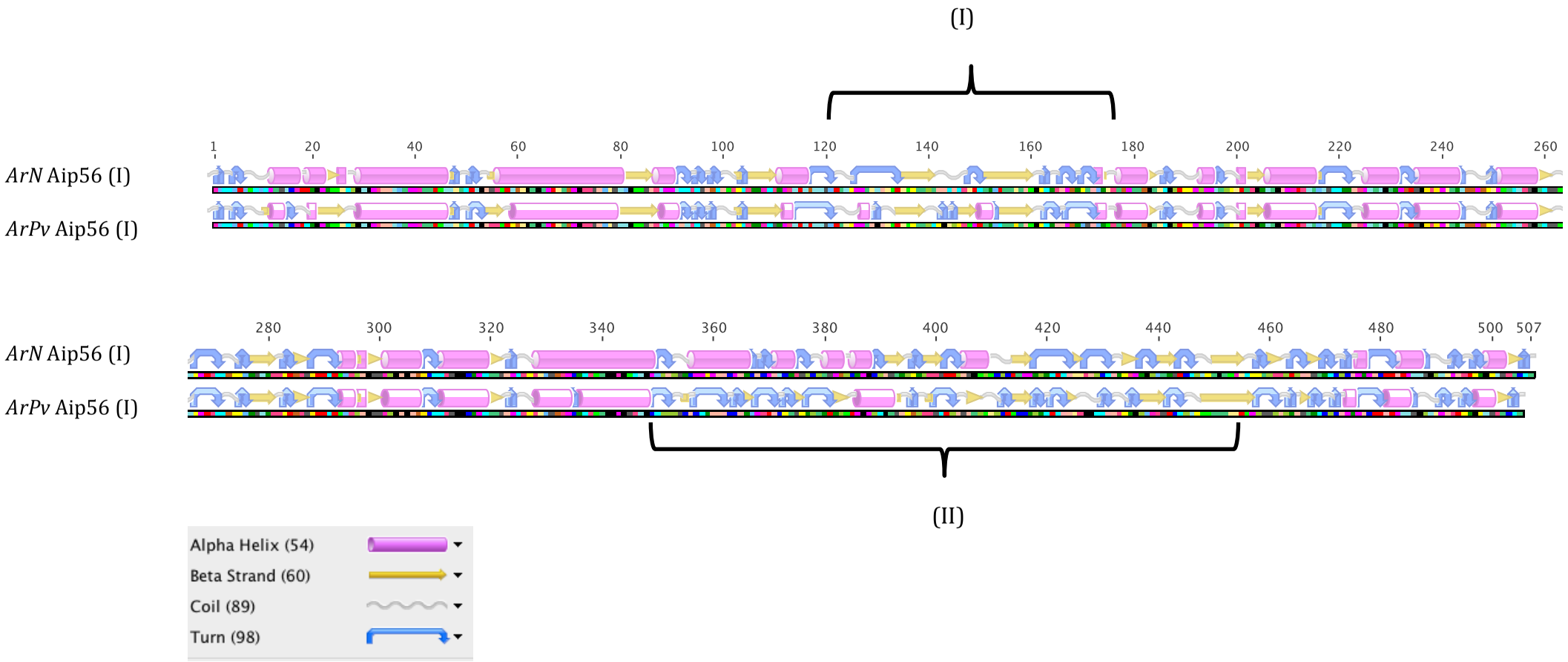


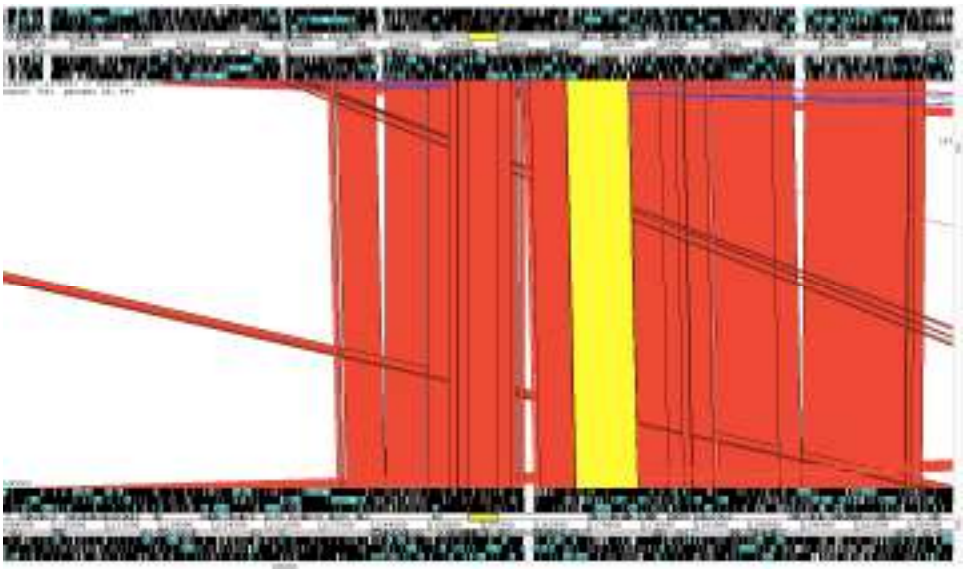
Figure 5.5: Predicted structural comparison of the apoptosis inducer associated aip56 gene from both *ArN* and *ArPv* (ARN_10220). The sequences share 89% nucleotide identity and 82.6% amino acid identity (aligned with geneious algorithm). Deviation is focused on two specific regions, indicated on the diagram as (I) and (II). Pfam searches and further structural modeling were inconclusive as to the adaptive function of the disparity. However, this is one of four aip56 ORF present in the genome of *ArN* whilst *ArPv* possesses only this single copy.

iii) Secretion apparatus:

ArN is known to possess two complete and one incomplete type 3 secretion systems (T3SS). Brief topological investigation identified counterpart regions of the *ArPv* genome that possess the same number of ORFs of similar size (Figure 5.9). These ORFs were also identified by automated annotation as being involved in T3SS assembly. Thus it appears that *ArPv* has all three T3SS islands intact.

Interestingly, *ArPv* also possess an island of 13 ORFs that were either reported as Type VI Secretion System (T6SS) associated by IMG/ER annotation, or were later linked to T6SS functionality by BLASTp searches (Appendix table 5.1). T6SSs are a relatively newly discovered secretion system, associated with microbe-microbe interactions (Russell *et al.*, 2011; Silverman *et al.*, 2012; Coulthurst, 2013). T6SS genes are notoriously difficult to identify by sequence alone (Silverman *et al.*, 2012), protein structure analysis will be required to verify functionality. No such T6SS is present in the genome of *ArN*, although closer inspection of the genetic architecture revealed that it may occupy a section of the genome that did not make it into the assembly presented by Wilkes *et al* (Figure 5.10).

(a)



(b)

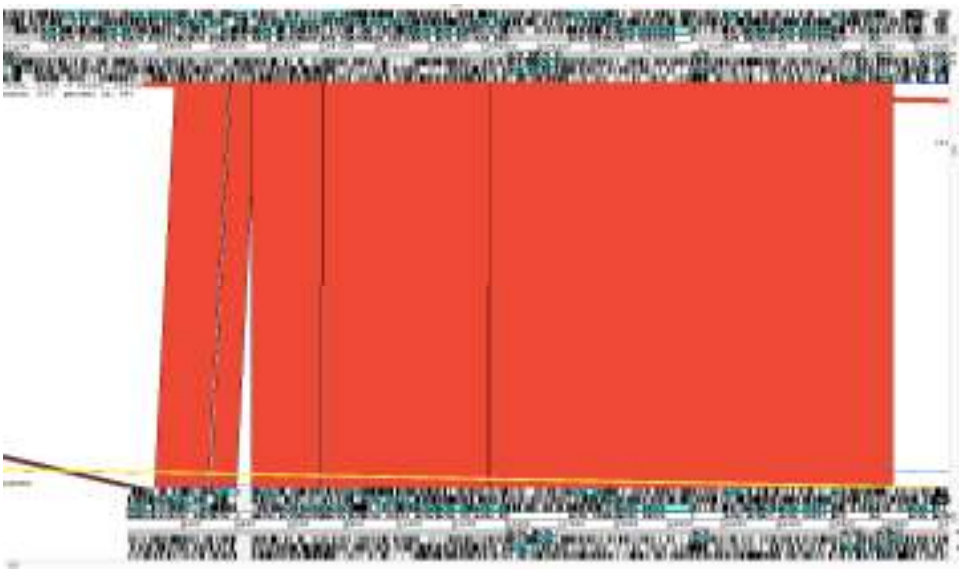


Figure 5.6: Corresponding T3SS ORFs in *ArN* and *ArPv*. Both number and order of T3SS associated ORFs match between the two genomes. (a) Yersinia-like T3SS, (b) Salmonella-like T3SS. Figures are ACT comparisons of the two *A. nasoniae* genomes. Red and yellow bars identify regions of high identity. In this case, the connections cover the T3SS islands.

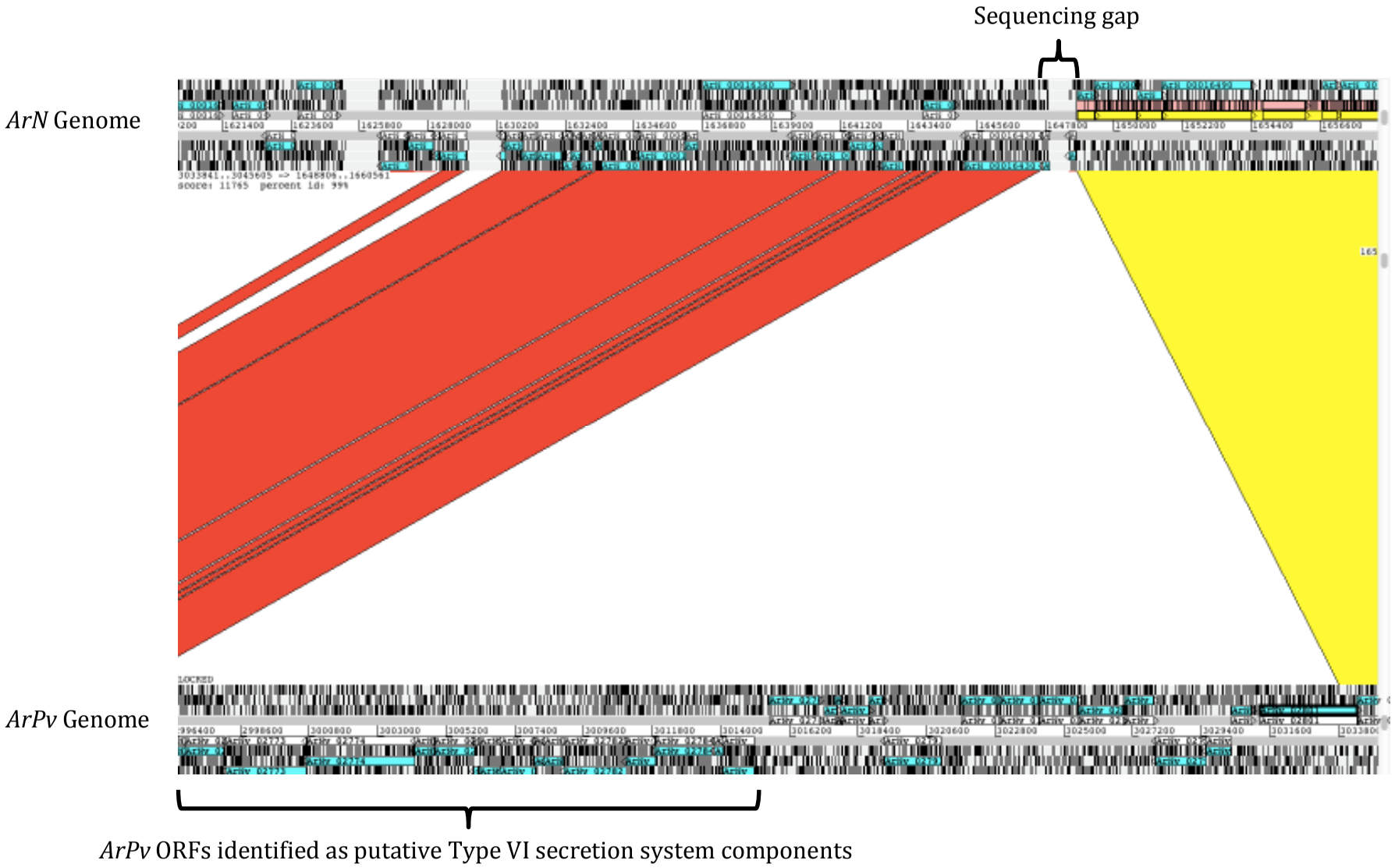


Figure 5.7: Artemis Comparison tool (ACT) output showing aligned genome sequences of male-killing *ArN* and *ArPv*. Thirteen *ArPv* ORFs were identified as components of a type six secretion system (T6SS), a system lacking in *ArN*. However, the comparison above shows that the relative address of these ORFs in *ArN* is missing due to a sequencing gap.

5.4 Discussion:

In this chapter I demonstrated first that the natural *Arsenophonus nasoniae* infection of *Pachycrepoideus vindemmiae, ArPv,* is highly disparate at the phenotypic level to its male-killing counter part (*ArN*), both in terms of phenotype within its host, and ability to transfer into novel hosts. Second, I demonstrated the strains were very closely related in terms of the sequence of core genome apparatus such as ribosomal proteins. However I also noted that the accessory genome was distinct, with different toxin and effector complements.

Like all other heritable bacterial endosymbionts*, A. nasoniae (ArPv)* is inherited through the maternal lines of its host. However, it exerts no overt sex ratio distortion on its host under laboratory conditions, and there is no evidence for a CI phenotype. In addition, fitness assays detected no effect of infection on either offspring production or survival. Unlike its male-killing counterpart, horizontal transmission does not appear to play a vital role in the epidemiology of the infection, although it has comparable, imperfect vertical transmission efficiency. *ArPv* also performs poorly when transinfected into a novel host (*Nasonia vitripennis*) and was not observed to persist beyond two generations. This is in contrast with the male-killing sister-strain, which has been demonstrated to infect and persist in a number of disparate host wasps (Duron *et al.*, 2010).

ArPv appears to be highly related to the male-killing *ArN* described by Wilkes *et al*, as it shares identical/near identical nucleotide sequences at over fifty conserved 'housekeeping' loci. Counter to what we would expect from a microbe undergoing selection for mutualism and specialization, *ArPv* appears to have a larger genome than its male-killing relative by 0.5Mb. However, this disparity may be a product of superior sequencing coverage. Furthermore, *ArPv* appears to possess the same capacity for toxin delivery through T3SS as its male-killing relative. However, there is a marked divergence between the genomes in terms of toxin complement.

5.3.1: Transmission biology of ArPv

ArPv exhibits strong maternal transmission efficiency with no evidence of additional paternal transmission. This is unsurprising, as it is likely that the microbe shares the same obligate per-oral route of transmission as its male-killing cousin, which is

dependent upon oviposition by the female host. The efficiency of vertical transmission is high, although not perfect and so we hypothesize that there should be some form of drive mechanism in place to facilitate the bacterium's maintenance in host populations. There is no evidence here for infectious transmission of the bacterium as there is for *ArN* in *N.vitripennis*. This may be because the bacterium has lost the capacity to horizontally transmit and so relies on vertical infection. Alternatively, it may well be a product of *P. vindemmiae's* pseudo-solitary reproduction. Only one wasp egg is laid per host, and so there will be little opportunity for infectious transmission. *Experiment 3* allows both infected and uninfected mothers to oviposit into hosts on the same patch, and infection prevalence of the offspring does not deviate from the expected 50%. However, it may be that the number of hosts provided (100+ per patch) were surplus to the point that infected and uninfected mothers never oviposited in the same host. Thus, horizontal transmission was not permitted. Casual observation implied that the majority of hosts were used in each vial when four mothers were present, as very few *Drosophila* successfully developed to adulthood. However, closer study of transmission under enforced co-parasitism, not just co-habitation is necessary in order to rule out infectious transmission as a key part of its epidemiology.

Vertical transmission of *ArPv* may be sensitive to the fly species parasitised by their wasp host. In *Experiment 4* I demonstrated that *ArPv* failed to vertically transmit in Nasonia vitripennis that were utilizing *Sarcophaga bullata* pupae for oviposition. This failure may be maladaptation to the wasp species, or maladaptation to the fly.

5.4.2 Evolution towards mutualism

The data presented here implies that *ArPv* may have lost the capacity for reproductive manipulation and is undergoing an evolutionary transition towards a mutualistic, exclusive relationship with *P. vindemmiae*.

Experiment 1 and *Experiment 2* allowed us to directly compare the sex ratio of offspring produced by infected and uninfected *P. vindemmiae* and assess for incompatibilities between differentially infected parents. There was no evidence for sex ratio distorting in either experiment. Indeed, *P. vindemmiae* has a more balanced natural sex ratio than *N. vitripennis* (c 40% male) and so we would expect that the effects of male-killing would be quite overt if it were present. The lack of male-killing makes sense in light of *P. vindemmiae's* reproductive biology. As stated above, *P. vindemmiae* only lays a single egg per host. Therefore, there is no sib-sib competition, a pre-requisite for the best

evidenced theory of male-killing to function; resource reallocation (Hurst, 1991). Potentially, male-killing is a plastic or host-specific trait, only expressed in hosts where it is potentially adaptive, such as *N.vitripennis*. Alternatively, selection has removed the capacity to male-kill form *ArPv*, irrespective of its host environment. Unfortunately, the bacterium's failure to establish in lines of *N. vitripennis* rendered testing of this hypothesis impossible.

Experiment 1 also provides evidence that *ArPv* does not induce cytoplasmic incompatibility between differentially infected parents. Significant differences were observed in both sex ratio and clutch viability but a) differences did not occur in the manner expected from CI (i.e high mortality/many males in A- female x A+ males) b) differences were relatively minor, unlike the near complete inviability we would expect from the strong drive phenotype of CI. Thus, it is reasonable to state that there was no evidence of reproductive manipulation phenotypes in this system.

Experiment 3 indicated that vertical transmission of *ArPv* was high, but not perfect, and casual observation informs us that *ArPv* is relatively stable in laboratory stock of *P. vindemmiae* without the need for selection. Therefore, we expect some form of drive phenotype to be responsible for its persistence. In the absence of reproductive manipulation, and infectious transfer, this phenotype is assumed to be a mutualism. However, *Experiment 2* failed to find any significant cost or benefit of *ArPv* infection in its natural host, both in terms of reproductive output and longevity. Whilst this offers no basis for a mutualism, it is in contrast to the highly costly nature of the *ArN/N. vitripennis* association detailed in Chapter 3. The fitness assays presented here are relatively crude, and unlikely to pick up the signature of marginal, but significant, fitness gains as result of infection. Indeed, the benefits may be contingent upon a factor that is not present in our experimental set-up.

Further evidence for *ArPv*'s descent towards mutualism is its inability to infect the host of its close relative, *N. vitripennis*. *Experiment 4* demonstrated that whilst initial infection was possible, it was unstable even under conditions that have been shown to promote *A. nasoniae* spread (specifically co-parasitism, see Chapter 4). This is in contrast to its sister strain, *ArN*, which readily transmits between disparate wasp species (Duron *et al.*, 2010), and so is indicative of specialization of *ArPv* upon a specific host. This also demonstrates that the phenotypic differences between the strains are

unlikely to be the result of plasticity or host-specific expression, but rather the result of divergent adaptation.

Comparative analysis of the *ArN*/*ArPv* genomes supports the hypothesis that the two strains have derived relatively recently, but swiftly undergone selection for different lifestyles. Six loci used to distinguish between *A.nasoniae* strains (Duron *et al.*, 2010; Taylor *et al.*, 2011) and a further fifty ribosome synthesis associated loci showed near identical sequence similarity between strains . The one exception to this was the zapA gene which was truncated due to a 1000bp deletion at the 5' end. It should be noted that zapA encodes a metalloprotease, an enzyme not required for core function, and enforces the contrast between core genome and accessory in terms of divergence. In contrast, the toxin compliment of the two genomes varied dramatically, with several putative toxins identified by Wilkes *et al* proving to be absent, potentially pseudogenising, or highly variable in nucleotide and amino acid sequence. It is likely that toxins and secretion systems are the first point at which divergent selection will act, as they are the implements with which the bacterium interacts with its host environment (Galan and Bliska, 1996). The specific nature of the divergence is difficult to discern from the brief survey of virulence/symbiosis factors carried out here. However, the fact that *ArPv* appears to have a smaller toxin complement, notably lacking several copies of the apoptosis inducer aip56, apoptosis regulator YopM implies that the bacterium is losing pathogenic functionality.

Interestingly, *ArPv* also lacks the colicin 1b associated ORF found in *ArN* (ARN_35480). Colicins are bactericidal molecules that are used to regulate the growth of competing microbes (Nomura, 1967). That there is no homologue for this ORF in *ArPv* suggests that it may be unable to form a co-infection with *ArN*. Further molecular verification work through knock-out transformation and analysis of protein structures are required to unravel the true nature of these disparities.

Typically, the transition from a free-living or parasitic life-style to a mutualism is accompanied by genomic reduction (Moran *et al.*, 2008). This is hypothesized to be an inherent byproduct of obligate vertical transmission, and may be driven by two non mutually exclusive processes. First, deleterious mutations, including deletions will accrue in the symbionts genome without purging as it repeatedly undergoes population bottlenecks during intergenerational transmission (Rispe and Moran, 2000). Secondly, as the symbiont becomes less dependent upon a free-living stage, it loses the necessity

for some genetic material and essentially outsources other functions to the genome of its host. In this scenario, the extra genetic load becomes deleterious and loss is favoured by selection. This process of adaptive gene loss facilitated by multi-organism dependency is referred to as the 'Black Queen Hypothesis' (Morris *et al.*, 2012).

These processes of genome erosion are postulated to be the reason that some of the smallest genomes recorded have belonged to obligate mutualists. For example *Carsonella rudii*, a primary endosymbionts of psyllids has a genome of just 160 kilobases (Nakabachi *et al.*, 2006). Recent work has directly compared the recently derived secondary symbiont *Serratia symbiotica* with its closest free-living relatives, *S. proteamaculans* and *S marcescens,* and demonstrated that erosion happens quickly and is accompanied by gene pseudogenisation, accruing of random mutations and large scale rearrangements (Burke and Moran, 2011). In the study presented here, we do not see evidence of widespread genomic decay. *ArPv* in fact has a larger assembled genome than its more parasitic relative with a higher compliment of ORFs with a predicted coding function. Potentially these differences in size are not of significance as the library preparation of *ArPv* was more robust, using an 8kb mate-pair compared to *ArN*'s 2kb mate-pair library. However, the genomes are at least comparable in size. The lack of size disparity seen here may be because the transition towards mutualism/specialization is still in its infancy and genome erosion has yet to make a significant impact. Alternatively, the unusual transmission route of *A. nasoniae*, whereby it must infect the wasp host, the dipteran host and then re-infect the wasp larvae may select for a minimum gene content and robust ability to exist outside of its primary host's body. Indeed, it is possible to culture *ArPv* in cell free media, albeit somewhat inefficiently (*pers obs*), and so it may be under selection to maintain the genetic complement, and by extension the genome size, with which to achieve this. If this is true, then potentially *A. nasoniae* is constrained in its potential trajectory towards mutualism by its own transmission route.

5.5 Conclusion

It is clear that *ArPv* represents a distinct, but closely related symbiont strain to the male-killing *ArN* discussed in the rest of this thesis. The evidence presented here certainly combines to illustrate that this strain is not a reproductive parasite and is maladapted to interspecific transmission into a common *A. nasoniae* host, *N. vitripennis.* I postulate that this maladaptation is the result of divergent selection that has acted on

the bacterium following a recent host shift, and that it is now on an evolutionary trajectory towards specialism/mutualism with *P. vindemmiae* that precludes previously held parasitic phenotypes. The contrast between the high level of genetic relatedness and key disparities in toxin complement in the genomes of *ArN* and *ArPv* support the hypothesis that it is selective forces, and not phenotypic plasticity, whcih has resulted in the disparity between strains. Importantly, here we can directly marry transmission ability/strategy with phenotype.

Chapter 6:

General Discussion

6.1 Summary of findings:

In this thesis I have addressed the importance of infectious (horizontal) transmission in the biology of *Arsenophonus nasoniae* and its parasitoid wasp hosts. I have demonstrated both theoretically and empirically that current theories on the adaptive benefit of male-killing are insufficient to explain the spread of *A. nasoniae* in its native host *Nasonia vitripennis*. I have also demonstrated that infectious transmission can overcome these shortcomings. The utility of infectious transmission is almost certainly a product of host population density and structure, factors that, reciprocally, the symbiont can also influence when at high prevalence. I further demonstrate that, counter to current dogma, infection with this heritable symbiont can confer high direct costs to host fitness. Finally, I expanded upon *A. nasoniae's* known capacity to host-shift and demonstrated that interspecific transmission can lead to rapid changes in symbiont biology, underpinned by diversification of the accessory (rather than core) genome.

6.1.1 Chapter 2: Theoretical models of male-killer spread.

In Chapter 2 I elucidate some of the shortcomings in the current theories proposed to describe the adaptive benefit of male-killing in insect symbiosis. Briefly, this dictates that a male-killing microbe should spread through maternal inheritance alone via a process of fitness compensation. The death of males should benefit their infected sisters or the offspring of their sisters, resulting in higher vertical transmission potential for the bacterium. Evidence for this phenomenon, through either a process of reallocating resources from dead males to their sisters or alleviating inbreeding within a brood, is relatively scarce and almost unanimously suggests that killing is a weak drive mechanism that is sensitive to direct costs of infection. In my model of male-killer dynamics I invoke two factors that are rarely incorporated formally in the spread of sex ratio distorters.

a) Symbiont infections may impose direct costs (outside those costs caused by the reproductive manipulation itself).

b) Infectious transmission of the symbiont between unrelated individuals may regularly occur.

Both factors have been alluded to in existing literature but hardly, if at all, formally incorporated into models. I demonstrate that direct cost is a major determinant of male-killer spread within a strictly vertical transmission based framework, by virtue of

male-killing's weak drive. Conversely, infectious transmission, even at low rates, all but negates the effects of physiological cost and allows for invasion over a far broader range of symbiont strains. This has important implications, not just for the system modeled, *A. nasoniae* in *N. vitripennis*, but for the numerous other heritable symbioses that have shown evidence of infectious transmission and cost. It may help explain why heritable symbionts are so widespread, as it allows for the invasion of even maladapted, costly symbionts into novel host populations.

6.1.2 Chapter 3: Evidence for direct costs of A. nasoniae infection.

In Chapter 3, I empirically explore one of the parameters modeled in Chapter 2; direct cost of infection. Symbiont transmission theory states that direct costs should be ameliorated by selection or adequately compensated for by the drive enhancing phenotype of the symbiont. Otherwise, the adaptive benefit of the symbiotic phenotype is eroded and vertical transmission efficiency suffers. Data presented here show that *A. nasoniae* does impart an overt, direct cost to its host *N vitripennis*. This manifests as reduced production of female offspring, extended development time, smaller female size and malformed wings. This brings into question current fitness compensation based theories of male-killer evolution. Further, I discuss the nature of this cost in the context of *N. vitripennis* population biology and how it may affect the spread of *A. nasoniae* by influencing host population structure. Importantly, in the model presented in Chapter 2, even minor costs prevented *A. nasoniae* spread. So the costs demonstrated here should negatively affect vertical transmission efficiency.

6.1.3 Chapter 4: Evidence for the importance of horizontal transmission.

In Chapter 4, I empirically test the influence of the second factor introduced in Chapter 2 - infectious transmission. I demonstrate that vertical transmission is indeed insufficient to drive *A. nasoniae* in experimental populations of *N. vitripennis*. However, a mixture of vertical and infectious transmission (through co-parasitism) facilitates *A. nasoniae* spread, even from very low initial prevalence. Furthermore, the penetrance of *A. nasoniae* in any given population appears to be dependent upon the horizontal transmission potential of that population, although it is unclear from the data if this is a linear relationship or if there is a threshold level of co-parasitism required to facilitate spread. Finally, I demonstrate that when drive of *A. nasoniae* is driven to high prevalence and host populations are resource stressed, the male-killing phenotype can cause extinction through global virginity.

One critique of the work in Chapters 3 & 4 of this thesis is that they use a single strain of *A. nasoniae* against a homogenous, lab reared host background. Thus, it is arguable that the effects observed are a product of the unusual combination of wasp and host, and that we are ignoring the effect of mosaic genotype by genotype interactions seen in nature that may produce variability in resistance and virulence. Furthermore, in these studies I do not account for or allow local adaptation of the microbe, which will occur in the face of differing selection pressures in disparate host populations. These criticisms are not without merit, however certainly not terminal to the conclusions of this study.

Firstly, the very conclusion that *A. nasoniae* regularly utilizes infectious transmission, implies that it will consistently find itself infecting novel host genetic backgrounds and move into populations with varying ecological characteristics. Thus, the combination of host and symbiont used in this study do represent an ecologically valid pairing. Furthermore, studies of the genetic structure of *N. vitripennis* populations have found very little differentiation between populations (Grillenberger *et al.*, 2008). Thus it is reasonable to assume that any given microbe will find itself moving between host sub-populations that are very similar to its origin.

That stated, the question of local adaptation is an enigma. We currently do not know the extent of phenotypic or genetic diversity within *A. nasoniae* infecting *N. vitripennis* populations. To determine this it will be necessary to obtain isolates from distinct populations, assay them for transmission, virulence and male-killing. Furthermore, the advent of next generation sequencing technology will allow us to assess fine-scale genetic differentiation between strains.

6.1.4 Chapter 5: Consequences of interspecific transmission.

Symbionts that regularly use infectious transmission within a species are more likely to switch between host species. In Chapter 5 I address the consequences of a host switch in terms of the phenotypic and genetic diversity it can create through disparate parallel co-evolution. I demonstrated that following a host shift to a species with markedly different reproductive biology (solitary vs. gregarious parasitism), selection has quickly eroded the symbionts manipulative phenotype, as well as its capacity to infectiously transmit. I also present the genome of this closely related, but phenotypically distinct strain of *A. nasoniae* and discuss this in the context of host-specialisation and symbiont diversity.

6.2 - Infectious transmission in heritable symbionts:

Aresenophonus nasoniae's ability to infectiously transmit is unusual but by no means unique amongst heritable symbionts (Schilthuizen and Stouthamer, 1997; Huigens *et al.*, 2000; Caspi-Fluger *et al.*, 2012). It is therefore important that we understand the role horizontal infection plays in driving symbiont spread within their host populations and its consequences for symbiont evolution.

Vertical infection of secondary symbionts is a fickle phenomenon; particularly where drive phenotypes are weak and the potential for direct costs of infection are high (Werren, 1987; Hurst, 1991; Kwiatkowski and Vorburger, 2012). Thus it is not surprising that additional infectious transmission is necessary to drive symbiont spread in some cases. Evidence has shown that symbionts can utilize a mixture of vertical and horizontal transmission in their epidemiology at different host densities and in different seasons (Watts *et al.*, 1973; Vilaplana *et al.*, 2008) and theoretical work postulates that the capacity for infectious transmission should rarely be lost by reproductive manipulators (Ironside *et al.*, 2011). However, infectious transmission is rarely incorporated into models of insect symbiont dynamics and is almost completely overlooked as a key component of the dynamics of heritable bacterial endosymbionts. The data presented here suggest that ignoring intraspecific horizontal transmission may well be omitting and integral part of the symbionts' biology. That *A. nasoniae* has maintained both vertical and horizontal transmission, and uses both in its epidemiology, is interesting. Given the costs of infection and the necessity for infectious transmission demonstrated in Chapters 3 & 4, it would appear that *A. nasoniae* (*ArN*) is trading off virulence with infectivity. This is what would be expected of an outright parasite (van Baalen, 2002). Indeed, *A. nasoniae* appears to be a missing link between heritable symbionts and outright pathogens, mixed vertical and horizontal transmission, and reproductive manipulation phenotypes.

Indeed, the genus *Arsenophonus* represents an unusual symbiotic clade, whose members range from P-symbionts, those that are secondary symbionts without reproductive parasitism, reproductive parasites, and strains that transfer both vertically and through plants (See Chapter 1, Table 1.2). Closely related genera are commonly gut associated bacteria (*Photorhabdus*, *Proteus*) of invertebrates. Vertical transmission has been shown in *Photorhabdus* (Ciche *et al.*, 2008), with this being produced, as in *A. nasoniae*, through oral infection. Because symbionts that are

transmitted through eggs rarely have the capacity or machinery for survival outside of host cells, it is likely that the infectious ability of *A. nasoniae* is ancestral, rather than secondarily derived. Gut symbiosis is potentially the ancestral state and that ecological circumstance (the parasitic lifestyle) generated vertical transmission by accident (the bacterium was placed in the environment alongside the progeny of the host), with infection occurring when co-parasitism occurred. The ecological scenario then favoured male-killing as an extra form of drive – male hosts do not oviposit so do not transmit symbionts onward. In this scenario, male-killing is not a sufficient drive to allow spread, but has evolved as an extra form of drive

The consequences of infectious transmission are profound. As demonstrated here, it may allow symbionts to rapidly invade susceptible populations, thus quickly introducing a novel phenotype to a population of hosts. In Chapter 4 I demonstrated that in extreme incidences, such spread might even result in extinction of the host population. Horizontal transmission also allows co-infections of distinct symbionts to occur in the same host individual. This may promote virulence, as symbiont strains compete for representation in the host tissues (Lively, 2009). It is unclear if the costs seen in Chapter 3 are therefore a result of *A. nasoniae* retaining a competitive ability and thus virulence. Co-infection also allows symbionts of different lineages to exchange genetic material, and so may be an important source of novel innovation for the symbionts themselves, as well as the hosts that acquire them. We therefore expect a symbiont that exhibits regular co-infection to have a vastly different and more varied evolutionary trajectory than one that relies solely on vertical transmission.

6.3 Divergent evolution following a host shift:

Host shift events have been regularly inferred from molecular data (e.g. Russell and Moran, 2005; Baldo *et al.*, 2006) and artificially induced under laboratory conditions (McGraw *et al.*, 2002; Huigens *et al.*, 2004; Duron *et al.*, 2010). However, we are lacking an understanding of how selection divergently acts on novel host-symbiont complexes. It has been documented that symbiotic phenotypes may change following a host shift, even descending from a parasitic to mutualistic association (Weeks *et al.*, 2007). How these changes manifest at the genetic level, particularly over short periods of evolutionary time is especially unexplored.

In this thesis I presented a novel *A. nasoniae*/wasp system that shows hallmark traits of a specialized mutualism and loss of reproductive parasitism. The benign symbiotic phenotype of *ArPv* shown in Chapter 5, compared to the costly and infectious strain described in Chapter 3 & 4, are testament to the speed with which diversity can be generated within symbiont groups. Genetic comparisons of this strain of symbiont with the male-killing relative found in *N. vitripennis* found little to no sequence variation at several conserved loci used to establish phylogenies, indicating a very recent divergence. In contrast, genes putatively associated with virulence and symbiosis were disparate between strains. This indicates that recent host switches can rapidly alter the phenotype of a symbiont through direct selection on symbiosis-associated factors. The large scale, whole genome erosion found when comparing mutualists with their closest recent free-living relative (Burke and Moran, 2011) was absent, indicating that this is a secondary process of symbiont evolution.

As a strain that apparently has no male-killing ability and also lost infectious transmission ability, *ArPv* presents a useful case study into the genetic basis of symbiotic traits. The *ArN* genome can usefully be dissected to suggest candidate systems involved in male-killing, and infectious transmission (Wilkes *et al.*, 2010). These factors can then either be introduced into *ArPv*, or function ablated from *ArN* to test hypotheses. Indeed, the combination of culturability, transferability and symbiotic diversity makes *A. nasoniae* an ideal system in which to investigate the elusive microbial systems underlying reproductive parasitism.

As the reproductive biology of *P. vindemmiae* negates any benefit of male-killing one high risk but high gain approach would be to transinfect *P. vindemmiae* with the male-killer *ArN*. We could then follow the evolution of the strain over several host generations and asses its symbiotic phenotype and genetic differentiation. Here, the systems associated with male death and infectious transmission should be redundant. Recovery of the bacterium and genetic analysis after passage would reveal pathways that underlay these traits.

What is yet to be revealed is how *ArPv* is maintained in its host. I detailed a lack of manipulative or beneficial phenotype but imperfect transmission (*c.*98% efficient) in the novel *A. nasoniae* strain *ArPv*. This should result in the symbiont being lost from the population, however casual observations of *A+* stocks kept in laboratory conditions show the infection to be stable. There must be a drive mechanisms operating in this

system at some level, however the metrics of fitness used in Chapter 5 failed to detect it. Future work could focus on identifying the symbiotic phenotype of this infection with more sensitive metric of fitness. It would be particularly interesting to perform invasion assays, in which a population is initiated at 50% prevalence, and then symbiont frequency monitored over time (as was done with *N. vitripennis*/*ArN* in Chapter 4). This would establish the presence and magnitude of drive, even if not its nature.

6.4 Theories of male-killing:

The evidence presented here show that an adaptive benefit of male-killing through fitness compensation is not sufficient to permit spread in this system. Benefit through the reallocation of resources from dead males to their infected sisters (Hurst, 1991) is shown to be insufficient to drive the spread of *A. nasoniae* (Chapter 4). This is most likely a combination of the sub-perfect transmission efficiency of the bacterium (Chapter 4) and the direct costs suffered by infected females (Chapter 3). It has been argued throughout this thesis that male-killing has a weak drive effect. In this instance it would appear that it is too weak to promote symbiont spread within a framework of pure vertical transmission.

I do not explicitly investigate the effects of inbreeding avoidance (Werren, 1987) in this thesis. Indeed, for most experiments in Chapter 4 inbreeding is enforced and host genetic background is kept homogenous. However, other work on the deleterious effects of inbreeding have shown them to be minor in the short term, only manifesting as an observable cost after several generations of enforced sib-sib mating (Luna and Hawkins, 2004). Furthermore, the negative effects are only marginal, and unlikely sufficient to drive *A. nasoniae* spread according to Werren's 1987 model (Werren, 1987). Nor would avoidance of inbreeding create a strong enough drive to overcome the costs presented in Chapter 3. Thus it seems unlikely that inbreeding avoidance is operating to sufficiently drive symbiont prevalence here.

However, the data presented in this thesis does not render the act of male-killing wholly maladaptive. It may still be beneficial in a framework of necessary infectious transmission as it alleviates crowding under co-parasitism. In Chapter 2 I discuss how the action of male-killing does not need to benefit just the sisters of killed males, but any female broodmate who is a vector for the infection. Classic male-killer theory

requires sib-sib competition in order for spread to occur (Hurst, 1991). Here, there only needs to be competition between any infected females and killed males (as occurs during co-parasitism) in order for resource release to have a benefit. Thus, the reproductive manipulation in this instance may enhance both vertical and horizontal transmission. Direct empirical evidence for a benefit of male-killing is also lacking in other systems, with only one explicit study (Koop *et al.*, 2009). Thus it may be necessary to apply the findings here to all instances of male-killing where infectious transmission is possible.

6.5 Future work beyond evolution of Arsenophonus: do studies of the gut microbiota require incorporation of Arsenophonus impacts?

One interesting area of recent research has utilized *Nasonia* as a model for the development and function of the gut microbiome (Zouache *et al.*, 2009). Indeed, hybrid *Nasonia* death is associated with pathogenic activity during gut microbiome development (Brucker and Bordenstein, 2012). It is possible that *A. nasoniae* costs and virulence observed in Chapter 3 may be associated with gut microbiome perturbation. It is certainly likely that an additional high titre microbe, adapted to gut living, will perturb the gut microbiome. It is notable that *ArN* genome contains colicin genes, and that the bacterium is thus likely capable of direct interference with competitors, as well as indirect (resource based) interference. Future work could be aimed at characterizing this perturbation, and in ascertaining if it is causing the developmental delay and abnormalities observed in Chapter 3. It is also possible that adaptation of *Nasonia* to *Arsenophonus* has played a role in the shaping of the gut microbiome, and that divergence in the genetic systems that create hybrid inviability may have been driven by this gut symbiont. In summary, it is worth considering how *Arsenophonus* impacts the *Nasonia* gut microbiome, how the gut microbiome may influence it, and how this may have affected the evolution of host systems for handling the gut microbiota.

Appendix:

Chapter 2:

The equations presented in Chapter 2 do not allow for the cost of crowding to be completely negated by the benefit of male-killing in scenarios where they both occur (*3* & *4*). Therefore below are presented the same equation but for the special case where $S = k$. This scenario is almost certainly biologically unrealistic and so is taken no further. This distinction is also not relevant for scenarios where a = 0 because there will be no cost of crowding.

#	Scenario	A+ Daughter Production	A- Daughter Production
1	Single I	$P(1-a)(1-u)(1+k)$	$Pu(1-a)(1+k)$
2	Single U	-	$(1-P)(1-a)$
3	UI Co-lay	$P(1-P)a(1-y)$	$P(1-P)ay$
4	IU Co-lay	$P(1-P)a(1-u)$	$P(1-P)au$
5	II Co-lay	$P^2a(1-uy)(1+k)$	$P^2auy(1+k)$
6	UU Co-lay	-	$(1-P)^2a(1-k)$

Recursion:

P'

$$= \frac{P + Pk - Pu - Puk + Pa - Pak + Pauk + P^2ak - P^2uya - P^2uyak - P^2a + P^2au - Pay + P^2ay}{Pk + 1 - aK + Pka}$$

Equilibrium for $S = k$ given by:

$$\hat{P} = \frac{(k - u - uk + a + auk - ay + 1)}{(k + uya + uyak + a - au - ay)}$$

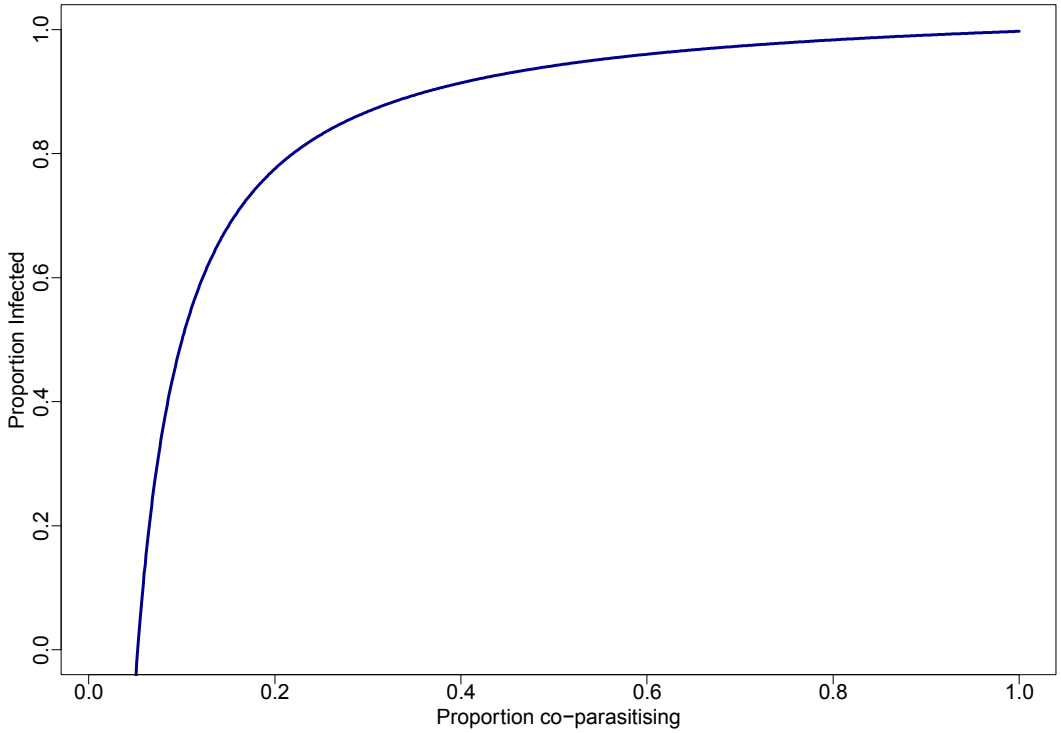


Figure 2.A1: Equilibria conditions for the special condition where S=k (fitness lost through crowding are identical to that gained through male-killing). There is a threshold of c 5% multiparasitims required before *A.nasoniae* establishes. The level of *A.nasoniae* is not linear with multiparasitism rate.

Equilibrium equation when direct costs of infection (1-*j*) are included into the model of symbiont spread:

$$(a+k+2Sa -2ak-(2ak-2a^2u -2a^2y+a^2+k^2 +a^2u^2+a^2y^2+4a^2uy+4a^2k^2u-2a^2uy^2-2a^2u^2y+4Sau-6aku-2aky+a^2u^2y^2-4Sa^2u-4ak^2u+4a^2ku+2akuy+2a^2ku^2y^2-4Sa^2ku+4Sa^2uy+2ak^2uy-2a^2kuy+a^2k^2u^2y^2-2a^2kuy^2-2a^2ku^2y-4a^2k^2uy+4Saku+4Sa^2kuy)^{(½)}-au-ay+auy+akuy)$$
$$/$$
$$(2(Sa-ak))$$

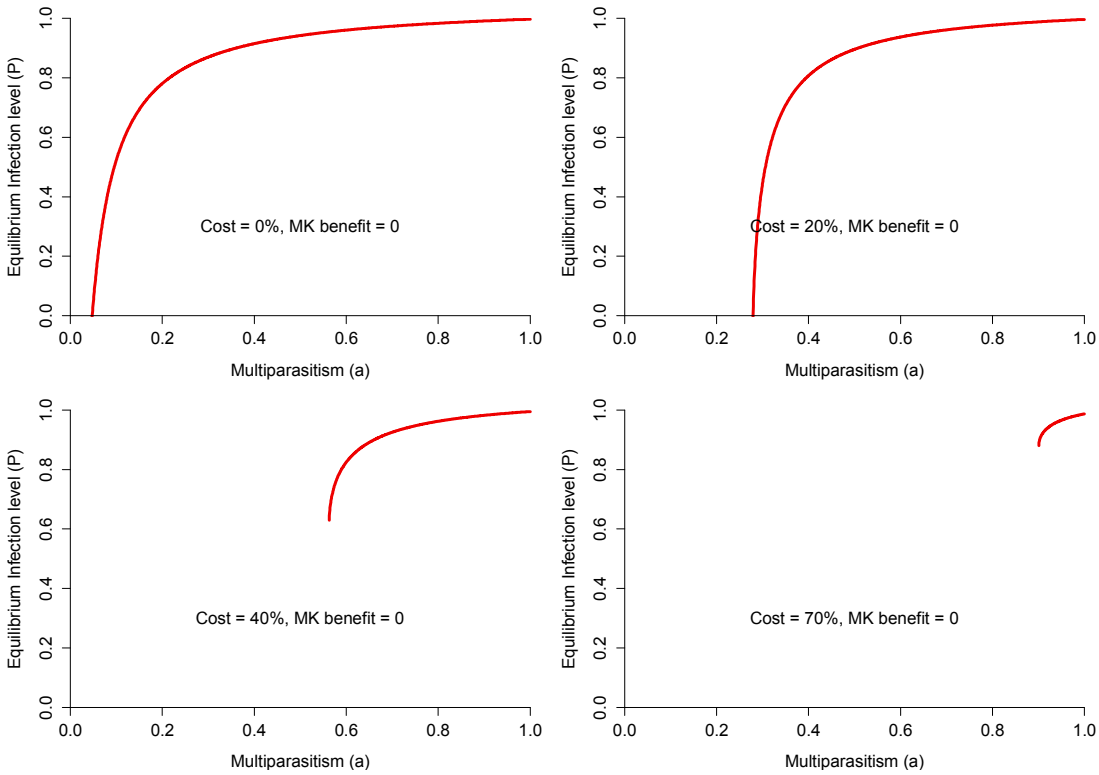


Figure 2.A2: Spread of *A.nasoniae* through both vertical and horizontal transmission in the absence of a male-killing derived drive (1-*k*=0). Such is the strength of infectious transmission, it can drive A.nasoniae into populations even without the benefit of male-killing through resource release and crowding alleviation.

Chapter 3:

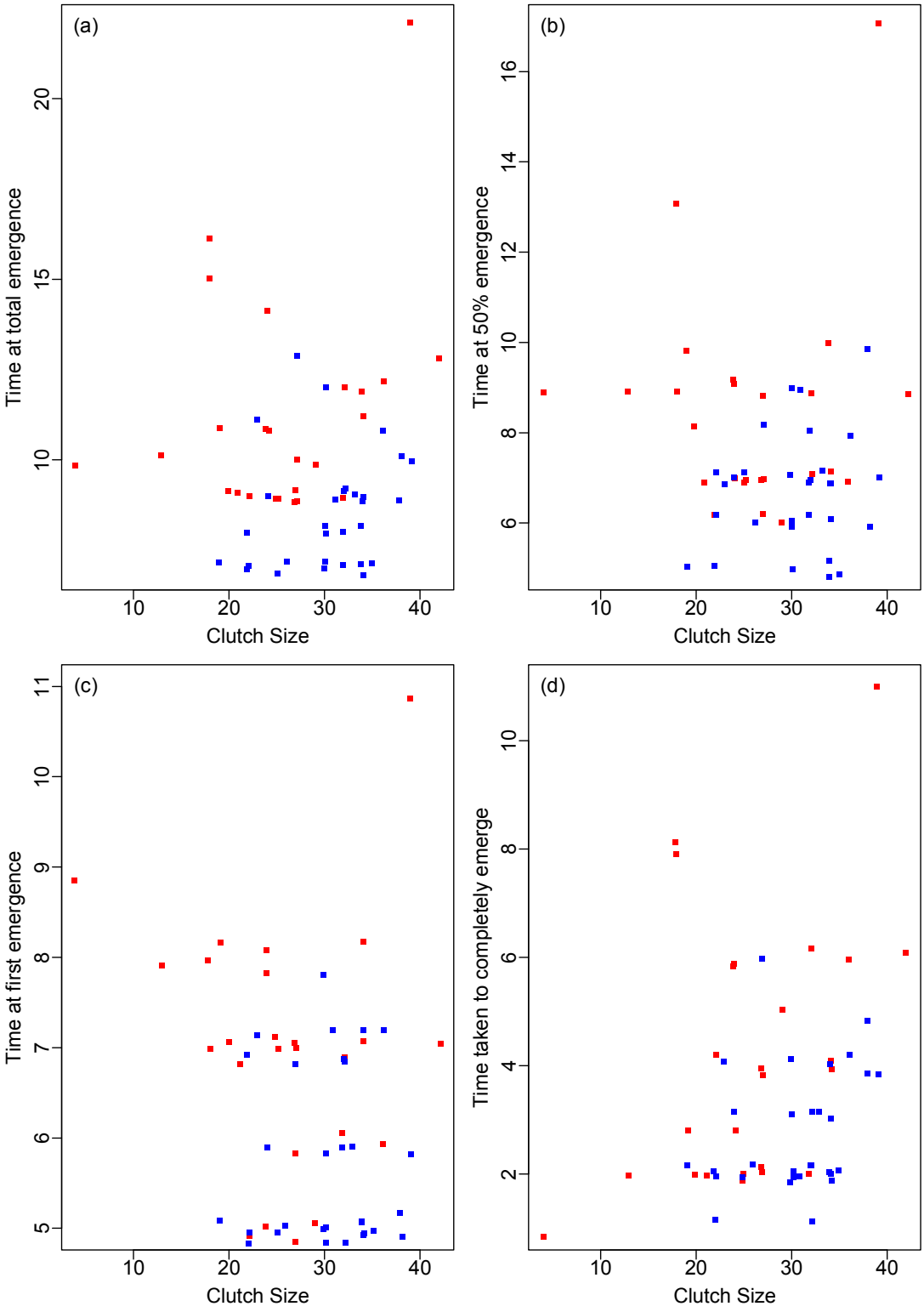


Fig 3.A1: Metrics of *N.vitripennis* fitness against relative clutch size. Results are discussed in the chapter.

Chapter 4:

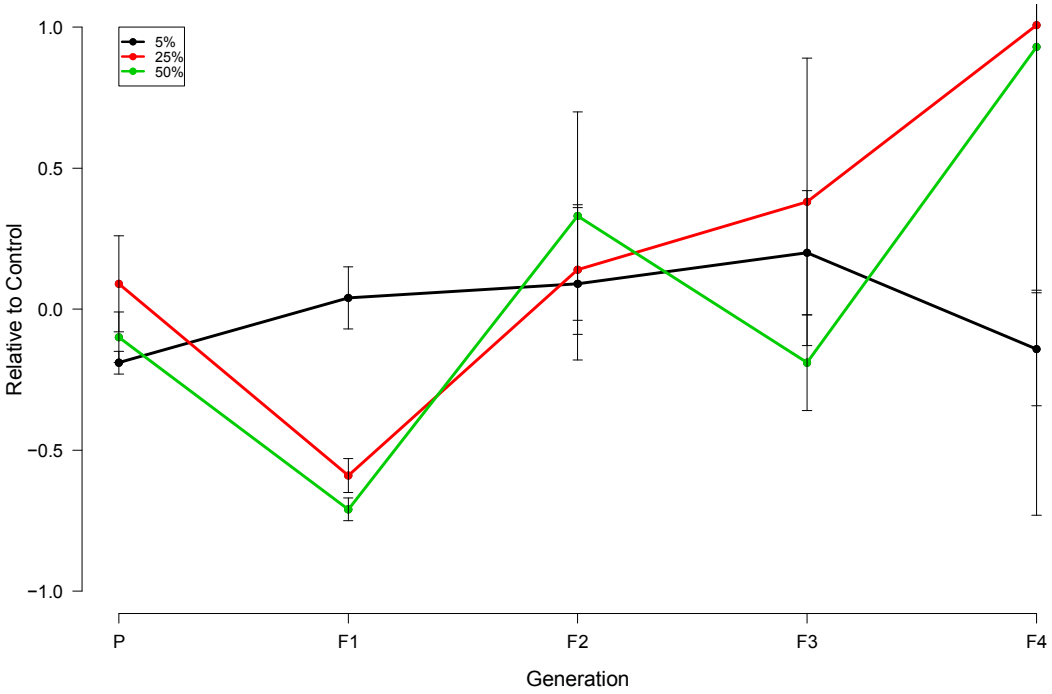


Figure 4.A1: Sex ratio of populations relative to uninfected controls from experiment 2. Where *A.nasonaie* infection starts at high prevalence (25% and 50%) sex ratio fluctuates, similarly to the results form *Experiment 1*. However, interestingly it remains roughly stable when infection is started at lower prevelance (5%)

Chapter 5:

Experiment 2: Fitness effects of ArPv on P.vindemmiae:

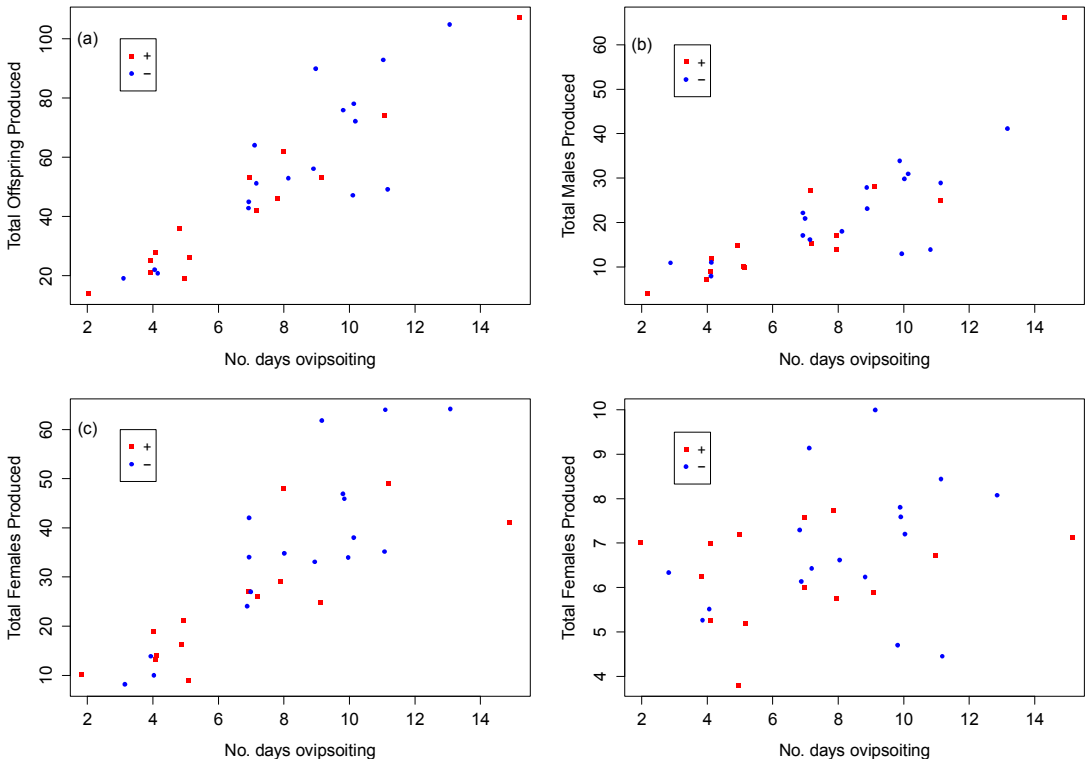


Figure 5.A1: Rudimentary measures of fitness (as offspring produced) against time ovipositing by females before they died. (a) total offspring, (b) Total males produced, (c) total females produced, (d) mean offspring produced per day. *A b* & *c* all show linear relationships, implying that females did not become fatigued or sperm depleted after 14 days. *(d)* demonstrates that daily offspring rate is relatively variable.

Table 5.7: ORFs called as ribosome associated by IMG/ER and their identity scores for the closest hit with the genome or ArN. All 50 ORFs have 99% or greater identity, indicating exact homology.

ArPv ORF	Cover	ID	Length	Bit score	E score
ArPv_00045	100	100	414	765	0.00E+00
ArPv_00046	100	100	2690	5376	0.00E+00
ArPv_00047	100	99	1513	2724	0.00E+00
ArPv_00057	100	100	477	861	0.00E+00
ArPv_00060	100	100	258	466	4.00E-128
ArPv_00061	100	100	309	558	1.00E-155
ArPv_00079	100	100	450	812	0.00E+00
ArPv_00097	100	99	1050	1880	0.00E+00
ArPv_00082	100	100	228	412	6.00E-112
ArPv_00080	100	100	393	710	0.00E+00
ArPv_00182	100	100	144	260	1.00E-66
ArPv_00254	100	100	216	385	8.00E-104
ArPv_00347	100	99	374	672	0.00E+00
ArPv_00348	100	100	471	850	0.00E+00
ArPv_00353	100	100	429	774	0.00E+00
ArPv_00354	100	99	701	1261	0.00E+00
ArPv_00355	100	100	498	899	0.00E+00
ArPv_00356	100	99	368	661	0.00E+00
ArPv_00436	100	99	888	1584	0.00E+00
ArPv_00459	100	100	288	520	2.00E-144
ArPv_00479	100	99	386	769	0.00E+00
ArPv_00480	100	100	339	710	6.00E-128
ArPv_00637	100	100	312	563	2.00E-157
ArPv_00638	100	100	630	1137	0.00E+00
ArPv_00639	100	99	606	1088	0.00E+00
ArPv_00640	100	99	356	542.00	7.00E-151
ArPv_00641	100	99	825	1483	0.00E+00
ArPv_00642	100	100	279	504	2.00E-139
ArPv_00643	100	100	333	601	1.00E-168
ArPv_00644	100	99	711	1274	0.00E+00
ArPv_00645	100	99	411	737	0.00E+00
ArPv_00646	100	100	192	347	2.00E-92
ArPv_00647	100	100	255	461	2.00E-126
ArNv_01631	100	100	729	1315	0.00E+00
ArNv_03061	100	100	1482	2673	0.00E+00
ArNv_03188	100	100	318	574	1.00E-160
ArNv_01232	100	100	978	1764	0.00E+00
ArNv_01234	100	99	232	410	2.00E-111

ArNv_01238	100	100	354	639	4.00E-180
ArNv_01241	100	100	249	450	3.00E-123
ArNv_01432	100	99	939	1676	0.00E+00
ArNv_02601	100	100	282	509	4.00E-141
ArNv_01676	100	99	648	1160	0.00E+00
ArNv_01707	100	99	144	385	8.00E-104
ArNv_02034	100	99	1710	3079	0.00E+00

Table 5.A2: ORFs identified through automated annotation or manual BLAST alignments as being involved with T6SS functionality. Note that all these ORFs showed multiple blast hits to T6SS factors, and in all cases *Photorhabdus spp* were the top hit.

ArPv ORF	Predicted Function	Best blast hit	Potential T6SS part
ArPv_02770	abc like periplasmic domain	none	Perisplasmic protein
ArPv_02771	LuxR family transcription regulator	Photorhabdus asymbiotica	pvp response regulator
ArPv_02772	Hypothetical protein	Conserved Photorhabdus protein	Unknown
ArPv_02773	Type VI secretion ATPase, ClpVI	Photorhabdus asymbiotica	ClpV1
ArPv_02774	Hypothetical protein	Photorhabdus hits	Some identity to TssM
ArPv_02775	Type IV/VI DotU	Photorhabdus	Type IV like dotU, possibly TssK,
ArPv_02776	Type VI secretion protein, VC_A0114 family	Photorhabdus	Type VI secretion protein, VC_A0114 family, TssK
ArPv_02777	Hypothetical protein	Photorhabdus	Type VI secretion protein, VC_A0114 family, TssK
ArPv_02778	Hypothetical protein	Photorhabdus	Type VI secretion lipoprotein
ArPv_02779	Type VI ImpA family protein	Photorhabdus	Type VI ImpA family protein
ArPv_02780	Uncharacterised	Photorhabdus	Type VI secretion protein
ArPv_02781	Hypothetical protein	Photorhabdus	Type VI secretion protein
ArPv_02782	Vgr element	Photorhabdus	VgrG, ImpA type VI protein
ArPv_02783	Type VI secretion protein, VC_A0111 family	Photorhabdus	Type VI secretion protein
ArPv_02784	Type VI secretion protein, VC_A0110 family	Photorhabdus	ImpG, VasA, EvpF TypeVI protein
ArPv_02785	Hypothetical protein	Clostridium/Facklamia	ABC transporter ATP-binding protein
ArPv_02786	ABC-type metal ion transport system, ATPase	Clostridium	ABC-type metal ion transport system, ATPase

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