The Interaction between *Arsenophonus* and its Eusocial Host

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

June 19, 2019

Abstract

Endosymbiotic bacteria of the genus *Arsenophonus* form associations with a diverse range of arthropod hosts. These symbiotic interactions are varied, ranging from reproductive parasites to coevolving obligate mutualists, and the predominant mode of symbiont transmission within the clade is vertical. Previous metagenomic studies indicate that *Arsenophonus* forms associations with the European honey bee, *Apis mellifera*. Despite the evolutionary and ecological significance of *Apis mellifera*, and the potentially important impacts of *Arsenophonus* on *Apis* biology, interactions between these players have remained uncharacterised. This thesis reports *Arsenophonus* is common within UK populations of *A. mellifera* and the genetic diversity of circulating strains appears to be low. Phylogenomic analysis confirms the position of the symbiont within the genus *Arsenophonus*, most closely related to the male-killer strain associated with the parasitoid wasp, *Nasonia vitripennis*. Epidemiological patterns are detected, with *Arsenophonus* occurrence varying over space and time. Spatial variation is evident at local scales but infection prevalence remains uniform with larger geography. Conserved seasonal dynamics, with prevalence low in spring and increasing into autumn, hint at the role of an environmental reservoir driving infection dynamics and argue against vertical transmission. The low prevalence of *Arsenophonus* among solitary *Colletes* spp. suggests spill over risk is low and associations are uncommon, perhaps mediated by the fixation of another endosymbiont, *Wolbachia*, in solitary Anthophila populations. FISH analysis indicates *Arsenophonus* is localised within the *A. mellifera* gut and forms diffuse

infections, however associations are highly dynamic. Infections can be lost rapidly under both field and laboratory conditions and correlate with reduced exposure to the environment. Absence in spring samples, in addition to the eggs, larvae and newly emerged workers argues against direct vertical transmission of *Arsenophonus* in *A. mellifera*, contrasting markedly with other *Arsenophonus*-host interactions examined to date. Horizontal transmission via social interactions can occur, but with variable success. Multiple lines of evidence suggest environmental exposure is key to infection maintenance and social transmission may further drive the dissemination of *Arsenophonus* within a colony. These findings demonstrate evolutionary lability within this common clade of insect symbionts and have repercussions for studying transitions in symbiotic lifestyle.

Acknowledgements

The completion of this thesis hinged on a great number of people, for whom I am endlessly grateful to. Firstly, thank you to all my supervisors. To Greg Hurst, despite burdening me with a great fondness for symbionts, I cannot thank you enough for your time and support throughout the past 4 years. Thank you to Giles Budge for all your knowledge and guidance at Fera, and to Alistair Darby in Liverpool. Additional thanks to Katherine Roberts, Kirsty Stainton and Crystal Frost for your invaluable help and general encouragement, and also to Bernard Diaper at the BDI. The BBSRC and Bee Disease Insurance Ltd generously funded this project, and I am very grateful for the opportunity.

A significant number of beekeepers, including Jack Wilford, shared their knowledge and time and played pivotal roles in sampling efforts. I am indebted to many members of IIB, for propagating skills, conversations and fostering a place that was always fun to work in. I'd like to extend additional gratitude to Olga's chocolate drawer and also to Andri, for sharing a little of his statistical wizardry and providing motivational speeches. To all my friends, thank you for helping me climb and picnic my way through the 4 years, and thank you, of course, to my family. Largely for both their unwavering support, and lyrical commitment to finding words that rhyme with *Arsenophonus* - their task was far harder than mine.

Finally, thanks to my bacterium of interest, you were never really there when I needed you, but your enigmatic nature and erratic ecology was interesting - in the end.

Contents

Chapter 1

General Introduction

1.1 The Evolutionary Significance of Symbiosis

Biological innovation is fundamental to the existence of life. Yet how organisms innovate new functions whilst retaining robust phenotypes is a central challenge in evolution. Symbiosis, the intimate interaction of two or more distinct biological entities (de Bary 1879; Lewis 1985), constitutes a route by which organisms arrive at new evolutionary innovations whilst minimising functional risk. Interactions between disparate organisms, with differing evolutionary histories, can unite novel functions and allow increased adaptability, and in this way symbiosis reduces the evolutionary constraints imposed on individual units. The symbionts best placed to occupy these nodes are microbial ones, with large population sizes, short generation times, flexible genomes, and rapid mutation rates. Microbes can associate intimately with host organisms and in some cases are vertically transmitted, thus providing direct sources of heritable variation upon which natural selection can subsequently act (Hurst 2017, Moran et al. 2008, Rosenberg & Zilber-Rosenberg 2011, Werren et al. 2008, Zilber-Rosenberg & Rosenberg 2008).

Some of the greatest examples of cooperation in nature are examples of symbiosis with a microbial partner. Mutualistic symbioses include the formation of heterospecific individuals, exemplified by the assembly of fungi and autotrophic

algae into lichen chimeras, or the mitochondria found within all eukaryotic cells - the relics of an ancient symbiosis credited with the emergence of complex life (Margulis 1970). However, interactions between different players often entail interests and demands that are asymmetric to one another, giving rise to an extensive stage of conflict that can be contingent on the context in which the interacting partners find themselves. Ultimately symbiosis occupies a dynamic continuum from mutualism to parasitism (Ewald 1987), underpinned by mechanisms of conflict and cooperation that play pivotal roles in the generation of global biodiversity.

The evolutionary significance of symbiosis has gained increasing attention in past years, with the hologenome theory of evolution gaining increasing popularity. This theory goes so far as to consider the 'holobiont', that is the host and all its microbial associates, as a level of selection (Mindell 1992, Zilber-Rosenberg & Rosenberg 2008) that can evolve by adaptive Lamarckian mechanisms within a Darwinian framework (Rosenberg et al. 2009). The theory remains controversial and reflects a growing trend in the rejection of a classical view of individuality (Hurst 2017, Queller & Strassmann 2016). In many cases, aspects of host biology are encoded by symbionts that are taxonomically variable but functionally equivalent, leading to suggestions that the metabolic function itself should be considered the unit of selection ("It is the song, not the singers") (Doolittle & Booth 2017).

1.2 Overview of Bacterial Symbioses

For one billion years, before the emergence of eukaryotes, the world was dominated by Archaea and Eubacteria. Today a vast diversity of eukaryotic hosts remain engaged in intimate symbioses with eubacterial partners. Here, and throughout this thesis, the term symbiont is used to refer to any agent that forms intimate and potentially long-lasting associations with a host organism, regardless of the implications for either party's fitness (Lewis 1985). Persistence of bacterial symbionts in host populations is enabled through either horizontal (non-heritable) or vertical

(heritable) transmission. In both cases the host organism can be viewed as the resource base, with bacterial symbionts acting as consumers that may also provide services in return (Leung & Poulin 2008). If services exist, they generally involve some level of protection to the host against biotic or abiotic stressors. Describing these interactions accurately can be difficult; nevertheless, the field has classically partitioned symbioses based on the cost/benefits experienced by the partners and the degree of dependence on host association. Interactions where all partners benefit represent mutualisms, a scenario that is widespread among bacterial symbionts with varying degrees of co-evolutionary interaction. Those where one partner benefits and the other neither profits nor gains is commensalism; here physiologic interaction or dependency between the partners is generally absent (or at least undetectable). Interactions where one partner benefits to the detriment of the other is viewed as parasitism; interactions here include heritable reproductive parasites that persist across the entire host life history through to infectious disease agents that exploit hosts for short time periods. While these interaction categories remain useful, there is an increasing recognition that the space from parasitism to mutualism can be dynamic and is better viewed as a continuum.

1.2.1 Horizontally acquired symbionts

Symbioses that rely on the horizontal transmission of their microbial partner are reformed each host generation, via contact with infected conspecifics or environmental reservoirs. Free-living bacterial populations are often the source of such horizontally transmitted symbionts, thus symbiotic life is often facultative for these bacteria (Bright & Bulgheresi 2010). However, dependence of the host can be high. Low host-dependence and absence of vertical transmission means these host-symbiont interactions are often more promiscuous than those with heritable symbionts, with lower partner specificity and higher rates of partner switching (Buchner 1967, Moran et al. 2008). As a result, evidence of tight co-evolutionary dynamics are often lacking in these systems. Despite this, evolutionary stable interactions between hosts

and horizontally transmitted symbionts are observed. The long-term symbiosis between bobtail squid and the environmentally acquired bioluminescent symbiont *Vibrio fischeri* (McFall-Ngai 2014) provides such an example.

Populations of environmentally acquired symbionts are genetically diverse with large effective population sizes, protecting against the accumulation and subsequent fixation of deleterious mutations. These symbionts must also maintain the genes required for host-association in addition to a free-living lifestyle, and under this constraint selection does not act to reduce genome size. The genomes of giant tubeworm endosymbionts have a large defence and signal transduction repertoire and show a marked ability with respect to chemotaxis and motility. These properties likely reflect demands on the symbiont to exist freely in hydrothermal vents, form an obligate nutritional association and migrate towards its sessile host (Bright & Bulgheresi 2010, Li et al. 2018, Robidart et al. 2008).

However, the benefits of maintaining independence may come at the cost of obligate synchronisation between host and symbiont. The acquisition of non-heritable symbionts relies on spatial proximity and subsequent direct contact between the free-living partners. Symbionts must additionally migrate to the colonisation or entry point upon the host surface and in some cases, infection is only possible during specific stages of the host's life cycle (Bright & Bulgheresi 2010). For example, the capture of planktonic *V. fischeri* cells by squid paralarvae is dependent on the bacteria's proximity to ciliated appendages of the light organ (Nyholm & Graf 2012). Asynchronisation may prevent acquisition of symbionts, with potentially detrimental effects for one or both partners.

1.2.2 Heritable symbionts

Heritable bacterial symbionts are transmitted vertically from parent to offspring. They are widely present in invertebrate animals, particularly insects, and are predominantly maternally inherited, passing from mother to offspring (Douglas 2010, Werren & O'Neill 1997). This matrilineal pattern is associated with asymmetry in

		HOST (H)	
		Dependent on symbiont	***Not** dependent on symbiont*
SYMBIONT (S)	*Dependent on host*	Obligate(S) – Obligate(H) *Blochmannia* & Carpenter ant	Obligate(S) – Facultative(H) *Hamiltonella* & Aphid
	***Not** dependent on host*	Facultative(S) - Obligate(H) *Zooxanthellae* & Coral	Facultative(S) – Facultative(H) *Vibrio* & Bobtail squid

Table 1.1. Patterns of symbiont – host dependence, with examples

both host gamete size (sperm size limits inclusion of microbial symbionts), and in the contact between parent and offspring (common for females, less common for male hosts). Indeed, whilst there are many accounts of maternally inherited bacteria, paternal inheritance is rarely documented (see De Vooght et al. 2015 for exception). Most heritable microbes of insects are considered to be fully dependent on hosts, lacking replicative or dormant phases outside of host organisms i.e. they are obligately symbiotic. However, these microbes vary with respect to whether or not the host is dependent on them for survival and reproduction. (For an overview of all possible host – symbiont dependence patterns, see Table 1.1). There are two major classes of heritable bacteria, namely obligate and facultative, these are defined by whether the host requires them for function (obligate), or can survive and reproduce autonomously (facultative) (Bennett & Moran 2015, Law & Dieckmann 1998, Moran et al. 2008).

1.2.3 Obligate associations

Obligate (primary) symbionts are those considered essential for host functioning. The microbe can confer a range of services to host organisms with nutritional symbioses being particularly well documented. For example, the bacterium *Wigglesworthia glossinidia* provisions essential B-vitamins for tsetse flies, which the host would otherwise lack in its blood based diet (Akman et al. 2002). This class of symbiont

is generally vertically transmitted from parent to offspring, representing heritable components of host biology (Douglas 1998, Werren & O'Neill 1997). Strict spatial structuring of the symbiont often occurs within the host and this is exemplified by the bacteriomes of many insects, a specialized organ that holds endosymbiotic bacteria in bacteriocyte cells and can facilitate domestication by the host (Moran et al. 2008). Obligate symbionts commonly have ancient associations with host lineages and often show patterns of co-cladogenesis that suggests host-shift events may be rare (Takiya et al. 2006).

The long history of association and dependence is reflected in the size and composition of obligate symbiont genomes. Vertical transmission creates bottlenecks, as the symbiont's effective population size is only as large as the number of cells that an egg, or other carrier, will accommodate. Repeated bottlenecks foster the accumulation and fixation of deleterious mutations in symbiont population in a process known as Muller's ratchet (Moran 1996). Additionally a journey of adaptive gene loss can occur via the Black Queen Hypothesis, whereby symbionts purge costly genes rendered obsolete by dependency on the host environment (Morris et al. 2012). These evolutionary forces leave distinct genomic signatures on obligate symbionts including reduced genomes with high gene densities (often biased towards specific functions) and AT bias. Evidence of foreign gene acquisition and homologous recombination between strains is also rare (Dale et al. 2006). The tiny number of coding DNA sequences (CDSs) found in some genomes classes them as simpler even than certain organelles and viruses (McCutcheon & Moran 2010). An extreme example is provided by *Nasuia deltocephalinicola*, a symbiont of the leafhopper *Macrosteles quadrilineatus*. The 112 kb genome is the smallest bacterial genome sequenced to date (Bennett & Moran 2013) and bares all the hallmarks of an ancient association that has existed for over 200 million years (Bennett & Moran 2013). For bacteria, genome size correlates almost perfectly with gene number, meaning that such reductions in genome size will ultimately come at the cost of lost functionality, and inability to survive away from the host (Giovannoni et al. 2005, Mira et al.

2001, Ochman 2005)

1.2.4 Facultative associations

Facultative (secondary) symbionts differ from obligate symbionts in that they are not essential for host fitness, although in many cases they may confer beneficial effects. Facultative heritable symbionts are also vertically transmitted, but horizontal transmission may nevertheless be important in maintaining the symbiont in host populations and facilitating host-switch events between species (Caspi-Fluger et al. 2012, Duron et al. 2010, Nakayama et al. 2015, Werren & O'Neill 1997). These factors often contribute to facultative symbionts having shorter evolutionary histories in host lineages and can contribute to a greater scope for conflict. Facultative symbionts may enter host bacteriocytes, coinfecting with obligate symbionts or even outcompeting them. Bacteriocytes of the whitefly *Bemisia tabaci* harbour five facultative symbionts (*Hamiltonella*, *Arsenophonus*, *Cardinium*, *Wolbachia*, *Rickettsia*) in addition to the obligate primary symbiont *Portiera* (Gottlieb et al. 2008). This tropism may represent an evolutionary strategy that allows the facultative symbionts to hitchhike with vertically transmitted *Portiera* and be transferred efficiently to the next generation of host offspring (Gottlieb et al. 2008). However, facultative symbionts generally infect a greater diversity of cell types and may also be found extracellularly (Rasgon et al. 2006, Salem et al. 2015).

The distributions of this class of symbiont are often more erratic, and they often infect just a fraction of individuals in host populations, which contrasts with obligate symbionts that infect every individual. The lifestyles of facultative symbionts often share greater similarities with invasive pathogens, such as maintaining the ability to evade host immune defences (Moran et al. 2008). Like their obligate counterparts, the genomes of facultative symbionts are also characterised by pseudogenization and genome reduction, but to a much lesser extent. Facultative symbiont genomes are greater than 1MB in nearly all cases, but rarely exceed 3.6 MB, and are often dynamic with extensive contributions from bacteriophage and mobile

genetic elements (Lo et al. 2016). Homologous recombination also remains an important process for facultative symbiont genomes and has shown to occur widely across *Wolbachia* strains (Baldo et al. 2006).

Within this discussion of the general features of facultative symbionts, two sub categories are recognised: facultative mutualists and reproductive parasites:

Facultative mutualists confer a fitness benefit to host, promoting host survival and reproduction. Common beneficial effects include anabolic contributions and protection against natural enemies (eg. parasites) or abiotic stress (eg. temperature) (Douglas 1998). Beneficial phenotypes increase the chance that the host will survive to reproduce in comparison to uninfected hosts, driving the spread of infection through the host population. For example, *Hamiltonella defensa* and *Regiella insecticola* have respectively driven themselves into aphid host populations by conferring protection against parasitoid wasp (Oliver et al. 2005) and fungal attack (Scarborough et al. 2005).

Reproductive parasites manipulate host reproduction to benefit their own transmission. Transmission enhancement is achieved by increasing the proportion of symbiont transmitting female hosts within a population or else reducing the comparative fitness of uninfected females (to the detriment of overall host fitness). Two major strategies are observed: distorting sex ratios of infected hosts towards production/survival of female hosts (male-killing, parthenogenesis, feminization) and inducing conditional sterility (cytoplasmic incompatibility) (for review, see Engelstädter & Hurst (2009)). The evolutionary rational for reproductive parasitism is that male hosts constitute evolutionary dead ends for these maternally transmitted symbionts (Cosmides & Tooby 1981), thus mechanisms that promote the production and survival of female hosts are favoured. Heritable symbionts that reduce female fitness are reported (Sakurai et al. 2005), but these are generally considered atypical as infected matrilines would disappear from the population due to selection (Engelstädter & Hurst 2009, Moran et al. 2008). Reproductive parasitism appears to be a successful strategy, evolving repeatedly across several genera of insect

symbiont, including *Arsenophonus*, *Rickettsia*, *Cardinium*, *Wolbachia* and *Spiroplasma*. Notably though, heritable symbionts classically associated with reproductive parasitism are increasingly found in the absence of manipulation phenotypes, indicating that beneficial phenotypes may be an underappreciated driver in the invasion and maintenance of these symbionts (Drew et al. 2019, Zug & Hammerstein 2015, 2018).

1.3 The Symbiosis Continuum

1.3.1 Transitions in symbiotic lifestyle

It is clear there is a continuum in the degree of host-association exhibited by symbionts. But what processes underlie transitions in symbiotic lifestyle? (See Figure 1.1 for a generalised depiction). The ancestral state of all host-associated symbionts must be free-living (Sachs et al. 2011). Thus bacteria that occupy positions as obligate endosymbionts, with extreme genome reduction, are likely the product of an evolutionary trajectory that moved through the entire host-association continuum.

1.3.2 A generalised view

The trajectory is likely to start with free-living environmental bacteria evolving to form an intimate interaction with a host organism. This transition demands the bacterium to compete with other host symbionts, evade host defences, access host resources and ensure transmission to additional hosts. Bacteria such as these may have ecological or genetic preadaptations to host-association (Toft & Andersson 2010); alternately gene acquisition (via lateral transfer or mobile genetic elements) may allow novel interactions with host that were previously unobtainable (Carvalho et al. 2010, Sachs et al. 2011).

Changes to the relationship between symbiont and host, such as a heightened stringency in host association, evolution of vertical transmission or establishment within host cells constitute important transitional events. These events can lead to

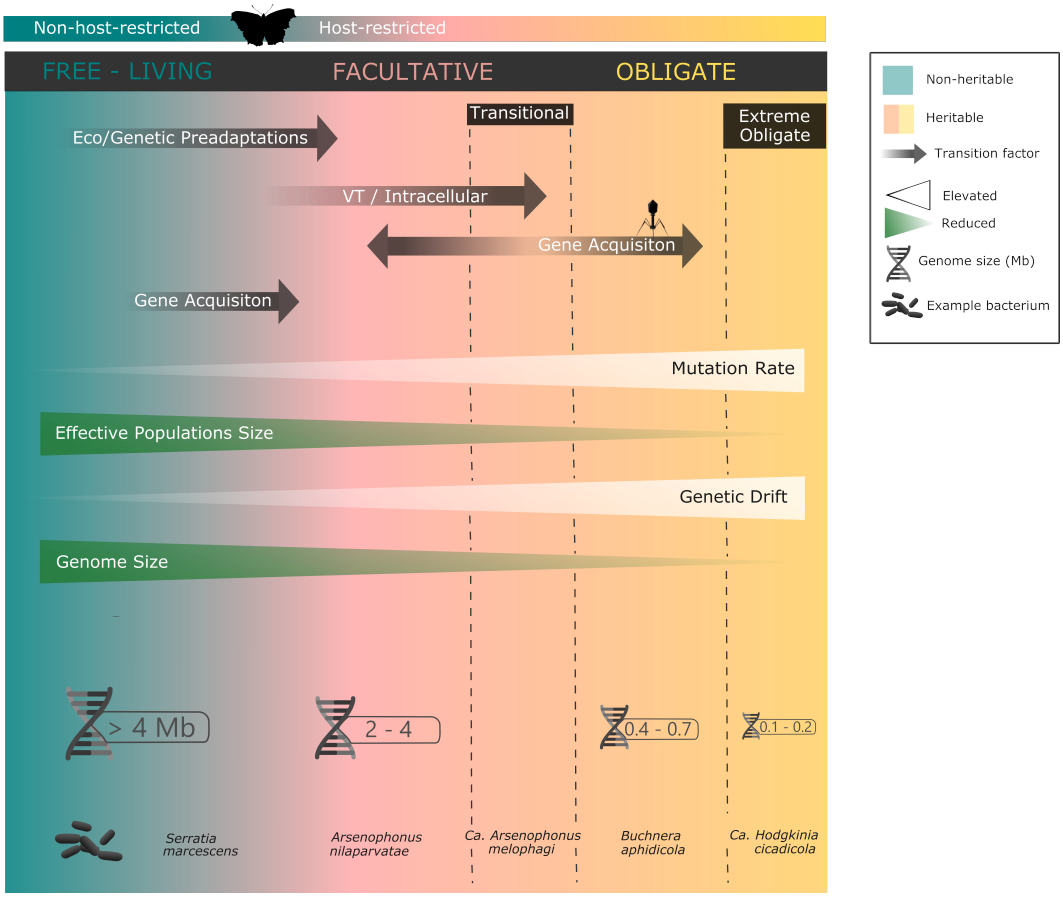

Figure 1.1. Evolutionary processes associated with symbiotic transitions along the continuum of host dependence. Free-living bacteria may evolve to associate intimately with a host, potentially mediated by ecological or genetic preadaptations or acquisition of novel genetic material that facilitates an interaction previously unobtainable. Adoption of strict vertical transmission or intracellular tropism will entrench the bacterium as facultative or further push its trajectory into a transitional stage towards obligate symbiosis. Effective population sizes decrease and non-adaptive mutations accumulate along trajectory, causing gene inactivations and reduction in genome size. Gene acquisition may rectify effects of inactivation and reverse steps along the trajectory or confer new functions that foster an obligate lifestyle. Constituent information drawn from Moran et al. (2008), Sachs et al. (2011), Lo et al. (2016)

the erosion of capacity for free living growth, and cement the microbe into life as a facultative symbiont or further push its trajectory into a transitional stage towards obligate symbiosis (Law & Dieckmann 1998, Sachs et al. 2011). These processes lead to a reduction in the effective population size of a symbiont. Consequently the selective constraints upon a large number of genes that were important in free-living ancestors are relaxed or removed and non-adaptive mutations may accumulate, ultimately leading to a process of pseudogenization and an associated drop in coding density (Mira et al. 2001). Over time, mutational bias towards deletions (Moran 1996, Moran et al. 2008, Wernegreen 2002, 2011) removes genes not maintained by selection, leading to smaller genomes with high coding densities. See (Lo et al. 2016) for review. As the cumulative effects of evolutionary processes take their toll, symbionts may be pushed to extreme genome reduction ($<$ 1Mb), retaining only fundamental genes (e.g. information processing, ribosomal structure, amino acid transport) and those essential for maintenance of the symbiosis. At this stage, far down the "evolutionary rabbit hole" (Bennett & Moran 2015) symbionts may share greater similarities with organelles than with free-living ancestors (Bennett & Moran 2013, McCutcheon & Moran 2010, 2012).

Genes lost & gained Acquisition of novel genetic material can facilitate movement in both directions on the host-dependence continuum. By rectifying the effects of gene inactivation and allowing a reversal of steps along the trajectory (e.g. the reinstatement of a metabolic pathway that reduces dependence on host-association) or by conferring new functions that facilitate transition to an obligate lifestyle. However, further down the trajectory towards obligate intracellular life, strict vertical transmission within host lineages and intracellular tropism of symbionts can genomically and physically restrict access to novel genetic material. This often occurs though loss of the DNA recombinational repair machinery, due to relaxed selection or an adaptive response to the intracellular niche (Dale et al. 2003). Further to this, the spatial separation of symbionts can reduce access to coinfecting bacte-

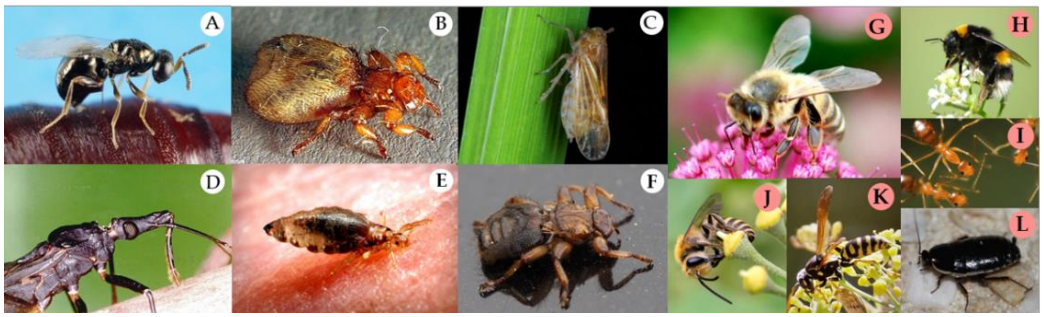

Figure 1.2. The diversity of insect taxa associated with *Arsenophonus*. A-F (white) hosts from Table 1.2 whose interactions with *Arsenophonus* spp. have been characterised phenotypically (to some degree) and examples G-L (red) of those that remain uncharacterised. *Apis mellifera* G, *Bombus terrestris* H, *Anoplolepis gracilipes* I, *Colletes hederae* J, *Polistes nimpha* K, *Loboptera decipiens* L. Photos A - F: © Peter Koomen, © Ken Gray Insect Image Collection, © Sylvia Villareal, © Marcelo de Campos Pereira. © James Gathany & Frank Collins, © Frank Vassen, © Georgia Drew, © Vera Buhl, © Stephen Belcher, Minden pictures, © Charlie Jackson, © Gail Hampshire, © Slim Guy, Wiki CC — Creative Commons 2.0

ria, limiting the innovation potential of symbionts and preventing the restoration of degraded genomes (Dale et al. 2006, Sachs et al. 2011, Toft & Andersson 2010).

1.4 The Genus *Arsenophonus*

One common group of bacterial symbionts that occupies all positions on the symbiosis continuum is *Arsenophonus*, a genus of gram-negative bacteria within the gamma-Proteobacteria). Bacteria belonging to the genus are estimated to infect 5% of arthropod species globally and have a truly vast host distribution (Duron et al. 2008). Insects carrying this symbiont include members of the Hymenoptera, Coleoptera, Diptera, Hemiptera, Blattoidea and (Figure 1.2), and it is likely that many other infected species remain to be identified (Duron et al. 2008, Nováková et al. 2009, Wilkes et al. 2011). However, the most intriguing aspect of the genus *Arsenophonus* is the diversity of symbiotic lifestyles observed across the clade, with members including highly coevolved obligate endosymbionts, reproductive parasites and facultative mutualists (Table 1.2). This diversity is further reflected by variation in transmission modes and genome size.

Strain	Host	Host Order	Host Ecology	Transmission	Phenotype	Genome	Localisation	Refs
A. nasoniae [A]	*Nasonia vitripennis*	Hymenoptera	Parasitoid	VT & HT	Facultative, Male-killing	3.5 Mb	Diffuse, invades via gut	[1]
Ca. A. melophagi [B]	*Melophagus ovinus*	Diptera	Hematophagous	VT	Obligate, Anabolic[†]	1.2 Mb	Bacteriome & extracellular in milk glands	[2]
Ca. A. nilaparvatae [C]	*Nilaparvata lugens*	Hemiptera	Phytophagous	VT	Facultative Anabolic[†]	2.9 Mb	Fat body mycetocytes	[3]
Ca. A. triatominarum [D]	*Triatoma infestans*	Hemiptera	Hematophagous	VT	Facultative, Beneficial[†]	2.2 Mb	Diffuse, cytoplasm of host cells	[4]
Ca. R. pediculicola [E]	*Pediculus humanus*	Phthiraptera	Hematophagous	VT	Obligate, B-vitamin synth.	0.57 Mb	Bacteriome	[5]
Ca. A. lipopteni [F]	*Lipoptena cervi*	Diptera	Hematophagous	VT	Obligate, B-vitamin synth.[†]	0.84 Mb	Bacteriome	[6]

[A – F] Corresponds to image in **Figure 1.2**, [VT] Vertical transmission, [HT] Horizontal transmission, [†] Hypothesised

[1] Gherna *et al.* 1991; Darby *et al.* 2010; Wilkes *et al.* 2010 [2] Chrudimský *et al.* 2012; Novakov´ *et al.* 2015
[3] Qu et al. 2013; Xue *et al.* 2014; Fan *et al.* 2016 [4] Hypša & Dale, 1997, [5] Perotti *et al.* 2007, [6] Nováková *et al.* 2016

Table 1.2. Frequently studied *Arsenophonus* strains with known or hypothesised associations with hosts

Arsenophonus is a relatively new genus taxonomically speaking, having been formerly recognised only in 1991 (Gherna et al. 1991). The type strain *Arsenophonus nasoniae* is a maternally inherited symbiont and was the first to be characterised as the causative agent of heritable heavily female biased offspring ratios observed in the parasitic wasp *Nasonia vitripennis* (Skinner 1985). Infection with *A. nasoniae* triggers the death of male embryos by inhibiting the formation of maternal centrosomes (Ferree et al. 2008) and killing $\sim$ 80% of male offspring (Skinner 1985, Werren et al. 1986). The evolutionary explanation for this phenotype is that killing male embryos reduces competition experienced by sibling female hosts within the fly pupa environment in which the *Nasonia* larvae feed. This enhances the survival of host individuals that can transmit the maternally inherited symbiont (females), above those that cannot (the males), facilitating the spread of *A. nasoniae* through host populations (Hurst & Majerus 1993, Hurst 1991, Werren 1987).

In its *N. vitripennis* host, *A. nasoniae* is maternally inherited, but is not transovarially transmitted from mother to offspring, as is common with many other heritable symbionts. Vertical transmission proceeds in an unusual manner. Female *Nasonia* parasitize fly pupal hosts and in doing so inoculate the pupa with *A. nasoniae*. *Nasonia* larvae developing in the fly host ingest *A. nasoniae* and the symbiont invades across the midgut and into the haemocoel where it persists until transmission to the next generation (Huger et al. 1985, Werren et al. 1986).

In addition to vertical transmission *A. nasoniae* also transmit infectiously (horizontal transmission) when an infected and uninfected female parasitise the same host individual (superparasitism) (Huger et al. 1985), as the offspring of the uninfected female consume the microbe transferred to the fly pupal environment by the infected female. *Arsenophonus nasoniae* almost reaches fixation in populations where superparasitism rates are high but is lost in under five generations from host populations if only solitary parasitism occurs (Parratt et al. 2016). These findings suggest that horizontal transmission is vital for the invasion and maintenance of *A. nasoniae* in host populations and that the male-killing phenotype may be of lesser

importance in infection dynamics (Parratt et al. 2016). The mixed mode transmission demands the symbiont invade and survive in two different host species. This capacity is reflected in the 3.5 Mb genome of *A. nasoniae* - large for a heritable insect endosymbiont - which contains genes encoding type III secretion systems, toxins and other machinery putatively associated with an infectious lifestyle (Darby et al. 2010, Wilkes et al. 2010). An only moderate reduction in genome size, relative to non-host dependent Enterobacteriaceae, presumably contributes to *A. nasoniae* being culturable on cell-free media (Werren et al. 1986).

Transmission both vertically and through plants is observed for *Arsenophonus* strains in Hemiptera (Cixiidae). *Arsenophonus phytopathogenicus* and *Phlomobacter fragariae* infect sugar beet and strawberry plants and are associated with varying disease phenotypes (Bressan 2014, Bressan et al. 2009, 2012). However, these phytopathogens are obligately transmitted between plants by planthopper hosts, where they colonise the salivary glands and reproductive organs of the insect and exhibit weak vertical transmission (Bressan et al. 2009). In this case, most of the seasonal lifecycle of *Arsenophonus* is associated with the insect vector, not the plant, and the symbiont persists through the entire lifespan of the insect. As a result it seems that infecting plants represents an additional route of horizontal transmission for *Arsenophonus* between insect hosts. The use of the host as a vector selects for the symbiont to reside in the insect host with minimal damage (Alizon et al. 2009, Anderson & May 1982, Ewald 1983) and potentially confer protection to the host. It also presents the symbiont with the opportunity to evolve vertical transmission as a means of maintenance. It thus presents a staging point in the evolutionary pathway between environmental acquisition and vertical transmission.

Situated at the other end of the symbiosis continuum that *Arsenophonus* spans is *Candidatus* Riesia pediculicola, an obligate mutualist of the human body louse (Perotti et al. 2007). *Ca.* R. pediculicola are hosted intracellularly within bacteriocytes of lice and synthesise required vitamins for its louse host which lacks B vitamins in its blood diet (Perotti et al. 2007, Sasaki-Fukatsu et al. 2006). With its 0.57

Mb genome, *Ca.* R. pediculicola is likely to be highly dependent on the host for metabolic functioning (Kirkness et al. 2010). In this case there is strong evidence of co-diversification between host and symbiont, something that is not observed between *A. nasoniae* and its wasp host, and Riesia lineages are typified by long branches that are indicative of the high substitution rates associated with an obligate lifestyle (Moran et al. 2008). This obligate lifestyle is additionally observed for *Ca.* Arsenophonus melophagi in the louse fly *Melophagus ovinus*; here there is evidence for an essential symbiont role in vitamin synthesis and vertical transmission via the milk glands (Chrudimský et al. 2012, Nováková et al. 2016).

In the *A. nasoniae* – *Nasonia* interaction, the bacterium retains cell free growth and has some infectious transmission. In the Riesia-louse interaction, vertical transmission is strict, there is no capacity for free-living growth of the microbe, and the host depends on the bacterium for function. Between these two scenarios lies the interaction between *A. triatominarum* and triatomine bugs. Within a species, all individuals are infected with *A. triatominarum*, which is vertically transmitted through eggs (Hypsa & Dale 1997). The symbiont cannot live in cell free culture, and thus requires the host. However, the host is viable and fertile in the absence of *Arsenophonus*, whose impact on the host remains elusive (Hypsa & Dale 1997).

The genus *Arsenophonus* is thus comprised of symbionts that show some degree of heritability and those that are host-restricted and only show vertical transmission. A high degree of diversity is observed across the clade, including diversity in ecological niche, genome size, tissue tropism and interactions with host organisms (Nováková et al. 2009). As more strains of *Arsenophonus* undergo phenotypic and genomic characterisation, the genus will become a fascinating and valuable source for studying the factors that mediate transitions in symbiotic lifestyle (Wilkes et al. 2011).

1.4.1 *Arsenophonus* & Anthophila

This thesis concerns interactions of *Arsenophonus* with Anthophila (bees). Members of the genus *Arsenophonus* have been recently identified in microbial screens of Anthophila. While they appear to represent non-core microbial taxa for these hosts, their presence has (in some cases) been associated with poor health outcomes. In 2012, metagenomic studies led to *Arsenophonus* being posited as a candidate agent of colony collapse disorder (CCD), a phenomenon describing large annual losses of *A. mellifera* colonies (Oldroyd & Fewell 2007). *Arsenophonus* related bacteria showed the highest proportional increase in CCD affected colonies compared to all other bacterial taxa (Cornman et al. 2012). *Arsenophonus* spp. had not previously been highlighted as common members of the bee microbiota, and nothing was known about the bacterium's interactions with Anthophila. Since this time, a number of studies have emerged reporting *Arsenophonus* spp. in *A. mellifera* colonies across the globe and in other Anthophila (Aizenberg-Gershtein et al. 2013, Babendreier et al. 2007, Corby-Harris et al. 2014, Gerth et al. 2015, Saeed & White 2015, Yaïez et al. 2016).

An extensive endosymbiont screen of Anthophila species in Germany uncovered new hosts for *Arsenophonus*. From 170 bee species tested, three species were observed to harbour *Arsenophonus* spp., all within the genus *Colletes*. The exclusivity of *Arsenophonus* to the *Colletes* genus in this study suggests that among Anthophila infection may sometimes be predictable by phylogeny. Similar screening efforts of solitary bee species in the US also suggest *Arsenophonus* is an uncommon endosymbiont of Anthophila. Among 29 species from across 5 families (Andrenidae, Apidae, Colletidae, Halictidae, Megachilidae) *Arsenophonus* was largely absent, aside from one *Lasioglosum pilosum* individual (N = 11), a species of sweat bee from the family Halictidae (Saeed & White 2015). Much like *Apis*, the solitary bee *Megachile rotundata* is an important species for crop pollination and is vulnerable to several of the same pathogens, including chalkbrood, a fungal disease affecting larvae. Interestingly the intestine of *M. rotundata* larvae can also harbour

Arsenophonus spp., which in one case has even been found to persist after treatment with antibiotics (McFrederick et al. 2014). Among *Apis* spp. the bacterium has been identified in *A. dorsata* (8.3%) and *A. florea* (8.3%) from Thailand, and in 24.2% of *A. mellifera* colonies in Switzerland (Yaïez et al. 2016).

Arsenophonus appears to have a global distribution in bees, yet it rarely infects all individuals in the species and populations in which it occurs, at least for the Anthophila populations studied thus far. However, as with many endosymbiont screens, several of the studies suffer from exceedingly low sampling power, such that it cannot be said with any degree of certainty that *Arsenophonus* is absent from these bee species. This problem is likely magnified by low infection frequencies often associated with *Arsenophonus* and other groups of facultative endosymbionts. While the links to colony health remain tentative it is important to characterise the basic biology of *Arsenophonus* spp. affecting Anthophila.

***Arsenophonus* associations with Anthophila health** A small number of studies have emerged explicitly linking *Arsenophonus* to poor colony health since the CCD study by Cornman et al., 2012. In the UK *Arsenophonus* occurs more frequently in unhealthy colonies and emerges as a predictor of colony size in statistical models, with infected colonies generally being smaller based on measures of the number of combs of adult bees (Budge et al. 2016). In bumblebees evidence is also mounting that *Arsenophonus* spp. may have important roles in disease dynamics. Recent work shows *Bombus terrestris* infected with the eukaryotic parasite *Apicystis bombi* have a higher relative abundance of *Arsenophonus* spp. in the fat body (Parmentier et al. 2018).

Arsenophonus has also been associated with a common ectoparasite of *Apis mellifera*, the mite *Varroa destructor*. Female *Varroa* mites retrieved from winter bee hive debris in the Czech Republic appear to harbour *Arsenophonus*, suggesting hive debris may constitute reservoirs of infection (Hubert et al. 2017). However, this inference assumes the *Arsenophonus* detected are viable, whilst the PCR assays that

conducted alone cannot establish the viability of *Arsenophonus*, nor its transmission to *A. mellifera*. Further genetic analysis is also required to assess if this is the same strain type commonly found infecting *A. mellifera*.

Later studies reported that *Arsenophonus* sometimes occurs as the predominant bacterial genus in Varroa mites, where it can occur with high relative abundance in 16S rRNA metagenomics screens (88.38 - 99.96%). However this domination of the microbiota was not a universal trend, with *Enterobacter, Pseudomonas* or *Proteus* dominating the bacteriome in other *Varroa* samples (Pakwan et al. 2018). *Arsenophonus* appears to be localised to the mite caecum (Pakwan et al. 2018) but its phenotype and effect on host fitness remains unknown. The mite has been suggested as a possible vector of *Arsenophonus* to *Apis*, but no correlation has been identified between levels of *Varroa* induced disease and *Arsenophonus* in *Apis* (Hubert et al. 2017).

Emerging questions *Arsenophonus* interaction with Anthophila clearly remain poorly understood. There are associations with poor health, observations of *Arsenophonus* in mites, but there has been little directed study, and all work has relied on PCR assay detection as means of understanding the interaction. This method does not allow differentiation between live and dead *Arsenophonus*. Further, there are no details that indicate the nature of the symbiosis – whether it is a gut symbiont or an endosymbiont, and if it is environmentally acquired or vertically transmitted.

A key emerging issue is transmission. Observations indicate that this bacterium can be associated with plant and plant-derived material. Flowers, pollen provisions of Megachilid bees and *A. mellifera* bee bread were found to screen positive for *Arsenophonus* (Donkersley et al. 2018, McFrederick et al. 2017*a*) and it has also been reported at high frequency in the corbicular pollen and crop of *A. mellifera* foragers (Corby-Harris et al. 2014). In this instance it is not clear if *Arsenophonus* is associated with pollen material, or if the signal may be a result of crop secretions used to pack pollen onto the hindleg corbiculae. This link to plant based material is im-

portant as it suggests *Arsenophonus* may be viable outside of an insect host and that environmental reservoirs may be an important source of infection to Anthophila individuals. While *Arsenophonus* spp. are largely considered heritable insect endosymbionts, perhaps this association with plant material should not be so unexpected, as several members of the genus are plant-associated pathogens that rely on insect hosts for transmission (see above) (Bressan 2014).

1.4.2 *Arsenophonus* in a social world

Despite identification of *Arsenophonus* in a number of important eusocial hosts (in addition to *Apis*), including the invasive *Anoplolepis gracilipes* (yellow crazy ant) (Sebastien et al. 2012), *Bombus terrestris* (Parmentier et al. 2018) and *Vespula* spp. (Loope et al. 2019), much of the previous work on this symbiont has focused on its interactions with solitary taxa. Symbionts in eusocial hosts are exposed to vastly different selection pressures from those in their solitary counterparts, largely due to a higher density of hosts, greater host relatedness and a homeostatic nest environment (Hughes et al. 2008, Keller & Genoud 1997, Schmid-Hempel 2017, Wilson 1971).

At one time these factors were presumed to heighten the risk of social hosts to parasite transmission and disease emergence (Freeland 1976, Hamilton 1987, Schmid-Hempel 1998), however this view is increasingly questioned (Hughes et al. 2008, Naug & Camazine 2002, Rifkin et al. 2012). For example, high host relatedness may lower infection barriers (Hughes & Boomsma 2006) but evolved mechanisms such as herd immunity and hygienic behaviour may strengthen barriers to symbiont invasion (Cremer et al. 2018, Hughes et al. 2002). It seems logical that host sociality may affect symbiont phenotypes (Onchuru et al. 2018, Rubin et al. 2017, Schmid-Hempel 2017) and in turn symbionts may affect host sociality (Biedermann & Rohlfs 2017, Lombardo 2008, Troyer 1984), yet the association between these remains unclear. With a wealth of solitary comparisons and the potential for genetic tractability (Gherna et al. 1991, Wilkes et al. 2011), interactions between

Arsenophonus and *A. mellifera* could provide valuable opportunities to explore the effect of host sociality on endosymbiont evolution.

1.5 Thesis Aims

This thesis aims to characterise interactions between *Arsenophonus* and its eusocial host (*Apis mellifera*) in an effort to improve our understanding of the ecology and evolution of this diverse clade of insect endosymbionts, and its potential impact on honey bees.

In **Chapter 2** the prevalence of *Arsenophonus* in the UK *A. mellifera* population is assessed over time and space. The genetic diversity of circulating strains is characterised and the phylogenetic position of the *Apis* strain is established within the *Arsenophonus* clade. The potential for *Arsenophonus* spill over to solitary bee populations is explored and the prevalence of another common endosymbiont (*Wolbachia*) is simultaneously examined.

In **Chapter 3** the persistence of *Arsenophonus* is explored at a colony level in the field and at an individual *A. mellifera* level within the laboratory. Localisation and detection of the bacterium in host tissues is established to assess the extent of infection within *A. mellifera*.

In **Chapter 4** efforts to characterise the transmission biology of *Arsenophonus* in *A. mellifera* are made. First, the capacity for vertical transmission is assessed by tracking the bacterium across host life history. Second, the ability to transmit horizontally is explored under differing social contexts.

The thesis ends (**Chapter 5**) with a general discussion of our current understanding of the *Arsenophonus* – *Apis* interaction in the light of thesis results. Implications for the broader understanding of *Arsenophonus* symbioses and directions for future study are also highlighted.

Chapter 2

Infection status of two common insect endosymbionts in wild & managed Anthophila

Abstract Whilst *Arsenophonus* is known from previous studies to be associated with the European honey bee, *Apis mellifera*, the interaction is uncharacterised. Further, whether the symbiont is exclusive to *A. mellifera* or found more widely in the Anthophila community is unknown. In this chapter, a phylogenomic analysis is presented that indicates the *Arsenophonus* strain from *A. mellifera* is most closely allied to strains found in parasitic wasps. A single MLST strain of Ars-Am was recorded widely across the UK with a pronounced seasonal pattern, being more common in workers collected towards August/September than those collected in May/June. *Arsenophonus* was not observed in live captured samples of solitary bees. Here, it was notable that *Arsenophonus* present in German *Colletes hederae* was distinct to that found in *A. mellifera* on MLST, but this infection was not observed in any live UK *C. hederae* specimens tested, which represents the invasive range. Thus, whilst other bees can harbour this symbiont, it is not established widely in the community in the UK currently. These data argue against both vertical trans-

mission of *Arsenophonus* in *Apis*, and against common exchange with other sympatric bee species tested. Finally, *Wolbachia* was commonly observed in UK bee species, a symbiont that was not found in *A. mellifera*.

2.1 Introduction

Upon discovery of a new host-microbe interaction, it is important to understand its occurrence and distribution within the natural environment. These data allow evaluation of the significance of the interaction, as those associations that occur rarely are less likely to merit interest in terms of host health, or as ecological or evolutionary drivers. Further to this, patterns of spatial (Chua et al. 2018, King & Lively 2009, Roe et al. 2011) and temporal variation (King et al. 2008, Poretsky et al. 2014) can provide clues as to the transmission biology and epidemiological drivers underpinning symbioses (Roe et al. 2011, Savage et al. 2011). Vertically transmitted symbionts can drive rapidly through host populations within relatively short evolutionary timescales (Himler et al. 2011, Turelli & Hoffmann 1991), but prevalence of these symbionts within a season remains relatively static across space and time. These heritable agents can reach fixation ($\sim$ 100%) in host populations, due to reproductive manipulation phenotypes and/or symbiont conferred fitness benefits driving their invasion and maintenance (Zug & Hammerstein 2018). In contrast, horizontally transmitted symbionts rarely reach such high infection frequencies in host populations (with some exceptions, e.g. Kikuchi et al. 2007), but often exhibit complex epidemic dynamics that can vary over short seasonal times scales (Lalzar et al. 2012, Lass & Ebert 2006, Yui et al. 2009).

Determining the genetic diversity of the focal symbiont is a key task when characterising host – symbiont associations. For both heritable and horizontally acquired symbionts, multiple strains may be circulating both within the host population and within host individuals (Anderson et al. 2016, Duron et al. 2008, Engel et al. 2014, Kaltenpoth 2017, Kwong et al. 2014). These strains can differ greatly in

their biology and thus potential interactions with hosts, with evidence of important variation in metabolic capacities even for closely related strains (Engel et al. 2014). While co-infections with multiple symbiont strains may be benign or even beneficial to host fitness in some scenarios (Xie et al. 2014), theory predicts heightened competition dynamics that may have detrimental impacts for host biology (Frank 1996*a*). For horizontally transmitted symbionts the scope for co-infections is generally considered greater than that of their heritable counterparts (Ebert 2013).

For many symbionts, both heritable and horizontally transmitted, spatial heterogeneity at both the species and strain level is also observed (Ahmed et al. 2015, Duron et al. 2008, Hosokawa et al. 2016, Russell, Goldman-Huertas, Moreau, Baldo, Stahlhut, Werren & Pierce 2009). Such spatial patterns can reflect local adaptation driven by selection that varies over space (e.g. local presence of a natural enemy, Oliver et al. (2005)) or local drivers of infection risk (e.g. transmission hotspots) (Paull et al. 2013). For the stinkbug, *Plautia stali*, this potential is exemplified by the extensive polymorphism of its *Pantoea* symbiont across the Japanese archipelago (Hosokawa et al. 2016). Intriguingly though, all strains appear to be functionally equivalent when rescuing *P. stali* development (Hosokawa et al. 2016), indicating strain divergence does not always equate to different outcomes. There is also evidence that changes to the spatial distribution of host populations can be reflected in their symbiont passengers, with a number of cases indicating the loss of symbionts from the invasive range of host species (Morrow et al. 2017, Nguyen et al. 2016, Rey et al. 2013).

Host - microbe interactions discovered in one host species also benefit from evaluation in an (ecological) community context (Fenton & Pedersen 2005, Holt et al. 2003, Johnson et al. 2015). For a heritable symbiont, presence of a focal strain in an additional host species is generally indicative of historical host shift events (Turelli et al. 2018). For certain symbiont lineages, including *Wolbachia* and *Arsenophonus*, host shift events occur over medium to long evolutionary times and are indicated by characteristic phylogenetic incongruences between host and sym-

biont (Moran et al. 2008). For infectiously transmitting agents, occurrence in multiple host taxa may be indicative of a symbiont that is utilizing multiple host species in the community (Gandon 2004, Holt et al. 2003, Webster et al. 2017). Presence in multiple host species may also highlight the capacity for spillover from the major host, in which symbiont dynamics are largely driven and confined. For *A. mellifera* parasites, suspected spillover events to other pollinator species are increasingly documented, a scenario that is probably confounded by the extensive distribution and polylectic status of *A. mellifera* (Graystock et al. 2014, 2016, Manley et al. 2015, Wilfert et al. 2016). Both cases (multi-host and spillover) can greatly complicate the epidemiology and life-cycles of symbionts, and have implications for their long term persistence in host populations (Gandon 2004, Webster et al. 2017). Thus, ascertaining these properties are vital for understanding evolutionary dynamics, as they determine whether the symbiont is coevolving with a single host species, or is circulating and evolving as a generalist in multiple host species (Rabajante et al. 2015).

Chapter Objectives *Arsenophonus* has been sporadically reported in *A. mellifera* across the globe (Aizenberg-Gershtein et al. 2013, Cornman et al. 2012, Yaïez et al. 2016). However, basic information is largely lacking on the prevalence, dynamics and diversity of *Arsenophonus* within *A. mellifera* populations and the wider Anthophila community. In this chapter spatial and temporal patterns are identified in *Arsenophonus* prevalence and the diversity of circulating strains is characterised. Solitary Anthophila, including one species in its invasive range, are screened for *Arsenophonus* and *Wolbachia* to establish a basic overview of symbiont association in the Anthophila community.

2.2 Methods

2.2.1 Phylogenomic position of *Arsenophonus* from *A. mellifera*

An Illumina paired end shotgun library, provided by Peter Neumann & Orlando Yanez (Institute of Bee Health, University of Bern), from *Arsenophonus*-infected *A. mellifera* (collected in Switzerland) was assembled and 53 core ribosomal protein coding genes extracted. The position of the *A. mellifera* - *Arsenophonus* was estimated compared to other *Arsenophonus* completed and draft genomes (Table 2.1). To this end, Bayesian inference was completed using Phylobayes (Lartillot et al. 2009) with two independent chains run for over 30,000 cycles each. Maximum likelihood (ML) analysis was completed using IQTREE (Hoang et al. 2018, Kalyaanamoorthy et al. 2017, Nguyen et al. 2015). The best fit model was determined automatically, and support values were based on 1,000 ultrafast bootstraps.

2.2.2 Screening of *Apis mellifera* colonies

Adult *A. mellifera* workers were collected directly from the outer frames of colonies in 10 counties across England. *Apis mellifera* were preserved in sterile tubes in 70 - 100% EtOH at -20°C until DNA extraction. For each colony, the posterior legs were pulled from *A. mellifera* workers (n = 12) using sterile forceps and pooled in groups of 4 for DNA extraction. Legs were exposed to ultra violet (UV) light for 10 minutes prior to molecular work to cross link DNA from surface microbes and render these refractory to PCR amplification. Samples were homogenised using a motorised micropestle and DNA was extracted using 180µl of 5% w/v Chelex solution and proteinase K incubated for 3 to 5 hours at 55°C and boiled for 10 minutes (Walsh et al. 1991). Samples were centrifuged at 14,800 rpm for 2 minutes and the supernatant retained and stored at -80°C until use. The presence of *Arsenophonus* spp. were detected by PCR assays using primers targeting fbaA (fructose-bisphosphate aldolase class II) adapted from (Duron et al. 2010), with extraction quality established by amplification of host DNA targeting *A. mellifera* EF1-α (elongation factor-

Bacterial strain	Host	Accession no.	Attribution
Ca. A. nilaparvatae	*Nilaparvata lugens*	JRLH01000001.1	Xue et al. 2014
Arsenophonus sp. ENCA	*Entylia carinata*	NHNG01000699.1	Mao et al. 2017
Ca. A. triatominarum	*Triatoma infestans*	LWMI01000001.1	A.C. Darby[1]
Arsenophonus sp. ARAF	*Aleurodicus floccissimus*	OUND01000011.1	Santos – Garcia et al., 2018
Arsenophonus sp. of *A. mellifera*	*Apis mellifera*	Unpublished	P. Neumann[2] & O. Yanez[2]
A. nasonaie (Pv)	*Pachycrepoideus vindemiae*	Unpublished	S. Parratt[1]
A. nasonaie (DSM 15247)	*Nasonia vitripennis*	AUCC01000001.1	Darby et al. 2010
A. nasonaie (ArsUK)	*Nasonia vitripennis*	Unpublished	S. Siozios[1] & G. Hurst[1]
A. nasonaie (ArsPerl)	*Nasonia vitripennis*	Unpublished	S. Siozios[1] & G. Hurst[1]
A. nasonaie (ArsLausanne)	*Nasonia vitripennis*	Unpublished	S. Siozios[1] & G. Hurst[1]
A. nasonaie (ArsFin)	*Nasonia vitripennis*	Unpublished	S. Siozios[1] & G. Hurst[1]
Providencia stuartii	Free-living	NC_017731.1	Clifford et al., 2012
Proteus mirabilis	Free-living	NC_010554.1	Pearson et al., 2008

[1] Institute of Integrative Biology, University of Liverpool (pers comm.)

[2] Institute of Bee Health, University of Bern (pers comm.)

Table 2.1. Bacterial strains used in phylogenomic analysis

1 alpha) (Table A1). PCR reaction conditions are described in section 2.2.4. Samples were determined positive only once Sanger sequencing of fbaA and BLASTn search confirmed allocation to the genus *Arsenophonus*. Samples were determined as negative only if they passed QC and were subsequently negative for *Arsenophonus* in PCR assays.

2.2.3 Screening of solitary bee taxa

Species Collection To explore endosymbiont prevalence in the wider Anthophila community, specimens (N = 339) of 11 species from the genera *Colletes, Andrena* and *Lasioglossum* were collected by collaborating entomologists or the authors in the UK between 2016 and 2017. The project was advertised via the BWARS (The Bees, Wasps and Ants Recording Society) and social media platforms to access the help of entomologists and collectors. A significant proportion of the collecting screening effort was completed by Lauren Noblet. An additional set of DNA aliquots from *Colletes hederae* collected in 2010 at sites in France, Greece and the UK were provided by Simon Dellicour (University of Leuven) and Nicolas Vereecken (Universit Libre de Bruxelles). These additional samples stemmed from a published study on the rapid range expansion of *C. hederae* and its effect on genetic diversity (Dellicour et al. 2014). Solitary bees were collected by hand, malaise or pan traps and specimens were stored in 70 - 100% EtOH at -20°C until DNA extraction. All specimens were alive on trapping with the exception of 15 *C. hederae* collected in Bath, UK.

DNA extraction from solitary bee taxa Specimens were halved along the anterior - posterior axis and DNA extracted using a Promega Wizard® genomic DNA purification kit with a protocol modified for insects. To this end, samples were homogenised in 250µl of nuclei lysis solution and incubated at 65°C for 30min. After incubation 80µl of protein precipitation solution was added and samples were vortexed and held on ice for 5 minutes. Samples were centrifuged at 14,725 rpm for 4 min and the supernatant removed and added to 150µl filtered isopropanol.

Samples were mixed via inversion and centrifuged at 14,725 rpm for 2 min, supernatants were discarded and 150µl of 70% EtOH added to pellets, before gentle vortexing and centrifugation at 14,800rpm for 1 minute. Supernatants were discarded and pellets airdried at 65°C for 30 - 45 min before resuspension in 60µl of molecular grade H_20 at 4°C overnight. For the additional European samples, provided by collaborators, half a thorax per specimen was extracted using a Qiagen DNeasy blood & tissue kit, as in (Dellicour et al. 2014).

2.2.4 Diagnostic PCR & sequencing conditions

Polymerase chain reaction assays (PCR) were based on a total volume of 15µl, containing 7.5µl GoTaq® Hot Start Green Master Mix 5.5µl nuclease free water, 0.5µl of 10µM primer (forward and reverse), 1µl template DNA. PCR amplifications were performed on Applied Biosystem® Veriti cycler under the following conditions: 95°C for 2 min, 35 cycles at 94°C for 30 s, 55°C to 58°C for 30 s (variable, depending on primer Tm, see Table A1), 72°C for 1 min with a final extension of 72°C for 10 min. Amplicons were visualised on 1.5% w/v agarose gel with 0.5µg/mL ethidium bromide, at 110 volts for 50 minutes. Every run included a positive, negative and no template control. Where samples were to be sequenced, PCR products were cleaned of unincorporated primers and nucleotides using 0.2µl of SAP (shrimp alkaline phosphatase), 0.05µl of Exonuclease I, 0.7µl of 10X RX Buffer and 1.05µl of molecular grade H_20 and incubated at 37°C for 45 min, followed by 80°C for 15 min. Purified products were Sanger sequenced through both strands in house on an ABI® Prism 3010x or outsourced to GATC Biotech.

***Arsenophonus* PCR assay sensitivity** Sensitivity of the detection method for *Arsenophonus* was assessed by serial dilution of *Arsenophonus* template with uninfected *A. mellifera* template DNA. In all tested cases (N = 6) *Arsenophonus* DNA remained detectable at 10^{-1} and 10^{-2} dilutions, in 4/6 cases *Arsenophonus* was detectable at 10^{-3} and 10^{-4} dilutions of *A. mellifera* template.

2.2.5 MLST typing & analysis

A multi locus sequence typing (MLST) scheme was implemented to assess the diversity of *Arsenophonus* strains in *A. mellifera* colonies. Sequences from five housekeeping (HK) genes were used to characterise each isolate of *Arsenophonus*. Three loci previously targeted in *A. nasoniae* studies were used, plus a further two HK genes chosen within this study. Primers amplified 400 - 800 bp fragments from *Arsenophonus* spp. genes encoding: fructose-bisphosphate aldolase class II (fbaA), outer membrane protein assembly factor (yaeT), DNA translocase cell division protein (ftsK), RNA polymerase subunit (RpoB) and translation initiation factor (infB). Two sets of primers were also designed to target genes that are not present in the *A. nasoniae* genome assembly, a surfactin synthase subunit (srfAA) and a type IV secretion system protein (VirB4-1) (primer details are shown in Table A1) . For each isolate MLST targets were sequenced as described in section 2.2.4, sequences were trimmed and aligned in MEGA 6.0 (Tamura et al. 2013) or Geneious (Biomatters Ltd.). Nucleotide base differences between each isolate were calculated and included in a distance matrix (Table 2.7)

2.2.6 Symbiont prevalence: statistical analyses

Infection Status of *A. mellifera* Colonies — GLMM The final data set for *Arsenophonus* prevalence across *A. mellifera* colonies included variation in sampling across space and time, pseudoreplication and nonnormally distributed presence/absence data. To account for these factors (Bolker et al. 2009, Paterson & Lello 2003) and examine the effect of seasonality and space, statistical analysis was completed using a generalized linear mixed model (GLMM) polynomial regression of day of the year, with a binomial error distribution and logit link function. GLMM parameter estimation was completed by maximum likelihood Laplace approximation (Raudenbush et al. 2000) and all models were constructed using the `lme4` package (Bates et al. 2015) in R version 3.3.1 (R Core Team 2013). During model selection

Arsenophonus status (0 = uninfected, 1 = infected) was used as a binary response variable and day of the season as an explanatory fixed effect. The variable of day of the season (0 – 232) was obtained by pooling all colony sampling dates and transforming them into day number of the season adjusted for the earliest (March 20th = 0) and latest (November 8th = 232) sampling date. The random effects tested were year + colony ID, and apiary and county nested. Due to power limitations colony ID and apiary could only be treated as random effects. Model selection using Akaikes information criterion (AIC) (Akaike 1974, Burnham & Anderson 2003) was implemented to identify the minimal model that explained the variation in *Arsenophonus* infection status (see Figure 2.2). The minimal degree polynomial curve, that both fitted the observed proportions and displayed a relatively low AIC (Akaike 1974, Burnham & Anderson 2003) was selected. In addition to AIC scores, likelihood ratio tests (LRT) were performed to compare models of greater complexity with nested (null) models and further inform selection (Lewis et al. 2011, Zuur et al. 2006). If the model of greater complexity, compared to the null model, was associated with better performance ($p < 0.05$) then the nested model was rejected. If this was not significant, then the nested model was taken forward (conditional upon AIC scores). Overdispersion in the models was assessed using the `Blemco` package in R version 3.3.1 (R Core Team 2013) and deemed acceptable if < 1.4.

Symbiont Prevalence among Solitary Bees — GLM The mean prevalence of *Arsenophonus* and *Wolbachia* was calculated for each Anthophila species and results were plotted in R version 3.3.1 (R Core Team 2013). Due to the exceedingly low prevalence of *Arsenophonus*, further statistical analysis focused on only *Wolbachia* prevalence in solitary bee species. An issue of separation (monotone likelihood) (Albert & Anderson 1984, Heinze & Schemper 2002) in the data set required modelling of *Wolbachia* prevalence using a generalized linear model (GLM) with bias reduction (`brglm` package, CRAN repository). Binomial error distributions were assumed.

The minimum adequate model (MAM) was selected by pairwise model comparisons using AIC values (Akaike 1974, Burnham & Anderson 2003). Models were assessed for overdispersion and fit was checked using a Hosmer-Lemeshow Goodness of Fit (GOF) test. *Wolbachia* status (0 = uninfected, 1 = infected) was used as a binary response variable, *Arsenophonus* status, species and sex were tested as explanatory variables. Species that did not meet a threshold of > 4 observations were excluded from the model, these were: *Andrena barbilabris*, *A. nigroaenaea*, *A. clarkella*, *Lasioglossum villosulum* and *Colletes fodiens*.

2.3 Results

2.3.1 Phylogenomic position of *Arsenophonus* – *Apis*

To establish the position of the *Arsenophonus* strain associated with *A. mellifera*, phylogenomic comparisons were made using genomes from available *Arsenophonus* strains. Within the *Arsenophonus* clade the *A. mellifera* strain was established as a sister strain to those infecting parasitoid wasps with strong support (Figure 2.1).

2.3.2 Geographic prevalence of *Arsenophonus*

To map the scale of *Arsenophonus* infection, managed *A. mellifera* colonies, i.e. non-wild populations, were screened for the presence of the bacterium. The total infection prevalence of *Arsenophonus* among *A. mellifera* colonies in England was 38.86% (95% CI: 32.5-45.6%), based on 229 observations from 159 colonies. *Arsenophonus* was widespread geographically and was identified in all 10 of the county regions sampled across England. An overview of results by region are presented in Figure 2.2.

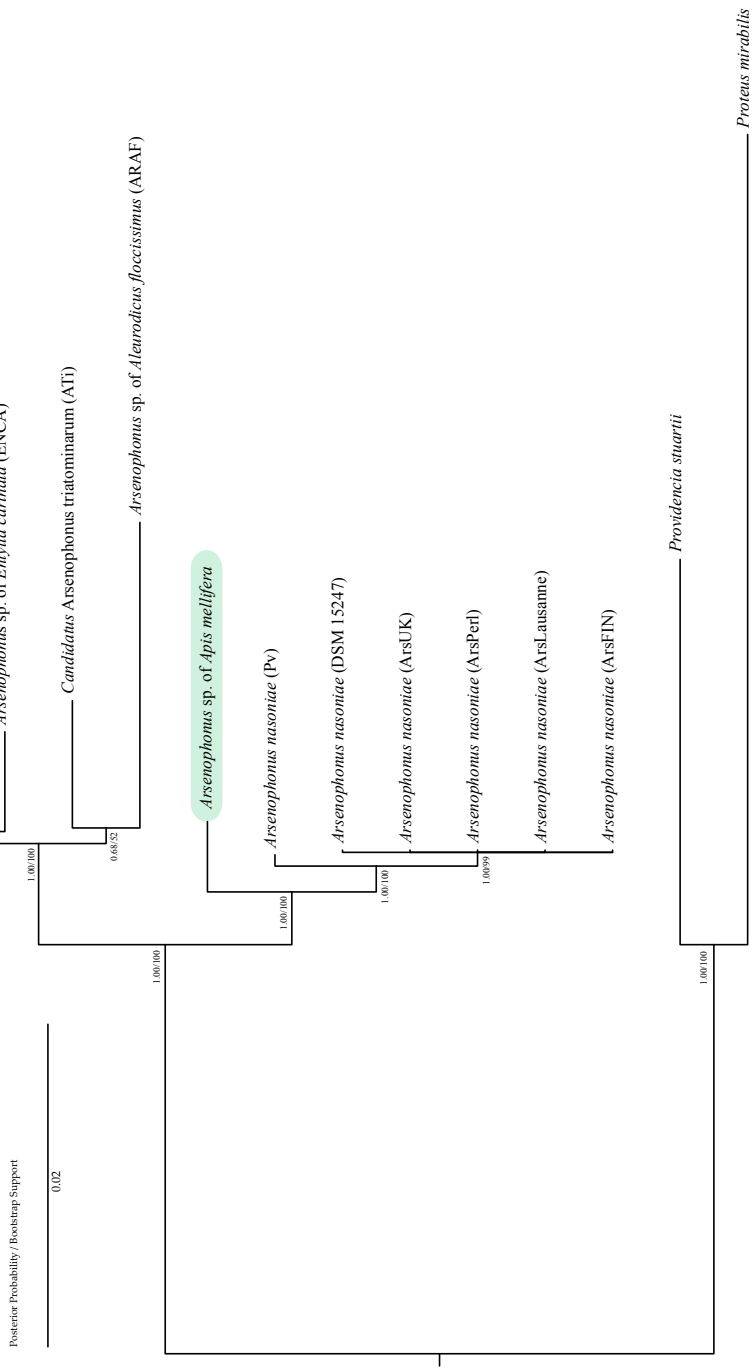

Figure 2.1. Phylogenomic position of *Arsenophonus* associated with *A. mellifera*. The analysis was completed using 53 ribosomal proteins (7067 amino acids) extracted from existing *Arsenophonus* genomes (see Table 2.1). Bacterial species names are given, if recognised, or else the associated host species is denoted. Support for both Bayesian (posterior probabilities) and Maximum likelihood (bootstrap support) inference is shown at nodes.

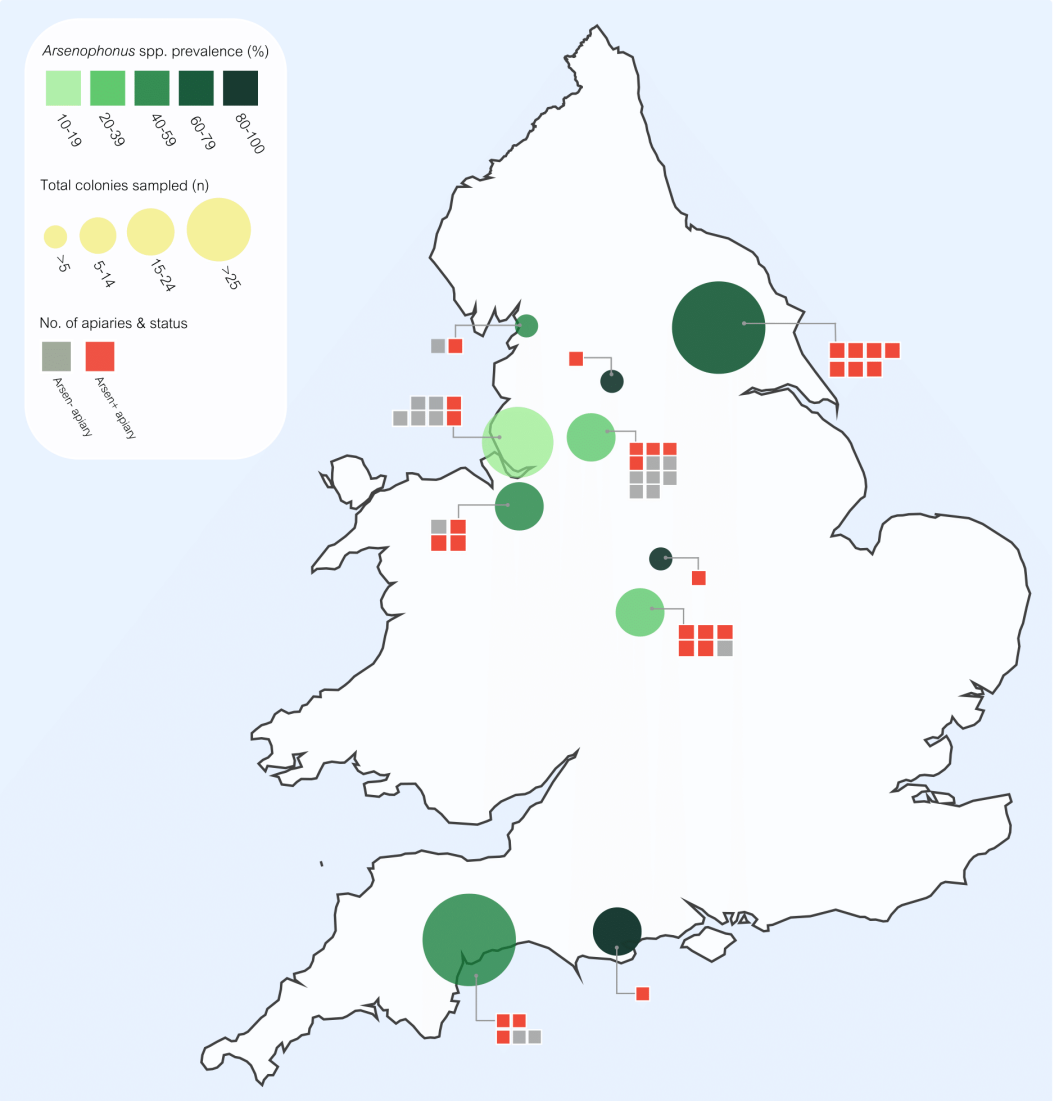

Figure 2.2. Prevalence of *Arsenophonus* associated with *Apis mellifera* colonies across England. Circle size represents the total number of colonies sampled and green shades reflect the *Arsenophonus* prevalence of colonies within the circled geographic area (county). Outlying grey/red boxes show the number of apiaries (N = 45) from which colonies (N = 159) stemmed and their *Arsenophonus* status. A grey box indicates an apiary where 0 colonies tested positive for *Arsenophonus*, red indicates an apiary where *Arsenophonus* was detected in 1 colony. Note 17 apiaries contain no infected colonies. Sampling occurred from 2014 – 2018 and *Arsenophonus* detection was based on standard PCR assay.

2.3.3 *Arsenophonus* prevalence across space & time

Arsenophonus* Space & Time — GLMM Random Effect Results** Neither county nor colony ID explained variation in *Arsenophonus* infection status with day of the season (county; variance = 0.00, colony ID; variance = 0.00). Inclusion of county (Model A1) did not alter the performance of the model (Likelihood ratio test (LRT), $X^2 = 0$, $df = 1$, $p = 1$), when compared to the nested model (A2) omitting county (model structure otherwise identical). Subsequent removal of colony ID from the model also did not have a significant effect (LRT: $X^2 = 0$, $df = 1$, $p = 0.999$). Apiary in contrast explained the greatest variation in *Arsenophonus* infection status (apiary; variance = 1.155, Std.Dev. = 1.075). The model (A3) including apiary as a random effect was significantly different (LRT: $X^2 = 13.81$, $df = 1$, $p = 0.002$) when compared to the nested model omitting apiary (A5). This indicated apiary was an important covariate for performance and fitting of the model. Year also accounted for variation in *Arsenophonus* prevalence (year; variance = 0.494, Std.Dev. = 0.703). Removal of year (A4) as a random effect (leaving only apiary) also had a significant effect (LRT: $X^2 = 3.871$, $df = 1$, $p = 0.049$*). However, this effect was less significant ($p < 0.05$) than the effect of apiary ($p < 0.001$). The model with the best AIC score included only year and apiary as random effects (see Table 2.2 for summary of all models). Based on these inferences, year and apiary are highlighted as the most important predictors of *Arsenophonus* prevalence across the season. In contrast, county and colony ID explained almost none of the observed variation in *Arsenophonus* prevalence.

Arsenophonus* Space & Time — GLMM Fixed Effect Results** For the fixed effect component of the space & time model, third-order (cubic) polynomial curves reflected the best fit for the observed data and had a low AIC score. The cubic polynomial fitted significantly better compared to a linear (B2) (LRT: $X^2 = 15.0$, $df = 2$, $p = 0.000554$) or quadratic (B3) polynomial (LRT: $X^2 = 4.51$, $df = 1$, $p = 0.0337$*). Curve fitting with a quartic polynomial (B5) marginally improved the AIC score

ID	Model[GLMM]	*df*	AIC
	Random Effect Structure		
A1	Year + County/Apiary/Colony.ID	8	256.7
A2	Year + Apiary/Colony.ID	7	254.7
A3	Year + Apiary [SM]	6	252.7
A4	Apiary	5	254.6
A5	Year	5	264.5
	Fixed Effect Structure		
B1	*Arsenophonus* status ~ 1	3	290.4
B2	*Arsenophonus* status ~ Day of season[1]	4	263.7
B3	*Arsenophonus* status ~ Day of season[2]	5	255.2
B4	*Arsenophonus* status ~ Day of season[3] [SM]	6	252.7
B5	*Arsenophonus* status ~ Day of season[4]	7	251.4

[ID] in-text reference [df] degrees of freedom, [AIC]Akaike's information criterion, [SM]selected model

Table 2.2. Model selection for spatial & temporal effects on *Arsenophonus* infection status

Fixed Effect	Estimate	Std. Error	Z- value	Pr (>|z|)
Intercept	-0.7423	0.4502	-1.649	0.099
Day of season^1	17.3738	4.5135	3.849	0.0001 ***
Day of season^2	-12.2590	4.1618	-2.946	0.003 **
Day of season^3	-7.7367	3.4953	-2.213	0.026 *

***P < 0.001 ,**0.01, *0.05

Table 2.3. GLMM (polynomial regression) parameter estimates for fixed effects (B4)

Random Effect	Variance	Std. Deviation
Apiary	1.155	1.075
Year	0.4944	0.7031

Table 2.4. Table 2.5 — GLMM estimates for random effects (A3)

but the difference was not significant (LRT: $X^2 = 3.27$, $df = 1$, $p = 0.071$). Day of the season was shown to be an important predictor of *Arsenophonus* prevalence, with model B4 performing significantly better than the model (B1) omitting day of the season as a fixed effect (LRT: $X^2 = 43.70$, $df = 3$, $p = 1.74 \times 10^{-9}$ ***).

***Arsenophonus* Space & Time — GLMM Final Predictions** The final model was comprised of: response variable = *Arsenophonus* status; fixed effect = 3rd-order polynomial of day of the year; random effects = year and apiary (random intercept) (AIC=252.7, *df*=6). Estimates for the fixed effects (Table 2.3) and random effects (Table 2.4) are shown. Predictions were generated for the overall temporal (March - November) trends in the prevalence of *Arsenophonus*, in addition to trends for each apiary (Figure 2.3) and each year (Figure 2.4).

Predictions from the final model highlight the seasonal dynamics in *Arsenophonus* infections, with prevalence of the bacterium changing in a non-linear fashion with respect to day of the season. *Arsenophonus* prevalence is lowest during the spring

Figure 2.3. Predictions of *Arsenophonus* prevalence by month and apiary. The overall model predictions for *Arsenophonus* prevalence across the seasons is shown by the solid black line, modelled using 229 binomial observations of *Arsenophonus* status (uninfected = 0, infected =1) from 159 colonies. The individual predictions for *Arsenophonus* prevalence by apiary (N = 45) are shown (grey lines). Orange circles represent the mean prevalence of *Arsenophonus* in each month, located at the mean of the sampling dates for that month (hence asymmetry). Circles are proportional to square root of the no. colony observations for the respective month and error bars represent binomial CI.

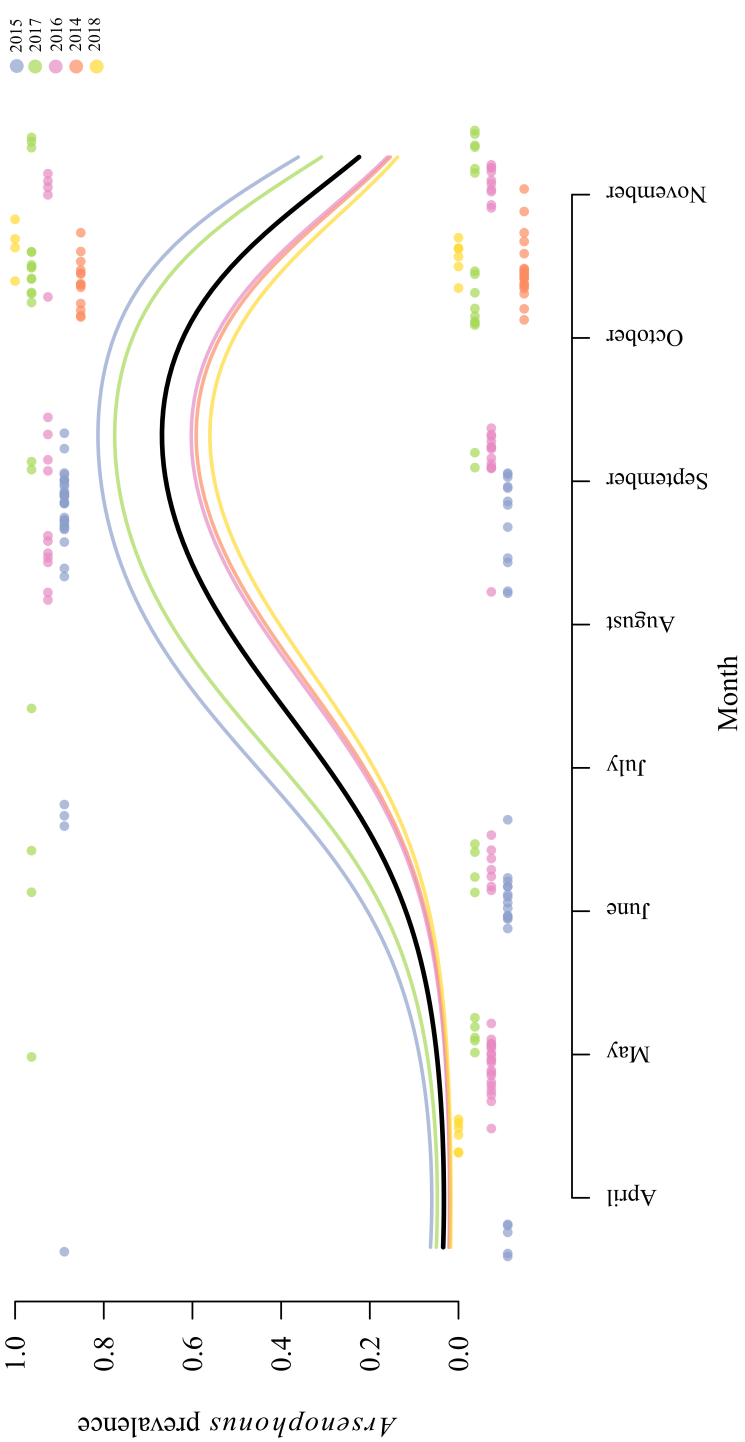

Figure 2.4. Seasonal predictions of *Arsenophonus* from 2014 to 2018. GLMM predictions of the overall seasonal dynamics in *Arsenophonus* prevalence, based on all colonies/all years (solid black line). The individual predictions for each year (N = 5) are indicated by different colours. Jittered data points (circles) represent colony observations by year, coloured accordingly. Data points in the top zone of the figure indicate infected colonies (1), points in the bottom zone indicate uninfected colonies (0).

(March, April & May) with ∼5% of colonies predicted to be infected. Prevalence rises during the early summer (June & July) before reaching peak prevalence (∼65% of colonies infected) at the transition from summer to early autumn (late August, early September). The prevalence of *Arsenophonus* drops with the onset of October and continues to do so into November at which ∼23% of colonies are predicted to be infected.

2.3.4 Symbiont prevalence in solitary bee taxa

***Arsenophonus* spp. & Solitary Bees** *Arsenophonus* spp. were absent from all 11 species of solitary bee tested (Figure 2.5), with the exception of *Colletes hederae*; the ivy mining bee. *Arsenophonus* has a low prevalence in the populations of *C. hederae* sampled, circulating at 3.76% (95% CI: 1.53 – 7.6, N = 186). However, this total included specimens (n = 4) previously identified as *Arsenophonus* positive from a population in Germany (rescreened with permission) (Gerth et al. 2015). Considering just UK populations of *C. hederae*, sampled in 2016/17, *Arsenophonus* is circulating at 1.90% (95% CI: 0.393 – 5.45, n = 158). The infected individuals (n = 3) were all male *C. hederae* collected in Bath, Somerset, and were dead at the time of collection.

***Wolbachia* spp. & Solitary Bees** *Wolbachia* spp. were common among the 11 species of solitary bee (Figure 2.5). From the genera *Andrena*, *Wolbachia* was identified in *A. clarkella* (100%, n = 3), *A. flavipes* (28.6%, n = 7) and *A. nigroaenaea* (50.0%, n = 2). From the genera *Colletes*, *Wolbachia* was identified in populations of *Colletes succinctus* (100%, n = 14), *C. cunicularius* (81.3%, n = 32), *C. daviesanus* (100%, n = 7) and *C. similis* (82.8%, n = 29). Across *C. hederae* populations *Wolbachia* was circulating at an overall prevalence of 72.6% (95% CI: 65.6 – 78.9, n = 186, females: 83.6%, males: 67.9%). This included *C. hederae* from populations across Europe, sampled in 2010. If we look exclusively within the UK populations of *C. hederae* (males & females), sampled in 2016/17, we see *Wolbachia* is circulating at a higher prevalence of 90.4% (95% CI: 84.4 – 94.7%). Among males exclusively, prevalence of the bac-

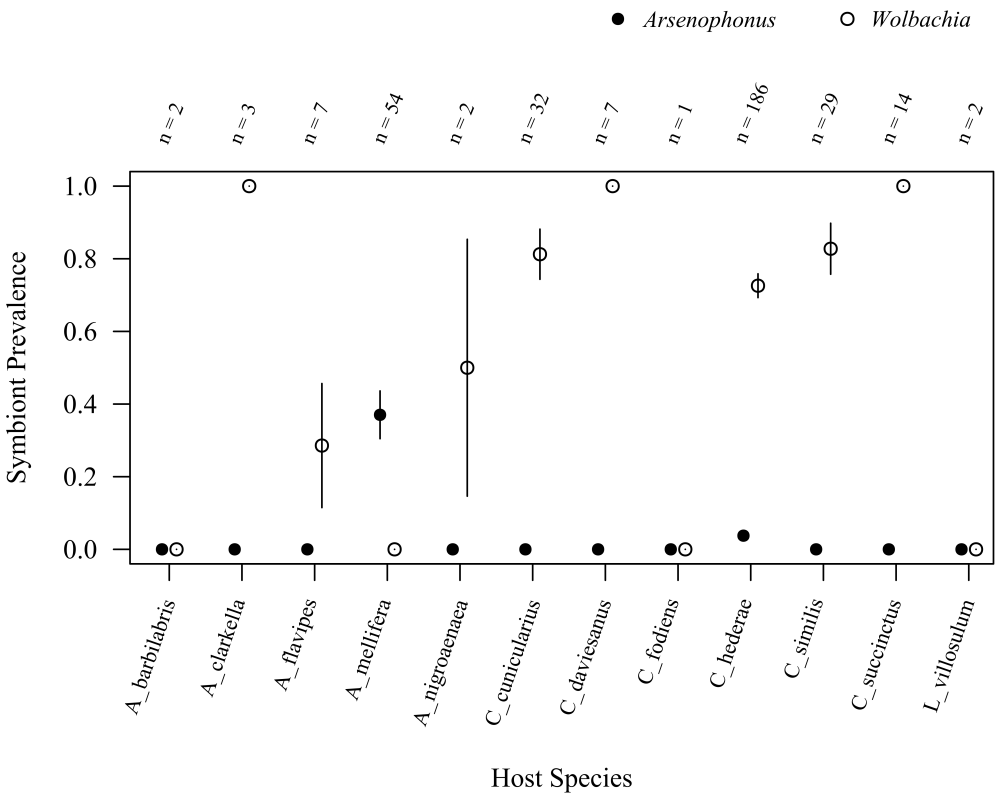

Figure 2.5. The prevalence of two common endosymbionts in surveyed solitary bee species and eusocial *A. mellifera*. The proportion of individuals infected with *Arsenophonus* spp. (black) and *Wolbachia* spp. (white) is shown with binomial CI. The number of individuals screened for each species is denoted on top axis, note the low number of observations for several species.

terium is 86.4% (95% CI: 78.2 – 92.4). Particularly notable though is the observation that *Wolbachia* is circulating at fixation in female *C. hederae* (100%, 95% CI: 91.8 – 100, n = 43) stemming from across 10 regions of the UK. *Wolbachia* was identified as coinfecting with *Arsenophonus* in the 3 male *C. hederae* from Bath, Somerset. *Wolbachia* was absent from solitary *Andrena barbilabris* (n = 2), *Lasioglossum villosulum* (n = 2), *Colletes fodiens* (n = 1), however for these species sample sizes are too small to be allow us to conclude infection is absent. *Wolbachia* was also not identified in the reference species, eusocial *Apis mellifera* (n = 54).

GLM Predictions: Symbionts & Solitary Bees An overview of the best explanatory variables for *Wolbachia* prevalence in solitary bees is shown in Table 2.5. As expected, *Arsenophonus* status was not a significant predictor of *Wolbachia* prevalence in solitary bees (LRT: $p = 0.660$). Sex, however, was a significant predictor of *Wolbachia* infection (LRT: $p = 0.022$*), with males associated with a lower prevalence of *Wolbachia* (Sol2, $p = 0.0312$*). Overall species and sex were identified as the best predictors of *Wolbachia* prevalence among solitary bees (Sol2, $df = 8$, AIC $= 316.6$). Coefficients of the final model are shown in Table 2.6 and *Wolbachia* predictions by species and sex are shown in Figure 2.6. The prevalence of *Wolbachia* was significantly higher for all *Colletes* species (intercept: *A. flavipes*, $p = 0.336$; *C. succinctus*, $p = 0.00525$**; *C. cunicularius*, $p = 0.00228$**; *C. daviesanus*, $p = 0.0230$*; *C. similis*, $p = 0.00398$**; *C. hederae*, $p = 0.00623$**) but significantly lower in eusocial *A. mellifera* ($p = 0.0163$*). The final GLM fitted the observed data well ($p > 0.05$) Hosmer & Lemeshow GOF test ($X^2 = 1.0798$, $p = 0.998$).

2.3.5 Genetic diversity of *Arsenophonus* strains circulating in Anthophila spp.

A single sequence type (MLST) of *Arsenophonus* was observed within each major host population (*A. mellifera* – UK, N=86 ; *A. mellifera* – Switzerland, N=1; *C. hederae* – UK, N=3; *C. hederae* – Germany, N=4). Whilst the strains identified in the differ-

ID	Model [GLM]	*df*	AIC
Sol1	*Wolbachi*a status ~ Species + Sex + *Arsenophonus*	9	299.9
Sol2	*Wolbachi*a status ~ Species + Sex [SM]	8	298.1
Sol3	*Wolbachi*a status ~ Species	7	301.3
Sol4	*Wolbachi*a status ~ 1	1	434.8

[ID] in-text reference [df] degrees of freedom, [AIC] Akaike's information criterion, [SM]selected model

Table 2.5. Model (MAM) selection: *Wolbachia* prevalence in Anthophila hosts

Variable	Estimate	Std. Error	Z- value	Pr (>|z|)
Intercept	-0.6952	0.8211	-0.847	0.39719
C. cunicularius	2.7583	0.9748	2.830	0.00466 **
C. daviesanus	3.8981	1.7841	2.185	0.02889 *
C. hederae	2.2175	0.8698	2.549	0.01079 *
C. similis	2.5934	0.9684	2.678	0.00741 **
C. succinctus	4.3929	1.7071	2.573	0.01007 *
A. mellifera	-3.9962	1.6523	-2.419	0.01558 *
Sex (Male)	-0.7569	0.3514	-2.154	0.03125 *

***P < 0.001 ,**0.01, *0.05

Table 2.6. GLM (Sol2) coefficients: *Wolbachia* prevalence in Anthophila hosts

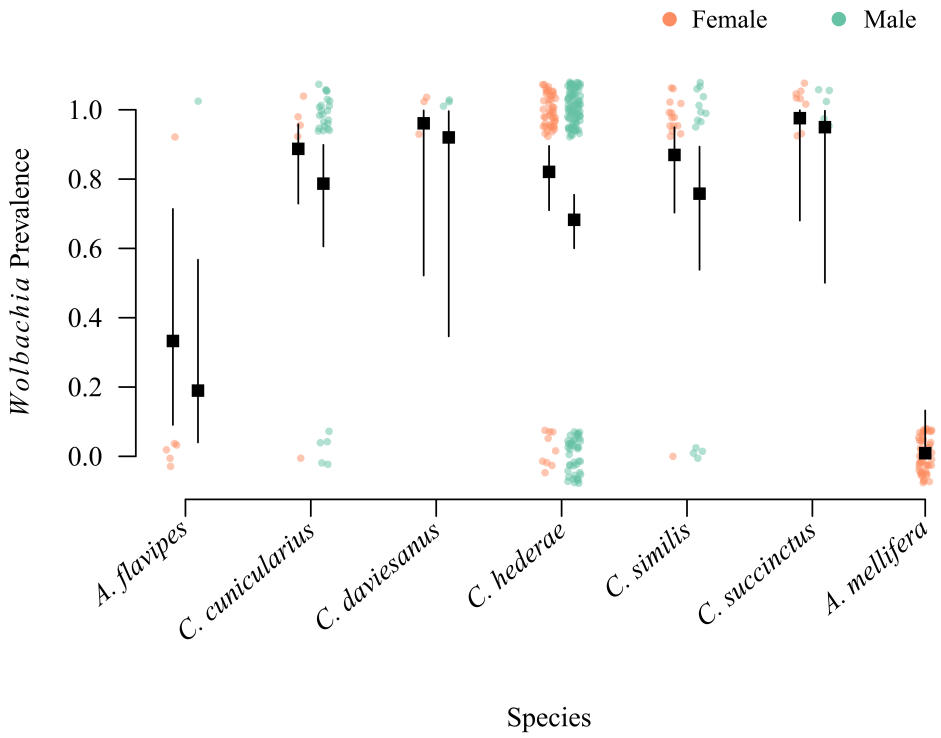

Figure 2.6. *Wolbachia* prevalence in Anthophila partitioned by host species and sex. Plotted square points represent predictions for the proportion of individuals infected with *Wolbachia* , modelled by a binomial GLM with bias reduction. The original data points are jittered (uninfected = 0, infected = 1) and coloured by sex (female = orange, male = green). No males were surveyed for *A. mellifera*. Error bars represent 95% CI. The final GLM fitted the observed data well ($p > 0.05$) Hosmer & Lemeshow GOF test ($X^2 = 1.0798$, $p = 0.998$).

ent species/populations are clearly closely related, a small degree of amongst-host species strain heterogeneity was observed across host species/countries (see Table 2.7).

With the exception of RpoB, nucleotide differences between the isolates were absent across all HK genes. For the non-HK targets (srfAA and VirB4-1) strain differences were observed more frequently and in greater number. Based on this screening, *Arsenophonus* isolated from UK *A. mellifera* were the same sequence type as that found in UK *C. hederae*. *Arsenophonus* from Swiss *A. mellifera* showed a single SNP in two of the seven markers, and that of *C. hederae* from Germany were distinct at three loci (total 14 SNP differences).

2.4 Discussion

2.4.1 Phylogenomic position of *Arsenophonus* & diversity of circulating strains in Anthophila

The *Arsenophonus* strain isolated from *A. mellifera* was established as a sister strain to *Arsenophonus Nasoniae* (Figure 2.1). This proximity is interesting, as the biology of *A. nasoniae* differs notably from the rest of the clade, which is largely heritable and intracellular. *Arsenophonus Nasoniae* strains associate with parasitoid wasps and are vertically transmitted when a female oviposits into a Diptera pupa (Huger et al. 1985, Werren et al. 1986). However, in addition to this unusual route of vertical transmission, *A. nasoniae* transmits infectiously between conspecifics and other Pteromalidae wasps that co-parasitise fly pupae (Duron et al. 2010, Parratt et al. 2016). This adaptability of this symbiont is remarkable, as it readily survives in two phylogenetically disparate hosts (the fly and the wasp), transmits easily between unrelated wasp hosts and survives as a gut symbiont for part of its lifecycle (Huger et al. 1985). Given its phylogenetic proximity to *A. nasoniae*, might the *A. mellifera* strain have an equally complex lifecycle with the potential to survive in multiple hosts?

	A. mellifera (Switzerland[1])							*C. hederae** (UK)							*C. hederae* (Germany[2])						
	fbaA	*ftsK*	*infB*	*RpoB*	*srfAA*	*virB4*	*yaeT*	*fbaA*	*ftsK*	*infB*	*RpoB*	*srfAA*	*virB4*	*yaeT*	*fbaA*	*ftsK*	*infB*	*RpoB*	*srfAA*	*virB4*	*yaeT*
A. mellifera (UK)	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	2	2	10	0
A. mellifera (Switzerland[1])								0	0	0	1	1	0	0	0	0	0	1	1	10	0
*C. hederae** (UK)															0	0	0	2	2	10	0

fbaA - fructose-bisphosphate aldolase class II (570bp); ftsk - DNA translocase cell division protein (355bp); infB - translation initiation factor (395bp); RpoB - RNA polymerase β subunit (449bp); srfaa - surfactin synthase subunit (369bp); virB4 - type IV secretion system protein (279bp); yaeT - outer membrane protein assembly factor (432bp)
*Host dead on collection
[1]Provided by P. Neumann & O. Yanez (University of Bern)
[2]Provided by M. Gerth

Table 2.7. *Arsenophonus* sequence distance matrix, denoting no. of base differences between isolates (note: matrix is cropped, 4 isolates are shown)

Based on an MLST approach, the *Arsenophonus* strains circulating in UK *Apis mellifera* are highly similar (100% sequence identity over four HK and two accessory genes), indicating there is a single *Arsenophonus* strain circulating. The identity of this strain to the (dead) UK *C. hederae* tested imply a common pool, with the low frequency in *C. hederae* suggesting this is a secondary host species. However, it should be noted that MLST identity may nevertheless still contain strain diversity when examined at the whole genome level (e.g. *w*Mel in *Drosophila* are identical at MLST but diverse at both a whole genome and phenotypic level) (Chrostek et al. 2013, Richardson et al. 2012). Thus, it is be essential to corroborate this with further genomic analyses of the respective host strains. The distinction of *Arsenophonus* in UK *Apis* and *Colletes* from continental samples implies that *C. hederae* infection in the UK did not establish during the invasion process. Further sampling efforts on *Arsenophonus* in continental European *Apis* are warranted to allow comparison with the UK strain, to both establish the range of strains circulating in Europe, and whether the low divergence observed here at genetic markers reflects biological differences in the symbiosis and transmission phenotypes.

2.4.2 *Arsenophonus* & *Apis mellifera*: prevalence across space & time

Seasonal dynamics in *Arsenophonus* – *Apis* interactions Marked seasonal dynamics were observed for the prevalence of *Arsenophonus* among *A. mellifera* colonies from 2014 to 2018. This epidemiological pattern is not expected for heritable symbionts, whose infection prevalence generally remain relatively static over short time periods (e.g. months). For horizontally transmitted agents, seasonal infection dynamics in the host population as observed here are common place (eg. Hosseini et al. 2004; Lass & Ebert 2006; Bressan et al. 2009; Lalzar et al. 2012; Schwarz et al. 2014) but the underlying causes are frequently elusive. Infection prevalence changes are often driven by environmental variables that change with season, such as food availability, temperature (Corbin 2017, Ebert et al. 1995, Kelly et al. 2002), host behaviour (Hosseini et al. 2004) and an interplay with intrinsic host – symbiont

traits (ie. transmission mode & immune defence) (Anderson & May 1979, Hosseini et al. 2004).

In the *A. mellifera* population *Arsenophonus* prevalence increased over the summer season (June – July), reaching peak prevalence in early autumn (ie. September). Vertical transmission is considered the predominant path within the *Arsenophonus* clade (Nováková et al. 2009, Wilkes et al. 2011), however given the temporal pattern observed in *A. mellifera* we may speculate on the role of a seasonal reservoir driving infection dynamics. The incidence of *Arsenophonus* could be tracking a biotic or abiotic reservoir whose peak activity falls around mid-summer/early autumn. This could be, for example, during the flowering period of a particular plant or flight period of an additional insect host/reservoir (eg. Bressan 2014; Graystock et al. 2015; McFrederick et al. 2017). The detection of *Arsenophonus* outside of this high prevalence window may reflect chronic infections or colonies unable to clear previous infections. Another explanation could be that *A. mellifera* continue to interact with the reservoir but at a lower rate outside of this period, generating seasonal patterns in *Arsenophonus* prevalence.

Populations of the symbiont *Spiroplasma* also show marked annual and seasonal fluctuations in *A. mellifera* (Schwarz et al. 2014), analogous to the *Arsenophonus* epidemiology observed here. Plants appear to be important reservoirs and drivers of this dynamic infection, with peak prevalence of *Spiroplasma melliferum* aligning with peak flowering periods (Clark 1978, Schwarz et al. 2014). Both *S. melliferum* and *S. apis* have also been isolated from plant species visited by *A. mellifera* (Clark 1978, Davis 1978, Mouches et al. 1984). Might a similar ecological variable be driving the periodicity of *Arsenophonus*? Notably, recent work has reported *Arsenophonus* from flowers (bagged to prevent Anthophila contact but allow access of small insects such as thrips), in addition to pollen taken from the scopa of the megachilid bee *Osmia chalybea* (McFrederick et al. 2017*b*). While these observations support the role of an environmental reservoir driving dynamics, further characterisation of *Arsenophonus* strains isolated from flowers vs Anthophila is required

and establishment of the viability of such a transmission route.

Interestingly though, such speculation reflects an emerging body of evidence that members of heritable intracellular genera, including *Rickettsia*, *Cardinium*, *Wolbachia*, are sometimes transmitted via plants (for review, see Chrostek et al. 2017). Live *Wolbachia* and *Rickettsia* have been identified within plant tissues (Caspi-Fluger et al. 2012, Li et al. 2016), and in one case localisation within the vacuole of a phloem cell was suggested (Li et al. 2016). Phytophagous insects carrying symbionts can inoculate plants, which act as infection reservoirs for new hosts (Caspi-Fluger et al. 2012, Gonella et al. 2015, Li et al. 2016) or facilitate transmission via contamination of the plant surface (Darby & Douglas 2003). If the former scenario is possible for *Arsenophonus*, could assimilation of the bacterium into nectar or pollen occur? This could be an effective, if seasonal, transmission route to pollinating Anthophila hosts. Preadaptations for such a lifestyle may even exist among *Arsenophonus* strains, as two species (*Phlomobacter fragariae & A. phytopathogenicus*) of obligately insect vectored plant pathogen are found within the clade (Bressan 2014, Bressan et al. 2009, 2012).

The apparent seasonal dynamics in *Arsenophonus* prevalence may also be explained by accumulation of the symbiont over the season. Accumulation may occur within the *A. mellifera* population as repeated exposures from spring to autumn build up to a peak prevalence observed in early autumn. Suppression of *Arsenophonus* dynamics at the onset of mid-autumn occurs due to extrinsic environmental and/or intrinsic host-symbiont factors, and *Arsenophonus* prevalence drops before the accumulation process starts again the following spring. The *Arsenophonus* infection pattern may also be affected considerably by fluctuations in *A. mellifera* population density over the course of the seasons (Winston 1987). Accumulation may also occur within an environmental reservoir, either independently and/or driven by feedback with increased prevalence in the *A. mellifera* population combined with shedding into the environment. Late in the season monarch butterflies *(Danaus plexippus)* have a higher risk of infection with the protozoan

parasite *(Ophryocystis elektroscirrha)*, with prevalence peaking in the autumn (Bartel et al. 2011). Here seasonality in infection is attributed to the shedding and accumulation of parasite spores on host plants over time (Bartel et al. 2011, Satterfield et al. 2017). A comparable process could explain *Arsenophonus* infection dynamics, however this would depend on longer term environmental viability to facilitate accumulation. Arguably some *Arsenophonus* spp. have properties that make this plausible, for instance their relatively large symbiont genomes, presence of extracellular infections and cell free cultivability all being found within the clade (Darby et al. 2010, Hypsa & Dale 1997).

Spatial patterns in *Arsenophonus* prevalence The prevalence of *Arsenophonus* did not vary significantly with county and the bacterium was detected in all ten counties that were included in the screening effort. At a much more local scale, variation in *Arsenophonus* prevalence emerged, with apiary (a site with multiple colonies) being a significant predictor of colony *Arsenophonus* status. This distinct spatial pattern suggests variation in drivers of infection likelihood across space, and two non-mutually exclusive explanations may be considered.

Significant differences in *Arsenophonus* prevalence at the apiary level may be explained by biotic/abiotic factors that vary at a local scale, such as types of foraging resource or the arthropod community assemblage. Such spatial variation is observed across many symbiont lineages and host associations (Hoffmann et al. 2011, Russell, Goldman-Huertas, Moreau, Baldo, Stahlhut, Werren & Pierce 2009), although the drivers often remain unclear. In Malaysian populations of the weaver ant (*Oecophylla smaragdina*) the structure of the symbiont communities varies with different foraging environments (forest vs urban) (Chua et al. 2018) and in Japan the infection frequencies of common endosymbionts (inc. *Sodalis*, *Wolbachia* & *Rickettsia*) correlate with local abiotic factors (Toju & Fukatsu 2011). Estimates of *Apis* foraging ranges vary, but averages of 2 – 3km are reported (Visscher & Seeley 1982). Given this range, *A. mellifera* originating from colonies within the same apiary (or

nearby apiaries) are likely to overlap more frequently in foraging range (Levin 1961), and thus encounter similar ecological conditions that may correlate with infection risk. In this context the spatial result supports inferences from seasonal patterns. The data argue against a stable vertically transmitted *Arsenophonus* strain and signal instead to infectious symbiont dynamics influenced by environmental factors.

An alternate process would derive from the movement of *A. mellifera* themselves. Within apiaries *A. mellifera* individuals commonly return to the incorrect colony in a process known as drifting. As symbiont fitness is dependent on both transmission within the colony and transmission to new colonies in eusocial hosts (Riesa & Amazineb 2001), drifting could prove advantageous for symbiont fitness. If *Arsenophonus* can transmit horizontally between *A. mellifera*, drifting within apiaries may drive inter-colonial transmission of *Arsenophonus* and explain the localised patterns. As drift levels vary between apiaries, depending on variables such as colony density, layout and hive type (Free & Spencer-Booth 1961, Bailey 1958, Natsopoulou et al. 2015), such factors may contribute to the observed variation in *Arsenophonus* prevalence at the apiary level and could warrant further investigation. Lower parasite levels in feral populations of *A. mellifera* have been attributed to reduced colony density and drift, relative to managed apiaries (Seeley 2007).

2.4.3 Contrasting populations: *Arsenophonus* in UK Colletes

The absence of *Arsenophonus* in UK populations of *C. cunicularius* and a low prevalence of the symbiont within *C. hederae* contrasts with the results from German populations where Gerth et al (2015) observed a high prevalence of *Arsenophonus* spp. in both *Colletes* species. Our lack of detection in *C. cunicularius* could be attributable to sample size, however, the large sampling effort for *C. hederae* allows us to be more confident in our low prevalence estimates for *Arsenophonus*. But what explains this geographic variation in *Arsenophonus* prevalence? Two possible explanations include: (I) *Arsenophonus* was lost under the invasive range hypothesis, or (II) there

is geographical variation in competition between endosymbionts

I. Range Expansion & Symbiont Loss The distribution of host genetic diversity can be heavily affected by range expansions, across and within populations (Ray et al. 2003). It thus seems plausible that endosymbiont diversity can be similarly affected. A phenomenon of symbiont loss has been recorded in the invasive range of several insect groups, including thrips, psyllids and multiple ant species (Morrow et al. 2017, Nguyen et al. 2016, Rey et al. 2013). This loss may be the result of a bottleneck associated with the expansion or variation in selection pressures, stochastic effects (Nguyen et al. 2016) or environmental changes.

Colletes hederae has undergone an astonishing range expansion from Western Europe. First confirmed in Southern England in 2001 (Cross, 2002) it has since spread rapidly through the UK and can be found as far north as Lancashire (BWARS). If, as evidence suggests, *Arsenophonus* was not at fixation in source populations on mainland European (Chapter 2 results & Gerth et al., 2015), the founding individuals may by chance not have been infected with *Arsenophonus*, or a low prevalence created a bottleneck for the symbiont. However, work by Dellicour et al. (2013) infers a high migration rate as the colonization mode of UK *C. hederae*. If migration rates and *Arsenophonus* prevalence are sufficiently low, the bottleneck theory could still hold true under this scenario. However, alterations to the environment and selection pressures (potentially in combination with genetic drift) may be having larger effects.

The carriage of facultative symbionts is always associated with costs, and sometimes benefits. New ranges can incur different abiotic (eg. temperature) and biotic (e.g. parasite presence) conditions that can relax selection or select against symbiont carriage (Dedeine et al. 2005). This scenario has been suggested for the loss of *Wolbachia* in the invasive range of another Hymenopteran species - *Wasmannia auropunctata* (the little fire ant), potentially mediated by a reduction in interspecific contact and competition with other ant species in the new range (Rey et al. 2013).

It is likely *C. hederae* has encountered novel conditions during the UK expansion, such as exposure to new parasites, thus it is possible factors intrinsic to the new environmental context has generated the loss of *Arsenophonus*.

II. Competing Endosymbionts A second theory of *Arsenophonus* loss may hinge on our findings of *Wolbachia* at fixation in the UK population of *C. hederae* females. Endosymbionts often occupy similar niches within hosts (e.g. Michalik et al. (2018), and resources are limited, leading to potential competition between different species and strains (Paredes et al. 2016, Šochová et al. 2017, Vautrin et al. 2008). This can create endosymbiont variation within or between host populations (Hosokawa et al. 2016), and also cause symbiont complementation and replacement dynamics (Michalik et al. 2018, Morrow et al. 2017). *Nilaparvata lugens* (brown planthoppers) for example are consistently infected with exclusively *Arsenophonus nilaparvatae* or a strain of *Wolbachia* (Qu et al. 2013). Complementarity suggests these symbionts may be competing for the same niche and/or be functionally redundant with respect to one another.

A similar interaction between *Arsenophonus* and *Wolbachia* may be occurring in *C. hederae*, and account for the disparity between European populations. An existing facultative symbiont could be compromised under new/different ecological conditions, (such as those associated with a range expansion) facilitating invasion of a new competing symbiont into the niche. In the longer-term, and especially for obligate interactions, complete symbiont replacement may occur.

While *Wolbachia* appears to dominate in the UK population, our work presents three cases of *Arsenophonus* and *Wolbachia* identification in the same individual, suggesting coinfections are possible under certain conditions. It would be interesting to determine if these bacteria co-localise within *C. hederae* tissues to determine the scope for competitive interactions and lateral gene transfer within the host. Putative lateral gene transfer events from *Wolbachia* to *Arsenophonus* have previously been reported (Darby et al. 2010, Duron et al. 2008). Notably all the coinfected UK

individuals, and the only *C. hederae* infected with *Arsenophonus*, were dead at the time of collection. With this information it is tempting to speculate on a saprophytic role for *Arsenophonus*, as such a stage has been identified in *A. nasoniae* (Werren et al. 1986). This role would also be unlikely to overlap in niche with generally obligate intracellular *Wolbachia*. However, coinfection with the two symbionts is hard to conclusively verify based on our methods and would benefit from additional work to establish if the symbionts are viable at the same time and if co-localisation occurs.

2.4.4 *Arsenophonus* distribution across Anthophila taxa

Within the survey, *Arsenophonus* was common in eusocial *A. mellifera* but absent from all sampled solitary bee species (with the exception of *C. hederae*) supporting the low prevalence of this symbiont noted in other solitary Anthophila surveys (Gerth et al., 2015). These results also indicate that despite the high prevalence in *A. mellifera*, the risk of *Arsenophonus* spillover to wild bees appears to be low (or does not occur) at least for the species surveyed. We know *Arsenophonus* is capable of infecting other Anthophila species (Gerth et al. 2015, McFrederick et al. 2014, Parmentier et al. 2018, Saeed & White 2015), so what factors mediate a disproportionately high prevalence of the symbiont in *A. mellifera* compared to UK solitary bee populations? These differences may be explained, in part, by traits linked to the sociality of Anthophila taxa.

Our seasonal pattern for *Arsenophonus* hints at a possible role of an environmental reservoir in determining infection prevalence. Given this, the high densities in *A. mellifera* colonies means many individuals are interacting with the environment, and potential *Arsenophonus* reservoirs, at a given time. This translates into an increased probability of the colony encountering the reservoir, and an increased probability of individuals becoming infected if horizontal transmission then occurs within the colony. Early in the association, a serial passaging effect within the eusocial colony may have allowed the rapid adaptation of *Arsenophonus* to *A. mellifera*, with successive hosts being highly similar genetically (Ebert 1998). Such social host

factors could contribute to the higher prevalence, and apparent invasion success, of *Arsenophonus* in *A. mellifera* populations.

In contrast, the potential effects of genetic proximity and reservoir exposure may be far lower in solitary species that appear to only be sporadically infected with *Arsenophonus*. Interestingly though, the solitary Anthophila most commonly infected are *Colletes* spp. that often nest in large aggregations of up to tens of thousands of individuals (C O'Toole, pers. comm.,BWARS)., Saxton 2009; Dellicour et al. 2014). While interactions in such aggregations clearly do not mirror the highly evolved and specialised behaviours within eusocial Anthophila (Wilson 1971), perhaps large nesting aggregations facilitate the horizontal transmission of *Arsenophonus* between solitary individuals via similar mechanisms that may occur within a colony environment.

While the social and behavioural traits aforementioned may feasibly explain *Arsenophonus* distribution across Anthophila taxa, it is likely that phylogenetic and ecological factors (ie. polylectic vs monolectic) may also explain symbiont distribution (Gerth et al. 2015).

2.4.5 *Wolbachia*: a common endosymbiont of Solitary Bees

Wolbachia prevalence in Anthophila has been suggested to be unusually high (Gerth et al. 2013, 2015) and the results of Chapter 2 corroborate this. *Wolbachia* was identified in 72% of solitary Anthophila species, which is within general model predictions for *Wolbachia* abundance among arthropod species (Hilgenboecker et al. 2008, Zug & Hammerstein 2012) and similar to previous Anthophila specific surveys (Gerth et al. 2013, 2015). Our prevalence estimate may also be conservative, as samples sizes were small for those species deemed uninfected.

Following on from screens of German populations (Gerth et al. 2013, 2015) we also identified *Wolbachia* in *C. hederae*, *C. cunicularius*, *C. daviesanus*, *C. succinctus*, *A. nigroaenaea* and *A. clarkella* showing presence of the symbiont is not just a local phenomenon in these species. However, several species (*C. cunicularius, A. flavipes & C.*

similis) showed an intermediate *Wolbachia* prevalence, contrasting with Gerth et als. results where *Wolbachia* was present in all or none of the infected individuals for a species. This medium prevalence may be accounted for by weak drive mechanisms and/or context-dependent maintenance of the symbiont, potentially influenced by population level differences. Males of all Anthophila species were infected less frequently with *Wolbachia*, and this pattern may be attributable to the dead end nature of male hosts for maternally transmitted *Wolbachia* infection (Hurst & Frost 2015).

A disparity was evident between *Wolbachia* presence in the UK screening (2016/17) of *C. hederae* and absence in European samples (2010) provided by collaborators. The disparity is most likely explained by methodological differences, as DNA from the 2010 samples was extracted only from thorax tissue. Our 2016/17 screening used half a bee (see methods 2.2.3), allowing detection of *Wolbachia* in abdominal tissues/organs. It is notable that UK populations did not test positive in 2010.

Evidence of drive? *Wolbachia* at fixation in solitary bees *Wolbachia* is circulating at a 100% infection prevalence in UK populations (2016/17) of three solitary bee species: *C. hederae* (females only), *C. succintus* and *C. daviesanus* (males & females). Although *Wolbachia* appears to be common in UK solitary bee populations generally, fixation (100% prevalence) in these 3 species suggests a drive phenotype. *Wolbachia* is well established as a reproductive parasite (RP) in a number of host species (Dyson & Hurst 2004, Engelstädter & Hurst 2009, Stouthamer & Huigens 2003, Werren et al. 2008). Manipulation of host reproduction facilitates transmission of *Wolbachia* (normally via the female line) and can drive the endosymbiont to fixation in host populations (Dobson et al. 2002, Turelli 1994, Turelli & Hoffmann 1991, Weeks et al. 2007). This can occur rapidly, as exemplified by a CI inducing *Wolbachia* in *Drosophila simulans* populations, spreading 700km in 10 years despite lowering the fecundity of infected females (Turelli and Hoffmann 1991; Weeks et al., 2007). As symbiont carriage is fundamentally always costly to hosts, infections at fixation in the three *Colletes* species are most likely driven by reproductive ma-

nipulation (RM) and/or symbiont conferred beneficial effects. Beneficial effects that occur alongside RM can allow symbionts to invade even when rare (Fenton et al. 2011) and also facilitate maintenance in the host population in the absence of RM (for reviews, see Zug and Hammerstein, 2018; Drew, Frost and Hurst, 2019). Potential direct beneficial effects conferred to hosts can include nutrient provisioning, environmental tolerance, reproductive benefits and protection against natural enemies (Elnagdy et al. 2013, Grenier et al. 2002, Unckless & Jaenike 2012, Xie et al. 2014). As wild bees are increasingly exposed to parasite spillover from managed *A. mellifera*, symbionts that protect against natural enemies may provide an important fitness advantage to hosts. *Apis* viruses, such as DWV for example, are now considered emerging pathogens in wild pollinator communities (Breeze et al. 2011, Fürst et al. 2015, Manley et al. 2015). *Wolbachia* is known to provide protection against RNA virus attack in *Drosophila* (Chrostek et al. 2013, Hedges et al. 2008), it may be interesting to investigate interactions between *Wolbachia* and common viruses in Anthophila hosts.

To deduce what is driving the fixation of *Wolbachia* in these *Colletes* species, further work should test for evidence of sex ratio distortion or cytoplasmic incompatibility, in addition to the implications for host fitness in the absence of the symbiont. Unfortunately however, such experiments may prove difficult, due to long generation times and sensitivity of solitary Anthophila to laboratory conditions.

Chapter 3

Persistence & Tropism

Abstract *Arsenophonus* is a common associate of honey bees in UK populations, and is also known from continental Europe and the USA. The interaction of *Arsenophonus* at the level of the individual host is, however, poorly characterized. In this chapter, sites of infection within individual *A. mellifera* are assessed by PCR and FISH, and persistence of infection is established at both the individual bee and colony level. At the individual level, infection was observed widely by PCR, including within legs, guts, thorax and mouthparts. FISH analysis revealed *Arsenophonus* within the gut community, and PCR revealed presence in faeces. Individual workers maintained in the lab gradually lost infection over a 14 day period. Overwintering was associated with declines in *Arsenophonus*, with the colonies infected in autumn presenting as uninfected the following spring. Altogether, these data reveal a highly dynamic symbiosis that implies gain and loss events occur, which contrast with the strictly vertically transmitted lifestyle of most other members of the genus.

3.1 Introduction

The spatial and temporal dynamics of a host - microbe interaction at the population level represents the sum of many thousands of acquisition and loss events occurring at the individual level. Where patterns of decline of a microbial symbiont are observed across a population, this implies either a reduction in new infections, or more individuals recovering from infection. Thus, understanding the patterns of change at the population level requires a detailed understanding of the processes occurring within individual hosts.

For social hosts, like *A. mellifera*, these processes occur additionally at the colony level. Here there is a requirement to track both the fate of infection in an individual, but also at the level of the colony, as colonies have internal transmission dynamics that makes them into an important level of organisation epidemiologically (Hughes et al. 2002, Naug & Camazine 2002, Rutrecht & Brown 2008). The colony may also represent a unit that has varying biological properties in terms of the environment in which it sits and the other pathogens and parasites that circulate within it (Cornman et al. 2012, Evans & Schwarz 2011).

This chapter presents data on *Arsenophonus - Apis* interactions at the level of the individual and of the colony. For the individual, two properties are examined. First, where is *Arsenophonus* infection detected in the individual? This property, known as tropism, is useful in developing hypotheses regarding transmission routes as well as potential pathology (Caspi-Fluger et al. 2012, Moran et al. 2008). Second, does infection persist in individual *A. mellifera* taken out of the colony and environmental context? This information establishes the stability of the association in the absence of external inputs. Lack of persistence at the individual level implies other factors must exist to stabilise infection at the population and colony level. At the colony level, a lack of symbiont persistence suggests that social interactions between infected and susceptible individuals, and the internal colony environment, are not sufficient to maintain infections (Breban et al. 2009). Thus factors extrinsic

to the colony may be implicated in driving stable associations. These additional drivers may include environmental sources or alternate reservoir host species that are subject to their own temporal dynamics and can cause loss or gain events in another host population (i.e. *A. mellifera*) (Pilosof et al. 2013).

The importance of tropism The tropism of symbionts within host tissues can provide important clues regarding symbiont phenotype and the nature of interactions with the host. A number of generalisations are made for heritable symbionts. For instance, microbes that are facultative for host fitness are often found diffusely across host tissues at low titres while obligate symbionts are often housed at high density in specialised bacteriomes (Buchner 1967, Moran et al. 2008). For symbionts that are acquired from the environment, the host gut represents a particularly important arena for symbiotic associations and constitutes a major site of invasion for many microbes (Engel & Moran 2013, Katsnelson 2015, Koch & Schmid-Hempel 2011, Martinson et al. 2011). The gut microbial community of *A. mellifera* consists of a distinctive and conserved gut microbiota that is transmitted largely socially (Kwong & Moran 2016, Powell et al. 2014), and these core symbionts are specialized and rarely found outside the hive environment (Anderson et al. 2013, Martinson et al. 2011). Central roles in maintaining bee health have been suggested for the gut microbiota, notably by protecting against microbes with pathogenic potential (Koch & Schmid-Hempel 2011, Kwong et al. 2014). Experimental evidence also supports this, with perturbations of the core gut microbiota often increasing susceptibility to biotic stressors (Schwarz et al. 2016).

Whilst the core gut symbionts of *A. mellifera* are well researched, non-core microbes such as *Arsenophonus* have received comparatively less attention. Nevertheless, there are important motivations for studying these players. Under certain conditions non-core microbes may confer direct or indirect beneficial effects to hosts (Ford et al. 2017, King et al. 2016), or they may act as opportunists that may be fundamental in generating additive pathological effects (Kwong & Moran 2015).

These microbes are not part of the conserved symbiont community in *A. mellifera* and their presence is by definition more sporadic, making interactions with hosts challenging to unveil.

Previous work has noted the presence of *Arsenophonus* in the gut (Babendreier et al. 2007, Corby-Harris et al. 2014), on the surface of individuals (Aizenberg-Gershtein et al. 2013) and also in the haemolymph of workers infected with the *Apis mellifera* filamentous virus (AmFv) (Gauthier et al. 2015). However, all these data derive largely from indirect observations – amplicons from PCR assays or DNA. These methods can be imprecise, poorly represent titre, and cannot distinguish live from dead microbes. To date, no images of the bacterium associating with *Apis* have been obtained.

Individual and colony persistence The previous chapter demonstrated strong seasonal patterns of *Arsenophonus* presence, being rare in spring and more common in autumn. These changes imply infections are gained during the active summer-autumn phase, but lost overwinter. The processes of gain will be a composite of acquisition of infection and the level of persistence in individual *A. mellifera*. In order to understand the acquisition and loss processes, it is vital to measure the core parameter of persistence within an individual, as all other processes are overlaid on this.

Further, the seasonal data indicate a particular effect of winter on *Arsenophonus* persistence. Winter conditions are very distinct for *A. mellifera*. Foraging and brood rearing ceases (temporarily), and the colony depends on accumulated hive resources (bee bread, honey). Winter bees are produced with differing physiological profiles and the size of the colony drops to form a tight thermoregulating cluster. Immune activation is also altered from the active summer state (Desai & Currie 2016, Johansson & Johansson 1979, Döke et al. 2015).

For the gut microbiota, associations during the winter period can be highly dynamic, with distinct changes occurring from the start of winter to the first spring

cleansing flight (Lyapunov et al. 2008). The rectum distends to store faecal matter and avoid defecation in the colony, and this is reflected in changes to the microbial gut community that must digest the accumulated matter (Sammataro & Yoder 2012). The major changes to host behaviour and population structure associated with the overwintering period (Döke et al. 2015, Johansson & Johansson 1979, Winston 1987) are likely to also impact the abundance and distribution of non-core microbes, via changes to transmission opportunities and competition dynamics between symbionts. Winter is particularly important in the *Apis* health cycle, with a large fraction of colony collapse observations occurring overwinter (Berthoud et al. 2010, Dainat et al. 2012). Thus, it is important to understand the fate of symbionts through this period.

Chapter Objectives To establish the stability of the *Arsenophonus* – *Apis* interaction, this chapter tracks focal colonies and individual workers over time. The persistence of *Arsenophonus* during the natural overwintering period and within a laboratory context are both explored. Clues as to the association between *A. mellifera* and *Arsenophonus* are gained by screening host tissues and imaging the gut environment.

3.2 Methods

3.2.1 Assessing tropism of *Arsenophonus* by PCR assays

To assess the distribution of *Arsenophonus* in *A. mellifera* tissues, workers from *Arsenophonus* infected colonies were dissected independently in sterile petri dishes using sterile forceps and scalpel blades. Tissues collected were as follows: posterior leg (n = 38), mid leg (n = 23), whole gut (n = 40), thorax tissue (n = 17), wings (n = 26), mouth parts (n = 15), abdomen case/cuticle (n = 22), faeces (n = 22).

Mouth parts were removed by pulling on the glossa tip, and included the maxillae, labial palps and glossa (but not mandibles). Thorax tissue was taken from within the thorax body (no cuticle). A section of abdominal cuticle was separated

from the empty case after removal of the whole gut (thus this tissue may include samples of fat body or gut traces). Faecal samples were collected by isolation of individual *A. mellifera* in petri dishes until defecation. Molecular grade H_20 ($\sim$ 50 µl) was added to the faecal sample to facilitate collection by pipette. Note, not all tissues were screened from all *A. mellifera* individuals, thus there is some bias in the dataset.

Tissues were placed directly into nuclei lysis solution (adjusted for tissue size) and DNA extracted using a Promega Wizard® kit as described previously (section 2.2.3). Detection of *Arsenophonus* in host tissues was completed by PCR assays, as previously described (section 2.2.4). The proportion of samples infected with *Arsenophonus* for each tissue class was plotted in R version 3.3.1 (R Core Team 2013) with binomial confidence intervals.

3.2.2 Localisation of *Arsenophonus* within the gut using FISH

For the localisation of *Arsenophonus* using fluorescence *in situ* hybridization (FISH), whole guts were dissected from live *A. mellifera* (on ice) and placed directly into Carnoys fixative (60% EtOH, 30% chloroform, 10% acetic acid). Tissue was fixed for 24 – 48hrs, washed with 100% EtOH (x 3) and incubated in the dark with hybridization buffer (20Mm Tris-HCL, 0.9M NaCl, 0.01% sodium dodecyl sulfate, 30% formamide, 100 pmol/ml Alexa Fluor® flurochrome) at room temperature overnight. See Table A2 for flurochrome details.

Tissues were washed with pre-heated ($\sim$48°C) washing buffer (20Mm Tris-HCL, 0.9M NaCl, 0.01% sodium dodecyl sulfate, 0.05M EDTA) three times for 10 mins (room temperature, dark). Wash buffer was replaced with cold molecular grade H_20 and the hybridized tissue mounted on glass slide. Excess water was drawn from the tissue with a laboratory wipe and ProLong Diamond anti-fade mountant with DAPI (Fisher Scientific) added. A glass coverslip was added and the tissue left to cure for 24 hrs. Fixed guts were visualised by confocal microscopy (ZEISS LSM 880) and resulting images were processed using ImageJ (Schneider et al. 2012).

Images that were comprised of multiple optical sections were assembled into Z – stacks under maximum intensity settings.

3.2.3 Determining persistence of *Arsenophonus* in colonies overwinter

To determine if *Arsenophonus* is lost from *A. mellifera* colonies during the overwintering process, the infection status of 25 colonies was tracked from autumn to spring. Colonies were drawn from 3 different counties and autumn sampling took place during September – November, and spring sampling between April and May. Samples of worker *A. mellifera* were collected from the outer frames of all hives in autumn and the respective spring that followed. A total of 15 *A. mellifera* were screened per colony (3 individual *A. mellifera* were pooled per extraction and 5 extractions completed per colony). DNA was extracted using a Promega® Wizard kit as before.

Colonies with a high prevalence of *Arsenophonus* within the worker group were selected as focal colonies for overwinter tracking. Thus infected colonies were selected if 80% of extractions (n = 5) returned a positive result for *Arsenophonus* based on standard PCR assays (section 2.2.4). Uninfected colonies were chosen on the premise that all extractions returned negative for *Arsenophonus*. Any colonies that did not meet these requirements were not included. A total of 19 infected colonies and 6 uninfected colonies were used in the final study (total =25). All colonies were left to overwinter, and the sampling process was repeated in the spring for each colony that overwintered successfully. To determine infection status in spring a total of 24 *A. mellifera* were screened per colony (pooled as above).

3.2.4 Monitoring infection persistence in *A. mellifera* in the laboratory

Experimental Design Worker *A. mellifera* were collected from colonies (N = 2) with a high prevalence of *Arsenophonus* ($>$ 90% individuals infected) in October 2016 (Cheshire) and September 2017 (Dorset). On entering the laboratory (day 1) *A. mellifera* were cooled to 4°C and the *Arsenophonus* status of individuals detected

Overwinter period	A+ colonies (N)†	A- colonies (N)	Location
September 2015 - April 2016	7	1	North Yorkshire
October 2015 - April 2016	2	-	Merseyside
November 2016 - May 2017	4	2	West midlands
October 2017 - April 2018	8	1	North Yorkshire

† Reflects autumn 'starting' *Arsenophonus* status of colony

Table 3.1. Dates and locations of focal colonies used for tracking overwintering persistence of *Arsenophonus*

by removal of posterior tarsus tissue and subsequent molecular analysis (as described earlier 2.2.2 & 2.2.4) (N = 76, Colony A = 36, Colony B = 40). A control group using individuals from an uninfected colony (N = 32, Colony C, Merseyside) was subjected to the same method. In addition, *A. mellifera* (N = 40) from the infected source colonies were treated in the same manner as the positive sample, but comprised of individuals that were not subjected to tarsus snips. These data were obtained to establish whether the initial leg snip (and any immune response resulting) affected infection persistence.

Individuals were marked with queen paint for subsequent identification and maintained in groups of 4. Groups were maintained on filter paper in plastic pot cages at 32°C $\pm$ 2 °C, incubators were kept dark and humidity levels were maintained using a tray of H_20. Ambrosia® bee fondant and 50% sucrose solution were fed ad libitum. To track the persistence of *Arsenophonus* with time, one randomly selected individual from each group was culled at each of days 4, 8 and 15. Individuals that died during the study were not included in the final analysis. Individual *Arsenophonus* status was determined using the same methodology as day 1, but using tarsus tissue from the opposing posterior leg. *A. mellifera* cadavers were retained in EtOH for subsequent dissections.

Arsenophonus persistence over time and under laboratory conditions was recorded by the proportion of *A. mellifera* infected at each time point. The persistence of infec-

tion was modelled by a generalized linear mixed model (GLMM) with a binomial error distribution in R version 3.3.1. Time (sampling day), colony and treatment (A+ test or A+ control) were modelled as fixed effects and pot ID as a random effect. Model construction, selection and assessments of overdispersion were performed as described previously (2.2.6).

3.3 Results

3.3.1 Elucidating *Arsenophonus* tropism

A diverse range of *A. mellifera* tissues tested positive for *Arsenophonus* spp., by PCR assay using individuals from colonies with a known A+ status (see Figure 3.1). Over 70% of dissected *A. mellifera* legs and guts were infected with *Arsenophonus* (posterior legs, 86.8%, n = 38; mid legs, 73.9%, n = 23; whole guts, 70%, n = 40). Internal thorax tissue and material from the abdominal cuticle tested positive in just under 50% of cases (thorax tissue, 47.1%, n = 17; abdo cuticle, 45.5%, n = 22). The lowest prevalence of *Arsenophonus* was detected in mouth parts and wings (mouth parts, 33.3%, n =15; wings, 15.4%, n = 26). Evidence for *Arsenophonus* shedding via faeces was also identified (faeces, 59.1%, n =22). It is possible these tissue results may be partially skewed by systemically infected individuals (n = 8) stemming from Colony A of the infection persistence study.

3.3.2 Localisation of *Arsenophonus* in *A. mellifera* gut

FISH images of the gut tissue from infected *A. mellifera* show clear aggregations of *Arsenophonus* (coloured red) in the midgut (Figure 3.2) . Large aggregations of *Arsenophonus* infection (red) were occasionally observed in the midgut, with more sporadic isolated infection being present in the ileum and hind gut (Figure 3.3 A – C). The midgut aggregations were sufficiently large that they imply colonisation and replication. Negative controls using *A. mellifera* that tested negative for *Arsenophonus* were clearly distinct in terms of autofluorescence and probe removal

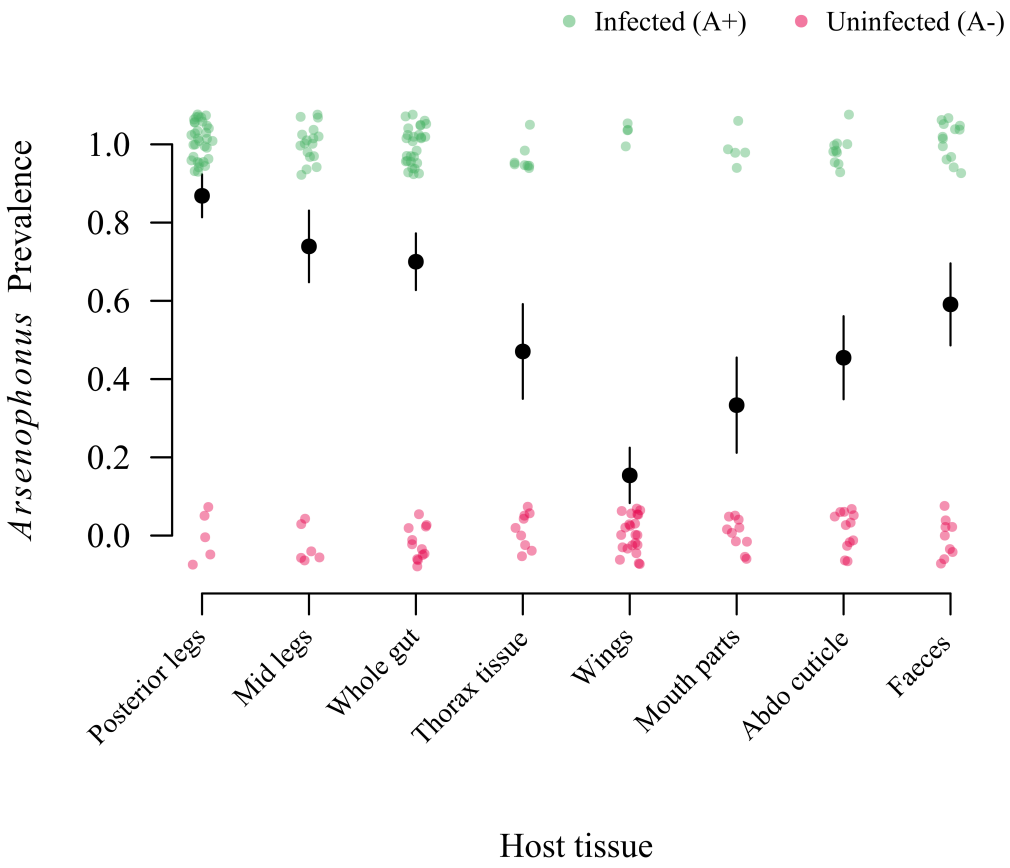

Figure 3.1. *Arsenophonus* is detectable in a broad range of *A. mellifera* tissues. All tissues classes (n = 8) were dissected from *A. mellifera* stemming from *Arsenophonus* infected colonies. A total of 203 tissues were screened by PCR and determined uninfected (0, col = pink) or infected (1, col = green). The proportion samples within each tissue class infected with *Arsenophonus* is plotted with binomial CI.

(Figure 3.3 C – F).

3.3.3 Overwintering generates infection loss

Previous work established the prevalence of *Arsenophonus* among *A. mellifera* colonies varies with season (see Chapter 2). The infection status of 25 *A. mellifera* colonies was tracked from autumn to spring to examine this trend with greater resolution (see Fig. 3.4). The bacterium was undetectable by the spring census for all colonies infected with *Arsenophonus* in the autumn (Group A, n = 19). Two colonies did not survive the winter (HA32, HA59). Of the colonies where *Arsenophonus* was not detectable in the autumn (Group B, n = 6), all colonies remained uninfected with the exception of one (WT2), which tested positive for *Arsenophonus* in the spring (May sampling) of 2017. Confidence in the status of this infected colony is high, as all extractions were positive for *Arsenophonus*.

3.3.4 Infection persistence under laboratory conditions

Arsenophonus was detected in all individuals from colony A (A+) and B (A+) at day 0, and none of negative control colony C (A-). Individuals from the A+ no manipulation groups (same colonies, no leg snip) were presumed to be infected at $>$ 90% on day 0, based on the 100% infection of the A+ test groups.

There was no significant effect of treatment (A+ with leg clip or A+ no manipulation) on the proportion of *A. mellifera* infected with *Arsenophonus* at each time point (Likelihood ratio test (LRT): 2 = 0.253, df = 1, p = 0.615). No significant interaction was evident between time and colony (LRT: X^2 = 0.583, df = 1, p = 0.445), however there was a significant effect of colony (LRT: X^2 = 20.23, df = 1, $p = 6.851 \times 10^{-06}$ ***). The minimum adequate model (IP3) included time and colony as fixed effects, and pot as a random effect (AIC 97.52, df 4, loglik -44.8, deviance 89.5, see Table 3.2). Model estimates for fixed (Table 3.3) and random effects (Table 3.4) are shown.

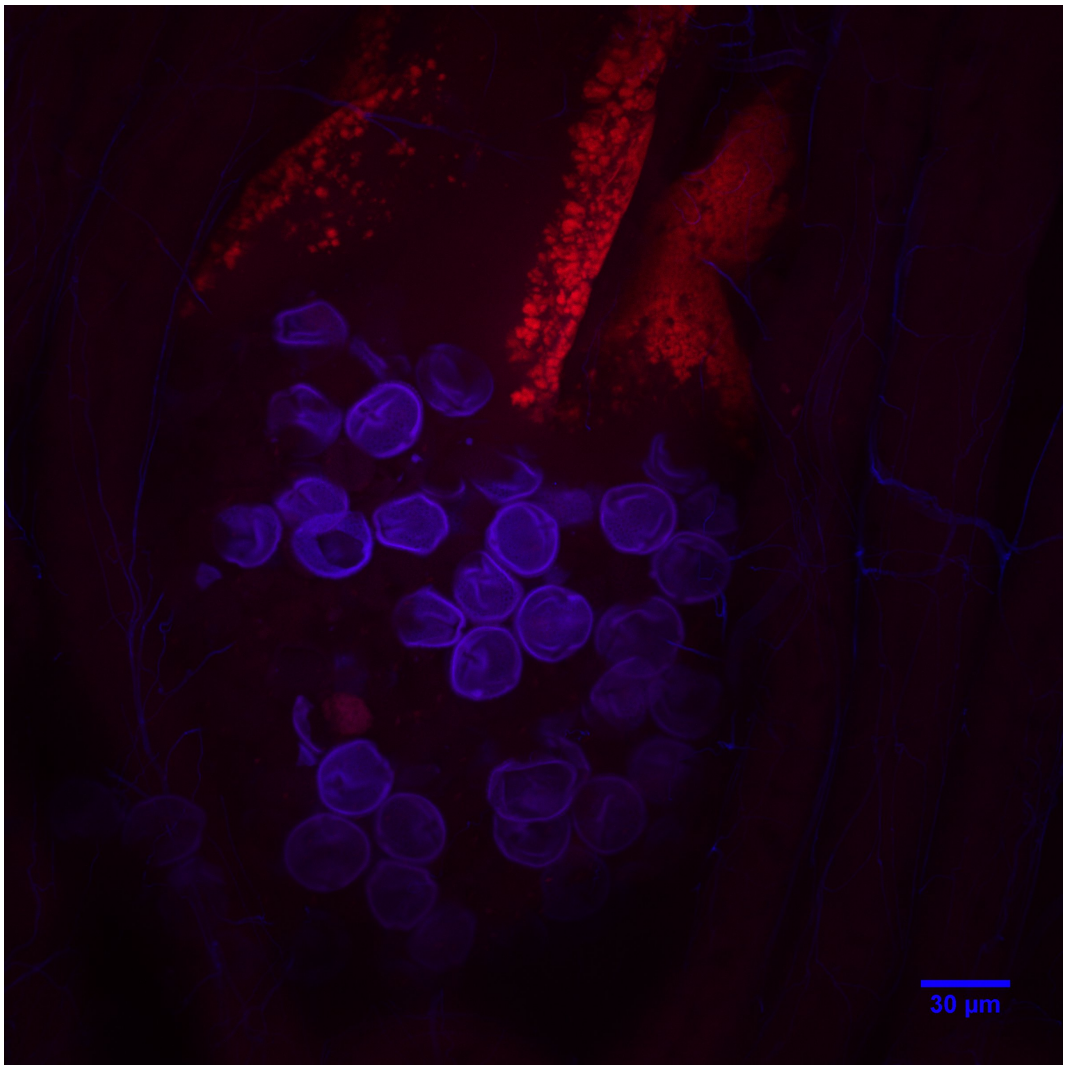

Figure 3.2. Localisation of *Arsenophonus* (coloured red) in the *Apis mellifera* midgut. Targeted using *Arsenophonus* specific fluorescent *in situ* hybridization using labelled probes (red), and DAPI counter staining (blue), on whole *A. mellifera* guts. Pollen grains can be seen in blue due to autofluorescence. Imaged by confocal microscopy (ZEISS LSM 880) at x 40 magnification. Image is a composite Z- stack comprised of 32 optical slices assembled in ImageJ under maximum intensity.

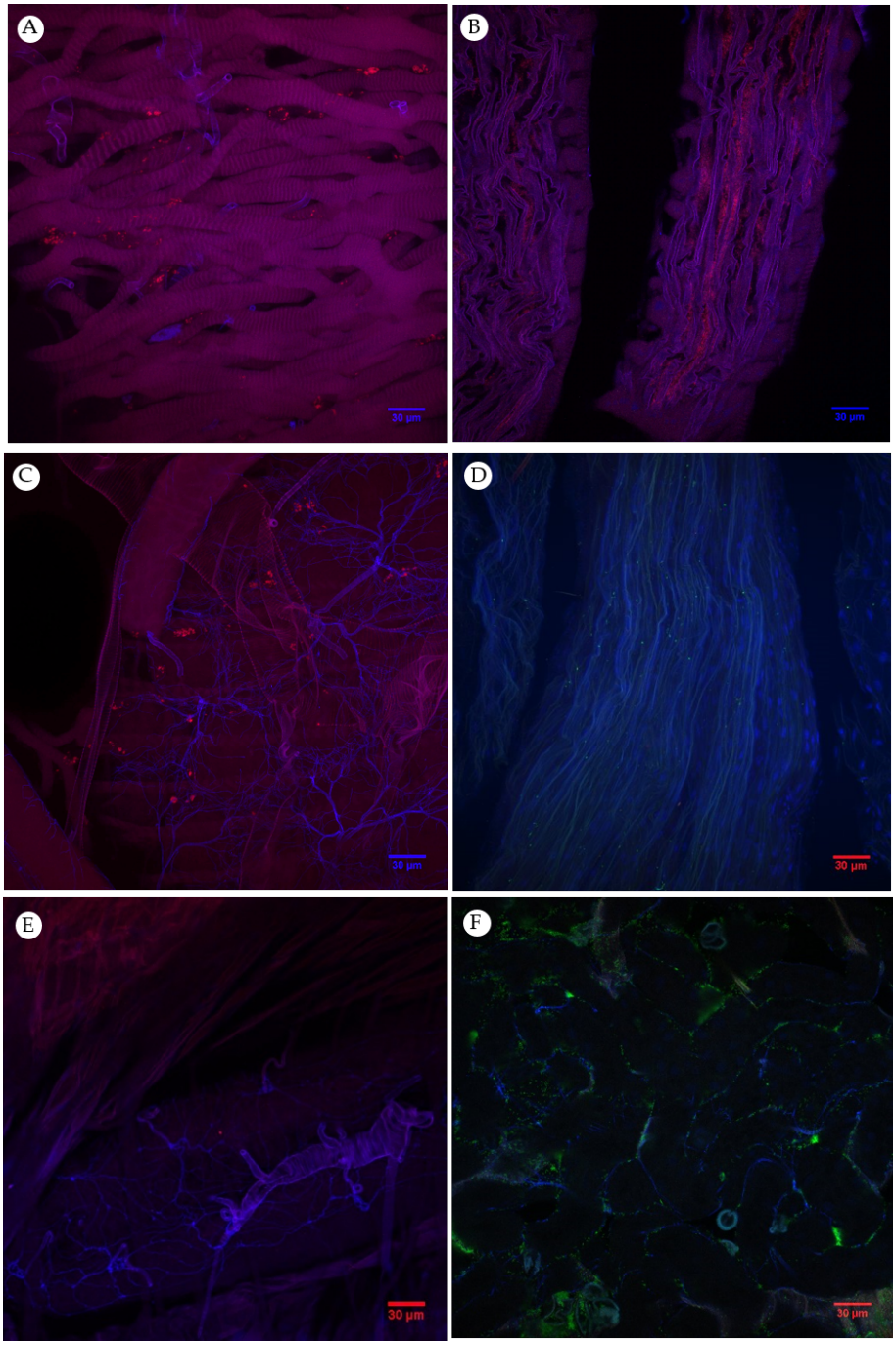

Figure 3.3. *In situ* identification & localisation of *Arsenophonus* in the *Apis mellifera* gut. *Arsenophonus* (coloured red) is shown localising in the *A. mellifera* hindgut (A), (C) and crop folds (B). Targeted using an *Arsenophonus* specific Alexa Fluor® 647 probe with DAPI counter stain. Images from the crop (D) and hindgut (E), (F) of *Arsenophonus* negative *A. mellifera* are shown. Imaged by confocal microscopy (ZEISS LSM 880) at x 40 magnification and processed using ImageJ.

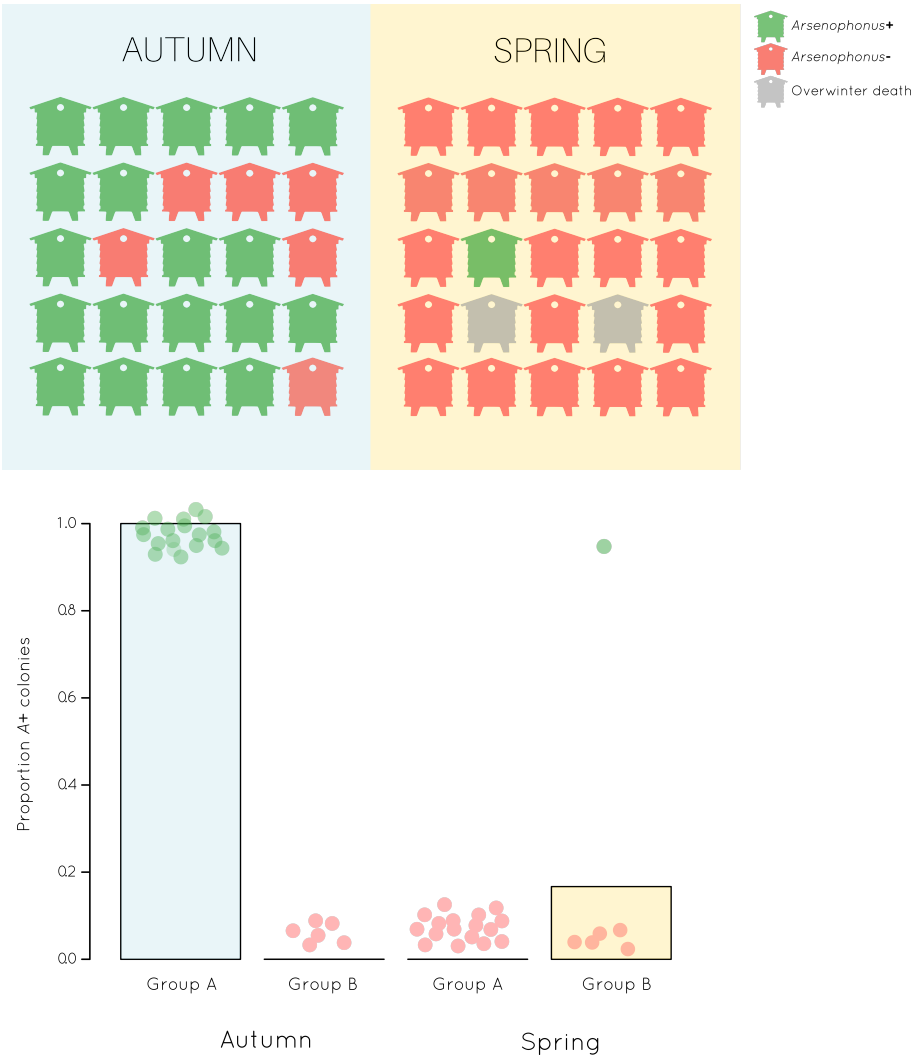

Figure 3.4. Overwinter loss of *Arsenophonus*. The schematic shows the prevalence of *Arsenophonus* across 25 focal colonies in the autumn and corresponding spring. Colonies that were infected in autumn are coloured green (Group A, n = 20) and uninfected colonies are coloured pink (Group B, n = 5). The infection status of each colony in the autumn and spring is presented in the matrix. The bar chart shows the total proportion of infected colonies in each group for each season. *Arsenophonus* detection by PCR assays.

ID	Model Structure[GLMM]	*df*	AIC
IP1	Arse_status ~ time * Colony + Treatment + (1 | Pot)	6	100.7
IP2	Arse_status ~ time * Colony + (1 | Pot)	5	98.94
IP3	Arse_status ~ time + Colony + (1 | Pot) [SM]	4	97.52
IP4	Arse_status ~ time + (1 | Pot)	3	115.8

[ID] in-text reference [df] degrees of freedom, [AIC]Akaike's information criterion, [SM]selected model

Table 3.2. Model (MAM) selection for the persistence of *Arsenophonus* infection

Fixed Effect	Estimate	Std. Error	Z- value	Pr (>|z|)
Intercept	6.6611	1.3840	4.813	$1.49x10^{-06}$ ***
Time	-0.3736	0.0739	-5.055	$4.30x10^{-07}$ ***
Colony (colony B)	-3.6148	1.0588	-3.414	0.00064 ***

***P < 0.001 ,**0.01, *0.05

Table 3.3. GLMM (IP3) parameter estimates for fixed effects

Table*. GLMM (IP3) estimates for random effects

Random Effect	Variance	Std. Dev
Pot	0.9725	0.9862

Table 3.4. GLMM (IP3) estimates for random effects

Overall the proportion of *A. mellifera* infected with *Arsenophonus* decreased significantly with time ($p < 0.001$) but there was variation between the colonies, with a significant negative correlation for the *Arsenophonus* status of colony B as a function of time ($p < 0.001$). The final model predictions with 95% confidence intervals are plotted in (Figure 3.5). For uninfected control *A. mellifera*, all pots maintained 0% infection throughout the study (data not plotted).

3.4 Discussion

In this chapter, *Arsenophonus* infection in individual *A. mellifera* has been established as being diffuse and to infect a wide variety of tissues. In contrast to the majority of members of the genus (Nováková et al. 2009), it was found as a gut inhabitant, and the numbers and aggregation observed in FISH analysis imply this bacterium can both reside and replicate in the gut. Infection of the gut environment is known for *A. Nasoniae*, is likely for the strains vectored by cixiids (Bressan 2014), but is not recorded in the remaining members of the clade. Further, most heritable microbes are either not found in guts, or are found but are considered unlikely to replicate outside of cells (though may remain transmissible) (Rasgon et al. 2006).

Further to this, *Arsenophonus* persistence in individual bees and colonies was examined. Colonies in which infection was present in autumn assayed negative the following spring, indicating infection has either been lost or has declined in titre to undetectable levels. Infection declined in workers maintained in the laboratory. These data in total indicate that the *Arsenophonus* interactions with *Apis* is distinct from the classic vertically transmitted symbiont model that has been established elsewhere in the genus. Aside *A. Nasoniae* in *Nasonia* wasps (Werren et al. 1986), *Arsenophonus* in insects are vertically transmitted from mother to offspring, and gut symbiosis is not recorded.

Three questions arise: i) What does the tropism of *Arsenophonus* tell us about the nature of the symbiosis, and how it may be distinct from other *Arsenophonus*-

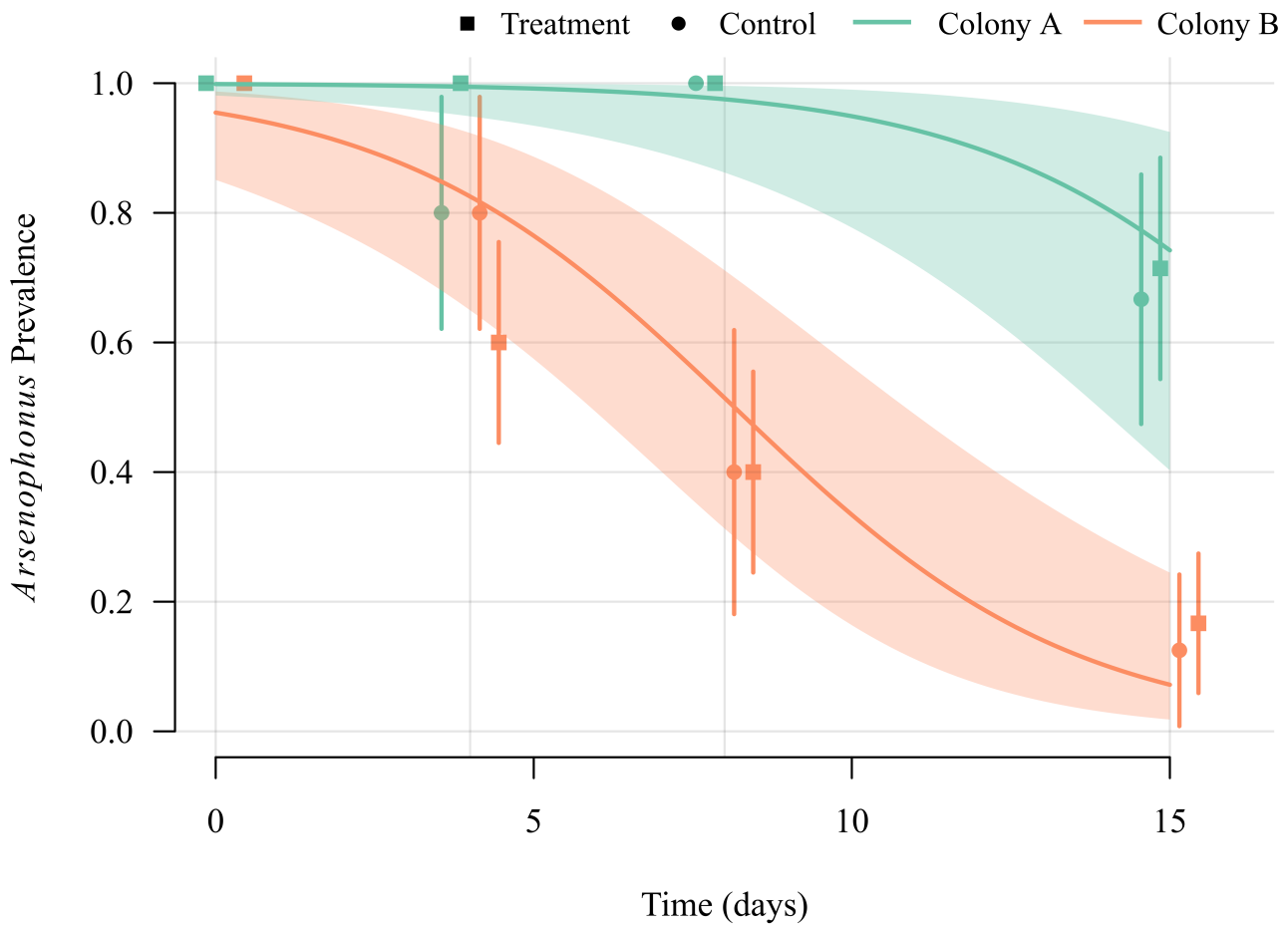

Figure 3.5. *Arsenophonus* persistence in *A. mellifera* over time under laboratory conditions. Model (GLMM) predictions and 95% CI are shown for the proportion of *A. mellifera* that remain infected with *Arsenophonus* over time under laboratory conditions. Predictions for colony A (green line) and colony B (orange line) are formed of observations from both treatment groups (test & control). The observed proportion of *A. mellifera* infected at day 0, 4, 8 and 15 is plotted independently for each colony & treatment (square = A+ test, circle = A+ control) with binomial CI. Observations from A+ controls (circles) are not shown at day 0, as their initial *Arsenophonus* status was not officially determined (legs not snipped). All negative control (A-) *A. mellifera* (colony C) remained uninfected for the duration of the study (data not shown).

host interactions? ii) What are the mechanisms underlying the patterns of loss in colonies overwinter? iii) What are the implications of the instability of symbiosis in individual *A. mellifera*?

3.4.1 Elucidating the nature of the symbiosis - what can we infer from the tropism of *Arsenophonus*?

***Arsenophonus* in the *A. mellifera* gut** The gut is recognised as a key habitat for symbionts, and the gut microbiota may influence host metabolism and other processes (Kinross et al. 2011, Li et al. 2008). *Apis mellifera* have a core gut community that is characterised by nine bacterial species clusters (Moran et al. 2012) and is extensively studied. In contrast, the role of generalist strains and those with an erratic presence remains largely unknown (Kwong & Moran 2016). *Arsenophonus* is not a core member of the *A. mellifera* gut community, thus its localisation here is potentially interesting. Observations of gut colonisation within the *Arsenophonus* clade are sparse. The closely related *A. nasoniae* functions as a gut inhabitant for part of its lifecycle (Huger et al. 1985, Werren et al. 1986). The *A. mellifera* strain may share this ecology, and in a eusocial host investment in gut colonisation could also prove highly adaptive. The gut is a key interface with the rest of the colony, the crop is employed in trophallaxis and the rectum in defecation (Wilson 1971). Thus, presence of the gut presents transmission opportunities for symbionts via oral - oral or faecal - oral routes. Core gut symbionts *Snodgrassella alvi*, *Gilliamella apicola* and *Frischella perrara* can all be acquired via faecal contact (Anderson et al. 2016, Powell et al. 2014).

In addition to localisation within the gut, *Arsenophonus* was identified in 50% of faecal samples from infected *A. mellifera* (Figure 3.1), indicating the bacterium can be shed via faeces. This observation echoes a similar pattern observed for *Wolbachia*, which is detectable in the gut and faeces of the ants *Acromyrmex echinatior* and *A. octospinosus* (Anderson et al. 2012, Frost et al. 2014). In general faecal – oral routes are common for symbionts in social insect groups such as Isoptera (termites)

and Blattaria (cockroaches) (Berlanga et al. 2009, Brune & Friedrich 2000, Schauer et al. 2012) and can have important implications for host fitness. In *Bombus terrestris* colonies faecally transmitted microbes confer protection against the virulent gut parasite *Crithidia bombi* (Koch & Schmid-Hempel 2011). However, this devastating gut parasite can itself be transmitted via faeces, highlighting the cost-benefit trade-offs of symbiont transmission in social hosts (Hughes et al. 2002). Regardless of the phenotype and initial acquisition route, further transmission via faecal – oral routes may be an effective strategy for *Arsenophonus* within the colony. However additional work is required to establish the infectivity of *Arsenophonus* cells in faeces.

Perturbations to gut communities Shifts in the core gut microbial community of hosts (dysbiosis) can be indicators of poor health or disease (Anderson & Ricigliano 2017). In *A. mellifera* such deviations are often associated with a rise in environmentally associated Enterobacteriaceae species (Corby-Harris et al. 2014, Kwong & Moran 2016, Moran et al. 2012, Tarpy et al. 2015). *Arsenophonus* spp. represent candidate symbionts for such an opportunistic phenotype, consistent with their placement within the Enterobacteriaceae and their association with poor colony health (Budge et al. 2016, Cornman et al. 2012). Further evidence for opportunism comes from *Bombus terrestris*, where individuals infected with the parasite *Apicystis bombi* also have higher levels of *Arsenophonus* (Parmentier et al. 2018).

As the core gut community is conserved across eusocial corbiculate species (Kwong & Moran 2016, Kwong et al. 2017), the response of *Bombus* and *Apis* spp. microbial communities to *Arsenophonus* infection may be similar. Further pathological similarities may be seen as changes to microbial gut composition affect the modulation of immune responses (Evans 2004), metabolic activity (Engel & Moran 2013) and susceptibility to additional parasites (Cariveau et al. 2014, Koch & Schmid-Hempel 2011, Schwarz et al. 2016). Solitary bee species, whose gut communities are often dominated by environmental bacteria (Martinson et al. 2011),

would constitute an interesting comparison. Do *Arsenophonus* spp. colonise similar niches in the gut of solitary species and have an opportunistic association? Or does the environmental gut community present different competition dynamics and select for different outcomes?

Systemic infections *Arsenophonus* was detected in a wide range of host tissues in infected *A. mellifera* (Figure 3.1). This distribution suggests infections are often systemic and the symbiont is likely circulating in the haemolymph. These data are consistent a previous study where *Arsenophonus* was found in the haemolymph of bees showing clinical symptoms of the *Apis mellifera* filamentous virus (AmFV) (Gauthier et al. 2015). The distribution of *Arsenophonus* in *A. mellifera* is probably typical of many facultative endosymbionts; in Hippoboscoidea symbionts are detected in the gut, fat body, milk glands, reproductive organs, bacteriome and haemolymph (Balmand et al. 2013, Dale & Maudlin 1999, Dale & Moran 2006, Nováková et al. 2016). This pattern contrasts with the distribution of coevolved core gut bacteria in *A. mellifera*, where symbionts occupy highly specialized locations and are rarely found invading other tissues (Donaldson et al. 2017).

Systemic infections in *A. mellifera* may result from *Arsenophonus* invading across the gut wall, analogous to the biology of *A. Nasoniae*. In *Nasonia* larvae *Arsenophonus* invades the haemolymph via the midgut, causing systemic infections in females and males (Huger et al. 1985, Werren et al. 1986). An invasion process like this could be costly to *A. mellifera*, as perforation of the gut by *Arsenophonus* could allow other symbionts/opportunists to spread to new tissues, creating opportunities for septicaemia, disease or the perturbation of resident microbial communities (Sammataro & Yoder 2012).

Another possibility is that the carriage of *Arsenophonus* across the gut is dependent on a coinfecting parasite or unrelated pathology causing damage. A requirement for coinfection could also account for the correlations observed between *Arsenophonus* infection and poor colony health (Budge et al. 2016, Cornman et al.

2012), as compromised health may be a prerequisite for systemic *Arsenophonus* infection. The migration of *A. bombi* sporozoites through the *B. terrestris* gut wall may have contributed to the high abundance of *Arsenophonus* spp. in the fat body (Parmentier et al. n.d.).

Extracellular & ectosymbiotic lifestyle Images from the gut (Fig 3.3 and 3.2) combined with the broad distribution observed in host tissues, suggests *Arsenophonus* has an extracellular lifestyle in *A. mellifera*. This is similar to vertically transmitted *A. Nasoniae*, which forms systemic infections but is not found within germ cells (Werren et al. 1986), yet contrasts with other intracellular members of the clade such as *Ca.* Arsenophonus arthropodicus (Dale & Moran 2006).

Based on this tropism, transmission in *A. mellifera* may also occur extracellularly, generating differing coevolutionary trajectories from strict intracellular symbionts (Bennett & Moran 2015, Moran et al. 2008). Extracellularly transmitted symbionts are defined by an ability to survive outside of hosts, at least for a short period of time (Salem et al. 2015), and are transmitted by routes such as faecal contact, environmental acquisition, egg smearing, capsules or social contact. While the transmission mode and route in *A. mellifera* remains unknown, *Arsenophonus* was detected in mouth part, wing and cuticle tissue. These data suggest the bacterium may exist as an ectosymbiont, in addition to having an extracellular lifestyle, or that surface contamination of *A. mellifera* may allow dispersal of the symbiont. The results could also be attributable to traces of additional material (eg. muscle tissue/haemolymph/faecal contamination), but interestingly another study also reported *Arsenophonus* from the cuticle of *A. mellifera* (Aizenberg-Gershtein et al. 2013).

Ectosymbionts are important for many hosts, including fungus-farming ants (Attini: *Formicidae*) that carry protective *Pseudonocardia* in cuticular crypts (Currie et al. 2006). The microbial communities of insect ectotissues are generally distinct from endotissues (eg. gut, fat body) (e.g. Durand et al. (2015)), thus if *Arsenophonus*

does colonise the *A. mellifera* cuticle this would be new for the genus and interesting generally from the perspective of ecto/endo lifestyle switching. This mode of colonisation may also facilitate social transmission through cuticle grooming between colony members.

3.4.2 What are the mechanisms underlying patterns of symbiont loss overwinter?

A. mellifera colonies infected with *Arsenophonus* in the autumn all tested negative for the bacterium the following spring. For heritable symbionts complete loss dynamics are rare over short time periods due to stable maintenance in individual hosts (Hurst & Frost 2015, Werren & O'Neill 1997). In contrast infectiously transmitted symbionts can experience interruptions to transmission and persistence, induced by a multitude of potential variables interacting with host and symbiont processes (Lalzar et al. 2012, Lass & Ebert 2006, Yui et al. 2009). Overwintering incurs marked environmental change in addition to physiological, behavioural and social changes in *A. mellifera* colonies (Döke et al. 2015, Fahrenholz et al. 1989, Winston 1987). These processes can also be reflected in changes to the host microbial community.

Changes to colony structure & symbiont community Colony size decreases over the winter months, reaching lows of $\sim$ 20,000 bees. This decline can represent a $\sim$ 50% reduction in the number of bees compared to the summer state (Winston 1987), where *Arsenophonus* is most prevalent in colonies. Seasonal drops in population size will be important for *Arsenophonus* infection dynamics, as symbiont spread can be related to the density of hosts (which may change with pop size) and persistence is only possible if host numbers are above a critical threshold (Anderson & May 1981, 1979). However, the effect on *Arsenophonus* dynamics will further depend on whether transmission is density or frequency-dependent (Begon et al. 2002).

Changes to the structure of the gut microbial community are reported with

overwintering, and may hold relevance for *Arsenophonus* arising from to its localisation in the *A. mellifera* gut. Species considered opportunistic, such as Providencia rettgeri, increase in prevalence overwinter from 25% to 83% of individuals (Lyapunov et al. 2008). This related genera of gamma-Proteobacteria (Darby et al. 2010) shows other commonalities with *Arsenophonus* in bees. *Providencia rettgeri* can be similarly found in the gut of perceived healthy *A. mellifera*, but under conditions of septicaemia invades the haemolymph (Sammataro & Yoder 2012, Tysset & Rousseau 1967). An apparent substitution of *Morganella morganii* for *Proteus vulgaris* was also observed in winter bees, and pathogenic *Hafnia* sp. and *Citrobacter* sp. present in autumn were absent by spring (Lyapunov et al. 2008).

Such changes in *A. mellifera* overwinter will alter competition dynamics within the gut community. With space and nutrition limited in the tissue, community changes may decrease the fitness of *Arsenophonus* – a microbe with a generally low abundance in the *A. mellifera* gut – through active interference or passive competition for resources. However, the extent of selection for competition will depend on cell density and metabolic overlap of *Arsenophonus* with other coinfecting microbes (Ghoul & Mitri 2016). Unfortunately information on these traits is currently lacking for the *A. mellifera* strain, and await a more complete genome sequence.

Overwintering Temperature Effects Temperature is well recognised as a modulator of host – microbe interactions, affecting the establishment and maintenance of both infectious parasites (Thomas & Blanford 2003) and heritable microbes (Corbin 2017). The transition to the winter state sees a deviation from the colonies summer temperature of 35.5°C (min 33.8°C, 37.0°C) (Fahrenholz et al. 1989). During the winter the core of the thermoregulating cluster is maintained at an average of 21.3°C (min 13°C , max 35.5°C) but bees on the cluster edge are maintained at 13°C, sometimes dropping as low as 6°C (Döke et al. 2015). This $\sim$14°C drop in temperature could affect the infection dynamics of *Arsenophonus*. Symbiont traits of latency, transmission, virulence and replication can all be affected by tempera-

ture (Fels & Kaltz 2006, Kelly et al. 2002), as can host resistance and recovery mechanisms (Linder et al. 2008). These changes have short term consequences for the individual players but can also affect long term coevolutionary dynamics in populations (Wolinska & King 2009). Even small changes to the thermal environment can affect infection outcomes via alterations to host or symbiont processes, or interplay between both players. For example, a 2°C rise in environmental temperature allows recovery of the grasshopper *Zonocerus variegatus* from the fungal pathogen *Entomophaga grylli* (Blanford et al. 2001, Chapman & Page 1979). Changes in temperature associated with overwintering may affect host and symbiont processes in diverse ways, such that colonies clear infections of non-beneficial symbionts accumulated over the foraging season.

Transmission efficiency is a key factor governing the spread and persistence of a symbiont and can be altered with changes to the thermal environment (Corbin 2017). In a number of cases transmission has become impaired with a drop-in temperature. In *Drosophila hydei* a 10°C reduction in temperature from the optimum (25°C) blocks the transmission of protective *Spiroplasma* HY1 (Osaka et al. 2008). In overwinter eggs produced by *Acyrthosiphon pisum* the symbiont *Regiella insecticola* suffers segregational loss, contrasting with perfect vertical transmission in summer eggs (Moran & Dunbar 2006). Here is an example of the overwintering process being associated with a change to the fundamental biology of symbionts, yet as Corbin et al., (2016) noted, the effects of overwintering on the transmission of symbionts is rarely explored. Over wintering processes can have important implications for the annual dynamics of symbionts, and on a broader level could inform our understanding (as a proxy) of environmental change (ie. temperature, humidity, weather effects) on host – symbiont interactions.

While the previous examples focused on heritable microbes, the effects of temperature on transmission can also hold true for horizontal (infectiously) transmitted symbionts (Stacey et al. 2003, Vale et al. 2008, Wolinska & King 2009). Lower temperatures are associated with lower parasite growth and infectivity; however the

net effect of temperature change can be complex, with impairment to traits sometimes offset by lowered parasite mortality (Lafferty 2009, Studer et al. 2010). The consequences of impaired transmission efficiency, due to reductions in temperature or other variables, could lead to elimination of symbionts from host population during the winter period.

Does overwintering block environmental acquisition? Alterations in the behaviour of the colony and changes to the biotic environment over the winter may also modulate exposure of *A. mellifera* to *Arsenophonus*. This could occur by overwintering blocking access to reservoirs of the symbiont. Foraging ceases and *A. mellifera* withdraw into the hive, causing a significant drop in the frequency and intensity with which *A. mellifera* interact with the natural environment (Johansson & Johansson 1979).

If *Arsenophonus* is an environmentally acquired symbiont (as several lines of evidence point to), or if this transmission path is important for the invasion/maintenance of the bacterium in host population, then this reduction in exposure may be sufficient for elimination of the infection. If there are additional effects on the symbiont, such as reduced within colony transmission, these could act synergistically with reduced reservoir exposure. These overwintering factors could feasibly affect transmission efficiency, contact rate and the duration of *Arsenophonus* infection, with ultimate implications for the symbiont fitness. If these processes are conserved across *A. mellifera* colonies it may explain the universal loss from colonies, with constriction/retreat of *Arsenophonus* population to an environmental or non-*Apis* host reservoir that then seeds the annual epidemics of the bacterium. Identification of *Arsenophonus* in the colonies by autumn may then be dependent on re-infection from the reservoir. This hypothesis is consistent with the observation of one colony that gained *Arsenophonus* by spring, as this colony was sampled later in the spring window (May), perhaps increasing chances of exposure or contact with the reservoir.

Typically heritable insect symbiont genera are not associated with complex epidemiology of mixed-mode transmission and multiple hosts/reservoirs. However this lifestyle is relatively common among infectious parasite species (Gandon 2004) and as we have seen *Arsenophonus*, although almost always heritable, is a diverse genus with respect to symbiotic lifestyle. The strain associated with *A. mellifera* may have one of the most complex and nuanced lifecycles identified within the clade to date.

The importance of overwintering dynamics Overwintering occupies a significant proportion of the year and is a critical time for *A. mellifera*. Colony losses are common over the winter and have risen to ∼30% over recent years (Neumann & Carreck 2010, VanEngelsdorp et al. 2009). As pressures mount, it will be increasingly important to understand the dynamics of all *A. mellifera* associated microbes (eg. pathogens through to mutualists) in the context of the behavioural, physiological and environmental changes experienced by the colony overwinter. If we achieve this, we will be better placed to adjust bee keeping practices within an evolutionary framework. By knowing, for example, the most effective time to deliver treatment in the annual dynamics of a target parasite and what changes mediate the emergence of opportunistic phenotypes. Or by identifying facultative symbionts that confer seasonally dependent benefits to *A. mellifera* and may be amenable for probiotic style applications.

Current beekeeping practices largely ignore evolutionary theory and, in some instances, even select for heightened virulence and more frequent disease outbreaks (Brosi et al. 2017). However, generating the most effective methods demands better resolution at the microbial and seasonal level. Overwintering dynamics of common *A. mellifera* pathogens (Desai & Currie 2016) and core microbiota (Ludvigsen et al. 2015) have been studied, but many non-core microbes of a commensal or unknown phenotype have been neglected. Despite increasing awareness that such common (but non-core) microbes can be important for direct or indirect com-

petition with pathogens, and in some instances can evolve to be significant extensions to host defence (King et al. 2016). Understanding the changes that occur in facultative symbiont communities during the critical winter period may be a valuable focal point and could see some of the greatest returns.

3.4.3 How does infection persist despite the instability of the host-microbe interaction?

The instability observed at individual and colony level imply that gain of infection events must be occurring. Environmental (horizontal) transmission is often considered to select for parasitic phenotypes (Sachs & Simms 2006) and hosts may require strict sanction mechanisms to avoid the emergence of cheater mutants (Douglas 2008, Kiersi & Denison 2008, Sachs et al. 2011). Yet marine hosts acquire bioluminescent bacteria and legumes take up nitrogen fixing bacteria in each life cycle (Bright & Bulgheresi 2010), showing this type of acquisition can be a stable strategy under certain conditions – often soil and marine habitats. But for insect hosts environmental acquisition of obligate endosymbionts appears to be uncommon (Kikuchi et al. 2007) . For facultative symbionts environmental acquisition occurs more frequently, but is normally predicted to occur through HT from another host species (biotic source) than an abiotic source (Gonella et al. 2015). Based on phylogenetic inference environmental acquisition has been suggested for facultative *Arsenophonus* strains infecting Hippoboscidae (louse flies) (Šochová et al. 2017) and *A. Nasoniae* can transmit interspecifically between parasitoid wasp host species (Duron et al. 2010). Generally however environmental acquisition *Arsenophonus* does not show regular infectious transmission within the clade.

However, outliers do emerge within clades of bacterial symbionts. The obligate bioluminescent symbionts of flashlight fish share many genomic features (ie. 1.11 Mb size) with obligate insect endosymbionts, yet they exist extracellularly and are released into seawater, where there is evidence for pseudo-VT (Haygood 1993). But the bacterium is unusual in being nested within a family of free-living or facultative

symbionts, with only HT (Hendry et al. 2016). The trend appears to be the inverse for the possibly environmentally acquired *A. mellifera Arsenophonus*, with the strain nested within a clade of largely vertically transmitted symbionts.

Environmental acquisition can be a key part of transitions in symbiotic lifestyle, as the ancestral state of all symbionts must be free-living (Sachs et al. 2011, 2013). A real-time case study by (Hosokawa et al. 2016) highlights this. Currently all evolutionary stages of symbiosis are observed among the coexisting *Pantoea* lineages in the stinkbug *P. stali*, from free-living mutualists to unculturable obligates circulating at fixation. The symbionts are vertically transmitted by egg smearing, and their removal severely impacts host fitness. But provisioning with environmentally sourced soil (containing *Pantoea* spp.) rescues the host phenotype and restores growth (with the exception of two obligate strains) (Hosokawa et al. 2016). Considering the potential for symbiotic interactions in the *Arsenophonus* clade, if the *A. mellifera* strain retains an environmental stage it may be well situated to replace or supplement existing symbionts.

Enterobacteriaceae genera appear to be well adapted for transitioning between ecological and symbiotic niches (Sachs et al. 2011, Walterson & Stavrinides 2015). *Pantoea* and *Sodalis* include representatives from soil and other biotic sources, plant hosts, clinical settings and insect hosts (Chrudimský et al. 2012, Clayton et al. 2012, Kenyon et al. 2015, Rubin et al. 2017). The *Arsenophonus* genus differs, in that all strains to date are believed to be arthropod host-restricted and environmental acquisition has not been shown to occur routinely (Nováková et al. 2009). However, the examples here show environmental acquisition can be fundamentally important for endosymbiont maintenance in host populations. This is the case even for some mutualistic associations in non-soil/aqueous habitats, despite the dogma of strict VT as representing the best conditions for fostering the evolution of beneficial phenotypes (Foster & Wenseleers 2006). In some cases microbes acquired directly from the environment can provide immediate benefits as well as costs (Sachs et al. 2011). It is increasingly apparent there is a dynamic continuum from free-living

environmental microbes to strict host associated symbionts. Evidence presented in this chapter suggest *Arsenophonus* may span more of this continuum than previously realised.

Future directions: does *Arsenophonus* utilise *A. mellifera* as a vector? The decline of *Arsenophonus* with overwintering and laboratory captivity suggest an unstable association vulnerable to rapid breakdown. It is likely this instability is explained by context-dependent infection outcomes and persistence. However, the possibility of *A. mellifera* functioning as only/or predominantly a vector must also be considered. Individuals may be determined positive for a symbiont even when the host is simply carrying the microbe as a passenger. However, such non-target host species can function as highly important vectors for dispersal of symbionts, with potentially underestimated implications for symbiont epidemiology (Graystock et al. 2015)

The cuticle and gut of bees may pick up microbes during foraging associated activities and disperse them to new sites, with experimental evidence showing such movements can occur rapidly. In under 6 hours parasites could be dispersed from *Bombus* colony to *Apis* colony via floral hubs (Graystock et al. 2015). When carriage only is occurring, it is possible symbionts are lost as rapidly as they are gained. This process is consistent with the abrupt loss of *Arsenophonus* in some *A. mellifera* individuals during our persistence studies.

Even if in some cases *Arsenophonus* does not establish infection in *Apis*, presence of the bacterium in the gut and faecal matter could be an effective strategy for dispersal. For example, the *Bombus* parasite *Crithidia bombi* can be ingested by *A. mellifera* but does not cause active infection in this species (Ruiz-GonzáLez & Brown 2006). Nevertheless, the parasite remains viable following transport through the gut, potentially allowing onward transmission (Ruiz-Gonzlez & Brown 2006).

Chapter 4

Phenotypic Divergence: Transmission of *Arsenophonus* in *Apis mellifera*

Abstract *Arsenophonus* is commonly associated with *A. mellifera* and can form systemic infections. Overwintering loss of infection combined with pronounced seasonal dynamics implies the classic mode of transmission of *Arsenophonus* – vertically from female to progeny through eggs – may not be operating in the interaction with *A. mellifera*. To determine presence/absence of vertical transmission, *Arsenophonus* infection was tracked across the host life history. To this end, the eggs, larvae, pupae and newly emerged workers of *Arsenophonus* infected colonies were screened for the presence of the symbiont. *Arsenophonus* was rarely detected through these life stages and the symbiont was consistently found only in workers and drones, with a lower prevalence in newly emerged workers compared to older siblings. These data indicate acquisition following emergence into adult phase. The capacity for horizontal transmission of *Arsenophonus* within colonies was assessed by exposure of uninfected workers to infected *A. mellifera* under differing social conditions. *Arsenophonus* was transmissible via stomodeal trophallaxis in a small

number of cases, but general social contact facilitated transmission more frequently. There was notable heterogeneity in transmission outcomes, potentially governed by super spreader effects. *Arsenophonus* was also lost from a notable proportion of infected *A. mellifera* during the transmission study, consistent with results from chapter 3. Together these result imply *Arsenophonus* is not strictly heritable in *A. mellifera*, instead environmental acquisition and social transmission govern symbiont dynamics. These data indicate a novel symbiotic phenotype for *A. mellifera* - *Arsenophonus* among the currently characterised members of the genus.

4.1 Introduction

From parasites through to mutualists, the transmission of symbionts is a fundamental driver of both their ecological and evolutionary dynamics. Two major modes of symbiont transmission are recognised, each with a multiplicity of potential routes and incurring differing evolutionary trajectories (Ewald 1987). Vertical (heritable) transmission (VT) involves the transfer of symbionts directly from parent to progeny often via the female line (Douglas 2010, Perlman et al. 2015, Werren & O'Neill 1997). Mechanistically, this may reflect intracellular transmission of symbionts within eggs (Purcell et al. 1986), transmission through gland secretions (Attardo et al. 2008), extracellular transmission via smearing/inoculation of eggs or pupae (Hosokawa et al. 2012, Kaltenpoth et al. 2012), and brood provisioning via capsules and jelly (Salem et al. 2015). Horizontal (infectious) transmission (HT) entails the transfer of symbionts between con- or hetero-specifics or from environmental sources (Gordon et al. 2016, Hosokawa et al. 2016, Kikuchi et al. 2007). Infectious transmission routes are diverse, and include sexual and social contact (Moran & Dunbar 2006, Smith & Mueller 2015), coprophagy (Nalepa 2015), soil/food/water (Slifko et al. 2000) and airborne (Hawker et al. 1998). In some cases the boundaries between VT and HT can be dynamic, with short-term switches in mode occurring in response to stimuli or longer-term evolutionary transitions in response to selec-

tion (Ebert 2013).

Transmission mode affects the evolution of host – symbiont interactions, generating a number of predictions (Ewald 1987). Vertical transmission tightly aligns the fitness of both host and symbiont, and thus associations are expected to evolve towards mutualism (Ebert 2013, Foster & Wenseleers 2006). For horizontally transmitted symbionts the correlation between host and symbiont fitness is less clear, and a trajectory towards parasitism is often assumed (Anderson & May 1982, Frank 1996*a*). This trajectory is driven by increased opportunities for co-infecting symbiont genotypes, cheater strains and trade-offs between symbiont replication and the likelihood of host mortality (the virulence – transmission trade-off)(Alizon et al. 2009, Anderson & May 1982). For HT symbionts exhibiting vector borne transmission, selection promotes low virulence in the vector (to maintain a healthy carrier host) but may favour high virulence in the static host (to enable acquisition by the vector)(Ewald 1983). For environmental transmission, virulence predictions are generally highest, as here a healthy host may not be required for infection, and may even reduce R_0 (Ewald 1983). However, exceptions to model predictions are widely evident, indicated by widespread VT parasites that have deleterious effects on host fitness (Bandi et al. 2001, Hurst & Frost 2015, Wenseleers et al. 2002) and a plethora of HT mutualists that form stable cooperative symbioses (Harmer et al. 2008, Kikuchi et al. 2007, McFall-Ngai 2014).

Within the *Arsenophonus* clade of symbionts, transmission across host generations is predominantly vertical, yet a remarkably diverse assortment of phenotypes from parasitism to mutualism are observed (Nováková et al. 2009). Two *Arsenophonus* species associated with Hippoboscidae (louse flies) are obligate for host functioning, probably conferring anabolic functions, and have genomes that are in the later stages of erosion that is characteristic of obligate symbionts (Chrudimský et al. 2012, Nováková et al. 2016). Observations of HT within the clade are sparse, and always occur in addition to VT. In contrast, the reproductive parasite *A. Nasoniae* can transmit horizontally, both intra- and interspecifically, between wasps

co-parasitizing dipteran pupae (Duron et al. 2010, Parratt et al. 2016); *Arsenophonus* spp. associated with Cixiidae are horizontally transmitted via plants where they induce disease symptoms (Bressan 2014). Thus, in line with theoretical predictions, it also appears that members of the *Arsenophonus* clade that do exhibit HT also shows traits associated with parasitism.

Arsenophonus is found in a diversity of arthropod species, including both solitary (e.g. Hypsa & Dale (1997), Perotti et al. (2007), Santos-Garcia et al. (2018)) and social taxa (Parmentier et al. 2018, Sebastien et al. 2012, Yaïez et al. 2016), and so far work has largely focused on solitary host species. For these taxa, overlapping generations and contact with conspecifics can be rare, demanding selection for high fidelity transmission routes that do not require direct contact. In contrast, eusocial insect hosts present special cases for symbiont transmission. Colonies of eusocial hosts are often characterised by high densities of related individuals and specialised social behaviours (Holldobler & Wilson 1990, Wilson 1971) that foster direct contact, such as proctodeal (anus – mouth feeding) and stomodeal trophallaxis (mouth - mouth feeding). These conditions represent an arena for the transmission of mutualists and parasites (Onchuru et al. 2018), with inevitable trade-offs for all parties (Hughes et al. 2002) (see Table 4.1 for more information, adapted from Onchuru et al. 2018).

Alongside *Arsenophonus*, which has been found in ants (Nováková et al. 2009), social bees (Budge et al. 2016, Cornman et al. 2012, Parmentier et al. 2018) and wasps (Loope et al. 2019), other bacteria that belong to classically considered genera of heritable endosymbionts are increasingly identified in eusocial hosts. *Wolbachia* and *Spiroplasma* are widely prevalent across ant species (Ballinger et al. 2018, Funaro et al. 2011, Russell et al. 2012, Wenseleers et al. 1998) and *Cardinium* has also been noted (Sirviö & Pamilo 2010). A wide range of termites are also associated with *Wolbachia*, however eusocial wasps and bees appear to be more uncommon (Andersen et al. 2012, Gerth et al. 2013, 2015, Lo & Evans 2007, Russell, Moreau, Goldman-Huertas, Fujiwara, Lohman & Pierce 2009, Russell et al. 2017). While

	Transmission Route			
	Trophallaxis	Coprophagy	Environmental	Transovarial
Requirement for transmission:				
Host adaptations	Advanced social behavioural	Behavioural	Behavioural & ecological	None clear, but more common in solitary hosts
Symbiont adaptations	Gut colonisation	Gut colonisation & survival in faeces	Survival in environment	Integration into ovarial machinery
Cost/benefit to host:				
Reliability	High	Low	Low	High
Parasite co-transmission risk	High	High	Low	Low
Opportunistic microbe risk	Low	High	High	Low
Predictions for symbiont evolution:				
Virulence predictions	Low	Medium	High	Low
Dependency on host	Medium	Medium	Low	High
Scope for co-infecting genotypes	Medium	Medium	High	Low

Table 4.1. Predicted prerequisites and potential cost/benefits associated with different symbiont transmission routes, adapted from Onchuru *et al.* 2018

basic phenotypic knowledge is lacking for most of these endosymbionts, interesting heterogeneities in infections are emerging based on caste (Anderson et al. 2012, Van Borm et al. 2001, Frost et al. 2010, Keller et al. 2001, Wenseleers et al. 2002) and degree of sociality (Rubin et al. 2017). These associations present valuable opportunities for exploring the effects of sociality on symbiont evolution.

Chapter Objectives The *Arsenophonus* strain associated with *Apis mellifera* is nested within a clade of VT symbionts and sister strain to a reproductive parasite. In this chapter I explore the transmission phenotype of *Arsenophonus* in its eusocial host.

4.2 Methods

4.2.1 Analysis of *Arsenophonus* infection across *A. mellifera* life history

The presence of *Arsenophonus* in eggs and brood in *A. mellifera* colonies where *Arsenophonus* is present in workers, and absence in eggs and brood in colonies where *Arsenophonus* is absent, would be consistent with vertical transmission. To assess the heritability of *Arsenophonus*, frames containing *A. mellifera* worker brood were removed from managed field colonies in Yorkshire and Cheshire ($N = 8$, A+ = 6, A- = 2) where *Arsenophonus* status had previously been determined (see method section 2.2.2 & 2.2.3). Eggs (n = 29), larvae (n = 67), pupae (n = 49) and newly emerged workers (NEWs) (n = 36) were collected from frames in the laboratory using sterilised forceps and grafting tools. Adult workers (n = 45), and where possible drones (n = 22), were collected concurrently from the same colonies. All samples were preserved in sterile tubes in 70 - 100% EtOH at -20°C until DNA extraction. Prior to testing for *Arsenophonus*, samples were removed from EtOH and exposed to ultra violet (UV) light for 10 minutes to cross link contaminating external DNA. DNA was extracted from eggs and early stage larvae using QIAGEN ® DNeasy blood & tissue kit according to manufacturers instructions. Later stage larvae (whole) and pupae, NEWs, drone and worker samples (head removed) were extracted individ-

ually using a Wizard® genomic DNA purification kit (see section 2.2.3). Molecular detection of *Arsenophonus* spp. was performed as previously described.

The prevalence of *Arsenophonus* across *A. mellifera* life stages and castes was analysed by a generalized linear model (GLM) with binomial error distribution implemented in R version 3.3.1 (R Core Team 2013). *Arsenophonus* status was used as a binary response variable and life stage/caste as an explanatory variable. Due to zero inflation in the observations for *Arsenophonus* in eggs (0%, n = 29), this level was excluded from analysis by the model. A Hosmer-Lemeshow Goodness of Fit (GOF) test was implemented to aid assessment of model fit. Model predictions were plotted for the proportion of each lifestage/caste infected with *Arsenophonus* with 95% CI intervals. The raw data points, pertaining to individual samples, were jittered with random noise and coloured by *Arsenophonus* status (uninfected [0] = red, infected [1] = blue).

4.2.2 Do workers become infected with *Arsenophonus* in the absence of contact with infected individuals?

To assess if *Arsenophonus* infection developed with worker age, NEWs (N = 75) from *Arsenophonus* infected colonies (N = 3) were allowed to emerge naturally from comb cells within the laboratory. On chewing through the cell cap *A. mellifera* were removed immediately using sterile forceps. NEWs were mixed in groups of 15 in sterile pots, no contact was allowed with other colony members and sterile 50% sucrose solution was supplied. At approximate forager age (25 $\pm$ 2 days post-emergence) *A. mellifera* were culled and screened for *Arsenophonus* (as section 2.2.3).

4.2.3 Is *Arsenophonus* horizontally transmitted between conspecifics?

To evaluate the capacity for the horizontal transmission of *Arsenophonus* in *A. mellifera*, workers and brood frames were removed from *Arsenophonus* infected (n = 4) and uninfected colonies (n = 3). Infected colonies were selected if over 85% of workers tested positive for *Arsenophonus*, using methods described earlier. NEWs,

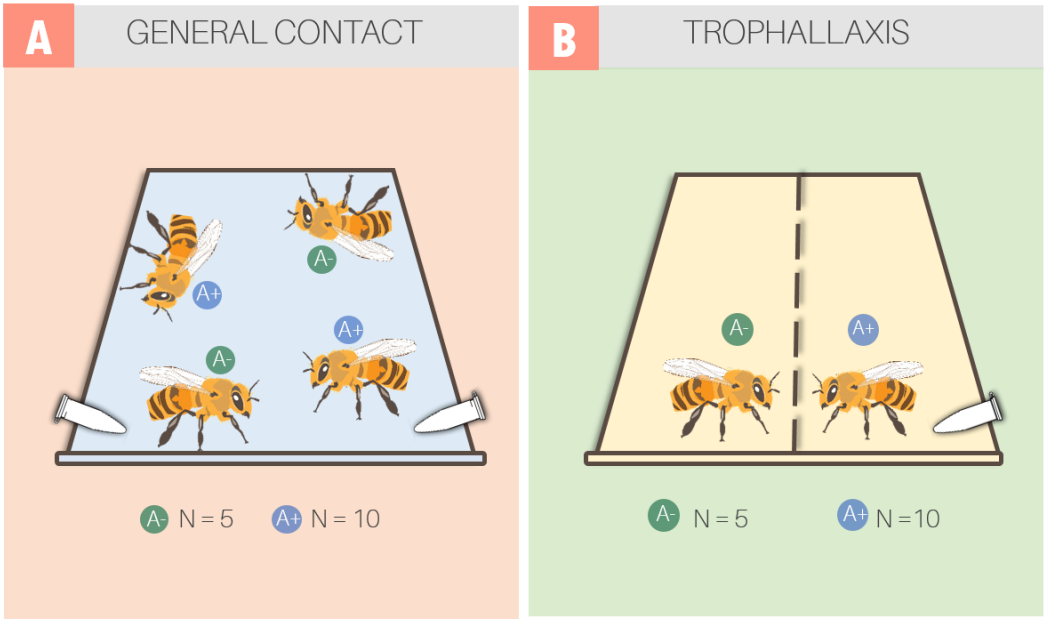

Figure 4.1. Horizontal transmission experimental setup. Workers (blue, A+) from *Arsenophonus* infected colonies were mixed with sterile newly emerged workers (NEWs) (green, A-) under differing social conditions. All interactions were allowed in general contact pots (A). In trophallaxis pots (B) infected *A. mellifera* were forced to feed the uninfected NEWs. Negative controls were established in the same manner with uninfected workers and NEWs.

which rarely carry *Arsenophonus*, were allowed to emerge naturally from the frame (within the laboratory) and marked to allow subsequent identification. Two transmission treatments were established (see Figure 4.1):

(A) General contact – workers were mixed with NEWs. All contacts were allowed between all individuals and two food sources were openly available to the whole pot.

(B) Trophallaxis - workers were placed in the same pot as NEWs, but separated by fine wire mesh through the pot centre. Food was available only on the workers side.

In each transmission treatment pot five NEWs (presumed A-) were mixed with 10 workers (presumed over 85% A+), with ten pot replicates for each transmission treatment. Negative controls were established using workers from *Arsenophonus*

uninfected colonies (A-) and NEWs (A-), with five pot replicates for each transmission treatment. Pots were 340ml deli pots with filter paper. Bees were fed 50% sucrose solution and maintained under laboratory conditions as previously described (4.2.2).

Contact was allowed for 5 days for all pots, after which all *A. mellifera* were culled. The fate (dead or alive) of all individuals at the end of the study was also recorded, i.e. those that had died during the course of the experiment (prior to culling) were deemed dead and analysed separately. *Arsenophonus* status of *A. mellifera* was assessed by PCR assay as detailed previously. Individual *A. mellifera* (test: n = 300, negative control: n = 150), excluding heads, were homogenised in nuclei lysis solution for 1 minute at 4000rpm and DNA was extracted using a Wizard ® genomic DNA purification kit (see section 2.2.3). Molecular detection of *Arsenophonus* spp. was performed as previously described (see section 2.2.4).

The horizontal transmission of *Arsenophonus*, via trophallaxis and general contact, was assessed using generalized linear mixed models (GLMMs) with binomial error distributions. GLMMs were constructed using the `lme4` package (Bates et al. 2015) in R version 3.3.1 (R Core Team 2013). *Arsenophonus* status (0 = uninfected, 1 = infected) was modelled as a binary response variable. Status (worker or NEW), transmission (general contact or trophallaxis), colony ID (4 levels) and fate (dead or alive) were modelled as fixed effects. Pot (10 levels per transmission treatment) was modelled as a random effect. Minimum adequate model selection (MAM) and checks were performed as described previously (section 2.2.6).

Evidence of within pot correlations between *Arsenophonus* prevalence in NEWs and workers prompted a further statistical analysis. For each pot the prevalence of *Arsenophonus* in NEWs was modelled as a function of *Arsenophonus* prevalence in workers, using a generalized linear model (GLM) with binomial error distributions. All analysis was completed in R version 3.3.1 (R Core Team 2013), model selection (MAM) and checks were performed as described previously (section 2.2.6).

4.3 Results

4.3.1 Analysis of *Arsenophonus* infection across *A. mellifera* life history

GLM predictions for the proportion of each life stage/caste infected with *Arsenophonus* are shown in Figure 4.3. There was no evidence of transovarial transmission of the bacterium in colonies where workers were *Arsenophonus* positive, with eggs from these colonies consistently testing negative (0%, 95% CI: 0 – 11.9%). Infection was observed in other life stages, but with low fraction of individuals infected compared to the 82.2% infection frequency recorded for mature workers (95% CI: 67.9 – 91.0). 5.97% of larvae tested positive for *Arsenophonus* infection (95% CI: 1.65 – 14.6, p = 3.28 x 10^{-11} ***) and 4.081% (95% CI: 0.498 – 14.0, p = 1.10x10^{-8} ***) of pupae were scored as *Arsenophonus* positive by PCR assays.

The proportion of newly emerged workers (NEWs) infected with *Arsenophonus* (2.78%, 95% CI: 0.07 – 14.5) was also significantly lower (p = 2.85x10^{-6}***) than the infection frequency observed in workers. Overall, the incidence of *Arsenophonus* was significantly lower ($p < 0.05$) among all early life stages compared to worker *A. mellifera*. Negative controls (brood from colonies without *Arsenophonus*) tested negative throughout. The incidence of *Arsenophonus* was notably higher in the adult life stages. 54.54% (95% CI: 32.2 – 75.6) of drones were infected with the bacterium. However the frequency with which drones were infected remained significantly lower ($p = 0.0198$ *) than the worker caste.

4.3.2 Do workers become infected with *Arsenophonus* in the absence of contact with infected individuals?

A. mellifera NEWs (final $N = 64$, deaths = 11) removed from *Arsenophonus* infected colonies (based on worker screens) did not develop *Arsenophonus* infection on reaching forager age in any case (25 ± 2 days post-emergence).

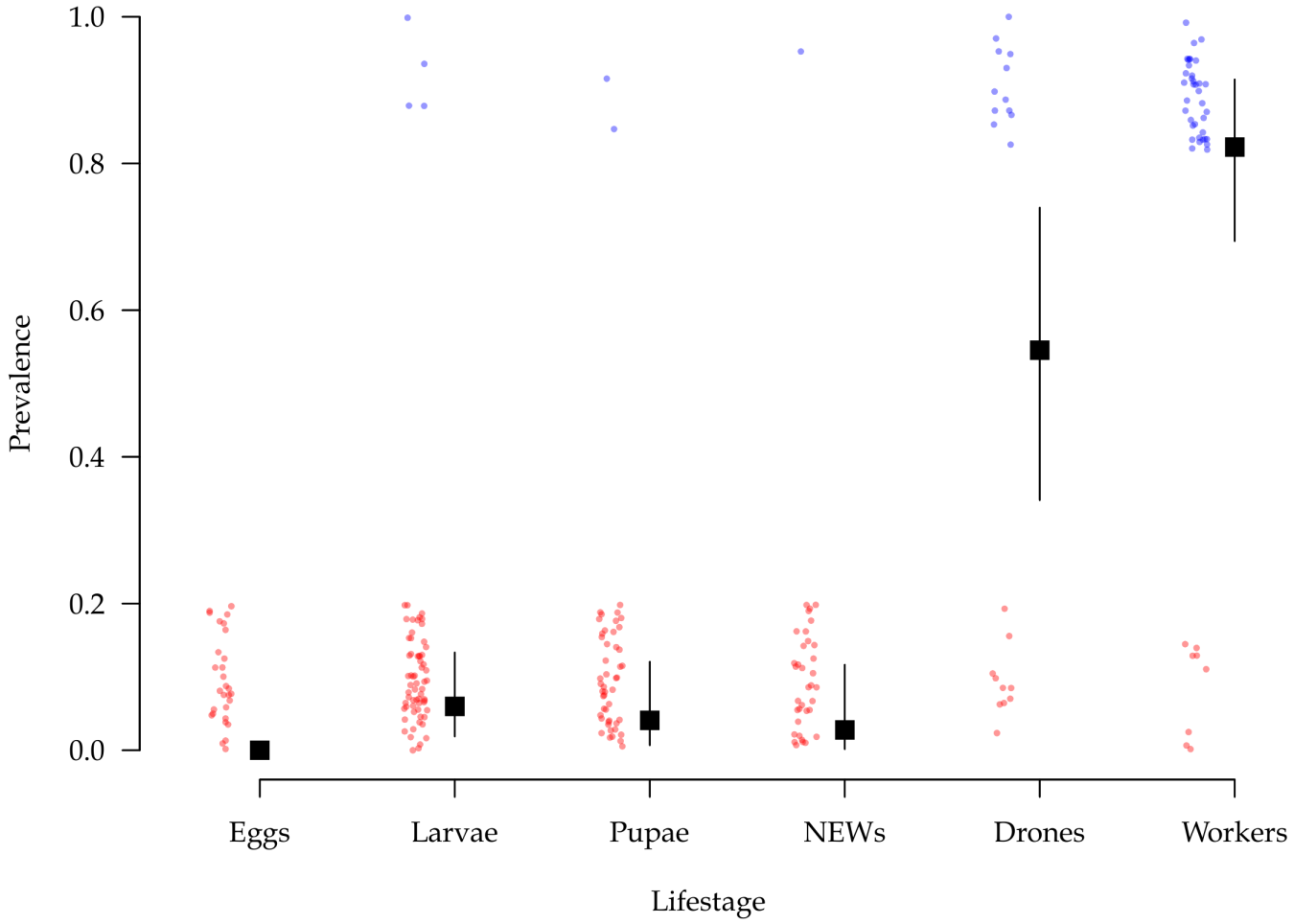

Figure 4.2. Prevalence of *Arsenophonus* among *Apis* mellifera lifestages and castes. All samples were taken from colonies (n = 6) previously identified as *Arsenophonus* positive and screened by diagnostic PCR. The results were analysed by a binomial generalized linear model, plotted points represent model predictions and error bars are 95% CI. Blue dots (infected = 1) and red dots (uninfected = 0) show the real data points jittered. Workers used as reference; Larvae***, Pupae***, NEWs*** $p > 0.001$, Drones* $p > 0.05$. The model fitted the observed data well ($p > 0.05$), Hosmer-Lemeshow GOF test (X^2 = 3.982x10-22, df = 8, p = 1).

4.3.3 Is *Arsenophonus* horizontally transmitted between conspecifics?

General contact & trophallaxis as transmission routes In contrast to experiments where NEWs were isolated on emergence, infection was observed to develop in NEWs when exposed to *Arsenophonus* infected older workers, either through general mixing or through trophallaxis alone (Figure 4.3). The final statistical model comprised of: response variable = *Arsenophonus* status (0 or 1); fixed effect = *A. mellifera* status (worker or NEW) and transmission type (gen. contact or trophallaxis); random effects = pot (AIC= 303.3, *df*=5). See Table 4.2 for a summary of model (GLMM) selection; estimates from the final model (Tr4) are shown for fixed effects in Table 4.3 and random effects Table 4.4. Model predictions for the proportion of individuals infected with *Arsenophonus* based on all possible combinations of the fixed effects is shown in Table 4.5.

The interaction term between *A. mellifera* status (worker or NEW) and transmission type (mixing or trophallaxis) was not significant (Likelihood ratio test Tr1:Tr2, $X^2 = 1.05$, $df = 1$, $p = 0.306$) and colony identity was also not significant as a fixed effect (Likelihood ratio test Tr2:Tr3, $X^2 = 3.13$, $df = 3$, $p = 0.372$). Fate (live/dead at end of experiment) emerged as an important predictor of infection status (Likelihood ratio test Tr3:Tr4, $X^2 = 7.64$, $df = 1$, $p = 0.006$**). Individuals that had died during the study had a significant positive association with *Arsenophonus* infection ($p = 0.009$ **). However observations of dead individuals were not evenly distributed across colony identities. Worker *A. mellifera* were associated with a slightly higher prevalence of *Arsenophonus* compared to NEWs, however this was not significant ($p = 0.125$). The trophallaxis only treatment resulted in a significantly lower *Arsenophonus* prevalence ($p = 0.01697$*) than the general contact treatment. Pot identity explained a notable amount of variation in *Arsenophonus* prevalence within the transmission study (variance = 2.371).

***Arsenophonus* prevalence in NEWs as a function of workers** The within pot prevalence of *Arsenophonus* in NEWs was modelled as a function of the correspond-

ID	Model [GLMM]	*df*	AIC
Tr1	*Arseno.* ~ Status * Transmission + Colony + Fate + (1| Pot)	9	307.2
Tr2	*Arseno.* ~ Status + Transmission + Colony + Fate + (1| Pot)	8	306.2
Tr3	*Arseno.* ~ Status + Transmission + Fate + (1| Pot) [SM]	5	303.3
Tr4	*Arseno.* ~ Status + Transmission + (1| Pot)	4	309.0
Tr5	*Arseno.* ~ Status + Fate + (1| Pot)	4	306.6

[ID] in-text reference [df] degrees of freedom, [AIC] Akaike's information criterion, [SM]selected model
Status = Worker or NEW, Transmission = Gen contact or Trophallaxis, Fate = Dead or Alive

Table 4.2. Model (MAM) selection: *Arsenophonus – A. mellifera* horizontal transmission

Variable	Estimate	Std. Error	Z - value	Pr (>|z|)
Intercept	-0.2764	0.5831	-0.474	0.63551
Status (Worker)	0.5058	0.3298	1.534	0.12511
Transmission (Trophall.)	-1.8417	0.7714	-2.387	0.01697 *
Fate (Dead)	1.9295	0.7385	2.613	0.00898 **

***P < 0.001 ,**0.01, *0.05

Table 4.3. GLMM (Tr3) fixed effect coefficients: *Arsenophonus – A. mellifera* horizontal transmission

Random Effect	Variance	Std. Deviation
Pot	2.371	1.54

Observations (n = 300), Pots (n = 20)

Table 4.4. GLMM (Tr3) estimates for random effects (Tr3)

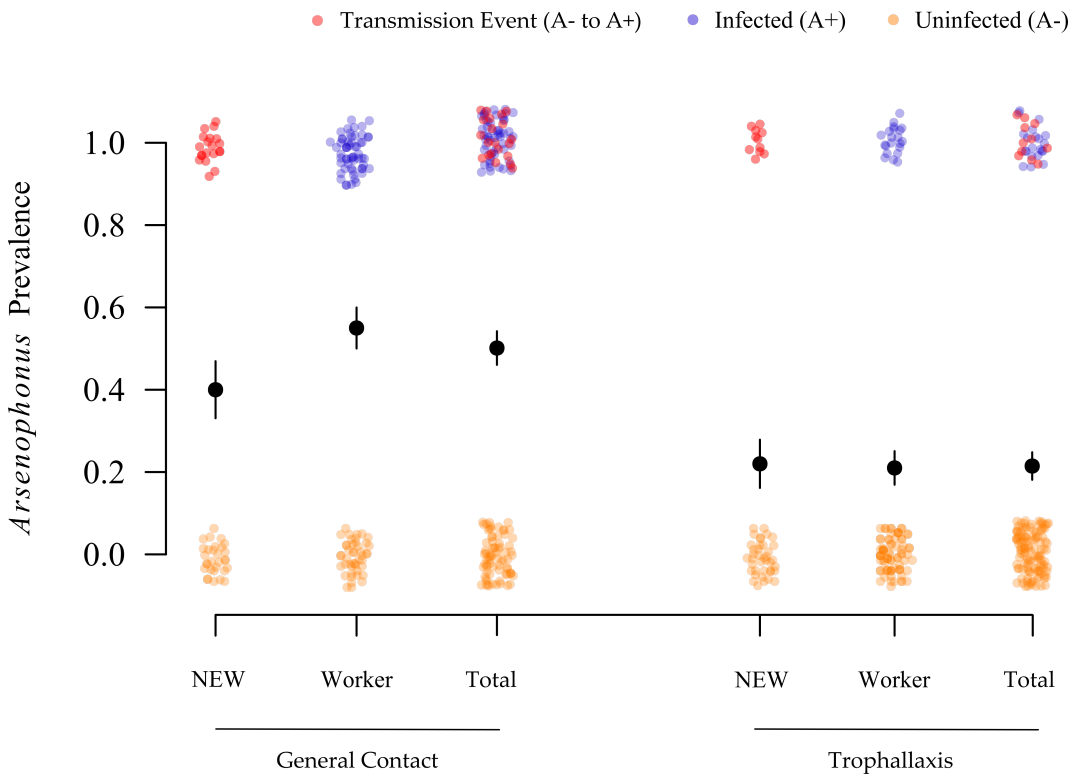

Figure 4.3. Total horizontal transmission of *Arsenophonus* under differing social conditions (general contact & trophallaxis only). The proportion of NEWs, workers and total (NEWs + workers) that tested positive for *Arsenophonus* after the 5 day transmission period (TP) is shown for each social condition. At the start of the transmission period NEWs were presumed 100% A- and over 85% of workers were presumed A+ (see methods). Coloured dots represent the raw binomial data jittered (orange, uninfected = 0 & blue, infected = 1), red dots indicate known transmission events and represent a NEW becoming infected with *Arsenophonus* by the end of the transmission period. For presentation purposes the results from all pot replicates are pooled and negative controls are not shown. Error bars represent binomial CIs and all detection was based on diagnostic PCR assays.

Transmission	Status	Fate	Predict. *Arseno.* proportion
General contact	NEW	Dead	0.84
	NEW	Alive	0.43
	Worker	Dead	0.90
	Worker	Alive	0.56
Trophallaxis	NEW	Dead	0.45
	NEW	Alive	0.11
	Worker	Dead	0.58
	Worker	Alive	0.17

Table 4.5. Model (Tr3) predictions for all fixed effect combinations: *Arsenophonus* – *A. mellifera* HT

ing prevalence of *Arsenophonus* in workers and an overview assessment of the best explanatory variables is shown in Table 4.6.

The colony that *A. mellifera* derived from was not a significant explanatory variable (Likelihood ratio test Co1:Co2, p = 0.298) for the observed data. Transmission type (general contact or trophallaxis) was not significant within the model, either as an interaction with *Arsenophonus* prevalence (LRT Co2:Co2a, p = 0.134) or as a standard explanatory variable (LRT Co2a:Co3, p = 0.147). As no significant difference was observed between the models the most minimal model (Co3) was selected (Arseno.Prev.NEWs $\sim$ Arseno.Prev.Workers), the final model coefficients are shown in Table 4.7. A strong positive correlation ($p = 9.44 \times 10^{-7***}$) was observed across the pots between *Arsenophonus* prevalence in NEWs and prevalence of the bacterium in workers, the model predictions are plotted in Figure 4.4. An overall loss of *Arsenophonus* from worker *A. mellifera* was observed across all pots (assuming $>$ 85% starting *Arsenophonus* prevalence).

ID	Model [GLM]	*df*	AIC
Co1	Arseno.Prev.NEWs ~ Arseno.Prev.Workers * Transmission + Colony	7	56.6
Co2	Arseno.Prev.NEWs ~ Arseno. Prev.Workers * Transmission	4	54.3
Co2a	Arseno.Prev.NEWs ~ Arseno. Prev.Workers + Transmission	3	54.5
Co3	Arseno.Prev.NEWs ~ Arseno Prev.Workers [SM]	2	54.6

[ID] in-text reference [df] degrees of freedom, [AIC] Akaike's information criterion, [SM]selected model

Table 4.6. Model (MAM) selection: NEW *Arsenophonus* prevalence as a function of worker *Arsenophonus* prevalence

Variable	Estimate	Std. Error	Z- value	Pr (>|z|)
Intercept	-2.7555	0.5087	-5.416	6.09×10^{-08} ***
Arseno. Prev. workers	4.4299	0.9035	4.903	9.44×10^{-07} ***

P < ***0.001 ,**0.01, *0.05

Table 4.7. GLM (Co3) coefficients:

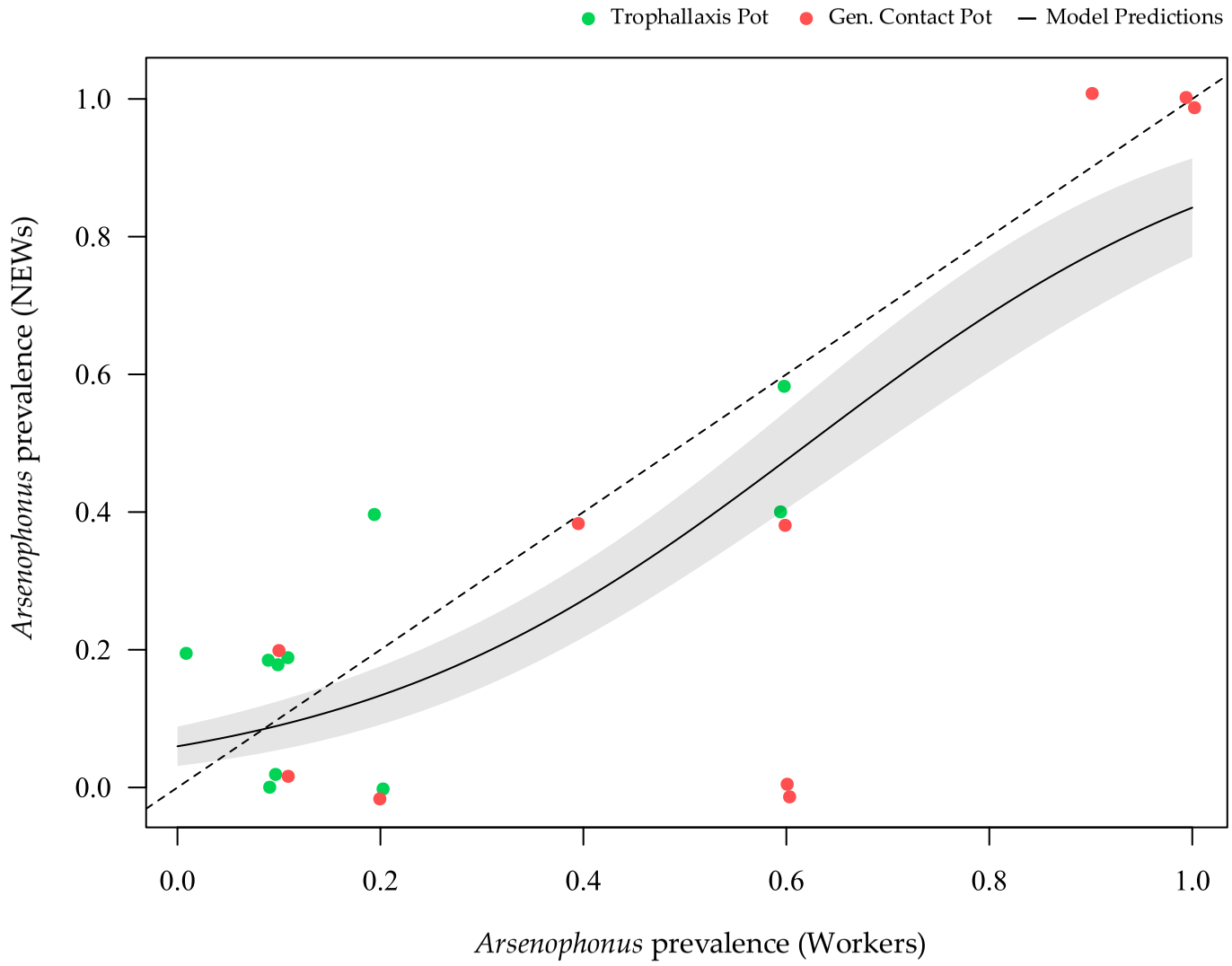

Figure 4.4. Horizontal transmission: *Arsenophonus* prevalence in NEWs as a function of *Arsenophonus* prevalence in workers is shown for each pot (circles) and each social treatment (green = trophallaxis only; red = general contact. Model predictions using observations from all pots (solid black line) shows a strong linear association between *Arsenophonus* prevalence in workers and detection of successful transmission events in NEWs, at the end of a 5 day mixing period. Analysis by a generalized linear model (GLM) in R, binomial confidence intervals are shown (grey shading).

4.4 Discussion

4.4.1 No heritability in *A. mellifera*: diversity within the *Arsenophonus* clade

Arsenophonus was absent from *A. mellifera* eggs within infected colonies, and detected at a very low incidence in larvae, pupae and NEWs. These results indicate strict vertical transmission through eggs is minimal in this system. Any vertical transmission that occurs is associated with transmission between conspecifics post emergence, and potentially *Arsenophonus* may be inherited by daughter colonies via swarming. However, loss of infection overwinter (Chapter 3) argues that even social pseudo-vertical transmission is incomplete, and interruptions to the social transmission cycle are common.

Lack of vertical transmission through eggs is striking for a genus that is comprised largely of heritable intracellular symbionts (Novakova et al. 2009). Across the clade, *Arsenophonus* spp. infect a diverse range of hosts, varied both phylogenetically and ecologically (Duron et al. 2008), and are transmitted vertically from parent to progeny, albeit via different mechanisms (Bressan et al. 2012, Huger et al. 1985, Nováková et al. 2016, Perotti et al. 2007).

Two other *Arsenophonus*-insect symbiosis show horizontal (infectious) transmission, but in each case vertical transmission is also known. *A. Nasoniae* is vertically transmitted, passing from a female wasp into the fly host at the point of oviposition, whereon it is taken up by the wasp larvae (Huger et al. 1985, Werren et al. 1986). This transmission mode additionally allows horizontal transmission between *Nasonia vitripennis* individuals (following superparasitism) and can drive fixation of the symbiont in host populations (Parratt et al. 2016). In addition, transmission between host species occurs during co-parasitism (Duron et al. 2010). The epidemiology of insect vectored *Arsenophonus* spp. also appears to be governed predominantly by horizontal transmission via host plants, but vertical transmission through eggs also occurs in the vector (Cixiidae planthoppers) (Bressan 2014, Bressan et al.

2009). In short, *Arsenophonus* spp. that demonstrate horizontal transmission show it in addition to vertical transmission.

Taken together, the *Apis* - *Arsenophonus* interaction is novel in having no evidence for vertical transmission and abundant evidence for infectious transmission. If *Arsenophonus* associated with *A. mellifera* is exclusively horizontally transmitted this will have implications for transitions in symbiotic lifestyle within the clade, and contribute to growing evidence of the importance of HT in heritable symbiont genera (e.g. Parratt et al. (2016))

4.4.2 Life stage susceptibility to *Arsenophonus*

Strict vertical transmission of *Arsenophonus* does not appear to occur in *A. mellifera*, however as young life stages are still exposed to both the colony environment and infected workers, the distribution of infection only in adult workers and drones merits further discussion. Early life stages did test positive for *Arsenophonus* in rare instances (larvae, n = 4; pupae, n = 2; NEW, n = 1), but these cases appear to represent sporadic and ephemeral acquisitions from nurse workers.

There are three possible explanations for low prevalence in early life stages. A) There is lower exposure of brood to infection. B) Infection loss in brood occurs during ecdysis. C) Adults and early life stages show different susceptibility to infection.

For the former hypothesis (low exposure), it is possible that the contact rate or intensity between larvae and infected workers may not be high enough for transmission in most cases, perhaps exacerbated by low *Arsenophonus* titres in nurse *A. mellifera*. The experiment exploring trophallactic transmission of *Arsenophonus* between workers produced a low transmission rate, and the same may be likely between workers and brood. For the second hypothesis (loss during ecdysis), it is notable (Chapter 3), that *Arsenophonus* is a gut resident. In *A. mellifera* the gut lining is shed during metamorphosis between life stages, such that any gut associated microbes that have accumulated within the life stage are lost, and newly

emerged workers (NEWs) are virtually sterile (Gilliam 1971). Thus, it may be that only adults can retain infection, as each ecdysis acts to resterilize the gut.

For the third hypothesis (differential susceptibility), the difference between adults and juvenile stages may reflect a greater risk of *Arsenophonus* infection to adult workers. Performing duties outside the hive, such as foraging, may increase exposure to possible reservoirs of *Arsenophonus* spp. and the higher energetic costs encountered by these members of the colony may all contribute to increased infection risk (Coulson et al. 2009, Koprivnikar & Penalva 2015). This may be seen as akin to how social status can be an important predictor of parasite risk in vertebrates, with more dominant individuals contracting more parasites, due to greater roaming distances, energetic costs and contact with other individuals (Habig & Archie 2015). This theory holds true particularly for parasites that are environmentally acquired or contact transmitted (Habig et al. 2018), both of which appear to be plausible transmission routes for *Arsenophonus* among *A. mellifera* based on results presented in this thesis and by others (Donkersley et al. 2018, McFrederick et al. 2017*a*).

Alternately, differential susceptibility may reflect evolved mechanisms for host driven modulation of symbiont titre across life stages, as has been observed in a number of insects (Laughton et al. 2014, Masson et al. 2014, Nishikori et al. 2009, Stoll et al. 2010). In this model, the data indicating low presence of *Arsenophonus* in eggs, larvae, pupae and NEWs would reflect low titre, rather than absence from the majority of individuals. However, when sterile NEWs were allowed to develop to forager age (in the company of other NEWs only) they showed no development of *Arsenophonus* infection, demonstrating that *Arsenophonus* infection, although possibly affected by age, does not develop as a programmed part of the titre control. Exposure post emergence to infected conspecifics, hive components, an environmental reservoir (or a combination) appears to be necessary for *Arsenophonus* infection, analogous to acquisition of the core *A. mellifera* gut bacteria (Powell et al. 2014).

4.4.3 Social acquisition & transmission heterogeneity

The finding that *Arsenophonus* can be horizontally (infectiously) transmitted between worker *A. mellifera* via social interactions is novel for a bacterium nested within a clade of heritable symbionts. The adaptive value of maintaining the ancestral transmission route makes evolutionary sense for the symbiont in the context of the eusocial host. The high host density, and comparatively low genetic diversity, of social insect colonies can facilitate the invasion and rapid spread of an infectiously transmitted symbiont (Hamilton 1987, Schmid-Hempel 1998). Further to this, social acquisition can prevent workers being the evolutionary dead ends they are often considered for heritable symbionts (Frost et al. 2014). Mechanisms of horizontal transfer of *Arsenophonus* within the colony likely extend to inter-colony transmission, potentially facilitated by drifting or robbing host individuals (e.g. Forfert et al. (2015), Nolan & Delaplane (2016)), allowing the symbiont to maximise fitness. Thus, the symbiont can achieve high transmission through the social route, which may in turn diminish selection to enhance vertical transmission.

However, many of the processes that promote the transmission of microbes, can also be alleviated by protective actions including hygienic behaviour and social immunity (Cremer et al. 2018, Theis et al. 2015). In addition to the successful transmission events, loss of *Arsenophonus* also occurred from a subset of worker *A. mellifera* that were infected at the start of the transmission treatments. It is possible these loss events are attributable to protective actions of *A. mellifera*, although they may also reflect the importance of an additional transmission route for infection maintenance that is absent within the study. For instance, infection may be maintained naturally from colony food stores (Donkersley et al. 2018) or environmental reservoirs. Social Anthophila are also known to acquire symbionts via shared flowers in addition to social acquisition within colonies (Koch et al. 2013).

Interestingly, both *Arsenophonus* transmission and loss/maintenance events were not homogenous across the treatment types or pots, indicating scope for context-dependent effects. Some pots exhibited full transmission to NEWs and complete

maintenance in workers, while other pots showed no transmission to NEWs and very low maintenance of *Arsenophonus* in workers. But what factors govern this significant variation? While stochastic factors may be at play, within host populations there is often heterogeneity in infection characteristics and susceptibility, which may play a part in the observed variation (Koopman 2004, Lloyd-Smith et al. 2005, Schmid-Hempel 2003, Wilson-Rich et al. 2008) Infection dynamics in pots with full transmission/maintenance of *Arsenophonus* may be driven by individual variation in infectiousness, with the chance of a highly infected superspreader individual entering a given pot. Superspreader hosts can contribute disproportionately to transmission via higher symbiont loads, shedding and contact rates (Lloyd-Smith et al. 2005). This type of host often affects disease spread in social taxa, as indicated in human populations with SARS (Shen et al. 2004) and potentially for the *Nosema ceranae* parasite in *A. mellifera* (Roberts & Hughes 2015). In turn, pots that lack superspreader individuals may clear/lose *Arsenophonus* infections at a similar rates, but with a lowered risk of reinfection from an individual with a higher symbiont load/shedding or contact rate (Lloyd-Smith et al. 2005, Roberts & Hughes 2015).

Despite the large variation in transmission and maintenance across pots, within each pot the efficiency of transmission remained similar (see Figure 4.4). At the end of the transmission period, the proportion of workers infected with *Arsenophonus* often mirrored the proportion of infected NEWs. This indicates the proportion of infected individuals can be an important predictor for *Arsenophonus* transmission events to susceptibles, as might be expected for an infectious agent transmitted by social contact. However, overall the transmission of *Arsenophonus* via social interactions was weak, with $< 40\%$ of susceptible *A. mellifera* becoming infected. While this may be attributable to other factors such as host density (Anderson & May 1981, McCallum et al. 2001) or immune changes (related to lab environment), the weak social transmission once again provides evidence *Arsenophonus* likely maximises fitness via additional routes to persist in *A. mellifera* populations. Additional transmission routes could include additional amplification/reservoir host species

or hotspot areas of infection, which are well recognised as contributors to transmission heterogeneity (Paull et al. 2013, Streicker et al. 2013, Johnson et al. 2015).

Symbiont transmission by general contact Overall transmission of *Arsenophonus* between *A. mellifera* occurred more frequently when additional social interactions (general contact) were allowed. This observation suggests transmission occurs via routes alongside trophallaxis. Within the general contact pots several potential routes were available for the transmission of *Arsenophonus* that are directly associated with sociality (Holldobler & Wilson 1990, Wilson 1971). These included stomodeal trophallaxis, proctodeal trophallaxis, coprophagy and shared food resources.

Social hosts benefit from the transmission of beneficial symbionts via both proctodeal trophallaxis (anus - mouth exchange of proctodeal fluid) and coprophagy (consumption of faeces). These routes are important for acquisition of core *A. mellifera* gut bacteria, including *Snodgrassella alvi, Gilliamella apicola* and *Frischella perrara* (Powell et al. 2014). However, these interactions are also easily co-opted for the transmission of parasites. For example, in pyrrhocorid firebugs the transmission of nutritional mutualists occurs via both VT and HT faecal deposition, but these routes are additionally hijacked by a co-transmitting and costly parasite (Salem et al. 2015). Regardless of its symbiotic phenotype, transmission of *Arsenophonus* via faeces would allow efficient transmission within a colony and also externally to other Anthophila via shared resources (Fürst et al. 2015, Graystock et al. 2014, 2015). The likelihood of this transmission route is further supported by the localisation of *Arsenophonus* in the *A. mellifera* gut and detection in faeces (Chapter 2), and is combined with knowledge that closely related *A. Nasoniae* infects across the gut of *Nasonia* larvae after ingestion (Huger et al. 1985, Werren et al. 1986). Exclusive testing of a faecal transmission route may benefit our understanding of *Arsenophonus* transmission dynamics within colonies.

The transmission opportunities available within the pots, but less directly cor-

related with sociality, included airborne transmission. Although this transmission route is rarely shown for insect endosymbionts, the high host density in a contained environment, combined with *A. mellifera* behaviour (eg. wing fanning), could facilitate symbiont dissemination. Insect symbionts, notably *Wolbachia*, have been identified at relatively high proportions in surveys of airborne microbial communities. This observation is probably driven by the sloughing of insect cells into the air (Yooseph et al. 2013), but may never the less represent an effective dispersal mechanism (Burrows et al. 2009) for symbionts if viability is retained. In *A. mellifera* colonies airborne transmission may work in concert with other routes (e.g. faecal deposition) to expose large proportions of the colony to *Arsenophonus*.

Symbiont transmission by stomodeal trophallaxis When only stomodeal trophallactic (mouth – mouth transmission) interactions were allowed between *Arsenophonus* infected and uninfected *A. mellifera*, the total number of transmission events was significantly lower than when all contacts were allowed. Stomodeal trophallaxis allows mutual feeding of conspecifics via regurgitation and is a highly evolved behaviour in social insects (Holldobler & Wilson 1990). Trophallaxis can be important in conferring social immunity (Hamilton et al. 2011), and in *Formicidae* (Marsh et al. 2014), Isoptera (Hongoh 2010, Nalepa 2015) and social Anthophila (Koch & Schmid-Hempel 2011, Powell et al. 2014) it contributes to transmission of core beneficial symbionts.

For *Arsenophonus*, stomodeal trophallaxis alone appears to be insufficient for a high rate of transmission and similar effects are observed for core *A. mellifera* symbionts. The gut community of NEWs shows lower diversity with absent core taxa if only stomodeal trophallaxis with nurse *A. mellifera* is allowed (Martinson et al. 2012, Powell et al. 2014). While transmission by stomodeal trophallaxis was only observed in a few cases, the finding remains interesting as it demonstrates the potential functional capacity of *Arsenophonus* to transmit via this social behaviour, and at a colony level may represent an important transmission mechanism

for *Arsenophonus*. *Wolbachia* has previously been detected in the salivary glands of solitary Anthophila, and as provisions left for offspring contain saliva this constitutes a possible vertical transmission route (see SI of Gerth et al. 2013). For social hosts, transmission of classically heritable symbionts could occur directly through saliva during stomodeal trophallaxis, in addition to inoculating colony stores. *Arsenophonus* has been identified in *A. mellifera* bee bread (Donkersley et al. 2018), and this may result from the incorporation of host saliva. The knowledge that *A. Nasoniae* also grows and survives in cell free media (Werren et al. 1986) strengthens speculation that in *A. mellifera*, *Arsenophonus* may be transmissible from abiotic source to host, in addition to host to host.

One caveat in experimental design should be noted, regarding these results. Although the size of mesh separation aimed to promote only stomodeal trophallaxis, we cannot rule out the possibility of proctodeal trophallaxis or faecal contamination occurring across the barrier.

Implications of transmission for predicting the symbiotic phenotype No significant vertical transmission of *Arsenophonus* was observed in *A. mellifera*. However, horizontal transmission via social interactions generated repeated transmission events. Transmission mode plays a crucial role in the evolution of host – symbiont interactions and a dichotomy exists between the two modes (Ewald 1987). Vertical transmission can align the fitness interests of host and symbiont and predicts a trajectory on the continuum towards mutualism (Foster & Wenseleers 2006). Horizontal transmission selects for the evolution of virulence as reproducing hosts are not required and competing coinfections can occur (Frank 1996*a*, Read 1994). Does then, the finding that *Arsenophonus* can be horizontally transmitted, affect predictions of its symbiotic phenotype in *A. mellifera*?

Due to HT virulence predictions, the finding is certainly pertinent to the assessment of the parasitic potential of *Arsenophonus* in Anthophila, and strengthens interpretations of previous work that correlated *Arsenophonus* with poor health out-

comes (Budge et al. 2016, Cornman et al. 2012, Parmentier et al. 2018). Of course, correlations may be explained by other third party factors, such as the maintenance of the bacterium in response to localised parasite pressure or other variables (e.g. if *Arsenophonus* conferred a fitness benefit in the presence of a stressor). *Arsenophonus* may also occur as a benign secondary infection (Budge et al. 2016) or titres may increase in colonies weakened by other factors, without being a significant contributor to *A. mellifera* pathology. These scenarios could produce a strong correlation of *Arsenophonus* with poor colony health, even in the absence of virulence.

However, eusocial hosts present an additional complication when considering transmission mode and the outcomes of host – microbe interactions. Due to the overlapping generations in eusocial insects, symbiont transmission between workers within a colony can be analogous to vertical transmission (ie. pseudo-vertical) when considering the evolution of virulence (Schmid-Hempel 1998). In contrast to solitary taxa, many potential routes of pseudo-vertical transmission are present in eusocial hosts. Combine this with a long-lived host colony characterised by fortress defence (Keller & Genoud 1997, Wilson 1971) and parasites may evolve toward avirulent or benign associations, as opposed to virulent horizontally transmitted epidemic infections (Hughes et al. 2008).

Conversely, selection on mutualistic symbionts may act to reduce host directed benefits in social hosts (Hughes et al. 2008). This pattern may be driven by more opportunities for strain diversity and/or reduced synchronization between host and symbiont reproduction. A fascinating example of this occurs in colonies of *Acromyrmex* leaf-cutting ants, where farmed fungal symbionts selfishly prevent the establishment of unrelated fungal cultivars (Poulsen & Boomsma 2005). This is achieved through fungal incompatibility compounds in ant faeces that are obligately applied to fungal gardens, thereby enforcing dependence of the colony on a single symbiont clone for its agricultural needs (Poulsen & Boomsma 2005).

We do not yet firmly know where *Arsenophonus* sits within the interplay between transmission mode and the evolution of outcomes in eusocial hosts. The

widespread occurrence and single strain type (Chapter 2) suggests an epidemic phenotype for *Arsenophonus* in *A. mellifera*. At some level, this does not follow the prediction of others (e.g. Hughes et al. 2008) who argue the evolution of epidemic parasites may be rare in social insect hosts. This disparity is probably because worker to worker transmission (ie. pseudo vertical transmission) is not the major route of transmission for *Arsenophonus*. Evidence is growing for the role of environmental acquisition and it is possible additional host/reservoirs exist (Duron et al. 2008, McFrederick et al. 2014, 2017*a*). A scenario such as this reduces the reliance of a symbiont on the homeostatic colony environment and generates a larger scope for conflict between *Arsenophonus* and colony fitness. It is possible that other major characteristics of social hosts, such as high host density and low genetic diversity (Schmid-Hempel 1998), may instead have facilitated the invasion of *Arsenophonus* into *A. mellifera* populations and contributed to the observed symbiont phenotype. An interesting comparison study will be to characterise the transmission phenotype, epidemiology and virulence outcomes for *Arsenophonus* associated with solitary Anthophila hosts.

4.4.4 Association of *Arsenophonus* with dead hosts

Individuals that died during the horizontal transmission study had a significant positive association with *Arsenophonus* infection and in Chapter 2 *Arsenophonus* was detected only in deceased *C. hederae* within the UK. This observation is intriguing, as obligately host-associated microbes are often only associated with living hosts (Bennett & Moran 2015, Pontes & Dale 2006). This detection could simply be attributable to residual *Arsenophonus* cells from the live infection that are no longer viable. However, the presence and possibly higher titre of *Arsenophonus* detected in dead individuals (PCR bands were disproportionately brighter, but no quantitative work has been completed) may also implicate the symbiont in their host's fate, or at least suggest opportunistic proliferation in a compromised host. This observation echoes previous studies that found a notable proportional increase of *Arsenophonus*

in colonies affected by CCD (Cornman et al. 2012) and those of poor health (Budge et al. 2016).

Another possibility is that *Arsenophonus* can transition to a saprophytic lifestyle. This is observed in *A. Nasoniae* as it grows saprophytically in the parasitized dead fly pupae prior to transmission to *Nasonia* (Werren et al. 1986). It is possible the *A. mellifera* strain shares this capacity. Large colony sizes (tens of thousands of individuals) (Wilson 1971) and a rise in mortality due to various pressures (Goulson et al. 2015, Potts et al. 2010) translates into a high number of dead hosts exiting *A. mellifera* colonies. A shift in lifestyle could prove evolutionary advantageous for *Arsenophonus*.

However, for saprophytic microbes there are issues associated with transmission from deceased hosts. For many entomopathogenic fungi this is a hurdle easily overcome by behavioural manipulation of the host and showers of infective spores (Roy et al. 2006), and spore forming bacteria may likewise easily exploit environmental transmission (Swick et al. 2016). However for bacteria endospore formation is a gram-positive associated trait (with some exceptions e.g. Thomas (2006), Poehlein et al. (2013)) and there is no evidence for endospore-formation within the gram-negative *Arsenophonus* genus. For the *Arsenophonus* population associated with *Apis* saprophytic growth will prove insignificant unless transmission can occur to new hosts or reservoirs. It is possible we are observing non-adaptive saprophytic growth in *A. mellifera* cadavers. This would be significant for the genus as a whole, departing from the assumption that *Arsenophonus* depends on a live host and contributing a trait that draws the clade further from its heritable endosymbiont classification into a more diverse assemblage. The capacity to survive in a dead host also increases the likelihood of survival in the environment. Further work should establish if dead *A. mellifera* constitute a source of infection to live *A. mellifera*.

Chapter 5

General Synthesis & Future Directions

How do free-living entities, with independent evolutionary agendas, come to associate and depend on one another? The drivers underpinning these major transitions in symbiotic lifestyle remain largely unknown (Law & Dieckmann 1998, Sachs et al. 2011, 2013). A significant contingent of the *Arsenophonus* clade is in the latter stages of this evolutionary trajectory towards obligate intracellular life (Chrudimský et al. 2012, Nováková et al. 2009, Perotti et al. 2007, Qu et al. 2013), and until now all strains within the genus showed a heritable lifestyle. Within this thesis, characterisation of the *Arsenophonus* strain associated with *Apis mellifera* highlights previously unrealised diversity within the clade. The *A. mellifera* strain shows several parallels to its sister strain, *A. nasoniae* – the strain can transmit intra-specifically (Chapter 4), it can reside in the gut (Chapter 3), and saprophytic growth may also occur (Chapter 2 & 4). Within its host population *A. nasoniae* is vertically transmitted, but contact with conspecifics and resulting infectious (horizontal) transmission is necessary for spread of the bacterium. The driver of *Arsenophonus* dynamics in *Apis* populations appear to be similarly multifactorial, but are shifted on the continuum towards an environmental drivers and possibly

a less-host restricted phenotype. In the *Apis* system, contact with conspecifics generates horizontal transmission events, but exposure to an unknown environmental reservoir (abiotic or biotic) appears to be important for maintenance of the bacteria in host populations (Chapter 2, 3 & 4). Here it appears that, at least in the context of the *A. mellifera* host, symbiotic life may actually be facultative for *Arsenophonus*.

Knowledge of the selective forces that drive the emergence of symbiotic diversity is imperative for understanding important transitions such as the emergence of pathogenic agents from commensal partners, and vice versa. For instance, the intracellular vertebrate pathogen *Coxiella burnetti* emerged from a clade of maternally inherited tick endosymbionts (Duron et al. 2015), losing dependence on its arthropod host and evolving mechanisms to persist in the abiotic environment and be airborne transmitted (Hawker et al. 1998). To understand the full variety of ecological and evolutionary mechanisms underpinning such remarkable transitions will require the study of different groups (Clayton et al. 2012, Duron et al. 2015, Perlman et al. 2006). The results from this thesis highlight that *Arsenophonus* – host interactions are evolutionary labile and span more of the symbiosis continuum than previously recognised. As others have previously noted, the presence of obligate symbionts alongside facultative, and beneficial symbionts alongside reproductive parasites, makes this genus an attractive one to study (Wilkes et al. 2011). The *Arsenophonus Apis* strain adds another layer of diversity to this, a strain that is both extracellular, a gut symbiont and endosymbiont, and apparently without vertical transmission.

5.1 Environmental transmission – implications for symbiont epidemiology & evolution?

Within this thesis, multiple lines of evidence suggest *Arsenophonus* dynamics in *A. mellifera* are governed by an interplay between environmental acquisition and social transmission. Microbial symbionts often evolve from environmental, free-

living, strains (Hosokawa et al. 2016, Sachs et al. 2011) such that the many host-associated microbes maintain the capacity to survive and transmit via the environment, whilst in a transitional period or otherwise (i.e. those that have not yet secondarily lost it). Despite this evolutionary trajectory and history, this potential capacity is often overlooked when considering transmission and epidemiology of major insect symbionts, and particularly for those considered heritable symbiont genera. At the initiation of this thesis, it was presumed that *Arsenophonus*, as for other strains in the genus, would likely be heritable microbe. However, the data presented indicate that it retains infectivity, potentially though an environmental medium.

While the environmental transmission of microbial endosymbionts is common place in marine communities this route is rarer in terrestrial habitats, particularly for the formation of insect – bacteria symbioses. A few cases are observed, demonstrated by the stable associations between stink bug hosts and strains of *Pantoea* and *Burkholderia* (Kikuchi et al. 2007; Hosokawa et al. 2016). However, these symbionts stem from diverse genera of Proteobacteria, that include free-living environmental bacteria and opportunistic pathogens of vertebrate hosts (Eberl & Vandamme 2016, Walterson & Stavrinides 2015). Here symbiotic life appears to be largely facultative from the perspective of the microbes. The clade in which *Arsenophonus* – *Apis* sits is one where strains are considered host-restricted and heritable, yet it appears that environmental acquisition (from an as yet unknown reservoir) plays a part in the epidemiology of *Arsenophonus* within the *A. mellifera* population.

For other insects the acquisition of environmentally borne symbionts often occurs through soil substrate (Hosokawa et al. 2016, Kikuchi et al. 2007), but the ecology of *Apis* suggests plants offer a more likely reservoir of infection. Here acquisition may occur directly, via assimilation of *Arsenophonus* into nectar or pollen (perhaps introduced by another host e.g. sap feeding insect) or via contamination of surface (e.g. faeces). These data accord with increasing findings of plants as sites for infectious transfer within insect communities (Chrostek et al. 2017, Graystock

et al. 2015, McFrederick et al. 2017*b*).

The contrast in transmission biology from heritable strains, reflected in distinct ecological dynamics of *Arsenophonus* in *Apis* (seasonality and overwinter loss) will be likely reflected in altered evolutionary dynamics. The lack of strong bottlenecks in symbiont number, combined with requirement for host-free survival, likely maintains a robust microbial genome both in terms of diversity of pathways, and reduced pseudogenization compared to other strains (Bennett & Moran 2015). In addition, the infectious transmission pathways may place *Arsenophonus* in competition with other strains within *A. mellifera* individuals, and indeed other microbes, such that competitive ability will be selected (Frank 1996*b*). Thus, the different transmission modes predict that the *Apis* strain will be distinct at the genome level from other sequenced *Arsenophonus* strains. A further question relates to its phenotype in bees. Unlike vertically transmitted relatives, *Arsenophonus* in *Apis* apparently does not depend on the long term success of its host. The presence of systemic infections and association with dead hosts, and previous work emphasising correlations with poor Anthophila health (Budge et al. 2016, Cornman et al. 2012, Parmentier et al. 2018) – are consistent with this. What is not clear is if these health outcomes are causal. Is *Arsenophonus* an opportunistic pathogen, or one where infection establishes strongly only in hosts compromised for other reasons? Further research is required to determine this.

Implications for surveys of *Arsenophonus* The genus *Arsenophonus* is considered one of the big four heritable microbe groups, present in 5% of species (Duron et al. 2008). Its incidence is commonly ascertained through PCR assay, and detection of *Arsenophonus* has been assumed to reflect presence of a heritable microbe. This thesis has established that this assumption requires validation, as strains can clearly retain infectious cycles and in the case of *Apis*, may depend upon these rather than vertical transmission.

The marked seasonal dynamics observed in Chapter 2 indicate *Arsenophonus*

infection risk may track with seasonal fluctuations in the activity or presence of environmental reservoirs. Of course these findings may also feedback with (or exclusively reflect) seasonal changes that are intrinsic to the host population (i.e. changes to host density or behaviour) (Hamilton 1987, Schmid-Hempel 1998). Yet regardless of the underlying basis for the observed seasonal heterogeneity, the results for *Arsenophonus* in *A. mellifera* highlight the importance of screening host populations at multiple time points across seasons. Without this broad sampling we lack a true picture of facultative symbiont communities, as many screens sample widely at a single time point and conclude the presence/absence of a given symbiont in a specific host species (without the caveat that symbiont prevalence may vary across time). Further to this, seasonal diversity or infection patterns may be missed that could valuably inform on the wider ecology and function of symbionts. For instance, a repeated annual peak in the prevalence of a symbiont could reflect capture by the host (or vice versa) at a time of high requirement (e.g. seasonal fluctuations in nutrient availability/parasite pressure for the host, lack of available host taxa for the symbiont).

5.2 Potential interactions between *Arsenophonus* & host sociality

Akin to symbiosis, sociality also lies along a continuum from transient and dynamic interactions between individuals of the same species to highly structured and permanent societies (Wilson 1971, 1975). Large variations in sociality are observed across the Anthophila clade, and interestingly *Arsenophonus* has emerged as a widespread associate of eusocial *Apis* (Chapter 2 & Budge et al. 2016; Yanez et al. 2016) and is also found in eusocial *Bombus* spp. (Parmentier et al. 2018). Have aspects of host sociality facilitated the invasion of *Arsenophonus* into these Anthophila populations?

The *Arsenophonus* strain from *A. mellifera* is the first within this symbiont clade

to show evidence of transmission via social interactions (Chapter 4). It is tempting to consider that the capacity of *Arsenophonus* to transmit via these routes has evolved in concert with social life. The presence in eusocial host taxa may reflect *Arsenophonus* transitioning investment from vertical to infectious transmission. Possibly in response to high host density and frequent direct contact, or maintaining these ancestral characteristics in eusocial hosts that support inter-individual infection. Another enabling factor for social transmission is the capacity to invade across the gut wall (as also observed in sister strain *A. nasoniae* (Huger et al. 1985, Werren et al. 1986)), which may generate infection via ingestion of *Arsenophonus* containing material (regardless of the route). These properties allow infection via feeding, grooming, trophallaxis, faecal shedding feedbacks etc. Within a social colony these exposure routes could multiply to create transmission frequencies that may be important for the annual fitness of *Arsenophonus*, even if *A. mellifera* is not the exclusive host (as suggested by Chapter 3).

More sporadic associations with *Arsenophonus* are evident across the rest of the Anthophila clade (Chapter 2 & McFrederick et al. (2014), Gerth et al. (2015), Saeed & White (2015)). Intriguingly though, the most frequent associations are with solitary *Colletes* bees. These species are well known for nesting in huge, dense, aggregations (of many thousands) and form large mating clusters (C O'Toole, pers. comm BWARS; Saxton 2009; Dellicour et al. 2014). This aspect of their biology may indeed foster similar opportunities for the horizontal transmission of *Arsenophonus* as occurs for social *Apis* and *Bombus* spp. For social taxa, large group sizes and high local densities can translate into heightened transmission (Freeland 1976, Hamilton 1987, Schmid-Hempel 1998), and (in some cases) are associated with lower thresholds for the infection and spread of infectious agents (Langwig et al. 2012). Thus it is notable that *Arsenophonus* is largely found in host Anthophila with these characteristics, perhaps reflecting an easier invasion trajectory for this horizontally transmitted strain. On a similar vein, recent work demonstrated a strong correlation between host sociality in the Halictidae family of bees and the abundance of *So-*

dalis, a genus of insect endosymbiont with many parallels to *Arsenophonus* (Rubin et al. 2017). These data further indicate that host sociality may be an important driver of symbiont polymorphism.

For social insect hosts, the prevailing focus on highly co-adapted partners have revealed fascinating and important interactions, from the ancient *Blochmannia* - ant symbiosis (Gil et al. 2003) to the conserved gut microbiota of eusocial corbiculate bees (Kwong et al. 2017). Nevertheless, there is also a distinct value to studying emerging symbiotic association. For here there may be more opportunities to observe the *de novo'* adaptation of symbionts to social life. Arguably, as the size and structure of human society changes, it will be increasingly important to understand the evolutionary trajectories of mutualists through to parasites in response to selection imposed by host sociality.

5.3 Future directions

As for many studies of symbiosis, experimentation on the *Arsenophonus – Apis* interaction has been impeded by the unculturability of the symbiont. A cultured symbiont would allow analysis of the complete genome and predict capacities for virulence and environmental survival. The ability to experimentally introduce infection would allow more defined analysis of transmission route, host response and virulence. Genetic manipulation, for instance, adding GFP markers, would allow in vivo tracking of symbiosis processes. The close relationship of *Arsenophonus* from bees to *A. nasonie* – which is culturable, has a closed genome, and has now been transformed with GFP (Darby et al. 2010, Gherna et al. 1991, Werren et al. 1986) – implies that this may be technically attainable for *Arsenophonus* interactions in bees.

Early attempts at *Arsenophonus* culture during this thesis work were unsuccessful, with only contaminating bacteria (Lactococcus) established from *Arsenophonus* infected hosts. Serendipitously, a US group recently established *Arsenophonus* on

DNase agar during investigations of *Serratia* diversity in bees. The *Arsenophonus* that was isolated is extremely slow growing (10 days to establish a microcolony), but has been sufficient to generate a complete genome scaffold which awaits annotation.

This culturability will enable future directed research on the interaction with Anthophila, and allow direct comparison to the *A. nasoniae* – *Nasonia* interaction. The biology of the *Arsenophonus* – *Apis* interaction establishes the strain as a key link in the clades trajectory from free-living ancestral life to one of obligate mutualism threatened by mutational decay. This diversity of lifestyles provides opportunities to use a comparative genomics approach to identify the genes lost, gained and altered in transitions between the symbiotic lifestyles. For example, will patterns of gene loss in metabolic pathways mirror that of sister strain *A. nasoniae* (Darby et al. 2010), or will signatures of low host dependence and environmental circulation be evident? It would be further predicted that systems for host invasion and effectors for host manipulation may be found. The origins of these genes may also be examined, and this may provide insight into whether the systems enabling the infectious life cycle of *Arsenophonus* in *A. mellifera* are derived from lateral gene transfer. Alternatively, the strain may simply maintain the ancestral gene set found in *Providencia, Proteus* and *Photorhabdus* (the most closely allied genera) with loss of function occurring in other *Arsenophonus* strains. A further prediction is that *Arsenophonus* from *Apis* may encode systems to aid in competition against other strains of *Arsenophonus* and other microbes, reflecting the increased importance of these interactions during the infection cycle.

Another interesting path will be to characterise the genetic and phenotypic diversity of *Arsenophonus* strains associated with other social insects and solitary Anthophila. The presence in a variety of solitary (Duron et al. 2008, Gerth et al. 2015) and eusocial species (Sebastien et al. 2012, Yanez et al. 2016, Parmentier et al. 2018) provides an opportunity to determine if the strains present and their transmission modes correlate with sociality, or whether effects of ecology or phylogeny are of

greater importance.

Aside comparative genomics, GFP labelling would provide a rich opportunity for studying transmission. Experiments such as those in Chapters 3 and 4 could be conducted via visualisation of live bacteria, rather than patterns of tropism and transmission being inferred post hoc by PCR assay or in fixed specimens. If *Arsenophonus* is shed into the environment (e.g. by foraging Anthophila) another key variable to elucidate will be the length of time which *Arsenophonus* can persist and remain viable. These data will be necessary for determining accurate models of infection dynamics, as under certain conditions environmental transmission is considered akin to direct transmission (if persistence in the environment is minimal, Breban (2013). Further to this, the relative contributions of social/HT and environmental acquisition from other hosts/reservoirs must be established.

Finally, culturing of *Arsenophonus* would allow titre-controlled infections in *Apis*. These experiments would have many uses – to reveal infectious dose; to study interactions of infection with other *Apis* parasites/pathogens such as deformed wing virus; to establish if *Arsenophonus* in *Apis* impacts host immune reactions. Beyond functional analysis, the ability to culture, infect and clone would allow experimental evolution approaches to symbiont-host interactions. If stable associations can be maintained between *Arsenophonus* and Anthophila then serial social passaging experiments (Ebert 1998) could reveal the evolutionary response of the symbiont to focal aspects of host sociality. For example, how might host population structure and contact affect symbiont investment in transmission?

Appendix

Gene	*Predicted Product*	*Primers (5′ – 3′)*	*Product (bp)*	*Tm (°C)*	*Ref*
yaeT	Outer membrane protein assembly factor	CCGTGTTATTACCCAACCTG	508	58	[1]
		ATAATCGCCCACCTAAACTG			
ftsK	Cell division protein	GTTGTTATGGTCGATGAATTTGC	444	59	[2]
		GCTCTTCATCACCATCATAACC			
fbaA	Fructose-bisphosphate aldolase class II	GCCGCTAAGGTTGGTTCTCC	658	63	[2]
		CCTGAACCACCATGGAAAACAAAA			
16S rRNA	Small ribosomal subunit	GGGTTGTAAAGTACTTTCAGTCGT	804	61	[3]
		GTAGCCCTRCTCGTAAGGGCC			
RpoB	RNA polymerase β subunit	ATCGCAGTCCTGGCGTATTT	536	59	[1]
		ACCTTGACTTAGACGTGCCA			
InfB	Translation initiation factor	AATCGAGGCGGAAAGGCTAC	416	60	[1]
		CGTGGTTCTGCGATTGCTTC			
ftsZ	Cell division protein	ATYATGGARCATATAAARGATAG	435	54	[4]
		TCRAGYAATGGATTRGATAT			
EF1-α	Elongation factor-1 α	GGAGATGCTGCCATCGTTAT	154	57	[5]
		CAGCAGCGTCCTTGAAAGTT			

[1] This study, [2] Adapted from Duron *et al*, 2010, [3] Duron *et al*, 2010. [4] Baldo et al. 2006, [5] Lourenço *et al*., 2008

Table A1. Primer used for the detection of symbiont and host DNA

Fluorochrome	Ab (nm)	Em (nm)	Em Colour	Target	Sequence	Ref
Alexa Fluor® 647	650	665	Far - red	*Arsenophonus* spp.	TCATGACCACAACCTCCA	[1]
Alexa Fluor® 555	555	565	Yellow	General bacteria	TGCTGCCTCCCGTAGGA	[2]
DAPI	345	455	Blue	Host DNA	-	-

[1] Labelled oligonucleotide probe, [2] DNA counter stain, [Ab] Absorbance, [Em] Emission
[1] Gottlieb *et al*.,2008 [2] Martinson *et al*., 2012

Table A2. Fluorochromes used for fluorescence *in situ* hybridization (FISH) of *A. mellifera* gut tissue

Bibliography

Ahmed, M. Z., Araujo-Jnr, E. V., Welch, J. J. & Kawahara, A. Y. (2015), '*Wolbachia* in butterflies and moths: Geographic structure in infection frequency', *Frontiers in Zoology* **12**(1), 16.

Aizenberg-Gershtein, Y., Izhaki, I. & Halpern, M. (2013), 'Do honeybees shape the bacterial community composition in floral nectar?', *PLoS ONE* **8**(7), 6–7.

Akaike, H. (1974), 'A new look at the statistical model identification', *IEEE Transactions on Automatic Control* **19**(6), 716–723.

Akman, L., Yamashita, A., Watanabe, H., Oshima, K., Shiba, T., Hattori, M. & Aksoy, S. (2002), 'Genome sequence of the endocellular obligate symbiont of tsetse flies, *Wigglesworthia glossinidia*.', *Nature genetics* **32**(3), 402–407.

Albert, A. & Anderson, J. A. (1984), 'On the existence of maximum likelihood estimates in logistic regression models', *Biometrika* **71**(1), 1–10.

Alizon, S., Hurford, A., Mideo, N. & Van Baalen, M. (2009), 'Virulence evolution and the trade-off hypothesis: history, current state of affairs and the future.', *Journal of Evolutionary Biology* **22**(2), 245–259.

Andersen, S. B., Boye, M., Nash, D. R. & Boomsma, J. J. (2012), 'Dynamic *Wolbachia* prevalence in *Acromyrmex* leaf-cutting ants: Potential for a nutritional symbiosis', *Journal of Evolutionary Biology* **25**(7), 1340–1350.

Anderson, K. E. & Ricigliano, V. A. (2017), 'Honey bee gut dysbiosis: a novel context of disease ecology', *Current Opinion in Insect Science* **22**, 125–132.

Anderson, K. E., Rodrigues, P. A. P., Mott, B. M., Maes, P. & Corby-Harris, V. (2016), 'Ecological succession in the honey bee gut: Shift in *Lactobacillus* strain dominance during early adult development.', *Microbial Ecology* **71**(4), 1008–1019.

Anderson, K. E., Russell, J. A., Moreau, C. S., Kautz, S., Sullam, K. E., Hu, Y., Basinger, U., Mott, B. M., Buck, N. & Wheeler, D. E. (2012), 'Highly similar microbial communities are shared among related and trophically similar ant species', *Molecular Ecology* **21**(9), 2282–2296.

Anderson, K. E., Sheehan, T. H., Mott, B. M., Maes, P., Snyder, L., Schwan, M. R., Walton, A., Jones, B. M. & Corby-Harris, V. (2013), 'Microbial ecology of the hive and pollination landscape: bacterial associates from floral nectar, the alimentary tract and stored food of honey bees (*Apis mellifera*)', *PLoS ONE* **8**(12), e83125.

Anderson, R. M. & May, R. M. (1979), 'Population biology of infectious diseases: Part I', *Nature* **280**, 361.

Anderson, R. M. & May, R. M. (1981), 'The population dynamics of microparasites and their invertebrate hosts', *Philosophical Transactions of the Royal Society B: Biological Sciences* **291**(1054), 451–524.

Anderson, R. M. & May, R. M. (1982), 'Coevolution of hosts and parasites', *Parasitology* **85**, 211 – 426.

Attardo, G. M., Lohs, C., Heddi, A., Alam, U. H., Yildirim, S. & Aksoy, S. (2008), 'Analysis of milk gland structure and function in *Glossina morsitans*: milk protein production, symbiont populations and fecundity.', *Journal of Insect Physiology* **54**(8), 1236–1242.

Babendreier, D., Joller, D., Romeis, J., Bigler, F. & Widmer, F. (2007), 'Bacterial community structures in honeybee intestines and their response to two insecticidal proteins', *FEMS Microbiology Ecology* **59**(3), 600–610.

Bailey, L. (1958), 'Wild honeybees and disease', *Bee World* **39**, 93 – 95.

Baldo, L., Bordenstein, S., Wernegreen, J. J. & Werren, J. H. (2006), 'Widespread recombination throughout *Wolbachia* genomes', *Molecular Biology and Evolution* **23**(2), 437–449.

Ballinger, M. J., Moore, L. D. & Perlman, S. J. (2018), 'Evolution and diversity of inherited *Spiroplasma* symbionts in *Myrmica* ants', *Applied and Environmental Microbiology* **84**(4), AEM.02299–17.

Balmand, S., Lohs, C., Aksoy, S. & Heddi, A. (2013), 'Tissue distribution and transmission routes for the tsetse fly endosymbionts.', *Journal of Invertebrate Pathology* **112 Suppl**, S116–22.

Bandi, C., Dunn, A. M., Hurst, G. D. & Rigaud, T. (2001), 'Inherited microorganisms, sex-specific virulence and reproductive parasitism', *Trends in Parasitology* **17**(2), 88–94.

Bartel, R. A., Oberhauser, K. S., de Roode, J. C. & Altizer, S. M. (2011), 'Monarch butterfly migration and parasite transmission in eastern North America', *Ecology* **92**(2), 342–351.

Bates, D., Mächler, M., Bolker, B. & Walker, S. (2015), 'Fitting Linear Mixed-Effects Models Using lme4', *Journal of Statistical Software; Vol 1, Issue 1 (2015)* .

Begon, M., Bennett, M., Bowers, R. G., French, N. P., Hazel, S. M. & Turner, J. (2002), 'A clarification of transmission terms in host-microparasite models: numbers, densities and areas', *Epidemiology and Infection* **129**(1), 147–153.

Bennett, G. M. & Moran, N. A. (2013), 'Small, smaller, smallest: The origins and evolution of ancient dual symbioses in a phloem-feeding insect', *Genome Biology and Evolution* **5**(9), 1675–1688.

Bennett, G. M. & Moran, N. A. (2015), 'Heritable symbiosis: The advantages and perils of an evolutionary rabbit hole', *Proceedings of the National Academy of Sciences of the United States of America* **112**(33), 10169–10176.

Berlanga, M., Paster, B. J. & Guerrero, R. (2009), 'The taxophysiological paradox: changes in the intestinal microbiota of the xylophagous cockroach *Spiroplasma* depending on the physiological state of the host.', *International microbiology : the official journal of the Spanish Society for Microbiology* **12**(4), 227–236.

Berthoud, H., Imdorf, A., Haueter, M., Radloff, S. & Neumann, P. (2010), 'Virus infections and winter losses of honey bee colonies (*Apis mellifera*)', *Journal of Apicultural Research* **49**(1), 60–65.

Biedermann, P. H. & Rohlfs, M. (2017), 'Evolutionary feedbacks between insect sociality and microbial management', *Current Opinion in Insect Science* **22**(Figure 1), 92–100.

Blanford, S., Thomas, M. B. & Langewald, J. (2001), 'Thermal ecology of *Zonocerus variegatus* and its effects on biocontrol using pathogens', *Agricultural and Forest Entomology* **2**(1), 3–10.

Bolker, B. M., Brooks, M. E., Clark, C. J., Geange, S. W., Poulsen, J. R., Stevens, M. H. H. & White, J. S. S. (2009), 'Generalized linear mixed models: a practical guide for ecology and evolution', *Trends in Ecology and Evolution* **24**(3), 127–135.

Breban, R. (2013), 'Role of environmental persistence in pathogen transmission: a mathematical modeling approach', *Journal of Mathematical Biology* **66**(3), 535–546.

Breban, R., Drake, J. M., Stallknecht, D. E. & Rohani, P. (2009), 'The Role of Environmental Transmission in Recurrent Avian Influenza Epidemics', *PLOS Computational Biology* **5**(4).

Breeze, T. D., Bailey, A. P., Balcombe, K. G. & Potts, S. G. (2011), 'Pollination services in the UK: How important are honeybees?', *Agriculture, Ecosystems & Environment* **142**(3), 137–143.

Bressan, A. (2014), 'Emergence and evolution of *Arsenophonus* bacteria as insect-vectored plant pathogens', *Infection, Genetics and Evolution* **22**, 81–90.

Bressan, A., Sémétey, O., Arneodo, J., Lherminier, J. & Boudon-Padieu, E. (2009), 'Vector Transmission of a Plant-Pathogenic Bacterium in the *Arsenophonus* Clade Sharing Ecological Traits with Facultative Insect Endosymbionts', *Phytopathology* **99**(11), 1289–1296.

Bressan, A., Terlizzi, F. & Credi, R. (2012), 'Independent Origins of Vectored Plant Pathogenic Bacteria from Arthropod-Associated *Arsenophonus* Endosymbionts', *Microbial Ecology* **63**(3), 628–638.

Bright, M. & Bulgheresi, S. (2010), 'A complex journey: transmisison of microbial symbionts', **51**(3), 505–510.

Brosi, B. J., Delaplane, K. S., Boots, M. & De Roode, J. C. (2017), 'Ecological and evolutionary approaches to managing honeybee disease', *Nature Ecology and Evolution* **1**(9), 1250–1262.

Brune, A. & Friedrich, M. (2000), 'Microecology of the termite gut: structure and function on a microscale', *Current Opinion in Microbiology* **3**(3), 263–269.

Buchner, P. (1967), 'Endosymbiosis of Animals with Plant Microorganisms', *Zeitschrift für allgemeine Mikrobiologie* **7**(2), 168.

Budge, G. E., Adams, I., Thwaites, R., Pietravalle, S., Drew, G. C., Hurst, G. D., Tomkies, V., Boonham, N. & Brown, M. (2016), 'Identifying bacterial predictors of honey bee health', *Journal of Invertebrate Pathology* **141**, 41–44.

Burnham, K. P. & Anderson, D. R. (2003), *Model selection and multimodel inference: a practical information-theoretic approach*, Springer Science & Business Media.

Cariveau, D. P., Elijah Powell, J., Koch, H., Winfree, R. & Moran, N. A. (2014), 'Variation in gut microbial communities and its association with pathogen infection in wild bumble bees (*Bombus*).', *The ISME journal* **8**(12), 2369–2379.

Carvalho, F. M., Souza, R. C., Barcellos, F. G., Hungria, M. & Vasconcelos, A. T. R. (2010), 'Genomic and evolutionary comparisons of diazotrophic and pathogenic bacteria of the order Rhizobiales', *BMC Microbiology* **10**(1), 37.

Caspi-Fluger, A., Inbar, M., Mozes-Daube, N., Katzir, N., Portnoy, V., Belausov, E., Hunter, M. S. & Zchori-Fein, E. (2012), 'Horizontal transmission of the insect symbiont *Rickettsia* is plant-mediated', *Proceedings of the Royal Society B: Biological Sciences* **279**(1734), 1791–1796.

Chapman, R. F. & Page, W. W. (1979), 'Factors Affecting the Mortality of the Grasshopper, *Zonocerus variegatus*, in Southern Nigeria', *Journal of Animal Ecology* **48**(1), 271–288.

Chrostek, E., Marialva, M. S. P., Esteves, S. S., Weinert, L. A., Martinez, J., Jiggins, F. M. & Teixeira, L. (2013), '*Wolbachia* Variants Induce Differential Protection to Viruses in *Drosophila melanogaster*: A Phenotypic and Phylogenomic Analysis', *PLOS Genetics* **9**(12), e1003896.

Chrostek, E., Pelz-stelinski, K., Hurst, G. D. D. & Hughes, G. L. (2017), 'Horizontal Transmission of Intracellular Insect Symbionts via Plants', **8**(November), 1–8.

Chrudimský, T., Husník, F., Nováková, E. & Hypša, V. (2012), '*Candidatus* Sodalis melophagi sp. nov.: phylogenetically independent comparative model to the tsetse ly symbiont *Sodalis glossinidius*', *PLoS ONE* **7**(7), e40354.

Chua, K.-o., Song, S.-l., Yong, H.-s. & Yin, W.-f. (2018), 'Microbial community composition reveals spatial variation and distinctive core microbiome of the weaver ant *Oecophylla smaragdina* in Malaysia', *Scientific Reports* (June), 1–11.

Clark, T. (1978), 'Honeybee spiroplasmosis , a new problem for beekeepers', *American Bee Journal* **118**, 18 – 19.

Clayton, A. L., Oakeson, K. F., Gutin, M., Pontes, A., Dunn, D. M., Von, A. C., Weiss, R. B., Fisher, M. & Dale, C. (2012), 'A novel human infection derived bacterium provides insights into the evolutionary origins of mutualistic insect bacterial symbioses', *PLOS Genetics* **8**(11).

Corbin, C. (2017), 'Thesis: The evolutionary ecology of an insect-bacterial mutualism', (September).

Corby-Harris, V., Maes, P. & Anderson, K. E. (2014), 'The bacterial communities associated with honey bee (*Apis mellifera*) foragers', *PLoS ONE* **9**(4), e95056.

Cornman, R. S., Tarpy, D. R., Chen, Y., Jeffreys, L., Lopez, D., Pettis, J. S., VanEngelsdorp, D. & Evans, J. D. (2012), 'Pathogen webs in collapsing honey bee colonies', *PLoS ONE* **7**(8).

Cosmides, L. M. & Tooby, J. (1981), 'Cytoplasmic inheritance and intergenomic conflict', *Journal of Theoretical Biology* **89**, 83–129.

Coulson, G., Elgar, M. A., Garnick, S. W. & Beveridge, I. (2009), 'Foraging efficiency and parasite risk in eastern grey kangaroos (*Macropus giganteus*)', *Behavioral Ecology* **21**(1), 129–137.

Cremer, S., Pull, C. D. & Fürst, M. A. (2018), 'Social Immunity: Emergence and Evolution of Colony-Level Disease Protection', *Annual Review of Entomology* **63**(1), annurev–ento–020117–043110.

Currie, C. R., Poulsen, M., Mendenhall, J., Boomsma, J. J. & Billen, J. (2006), 'Coevolved Crypts and Exocrine Glands Support Mutualistic Bacteria in Fungus-Growing Ants', *Science* **311**(5757), 81–83.

Dainat, B., Evans, J. D., Chen, Y. P., Gauthier, L. & Neumann, P. (2012), 'Predictive markers of honey bee colony collapse', *PLoS ONE* **7**(2).

Dale, C., Beeton, M., Harbison, C., Jones, T. & Pontes, M. (2006), 'Isolation, pure culture, and characterization of "*Candidatus Arsenophonus* arthropodicus," an intracellular secondary endosymbiont from the hippoboscid louse fly Pseudolynchia canariensis', *Applied and Environmental Microbiology* **72**(4), 2997–3004.

Dale, C. & Maudlin, I. (1999), 'Sodalis gen. nov. and *Sodalis glossinidius* sp. nov., a microaerophilic secondary endosymbiont of the tsetse fly *Glossina morsitans*', *International journal of systematic bacteriology* **49 Pt 1**, 267–275.

Dale, C. & Moran, N. A. (2006), 'Molecular Interactions between Bacterial Symbionts and Their Hosts', *Cell* **126**(3), 453–465.

Dale, C., Wang, B., Moran, N. & Ochman, H. (2003), 'Loss of DNA recombinational repair enzymes in the initial stages of genome degeneration.', *Molecular biology and evolution* **20**(8), 1188–1194.

Darby, A. C., Choi, J. H., Wilkes, T., Hughes, M. A., Werren, J. H., Hurst, G. D. D. & Colbourne, J. K. (2010), 'Characteristics of the genome of *Arsenophonus nasoniae*, son-killer bacterium of the wasp *Nasonia*', *Insect Molecular Biology* **19**(SUPPL. 1), 75–89.

Darby, A. C. & Douglas, A. E. (2003), 'Elucidation of the Transmission Patterns of an Insect-Borne Bacterium', **69**(8), 4403–4407.

Davis, R. E. (1978), '*Spiroplasma* associated with flowers of the tulip tree (Liriodendron tulipifera L.).', *Canadian journal of microbiology* **24**(8), 954–959.

Dedeine, F., Ahrens, M., Calcaterra, L. & Shoemaker, D. D. (2005), 'Social parasitism in fire ants (Solenopsis spp.): A potential mechanism for interspecies transfer of *Wolbachia*', *Molecular Ecology* **14**(5), 1543–1548.

Dellicour, S., Mardulyn, P., Hardy, O. J., Hardy, C., Roberts, S. P. & Vereecken, N. J. (2014), 'Inferring the mode of colonization of the rapid range expansion of a solitary bee from multilocus DNA sequence variation', *Journal of Evolutionary Biology* **27**(1), 116–132.

Desai, S. D. & Currie, R. W. (2016), 'Effects of wintering environment and parasite-Pathogen interactions on honey bee colony loss in north temperate regions', *PLoS ONE* **11**(7), 1–24.

Dobson, S. L., Marsland, E. J. & Rattanadechakul, W. (2002), 'Mutualistic *Wolbachia* infection in Aedes albopictus: Accelerating cytoplasmic drive', *Genetics* **160**(3), 1087–1094.

Döke, M. A., Frazier, M. & Grozinger, C. M. (2015), 'Overwintering honey bees: biology and management', *Current Opinion in Insect Science* **10**, 185–193.

Donaldson, G. P., Lee, S. M. & Mazmanian, S. K. (2017), 'Gut biogeography of the bacterial microbiota', **14**(1), 20–32.

Donkersley, P., Rhodes, G., Pickup, R. W., Jones, K. C. & Wilson, K. (2018), 'Bacterial communities associated with honeybee food stores are correlated with land use', *Ecology and Evolution* **8**(10), 4743–4756.

Doolittle, W. F. & Booth, A. (2017), 'It's the song, not the singer: an exploration of holobiosis and evolutionary theory', *Biology & Philosophy* **32**(1), 5–24.

Douglas, A. E. (1998), 'Host benefit and the evolution of specialization in symbiosis', *Heredity* **81**, 599.

Douglas, A. E. (2008), 'Conflict, cheats and the persistence of symbioses', *New Phytologist* **177**(4), 849–858.

Douglas, A. E. (2010), *The Symbiotic Habit*, Princeton University Press, Princeton, NJ.

Drew, G. C., Frost, C. L. & Hurst, G. D. (2019), 'Reproductive parasitism and positive fitness effects of heritable microbes', *eLS* .

Durand, A.-A., Bergeron, A., Constant, P., Buffet, J.-P., Déziel, E. & Guertin, C. (2015), 'Surveying the endomicrobiome and ectomicrobiome of bark beetles: The case of *Dendroctonus simplex*', *Scientific reports* **5**, 17190.

Duron, O., Bouchon, D., Boutin, S., Bellamy, L., Zhou, L., Engelstädter, J. & Hurst, G. D. (2008), 'The

diversity of reproductive parasites among arthropods: *Wolbachia* do not walk alone', *BMC Biology* **6**, 1–12.

Duron, O., Noël, V., Mccoy, K. D., Bonazzi, M., Sidi, K., Morel, O., Vavre, F., Zenner, L., Jourdain, E., Durand, P., Arnathau, C., Renaud, F., Trape, J.-f., Biguezoton, S., Cremaschi, J., Dietrich, M. & Léger, E. (2015), 'The Recent Evolution of a Maternally- Inherited Endosymbiont of Ticks Led to the Emergence of the Q Fever Pathogen , Coxiella burnetii', pp. 1–23.

Duron, O., Wilkes, T. E. & Hurst, G. D. D. (2010), 'Interspecific transmission of a male-killing bacterium on an ecological timescale', *Ecology Letters* **13**(9), 1139–1148.

Dyson, E. A. & Hurst, G. D. D. (2004), 'Persistence of an extreme sex-ratio bias in a natural population', *Proceedings of the National Academy of Sciences of the United States of America* **101**(17), 6520–6523.

Eberl, L. & Vandamme, P. (2016), 'Members of the genus *Burkholderia*: good and bad guys', *F1000Research* **5**, 1–7.

Ebert, D. (1998), 'Experimental Evolution of Parasites', **282**(November), 1432–1436.

Ebert, D. (2013), 'The Epidemiology and Evolution of Symbionts with Mixed-Mode Transmission', *Annual Review of Ecology, Evolution, and Systematics* **44**(1), 623–643.

Ebert, D., Journal, S., May, N. & Ebert, D. (1995), 'The ecological interactions between a microsporidian parasite and its host *Daphnia magna*', **64**(3), 361–369.

Elnagdy, S., Majerus, M. E. N., Gardener, M. & Lawson Handley, L. J. (2013), 'The direct effects of male killer infection on fitness of ladybird hosts (Coleoptera: Coccinellidae)', *Journal of Evolutionary Biology* **26**(8), 1816–1825.

Engel, P. & Moran, N. A. (2013), 'The gut microbiota of insects - diversity in structure and function', *FEMS Microbiology Reviews* **37**(5), 699–735.

Engel, P., Stepanauskas, R. & Moran, N. A. (2014), 'Hidden Diversity in Honey Bee Gut Symbionts Detected by Single-Cell Genomics', *PLoS Genetics* **10**(9).

Engelstädter, J. & Hurst, G. D. (2009), 'The Ecology and Evolution of Microbes that Manipulate Host Reproduction', *Annual Review of Ecology, Evolution, and Systematics* **40**(1), 127–149.
URL: *http://www.annualreviews.org/doi/10.1146/annurev.ecolsys.110308.120206*

Evans, J. D. (2004), 'Transcriptional immune responses by honey bee larvae during invasion by the bacterial pathogen, Paenibacillus larvae', *Journal of Invertebrate Pathology* **85**(2), 105–111.

Evans, J. D. & Schwarz, R. S. (2011), 'Bees brought to their knees: Microbes affecting honey bee health', *Trends in Microbiology* **19**(12), 614–620.
URL: *http://dx.doi.org/10.1016/j.tim.2011.09.003*

Ewald, P. W. (1983), 'Host-Parasite Relations, Vectors, and the Evolution of Disease Severity', *Annual Review of Ecology and Systematics* **14**, 465–485.

Ewald, P. W. (1987), 'Transmission modes and evolution of the parasitism-mutualism continuum.', *Annals of the New York Academy of Sciences* **503**, 295–306.

Fahrenholz, L., Lamprecht, I. & Schricker, B. (1989), 'Thermal investigations of a honey bee colony: thermoregulation of the hive during summer and winter and heat production of members of different bee castes', *Journal of Comparative Physiology B* **159**(5), 551–560.

Fels, D. & Kaltz, O. (2006), 'Temperature-dependent transmission and latency of Holospora undulata, a micronucleus-specific parasite of the ciliate *Paramecium caudatum*', *Proceedings of the Royal Society B: Biological Sciences* **273**(1589), 1031–1038.

Fenton, A., Johnson, K. N., Brownlie, J. C. & Hurst, G. D. D. (2011), 'Solving the *Wolbachia* Paradox: Modeling the Tripartite Interaction between Host, *Wolbachia* , and a Natural Enemy', *The American Naturalist* **178**(3), 333–342.

Fenton, A. & Pedersen, A. B. (2005), 'Community epidemiology framework for classifying disease threats.', *Emerging infectious diseases* **11**(12), 1815–1821.

Ferree, P. M., Avery, A., Azpurua, J., Wilkes, T. & Werren, J. H. (2008), 'A bacterium targets maternally inherited centrosomes to kill males in *Nasonia*.', *Current biology : CB* **18**(18), 1409–1414.

Ford, S. A., Williams, D., Paterson, S. & King, K. C. (2017), 'Co-evolutionary dynamics between a defensive microbe and a pathogen driven by fluctuating selection', *Molecular Ecology* **26**(7), 1778–1789.

Forfert, N., Natsopoulou, M. E., Frey, E., Rosenkranz, P., Paxton, R. J. & Moritz, R. F. A. (2015), 'Parasites and Pathogens of the Honeybee (*Apis mellifera*) and Their Influence on Inter-Colonial Transmission', *PLoS ONE* **10**(10), e0140337.

Foster, K. R. & Wenseleers, T. (2006), 'A general model for the evolution of mutualisms.', *Journal of evolutionary biology* **19**(4), 1283–1293.

Frank, S. A. (1996*a*), 'Host-symbiont conflict over the mixing of symbiotic lineages.', *Proceedings. Biological sciences* **263**(1368), 339–344.

Frank, S. A. (1996*b*), 'Models of parasite virulence.', *The Quarterly review of biology* **71**(1), 37–78.

Free, J. & Spencer-Booth, Y. (1961), *Further experiments on the drifting of honey-bees*, Vol. 57.

Freeland, W. J. (1976), 'Pathogens and the Evolution of Primate Sociality', *Biotropica* **8**(1), 12–24.

Frost, C. L., FernÁndez-MarÍn, H., Smith, J. E. & Hughes, W. O. (2010), 'Multiple gains and losses of *Wolbachia* symbionts across a tribe of fungus-growing ants', *Molecular Ecology* **19**(18), 4077–4085.

Frost, C. L., Pollock, S. W., Smith, J. E. & Hughes, W. O. (2014), '*Wolbachia* in the flesh: Symbiont intensities in germ-line and somatic tissues challenge the conventional view of *Wolbachia* transmission routes', *PLoS ONE* **9**(7).

Funaro, C. F., Kronauer, D. J. C., Moreau, C. S., Goldman-Huertas, B., Pierce, N. E. & Russell, J. A. (2011), 'Army Ants Harbor a Host-Specific Clade of Entomoplasmatales Bacteria', *Applied and Environmental Microbiology* **77**(1), 346 LP – 350.

Fürst, M. A., Mcmahon, D. P., Caspar, J., Theodorou, P., Brown, M. J. & Paxton, R. J. (2015), 'A sting in the spit: Widespread cross-infection of multiple RNA viruses across wild and managed bees', *Journal of Animal Ecology* **84**(3), 615–624.

Gandon, S. (2004), 'Evolution of multihost parasites', *Evolution* **58**, 455–469.

Gauthier, L., Cornman, S., Hartmann, U., Cousserans, F., Evans, J. D., De Miranda, J. R. & Neumann, P. (2015), 'The *Apis mellifera* filamentous virus genome', *Viruses* **7**(7), 3798–3815.

Gerth, M., Röthe, J. & Bleidorn, C. (2013), 'Tracing horizontal *Wolbachia* movements among bees (Anthophila): A combined approach using multilocus sequence typing data and host phylogeny', *Molecular Ecology* **22**(24), 6149–6162.

Gerth, M., Saeed, A., White, J. A. & Bleidorn, C. (2015), 'Extensive screen for bacterial endosymbionts reveals taxon-specific distribution patterns among bees (Hymenoptera, Anthophila)', *FEMS Microbiology Ecology* **91**(6), 1–12.

Gherna, R. L., Werren, J. H., Weisburg, W., Cote, R., Woese, C. R., Mandelco, L. & Brenner, D. J. (1991), '*Arsenophonus Nasonia*e gen. nov., sp. nov., the Causative Agent of the Son-Killer Trait in the Parasitic Wasp *Nasoniavitripennis*', *International Journal of Systematic Bacteriology* **41**(4), 563–565.

Ghoul, M. & Mitri, S. (2016), 'The ecology and evolution of microbial competition', *Trends in Microbiology* **24**(10), 833–845.

Gil, R., Silva, F. J., Zientz, E., Delmotte, F., González-Candelas, F., Latorre, A., Rausell, C., Kamerbeek, J., Gadau, J., Hölldobler, B., van Ham, R. C. H. J., Gross, R. & Moya, A. (2003), 'The genome sequence of *Blochmannia floridanus*: Comparative analysis of reduced genomes', *Proceedings of the National Academy of Sciences of the United States of America* **100**(16), 9388–9393.

Gilliam, M. (1971), 'Microbial Sterility of the Intestinal Content of the Immature Honey Bee, *Apis mellifera*1,2,3', *Annals of the Entomological Society of America* **64**(1), 315–316.

Giovannoni, S. J., Tripp, H. J., Givan, S., Podar, M., Vergin, K. L., Baptista, D., Bibbs, L., Eads, J., Richardson, T. H., Noordewier, M., Rappe, M. S., Short, J. M., Carrington, J. C. & Mathur, E. J. (2005), 'Genome streamlining in a cosmopolitan oceanic bacterium.', *Science (New York, N.Y.)* **309**(5738), 1242–1245.

Gonella, E., Pajoro, M., Marzorati, M., Crotti, E., Mandrioli, M., Pontini, M., Bulgari, D., Negri, I., Sacchi, L., Chouaia, B., Daffonchio, D. & Alma, A. (2015), 'Plant-mediated interspecific horizontal transmission of an intracellular symbiont in insects', *Scientific Reports* **5**, 15811.

Gordon, E. R. L., McFrederick, Q. S. & Weirauch, C. (2016), 'Comparative phylogenetic analysis of bacterial associates in Pyrrhocoroidea and evidence for ancient and persistent environmental symbiont reacquisition in Largidae (Hemiptera: Heteroptera).', *Applied and Environmental Microbiology* **82**(24), 064022.

Gottlieb, Y., Ghanim, M., Gueguen, G., Kontsedalov, S., Vavre, F., Fleury, F. & Zchori-Fein, E. (2008), 'Inherited intracellular ecosystem: symbiotic bacteria share bacteriocytes in whiteflies', *The FASEB Journal* **22**(7), 2591–2599.

Goulson, D., Nicholls, E., Botias, C. & Rotheray, E. L. (2015), 'Bee declines driven by combined stress from parasites, pesticides, and lack of flowers', *Science* **347**(6229).

Graystock, P., Blane, E. J., Mcfrederick, Q. S., Goulson, D. & Hughes, W. O. H. (2016), 'International Journal for Parasitology : Parasites and Wildlife Do managed bees drive parasite spread and emergence in wild bees ?', *International Journal for Parasitology: Parasites and Wildlife* **5**(1), 64–75.

Graystock, P., Goulson, D. & Hughes, W. O. (2015), 'Parasites in bloom: Flowers aid dispersal and transmission of pollinator parasites within and between bee species', *Proceedings of the Royal Society B: Biological Sciences* **282**(1813).

Graystock, P., Goulson, D. & Hughes, W. O. H. (2014), 'The relationship between managed bees and the prevalence of parasites in bumblebees', *PeerJ* **2**, e522–e522.

Grenier, S., Gomes, S. M., Pintureau, B., Lassablière, F. & Bolland, P. (2002), 'Use of tetracycline in larval diet to study the effect of *Wolbachia* on host fecundity and clarify taxonomic status of Trichogramma species in cured bisexual lines', *Journal of Invertebrate Pathology* **80**(1), 13–21.

Habig, B. & Archie, E. A. (2015), 'Social status, immune response and parasitism in males: A meta-analysis', *Philosophical Transactions of the Royal Society B: Biological Sciences* **370**(1669).

Habig, B., Doellman, M. M., Woods, K., Olansen, J. & Archie, E. A. (2018), 'Social status and parasitism in male and female vertebrates: a meta-analysis', *Scientific Reports* **8**(1), 3629.

Hamilton, C., Lejeune, B. T. & Rosengaus, R. B. (2011), 'Trophallaxis and prophylaxis: social immunity in the carpenter ant *Camponotus pennsylvanicus*', *Biology Letters* **7**(1), 89–92.

Hamilton, W. (1987), *Animal societies: theories and facts*, Japan Scientific Society Press., Tokyo.

Harmer, T. L., Rotjan, R. D., Nussbaumer, A. D., Bright, M., Ng, A. W., DeChaine, E. G. & Cavanaugh, C. M. (2008), 'Free-Living Tube Worm Endosymbionts Found at Deep-Sea Vents', *Applied and Environmental Microbiology* **74**(12), 3895 LP – 3898.

Hawker, J. I., Ayres, J. G., Blair, I., Evans, M. R., Smith, D. L., Smith, E. G., Burge, P. S., Carpenter, M. J., Caul, E. O., Coupland, B., Desselberger, U., Farrell, I. D., Saunders, P. J. & Wood, M. J. (1998), 'A large outbreak of Q fever in the West Midlands: windborne spread into a metropolitan area?', *Communicable disease and public health* **1**(3), 180–187.

Haygood, M. G. (1993), 'Light organ symbioses in fishes.', *Critical reviews in microbiology* **19**(4), 191–216.

Hedges, L. M., Brownlie, J. C., O'Neill, S. L. & Johnson, K. N. (2008), '*Wolbachia* and virus protection in insects', *Science* **322**(5902), 702.

Heinze, G. & Schemper, M. (2002), 'A solution to the problem of separation in logistic regression', *Statistics in Medicine* **21**(16), 2409–2419.

Hendry, T. A., De Wet, J. R., Dougan, K. E. & Dunlap, P. V. (2016), 'Genome evolution in the obligate but environmentally active luminous symbionts of flashlight fish', *Genome Biology and Evolution* **8**(7), 2203–2213.

Hilgenboecker, K., Hammerstein, P., Schlattmann, P., Telschow, A. & Werren, J. H. (2008), 'How many species are infected with *Wolbachia*? - A statistical analysis of current data', *FEMS Microbiology Letters* **281**(2), 215–220.

Himler, A. G., Adachi-Hagimori, T., Bergen, J. E., Kozuch, A., Kelly, S. E., Tabashnik, B. E., Chiel, E., Duckworth, V. E., Dennehy, T. J., Zchori-Fein, E. & Hunter, M. S. (2011), 'Rapid spread of a bacterial symbiont in an invasive whitefly is driven by fitness benefits and female bias', *Science* **332**(6026), 254–256.

Hoang, D. T., Chernomor, O., von Haeseler, A., Minh, B. Q. & Vinh, L. S. (2018), 'UFBoot2: Improving the Ultrafast Bootstrap Approximation', *Molecular Biology and Evolution* **35**(2), 518–522.

Hoffmann, A. A., Montgomery, B. L., Popovici, J., Iturbe-Ormaetxe, I., Johnson, P. H., Muzzi, F., Greenfield, M., Durkan, M., Leong, Y. S., Dong, Y., Cook, H., Axford, J., Callahan, A. G., Kenny, N., Omodei, C., McGraw, E. A., Ryan, P. A., Ritchie, S. A., Turelli, M. & O'Neill, S. L. (2011), 'Successful establishment of *Wolbachia* in *Aedes* populations to suppress dengue transmission', *Nature* **476**(7361), 454–459.

Holldobler, B. & Wilson, E. O. (1990), *The Ants*, Harvard University Press, Cambridge, Massachusetts.

Holt, R. D., Dobson, A. P., Begon, M., Bowers, R. G. & Schauber, E. M. (2003), 'Parasite establishment in host communities', *Ecology Letters* **6**(9), 837–842.

Hongoh, Y. (2010), 'Diversity and genomes of uncultured microbial symbionts in the termite gut.', *Bioscience, biotechnology, and biochemistry* **74**(6), 1145–1151.

Hosokawa, T., Hironaka, M., Mukai, H., Inadomi, K., Suzuki, N. & Fukatsu, T. (2012), 'Mothers

never miss the moment: A fine-tuned mechanism for vertical symbiont transmission in a subsocial insect', *Animal Behaviour* **83**(1), 293–300.

Hosokawa, T., Ishii, Y., Nikoh, N., Fujie, M., Satoh, N. & Fukatsu, T. (2016), 'Obligate bacterial mutualists evolving from environmental bacteria in natural insect populations', *Nature Microbiology* **1**(1), 1–7.

Hosseini, P. R., Dhondt, A. & Dobson, A. (2004), 'Seasonality and wildlife disease : how seasonal birth, aggregation and variation in immunity affect the dynamics of *Mycoplasma gallisepticum* in house finches', (December), 2569–2577.

Hubert, J., Bicianova, M., Ledvinka, O., Kamler, M., Lester, P. J., Nesvorna, M., Kopecky, J. & Erban, T. (2017), 'Changes in the Bacteriome of Honey Bees Associated with the Parasite *Varroa destructor*, and Pathogens *Nosema* and *Lotmaria passim*', *Microbial Ecology* **73**(3), 685–698.

Huger, A. M., Skinner, S. W. & Werren, J. H. (1985), 'Bacterial infections associated with the son-killer trait in the parasitoid wasp *Nasoniavitripennis* (Hymenoptera: Pteromalidae)', *Journal of Invertebrate Pathology* **46**(3), 272–280.

Hughes, D. P., Pierce, N. E. & Boomsma, J. J. (2008), 'Social insect symbionts: evolution in homeostatic fortresses', *Trends in Ecology and Evolution* **23**(12), 672–677.

Hughes, W. O. H. & Boomsma, J. J. (2006), 'Does genetic diversity hinder parasite evolution in social insect colonies?', *Journal of Evolutionary Biology* **19**(1), 132–143.

Hughes, W. O. H., Eilenberg, J. & Boomsma, J. J. (2002), 'Trade-offs in group living : transmission and disease resistance in leaf-cutting ants', (8), 1811–1819.

Hurst, G. D. (2017), 'Extended genomes: Symbiosis and evolution', *Proceedings of the Royal Society* **7**(5), 17–20.

Hurst, G. D. & Frost, C. L. (2015), 'Reproductive parasitism: Maternally inherited symbionts in a biparental world', *Cold Spring Harbor Perspectives in Biology* **7**(5), 1–21.

Hurst, G. D. & Majerus, M. E. (1993), 'Why do maternally inherited microorganisms kill males?', *Heredity* **71**(1), 81–95.

Hurst, L. D. (1991), 'The incidences and evolution of cytoplasmic male killers', *Proceedings of the Royal Society B: Biological Sciences* **244**(1310), 91–99.

Hypsa, V. & Dale, C. (1997), 'In vitro culture and phylogenetic analysis of "*Candidatus* Arsenophonus triatominarum," an intracellular bacterium from the triatomine bug, *Triatoma infestans*', *International Journal of Systematic Bacteriology* **47**(4), 1140–1144.

Johansson, T. S. K. & Johansson, M. P. (1979), 'The Honeybee Colony in Winter', *Bee World* **60**(4), 155–170.

Johnson, P. T. J., de Roode, J. C. & Fenton, A. (2015), 'Why infectious disease research needs community ecology', **349**(6252).

Kaltenpoth, M. (2017), 'Symbiont dynamics and strain diversity in the defensive mutualism between *Lagria* beetles and *Burkholderia*', **19**, 3674–3688.

Kaltenpoth, M., Yildirim, E., Gürbüz, M. F., Herzner, G. & Strohm, E. (2012), 'Refining the roots of the beewolf-streptomyces symbiosis: Antennal symbionts in the rare genus Philanthinus (Hymenoptera, Crabronidae)', *Applied and Environmental Microbiology* **78**(3), 822–827.

Kalyaanamoorthy, S., Minh, B. Q., Wong, T. K. F., von Haeseler, A. & Jermiin, L. S. (2017), 'ModelFinder: fast model selection for accurate phylogenetic estimates', *Nature Methods* **14**, 587.

Katsnelson, A. (2015), 'Microbiome: The puzzle in a bee's gut', *Nature* **521**(7552), S56.

Keller, L. & Genoud, M. (1997), 'Extraordinary lifespans in ants: a test of evolutionary theories of ageing', *Nature* **389**, 958.

Keller, L., Liautard, C., Reuter, M. A. X., Brown, W. D., Chapuisat, M. & Sundstro, L. (2001), 'Sex ratio and *Wolbachia* infection in the ant *Formica exsecta*', **87**(February), 227–233.

Kelly, A., Dunn, A. M. & Hatcher, M. J. (2002), 'Incomplete feminisation by the microsporidian sex ratio distorter, *Nosema granulosis* , and reduced transmission and feminisation efficiency at low temperatures', **32**, 825–831.

Kenyon, L. J., Meulia, T. & Sabree, Z. L. (2015), 'Habitat visualization and genomic analysis of "*Candidatus* pantoea carbekii," the primary symbiont of the brown marmorated stink bug', *Genome Biology and Evolution* **7**(2), 620–635.

Kiersi, E. T. & Denison, R. F. (2008), 'Sanctions, Cooperation, and the Stability of Plant-Rhizosphere Mutualisms', *Annual Review of Ecology, Evolution, and Systematics* **39**, 215–236.

Kikuchi, Y., Hosokawa, T. & Fukatsu, T. (2007), 'Insect-Microbe Mutualism without Vertical Transmission: a Stinkbug Acquires a Beneficial Gut Symbiont from the Environment Every Generation', *Applied and Environmental Microbiology* **73**(13), 4308 LP – 4316.

King, K. C., Brockhurst, M. A., Vasieva, O., Paterson, S., Betts, A., Ford, S. A., Frost, C. L., Horsburgh, M. J., Haldenby, S. & Hurst, G. D. (2016), 'Rapid evolution of microbe-mediated protection against pathogens in a worm host', *ISME Journal* **10**(8), 1915–1924.

King, K. C., Gendron, A. D., McLaughlin, J. D., Giroux, I., Brousseau, P., Cyr, D., Ruby, S. M., Fournier, M. & Marcogliese, D. J. (2008), 'Short-term seasonal changes in parasite community structure in northern leopard froglets (*Rana pipiens*) Inhabiting Agricultural Wetlands', *Journal of Parasitology* **94**(1), 13–22.

King, K. C. & Lively, C. M. (2009), 'Geographic variation in sterilizing parasite species and the Red Queen', *Oikos* **118**(9), 1416–1420.

Kinross, J. M., Darzi, A. W. & Nicholson, J. K. (2011), 'Gut microbiome-host interactions in health and disease', *Genome Medicine* **3**(3), 14.

Kirkness, E. F., Haas, B. J., Sun, W., Braig, H. R., Perotti, M. A., Clark, J. M., Lee, S. H., Robertson, H. M., Kennedy, R. C., Elhaik, E., Gerlach, D., Kriventseva, E. V., Elsik, C. G., Graur, D., Hill, C. A., Veenstra, J. A., Walenz, B., Tubio, J. M. C., Ribeiro, J. M. C., Rozas, J., Johnston, J. S., Reese, J. T., Popadic, A., Tojo, M., Raoult, D., Reed, D. L., Tomoyasu, Y., Kraus, E., Mittapalli, O., Margam, V. M., Li, H.-M., Meyer, J. M., Johnson, R. M., Romero-Severson, J., VanZee, J. P., Alvarez-Ponce, D., Vieira, F. G., Aguade, M., Guirao-Rico, S., Anzola, J. M., Yoon, K. S., Strycharz, J. P., Unger, M. F., Christley, S., Lobo, N. F., Seufferheld, M. J., Wang, N., Dasch, G. A., Struchiner, C. J., Madey, G., Hannick, L. I., Bidwell, S., Joardar, V., Caler, E., Shao, R., Barker, S. C., Cameron, S., Bruggner, R. V., Regier, A., Johnson, J., Viswanathan, L., Utterback, T. R., Sutton, G. G., Lawson, D., Waterhouse, R. M., Venter, J. C., Strausberg, R. L., Berenbaum, M. R., Collins, F. H., Zdobnov, E. M. & Pittendrigh, B. R. (2010), 'Genome sequences of the human body louse and its primary endosymbiont provide insights into the permanent parasitic lifestyle', *Proceedings of the National Academy of Sciences of the United States of America* **107**(27), 12168–12173.

Koch, H., Abrol, D. P., Li, J. & Schmid-Hempel, P. (2013), 'Diversity and evolutionary patterns of bacterial gut associates of corbiculate bees.', *Molecular ecology* **22**(7), 2028–2044.

Koch, H. & Schmid-Hempel, P. (2011), 'Socially transmitted gut microbiota protect bumble bees against an intestinal parasite', *Proceedings of the National Academy of Sciences of the United States of America* **108**(48), 19288 LP – 19292.

Koopman, J. (2004), 'Modeling infection transmission.', *Annual review of public health* **25**, 303–326.

Koprivnikar, J. & Penalva, L. (2015), 'Lesser of two evils? Foraging choices in response to threats of predation and parasitism', *PLoS ONE* **10**(1), e0116569–e0116569.

Kwong, W. K., Engel, P., Koch, H. & Moran, N. A. (2014), 'Genomics and host specialization of honey bee and bumble bee gut symbionts', *Proceedings of the National Academy of Sciences of the United States of America* **111**(31), 11509–11514.

Kwong, W. K., Medina, L. A., Koch, H., Sing, K. W., Soh, E. J. Y., Ascher, J. S., Jaffé, R. & Moran, N. A. (2017), 'Dynamic microbiome evolution in social bees', *Science Advances* **3**(3), e1600513.

Kwong, W. K. & Moran, N. A. (2015), 'Evolution of host specialization in gut microbes: The bee gut as a model', *Gut Microbes* **6**(3), 214–220.

Kwong, W. K. & Moran, N. A. (2016), 'Gut microbial communities of social bees', *Nature Reviews Microbiology* **14**(6), 374–384.

Lafferty, K. D. (2009), 'The ecology of climate change and infectious diseases', *Ecology* **90**(4), 888–900.

Lalzar, I., Harrus, S., Mumcuoglu, K. Y. & Gottlieb, Y. (2012), 'Composition and seasonal variation of Rhipicephalus turanicus and Rhipicephalus sanguineus bacterial communities', *Applied and Environmental Microbiology* **78**(12), 4110–4116.

Langwig, K. E., Frick, W. F., Bried, J. T., Hicks, A. C., Kunz, T. H. & Marm Kilpatrick, A. (2012), 'Sociality, density-dependence and microclimates determine the persistence of populations suffering from a novel fungal disease, white-nose syndrome', *Ecology Letters* **15**(9), 1050–1057.

Lartillot, N., Lepage, T. & Blanquart, S. (2009), 'PhyloBayes 3: a Bayesian software package for phylogenetic reconstruction and molecular dating', *Bioinformatics* **25**(17), 2286–2288.

Lass, S. & Ebert, D. (2006), 'Apparent seasonality of parasite dynamics : analysis of cyclic prevalence patterns', (November 2005), 199–206.

Laughton, A. M., Fan, M. H. & Gerardo, N. M. (2014), 'The combined effects of bacterial symbionts and aging on life history traits in the pea aphid, *Acyrthosiphon pisum*', *Applied and Environmental Microbiology* **80**(2), 470–477.

Law, R. & Dieckmann, U. (1998), 'Symbiosis through exploitation and the merger of lineages in evolution', *Proceedings of the Royal Society B* **265**(March).

Leung, T. L. F. & Poulin, R. (2008), 'Parasitism, commensalism, and mutualism: Exploring the many shades of symbioses', *Vie et Milieu* **58**(2), 107–115.

Levin, M. D. (1961), 'Interactions among foraging honey bees from different apiaries in the same field', *Insectes Sociaux* **8**(3), 195–201.

Lewis, D. H. (1985), 'Symbiosis and mutualism: crisp concepts and soggy semantics'.

Lewis, F., Butler, A. & Gilbert, L. (2011), 'A unified approach to model selection using the likelihood ratio test', *Methods in Ecology and Evolution* **2**(2), 155–162.

Li, M., Wang, B., Zhang, M., Rantalainen, M., Wang, S., Zhou, H., Zhang, Y., Shen, J., Pang, X., Zhang, M., Wei, H., Chen, Y., Lu, H., Zuo, J., Su, M., Qiu, Y., Jia, W., Xiao, C., Smith, L. M., Yang, S., Holmes, E., Tang, H., Zhao, G., Nicholson, J. K., Li, L. & Zhao, L. (2008), 'Symbiotic gut microbes modulate human metabolic phenotypes', *Proceedings of the National Academy of Sciences of the United States of America* **105**(6), 2117–2122.

Li, S.-j., Ahmed, M. Z., Lv, N., Shi, P.-q. & Wang, X.-m. (2016), 'Plant mediated horizontal transmission of *Wolbachia* between whiteflies', *ISME Journal* **11**(4), 1019–1028.

Li, Y., Liles, M. R. & Halanych, K. M. (2018), 'Endosymbiont genomes yield clues of tubeworm success', *ISME Journal* pp. 2785–2795.

Linder, J. E., Owers, K. A. & Promislow, D. E. L. (2008), 'The effects of temperature on host-pathogen interactions in *D. melanogaster*: who benefits?', *Journal of Insect Physiology* **54**(1), 297–308.

Lloyd-Smith, J. O., Cross, C. P., Briggs, J. C., Daugherty, M., Getz, M. W., Latto, J., Sanchez, M., Smith, A. B. & Swei, A. (2005), 'Should we expect population thresholds for wildlife disease?', *Trends in Ecology and Evolution* **20**, 511 – 519.

Lo, N. & Evans, T. A. (2007), 'Phylogenetic diversity of the intracellular symbiont *Wolbachia* in termites', *Molecular Phylogenetics and Evolution* **44**(1), 461–466.

Lo, W. S., Huang, Y. Y. & Kuo, C. H. (2016), 'Winding paths to simplicity: Genome evolution in facultative insect symbionts', *FEMS Microbiology Reviews* **40**(6), 855–874.

Lombardo, M. P. (2008), 'Access to mutualistic endosymbiotic microbes: An underappreciated benefit of group living', *Behavioral Ecology and Sociobiology* **62**(4), 479–497.

Loope, K. J., Baty, J. W., Lester, P. J. & Rankin, E. E. W. (2019), 'Pathogen shifts in a honeybee predator following the arrival of the Varroa mite', *Proceedings of the Royal Society B* **1**.

Ludvigsen, J., Rangberg, A., Avershina, E., Sekelja, M., Kreibich, C., Amdam, G. & Rudi, K. (2015), 'Shifts in the Midgut/Pyloric Microbiota Composition within a Honey Bee Apiary throughout a Season.', *Microbes and environments* **30**(3), 235–244.

Lyapunov, Y. E., Kuzyaev, R. Z., Khismatullin, R. G. & Bezgodova, O. A. (2008), 'Intestinal enterobacteria of the hibernating *Apis mellifera* L. bees', *Microbiology* **77**(3), 373–379.

Manley, R., Boots, M. & Wilfert, L. (2015), 'Emerging viral disease risk to pollinating insects: Ecological, evolutionary and anthropogenic factors', *Journal of Applied Ecology* **52**(2), 331–340.

Margulis, L. (1970), *Origin of eukaryotic cells*, Yale University Press, New Haven.

Marsh, S. E., Poulsen, M., Pinto-Tomás, A. & Currie, C. R. (2014), 'Interaction between workers during a short time window is required for bacterial symbiont transmission in Acromyrmex leaf-cutting ants', *PLoS ONE* **9**(7), 1–9.

Martinson, V. G., Danforth, B. N., Minckley, R. L., Rueppell, O., Tingek, S. & Moran, N. A. (2011), 'A simple and distinctive microbiota associated with honey bees and bumble bees', *Molecular Ecology* **20**(3), 619–628.

Martinson, V. G., Moy, J. & Moran, N. A. (2012), 'Establishment of characteristic gut bacteria during development of the honeybee worker', *Applied and Environmental Microbiology* **78**(8), 2830–2840.

Masson, F., Rey, M., Aksoy, E., Vincent-mone, C., Aubailly-giraud, E. & Zaidman-re, A. (2014), 'Report Insects Recycle Endosymbionts when the Benefit Is Over', *Current Biology* pp. 2267–2273.

McCallum, H., Barlow, N. & Hone, J. (2001), 'How should pathogen transmission be modelled?', *Trends in Ecology & Evolution* **16**(6), 295–300.

McCutcheon, J. P. & Moran, N. A. (2010), 'Functional convergence in reduced genomes of bacterial symbionts spanning 200 my of evolution', *Genome Biology and Evolution* **2**(1), 708–718.

McCutcheon, J. P. & Moran, N. A. (2012), 'Extreme genome reduction in symbiotic bacteria', *Nature Reviews Microbiology* **10**(1), 13–26.

McFall-Ngai, M. (2014), 'Divining the Essence of Symbiosis: Insights from the Squid-Vibrio Model', *PLOS Biology* **12**(2), e1001783.

McFrederick, Q. S., Mueller, U. G. & James, R. R. (2014), 'Interactions between fungi and bacteria influence microbial community structure in the Megachile rotundata larval gut', *Proceedings of the Royal Society B: Biological Sciences* **281**(1779), 20132653–20132653.

McFrederick, Q. S., Thomas, J. M., Neff, J. L., Vuong, H. Q., Russell, K. A., Hale, A. R. & Mueller, U. G. (2017*a*), 'Flowers and Wild Megachilid Bees Share Microbes', *Microbial Ecology* **73**(1), 188–200.
URL: *http://link.springer.com/10.1007/s00248-016-0838-1*

McFrederick, Q. S., Thomas, J. M., Neff, J. L., Vuong, H. Q., Russell, K. A., Hale, A. R. & Mueller, U. G. (2017*b*), 'Flowers and Wild Megachilid Bees Share Microbes', *Microbial Ecology* **73**(1), 188–200.

Michalik, A., Schulz, F., Michalik, K., Wascher, F., Horn, M. & Szklarzewicz, T. (2018), 'Coexistence of novel gammaproteobacterial and *Arsenophonus* symbionts in the scale insect Greenisca brachypodii (Hemiptera, Coccomorpha: Eriococcidae)', *Environmental Microbiology* **20**(3), 1148–1157.

Mindell, D. P. (1992), 'Phylogenetic consequences of symbioses: Eukarya and Eubacteria are not monophyletic taxa.', *Bio Systems* **27**(1), 53–62.

Mira, A., Ochman, H. & Moran, N. A. (2001), 'Deletional bias and the evolution of bacterial genomes', *Trends in Genetics* **17**(10), 589–596.

Moran, N. A. (1996), 'Accelerated evolution and Muller's rachet in endosymbiotic bacteria.', *Proceedings of the National Academy of Sciences of the United States of America* **93**(7), 2873–2878.

Moran, N. A. & Dunbar, H. E. (2006), 'Sexual acquisition of beneficial symbionts in aphids', *Proceedings of the National Academy of Sciences of the United States of America* **103**(34), 12803 LP – 12806.

Moran, N. A., Hansen, A. K., Powell, J. E. & Sabree, Z. L. (2012), 'Distinctive Gut Microbiota of Honey Bees Assessed Using Deep Sampling from Individual Worker Bees', *PLoS ONE* **7**(4), e36393.

Moran, N. A., McCutcheon, J. P. & Nakabachi, A. (2008), 'Genomics and Evolution of Heritable Bacterial Symbionts', *Annual Review of Genetics* **42**(1), 165–190.

Morris, J. J., Lenski, R. E. & Zinser, E. R. (2012), 'The Black Queen Hypothesis : Evolution of Dependencies through Adaptative Gene Loss', *Mbio* **3**(2), 1–7.

Morrow, J. L., Hall, A. A. & Riegler, M. (2017), 'Symbionts in waiting: the dynamics of incipient

endosymbiont complementation and replacement in minimal bacterial communities of psyllids', *Microbiome* **5**(1), 58.

Mouches, C., Bové, J. M. & Albisetti, J. (1984), 'Pathogenicity of *Spiroplasma apis* and other *Spiroplasmas* for honey-bees in Southwestern France', *Annales de l'Institut Pasteur Microbiology* **135**(1), 151–155.

Nakayama, S., Parratt, S. R., Hutchence, K. J., Lewis, Z., Price, T. A. & Hurst, G. D. (2015), 'Can maternally inherited endosymbionts adapt to a novel host? Direct costs of *Spiroplasma* infection, but not vertical transmission efficiency, evolve rapidly after horizontal transfer into *D. melanogaster*', *Heredity* **114**(6), 539–543.

Nalepa, C. A. (2015), 'Origin of termite eusociality: Trophallaxis integrates the social, nutritional, and microbial environments', *Ecological Entomology* **40**(4), 323–335.

Natsopoulou, M. E., Frey, E. & Rosenkranz, P. (2015), 'Parasites and Pathogens of the Honeybee (*Apis mellifera*) and their Influence on Inter- Colonial Transmission', *PLoS ONE* pp. 1–14.

Naug, D. & Camazine, S. (2002), 'The role of colony organization on pathogen transmission in social insects.', *Journal of theoretical biology* **215**(4), 427–439.

Neumann, P. & Carreck, N. L. (2010), 'Honey bee colony losses', *Journal of Apicultural Research* **49**(1), 1–6.

Nguyen, D. T., Spooner-Hart, R. N. & Riegler, M. (2016), 'Loss of *Wolbachia* but not *Cardinium* in the invasive range of the Australian thrips species, *Pezothrips kellyanus*', *Biological Invasions* **18**(1), 197–214.

Nguyen, L.-T., Schmidt, H. A., von Haeseler, A. & Minh, B. Q. (2015), 'IQ-TREE: A Fast and Effective Stochastic Algorithm for Estimating Maximum-Likelihood Phylogenies', *Molecular Biology and Evolution* **32**(1), 268–274.

Nishikori, K., Morioka, K., Kubo, T. & Morioka, M. (2009), 'Age- and morph-dependent activation of the lysosomal system and Buchnera degradation in aphid endosymbiosis.', *Journal of Insect Physiology* **55**(4), 351–357.

Nolan, M. P. & Delaplane, K. S. (2016), 'Distance Between Honey Bee *Apis mellifera* Colonies Regulates Populations of Varroa destructor at a Landscape Scale', *Apidologie* **2016**, 1–9.

Nováková, E., Hypša, V. & Moran, N. A. (2009), '*Arsenophonus*, an emerging clade of intracellular symbionts with a broad host distribution', *BMC Microbiology* **9**, 1–14.

Nováková, E., Hypša, V., Nguyen, P., Husník, F. & Darby, A. C. (2016), 'Genome sequence of *Candidatus Arsenophonus* lipopteni, the exclusive symbiont of a blood sucking fly *Lipoptena cervi* (Diptera: Hippoboscidae)', *Standards in genomic sciences* **11**, 72.

Nyholm, S. V. & Graf, J. (2012), 'Knowing your friends: Invertebrate innate immunity fosters benefi-

cial bacterial symbioses', *Nature Reviews Microbiology* **10**(12), 815–827.
URL: *http://dx.doi.org/10.1038/nrmicro2894*

Ochman, H. (2005), 'Genomes on the shrink.', *Proceedings of the National Academy of Sciences of the United States of America* **102**(34), 11959–11960.

Oldroyd, B. P. & Fewell, J. H. (2007), 'Genetic diversity promotes homeostasis in insect colonies', *Trends in Ecology and Evolution* **22**(8), 408–413.

Oliver, K. M., Moran, N. A. & Hunter, M. S. (2005), 'Variation in resistance to parasitism in aphids is due to symbionts not host genotype', *Proceedings of the National Academy of Sciences of the United States of America* **102**(36), 12795 LP – 12800.

Onchuru, T. O., Javier Martinez, A., Ingham, C. S. & Kaltenpoth, M. (2018), 'Transmission of mutualistic bacteria in social and gregarious insects', *Current Opinion in Insect Science* **28**, 50–58.

Osaka, R., Nomura, M., Watada, M. & Kageyama, D. (2008), 'Negative effects of low temperatures on the vertical transmission and infection density of a *Spiroplasma* endosymbiont in Drosophila hydei.', *Current microbiology* **57**(4), 335–339.

Pakwan, C., Kaltenpoth, M., Weiss, B., Chantawannakul, P., Jun, G. & Disayathanoowat, T. (2018), 'Bacterial communities associated with the ectoparasitic mites *Varroa destructor* and *Tropilaelaps mercedesae* of the honey bee (*Apis mellifera*)', *FEMS Microbiology Ecology* **94**(1).

Paredes, J. C., Herren, J. K., Schüpfer, F. & Lemaitre, B. (2016), 'The role of lipid competition for endosymbiont-mediated protection against parasitoid wasps in Drosophila', *mBio* **7**(4), 1–8.

Parmentier, A., Billiet, A., Smagghe, G., Vandamme, P., Deforce, D., Nieuwerburgh, F. V. & Meeus, I. (n.d.), 'A prokaryoticeukaryotic relation in the fat body of *Bombus* terrestris', *Environmental Microbiology Reports* .

Parmentier, A., Billiet, A., Smagghe, G., Vandamme, P., Deforce, D., Van Nieuwerburgh, F. & Meeus, I. (2018), 'A prokaryotic – eukaryotic relation in the fat body of *Bombus* terrestris', *Environmental Microbiology Reports* .

Parratt, S. R., Frost, C. L., Schenkel, M. A., Rice, A., Hurst, G. D. & King, K. C. (2016), 'Superparasitism Drives Heritable Symbiont Epidemiology and Host Sex Ratio in a Wasp', *PLoS Pathogens* **12**(6), 1–22.

Paterson, S. & Lello, J. (2003), 'Mixed models: Getting the best use of parasitological data', *Trends in Parasitology* **19**(8), 370–375.

Paull, S. H., Sejin, S., McClure, K. M., Sackett, L. C., Kilpatrick, Marm, A. & Johnson, P. T. J. (2013), 'From superspreaders to disease hotspots: linking transmission across hosts and space', *Frontiers in Ecology and the Environment* **10**(2), 75–82.

Perlman, S. J., Hodson, C. N., Hamilton, P. T., Opit, G. P. & Gowen, B. E. (2015), 'Maternal transmission, sex ratio distortion, and mitochondria', *Proceedings of the National Academy of Sciences of the United States of America* **112**(33), 10162–10168.
URL: *http://www.pnas.org/lookup/doi/10.1073/pnas.1421391112*

Perlman, S. J., Hunter, M. S. & Zchori-Fein, E. (2006), 'The emerging diversity of *Rickettsia*', *Proceedings. Biological sciences* **273**(1598), 2097–2106.
URL: *https://www.ncbi.nlm.nih.gov/pubmed/16901827 https://www.ncbi.nlm.nih.gov/pmc/PMC1635513/*

Perotti, M. A., Allen, J. M., Reed, D. L. & Braig, H. R. (2007), 'Host-symbiont interactions of the primary endosymbiont of human head and body lice.', *FASEB journal : official publication of the Federation of American Societies for Experimental Biology* **21**(4), 1058–1066.

Pilosof, S., Fortuna, M. A., Vinarski, M. V., Korallo-Vinarskaya, N. P. & Krasnov, B. R. (2013), 'Temporal dynamics of direct reciprocal and indirect effects in a hostparasite network', *Journal of Animal Ecology* **82**(5), 987–996.

Poehlein, A., Gottschalk, G. & Daniel, R. (2013), 'First Insights into the Genome of the Gram-Negative, Endospore-Forming Organism Sporomusa ovata Strain H1 DSM 2662', *Genome Announcements* **1**(5), e00734–13.

Pontes, M. H. & Dale, C. (2006), 'Culture and manipulation of insect facultative symbionts', *Trends in Microbiology* **14**(9), 406–412.

Poretsky, R., Rodriguez-R, L. M., Luo, C., Tsementzi, D. & Konstantinidis, K. T. (2014), 'Strengths and limitations of 16S rRNA gene amplicon sequencing in revealing temporal microbial community dynamics', *PLoS ONE* **9**(4).

Potts, S. G., Biesmeijer, J. C., Kremen, C., Neumann, P., Schweiger, O. & Kunin, W. E. (2010), 'Global pollinator declines: Trends, impacts and drivers', *Trends in Ecology and Evolution* **25**(6), 345–353.
URL: *http://dx.doi.org/10.1016/j.tree.2010.01.007*

Poulsen, M. & Boomsma, J. J. (2005), 'Mutualistic Fungi Control Crop Diversity in Fungus-Growing Ants', *Science* **307**(5710), 741 LP – 744.

Powell, J. E., Martinson, V. G., Urban-Mead, K. & Moran, N. A. (2014), 'Routes of acquisition of the gut microbiota of the honey bee *Apis mellifera*', *Applied and Environmental Microbiology* **80**(23), 7378–7387.

Purcell, A. H., Steiner, T., Mégraud, F. & Bové, J. (1986), 'In vitro isolation of a transovarially transmitted bacterium from the leafhopper *Euscelidius variegatus* (Hemiptera: Cicadellidae)', *Journal of Invertebrate Pathology* **48**(1), 66–73.

Qu, L. Y., Lou, Y. H., Fan, H. W., Ye, Y. X., Huang, H. J., Hu, M. Q., Zhu, Y. N. & Zhang, C. X. (2013),

'Two endosymbiotic bacteria, *Wolbachia* and *Arsenophonus*, in the brown planthopper *Nilaparvata lugens*', *Symbiosis* **61**(1), 47–53.

Queller, D. C. & Strassmann, J. E. (2016), 'Problems of multi-species organisms: endosymbionts to holobionts', *Biology & Philosophy* **31**(6), 855–873.

R Core Team (2013), 'A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL http://www.R-project.org/'.

Rabajante, J. F., Tubay, J. M., Uehara, T., Morita, S., Ebert, D. & Yoshimura, J. (2015), 'Red Queen dynamics in multi-host and multi-parasite interaction system', *Scientific reports* **5**, 10004.

Rasgon, J. L., Gamston, C. E. & Ren, X. (2006), 'Survival of *Wolbachia* pipientis in Cell-Free Medium', *Applied and Environmental Microbiology* **72**(11), 6934 LP – 6937.

Raudenbush, S. W., Yang, M.-L. & Yosef, M. (2000), 'Maximum Likelihood for Generalized Linear Models with Nested Random Effects via High-Order, Multivariate Laplace Approximation', *Journal of Computational and Graphical Statistics* **9**(1), 141–157.

Ray, N., Currat, M. & Excoffier, L. (2003), 'Intra-deme molecular diversity in spatially expanding populations.', *Molecular biology and evolution* **20**(1), 76–86.

Read, A. F. (1994), 'The evolution of virulence', *Trends in Microbiology* **2**(3), 2–5.

Rey, O., Estoup, A., Facon, B., Loiseau, A., Aebi, A., Duron, O., Vavre, F. & Foucaud, J. (2013), 'Distribution of endosymbiotic reproductive manipulators reflects invasion process and not reproductive system polymorphism in the little fire ant *Wasmannia auropunctata*', *PLoS ONE* **8**(3), e58467.

Richardson, M. F., Weinert, L. A., Welch, J. J., Linheiro, R. S., Magwire, M. M., Jiggins, F. M. & Bergman, C. M. (2012), 'Population genomics of the *Wolbachia* endosymbiont in *Drosophila melanogaster*', *PLOS Genetics* **8**(12), e1003129.

Riesa, I. F. & Amazineb, S. C. (2001), 'Review article Implications of horizontal and vertical pathogen transmission for honey bee epidemiology', **32**, 199–214.

Rifkin, J. L., Nunn, C. L. & Garamszegi, L. Z. (2012), 'Do Animals Living in Larger Groups Experience Greater Parasitism? A Meta-Analysis.', *The American Naturalist* **180**(1), 70–82.

Roberts, K. E. & Hughes, W. O. (2015), 'Horizontal transmission of a parasite is influenced by infected host phenotype and density', *Parasitology* **142**(2), 395–405.

Robidart, J. C., Bench, S. R., Feldman, R. A., Novoradovsky, A., Podell, S. B., Gaasterland, T., Allen, E. E. & Felbeck, H. (2008), 'Metabolic versatility of the *Riftia pachyptila* endosymbiont revealed through metagenomics', *Environmental Microbiology* **10**(3), 727–737.

Roe, A. D., James, P. M. A., Rice, A. V., Cooke, J. E. K. & Sperling, F. A. H. (2011), 'Spatial Commu-

nity Structure of Mountain Pine Beetle Fungal Symbionts Across a Latitudinal Gradient', *Microbial Ecology* **62**(2), 347–360.

Rosenberg, E., Sharon, G. & Zilber-Rosenberg, I. (2009), 'The hologenome theory of evolution contains Lamarckian aspects within a Darwinian framework.', *Environmental microbiology* **11**(12), 2959–2962.

Rosenberg, E. & Zilber-Rosenberg, I. (2011), 'Symbiosis and development: the hologenome concept.', *Birth defects research. Part C, Embryo today : reviews* **93**(1), 56–66.

Roy, H. E., Steinkraus, D. C., Eilenberg, J., Hajek, A. E. & Pell, J. K. (2006), 'Bizarre Interactions and Endgames: Entomopathogenic Fungi and Their Arthropod Hosts', *Annual Review of Entomology* **51**(1), 331–357.

Rubin, B. E., Sanders, J. G., Turner, K. M., Pierce, N. E. & Sarah, D. (2017), 'Social behavior in bees influences the abundance of *Sodalis* (Enterobacteriaceae) symbionts', *bioRxiv* .

Ruiz-GonzáLez, M. X. & Brown, M. J. F. (2006), 'Honey bee and bumblebee trypanosomatids: specificity and potential for transmission', *Ecological Entomology* **31**(6), 616–622.

Russell, J. A., Funaro, C. F., Giraldo, Y. M., Goldman-Huertas, B., Suh, D., Kronauer, D. J. C., Moreau, C. S. & Pierce, N. E. (2012), 'A Veritable Menagerie of Heritable Bacteria from Ants, Butterflies, and Beyond: Broad Molecular Surveys and a Systematic Review', *PLoS ONE* **7**(12).

Russell, J. A., Goldman-Huertas, B., Moreau, C. S., Baldo, L., Stahlhut, J. K., Werren, J. H. & Pierce, N. E. (2009), 'Specialization and geographic isolation among *Wolbachia* symbionts from ants and lycaenid butterflies', *Evolution* **63**(3), 624–640.

Russell, J. A., Moreau, C. S., Goldman-Huertas, B., Fujiwara, M., Lohman, D. J. & Pierce, N. E. (2009), 'Bacterial gut symbionts are tightly linked with the evolution of herbivory in ants', *Proceedings of the National Academy of Sciences of the United States of America* **106**(50), 21236–21241.

Russell, J. A., Sanders, J. G. & Moreau, C. S. (2017), 'Hotspots for symbiosis: Function, evolution, and specificity of ant-microbe associations from trunk to tips of the ant phylogeny (Hymenoptera: Formicidae)', *Myrmecological News* **24**(January), 43–69.

Rutrecht, S. & Brown, M. (2008), 'Within colony dynamics of *Nosema bombi* infections: disease establishment, epidemiology and potential vertical transmission', *Apidologie* **39**(5), 504–514.

Sachs, J. L. & Simms, E. L. (2006), 'Pathways to mutualism breakdown', *Trends in Ecology and Evolution* **21**(10), 585–592.

Sachs, J. L., Skophammer, R. G., Bansal, N., Stajich, J. E. & Sachs, J. L. (2013), 'Evolutionary origins and diversification of proteobacterial mutualists', *Proceedings of the Royal Society B Biological Science* .

Sachs, J. L., Skophammer, R. G. & Regus, J. U. (2011), 'Evolutionary transitions in bacterial symbiosis', *Proceedings of the National Academy of Sciences of the United States of America* **108**(2), 10800–10807.

Saeed, A. & White, J. A. (2015), 'Surveys for maternally-inherited endosymbionts reveal novel and variable infections within solitary bee species', *Journal of Invertebrate Pathology* **132**, 111–114.

Sakurai, M., Koga, R., Tsuchida, T., Meng, X.-Y. & Fukatsu, T. (2005), '*Rickettsia* symbiont in the pea aphid *Acyrthosiphon pisum*: novel cellular tropism, effect on host fitness, and interaction with the essential symbiont Buchnera.', *Applied and environmental microbiology* **71**(7), 4069–4075.

Salem, H., Florez, L., Gerardo, N. & Kaltenpoth, M. (2015), 'An out-of-body experience : the extracellular dimension for the transmission of mutualistic bacteria in insects'.

Sammataro, D. & Yoder, J. A. (2012), *Honey bee colony health: challenges and sustainable solutions*, CRC Press.

Santos-Garcia, D., Juravel, K., Freilich, S., Zchori-Fein, E., Latorre, A., Moya, A., Morin, S. & Silva, F. J. (2018), 'To B or not to B: *Arsenophonus* as a source of B-vitamins in whiteflies.', *bioRxiv* p. 311076.

Sasaki-Fukatsu, K., Koga, R., Nikoh, N., Yoshizawa, K., Kasai, S., Mihara, M., Kobayashi, M., Tomita, T. & Fukatsu, T. (2006), 'Symbiotic bacteria associated with stomach discs of human lice.', *Applied and environmental microbiology* **72**(11), 7349–7352.

Satterfield, D. A., Altizer, S., Williams, M. K. & Hall, R. J. (2017), 'Environmental persistence influences infection dynamics for a butterfly pathogen', *PLoS ONE* **12**(1), 1–16.

Savage, A. E., Sredl, M. J. & Zamudio, K. R. (2011), 'Disease dynamics vary spatially and temporally in a North American amphibian', *Biological Conservation* **144**(6), 1910–1915.

Scarborough, C. L., Ferrari, J. & Godfray, H. C. J. (2005), 'Aphid protected from pathogen by endosymbiont.', *Science (New York, N.Y.)* **310**(5755), 1781.

Schauer, C., Thompson, C. L. & Brune, A. (2012), 'The bacterial community in the gut of the Cockroach *Shelfordella lateralis* reflects the close evolutionary relatedness of cockroaches and termites', *Applied and environmental microbiology* **78**(8), 2758–2767.

Schmid-Hempel, P. (1998), *Parasites in Social Insects*, Princeton University Press.

Schmid-Hempel, P. (2003), 'Variation in immune defence as a question of evolutionary ecology', *Proceedings of the Royal Society of London. Series B: Biological Sciences* **270**(1513), 357–366.

Schmid-Hempel, P. (2017), 'Parasites and Their Social Hosts', *Trends in Parasitology* **33**(6), 453–462.

Schneider, C. A., Rasband, W. S. & Eliceiri, K. W. (2012), 'NIH Image to ImageJ: 25 years of image analysis.', *Nature methods* **9**(7), 671–675.

Schwarz, R. S., Moran, N. A. & Evans, J. D. (2016), 'Early gut colonizers shape parasite susceptibility

and microbiota composition in honey bee workers', *Proceedings of the National Academy of Sciences of the United States of America* **113**(33), 9345–9350.

Schwarz, R. S., Teixeira, É. W., Tauber, J. P., Birke, J. M., Martins, M. F., Fonseca, I. & Evans, J. D. (2014), 'Honey bee colonies act as reservoirs for two *Spiroplasma* facultative symbionts and incur complex, multiyear infection dynamics', *MicrobiologyOpen* **3**(3), 341–355.

Sebastien, A., Gruber, M. A. & Lester, P. J. (2012), 'Prevalence and genetic diversity of three bacterial endosymbionts (*Wolbachia*, *Arsenophonus*, and *Rhizobiales*) associated with the invasive yellow crazy ant (*Anoplolepis gracilipes*)', *Insectes Sociaux* **59**(1), 33–40.

Seeley, T. D. (2007), 'Honey bees of the Arnot Forest: a population of feral colonies persisting with Varroa destructor in the northeastern United States', *Apidologie* **38**(1), 19–29.

Sirviö, A. & Pamilo, P. (2010), 'Multiple endosymbionts in populations of the ant *Formica cinerea*', *BMC Evolutionary Biology* **10**(1), 335.

Skinner, S. W. (1985), 'Son-killer: a third extrachromosomal factor affecting the sex ratio in the parasitoid wasp, *Nasoniavitripennis*.', *Genetics* **109**(4), 745–759.

Slifko, T. R., Smith, H. V. & Rose, J. B. (2000), 'Emerging parasite zoonoses associated with water and food', *International Journal for Parasitology* **30**(12), 1379–1393.

Smith, C. C. & Mueller, U. G. (2015), 'Sexual transmission of beneficial microbes', *Trends in Ecology and Evolution* **30**(8), 438–440.

Šochová, E., Husník, F., Nováková, E., Halajian, A. & Hypša, V. (2017), '*Arsenophonus* and *Sodalis* replacements shape evolution of symbiosis in louse flies', *PeerJ* **5**, e4099.

Stacey, D. A., Thomas, M. B., Blanford, S., Pell, J. K., Pugh, C. & Fellowes, M. D. E. (2003), 'Genotype and temperature influence pea aphid resistance to a fungal entomopathogen', *Physiological Entomology* **28**(2), 75–81.

Stoll, S., Feldhaar, H., Fraunholz, M. J. & Gross, R. (2010), 'Bacteriocyte dynamics during development of a holometabolous insect, the carpenter ant *Camponotus floridanus*', *BMC Microbiology* **10**(1), 308.

Stouthamer, R. & Huigens, M. E. (2003), Parthenogenesis associated with *Wolbachia*, *in* K. Bourtzi & T. A. Miller, eds, 'Insect symbiosis', pp. 247–266.

Streicker, D. G., Fenton, A. & Pedersen, A. B. (2013), 'Differential sources of host species heterogeneity influence the transmission and control of multihost parasites', *Ecology Letters* (16), 975–984.

Studer, A., DW, T. & Poulin, R. (2010), 'Parasites and global warming: net effects of temperature on an intertidal hostparasite system ', *Marine Ecology Progress Series* **415**, 11–22.

Swick, M. C., Koehler, T. M. & Driks, A. (2016), 'Surviving Between Hosts: Sporulation and Transmission', *Microbiology spectrum* **4**(4), 10.1128/microbiolspec.VMBF–0029–2015.

Takiya, D. M., Tran, P. L., Dietrich, C. H. & Moran, N. A. (2006), 'Co-cladogenesis spanning three phyla: leafhoppers (Insecta: Hemiptera: Cicadellidae) and their dual bacterial symbionts.', *Molecular ecology* **15**(13), 4175–4191.

Tamura, K., Stecher, G., Peterson, D., Filipski, A. & Kumar, S. (2013), 'MEGA6: Molecular evolutionary genetics analysis version 6.0', *Molecular Biology and Evolution* **30**(12), 2725–2729.

Tarpy, D. R., Mattila, H. R. & Newton, I. L. G. (2015), 'Development of the Honey Bee Gut Microbiome throughout the Queen-Rearing Process', *Applied and Environmental Microbiology* **81**(9), 3182 LP – 3191.

Theis, F. J., Ugelvig, L. V., Marr, C. & Cremer, S. (2015), 'Opposing effects of allogrooming on disease transmission in ant societies', *Philosophical Transactions of the Royal Society B: Biological Sciences* **370**(1669), 20140108.
URL: *http://rstb.royalsocietypublishing.org/content/370/1669/20140108.abstract*

Thomas, M. B. & Blanford, S. (2003), 'Thermal biology in insect-parasite interactions', *Trends in Ecology and Evolution* **18**(7), 344–350.

Thomas, P. (2006), 'Isolation of an ethanol-tolerant endospore-forming Gram-negative *Brevibacillus* sp. as a covert contaminant in grape tissue cultures.', *Journal of applied microbiology* **101**(4), 764–774.

Toft, C. & Andersson, S. G. E. (2010), 'Insights into bacterial host adaptation', *Nature Publishing Group* **11**(7), 465–475.

Toju, H. & Fukatsu, T. (2011), 'Diversity and infection prevalence of endosymbionts in natural populations of the chestnut weevil : relevance of local climate and host plants', *Molecular Ecology* pp. 853–868.

Troyer, K. (1984), 'Microbes, herbivory and the evolution of social behavior', *Journal of Theoretical Biology* **106**(2), 157–169.

Turelli, M. (1994), 'Evolution of Incompatibility-Inducing Microbes and Their Hosts', *Evolution* **48**(5), 1500.

Turelli, M., Cooper, B. S., Richardson, K. M., Ginsberg, P. S., Peckenpaugh, B., Antelope, C. X., Kim, K. J., May, M. R., Abrieux, A., Wilson, D. A., Bronski, M. J., Moore, B. R., Gao, J. J., Eisen, M. B., Chiu, J. C., Conner, W. R. & Hoffmann, A. A. (2018), 'Rapid global spread of wRi-like *Wolbachia* across multiple *Drosophila*', *Current Biology* **28**(6), 963–971.e8.

Turelli, M. & Hoffmann, A. A. (1991), 'Rapid spread of an inherited incompatibility factor in California *Drosophila*', *Nature* **353**(6343), 440–442.

Tysset, C. & Rousseau, M. (1967), 'Des germes phytopathogenes du genre Erwinia commensaux du tractus intestinal de'labeille adulte (Apis mellifer L.)', *B. Tech. Apic* (2), 175–194.

Unckless, R. L. & Jaenike, J. (2012), 'Maintenance of a male-killing *Wolbachia* in *Drosophila innubila* by male-killing dependent and male-killing independent mechanisms', *Evolution* **66**(3), 678–689.

Vale, P. F., Stjernman, M. & Little, T. J. (2008), 'Temperature-dependent costs of parasitism and maintenance of polymorphism under genotype-by-environment interactions.', *Journal of evolutionary biology* **21**(5), 1418–1427.

Van Borm, S., Wenseleers, T., Billen, J. & Boomsma, J. J. (2001), '*Wolbachia* in leafcutter ants: A widespread symbiont that may induce male killing or incompatible matings', *Journal of Evolutionary Biology* **14**(5), 805–814.

VanEngelsdorp, D., Hayes Jr., J., Underwood, R. M. & Pettis, J. (2009), 'A Survey of Honey Bee Colony Losses in the U.S., Fall 2007 to Spring 2008', *PLoS ONE* **3**(12), e4071.

Vautrin, E., Genieys, S., Charles, S. & Vavre, F. (2008), 'Do vertically transmitted symbionts co-existing in a single host compete or cooperate? A modelling approach', *Journal of Evolutionary Biology* **21**(1), 145–161.

Visscher, P. K. & Seeley, T. D. (1982), 'Foraging Strategy of Honeybee Colonies in a Temperate Deciduous Forest', *Ecology* **63**(6), 1790–1801.

Walsh, P. S., Metzger, D. A. & Higuchi, R. (1991), 'Chelex 100 as a medium for simple extraction of DNA for PCR-based typing from forensic material.', *BioTechniques* **10**(4), 506–513.

Walterson, A. M. & Stavrinides, J. (2015), ' *Pantoea*: insights into a highly versatile and diverse genus within the Enterobacteriaceae.', *FEMS microbiology reviews* **39**(6), 968–984.

Webster, J. P., Borlase, A. & Rudge, J. W. (2017), 'Who acquires infection from whom and how? Disentangling multi-host and multimode transmission dynamics in the elimination' era', *Philosophical Transactions of the Royal Society B: Biological Sciences* **372**(1719).

Weeks, A. R., Turelli, M., Harcombe, W. R., Reynolds, K. T. & Hoffmann, A. A. (2007), 'From parasite to mutualist: Rapid evolution of *Wolbachia* in natural populations of *Drosophila*', *PLoS Biology* **5**(5), 0997–1005.

Wenseleers, T., Ito, F., Van Borm, S., Huybrechts, R., Volckaert, F. & Billen, J. (1998), 'Widespread occurrence of the micro-organism *Wolbachia* in ants', *Proceedings of the Royal Society B: Biological Sciences* **265**(1404), 1447–1452.

Wenseleers, T., Sundström, L. & Billen, J. (2002), 'Deleterious *Wolbachia* in the ant *Formica truncorum*', *Proceedings of the Royal Society B: Biological Sciences* **269**(1491), 623–629.

Wernegreen, J. J. (2002), 'Genome evolution in bacterial endosymbionts of insects.', *Nature reviews. Genetics* **3**(11), 850–861.

Wernegreen, J. J. (2011), 'Reduced selective constraint in endosymbionts: elevation in radical amino acid replacements occurs genome-wide.', *PLoS ONE* **6**(12), e28905.

Werren, J. H. (1987), 'The coevolution of autosomal and cytoplasmic sex ratio factors', *Journal of Theoretical Biology* **124**(3), 317–334.

Werren, J. H., Baldo, L. & Clark, M. E. (2008), '*Wolbachia*: Master manipulators of invertebrate biology', *Nature Reviews Microbiology* **6**(10), 741–751.

Werren, J. H. & O'Neill, S. L. (1997), 'The evolution of heritable symbionts', *Influential Passengers: Inherited Microorganisms and Arthropod Reproduction* pp. 3–41.

Werren, J. H., Skinner, S. W. & Huger, A. M. (1986), 'Male-killing bacteria in a parasitic wasp'.

Wilfert, L., Long, G., Leggett, H. C., Schmid-Hempel, P., Butlin, R., Martin, S. J. M. & Boots, M. (2016), 'Deformed wing virus is a recent global epidemic in honeybees driven by *Varroa mites*', *Science* **351**(6273), 594 LP – 597.

Wilkes, T. E., Darby, A. C., Choi, J. H., Colbourne, J. K., Werren, J. H. & Hurst, G. D. D. (2010), 'The draft genome sequence of *Arsenophonus nasoniae*, son-killer bacterium of *Nasonia vitripennis*, reveals genes associated with virulence and symbiosis', *Insect Molecular Biology* **19**(SUPPL. 1), 59–73.

Wilkes, T. E., Duron, O., Darby, A. C., Hypša, V., Nováková, E. & Hurst, G. D. D. (2011), 'The Genus *Arsenophonus*', *Manipulative Tenants: Bacteria Associated with Arthropods* (March 2017), 225–244.

Wilson, E. O. (1971), *The Insect Societies*, Harvard University Press.

Wilson, E. O. (1975), Sociobiology: the new synthesis, *in* 'Philosophy of Biology: An Anthology', Harvard University Press.

Wilson-Rich, N., Dres, S. T. & Starks, P. T. (2008), 'The ontogeny of immunity: Development of innate immune strength in the honey bee (*Apis mellifera*)', *Journal of Insect Physiology* **54**(10-11), 1392–1399.

Winston, M. (1987), *The biology of the honey bee*, Harvard University Press.

Wolinska, J. & King, K. C. (2009), 'Environment can alter selection in host-parasite interactions', *Trends in Parasitology* **25**(5), 236–244.

Xie, J., Butler, S., Sanchez, G. & Mateos, M. (2014), 'Male killing *Spiroplasma* protects Drosophila melanogaster against two parasitoid wasps', *Heredity* **112**(4), 399–408.
URL: *http://dx.doi.org/10.1038/hdy.2013.118*

Yaïez, O., Gauthier, L., Chantawannakul, P. & Neumann, P. (2016), 'Endosymbiotic bacteria in honey bees: *Arsenophonus* spp. are not transmitted transovarially', *FEMS Microbiology Letters* **363**(14), 1–24.

Yanez, O., Gauthier, L., Chantawannakul, P. & Neumann, P. (2016), 'Endosymbiotic bacteria in honey bees: *Arsenophonus* spp. are not transmitted transovarially', *FEMS Microbiology Letters* **363**(14), 1–7.

Yui, S., Bourne, D. G. & Willis Bette, L. (2009), 'Dynamics of seasonal outbreaks of black band disease in an assemblage of *Montipora* species at Pelorus Island (Great Barrier Reef, Australia)', *Proceedings of the Royal Society B: Biological Sciences* **276**(1668), 2795–2803.

Zilber-Rosenberg, I. & Rosenberg, E. (2008), 'Role of microorganisms in the evolution of animals and plants: the hologenome theory of evolution.', *FEMS microbiology reviews* **32**(5), 723–735.

Zug, R. & Hammerstein, P. (2012), 'Still a host of hosts for *Wolbachia*: Analysis of recent data suggests that 40% of terrestrial arthropod species are infected', *PLoS ONE* **7**(6), 7–9.

Zug, R. & Hammerstein, P. (2015), 'Bad guys turned nice? A critical assessment of ¡i¿ Wolbachia ¡/i¿ mutualisms in arthropod hosts', *Biological reviews of the Cambridge Philosophical Society* **90**(1), 89–111.

Zug, R. & Hammerstein, P. (2018), 'Evolution of reproductive parasites with direct fitness benefits', *Heredity* **120**(3), 266–281.

Zuur, A., Ieno, E., Walker, N., Saveliev, A. & Smith, G. (2006), Mixed effects models and extensions in ecology with R, *in* 'New York Science and Business Media'.