Symbionts in Societies

the biology of *Wolbachia* in social insects

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Declaration

I hereby declare that this thesis has not been, and will not be, submitted in whole or in part to another university for the award of any other degree. Below, details of contributions by others towards work presented in this thesis are listed.

Chapter 2

Tobias Pamminger and Craig Perl assisted in the collection and counting of ant colonies

Chapter 3

Tobias Pamminger assisted in the collection and counting of ant colonies

Chapter 4

This chapter arose in part from discussions with Tobias Pamminger.

Chapter 5

The majority of ant specimens used in the second part of this study were provided by Magdalena Witek, Balint Markó, Enikő Csata, Luca Casacci and Michal Woyciechowski.

Chapter 6

The majority of ant specimens used in this study were provided by Jes Pedersen, Luigi Pontieri and Helen Theron

Signed:

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UNIVERSITY OF SUSSEX

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Summary

Heritable bacterial symbionts are astonishingly common in insects, yet relatively little is known about how heritable symbionts influence the biology of social insects such as ants, bees, wasps and termites. In this thesis I investigate various aspects of the biology of heritable symbionts in social insects, principally focusing on the relationship between ants, the largest group of social insects, and the symbiont *Wolbachia*, the archetypal reproductive parasite. In **Chapter 1**, I begin by reviewing the biology of *Wolbachia*. In **Chapter 2**, I show that the sex, caste and size of an individual's colony determine the likelihood that it is infected with *Wolbachia,* and I provide correlational evidence that *Wolbachia* provides small increases in colony productivity in the ant *Temnothorax crassispinus*. In **Chapter 3**, I combine colony censuses and antibiotic treatment experiments, finding that *Wolbachia* neither distorts host sex ratios nor causes strong female mortality type mating incompatibilities in the ant *Myrmica scabrinodis*. In **Chapter 4**, I critically evaluate the theory that heritable symbionts should evolve to manipulate caste-fate in social insects, outlining three distinct evolutionary scenarios under which this might occur. In **Chapter 5**, I provide evidence for negative interactions between *Wolbachia* and both *Spiroplasma* and *Arsenophonus* in *M. scabrinodis* hosts, and I show that multiple unrelated strains of both *Wolbachia* and *Spiroplasma* occur across the Palaearctic. In **Chapter 6**, I show that one of two strains of *Wolbachia* infecting the ant *Monomorium pharaonis* was acquired by hybrid introgression. In **Chapter 7**, I find that ant species with limited queen dispersal are almost twice as likely to be infected with *Wolbachia* relative to other ant species, supporting the hypothesis that population structure influences the invasion ability of *Wolbachia*. Finally, in **Chapter 8,** I discuss the broader significance of my findings.

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"Some 45 years after the seminal work of Yenn and Barr, the interest of the research community in *Wolbachia* has never been greater. The field has gone from being an esoteric example of evolution to underpinning many aspects of insect biology and even being the basis of a major public health program"

- Francis Jiggins. (2016) Open questions: how does *Wolbachia* do what it does? *BMC Biology*, **14**

"To a degree seldom grasped even by entomologists, the modern insect fauna has become predominantly social"

- Bert Hölldobler and Edward O. Wilson. (1990) *The Ants*.

1 General introduction

The increasing ease and economy of molecular screening for bacterial infections has uncovered a huge diversity of bacterial symbionts in animals. However, one group stands head and shoulders above the rest in terms of its prevalence, diversity, and evolutionary importance – *Wolbachia*. These intracellular bacteria from the order Rickettsiales are extremely prevalent bacterial symbionts of arthropods and filarial nematodes, and are thought to infect roughly half of all arthropod species (Hilgenboecker et al., 2008; Zug & Hammerstein, 2012; Weinert et al., 2015). Furthermore, *Wolbachia* also exhibits a greater variety of strategies to ensure its spread than any other bacterial symbiont (Engelstädter & Hurst, 2009a). Whilst in nematodes it exclusively behaves as an obligate mutualist, the biology of *Wolbachia* in arthropods is astonishingly variable, and it induces a suite of parasitic and mutualistic phenotypes in its host organisms (Taylor et al., 2005; Zug & Hammerstein, 2015). In this introduction, I discuss how the mode of transmission of *Wolbachia* shapes its phenotypic effects, describe the phenotypic effects of *Wolbachia* in arthropods, explain how *Wolbachia* is gained and lost by taxa on evolutionary timescales, and outline the major evolutionary consequences of *Wolbachia* infection. I then introduce the social insects, and discuss what is currently known about the

prevalence, population genetics, transmission, and phenotypic effects of *Wolbachia* in social insects.

1.1 Setting the evolutionary stage for Wolbachia: symbiosis and mode of transmission

The multifarious evolutionary strategies employed by *Wolbachia* are all ultimately a result of its particular mode of transmission. Bacterial symbionts will always evolve to maximise their transmission to new hosts, but some are transmitted in fundamentally different ways to others. As a consequence, this maximisation of transmission is achieved by very different means across a variety of bacterial symbionts. Most overtly pathogenic bacteria are horizontally transmitted between unrelated hosts, either via direct or indirect physical contact, through airborne transmission, or via a common vehicle or vector (Chin, 2000) and so the reproductive success of the pathogen is not highly dependent upon the long term survival of the host. Furthermore, because successful horizontal transmission is often assured by the production of a large number of propagules that can infect new hosts, horizontally transmitted bacteria frequently evolve to destructively utilise host resources in order to reproduce, leading to the damage or death of the

host (Anderson & May, 1982; Ewald, 1983). In contrast, many other bacterial symbionts are predominantly vertically transmitted, passed on from parents to offspring (Moran et al., 2008). The reproduction of a vertically transmitted symbiont is contingent upon the successful reproduction of its host and so vertical transmission aligns, at least partially, the fitness interests of bacterial symbionts and their hosts (Lipsitch et al., 1996; Herre et al., 1999). Vertically transmitted symbionts are thus expected to evolve to benefit their hosts, rather than harm them (Stewart et al., 2005).

The mode of transmission of a symbiont is itself often related to the location of the symbiont within its host. Many vertically transmitted bacterial symbionts are endocellular, residing within host cells, and transmission from one generation to the next occurs naturally due to the presence of the symbiont within eggs (Bright & Bulgheresi, 2010). Such symbionts are thus maternally transmitted, as they pass exclusively through the female line. Furthermore, many other symbionts that are not transmitted transovarially are nonetheless maternally transmitted through other routes (Bright & Bulgheresi, 2010; Ebert, 2013). This uniparental inheritance of symbionts can be significantly advantageous to host organisms. For instance, it ensures that multiple symbiont lineages do not compete within the same host, and restricts the spread of deleterious cytoplasmic elements that can increase in frequency despite reducing host fitness (Cosmides & Tooby, 1981;

Hurst, 1992; Law & Hutson, 1992). However, maternal transmission of bacterial symbionts also partially misaligns the evolutionary interests of nuclear and symbiont genes, and *Wolbachia* is a classic example of this. Autosomal nuclear genes benefit from increased production of both male and female offspring of the organism they inhabit, but males are a dead end from the perspective of a maternally transmitted cytoplasmic element, and so maternally inherited symbionts only benefit from the production of female offspring of their host (Hurst & Frost, 2015). As such, maternally transmitted symbionts such as *Wolbachia* are in conflict with the bulk of the host's genetic parliament over the production and importance of males, leading these symbionts along profoundly different evolutionary trajectories whereby they evolve exclusively to enhance their transmission through female hosts.

1.2 The phenotypic effects of *Wolbachia*: a quartet of strategies to enhance transmission

Maternally transmitted symbionts such as *Wolbachia* can enhance their transmission through female hosts using four basic strategies; i) increasing the fitness of infected females ii) increasing the proportion of female offspring

produced by infected females iii) decreasing the fitness of uninfected females iv) decreasing the proportion of female offspring produced by uninfected females (Werren & O'Niell, 1997). Ever the versatile symbiont, *Wolbachia* makes use of each of these different strategies in different arthropod hosts.

1.2.1 Increasing the fitness of infected females

Many strains of *Wolbachia* have evolved to increase the fitness of their female hosts, although different strains achieve this goal through quite distinct means (Zug & Hammerstein, 2015). By increasing the fitness of females, *Wolbachia* enhances host production of offspring, which is its principal or exclusive means of transmission. Male fitness may also be enhanced as a side effect, or deliberately reduced to benefit females, but only phenotypes that increase female fitness will be subject to positive selection (Werren & O'Niell, 1997). There are three main ways in which *Wolbachia* is thought to enhance the fitness of infected females; by protecting hosts against natural enemies, by providing hosts with essential nutrients or metabolites, or by inducing the death of male-offspring at an early embryonic stage.

1.2.1.1 Protection against natural enemies

Roughly a decade ago, two independent studies demonstrated that *Wolbachia* provides *Drosophila melanogaster* with resistance to *Drosophila* C virus (Hedges et

al., 2008; Teixeira et al., 2008), and a plethora of subsequent reports have presented evidence suggesting that *Wolbachia* provides resistance to a broad range of parasites and pathogens (Kambris et al., 2009; Moreira et al., 2009; Unckless & Jaenike, 2012; Ye et al., 2013; Gupta et al., 2017). Under certain ecological circumstances, increased disease resistance could substantially enhance host fitness and lead to the spread of protective *Wolbachia* strains (Brownlie & Johnson, 2009). However, many of these studies involved the transfection of *Wolbachia* into naturally uninfected species, which often results in upregulation of the host immune system (Wong et al., 2011; Rancès et al., 2012). This artificial immune system activation may account for the observed anti-pathogenic effects, particularly those relating to non-viral pathogens, and so positive results in many studies evaluating *Wolbachia*-associated protection may in fact be experimental artefacts (Zélé et al., 2012; Zug & Hammerstein, 2015). Nonetheless, in at least some cases, the protective effects of *Wolbachia* are not a result of immune upregulation (even in some transfection experiments; see Martinez et al., (2014)) and protection is directly associated with an increase in host fitness (Teixeira et al., 2008; Zélé et al., 2012).

1.2.1.2 Nutritional and metabolic provisioning

Many studies of the phenotypic effects of *Wolbachia* have shown that infected individuals exhibit increased survival and fecundity relative to uninfected

individuals (Dobson et al., 2004; Fry et al., 2004; Dong et al., 2007; Weeks et al., 2007; Zhang et al., 2010; Xue et al., 2012). However, in most cases the mechanistic basis of these effects is either unclear or was not subject to investigation. It may be that these cryptic fitness benefits associated with infection are at least partially due to nutritional benefits provided by *Wolbachia*. Obligate symbionts of various insects with nutritionally unbalanced diets are known to provide their hosts with essential nutrients and metabolic products (Zientz et al., 2004), and strains of *Wolbachia* that provide their hosts with important nutrients may experience a selective advantage in nutrient deprived environments. For instance, *Wolbachia* enhance the fecundity of infected females of *Drosophila innubila* reared on a nutrient deprived growth medium (Unckless & Jaenike, 2012).

One possibility is that *Wolbachia* assists its host with iron metabolism. For example, *Wolbachia* infected *Drosophila melanogaster* reared on iron-poor diets show increased fecundity relative to uninfected flies (Brownlie et al., 2009). Alternatively (or additionally), the provision of B vitamins may be a widespread strategy used by *Wolbachia* to increase host fitness. In the bedbug *Cimex lectularius*, *Wolbachia* is an obligate nutritional mutualist. It resides in bacteriocytes in a manner akin to the obligate symbionts of aphids and tsetse flies, and like these symbionts, it synthesises essential compounds for its host. Individuals cured of *Wolbachia* suffer from biotin deficiencies, and consequently experience growth deficiencies and

sterility (Hosokawa et al., 2010). However, the ability of this strain to synthesise biotin is not necessarily common amongst *Wolbachia* generally, and the requisite genetic machinery was acquired by lateral gene transfer from another symbiont (Nikoh et al., 2014). In a comparison of 21 *Wolbachia* genomes from a diverse range of hosts, only the strain infecting *C. lectularis* possessed the genes responsible for the complete synthesis pathway of biotin (Moriyama et al., 2015). In contrast, the same study found that 16 out of 21 *Wolbachia* genomes contained the entire biosynthetic pathway for riboflavin (another B-vitamin), and the remaining 5 contained a portion of the pathway. In addition, it was shown using the bedbug-*Wolbachia* system that the provision of riboflavin by *Wolbachia* also plays an important role in host survival and fecundity. Moriyama et al., (2015) suggest that widespread synthesis of riboflavin by *Wolbachia* may thus explain cryptic fitness benefits found in a diversity of *Wolbachia*-infected insects.

1.2.1.3 Male-killing

Male-killing refers to the symbiont-induced death of males at an early embryonic stage, and although it is often thought of as a means of distorting sex ratios (i.e. increasing the production of infected females), the actual selective advantage occurs due to the increased fitness of surviving females following the death of their brothers (Werren & O'Niell, 1997). The exact nature of this beneficial effect of male death probably varies between species. For instance, in some species, such as

ladybirds, females cannibalise their dead brothers, and this additional food significantly enhances larval survival (Elnagdy et al., 2011). In other species, male-killing *Wolbachia* may spread because the death of males reduces intra-brood competition, leaving more resources for surviving females (Skinner, 1985; Hurst, 1991a). It has also been suggested that male-killing may evolve as a mechanism of inbreeding avoidance (Werren, 1987) but a more recent model suggests that inbreeding may actually hinder the spread of male-killing bacteria (Dannowski et al., 2009).

Male-killing *Wolbachia* have been found to infect a number of species, but appear to be largely restricted to beetles, butterflies, and flies (Hurst et al., 1999, 2000; Fialho & Stevens, 2000; Jiggins et al., 2000; Mitsuhashi et al., 2004; Zeh et al., 2005; Sheeley & McAllister, 2009; Graham & Wilson, 2012). However it is not clear whether this is a genuine pattern or more of a reflection of taxonomically focussed research efforts coupled with a preference for model organisms (at least in the case of *Drosophila*). Nonetheless, one factor that is sure to determine the taxonomic distribution of male-killing *Wolbachia* to a large extent is the propensity of hosts to lay large clutches of eggs. Only then will there be sufficient intrabrood competition for male-killing agents to provide a selective advantage to females (Hurst & Jiggins, 2000).

1.2.2 Increasing the primary sex ratio of infected females

Instead of enhancing the fitness of infected females, some strains of *Wolbachia* directly alter primary sex-ratios, thereby increasing host investment in female offspring at the expense of males. This is either achieved by *Wolbachia*-induced feminization of genetically male offspring, or *Wolbachia*-induced parthenogenesis.

1.2.2.1 Feminization

In some species, *Wolbachia* causes genetically male offspring to develop into phenotypic females (Bouchon et al., 2008). For instance, the isopod crustacean *Armadillidium vulgare* exhibits female-heterogametic sex-determination, in which ZZ individuals ordinarily develop into males and ZW individuals develop into females. However, *Wolbachia* causes genetically male ZZ individuals to develop into females (Rigaud, 1997). Whilst *Wolbachia*-induced feminization has only been conclusively demonstrated in a small number of other isopod crustaceans, *Wolbachia* is widespread amongst isopods and feminization is thought to occur commonly in this group (Cordaux et al., 2011). *Wolbachia*-induced feminization has also been demonstrated in the leafhopper *Zyginidia pullula* and the butterfly *Eurema hecabe*, and other symbionts are known to induce feminization in mites and wasps (Weeks et al., 2001; Hiroki et al., 2002; Negri et al., 2006; Giorgini et al., 2009). Symbiont-induced feminization is thus not limited to species with female-heterogametic sex-determination, and symbionts have clearly evolved to

target very different sex-determination processes to achieve the same end, although a complete picture of how this is achieved is currently lacking. In isopods, *Wolbachia* appears to inhibit the differentiation of the androgenic gland, which is necessary for the development of males, whilst in the moth *Ostrinia scapulalis*, *Wolbachia* has been shown to alter the sex-specific splicing of the *doublsex* homologue, a conserved sex-determining gene in insects (Rigaud, 1997; Sugimoto et al., 2010) but the precise molecular mechanisms behind *Wolbachia*-induced feminization are unclear.

1.2.2.2 Parthenogenesis induction

In its most conceptually simple attempt to boost host production of females, some strains of *Wolbachia* cause their previously sexual hosts to reproduce parthenogenetically (Stouthamer et al., 1990; Werren, 1997a; Stouthamer et al., 1999). In the most dramatic experimental demonstrations of the influence of parthenogenesis-inducing *Wolbachia*, the simple treatment of infected species with antibiotics or high temperatures can cause an entirely parthenogenetic species, consisting only of females, to suddenly begin producing males (Stouthamer et al., 1990). Different strains of *Wolbachia* employ a variety of cellular mechanisms to induce parthenogenesis in their hosts, but the end result is the same – infected hosts produce only female eggs (Cordaux et al., 2011; Ma & Schwander, 2017). This contrasts with *Wolbachia*-induced feminization, where male eggs are laid, but

develop into females, and where sex is still required for the production of offspring. Based on species for which clear evidence is available, parthenogenesis-inducing *Wolbachia* appear to be restricted to mites, thrips and wasps, all of which are haplodiploid (Werren et al., 2008). However, this probably reflects the difficulty of obtaining conclusive experimental evidence of symbiont-induced parthenogenesis in diplodiploids, and it may well be the case that parthenogenesis-inducing *Wolbachia* are in fact far more taxonomically widespread (Ma & Schwander, 2017).

1.2.3 Decreasing the fitness of uninfected females

1.2.3.1 Cytoplasmic incompatibility

Of the four main ways in which *Wolbachia* manipulates host reproduction, cytoplasmic incompatibility (CI) is the most common (Werren et al., 2008; Engelstädter & Hurst, 2009a). Whilst the other reproductive manipulations are ways of increasing the fitness or number of female offspring produced by infected hosts, CI is perhaps better understood as spiteful behaviour by *Wolbachia* in which related bacteria in infected females benefit from the reduced fitness of uninfected females (Hurst, 1991b). When a male infected with CI-inducing *Wolbachia* mates with an uninfected female, a proportion of the females eggs fail to hatch. In contrast, when an infected male mates with an infected female, the female's eggs

hatch as normal. Because both infected and uninfected females can also mate with uninfected males without suffering any adverse effects, the presence of CI-inducing *Wolbachia* causes an overall reduction in the fecundity of uninfected females. The higher relative fecundity of infected females thus causes the infection to spread through the population (Werren et al., 2008). Whilst the precise molecular mechanisms underpinning CI are not understood, its action appears to involve a two-step process on the part of *Wolbachia* (Charlat et al., 2001; Serbus et al., 2008). Firstly, *Wolbachia* modifies male sperm, which prevents paternal chromosomes from properly segregating during anaphase early in the development of embryos, rendering fertilised eggs haploid (Tram et al., 2006). However, when the same strain of *Wolbachia* is present in females, the modification is 'rescued' allowing development to occur normally (Bourtzis et al., 1998; Charlat et al., 2001).

The spread of CI-*Wolbachia* is subject to positive frequency dependent selection because the more individuals within a population that are infected, the greater the risk to an uninfected female of mating with an infected male (Jansen et al., 2008; Engelstädter & Telschow, 2009). When the infection is at a low frequency within a population, the selective benefit of infection may be outweighed by fixed fitness costs of infection or imperfect maternal transmission, leading to the loss of *Wolbachia* from the population. Thus, for strains associated with a fecundity cost or

imperfect transmission, the frequency of *Wolbachia* in a population needs to be above a certain threshold, termed the invasion threshold, for its frequency to increase deterministically (Caspari & Watson, 1959; Hoffmann et al., 1990; Engelstädter & Telschow, 2009). When CI-*Wolbachia* first become established in a new population, the number of infected individuals is likely to be very small, and likely below the invasion threshold. It is thought that stochastic demographic processes or the provision of context-dependent fitness benefits to infected hosts (e.g. antiviral effects) help CI strains overcome these invasion thresholds (Stouthamer et al., 1999; Egas et al., 2002; Jansen et al., 2008; Reuter et al., 2008; Fenton et al., 2011).

Generally speaking, the ability to rescue a particular modification is strain specific, and this factor underlies bidirectional cytoplasmic incompatibility. When two *Wolbachia* strains, either within a species or found in two closely related species, are mutually incompatible, females infected with the first strain produce inviable offspring when mated with males infected with a second strain and vice-versa (Werren, 1997a). However, any female that acquires both strains will be compatible with males of any infection status. This will lead to the rapid spread of coinfected individuals within a population, and may explain the frequent occurrence of *Wolbachia* superinfections in natural populations (Sinkins et al., 1995). Finally, some strains of *Wolbachia* appear to have lost the ability to modify

male sperm, but nonetheless retain the ability to rescue the modification. These so called 'rescuing' strains may spread at the expense of CI-inducing strains (Bourtzis et al., 1998; Zabalou, 2004) and I discuss this further in section 1.3.3.

1.2.3.2 Host dependence

A number of arthropod species have become dependent on *Wolbachia* for successful reproduction or survival. For instance, removal of *Wolbachia* by antibiotic treatment from females of the wasp *Asobara tabida* leaves them unable to produce mature oocytes, thus rendering them sterile (Dedeine et al., 2001). Dependence on *Wolbachia* could evolve as result of relaxed selection on genes whose essential function is also fulfilled by bacterial genes. Mutations in essential host genes would thus render the host dependent on *Wolbachia* (Starr & Cline, 2002). Alternatively, dependence may arise as a result of antagonistic coevolution between *Wolbachia* and its host (Aanen & Hoekstra, 2007; Zug & Hammerstein, 2015). Pannebakker et al., (2007) demonstrated that *Wolbachia* downregulates apoptosis in *A. tabida,* and this accounts for the infertility of aposymbiotic females as targeted apoptosis is required for successful oogenesis. The authors suggest that *A. tabida* responds to infection by upregulating apoptosis in an attempt to destroy infected cells. *Wolbachia* responds by downregulating apoptosis to counteract this host immune response. However, because apoptosis is necessary for successful oogenesis, the host evolves to further upregulate apoptosis, simply to restore

normal levels. As such, in the absence of *Wolbachia,* excessive upregulation of apoptosis, which was selected for in the presence of *Wolbachia,* leads to the failure of oogenesis and thus host sterility (Pannebakker et al., 2007). In either case, host dependence cannot be employed by *Wolbachia* to secure its initial spread through a naïve population. However, once dependence evolves, the dramatic reduction in the fitness of uninfected individuals presumably secures the long term survival of the bacteria in a dependent host.

1.2.4 Decreasing the primary sex ratio of uninfected females

1.2.4.1 Cytoplasmic incompatibility in haplodiploids

As discussed in the previous section, mating incompatibilities between infected males and uninfected females leads to the improper segregation of paternal chromosomes, and so fertilised eggs remain haploid (Hoffmann & Turelli, 1997; Tram et al., 2006). In diplodiploids, haploid eggs fail to develop, but in haplodiploids, sex-determination occurs in response to the ploidy of offspring, and haploid eggs develop into males. Consequently, CI-*Wolbachia* do not spread in haplodiploids because they reduce the fitness of uninfected females, but rather because they alter the primary sex-ratio of uninfected female's offspring, causing them to overproduce males at the expense of female offspring.

Two different forms of cytoplasmic incompatibility occur in haplodiploids. In the simplest form, termed 'male development', fertilised eggs are rendered haploid and develop into males. In addition, unfertilised eggs also develop into males, and so females expressing mating incompatibilities produce normal numbers of offspring but with a male-biased sex-ratio (Breeuwer & Werren, 1990; Vavre et al., 2000). However, in a second and more common type, called 'female mortality', fertilised eggs fail to develop at all, whilst unfertilised eggs develop into males as in the first case (Vavre et al., 2003; Tram et al., 2006). However, in either case, females mated within incompatible males are not incapable of reproducing, but simply produce fewer or no female offspring, resulting in the spread of the infected cytotype through a population.

1.2.5 Combining strategies

Thus far, the different strategies employed by *Wolbachia* have been discussed separately, but it is important to note that some strains of *Wolbachia* make use of multiple strategies to efficiently spread within host populations, often combining reproductive parasitism (CI, parthenogenesis induction, feminization and male-killing) with mutualistic traits (Zug & Hammerstein, 2015). Mutant strains that provide mutualistic benefits to their hosts in addition to manipulating host reproduction will spread at the expense of strains only engaging in reproductive

parasitism (Turelli, 1994). A dramatic example of this was observed in natural populations of *Drosophila simulans,* in which a CI-inducing strain of *Wolbachia* that initially reduced the fecundity of its host evolved to markedly enhance host fecundity over the course of just a few decades (Weeks et al., 2007). Indeed, as discussed previously, it has been suggested that increasing host fitness may be a general explanation of how CI-inducing *Wolbachia* can overcome invasion thresholds and spread through natural populations (Fenton et al., 2011).

1.2.6 Strains that reduce host fitness

Strictly maternally transmitted *Wolbachia* that reduce host fitness without inducing any other effects are unlikely to spread in nature (Werren, 1997b), yet many strains of *Wolbachia* have been shown to negatively affect proxies of host fitness such as fecundity and longevity (Hoffmann et al., 1990; Fleury et al., 2000; Perrot-Minnot et al., 2002; Fytrou et al., 2006; Ros & Breeuwer, 2009; Vasquez et al., 2011; Joshi et al., 2014; Russell et al., 2017). Such adverse effects of *Wolbachia* may occur as a consequence of harbouring intracellular bacteria that require host metabolic products, or as a side effect of reproductive parasitism or facultative mutualism (Poinsot & Merçot, 1997; Fleury et al., 2000; Weeks et al., 2007; Martinez et al., 2015; Zug & Hammerstein, 2015). For instance, the transfer of different strains of *Wolbachia* infecting various *Drosophila* species into a common host genetic

background has shown that the strength of antiviral protection is negatively correlated with host fecundity; this is probably because high *Wolbachia* titres are required for effective protection against viruses (Martinez et al., 2015). Alternatively, horizontal transmission of *Wolbachia*, even at low levels, may also select for traits that reduce host fecundity if they also increase the rate of horizontal transmission (Werren, 1997b; Bandi et al., 2001). For example, *Wolbachia* subject to experimental horizontal transfer in *Armadillidium vulgare* rapidly evolved into a highly virulent pathogen (Le Clec'h et al., 2017) and whilst *Wolbachia* is generally maternally transmitted, there are examples of horizontal transmission of *Wolbachia* occurring on ecological timescales (Duron et al., 2010). Finally, some virulent strains of *Wolbachia* may be maladaptive laboratory artefacts that would not spread under natural conditions (Min & Benzer, 1997; Werren, 1997b).

1.3 Here today, gone tomorrow: the acquisition and extinction of *Wolbachia* on evolutionary timescales

In the case of obligate nutritional mutualists, symbiont phylogenies tend to closely reflect host phylogenies as a result of co-cladogenesis (Moran et al., 2008). In contrast, phylogenies of *Wolbachia* and its hosts are generally quite discordant;

closely related species are often infected with distantly related strains, and distantly related species are frequently infected with closely related strains (Werren et al., 1995, 2008; Schilthuizen & Stouthamer, 1997; Frost et al., 2010; Gerth et al., 2013). The obvious conclusion is that *Wolbachia* is capable of moving between unrelated species, at least on evolutionary timescales. This can occur either through horizontal transfer of the infection, or by introgression of *Wolbachia* into a related species (Raychoudhury et al., 2009).

1.3.1 Horizontal transfer

Although *Wolbachia* is clearly capable of naturally transferring between distantly related species, precisely how this occurs is not clear. A number of routes for horizontal transmission have been suggested, such as parasitoidism, the sharing of food sources, predation and cannibalism (Schilthuizen & Stouthamer, 1997; Huigens et al., 2000; Haine et al., 2005). In most cases, the evidence in favour of a particular route of transmission is based on the phylogenetic patterns of infection. For instance, *Drosophila tristis* and *D. ambigua* share a common food source and are attacked by the same parasitoids, and also share an almost identical strain of *Wolbachia* (Haine et al., 2005). However, in the case of parasitoid-mediated horizontal transfer, laboratory observations also support the notion that *Wolbachia* can be transferred between unrelated species; when infected and uninfected

parasitoid wasp larvae from the genus *Trichogramma* shared a host egg, uninfected larvae were shown to acquire *Wolbachia* from infected larvae (Huigens et al., 2004) Phylogenetic evidence for the horizontal transfer of *Wolbachia* is also complemented by microinjection experiments which show that *Wolbachia* from one host can be stably transferred into another species. This was first achieved by transferring *Wolbachia* between two species of *Drosophila* (Boyle et al., 1993) but *Wolbachia* have since been transferred between a range of more distantly related species (Van Meer & Stouthamer, 1999; Kang et al., 2003). Horizontal transfer between more distantly related hosts appears to be generally less successful than transfer between closely related host species (Huigens et al., 2004; Tinsley & Majerus, 2007) but theoretical work suggests that even occasional transfer of *Wolbachia* over long phylogenetic distances may be sufficient to maintain its remarkably high incidence across species (Zug et al., 2012).

1.3.2 Natural introgression of *Wolbachia*

Although unlikely to be as general an explanation for the breadth of the *Wolbachia* pandemic as horizontal transfer, naïve species can also acquire *Wolbachia* through introgression of infected cytoplasm from a related species. Hybridisation between a male of an uninfected species and a female of related species infected with *Wolbachia* will produce an infected hybrid, and subsequent backcrossing of the

hybrid with the previously uninfected species will lead to the introgression of both heterospecific mtDNA and *Wolbachia* with comparatively little impact on host nuclear DNA (Hurst & Jiggins, 2005; Raychoudhury et al., 2009). The historical introgression of *Wolbachia* has been inferred in a number of species based on striking relationships between infection and mtDNA haplotypes and the discordance between host mtDNA and nuclear DNA (Jiggins, 2003; Charlat et al., 2009; Raychoudhury et al., 2009; Xiao et al., 2012). For instance, in the fig wasp *Ceratosolen solmsi*, *Wolbachia*-infected individuals harbour a radically different mtDNA haplotype in comparison to uninfected individuals, but there are no substantial differences in sequences of the nuclear genes *its2* and *opsin* in infected and uninfected individuals; this implies that cytoplasmic and nuclear genes have different evolutionary histories in this species (Xiao et al., 2012)

1.3.3 Loss of *Wolbachia* on evolutionary timescales

Whilst *Wolbachia* is clearly adept at infecting new and unrelated species, the corollary of this is that if global infection levels have reached anything approximating an equilibrium, infections must be lost from species as well as gained. A number of possible evolutionary scenarios could result in the loss of *Wolbachia* that engage in reproductive parasitism, largely due to conflicts between reproductive parasites and nuclear genes. Perhaps the most obvious is the

evolution of nuclear genes that simply prevent or reduce the transmission of *Wolbachia* to the next generation (Jaenike, 2007a). Alternatively, nuclear suppressor genes that negate the deleterious effects of *Wolbachia* could evolve and spread thorough infected host populations. The evolution of reduced transmission or suppression has been predicted to occur most easily in species infected with strains that induce male-killing, feminization, or parthenogenesis, and accordingly suppressor genes have been discovered in natural populations of the isopod *Armadillidium vulgare* and the butterfly *Hypolimnas bolina* infected with feminizing and male-killing *Wolbachia,* respectively (Rigaud & Juchault, 1993; Hornett et al., 2006; Charlat et al., 2007). Evidence for suppressor genes was also uncovered in *Drosophila subquinaria* following the introgression of *Wolbachia* from its sister species *D. recens* (Jaenike, 2007b).

The evolution of transmission-reducers or suppressors of CI-inducing *Wolbachia* is likely to be more complicated. Once CI-inducing *Wolbachia* are sufficiently common in a population, females actually experience a net benefit from infection because they avoid the fitness reduction associated with mating incompatibilities. Consequently, genes that cause general transmission reduction or suppression of the CI-phenotype will be selected against. Instead, sex-specific suppression of *Wolbachia* in males must occur first. Only once suppression of *Wolbachia* in males has spread, and uninfected females are at a sufficiently low risk

of mating incompatibilities, will suppression in females will be selected for (Werren et al., 2008; Koehncke et al., 2009).

Whilst CI-Inducing *Wolbachia* may be more resistant to the evolution of nuclear suppressors than sex-ratio distorters or male-killers, they may be susceptible to eventual extinction through other means. For instance, theoretical work suggests that CI-inducing *Wolbachia* are vulnerable to invasion by other strains causing male-killing, feminization, and parthenogenesis (Hurst et al., 2002). CI-inducing *Wolbachia* may also become extinct in a species because of an evolutionary trajectory taken by the bacteria itself. Suppose that both the induction of mating incompatibilities and the presence of *Wolbachia* itself carries a fitness cost to the host. A mutant strain that no longer induces mating incompatibilities but is still capable of allowing its host to mate with infected males (so called 'rescuing *Wolbachia*' mentioned in section 1.2.3.1) will spread through an infected population, excluding the original CI strain. In turn, uninfected individuals, either generated by transmission failure or introduced by migration from an adjacent population, will outcompete individuals infected with the rescuing strain, because uninfected females will not suffer from mating incompatibilities when mated to a male infected with a rescuing strain. A two-step process can thus lead to the extinction of CI-*Wolbachia* from a population (Turelli, 1994; Hurst & Mcvean, 1996).

Interestingly, such rescuing *Wolbachia* have been identified in *Drosophila* (Bourtzis et al., 1998; Zabalou, 2004).

Finally, male-killing and feminizing strains of *Wolbachia* with perfect transmission may simply drive themselves, and their hosts, to extinction due to the elimination of males in species which is only capable of reproducing sexually (Hurst & Jiggins, 2000). In many cases, this appears not to happen because male-killing strains often display imperfect transmission, unlike CI-inducing *Wolbachia*. Imperfect transmission may exist due to environmental factors, such as temperature and naturally-occurring antibiotics that lead to natural curing of infection, or due to the evolution of nuclear genes that reduce transmission (Hurst & Jiggins, 2000; Hurst et al., 2001).

1.4 The evolutionary consequences of *Wolbachia* infection

1.4.1 Speciation

It has been suggested that *Wolbachia,* and in particular CI-inducing strains, may cause or enhance the process of speciation in arthropods (Werren et al., 2008; Brucker & Bordenstein, 2012), and the extreme prevalence of *Wolbachia* across species coupled with the strength of mating incompatibilities in many systems has

made *Wolbachia* an attractive prospect as a general cause of speciation. Bidirectional CI intuitively seems to have the most potential to act as a cause of reproductive isolation. If two populations acquire different, mutually incompatible strains of *Wolbachia,* interpopulation crosses will fail to produce viable offspring (in the case of complete incompatibility) leading to reproductive isolation (Brucker & Bordenstein, 2012). In the parasitoid wasp sister-species *Nasonia giraulti* and *N. longicornis*, such *Wolbachia*-induced bidirectional incompatibilities are the major cause of reproductive isolation between the two species (Bordenstein et al., 2001). However, these two species do not actually exist in sympatry (Weeks et al., 2002), and few other clear examples of bidirectional CI acting as the main cause of reproductive isolation between have been identified. Although CI-inducing *Wolbachia* are common, bidirectional CI is comparatively rare (Werren et al., 2008). Furthermore, in a bidirectional CI system, the presence of uninfected individuals due to imperfect vertical transmission of *Wolbachia* will lead to gene flow between the two cytotypes, and the creation of individuals infected with both strains via horizontal transfer will lead to the complete loss of incompatibility between hybrids (Hurst & Schilthuizen, 1998).

Alternatively, it has been argued that unidirectional CI may act as one of multiple mechanisms accounting for reproductive isolation between species, acting in concert with other factors responsible for pre- or post-zygotic reproductive

isolation (Shoemaker et al., 1999). Theoretical work also implies that mating incompatibilities, in addition to acting as barriers to gene flow, lead to selection for reinforcement (Telschow et al., 2005). Although the model of Telschow et al., (2005) focussed on the role of bidirectional CI in the evolution of reinforcement, empirical evidence for reinforcement as a response to *Wolbachia*-induced unidirectional CI exists in *Drosophila. Drosophila recens* is infected with a CI-inducing strain of *Wolbachia,* whilst its sister species *D. subquinaria* is uninfected, and so *D. subquinaria* females mated with *D. recens* males exhibit low egg hatch rates. *D. subquinaria* females originating from populations where both species exist in sympatry are far less inclined to mate with *D. recens* males than females originating from populations where the two species do not coexist. In contrast, *D. recens* females did not show mating preferences specific to their population of origin. This suggests that mating discrimination exhibited by *D. subquinaria* females has evolved in response to the deleterious effects of mating with *D. recens* males. *Wolbachia* may thus have been responsible for initial post-zygotic isolation between the two species, with additional pre-zygotic isolation evolving in response (Jaenike et al., 2006).

Parthenogenesis-inducing *Wolbachia* may also cause speciation in their hosts. At first, infected parthenogenetically reproducing females may retain the ability to reproduce sexually. However, in populations where the infection has

spread to fixation, the continued absence of males may relax selection on genes related to sexual reproduction. Subsequent accumulation of deleterious mutations in these genes may lead to a loss of sexual function, resulting in irreversible parthenogenesis and complete reproductive isolation from conspecific sexual populations (Gottlieb & Zchori-Fein, 2001; Brucker & Bordenstein, 2012).

Finally, genetic conflict between *Wolbachia* and its host may also result in reproductive isolation. In section *1.2.3.2*, I discussed how coevolutionary processes can result in the dependence of a host on *Wolbachia*, and similar processes could result in hybrid inviability, and thus reproductive isolation. Suppose a costly strain of *Wolbachia* invades a species, and host nuclear genes evolve to supress replication of *Wolbachia* or ameliorate any deleterious phenotypic effects. *Wolbachia* then evolves to increase its replication rate or enhance its effects, and the cycle continues. However, if the host hybridises with an uninfected species, the hybrid will have failed to inherit at least some of the genes involved in controlling the infection. *Wolbachia* may thus go into overdrive, drastically reducing the fitness of its hybrid host (Crespi & Nosil, 2013). Such effects have been observed in both *Nasonia* and *Drosophila,* where *Wolbachia* over-replicates and reduces host fecundity in hybrids (Miller et al., 2010; Chafee et al., 2011).

1.4.2 Evolution of sex determination

Wolbachia clearly plays a central role in sex determination in certain species. In species infected with parthenogenesis inducing strains, *Wolbachia* is responsible for the absence of the male sex, and in species infected with feminizing strains, *Wolbachia* causes otherwise genetically male offspring to develop into phenotypic females (Werren et al., 2008; Ma & Schwander, 2017). In *Armadillidum vulgare*, where females are the heterogametic sex, the presence of feminizing *Wolbachia* can rapidly lead to the loss of the W chromosome, which would normally trigger female development. Instead, all individuals are ZZ, and *Wolbachia* becomes the sex-determining locus; infected individuals develop into females and uninfected individuals, generated via imperfect transmission, develop into males (Rigaud & Juchault, 1992; Rigaud, 1997; Bouchon et al., 2008). However, some lines of *A. vulgare* produce female biased sex-ratios despite the absence of *Wolbachia* infection. This is due to the insertion of a fragment of the feminizing *Wolbachia* genome into the genome of *A. vulgare*, essentially giving birth to a new W chromosome and restoring nuclear sex determination (Leclercq et al., 2016). Not only is this interesting in its own right, it also highlights the potential importance of genetic conflict in the turnover of sex-determination systems generally. Sex-determination is remarkably labile across animals, and sex-determination mechanisms clearly change relatively frequently on evolutionary timescales (Bachtrog et al., 2014).

Sex-ratio conflict between nuclear and cytoplasmic genes may play an important role in the turnover of sex-chromosomes and transitions between sex-determination mechanisms, as it can cause strong selection for a change in the mechanism of sex-determination to restore unbiased sex-ratios (Bachtrog et al., 2014; Mank et al., 2014).

1.4.3 Extinction

Strains of *Wolbachia* that engage in reproductive parasitism may contribute to the extinction of their hosts in various ways (Charlat et al., 2003). A switch to parthenogenetic reproduction is likely beneficial in the short term, as it circumvents the various costs of sex (Maynard Smith, 1978; Lehtonen et al., 2012). However, it is thought that asexual species are more prone to extinction in the long run due to factors such as the accumulation of deleterious mutations and a reduced ability to adapt to fluctuating biotic and abiotic environmental factors (Maynard Smith, 1978; Normark et al., 2003; Ross et al., 2013) and so parthenogenesis-inducing *Wolbachia* may increase the risk of host extinction. In a rather more straightforward manner, male-killing and feminizing strains of *Wolbachia* could potentially drive their hosts to extinction by eliminating males entirely from infected populations, as discussed in section *1.3.3*. Finally, through their effects on host population size and sex-ratio, CI-inducing and sex-ratio

distorting *Wolbachia* may significantly decrease genetic diversity within host populations, increasing the risk of host extinction (Dobson et al., 2002a; Charlat et al., 2003; Engelstadter & Hurst, 2006).

1.5 *Wolbachia* in social insects

1.5.1 Social insects

Many animals form relatively loose aggregations or groups, which may help mitigate the risks posed by predators and parasites, increase the efficiency of foraging, and improve the odds of finding a mate (Hamilton, 1971; Macdonald, 1983; Krause & Ruxton, 2002). Some species exhibit a more tightly knit societal structure, where members cooperate in breeding and parental care, and in some cases significant reproductive skew exists (Brockman, 1997; Clutton-Brock, 2002; Arnold & Martin, 2009). However, in obligately eusocial species cooperation is no longer a choice, and most individuals in the group are members of irreversibly sterile helper castes that assist in the care of their relative's offspring (Crespi & Yanega, 1995; Boomsma & Gawne, 2016). Eusociality has a surprisingly broad phylogenetic distribution, and even according to the strict definition of eusociality *sensu* (Crespi & Yanega, 1995), examples exist of eusocial ants (Hölldobler &

Wilson, 1990), bees (Michener, 2000), wasps (Hines et al., 2007), termites (Korb, 2008), aphids (Stern & Foster, 1996), thrips (Crespi, 1992), beetles (Kent & Simpson, 1992) and shrimp (Duffy, 1996). Accordingly, eusociality has evolved convergently many times, and yet the majority of eusocial species are concentrated in the social Hymenoptera – ants, bees and wasps (Wilson, 1971; Hughes et al., 2008). The social Hymenoptera, along with termites, clearly represent the pinnacle of social behaviour, with colonies comprising thousands or even millions of workers collectively foraging, defending and caring for the offspring of one or a small number of reproductive individuals (Hölldobler & Wilson, 2008). However, insect societies are not as harmonious as they are often portrayed, and like genes within cells, and cells within organisms, individuals within societies frequently act selfishly to facilitate their own reproduction or the reproduction of individuals they are more closely related to (Ratnieks et al., 2006; Bourke, 2011).

As well as being fascinating evolutionary case-studies, social insects are of huge ecological and economic importance (Chapman & Bourke, 2001; Hölldobler & Wilson, 2008). However, comparatively little is known about the biology of *Wolbachia* in social insects relative to solitary species, particularly in terms of its phenotypic effects. The unique biology of social insects may significantly alter the success of different *Wolbachia*-induced phenotypes, as well as affecting the prevalence of *Wolbachia* and the manner of its transmission between species

(Russell, 2012). Furthermore, social insects as a group vary enormously in their biology and life-history, and so our expectations about the biology of *Wolbachia* in social insects may vary considerably across species.

1.5.2 The prevalence of *Wolbachia* in social insects

Generally speaking, *Wolbachia* is no less prevalent amongst social insects than solitary insects, although there is significant variation in the incidence of *Wolbachia* across different groups (Russell, 2012). Many ant species are infected with *Wolbachia*, but not significantly more than other insect groups (Wenseleers et al., 1998; Shoemaker et al., 2000; Russell et al., 2009, 2012; Frost et al., 2010; Russell, 2012; Kautz et al., 2013; Wang et al., 2016). However, the prevalence of *Wolbachia* in ants varies enormously at various taxonomic scales. At the subfamilial level, Tsoi, (2013) found that 41% of Cerapachyine ants screened for *Wolbachia* were infected, whilst only 2% of Amblyoponine ants were infected. Substantial variation also occurs at the generic level. For instance, in the Dorylinae, some genera appear completely free from infection; all 16 species of *Dorylus* and all 5 species of *Eciton* that have been screened for *Wolbachia* appear to be uninfected, whilst all 8 species of *Aenictus* and 6/7 species of *Neivamyrmex* were found to be infected with *Wolbachia* (Russell et al., 2009, 2012). Clearly there are taxon-specific traits that influence the incidence of *Wolbachia* across taxa (Russell, 2012). One strong

candidate is the mode of colony foundation (Wenseleers et al., 1998; Russell, 2012), and perhaps other forms of dispersal-related traits, which I discuss further in Chapter 6.

Few species of social bees have been screened for *Wolbachia* infection, but broad screening of the German fauna suggests that *Wolbachia* may be extremely common in bees generally (Gerth et al., 2015). As with ants, the incidence of *Wolbachia* was shown to vary considerably between different groups (Gerth et al., 2015). Again, few species of social wasps have been screened for infection with *Wolbachia*; conventional PCR screens have detected no infected Vespid wasps (the family containing both origins of sociality in wasps), with the exception of a study of *Polistes dominula,* but a more recent study of *Wolbachia* infection in Brazilian insects using more sensitive qPCR assays identified a number of infected Vespids (Stahlhut et al., 2006; Russell, 2012; de Oliveira et al., 2015).

Termites present an unusual case with regards to the phylogenetic affiliation of the *Wolbachia* they harbour. The vast majority of *Wolbachia* strains infecting arthropods are from supergroups A and B, and strains identified in ants, bees and wasps are no exception to this (Russell, 2012; Gerth et al., 2014, 2015). However, the majority of infections in termites are from supergroups F and H; the former is unusual as it is the only supergroup found in both arthropods and nematodes, whilst the latter has so far only been identified from termites (Bandi et

al., 1997; Bordenstein & Rosengaus, 2005; Casiraghi et al., 2005; Salunke et al., 2010).

1.5.3 The Phenotypic effects of *Wolbachia* in social insects

Elucidating the phenotypic effects of *Wolbachia* presents a special difficulty in the social insects. The gold-standard demonstration of CI involves performing all four potential crosses between infected and uninfected males and females, preferably with infected and uninfected individuals from multiple genetic lines (Weeks et al., 2002). Similarly, demonstrating male-killing, parthenogenesis induction or feminization generally involves breeding infected and uninfected lines for at least one generation. However, the large majority of social insects are difficult or impossible to breed in the laboratory and have long generation times (Jemielity et al., 2005) and it is difficult to encourage many species to even produce sexual offspring in captivity, let alone mate. As such, identifying the phenotypic effects of *Wolbachia,* particularly those related to reproduction, has proved extremely difficult (Bouwma & Shoemaker, 2011; Russell, 2012).

Of the four manipulations of host reproduction performed by *Wolbachia,* it has been suggested that feminization and parthenogenesis induction are unlikely to occur in the social Hymenoptera, which represent the majority of social insects

(Wenseleers et al., 1998; Shoemaker et al., 2000; Russell, 2012). Feminized males would remain haploid and thus be sterile, and feminization has been identified in a fairly narrow range of insect species (Wenseleers et al., 1998; Hurst & Frost, 2015). Initially, *Wolbachia*-induced parthenogenesis was thought to proceed only via gamete duplication, which eliminates genomic heterozygosity entirely (Ma & Schwander, 2017). Because the majority of social hymenopterans utilise single-locus complimentary sex determination, gamete duplication would lead to the production of sterile diploid males instead of females, and so PI strains were assumed to be unable to invade social hymenopterans (van Wilgenburg et al., 2006). However, it is now understood that a variety of cellular mechanisms underlie PI, some of which preserve heterozygosity (Ma & Schwander, 2017) and so PI strains could in theory spread in social hymenopterans. Nonetheless, a number of obligately and facultatively parthenogenetic social hymenopterans have been screened for *Wolbachia*, none of which appear to be infected (Grasso et al., 2000; Wenseleers & Billen, 2000; Himler et al., 2009; Kronauer et al., 2012; Martinez-Rodriguez et al., 2013; Rabeling & Kronauer, 2013). On the other hand, conditions in social insect colonies seem ripe for the spread of male-killers. Competition between siblings, which is essentially a pre-requisite for the successful invasion of male-killing *Wolbachia*, is likely intense in social insects, and female larvae have the potential to benefit considerably from the death of males

(Rüger et al., 2008; Schultner et al., 2013). Indeed, in a sense, male-killing has already evolved in social insects themselves; in *Formica exsecta* colonies where the queen has mated with a single male, workers kill their brothers, to whom they are relatively less related, in order free up resources for investment in their sisters (Sundström, 1994; Sundström et al., 1996)

A number of authors have attempted to identify *Wolbachia*-induced sex-ratio distortion in social insects. Two surveys in populations of *Formica* ants exhibiting endemic *Wolbachia* infections found no correlation between colony sex-ratio and the proportion of workers infected by *Wolbachia*, suggesting the absence of indirect sex-ratio manipulation by infected workers (Keller et al., 2001; Wenseleers et al., 2002). In both cases, all colonies contained at least some infected workers, and so no comparisons between categorically infected and uninfected colonies could be made to test for a direct effect of *Wolbachia* on colony sex-ratios by e.g. male-killing. However Wenseleers et al., (2002) ruled out a direct effect of *Wolbachia* on the sex ratio via male-killing in *F. truncorum*, as males were no less likely to be infected by *Wolbachia* than gynes; if *Wolbachia* engaged in male-killing, uninfected males would be disproportionately represented in the survivors. Interestingly, (Van Borm et al., 2001) found indirect evidence of male-killing in two species of *Acromyrmex* ants, where the frequency of infection in males was markedly lower than that of gynes. However this pattern could also be explained by sex-specific differences in parasite

resistance or adaptive loss of infection in males. In a recent study of sex- and caste-ratios in *Monomorium pharaonis*, one of the few ant species which can be bred under laboratory conditions, Pontieri et al., (2016) were able to breed infected and uninfected lines, uncovering a correlation between *Wolbachia* infection and female-biased sex-ratios in the process. However, further work is required to identify the details of the *Wolbachia* phenotype responsible for sex-ratio distortion in this species.

It has been suggested that CI-inducing *Wolbachia* may spread particularly easily within social insects. Firstly, social insects have relatively small effective population sizes due to their particular life-histories (Romiguier et al., 2014). This is expected to facilitate the invasion of CI-inducing *Wolbachia* with invasion thresholds above zero (i.e. those with a fitness cost or imperfect maternal transmission), because it will be easier for them to exceed the threshold frequency through drift and subsequently spread deterministically within the host population (Reuter & Keller, 2003). Secondly, the expression of mating incompatibilities may carry an additional cost in the social Hymenoptera, because the reduced production of female offspring will interfere with a queen's ability to rear workers and thus establish a successful colony, which is required for the rearing of sexual offspring. Thus, whereas the expression of CI in a solitary haplodiploid will reduce the production of female offspring, the expression of CI

in a social insect may prevent the host from rearing any sexual offspring at all (Wenseleers et al., 1998).

Unfortunately, little progress has been made in identifying *Wolbachia*-induced mating incompatibilities in social insects. Van Borm et al., (2001) and Wenseleers et al., (2002) suggested that the lower infection rates of virgin queens relative to established colonies in the ant species *Acromyrmex insinuator* and *Formica truncorum,* respectively, may result from the failure of uninfected queens in establishing successful colonies due to the expression of mating incompatibilities. (Bouwma & Shoemaker, 2011) performed a direct test of *Wolbachia*-induced CI in the ant *Solenopsis invicta,* but their analyses of the brood production patterns of sympatric infected and uninfected queens did not correlate with the expectations of either male development or female mortality type CI. Specifically, most queens produced normal amounts of brood regardless of infection status.

Again, very few studies have assessed the fitness effects of *Wolbachia* in social insects. (Bouwma & Shoemaker, 2011) found no relationship between *Wolbachia* infection and an array of survival and fecundity-related traits in *S. invicta.* Interestingly, Wenseleers et al., (2002) uncovered a strong negative correlation between colony productivity and the proportion of infected workers within a colony in *F. truncorum.* They suggest that the loss of infection observed in

many workers during development from pupae to adult may thus be adaptive, as it reduces the prevalence of an apparently costly infection within colonies.

1.5.4 The population biology and phylogeography of *Wolbachia* in social insects

Whilst unravelling the phenotypic effects of *Wolbachia* in social insects has proved challenging, a few studies have investigated aspects of the population biology, genetics and transmission of *Wolbachia* within and between species, again largely in ants. A few general results have emerged from these studies. Firstly, the presence of multiple strains of *Wolbachia* within species appears to be common; species of *Formica* (Reuter & Keller, 2003), *Acromyrmex* (Van Borm et al., 2003) and *Solenopsis* (Shoemaker et al., 2000, 2003; Ahrens & Shoemaker, 2005; Dedeine et al., 2005) are all co-infected with multiple strains of *Wolbachia.* Indeed, single individuals of the social parasite *Solenopsis daguerrei* were found to be infected with up to eight different strains of *Wolbachia,* more than any other arthropod species (Dedeine et al., 2005) and *Acromyrmex* and *Formica spp.* were shown to be infected with up to four and five different strains, respectively. (Reuter & Keller, 2003; Van Borm et al., 2003). In *S. daguerrei,* many of these strains are genetically divergent, and so were probably independently acquired, and the same appears to be true of other *Solenopsis* species, which are infected with *Wolbachia* from both the A and B

supergroups (Ahrens & Shoemaker, 2005; Dedeine et al., 2005). In contrast, three of the five strains of *Wolbachia* found in *Formica exsecta* appear to have arisen by homologous recombination (Reuter & Keller, 2003).

Secondly, the sharing of related strains between species suggests that acquisition has occurred via horizontal transmission between different species within a genus. Various *Solenopsis* species share very similar strains of *Wolbachia* that are unlikely to have been acquired by descent (Ahrens & Shoemaker, 2005; Martins et al., 2012). Interestingly, some strains of *Wolbachia* that infect *S. daguerrei,* a social parasite of *S. invicta* and *S. richteri,* are identical to strains isolated from its hosts, despite the fact that *S. daguerrei* is not closely related to *S. invicta* or *S. richteri*; this strongly suggests that they were either acquired from, or transmitted to, the hosts via horizontal transmission (Dedeine et al., 2005). Similar patterns have been identified in *Acromymrex* and *Formica*; in the case of the latter, the dissociation of mtDNA and *Wolbachia* surface protein haplotypes supports the hypothesis of horizontal transmission of *Wolbachia* between related species, as opposed to the sharing of strains due to common ancestry (Van Borm et al., 2003; Viljakainen et al., 2008).

Thirdly, global screening data from three different ant genera show that *Wolbachia* is often lost during the spread of species outside their native range (Shoemaker et al., 2000; Tsutsui et al., 2003; Reuter et al., 2005; Rey et al., 2013). For

instance, two separate studies showed that a distinctly larger proportion of native populations of the invasive ant *Linepithema humile* are infected with *Wolbachia* than introduced populations (Tsutsui et al., 2003; Reuter et al., 2005). Similarly, *Wolbachia* appears to be more common in the native range of *S. invicta* and *S. richteri* (i.e. South America) than in populations from the USA (Shoemaker et al., 2000). Whether these patterns occur because of the more successful invasion of uninfected species (i.e. the enemy-release hypothesis), adaptive curing due to high temperatures outside native ranges, relaxed selection on infection status, or some other factor, is not clear (Reuter et al., 2005; Rey et al., 2013).

1.6 Thesis aims

In this thesis I combine molecular techniques, extensive ecological sampling, laboratory experiments and comparative analyses to explore various aspects of the biology of heritable symbionts in social insects, principally focusing on the relationship between ants, the largest group of social insects, and the symbiont *Wolbachia*.

The first part of this thesis is mainly concerned with the phenotypic effects of *Wolbachia* in social insects. In Chapter 2, I investigate patterns in the prevalence of

Wolbachia in a population of the ant *Temnothorax crassispinus* and attempt to identify colony-level effects of infection. In Chapter 3, I test for effects of *Wolbachia* on host reproduction in the ant *Myrmica scabrinodis*, and investigate whether *Wolbachia* induces mating incompatibilities in this species. In Chapter 4, I discuss the theory that maternally transmitted symbionts might evolve to manipulate the caste of their hosts in social insects, and identify three distinct selective reasons as to why this might occur.

The second part of this thesis is largely concerned with different aspects of the movement of *Wolbachia* between species. In Chapter 5, I examine the prevalence of *Wolbachia*, and two other symbionts, *Spiroplasma* and *Arsenophonus*, in multiple populations of *M. scabrinodis* across the Palaearctic, and I identify non-random patterns of symbiont co-infection and present evidence of multiple introductions of both *Wolbachia* and *Spiroplasma* into this species. In Chapter 6, I investigate the acquisition of *Wolbachia* in the ant *Monomorium pharaonis*, finding that one of two strains infecting this species was likely acquired by introgressive hybridisation from a related species. In Chapter 7, I re-examine the relationship between *Wolbachia* and dispersal-related life-history traits in ants, improving on previous analyses by curating a larger dataset, including multiple dispersal-related life-history traits, and conducting a phylogenetically controlled comparative analysis of the dataset; my results suggest that host effective population size may

be an important determining factor in the ability of *Wolbachia* to infect new host species. Finally in Chapter 8, I discuss the findings of this thesis as a whole.

2 Sex, caste, and colony size specific patterns of *Wolbachia* infection in the ant *Temnothorax crassispinus*

2.1 Abstract

Wolbachia is a common symbiont of arthropods and filarial nematodes, yet comparatively little is known about its biology in social insects, particularly regarding the phenotypic effects of infection and the causes of intra- and inter-colonial patterns of infection. Here, I report the results of a detailed population study of *Wolbachia* infection in the common eastern European ant *Temnothorax crassispinus*. I show that whilst *Wolbachia* infection has no effect on the production of new workers, colonies with a higher prevalence of infection show slightly increased sexual productivity. In addition, I find a large degree of individual and colony-level variation in the likelihood of infection, and show that this is partially explained by the sex and caste of host individuals – a higher proportion of queens and virgin queens were infected than workers, and in turn,

more workers were infected than males. In the case of the worker caste, colony size was also related to the likelihood of infection, with larger colonies containing a higher proportion of infected workers. In contrast, colony usurpation and colony fusion failed to account for intra-colonial variation in infection. Seen in the context of other studies, my results suggest that complex patterns of *Wolbachia* infection may be a widespread feature across social insects.

2.2 Introduction

Wolbachia is an extremely widespread symbiont of arthropods and filarial nematodes (Werren, 1997a; Taylor et al., 2005). One of the reasons for the astonishing success of *Wolbachia* is the wide variety of strategies, ranging from mutualistic to parasitic, that it is capable of utilising (Werren et al., 2008). Whilst it is an obligate mutualist in its nematode hosts, the biology of *Wolbachia* in arthropods is considerably more variable, where it often manipulates the reproduction of its hosts in various ways (Taylor et al., 2013; Zug & Hammerstein, 2015). In many species, *Wolbachia* induces incompatible matings between uninfected females and infected males, leading to the production of inviable eggs. The consequent reduction in the fitness of uninfected females relative to infected

females enables *Wolbachia* to spread through populations (Werren, 1997a; Stouthamer et al., 1999). In other species, *Wolbachia* distorts host sex-ratios by causing the early death of male embryos, a transition to parthenogenetic reproduction in its host, or the feminization of genetic males (Werren et al., 2008). However, *Wolbachia* exhibits an array of other phenotypes in arthropods. For example, it confers resistance to viral infections in *Drosophila melanogaster* (Hedges et al., 2008), behaves as a nutritional mutualist in bedbugs and *D. melanogaster* (Brownlie et al., 2009; Hosokawa et al., 2010) and is required for oogenesis in *Asobara tabida* (Dedeine et al., 2001). In addition, *Wolbachia* infections can positively or negatively impact host fitness in parallel with manipulating host reproduction (Fleury et al., 2000; Fytrou et al., 2006; Weeks et al., 2007).

Wolbachia is no less common in social insects (Bandi et al., 1997; Wenseleers et al., 1998; Stahlhut et al., 2006; Evison et al., 2012; Russell, 2012; Russell et al., 2012; Kautz et al., 2013; Gerth et al., 2015) and a number of studies have revealed complex and intriguing interactions between *Wolbachia* and social insects, including variability in the patterns of infection dependent on sex and caste (Van Borm et al., 2001; Wenseleers et al., 2002; Roy et al., 2015) and remarkably high numbers of co-infecting strains (Reuter & Keller, 2003; Van Borm et al., 2003; Dedeine et al., 2005). However, the vast majority of studies of *Wolbachia* in social insects have focussed on distributional patterns and transmission dynamics

(Ahrens & Shoemaker, 2005; Viljakainen et al., 2008; Gerth et al., 2015) and consequently there remains a paucity of data concerning the phenotypic effects of *Wolbachia* in social insects. It remains unclear whether *Wolbachia* commonly influences the reproductive biology of social insects, and there is no clear evidence from social insects of *Wolbachia*-induced cytoplasmic incompatibility (Van Borm et al., 2001; Wenseleers et al., 2002; Bouwma & Shoemaker, 2011), parthenogenesis (Grasso et al., 2000; Wenseleers & Billen, 2000; Himler et al., 2009; Martinez-Rodriguez et al., 2013; Rey et al., 2013) feminization or male-killing (Keller et al., 2001; Wenseleers et al., 2002; Russell, 2012). However, a recent study in the ant *Monomorium pharaonis* found that *Wolbachia* infection is correlated with female-biased sex-ratios (Pontieri et al., 2016). Only one other study has demonstrated a phenotypic effect of *Wolbachia* in ants; (Wenseleers et al., 2002) documented a negative relationship between infection prevalence within a colony and the production of sexual offspring.

Here, I aim to address this gap by investigating the relationship between *Wolbachia* infection, sex-ratio and colony-productivity in *Temnothorax crassispinus*, a common Palearctic ant species. *T. crassispinus* is an ideal species for examining the relationship between *Wolbachia* infection and colony traits because large numbers of entire nests can be easily collected and censused due to their small size, high population density, and their location within collectible twigs and acorns, allowing

for both a large sample size and the accurate determination of the sex-ratio and productivity of colonies. In addition, I aim to elucidate the causes of intra- and inter-colonial variation in *Wolbachia* infection by examining the relationship between the infection status of individuals and sex, caste, colony-size and queen presence. Finally, whilst *T. crassispinus* is monogynous and monandrous, colonies can contain multiple matrilines due colony usurpation and colony fusion (Tichá & Styš, 2002; Strätz & Heinze, 2004; Pusch et al., 2006b, 2006a). As such, I test the hypothesis that the presence of multiple matrilines within colonies might explain the co-existence of infected and uninfected individuals within a single colony.

2.3 Materials and methods

Nests of *Temnothorax crassispinus* were collected from Hoia-Baciu forest on the outskirts of Cluj-Napoca, Romania in July 2015. *T.crassispinus* is polydomous and so nests were sampled a minimum of 3 metres apart, to avoid sampling the same colony multiple times. All queens, workers, gynes, males, worker pupae, gyne pupae, male pupae and larvae were counted and transferred to 100% ethanol. Two measures of productivity were calculated for each nest. Sexual productivity was defined as the total number of gynes, gyne pupae, males and male pupae, with the

total for gynes and gyne pupae multiplied by a weighting factor of 3 to account for the greater cost of rearing gynes (Strätz & Heinze, 2004). Non-sexual productivity was defined as the total number of callow workers and worker pupae.

To estimate the prevalence of *Wolbachia* infection within nests, eight workers and, when present, eight gynes and males, per colony were screened for *Wolbachia* infection using diagnostic PCR. If nests comprised fewer than eight workers, gynes or males, all individuals in the nest were screened. Queens from queenright colonies were also screened for *Wolbachia* infection. DNA was extracted from whole ants in 100 μl of 5% Chelex® solution with 5 μl of 10 mg/ml Proteinase K. PCR reactions were performed using 10 μl reaction mixtures, consisting of 4.8 μl molecular grade H_2O, 2 μl Promega GoTaq Flexi green buffer, 1.25 μl $MgCl_2$, 0.5 μl dNTP's (2.5 mM each), 0.2 μl each of the forward and reverse primer (10 μM), 0.05 μl Promega GoTaq DNA Polymerase and 1 μl DNA extract. The primers *wsp_F1* and *wsp_R1* (Baldo et al., 2006) were used to detect the presence of *Wolbachia* and each individual was screened twice to control for errors during the PCR stage. In addition, the primers *F1-1424F* and *F1-1829R* (Brady et al., 2006) were used to amplify a segment of the gene *EF1αF1* for each individual as a host control. Infected *Acromyrmex octospinosus* individuals were used as positive controls, and molecular grade H_2O was used for negative controls. PCR reactions consisted of 95°C for 2 min followed by 35 cycles of 95°C for 30 s, 55°C for 30 s and 72°C for 1

min, and finally 72°C for 7 min. PCR products were run on 1% agarose gels, visualised under UV light, and bands were scored as present or absent by eye. PCR's performed on serially diluted DNA extractions showed that *Wolbachia* infection could be detected at 1000x dilution of gyne template DNA, and so the PCR assay used here is likely to be sufficiently sensitive to detect all but extremely low titre infections.

The five *Wolbachia* MLST genes (Baldo et al., 2006) were sequenced to assess both the phylogenetic position of the *Wolbachia* infecting *T. crassispinus* and to determine whether multiple strains of *Wolbachia* co-occur in this population. PCR reactions were performed using 20 μl reaction mixtures, consisting of 9.6 μl molecular grade H_2O, 4 μl Promega GoTaq Flexi green buffer, 2.5 μl $MgCl_2$, 1 μl dNTP's (2.5 mM each), 0.4 μl each of the forward and reverse primer (10 μM), 0.1 μl Promega GoTaq DNA Polymerase and 2 μl DNA extract. PCR conditions were as described above, except that the annealing temperature was 54°C, 50°C, 54°C, 53°C, 58°C for the genes *gatB*, *coxA*, *hcpA*, *ftsZ* and *fbpA*, respectively. PCR products were purified using a QIAquick PCR purification kit as per the manufacturer's instructions and sequenced by Eurofins Genomics.

To investigate the possibility that the presence of both infected and uninfected workers in a colony was due to the presence of multiple matrilines, 8 workers from 24 colonies containing both infected and uninfected workers were

genotyped at five polymorphic microsatellite loci: *Ant1343*, *Ant2936*, *Ant3993*, *Ant8424*, and *Ant11893* (Butler et al., 2014). PCR reactions were performed using 10 μl reaction mixtures, as described above. PCR conditions were as described above, except that the annealing temperature was 50°C. DNA sequencing was performed by DNA Sequencing & Services (MRC I PPU, College of Life Sciences, University of Dundee, Scotland, www.dnaseq.co.uk) using Applied Biosystems Big-Dye Ver 3.1 chemistry on an Applied Biosystems model 3730 automated capillary DNA sequencer. Microsatellite alleles were scored using GeneMapper v3.7, and binned using TANDEM (Matschiner & Salzburger, 2009).

All statistics were performed using SPSS 22 (IBM-SPSS Statistics, Armonk, NY, USA). The relationship between the infection status of individuals and caste, colony-size (i.e. number of non-callow workers) and queen presence was analysed using a mixed effects binomial logistic regression, with the model including an interaction between caste and colony size, and with colony included as a random factor. Post-hoc comparisons of castes were made using the sequential Bonferroni method to control for multiple comparisons. The relationship between colony productivity and infection prevalence within the worker caste, colony size and queen presence was analysed using Poisson regressions. The relationship between sex-ratio and colony infection prevalence, colony size, queen presence and nest material was analysed using a binomial logistic regression.

2.4 Results

2.4.1 Infection status of colonies and individuals

87 out of 88 of nests were infected with *Wolbachia,* with each nest containing at least one infected individual. Sequencing of the five *Wolbachia* MLST genes suggests that this population of *T. crassispinus* is infected with multiple strains of *Wolbachia*, as evidenced by consistent double peaks seen on DNA chromatograms at a small number of sites. The likelihood of infection in a given individual was significantly related to its caste ($F_{3,\ 1017} = 5.169$, $p = 0.002$). Whilst there was no difference in infection rate between queens and gynes ($p = 0.745$), queens and gynes were significantly more likely to be infected than workers and males ($p < 0.001$), and workers in turn were significantly more likely to be infected than males ($p < 0.001$; see Fig. 2.1). In addition, whilst there was no significant relationship between colony size and likelihood of infection ($F_{1,\ 1017} = 3.085$, $p = 0.079$) there was a significant interaction between caste and colony size ($F_{3,\ 1017} = 3.506$, $p = 0.015$). Separate analyses for each caste showed that workers from larger colonies were more likely to be infected with *Wolbachia* ($F_{1,\ 627} = 28.595$, $p < 0.001$) whereas the likelihood of infection in queens ($F_{1,\ 45} = 0.389$, $p = 0.536$), gynes ($F_{1,\ 146} = 0.061$, $p = 0.805$) and males ($F_{1,\ 199} = 0.270$, $p = 0.604$) was unrelated to colony size (Fig. 2).

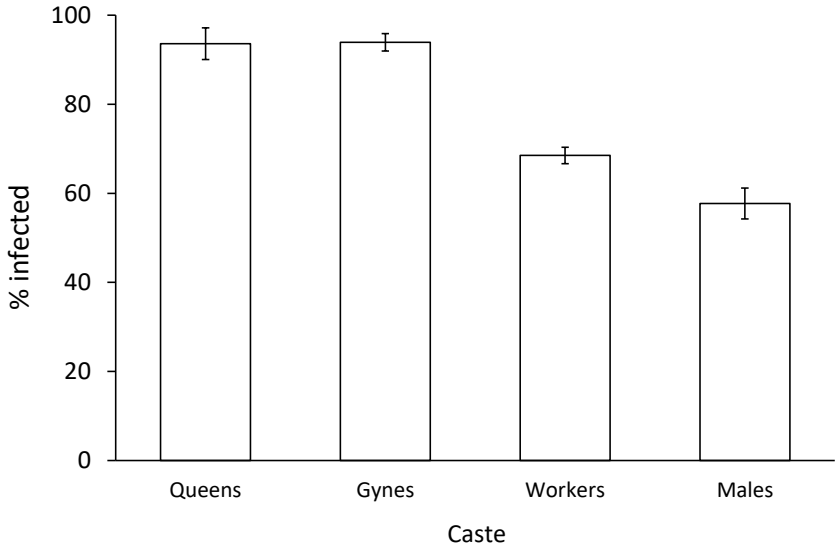

Figure 2.1 *Proportion (± SE) of individuals of different castes infected with* Wolbachia. *Queens and gynes were significantly more likely to be infected with* Wolbachia *than workers ($p = 0.005$ and $p < 0.001$, respectively), and workers in turn were significantly more likely to be infected than males ($p = 0.032$). No significant difference in the infection rates of queens and gynes was observed ($p = 0.783$).*

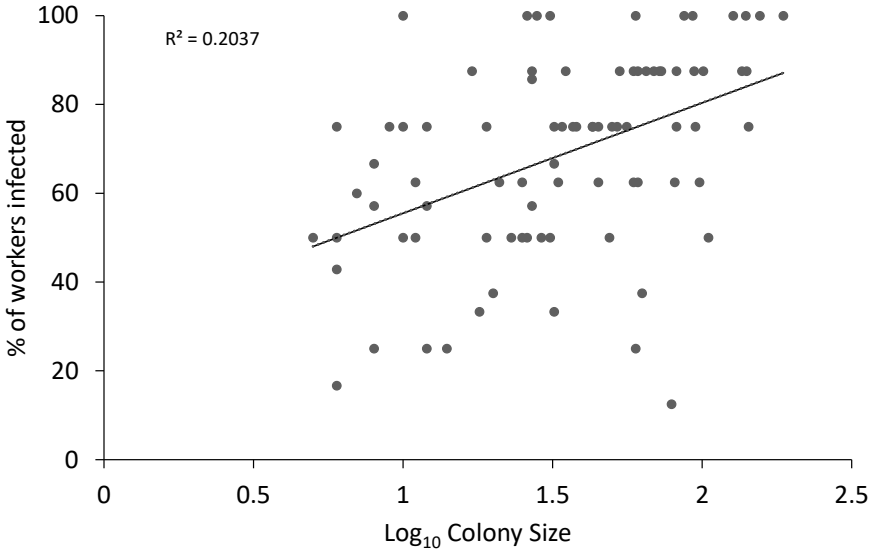

Figure 2.2 *Colony size (specifically the number of non-callow workers per colony) was significantly correlated with the percentage of sampled workers within a colony infected with* Wolbachia *($F_{1, 627} = 28.595$, $p < 0.001$).*

2.4.2 Productivity and sex-ratio analyses

Larger colonies and colonies with a higher proportion of infected workers produced more sexual biomass ($\chi^2 = 24.891$, df = 84, $p < 0.001$ and $\chi^2 = 5.322$, df = 84, $p = 0.021$, respectively; see Fig. 2.3), whilst queen presence was not related to sexual productivity ($\chi^2 = 0.016$, df = 84, $p = 0.900$). Larger colonies and queenright colonies produced more worker biomass ($\chi^2 = 5.667$, df = 84, $p = 0.017$ and $\chi^2 = 4.788$, df = 84, $p = 0.029$, respectively), whilst the prevalence of infection in the worker caste was not significantly related to the production of worker biomass ($\chi^2 = 0.889$, df = 84, $p = 0.346$). Larger colonies produced a more female-biased sex-ratio ($\chi^2 = 13.640$, df = 46, $p < 0.001$), whilst queen presence and the prevalence of infection in the worker caste failed to explain variation in colony sex-ratios ($\chi^2 = 0.111$, df = 46, $p = 0.739$ and $\chi^2 = 0.058$, df = 46, $p = 0.810$, respectively).

2.4.3 Colony genetic structure and infection

Worker genotypes from 18 out of 24 colonies were consistent with the presence of a single matriline, whilst in the remaining 6 colonies, worker genotypes were best explained by the presence of two (five colonies) or three (one colony) matrilines. In the 6 colonies with multiple matrilines, there was no apparent association between matriline and infection status in workers; of the 31 matrilines across 24 colonies, only one was uninfected, and this matriline was only represented by a single worker.

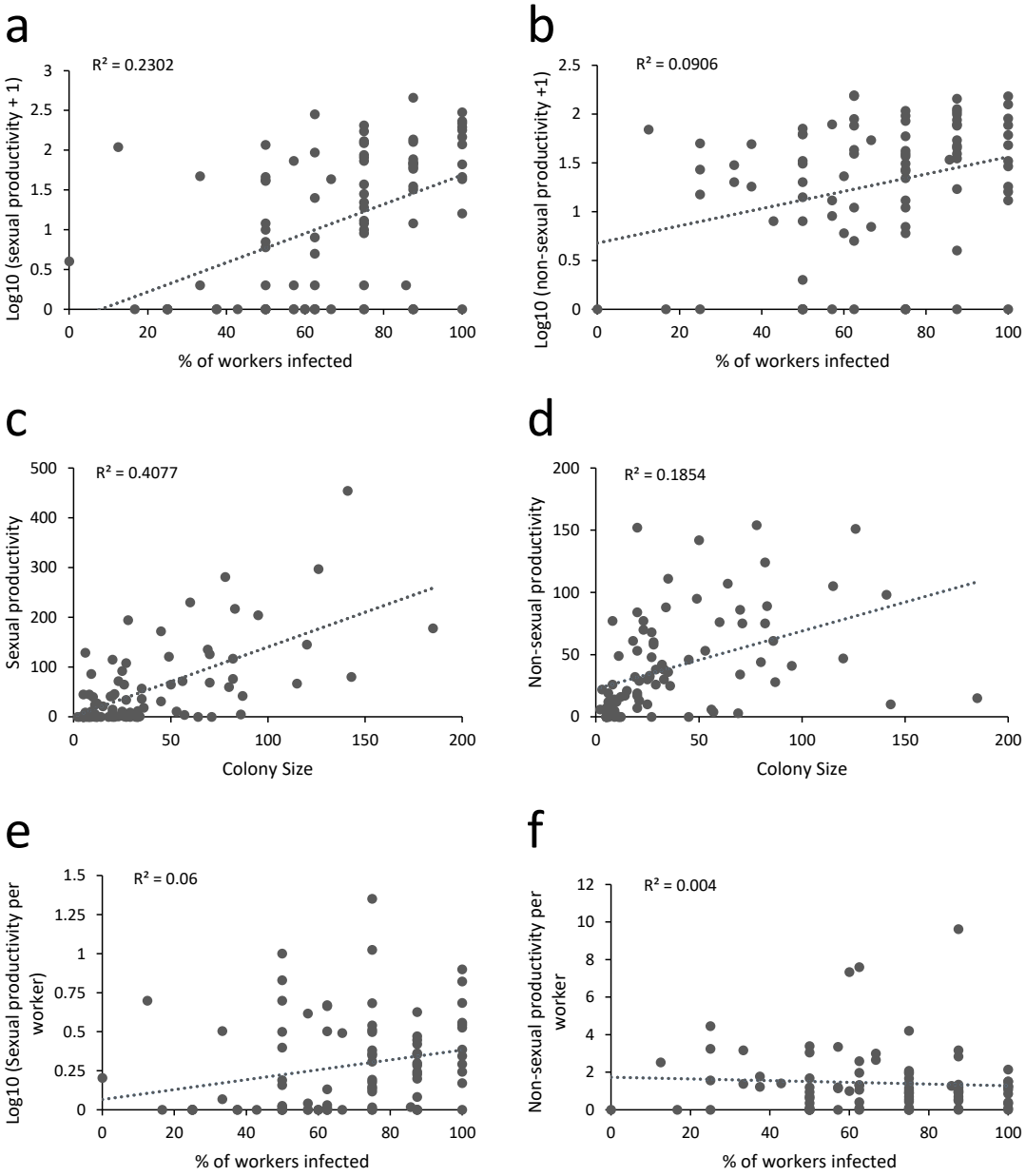

***Figure* 2.3** *The prevalence of* Wolbachia *infection in the worker caste positively correlates with colony productivity, both in terms of the production of new sexuals (Fig 2.3a) and new workers (Fig 2.3b). However, as shown in Fig 2.2, prevalence of infection in the worker caste also positively correlates with colony size, and colony size in turn is positively correlated with both the production of new sexuals (Fig. 2.3c) and new workers (Fig. 2.3d). As such, the relationship between infection in the worker caste and colony productivity may be confounded by colony size. Accordingly, the relationships between infection in the worker caste and per worker production of new sexuals (Fig 2.3e) and new workers (Fig 2.3f) are far weaker. Nonetheless, a Poisson regression incorporating colony size, queen number and prevalence of infection in the worker caste suggests that the latter is still significantly positively correlated with the production of new sexuals ($\chi^2 = 5.322$, $df = 84$, $p = 0.021$) but not new workers ($\chi^2 = 0.889$, $df = 84$, $p = 0.346$).*

2.5 Discussion

In this study, I show that *Wolbachia* is widespread in a dense natural population of *T. crassispinus*. The prevalence of *Wolbachia* within colonies was extremely variable, with some colonies containing mostly infected individuals and others very few. My data clearly show that this variation in the infection status of individuals is explained in part by both the sex and caste of the individual, and in the case of workers, the size of the colony an individual inhabited. In contrast, my findings imply that colony fusion and usurpation are unlikely to be important factors contributing to variation in infection prevalence between colonies. I also show that, whilst the prevalence of infection is uncorrelated to colony-sex ratios, more heavily infected colonies rear a greater number of sexual offspring, although this relationship is fairly weak.

In accordance with studies conducted on populations of *Acromyrmex* and *Formica* ants and *Cubitermes* termites (Van Borm et al., 2001; Wenseleers et al., 2002; Roy et al., 2015), I found that workers were considerably less likely to be infected than queens and gynes. It has been suggested that this might be due to a physiological cost of infection in the worker caste. Since workers cannot produce female offspring, they are a reproductive dead-end from the perspective of a maternally transmitted symbiont, and so both host and symbiont would benefit from clearance of the symbiont if it exerted a physiological cost upon host workers

(Wenseleers et al., 2002; Russell, 2012). However, my data suggest that there is no apparent cost of infection in *T. crassispinus.* Instead, lower infection rates in the worker caste may simply be a result of age-dependent or task-dependent regression of worker ovaries, given that *Wolbachia* is sometimes localized to reproductive tissue and a reduction in ovary tissue with age is a common phenomenon in worker ants (Dobson et al., 1999; Van Borm et al., 2001; Wenseleers et al., 2002; Dolezal et al., 2013; Pamminger & Hughes, 2016). I also considered the possibility that a mixture of infected and uninfected matrilines within a colony might explain the coexistence of infected and uninfected workers within a colony. However, my analysis of colony genetic structure suggested that only a quarter of colonies contain multiple matrilines, which is broadly in line with estimates from a previous study (Strätz & Heinze, 2004). Furthermore, in colonies with evidence of multiple matrilines, all well-sampled matrilines were represented by at least some infected workers. As such, the presence of multiple matrilines within colonies fails to explain large differences in the proportion of workers infected with *Wolbachia* between colonies.

The relationship between sex and *Wolbachia* infection in social insects is less clear. Whilst gynes appear more likely to be infected than males in *Acromyrmex echinatior* and *A. octospinosus*, this is not the case in *A. insinuator* or *Formica truncorum* (Van Borm et al., 2001; Wenseleers et al., 2002). Here, I find that the

situation in *T. crassispinus* appears to mirror that of *A. echinatior* and *A. octospinosus,* in that gynes have a much higher infection rate than males. One possible explanation for the considerably lower infection rate of males is that infected males experience greater mortality than uninfected males, as in the case of a male-killing *Wolbachia* strain (Van Borm et al., 2001). However, the relatively high infection rate of males in this study (57.7%) could only be explained by a remarkably inefficient male killer. Alternatively, this pattern could be caused by sex-specific selection on the transmission of *Wolbachia* to progeny; if a *Wolbachia* strain causing cytoplasmic incompatibility is common in a population, hosts can be selected to ensure the transmission of *Wolbachia* to their daughters. This allows daughters to mate successfully with infected males, however no such benefit would be provided to male offspring, and so we might expect simultaneous selection *for* transmission to female offspring and *against* transmission to male offspring (Werren & O'Niell, 1997).

The strong positive relationship between colony size and infection prevalence in the worker caste is an exciting finding, though difficult to explain. In particular it is not clear whether larger colonies are more prone to higher infection prevalences, or whether more heavily infected colonies tend to grow larger. The fact that infection prevalence is not positively correlated with the production of new workers suggests that the latter is unlikely, but cannot be ruled out. For

instance, it may be that *Wolbachia* provides protection from parasites or parasitoids, or enhances worker longevity, as has been shown in solitary insects (Hedges et al., 2008; Maistrenko et al., 2016), thus reducing worker mortality rate and allowing colonies to reach larger sizes. Alternatively, larger colonies may be more susceptible to higher infection prevalences. In *Acromyrmex echinatior* and *A. octospinosus*, *Wolbachia* is present in the gut and faeces, which opens up the possibility for intra-colonial horizontal transmission of *Wolbachia* (Andersen et al., 2012; Frost et al., 2014). Larger colonies would likely experience more total worker-worker interactions and instances of behaviour such as proctodeal trophallaxis and thus horizontal transmission of infections might be greater in larger colonies.

Due to the fact that almost every colony was infected with *Wolbachia,* I was unable to assess the effect of *Wolbachia* on sex ratios by comparing infected and uninfected colonies. As in the ant *Formica truncorum* (Wenseleers et al., 2002), I found no relationship between colony sex ratios and the proportion of infected workers within a colony, suggesting that *Wolbachia* does not exert an indirect effect on sex-ratios by influencing worker behaviour. In contrast, I did find a positive relationship between the prevalence of infection within the worker caste and sexual productivity of a colony. Interestingly, this is the opposite relationship to that found in *F. truncorum,* in which more heavily infected colonies produce fewer

sexuals (Wenseleers et al., 2002), but is not unprecedented in the context of studies on solitary insects, in which *Wolbachia* infection can enhance host fecundity or provide other mutualistic benefits (Fry et al., 2004; Weeks et al., 2007; Brownlie et al., 2009; Hosokawa et al., 2010). However, it should be noted that the relationship between infection prevalence within the worker caste and sexual productivity was rather weak (see Fig. 2.3e)

In conclusion, I show that *T. crassispinus* exhibits sex, caste and colony size specific patterns of *Wolbachia* infection. The positive relationship between the prevalence of infection in the worker caste and both colony size and sexual productivity suggests that *Wolbachia* exerts no discernible costs in this species, and may even provide minor fitness benefits to *T. crassispinus*. Sex and caste specific patterns of infection have now been described from several social insect species, and may thus be a general feature of *Wolbachia* infection across many social insects. Additionally, the presence of multiple strains, as observed in this and other studies, may also contribute to complexity in the patterns of *Wolbachia* infection and its consequent phenotypic effects (Wenseleers et al., 2002; Reuter & Keller, 2003). Future work should aim to clarify the causes of variable patterns of *Wolbachia* infection, and whether these patterns ultimately depend on the particular phenotypic effects induced by *Wolbachia.*

3 The phenotypic effects of *Wolbachia* in the ant *Myrmica scabrinodis*

3.1 Abstract

Wolbachia is an extremely widespread heritable symbiont, and is renowned for its ability to manipulate the reproductive biology of its arthropod hosts. However, whilst a great deal is known about the reproductive effects of *Wolbachia* in a range of solitary insects, very little is known about how it influences the biology of social insects, despite the fact that social insects are of enormous economic and ecological importance, and are commonly hosts of *Wolbachia* infections. I investigated the biology of *Wolbachia* in the common Palaearctic ant *Myrmica scabrinodis*, in an effort to uncover the phenotypic effects of infection. Diagnostic PCR assays revealed that the majority of colonies of a dense *M. scabrinodis* population were infected with *Wolbachia*. Measurement of the production of sexual offspring in 66 colonies showed no differences in sex-ratio between infected and uninfected colonies. An antibiotic treatment experiment designed to test for the presence of incompatibility-inducing *Wolbachia* of the female-mortality type showed that

antibiotic treatments generally reduced production of new larvae by queenright sub-colonies. However, the negative effect of antibiotic treatment on production of larvae was of a similar magnitude in infected and uninfected colonies. This result is inconsistent with the presence of female-mortality type incompatibility-inducing *Wolbachia,* as antibiotic treatments are expected to lead to the expression of incompatible matings in the offspring of infected queens but not uninfected queens, reducing the supply of viable eggs and hampering the ability of sub-colonies to rear larvae. In conclusion, it seems that *Wolbachia* is responsible for neither sex-ratio distortion nor strong female-mortality type mating incompatibilities in *M. scabrinodis.*

3.2 Introduction

Amongst heritable symbionts, *Wolbachia* has become infamous thanks to its ubiquity and the enormously varied effects it has upon its hosts (Werren et al., 2008). Whilst strains infecting filarial nematodes generally behave as obligate mutualists, its phenotypic effects in arthropods are far more wide-ranging (Taylor et al., 2013; Zug & Hammerstein, 2015). Initially, *Wolbachia* developed a reputation as the archetypal reproductive parasite, capable of manipulating the reproductive

biology of its hosts in numerous ways. Some strains feminize their hosts, causing genetically male offspring to develop into phenotypic females, whilst other strains induce parthenogenesis in their hosts, resulting in the disappearance of males from a species altogether (Stouthamer et al., 1999; Werren et al., 2008). This benefits the symbiont as, under most circumstances, *Wolbachia* is exclusively maternally transmitted, and these distortions of host sex-ratios redirect host resources away from investment in the non-transmitting sex in favour of the sex that can transmit the infection to the next generation (Engelstädter & Hurst, 2009a). Other strains of *Wolbachia* kill male offspring at an early developmental stage, which again distorts host-sex ratios in favour of female offspring (Hurst et al., 1999; Hurst & Jiggins, 2000) but does not directly result in the production of more females. Instead, this phenotype has probably evolved because of benefits accrued by surviving females in the form of reduced intra-brood competition, consumption of dead males, or avoidance of inbreeding (Hurst & Majerus, 1993; Engelstädter & Hurst, 2009a).

However, the most commonly observed reproductive manipulation caused by *Wolbachia* is the induction of mating incompatibilities (Werren et al., 2008). When infected males mate with uninfected females, *Wolbachia* 'modifies' male sperm, preventing paternal chromosomes from properly segregating during anaphase early in the development of embryos (Tram et al., 2006). However, infected females are capable of 'rescuing' this modification, and so embryonic

development occurs normally (Bourtzis et al., 1998). In diploids, this failure of paternal chromosomal segregation results in the production of haploid eggs, which in turn leads to significant embryonic mortality (Hoffmann & Turelli, 1997). As such, infected females more efficiently produce female offspring, and so the maternally inherited *Wolbachia* increases in frequency in the host population to fixation or a stable equilibrium threshold (Hurst & Frost, 2015). In haplodiploids, where males typically develop from unfertilized haploid eggs, two different incompatibility types occur. In the first, termed 'male development' (MD), paternal chromosomes are lost following fertilization, resulting in haploid eggs (Breeuwer & Werren, 1990; Vavre et al., 2000). However, these haploid eggs develop into males rather than dying early in their development as occurs in diploid organisms. In addition, unfertilized haploid eggs laid by the host also develop into males as normal, and so the MD type does not affect overall fecundity. Instead, it reduces or entirely prevents the production of female offspring by uninfected females, and consequently increases the production of male offspring (Vavre et al., 2000). In the second and apparently more common type, termed 'female mortality' (FM), fertilized eggs from incompatible crosses display embryonic mortality as in diploids, whilst haploid eggs develop normally into males. Consequently, the FM type reduces the overall fecundity of its host by preventing the development of fertilised eggs into females (Vavre et al., 2000, 2003; Tram et al., 2006).

Despite its reputation as a reproductive parasite, recent research has left us with a more nuanced perspective on the relationship between *Wolbachia* and its arthropod hosts. Whilst uniparental transmission of *Wolbachia* is the fundamental cause of the reproductive conflicts between it and its hosts, vertical transmission also aligns the interests of hosts and symbionts to a large extent (Zug & Hammerstein, 2015). Consequently, *Wolbachia* should also be subject to selection to benefit its hosts, and numerous examples of beneficial infections have now emerged (Duron & Hurst, 2013). Various case studies have shown that *Wolbachia* can enhance host disease resistance (Hedges et al., 2008; Teixeira et al., 2008; Martinez et al., 2014), provide nutritional benefits (Brownlie et al., 2009; Hosokawa et al., 2010), and increase host fecundity and longevity (Dobson et al., 2002b, 2004; Dong et al., 2007; Weeks et al., 2007). In some cases, a single strain of *Wolbachia* combines facultative mutualism with reproductive parasitism, both helping and harming its host (Zug & Hammerstein, 2015).

Whilst a wealth of data exists concerning the phenotypic effects of *Wolbachia* in most arthropods, very little is known about how it influences the biology of social insects, in spite of the fact that ants, termites and social bees are commonly infected (Bandi et al., 1997; Wenseleers et al., 1998; Evison et al., 2012; Russell, 2012; Russell et al., 2012; Kautz et al., 2013; Gerth et al., 2015). Of the four reproductive manipulations performed by *Wolbachia*, parthenogenesis induction and

feminization are thought to be unlikely to occur in the social hymenoptera. In many (though certainly not all) cases, *Wolbachia* induces parthenogenesis via gamete duplication (van Wilgenburg et al., 2006). The majority of social hymenopterans are thought to utilise single-locus complimentary sex-determination, and so gamete duplication would lead to homozygosity at the sex-determining locus, and thus the production sterile diploid males rather than females (Hurst & Peck, 1996; van Wilgenburg et al., 2006). Similarly, feminized males would still be haploid and so also be sterile (Wenseleers et al., 1998; Van Borm et al., 2001). Furthermore, all empirical investigations of the causes of parthenogenesis in social hymenopterans have found no evidence of *Wolbachia* infection in parthenogenetic species (Grasso et al., 2000; Wenseleers & Billen, 2000; Himler et al., 2009; Martinez-Rodriguez et al., 2013; Rey et al., 2013).

A handful of studies have searched for other phenotypic effects of *Wolbachia* in social insects. (Keller et al., 2001; Wenseleers et al., 2002) tested for a relationship between sex-ratio and *Wolbachia* infection in *Formica* ants. However, whilst both studies found no evidence for *Wolbachia* induced sex-ratio distortion, the extremely high prevalence of *Wolbachia* in the study populations limited the statistical power of comparisons between infected and uninfected colonies. Bouwma & Shoemaker, (2011) examined measures of queen fitness and brood production in *Solenopsis invicta* in relation to *Wolbachia* infection, but found no evidence of any

Wolbachia-induced reproductive manipulation or other fitness effects. However, more recently, Pontieri et al., (2016) identified a correlation between *Wolbachia* infection and female-biased sex-ratios in the ant *Monomorium pharaonis* during an artificial selection experiment designed to investigate the heritability of caste-ratios. Futhermore, they ruled out the possibility that *Wolbachia* causes mating incompatibilities in this species based on the fertility of crosses between infected males and uninfected females. Thus far, this is the only clear demonstration of any phenotypic effect of *Wolbachia* in a social insect.

In this study I investigated the prevalence and phenotypic effects of *Wolbachia* in the Palaearctic ant *Myrmica scabrinodis*. Preliminary screening for *Wolbachia* demonstrated that this species is infected with *Wolbachia,* but the infection has not spread to fixation and uninfected colonies can also be found. Furthermore, this species is common and easily sampled, and colonies are relatively small and easily to collect in their entirety, allowing more accurate determination of any colony-level effects of infection. Finally, colonies are facultatively polygynous, allowing the creation of multiple queenright sub-colonies from stock colonies. Here, I combine molecular surveys for *Wolbachia* infection, colony censuses, and laboratory growth and antibiotic treatment experiments in an effort to elucidate the phenotypic effects of *Wolbachia* in *M. scabrinodis.*

3.3 Materials & methods

3.3.1 Population screening

All samples were collected from a dense population of *M. scabrinodis* on the campus of the University of Sussex, UK during the summers of 2015 and 2016. Five workers each from 215 colonies were collected and stored at -20°C. DNA was extracted from whole ants using a mixture 100 µl of 5% Chelex® solution and 5 µl of 10 mg/ml Proteinase K. The product was then centrifuged at 4680 rpm for 20min and the supernatant was pipetted off. PCR reactions were performed using 10µl reaction mixtures, consisting of 4.8 µl molecular grade H_2O, 2 µl Promega GoTaq Flexi green buffer, 1.25 µl $MgCl_2$, 2.5 µl dNTP's (2.5mM each), 0.2 µl each of the forward and reverse primer (10µM), 0.05 µl Promega GoTaq DNA Polymerase and 1 µl DNA extract. The primers *wsp_F1* and *wsp_R1* (Baldo et al., 2006) were used to detect the presence of *Wolbachia* and each individual was screened twice to control for errors during the PCR stage. In addition, the primers *F1-1424F* and *F1-1829R* (Brady et al., 2006) were used to amplify a fragment of the gene *EF1αF1* for each individual as a host control. DNA extracts from infected *Acromyrmex octospinosus* individuals were used as positive controls, and molecular grade H_2O was used for negative controls. PCR reactions consisted of 95°C for 2 mins followed by 35 cycles of 95°C for 30s, 55°C for 30s and 72°C for 1 min, and finally 72°C for 7 mins. PCR

products were run on 1% agarose gels, visualised under UV light, and bands were scored as present or absent by eye.

To determine how many strains of *Wolbachia* were present in this population, a 426bp fragment of the *Wolbachia* gene *coxA* was sequenced from a single individual from each of 20 colonies. PCR reactions were performed using 30μl reaction mixtures, consisting of 14.4 μl molecular grade H_2O, 6 μl Promega GoTaq Flexi green buffer, 3.75 μl $MgCl_2$, 7.5 μl dNTP's (2.5mM each), 0.6 μl each of the forward and reverse primer (10μM), 0.15 μl Promega GoTaq DNA Polymerase and 3 μl DNA extract. PCR products were run on 1% agarose gels, and visualised under UV light. The remaining PCR product was purified using a Qiaquick PCR purification kit, as per the manufacturer's instructions. Purified DNA was then sent to Eurofins Genomics for sequencing. Sequences were aligned using MUSCLE.

It has been suggested that *M. scabrinodis* is in fact a complex of cryptic species (Radchenko & Elmes, 2010; Seifert et al., 2014). To examine whether intra-population variation in infection status was due to the inadvertent sampling of multiple cryptic species, I sequenced fragments of six nuclear genes (*18s*, *28s*, *Abdominal-A*, *EF1αF1*, *EF1αF2* and *Wingless*) from one individual each from five *Wolbachia* infected colonies and five uninfected colonies. PCR reactions and

purification were performed as described above, and purified DNA was sent to Eurofins Genomics for sequencing. Sequences were aligned using MUSCLE.

3.3.2 Colony collection and censusing

M. scabrinodis colonies were collected with the aim of investigating colony-level effects of *Wolbachia* infection. In particular, the presence of a sex-ratio distorting strain should be detectable by analysing sex-ratios produced by infected and uninfected colonies. On the other hand *Wolbachia*-induced mating incompatibilities or fitness enhancing *Wolbachia* could result in differences in colony size or the amount of brood reared by infected and uninfected colonies. Across both years, a total of 66 colonies of *M. scabrinodis* were collected. 36 of these were infected with *Wolbachia* and 30 were uninfected. Colonies were collected by excavating a large amount of soil around an identified nest mound. Subsequently, excavation around this area continued until no more nest chambers or ants could be found. Colonies were then taken to the laboratory where all queens, workers, larvae, pupae, gynes and males were counted. The relationship between the number of workers per colony and infection with *Wolbachia* was analysed using a Mann-Whitney U Test. Associations between measures of colony productivity (i.e. numbers of larvae and pupae) and infection status were analysed using mixed-effects negative binomial regressions with worker number included as a random factor in the model. The

relationship between sex-ratio and infection status was analysed using a mixed-effects logistic regression with colony included as a random factor. All statistics were performed in SPSS 22 (IBM-SPSS Statistics, Armonk, NY, USA).

3.3.3 Antibiotic treatment experiment

The purpose of this experiment was to test for the presence of *Wolbachia*-induced mating incompatibilities, specifically of the female mortality type. Most ants, including *M. scabrinodis*, cannot be mated easily in the laboratory, which precludes testing for mating incompatibilities by performing experimental crosses between infected and uninfected males and females. Nonetheless, treating infected females with antibiotics can result in the production of uninfected eggs without affecting the compatibility type of sperm, which is determined in immature sperm and retained in the absence of *Wolbachia* (Breeuwer & Werren, 1993; Hoffmann & Turelli, 1997). As such when infected females mated with infected males are treated with antibiotics, any resulting fertilized eggs should be subject to the effects of an incompatible cross. If the *Wolbachia* strain present causes the MD type then females treated with antibiotics will produce mostly male broods, whereas a strain causing the more common FM type incompatibility is expected to lead to reduced production of brood generally, as fertilised eggs will suffer from partial or

complete mortality. In contrast, antibiotic treatment should have comparatively little effect on uninfected females.

The following experiment was designed to examine whether patterns of brood production following antibiotic treatment were consistent with the presence of *Wolbachia*-induced FM type mating incompatibilities. 10 infected and 10 uninfected polygynous colonies were selected and three sub-colonies were created from each colony, containing workers, brood and one queen, resulting in a total of 60 sub-colonies. Sub-colonies were grouped in blocks of six, such that each block contained three infected sub-colonies and three uninfected sub-colonies, each created from one original infected or uninfected colony, respectively. The composition of sub-colonies (i.e. numbers of workers and brood) differed between blocks of six, but was identical within each block of six.

Each sub-colony was then assigned to one of three treatments i) a control diet, consisting of a slightly modified version of the bhatkar diet (Bhatkar & Whitcomb, 1970) and chopped up *Tenebrio molitor* larvae provided *ad libitum* three times per week ii) the same diet with 5mg/ml tetracycline added to the bhatkar mixture or iii) the same diet with 0.5mg/ml rifampicin added to the bhatkar mixture. Tetracycline and rifampicin are broad spectrum antibiotics commonly used to target *Wolbachia* (Li et al., 2014). Sub-colonies were fed this diet and allowed to grow for 8 weeks. After 8 weeks, all larvae produced by each of the 60

sub-colonies were counted. The effects of antibiotic treatment on larval production across groups was analysed using a mixed-effects negative binomial regression. Antibiotic treatment, infection status, and a treatment by infection status interaction were included in the model as fixed effects, and block (i.e. groups of six colonies) was included as a random effect. All statistics were performed in SPSS 22 (IBM-SPSS Statistics, Armonk, NY, USA).

3.4 Results

3.4.1 Population screening

176 out of 215 (82%) *M. scabrinodis* colonies screened for *Wolbachia* were infected, containing at least one infected worker. In all but 4 of the 176 infected colonies, all 5 workers screened were infected. In contrast, 39 colonies contained no infected workers. All *coxA* sequences obtained were identical, and no mixed peaks were present in any chromatograms, suggesting the presence of a single strain of *Wolbachia.*

Fragments of 6 nuclear genes were successfully sequenced for all 10 colonies, yielding a total of 2,801bp. No sequence variation was detected between individuals in five of these genes (*18s, 28s, Abdominal-A, EF1αF1* and *Wingless*), regardless of infection status. Three variable nucleotide positions were identified in *EF1αF2,* two present only in one individual and a third present only in a second

individual, whilst sequences obtained from the remaining 8 individuals were identical. In all three variable nucleotide positions, distinct mixed peaks were present, implying that these individuals were heterozygotes with respect to *EF1αF2.*

3.4.2 Colony-level effects of infection

Infection with *Wolbachia* did not correlate with any of the colony-level characteristics measured (see Fig. 3.1), including colony size ($F_{1, 64} = 0.901$, $p = 0.367$) numbers of larvae or pupae ($F_{1, 64} = 0.754$, $p = 0.389$ and $F_{1, 64} = 2.148$, $p = 0.125$, respectively) or sex-ratio ($F_{1, 2268} = 1.401$, $p = 0.237$).

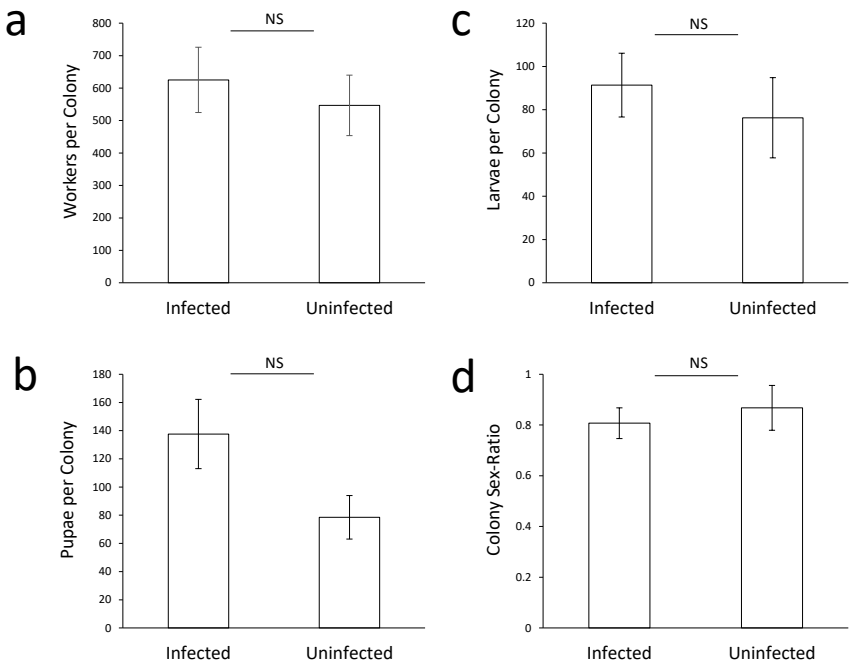

Figure 3.1 *The relationship between* Wolbachia *infection and a) numbers of workers per colony, b) numbers of larvae per colony c) numbers of pupae per colony, d) sex-ratio (all mean ± SE). NS indicates no statistically significant difference.*

3.4.3 Antibiotic treatment experiment

Antibiotic treatment significantly reduced the production of larvae across sub-colonies ($F_{2, 54}$ = 3.952, p = 0.025; see Fig 3.2), whilst infection status did not significantly affect the production of larvae ($F_{1, 54}$ = 0.791, p = 0.378). Although infected sub-colonies subjected to antibiotic treatment produced fewer larvae than uninfected sub-colonies subjected to the same treatments, the interaction term between treatment and infection status was not significant ($F_{2, 54}$ = 0.358, p = 0.701)

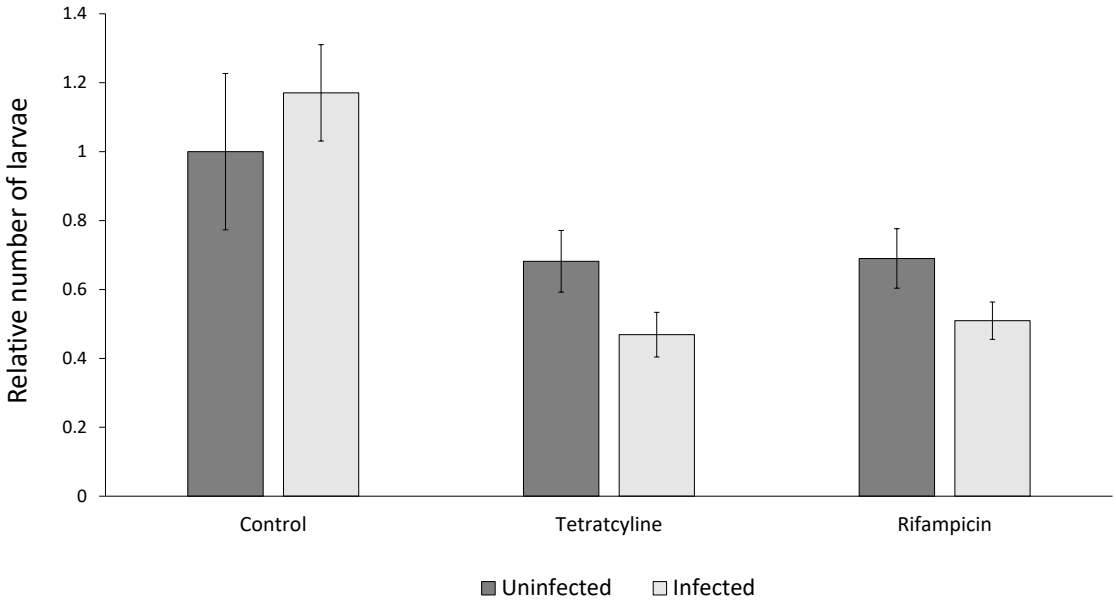

Figure 3.2 *Mean production of larvae ± SE, relative to uninfected control sub-colonies, by size-matched sub-colonies over an 8 week period of provision with antibiotic-supplemented or control diets. Dark grey bars represent uninfected sub-colonies and light grey bars represent infected sub-colonies.*

3.5 Discussion

Through extensive screening for *Wolbachia* infection by diagnostic PCR I found that a single strain of *Wolbachia* is widespread in a dense population of the common Palaearctic ant *Myrmica scabrinodis*. Infection appeared to be a colony-level trait, as in almost all colonies sampled (98.14%) either all sampled workers or no sampled workers were infected. Whilst the majority of colonies were infected, *Wolbachia* has not spread to fixation in this population, and a minority of colonies were free from infection. The distinct lack of variation at sequenced nuclear loci and the absence of any correlation between nuclear genetic variation and *Wolbachia* infection strongly suggests that infected and uninfected colonies are not representatives of sympatric cryptic species, but that polymorphism in infection status is a genuine characteristic of this population of *M. scabrinodis.*

Collection and censusing of infected and uninfected *M. scabrinodis* colonies did not reveal any clear colony-level effects of infection (see Fig. 3.1). In particular, there were no apparent differences in sex-ratio between *Wolbachia* infected and uninfected colonies. As such, it would appear that this strain of *Wolbachia* does not engage in male-killing or other means of sex-ratio distortion. Furthermore, no significant differences in colony size, or in the numbers of larvae and pupae produced, were observed between infected and uninfected colonies.

Because colony-censuses provided no evidence for *Wolbachia*-mediated sex-ratio distortion in *M. scabrinodis*, an antibiotic treatment experiment was conducted to determine whether *Wolbachia* induces FM-type mating incompatibilities in this species. Like most ants, *M. scabrinodis* queens only mate once or a few times before founding a colony and store sperm from these matings in the spermatheca for the remainder of their lives (Boomsma et al., 2005; Śliwińska et al., 2008). The compatibility type of sperm is determined early in their development and retained in the absence of *Wolbachia*, and so antibiotic treatment will not affect the compatibility type of these sperm (Breeuwer & Werren, 1993; Hoffmann & Turelli, 1997). Given that the majority of colonies in the population are infected, it seems reasonable to expect that the majority of queens will have mated with infected males. If *Wolbachia* causes mating incompatibilities in *M. scabrinodis*, antibiotic treatment will likely cause infected queens to produce some proportion of uninfected eggs, and if stored sperm from infected males are of the FM incompatibility type, fertilized eggs will die at an early embryonic stage. Any reduction in the supply of viable eggs, especially given that the experimental sub-colonies contained only a single queen, will likely impact the ability of colonies to rear new larvae.

In this experiment, the provision of tetracycline and rifampicin to queenright sub-colonies significantly reduced the production of larvae, regardless

of the presence or absence of *Wolbachia* (see Fig. 3.2). This implies that antibiotic treatments had substantial off-target effects, probably due to their impact on the wider bacterial community present in *M. scabrinodis* or through antibiotic-induced mitochondrial dysfunction (Southamer & Mak, 2002; Ballard & Melvin, 2007). Infection status alone did not significantly predict differences in larval production across sub-colonies, implying that uninfected colonies do not suffer from a substantial deficit in productivity relative to infected colonies. Furthermore, whilst infected colonies subject to antibiotic treatment did produce slightly fewer larvae than uninfected colonies subject to the same treatment, this effect was comparatively weak, as indicated by the non-significant infection status by treatment interaction term included in the model.

Taking into account the results of both the colony censuses and the antibiotic treatment experiment, it seems likely that *Wolbachia* does not cause mating incompatibilities in *M. scabrinodis*. No significant differences in colony composition or productivity between infected and uninfected colonies were observed in either colony censuses or laboratory experiments, and antibiotics did not have a disproportionate effect in infected colonies, as would be predicted if this strain of *Wolbachia* induces mating incompatibilities. It may be the case that *Wolbachia* is thus a largely inert passenger in this species, or may instead provide minor cryptic fitness benefits that ensure its long term success. Alternatively, given

that, in populations where incompatibility-inducing *Wolbachia* have not spread to fixation, selection on host nuclear genes is expected to reduce the intensity of mating incompatibilities (Turelli, 1994), it may be that *Wolbachia* simply induces very weak mating incompatibilities that I was unable to detect with the relatively small sample size of this study. Finally, the results of the antibiotic treatment experiment do not rule out the possibility that *Wolbachia* induces the apparently rarer 'male-development' form of hymenopteran mating incompatibilities, where incompatible crosses lead to male development instead of embryonic mortality (Vavre et al. 2003). Experiments combining antibiotic treatments with genetic or microscopic determination of egg ploidy would be required to determine whether this is the case.

Elucidating the phenotypic effects of *Wolbachia* in social insects has proved difficult due to a combination of factors, including the difficulty of breeding many social insects in the laboratory and the apparent fixation of *Wolbachia* in species targeted for study (Keller et al., 2001; Wenseleers et al., 2002; Russell, 2012). However *M. scabrinodis* clearly displays colony-level polymorphism in infection status, allowing detailed investigation into the biology of *Wolbachia* in this species. This polymorphism in infection status, in addition to its prevalence and the status of *Myrmica* ants generally as common subjects of scientific study in social insect

research make *M. scabrinodis* a promising candidate for further investigations of the biology of *Wolbachia* in social insects.

4 The evolution of caste-biasing symbionts in social insects

4.1 Abstract

The separation of individuals into reproductive and worker castes is the defining feature of insect societies. However, caste determination is itself a complex phenomenon, dependent on interacting genetic and environmental factors. It has been suggested by some authors that widespread maternally transmitted symbionts such as *Wolbachia* may be selected to interfere with caste determination, whilst others have discounted this possibility on theoretical grounds. I argue that there are in fact three distinct evolutionary scenarios in which maternally transmitted symbionts might be selected to influence the process of caste determination in a social insect host. Each of these scenarios generate testable predictions which I outline here. Given the increasing recognition of the complexity and multi-faceted nature of caste determination in social insects, I argue that maternally transmitted symbionts should also be considered as possible factors influencing the development of social insects.

4.2 Introduction

The defining feature of eusociality is reproductive division of labor (Oster & Wilson, 1979; Crespi & Yanega, 1995). Within a colony, individuals of different castes specialize in either reproduction (i.e. reproductives) or parental care and foraging (i.e. workers), and in many social insects these behavioral castes also exhibit irreversible morphological differentiation (Wilson, 1971). As a consequence, social insect colonies have to produce a mixture of new reproductives and workers from offspring of the same sex, and a major theme of research in social insect biology concerns the developmental processes underlying this. Until relatively recently, the prevailing view was that the development of totipotent larvae into either reproductives or workers was determined largely or entirely by environmental effects, but a number of studies have now shown effects of genetic background on caste determination across a range of social insects (Anderson et al., 2008; Schwander et al., 2010). In some species these genetic effects are plastic, with individuals of certain genotypes displaying a moderate propensity towards developing into either reproductives or workers (Hughes & Boomsma, 2007, 2008; Schwander & Keller, 2008) whilst in other species, caste is determined almost entirely by genetic factors (Julian et al., 2002; Fournier et al., 2005).

Here, I discuss how natural selection may cause maternally transmitted symbionts (MTSs) to influence caste determination in social insects. Heritable

symbionts are extremely widespread in insects and vary enormously in terms of their effects on host organisms (Moran et al., 2008; Weinert et al., 2015). Some symbionts, such as *Buchnera* in aphids, are obligate mutualists of their hosts, providing essential metabolic functions (Shigenobu et al., 2000), whilst others enhance host fitness without being essential for survival (Oliver et al., 2003; Scarborough et al., 2005). Nevertheless, many MTSs have fundamentally selfish interests that differ from their hosts, because they can only be transmitted by females (Engelstädter & Hurst, 2009; Duron & Hurst, 2013; Bennett & Moran, 2015; Hurst & Frost, 2015). This simple fact has led to the evolution of a profusion of manipulative traits in MTSs that ensure the preferential production of females (Werren et al., 2008). However, in social insects, such manipulations may also extend to caste determination; only individuals of the reproductive caste are capable of propagating MTSs, and so these symbionts have a strong evolutionary interest in ensuring that first, they preferentially *infect* queens, and second, that their hosts preferentially *produce* queens.

4.3 Three distinct selective pressures can lead to the evolution of caste-biasing by symbionts

Bourke & Ratnieks, (1999) suggested that MTSs such as *Wolbachia* might be party to social conflicts concerning caste determination in insect societies. They argued that, because symbionts such as *Wolbachia* cannot be transmitted through sterile workers, symbionts should evolve to manipulate the development of females so they are more likely to develop into queens. These queens will then be capable of transmitting the infection to the next generation. On the other hand, Wenseleers, (2001) argued that, since all females in a colony are clonally related from the perspective of an MTS, the MTS will be selected to mutualistically maximize colony productivity rather than bias the caste-fate of developing larvae.

Whilst this logic is sound, it rests on three important assumptions; 1) that the fidelity of transmission of the symbiont from parent to offspring is perfect i.e. infected queens produce only infected offspring; 2) that only a single maternal lineage is present within a colony; 3) that any symbiont present has no interest in distorting the sex-ratio of its host. In reality, each of these assumptions is violated in nature and each creates a different arena for conflict over caste determination between MTSs and their social insect hosts. Here, I discuss each of these scenarios in turn and describe how they might be experimentally investigated.

4.3.1 Caste-biasing due to imperfect vertical transmission of symbionts

In many instances, the fidelity of transmission of MTSs to the offspring of their host is impressively high, and essentially all the offspring of an infected female are themselves infected (e.g. Shoemaker *et al.*, 2003). Nonetheless, there are many cases in which the fidelity of transmission of MTSs is less than perfect; in other words, infected females produce some uninfected offspring (Hoffmann et al., 1990; Hurst et al., 2001; Wenseleers et al., 2002; Graham & Wilson, 2012; Dykstra et al., 2014; Oliver et al., 2014), despite the fact that ensuring a high transmission rate is clearly in the evolutionary interests of MTSs. In solitary insects, MTSs will be under selection to maximize their transmission to the female offspring of their host, because only female offspring are capable of propagating the symbiont. However, in social insects, only queens can reproduce, and workers are either completely sterile or only capable of producing male offspring. As such, in social insects, MTSs will be subject to selection to maximize the infection rate of queens, rather than females generally. Consequently, an MTS infecting a social insect could maximize the infection rate of queens by increasing the likelihood that infected female offspring develop into queens rather than workers. In a simple scenario, a caste-biasing MTS might alter larval begging or feeding behavior, lengthen the developmental period of larvae, or reduce the effect of queen pheromones that prevent the development of female larvae into new queens.

In a more sophisticated scenario, the MTS itself could become an additional caste-determining locus, being necessary but not sufficient for the development of female larvae into reproductives. MTSs might interfere with caste determination at a molecular level, perhaps even becoming an essential part of signaling pathways that lead to the development of larvae into queens. Such fundamental effects of MTSs on host biology is not without precedent; in some lineages of the isopod crustacean *Armadillidium vulgare*, *Wolbachia* has become the sex-determining locus, supplanting nuclear sex-determination (Cordaux et al., 2011). An alternative mechanism might involve a parallel with the Medea phenotype found in *Trilobium* beetles. When a female has a copy of the gene *medea,* any of her offspring that fail to inherit a copy die as zygotes. Thus, the *medea* gene ensures that all the offspring produced by a female contain copies of itself (Beeman et al., 1992). Werren & O'Niell, (1997) suggested that MTSs are likely to cause similar phenotypes, in which uninfected offspring of infected hosts will die or suffer reduced fitness. An equivalent situation could occur with regards to the caste fate of larvae. Instead of an MTS causing mortality in offspring that do not inherit it, it would cause uninfected offspring to develop into workers instead of queens.

A clear prediction of this hypothesis is that in species with a caste-biasing MTS, the prevalence of infection will be lower in the worker caste than in queens. Interestingly, this pattern has been observed for *Wolbachia* infection in a number of

social insect species (Keller et al., 2001; Van Borm et al., 2001; Wenseleers et al., 2002; Frost et al., 2010; Roy et al., 2015). In the past, this has been attributed to adaptive loss from the worker caste, ovarial regression, or age-dependent changes in symbiont titres (Keller et al., 2001; Wenseleers et al., 2002; Russell, 2012), but such relationships could alternatively be explained by the presence of a caste-biasing MTS with imperfect vertical transmission. Unfortunately, an experimental investigation of this variety of caste-biasing symbiont would require the ability to ascertain the infection status of larvae prior to the determination of caste fate, and subsequently follow their development into either workers or queens. This rules out the PCR and microscopy-based methods that are commonly used to determine infection status, because they require destructive sampling. However, it has recently been shown that *Wolbachia* infection in *Aedes aegypti* mosquitoes can be determined non-lethally using near infrared spectroscopy (Sikulu-Lord et al., 2016). If applied to social insects, this would allow the developmental fate of larvae of known infection status to be tracked, providing a means of directly testing for the presence of a caste-biasing MTS.

4.3.2 Caste-biasing due to the coexistence of multiple maternal lineages

In a monogynous social insect colony, all offspring generally have the same mother. As such, all offspring will share the same strain of any MTS present

infecting their mother. In this case, as noted by Wenseleers, (2001), all individuals within such a colony are clonally related from the perspective of an MTS, so there should be no selection on MTSs to manipulate larval caste fate. The same reasoning also holds for secondarily polygynous colonies that readopt related queens. However, there are numerous examples of social insects with polygynous colonies formed of unrelated queens (Stille & Stille, 1992; Evans, 1996; Carew et al., 1997; Heinze & Keller, 2000; Brown et al., 2003; Hacker et al., 2005; Holzer et al., 2008; Helantera et al., 2013). As a consequence, colonies will consist of multiple matrilines. The presence of multiple maternal lineages introduces the potential for intra-colony conflict between infected and uninfected lineages. Any strain that causes its host to preferentially produce queens will be at a considerable advantage compared to queens that are uninfected or infected with a non-biasing strain; they will not have to pay the cost of producing workers but will still be able to produce queens, and so will have a greater reproductive output.

This situation is not without precedent. At its simplest, a subset of queens in certain species appear to selfishly contribute more to the production of sexual offspring, and less to the production of new workers (Ross, 1988; Fournier et al., 2004). In addition, alternative reproductive morphs, including varying degrees of queen dimorphism, are surprisingly common in ants (Heinze & Tsuji, 1995; Heinze & Keller, 2000). Whilst this might occur for a number of reasons, in at least some

cases smaller morphs called microgynes behave as intraspecific social parasites of colonies also inhabited by larger queens called macrogynes (Wolf & Seppä, 2016). For instance, in the ant *Myrmica rubra*, microgynes produce a much higher proportion of queens relative to macrogynes, essentially parasitizing the production of workers by macrogynes (Elmes, 1976; Pearson & Child, 1980; Leppänen, 2012; Schär & Nash, 2014). Furthermore, whilst some gene flow still occurs between the microgyne and macrogyne lineages, there is evidence of genetic divergence between the two (Leppänen et al., 2015, 2016). The social parasite *Mycocepurus castrator* has taken this a step further; it appears to have originated as an intraspecific social parasite of *M. goeldii* but has subsequently become entirely reproductively isolated from its host, and the two are now considered to be distinct species (Rabeling et al., 2014).

It may even be the case that MTSs assist in the sympatric speciation of hosts and their social parasites, as well as being a causal factor in the initial selfish production of queens instead of workers. For instance, *Wolbachia*-induced mating incompatibilities contribute to reproductive isolation between *Drosophila recens* and its sister species *D. subquinaria,* acting in concert with behavioral isolation (Shoemaker et al., 1999; Jaenike et al., 2006). In *M. rubra*, the presence of a parasite queen generally prevents the production of males by host queens, but hosts do still produce males occasionally, and whilst these males appear less inclined to mate

with parasite females, they occasionally do so (Leppänen et al., 2016). Infection with an MTS that also induces mating incompatibilities might explain how social parasites can genetically diverge from their hosts in sympatry, as appears to be the case in *M. rubra,* even when behavioral isolation is incomplete. MTSs could thus not only drive the initial evolution of selfish reproductive behavior, but also assist in the progression from intraspecific parasite to interspecific parasite. I suggest that alternative reproductive morphs, intraspecific social parasites, inquilines and their hosts should be screened for the presence of reproductive parasites to examine this possibility. Subsequent experiments combining antibiotic treatments, quantification of caste ratios and controlled mating between hosts and parasites could reveal whether social insect lineages are infected with caste-biasing MTSs.

4.3.3 Caste-biasing as a means of distorting host sex-ratios

A number of sex-ratio distorting MTSs are found across arthropods (Duron et al., 2008; Engelstädter & Hurst, 2009a; Hurst & Frost, 2015). Some cause the death of host males at an early embryonic stage, some feminize genetic males, and others induce parthenogenesis in their hosts, all of which lead to female biased host sex-ratios (Werren et al., 2008). These phenotypes have evolved because males are a reproductive dead end from the perspective of maternally transmitted symbionts, so it is not surprising that they all involve *reducing* the number of males produced

in order to increase the production of females.

However sex ratios in social insects are not determined simply by the relative proportion of male and female eggs that are laid, because eggs of the same sex can develop into both workers, which are irrelevant with respect to sex ratio, and queens. For instance, in the social Hymenoptera, in which female eggs can develop into workers or queens, the sex ratio of reproductives is determined as much by the proportion of diploid eggs that develop into queens rather than workers (i.e. the caste ratio) as it is by the relative proportion of male and female eggs. As discussed earlier, there is mounting evidence that such caste ratios can have a genetic component (Anderson et al., 2008; Hughes & Boomsma, 2008; Schwander et al., 2010).

In some species, genetic effects on caste determination, and thus caste ratios, can go on to affect colony sex ratios in social insects. In the ant *Cardiocondyla kagutsuchi*, different genetic lines produce markedly different sex ratios; interestingly, this is due to differences between genetic lines in the likelihood of female larvae developing into reproductives rather than differences in the primary sex ratio between genetic lines (Frohschammer & Heinze, 2009). MTSs in social insects could employ a very similar strategy, distorting host sex ratios by altering the caste fate of developing larvae such that female larvae are more likely to develop into queens than workers. Evidence for such an effect has recently been

found in the ant *Monomorium pharaonis.* Experimental crosses between *Wolbachia*-infected and uninfected lineages have shown that infected colonies have more female biased sex ratios than uninfected colonies. Whilst this is largely driven by reduced production of males in infected colonies, there also appeared to be a weaker effect of infection on caste ratio, with infected colonies producing more reproductive females relative to workers (Pontieri et al., 2016). Future tests of caste-biasing by MTSs as a means of distorting host sex ratios will require further comparisons of caste and sex ratios either in natural populations of mixed infection status, or in experimental populations in which infection status has been manipulated to allow caste and sex ratios to be compared whilst controlling for both host genetic background and environmental effects.

4.4 Is caste-biasing likely to evolve in practice?

It could be argued that MTSs have only evolved a limited number of manipulative phenotypes i.e. cytoplasmic incompatibility, male-killing, feminization and parthenogenesis induction, during the course of tens of millions of years of evolutionary history in association with an enormous number of arthropod hosts (Engelstädter & Hurst, 2009a; Gerth et al., 2014); perhaps then, it is unparsimonious to propose another origin of a manipulative phenotype. However,

it is important to note that the different manipulative phenotypes are, in reality, broad categories describing outcomes in the host, and the cellular and molecular mechanisms underlying reproductive manipulations vary enormously across hosts and MTS strains (Hurst & Frost, 2015). For instance, symbiont-induced parthenogenesis proceeds through at least four different cellular mechanisms in arthropods (Ma & Schwander, 2017) suggesting independent origins of the phenotype. Furthermore, even when the cellular mechanisms of a manipulative phenotype are the same, the underlying molecular mechanisms may vary, again due to independent origins of the manipulative phenotype (Ma et al., 2014). For example, symbiont induced cytoplasmic incompatibility is known to occur in at least three distinct bacterial taxa (Engelstädter & Hurst, 2009a; Takano et al., 2017). The cytological mechanisms of these mating incompatibilities have been studied in hosts infected with *Wolbachia* and *Cardinium,* and are remarkably similar regardless of which symbiont the host is infected with (Gebiola et al., 2017) but comparisons of the genomes of incompatibility-inducing *Wolbachia* and *Cardinium* provide no evidence for shared genes underlying the induction of mating incompatibilities; cytoplasmic incompatibility thus appears to have (at the very least) two independent evolutionary origins, rather than a single origin followed by horizontal gene transfer between taxa (Penz et al., 2012). The limited number of categories of manipulative phenotypes are thus likely to represent many

independent origins of manipulative phenotypes, and should not be taken to suggest that MTSs have limited evolutionary potential. In fact, quite the opposite appears to be the case; when the selective conditions are appropriate, MTSs appear readily able to evolve the ability to manipulate host biology through a range of different cellular and molecular mechanisms.

4.5 Conclusions

Caste determination in social insects is increasingly recognized as a complex phenomenon, with multiple interacting causes. I suggest that heritable symbionts should also be considered as additional factors that may influence caste determination, because there are at least three distinct evolutionary scenarios that could select for caste-biasing genes in MTSs. Such symbionts are extremely widespread, easy to detect using a range of standard laboratory techniques, and all three evolutionary scenarios outlined here generate testable predictions concerning caste determination in social insects. Maternally transmitted symbionts are already renowned for their ability to influence fundamental aspects of metabolism, immunity, behavior and reproduction in their hosts and I see no reason why caste determination should be any exception to this.

5 The population biology and biogeography of bacterial symbiont communities in *Myrmica scabrinodis*

5.1 Abstract

Bacterial symbionts are extremely common in insects, and affect their hosts in a multitude of different ways, acting as pathogens, reproductive parasites, and facultative or obligate mutualists. Frequently, a single symbiont is studied in isolation, and the host organism is drawn from a single population or laboratory stock. However, screening for multiple symbionts across multiple populations can reveal interesting details about the biology of symbiosis that would otherwise be missed. Following on from my investigation of *Wolbachia* infection in the ant *Myrmica scabrinodis* in Chapter 3, I investigated the prevalence and genetic diversity of two additional bacterial symbionts, *Spiroplasma* and *Arsenophonus*, in 472 workers sampled from 97 colonies that had previously been screened for *Wolbachia*. I found that a single strain each of *Spiroplasma* and *Arsenophonus* was

present in this population, and that individuals infected with *Wolbachia* were significantly less likely to be infected with *Spiroplasma* and *Arsenophonus.* I then screened for *Wolbachia, Spiroplasma* and *Arsenophonus* in 186 colonies from 23 *M. scabrinodis* populations across Europe and Russia. I identified multiple genetically distinct strains of both *Wolbachia* and *Spiroplasma* that were likely acquired via independent horizontal transmission events. In addition, the prevalence of *Wolbachia*, as well as the strain of *Wolbachia* and *Spiroplasma* present, was not uniform across populations, but instead varied considerably across Europe. My results suggest that interactions between bacterial symbionts may be important in determining the prevalence of individual symbionts, and illustrate how symbiont communities can vary dramatically across the range of a species.

5.2 Introduction

Bacterial symbionts of insects are as diverse as they are ubiquitous, and in many cases fundamentally alter the biology of their hosts (McFall-Ngai et al., 2013; Bennett & Moran, 2015; Hurst & Frost, 2015). Some bacteria play familiar roles as entomopathogens with predictably dire consequences for their hosts (de Maagd et al., 2001; Regassa & Gasparich, 2006; Vodovar et al., 2006) but many bacterial

symbionts of insects either do not harm their hosts, or exert their parasitic effects more subtly. A wealth of studies utilising *16s* amplicon sequencing have revealed that insects commonly harbour surprisingly diverse microbial communities that vary considerably across taxa (Engel & Moran, 2013). Individual insects can harbour more than 100 bacterial taxa in their digestive tracts, and these residents of the gut synthesise important nutrients, assist in digestion and detoxification of food, and provide resistance to colonisation by pathogens (Dillon & Dillon, 2004; Dillon et al., 2005; Douglas, 2009; Koch & Schmid-Hempel, 2011; Yun et al., 2014). In addition to gut microbial symbionts, the majority of insect species also host heritable intracellular bacteria (Zug & Hammerstein, 2012; Weinert et al., 2015). In some cases, the host cannot survive without these symbionts; for instance, species with nutritionally unbalanced diets, such as plant-sap feeding hemipterans and blood-feeding tsetse flies, often harbour obligate endosymbiotic bacteria that synthesise essential components of the insects diet (Douglas, 1998; Shigenobu et al., 2000; Akman et al., 2002). However, in many cases, heritable symbionts are not required for host survival. Some of these facultative symbionts provide non-essential benefits to their hosts such as resistance to parasites and heat stress (Montllor et al., 2002; Oliver et al., 2003; Scarborough et al., 2005; Martinez et al., 2014). Others act as reproductive parasites that alter the reproductive biology of their hosts, either by distorting sex-ratios in favour of the transmitting sex or

causing mating incompatibilities that reduce the relative fitness of uninfected females (Engelstädter & Hurst, 2009a). It is also increasingly recognised that many symbionts may combine both mutualism and reproductive parasitism to spread more efficiently within and amongst host populations (Duron & Hurst, 2013; Zug & Hammerstein, 2015).

It has been demonstrated in numerous taxa that single individuals can be infected with multiple different heritable bacteria (Montenegro et al., 2005; Duron et al., 2008; Ferrari et al., 2012; Kautz et al., 2013). However, detailed information regarding the population level patterns of co-infection exists for only a relatively small number of taxa (Jaenike et al., 2010a; Toju & Fukatsu, 2011; Ferrari et al., 2012; Henry et al., 2013). This is despite the fact that non-random associations between symbionts seem likely, due to either competitive or cooperative interactions between symbionts, or selection upon hosts for a particular complement of symbionts (Kondo et al., 2005; Vautrin & Vavre, 2009; Jaenike et al., 2010a; Ferrari & Vavre, 2011; McLean et al., 2017). Furthermore, most studies regarding the biology of symbiont communities either examine a broad range of species with often just a few individuals sampled per species, or instead focus on the phenotypic effects induced by symbionts in a small number of host populations or laboratory lineages. However, studies bridging the gap between these two extremes often reveal interesting and unexpected details regarding the

prevalence and diversity of host-symbiont associations that might not be observed through examining a single host population, such as the presence of extensive symbiont genetic diversity, the invasion of multiple symbiont strains, and variation in the prevalence of symbionts between populations (Ahrens & Shoemaker, 2005; Watts et al., 2009; Atyame et al., 2011; Doudoumis et al., 2012; Ferrari et al., 2012; Zhang et al., 2013). In Chapter 3, a single strain of *Wolbachia* was found to infect the majority of colonies in a population of the ant *Myrmica scabrinodis*. My aim in this study is to expand the understanding of symbiont communities in *M. scabrinodis* by screening for multiple symbionts in multiple populations across the geographic range of the species, and sequencing taxonomically informative genes from detected symbionts.

5.3 Materials and methods

5.3.1 Molecular screening for symbionts in a UK population

Five workers were sampled from each of 97 colonies of *M. scabrinodis* as part of an earlier study of *Wolbachia* infection in a population in the South-East of England. As such, the infection status of all individuals with respect to *Wolbachia* was already determined. DNA extracts from the second year of this study were used to

screen all individuals (472 in total, after excluding 13 for which DNA extractions were unsuccessful) by diagnostic PCR for *Spiroplasma* and *Arsenophonus*. PCR reactions were performed using 10 μl reaction mixtures, consisting of 4.8 μl molecular grade H_2O, 2 μl Promega GoTaq Flexi green buffer, 1.25 μl $MgCl_2$, 0.5 μl dNTP's (2.5 mM each), 0.2 μl each of the forward and reverse primer (10 μM), 0.05 μl Promega GoTaq DNA Polymerase and 1 μl DNA extract. The primers SpoulF and SpoulR (Montenegro et al., 2005) and ArsF and ArsR3 (Duron et al., 2008) were used to detect *Spiroplasma* and *Arsenophonus*, respectively. Positive and negative controls were included in each plate. PCR reactions consisted of 95°C for 2 min followed by 35 cycles of 95°C for 30 s, 50-54°C for 30 s and 72°C for 1 min, and finally 72°C for 7 min. PCR products were run on 1% agarose gels, visualised under UV light, and bands were scored as present or absent by eye. Binary logistic regressions were used to assess whether the prevalence of *Spiroplasma* and *Arsenophonus* varied significantly between colonies. The relationship between infection with *Arsenophonus*, *Spiroplasma,* and *Wolbachia* was assessed using hierarchical log-linear analysis, and a backward elimination procedure was used to determine the final model. All statistics were performed using SPSS 22 (IBM-SPSS Statistics, Armonk, NY, USA).

20 workers infected with *Spiroplasma* and 19 workers infected with *Arsenophonus* were then randomly selected for sequencing to determine the

phylogenetic affiliation of the symbionts and assess whether individuals were infected by a single strain or multiple symbiont strains. The primers SpoulF/SpoulR and ArsF/ArsR3 described previously were used to amplify a fragment of the *16s* rRNA gene from *Spiroplasma* and *Arsenophonus*, respectively. PCR reactions were performed using 30 μl reaction mixtures, consisting of 14.4 μl molecular grade H_2O, 6 μl Promega GoTaq Flexi green buffer, 3.75 μl $MgCl_2$, 1.5 μl dNTP's (2.5 mM each), 0.6 μl each of the forward and reverse primer (10 μM), 0.15 μl Promega GoTaq DNA Polymerase and 3 μl DNA extract. PCR products were purified using a QIAquick PCR purification kit as per the manufacturer's instructions and sequenced by Eurofins Genomics. Sequences were aligned using MUSCLE.

5.3.2 Molecular screening for symbionts across the Palaearctic

M. scabrinodis workers were sampled from 23 additional populations in the UK, Italy, Germany, Poland, the Czech Republic, Slovakia, Hungary, Romania, Ukraine and Russia (Table 1) in order to assess the prevalence of *Wolbachia*, *Spiroplasma* and *Arsenophonus* across the range of *M. scabrinodis*. 1-18 colonies were sampled from each population (186 colonies in total) and one worker was sampled from each colony. DNA was extracted from whole ants in 100 μl of 5% Chelex® solution with 5 μl of 10 mg/ml Proteinase K. PCR reactions were performed as described above

for *Spiroplasma* and *Arsenophonus*, with the addition of PCR's to detect *Wolbachia* infection. These were performed using 30 μl reaction mixtures as described above, with the primers coxA_F1 and coxA_R1 used to detect *Wolbachia*. PCR products were run on 1% agarose gels, visualised under UV light, and bands were scored as present or absent by eye. For each of the three symbionts, 1-6 infected workers from each infected population were then randomly selected for sequencing. The primers SpoulF/SpoulR and ArsF/ArsR3 described previously were used to amplify a fragment of the *16s* rRNA gene from *Spiroplasma* and *Arsenophonus,* respectively, whilst the primers coxA_F1/coxA_R1 were used to amplify a fragment of the *cytochrome* c *oxidase subunit I* gene, hereafter *coxA,* from *Wolbachia*. PCR products were purified using a QIAquick PCR purification kit as per the manufacturer's instructions and sequenced by Eurofins Genomics. Sequences were aligned using MUSCLE, and maximum likelihood (ML) trees for the three genes were constructed using MEGA7 (Kumar et al., 2016), including sequences obtained in this study and related sequences derived from Genbank. The ML tree for *coxA* sequences was based on the HKY substitution model, whilst the ML trees for *16s* rRNA sequences from *Spiroplasma* and *Arsenophonus* were based on the Kimura 2-parameter model.

Since multiple *coxA* haplotypes were detected, one individual infected with *Wolbachia* corresponding to each haplotype was selected for multi-locus sequence

typing (MLST), involving the sequencing of five conserved genes using *Wolbachia* specific primers (Baldo et al., 2006). PCR reactions were performed using 30 μl reaction mixtures, consisting of 14.4 μl molecular grade H_2O, 6 μl Promega GoTaq Flexi green buffer, 3.75 μl $MgCl_2$, 1.5 μl dNTP's (2.5 mM each), 0.6 μl each of the forward and reverse primer (10 μM), 0.15 μl Promega GoTaq DNA Polymerase and 3 μl DNA extract. Infected *Acromyrmex octospinosus* individuals were used as positive controls, and molecular grade H_2O was used for negative controls. PCR reactions consisted of 95°C for 2 min followed by 35 cycles of 95°C for 30 s, 50-64°C for 30 s and 72°C for 1 min, and finally 72°C for 7 min. PCR products were run on 1% agarose gels, and visualised under UV light. The remaining PCR product was purified using a Qiaquick PCR purification kit, as per the manufacturer's instructions. Purified DNA was then sent to Eurofins Genomics for sequencing.

Sequences of the five MLST genes were aligned in MUSCLE along with sequences from 40 strains of supergroup A *Wolbachia* and four strains of supergroup B *Wolbachia*. ClonalFrame 1.1 was then used to construct a Bayesian phylogeny that accounts for recombination events between strains (Didelot & Falush, 2006). The program was run twice with 350,000 generations for burnin and 350,000 post burnin generations. The convergence of the two runs, which began at different random configurations, was assessed using methods described in Gelman & Rubin, (1992) and implemented in ClonalFrame; Gelman and Rubin statistics for

θ, R, v, δ and the TMRCA were all <1.2, indicating convergence of independent runs. Post-burnin trees from both runs were combined to build a majority-rule consensus tree.

5.4 Results

5.4.1 Molecular screening for symbionts in a UK population

Spiroplasma was extremely common, infecting 86% of individuals, and was found in 100% of colonies. In contrast, *Arsenophonus* was only detected in 19% of individuals, and found in at least one worker in 49% of colonies. Significant inter-colony variation in the number of workers infected per colony was detected for both *Spiroplasma* and *Arsenophonus* (χ^2 = 186.10, df = 96, p < 0.0001 and χ^2 = 216.208, df = 96, p < 0.0001, respectively). The final log-linear model obtained from a backward elimination procedure indicated significant interactions between infection with *Wolbachia* and *Spiroplasma* (χ^2 = 31.779, df = 1, p < 0.001) and *Wolbachia* and *Arsenophonus* (χ^2 = 3.923, df = 1, p = 0.048). Specifically, infection with *Wolbachia* was associated with reduced likelihood of infection with *Spiroplasma* (81.4% vs. 99.1%; see Fig. 5.1a) and *Arsenophonus* (17.2% vs. 25.9%; see Fig. 5.1b). No genetic variation was detected in in sequenced fragments of *16s* rRNA from

Spiroplasma and *Arsenophonus*, and no mixed-peaks were observed in chromatograms, suggesting the presence of a single strain each of *Spiroplasma* and *Arsenophonus* in this population.

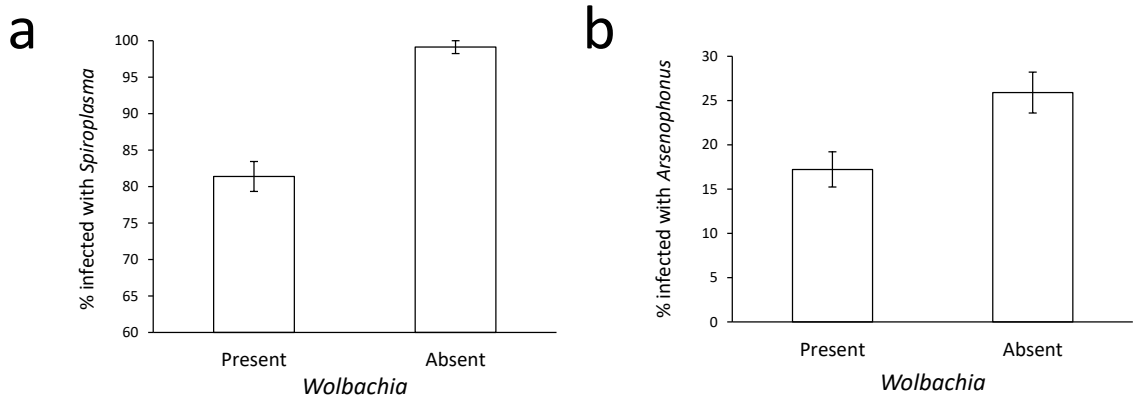

Figure 5.1 *Workers infected with* Wolbachia *were less likely to be infected with a)* Spiroplasma (χ^2 = *31.779, df = 1, p < 0.001; proportion ± SE) and b)* Arsenophonus (χ^2 = *3.923, df = 1, p = 0.048; proportion ± SE).*

5.4.2 Molecular screening for symbionts across the Palaearctic

Screening for *Wolbachia, Spiroplasma* and *Arsenophonus* across the Palaearctic revealed different patterns of infection for each of three symbionts (see Table 1). All 186 colonies from 23 populations were infected with *Spiroplasma*, whilst only two colonies, one in the UK and one in Germany, were infected with *Arsenophonus*. *Wolbachia* showed a more complex pattern of infection across the Palaearctic (see Fig. 5.2). Across all 23 populations, 33.87% of colonies were infected, however significant variation in the prevalence of infection between populations was detected ($p < 0.0001$). *Wolbachia* was not detected at all in 12/23 populations, whilst

an average of 69.85% of colonies were infected in the remaining 11 populations. If populations where fewer than 10 colonies were screened are excluded, an average of 85.34% of colonies from infected populations were infected.

Sequencing of *coxA* and *16s* identified three haplotypes each of *Wolbachia* and *Spiroplasma,* respectively, occupying distinct geographical areas (Fig. 5.2). The most common strain of *Wolbachia,* designated here *w*MscaA_1, was found as a single infection in 7 out 23 populations located in the UK, Germany, Poland and Ukraine. The strain *w*MscaA_2 was found as a single infection in two populations in Hungary. In two populations in Romania, otherwise clear chromatograms with two peaks at each of four nucleotide positions were obtained from all 7 individuals subject to coxA sequencing. At each position with multiple peaks, one of the two nucleotides corresponded to the nucleotide found in *w*MscaA_1 sequences. As such, these sequences are likely the result of infection with *w*MscaA_1 and a third strain of *Wolbachia,* designated *w*MscaA_3. Maximum likelihood analysis suggested that the three *coxA* haplotypes were not monophyletic (Fig. 5.3) and the presence of three non-monophyletic *Wolbachia* strains was confirmed by an analysis of 5 MLST loci sequenced for each putative *Wolbachia* strain (Fig. 5.4). The most common *Spiroplasma* strain, *s*Msca_1 was found in all populations, with the exception of one German and one Polish population infected with *s*Msca_2, and one Romanian population harbouring *s*Msca_3. *s*Msca_1 and *s*Msca_2 formed a

monophyletic group after the inclusion of the most closely related *16s* sequences in GenBank, and differed by only a single base pair, whilst *s*Msca_3 was not closely related to *s*Msca_1 and *s*Msca_2 (Fig. 5.5). Only a single haplotype of *Arsenophonus* was found (Fig. 5.6).

Country	**Population**	**Colonies Screened**	**Wolbachia**	**Spiroplasma**	**Arsenophonus**
UK	Seaford Head	4	75%	100%	25%
UK	Frensham	1	0%	100%	0%
UK	Denton Downs	1	100%	100%	0%
Germany	Gessertshausen	8	75%	100%	12.5%
Italy	Monte Musinè	9	0%	100%	0%
Poland	Kosyń	18	77.78%	100%	0%
Poland	Kraków	17	0%	100%	0%
Poland	Sliwa	5	0%	100%	0%
Czech Republic	Přelouč	8	0%	100%	0%
Slovakia	Stefanova	10	0%	100%	0%
Hungary	Aggtelek	6	0%	100%	0%
Hungary	Csíkgát	6	33.33%	100%	0%
Hungary	Rakaca	6	0%	100%	0%
Hungary	Újléta	6	33.33%	100%	0%
Romania	Kendilóna	14	0%	100%	0%
Romania	Magyarderzse	9	0%	100%	0%
Romania	Sárd	4	25%	100%	0%
Romania	Szenéte	9	88.89%	100%	0%
Romania	Cluj-Napoca	12	0%	100%	0%
Ukraine	Rudne	10	70%	100%	0%
Ukraine	Rudniki	10	90%	100%	0%
Ukraine	Novobarovo	10	100%	100%	0%
Russia	Novosibirsk	3	0%	100%	0%

Table 1. *A summary of molecular screening for* Wolbachia, Spiroplasma, Arsenophonus *across the Palaearctic.*

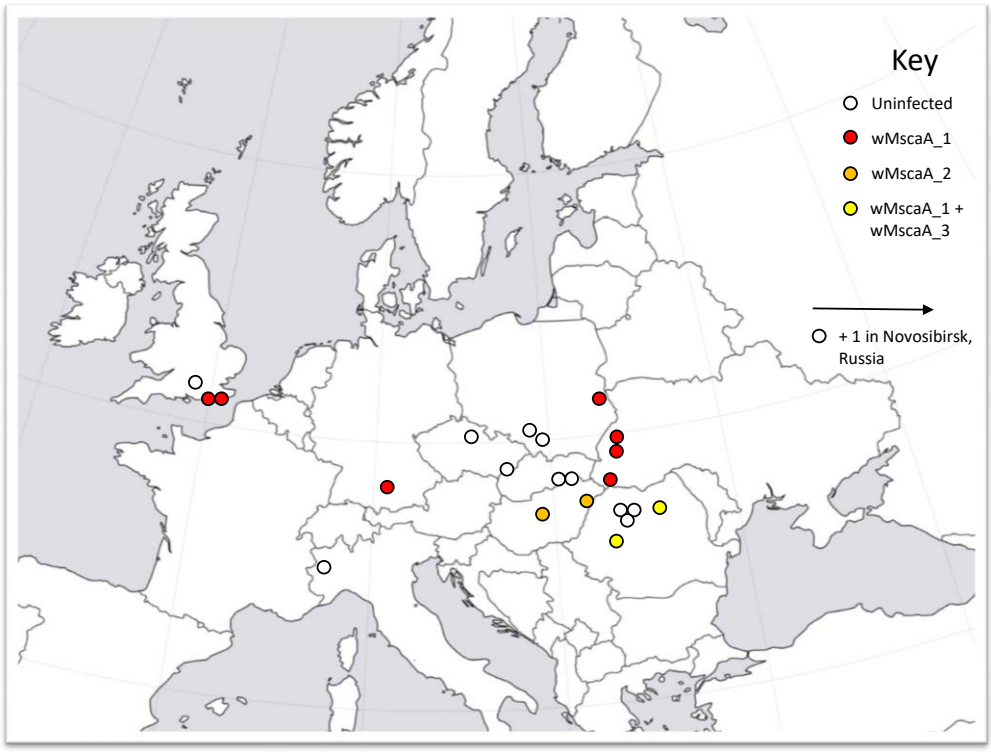

Figure 5.2 *Three distinct strains of* Wolbachia *appear to be found in geographically distinct locations across Europe. However,* Wolbachia *was not detected in 12/23 populations.*

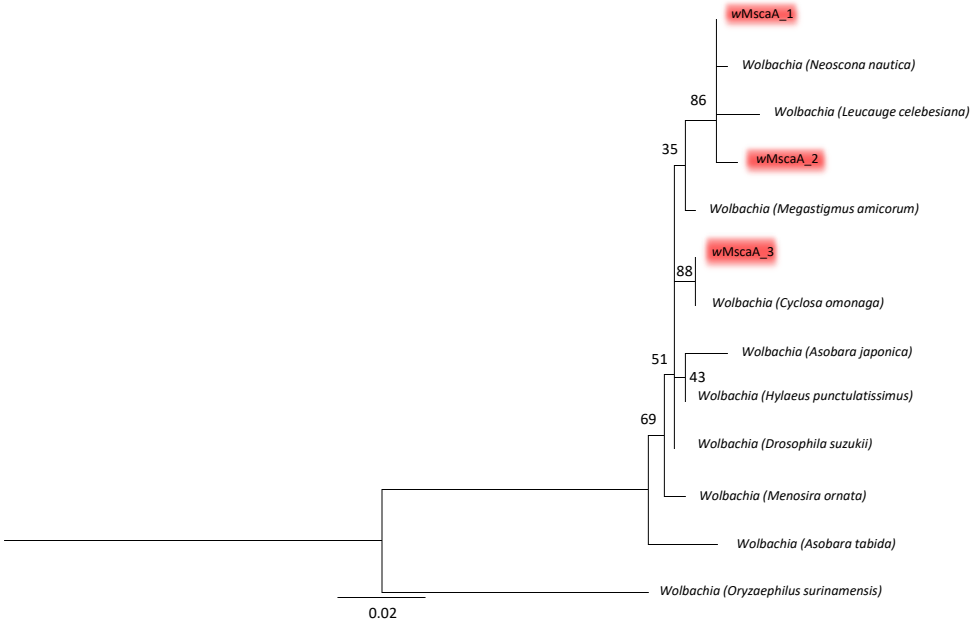

Figure 5.3 *A maximum likelihood phylogeny based on coxA sequences obtained in this study (highlighted in red) and similar sequences obtained from Genbank. The scale bar indicates the number of substitutions per site. Bootstrap values are shown next to nodes in the tree and were calculated using 5000 replicates.*

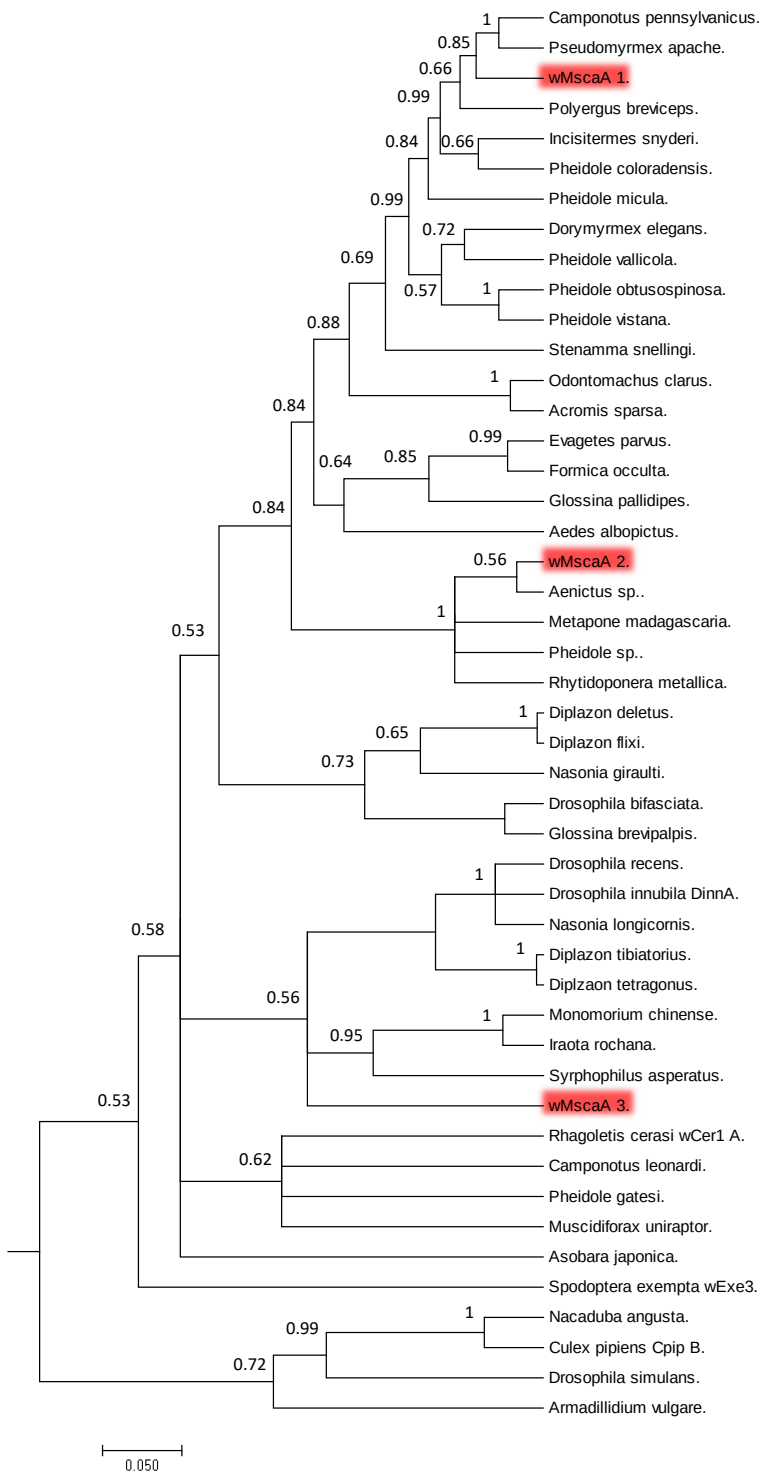

Figure 5.4 *A Bayesian phylogeny of* Wolbachia *strains infecting* Myrmica scabrinodis *(highlighted in red) and other arthropod species produced using ClonalFrame. Numbers adjacent to nodes correspond to Bayesian posterior probabilities. The scale bar represents the number of substitutions per site.*

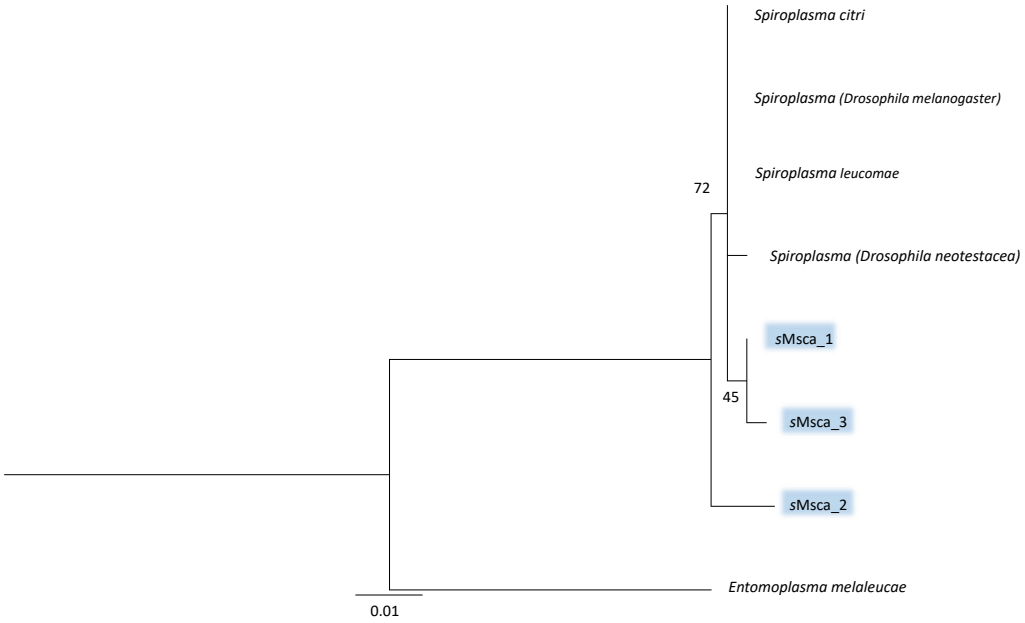

Figure 5.5 *A maximum likelihood phylogeny based on* Spiroplasma 16s *sequences obtained in this study (highlighted in blue) and related sequences obtained from Genbank. The scale bar indicates the number of substitutions per site. Bootstrap values are shown next to nodes in the tree and were calculated using 5000 replicates.*

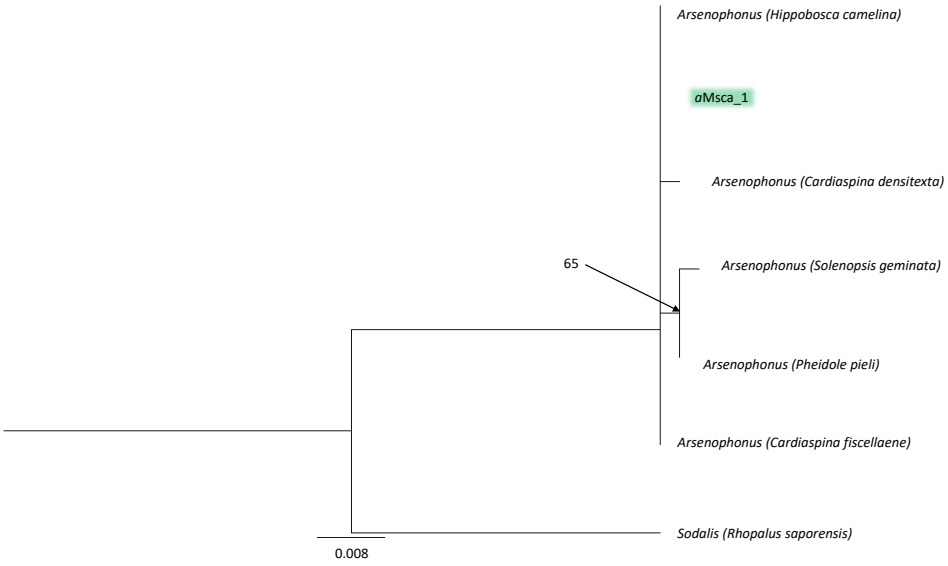

Figure 5.6 *A maximum likelihood phylogeny based on* Arsenophonus 16s *sequences obtained in this study (highlighted in green) and related sequences obtained from Genbank. The scale bar indicates the number of substitutions per site. Bootstrap values are shown next to nodes in the tree and were calculated using 5000 replicates.*

5.5 Discussion

In this study I aimed to extend the understanding of the symbiotic community associated with *M. scabrinodis* by screening for multiple bacterial symbionts from populations across Europe and Russia. In the first part of this study, I screened a large number of individuals from a single UK population, already known to be infected with the symbiont *Wolbachia,* for *Spiroplasma* and *Arsenophonus* infections. *Spiroplasma* was shown to be extremely common, with at least some infected individuals in every colony. Conversely, only a minority of individuals were infected with *Arsenophonus*. Furthermore, multiple infections were not uncommon, and approximately 11% of individuals were infected with all three symbionts. However, symbiont screening in this population clearly revealed non-random patterns of coinfection. Specifically, individuals infected with *Wolbachia* were less likely to be infected with *Spiroplasma* and *Arsenophonus*. Negative correlations between different symbionts might arise for a number of reasons. Competition between symbionts for limited host resources may lead to competitive exclusion (Goto et al., 2006; Vautrin & Vavre, 2009). Alternatively, such patterns may arise due to selection acting upon hosts infected with particular combinations of symbionts, where the benefits of multiple infection are outweighed by the cost (Ferrari & Vavre, 2011). For instance, in pea aphids both *Spiroplasma* and *Regiella* provide protection against the fungal parasite *Pandora*. However, infection with

both symbionts provides no additional protection than do single infections, whilst control individuals infected with both symbionts experience lower survival than those infected with *Spiroplasma* alone (McLean et al., 2017).

Sequencing of infected individuals strongly implied that a single strain each of *Spiroplasma* and *Arsenophonus* is present in the focal UK population, and previous work has demonstrated that only a single strain of *Wolbachia* exists in this population (Chapter 3). However, my results show that a more complex pattern exists across the range of *M. scabrinodis*. The same strain of *Wolbachia* as in the focal UK population, *w*MscaA_1, was found in 9 out of 23 populations, ranging from the UK to Ukraine, but two additional strains of *Wolbachia*, *w*MscaA_2 and *w*MscaA_3 were identified from two Hungarian and two Romanian populations, respectively. *w*MscaA_2 existed as a single infection, and *w*MscaA_1 was not found in any Hungarian populations. *w*MscaA_3 was only found in individuals also infected with *w*MscaA_1. In some cases, symbiont genetic diversity within a species can be due to divergence following a single introduction into a species, as in *w*Pip infections in *Culex pipiens* mosquitoes (Atyame et al., 2011) but this does not appear to be the case in *M. scabrinodis*. Both a maximum likelihood analysis of *coxA* sequences obtained from all 11 infected populations, as well as an MLST phylogeny constructed in ClonalFrame, suggest that the three strains of *Wolbachia* in *M. scabrinodis* do not form a monophyletic clade. As such, it seems likely that

there were three separate introductions of *Wolbachia* into this species. Similarly, I detected three distinct strains of *Spiroplasma* that did not appear to form a monophyletic clade based on *16s* sequences, and so it would seem that there have been at least two, and probably three, separate introductions of *Spiroplasma* symbionts. The fact that *w*MscaA_3 was only found in concert with *w*MscaA_1, whilst latter frequently occurred in isolation, implies that *w*MscaA_3 first infected individuals already infected with *w*MscaA_1. In contrast, the fact that *w*MscaA_2 is found alone suggests that its initial transmission into *M. scabrinodis* likely occurred in uninfected individuals. However, alternative scenarios are possible, and future work combining symbiont screening in additional populations with sequencing of nuclear and mitochondrial loci would help resolve the evolutionary history of these putative symbiont invasion events.

In addition to geographic variation in symbiont genotype, I also uncovered variation in the prevalence of some symbionts between populations. Whilst *Spiroplasma* was very common in the focal UK population, I detected infections in all 186 individuals sampled from additional populations, regardless of infection with *Wolbachia*. *Arsenophonus*, relatively uncommon in the UK population, was only identified in two individuals from additional populations, one each from the UK and Germany. However, the most interesting patterns of infection prevalence were seen for *Wolbachia*, where I detected highly significant variation in the

prevalence of infection between populations. Whilst *Wolbachia* was not detected in more than half of populations, it was highly prevalent in infected populations, present in almost 70% of colonies. This rises to over 85% if populations with fewer than 10 sampled colonies are excluded. This pattern poses two obvious questions; firstly, why has *Wolbachia* failed to spread to fixation in all infected populations sampled, and secondly, why is *Wolbachia* found in some populations and not others?

The most common phenotypic effect of *Wolbachia* in insects is the induction of mating incompatibilities (Werren et al., 2008). Imperfect transmission of incompatibility-inducing *Wolbachia* can lead to the continued presence of uninfected individuals in infected populations (Hoffmann & Turelli, 1997), however data regarding the transmission efficiency of *Wolbachia* in *M. scabrinodis* suggests that infected females transmit *Wolbachia* to an extremely high proportion of their offspring (Treanor et al. unpublished data). Instead, it may be that *M. scabrinodis* exists as a metapopulation, and queens migrate into infected populations from uninfected populations, with the steady influx of uninfected individuals preventing *Wolbachia* from spreading to fixation. Interestingly, this metapopulation perspective may also answer the second of my questions, accounting for the presence of both infected and uninfected populations across my sampling range. Both deterministic models and cage experiments concerning the

spread of cytoplasmic incompatibility-inducing *Wolbachia* in a single panmictic population show that, following initial establishment, the infection will either spread to a high equilibrium prevalence or be lost entirely, depending on the strength of CI, fecundity effects of infection, and transmission efficiency (Engelstädter & Telschow, 2009). In contrast, models incorporating population substructure suggest that, even without any variation in these crucial parameters, infected and uninfected populations can coexist in a larger metapopulation (Egas et al., 2002; Flor et al., 2007). Variation in infection status both within and amongst populations could thus be due to the fragmentation of *M. scabrinodis* into many populations linked by migration.

However, it may also be that the coexistence of infected and uninfected populations across the Palaearctic is due to large scale geographic patterns of *Wolbachia* infection rather than a patchwork of closely situated populations of differing infection status, and that at medium geographic scales (i.e. 10-100km) infection status is uniform across populations. Populations of similar infection status, and those infected with a particular strain of *Wolbachia,* do appear to cluster together to some degree (see Fig. 5.2). Such spatial variation could be due to differences in climate or land use, as well as the geographic history of the species following the post-glacial recolonisation of the Paelaearctic. More evenly spaced sampling as part of future work may help to resolve this issue. Finally, the

inadvertent sampling of multiple sympatric cryptic species of differing infection status could provide an alternate explanation for the coexistence of infected and uninfected colonies, as there is some evidence that *M. scabrinodis* is actually a complex of cryptic species (Radchenko & Elmes, 2010; Seifert et al., 2014). However, this is unlikely to be the case, as sequencing of six nuclear loci from infected and uninfected colonies identified very little genetic variation and no association between genotype and *Wolbachia* infection in a previous study of the focal UK population (see Chapter 3)

Whilst previous evidence suggests the *w*MscaA_1 strain of *Wolbachia* causes mating incompatibilities in *M. scabrinodis*, nothing is known about the phenotypic effects of *Spiroplasma* and *Arsenophonus* in this species, and both symbionts exhibit remarkably variable biology. *Spiroplasma* has been variously described as the causative agent of disease in both plants and animals (Regassa & Gasparich, 2006; Cisak et al., 2015), as a male-killing reproductive parasite (Jiggins et al., 2000; Montenegro et al., 2005), and as a defensive mutualist protecting against parasites and parasitoids (Jaenike et al., 2010b; Xie et al., 2010; Łukasik et al., 2013). However, in most insects *Spiroplasma* appears to be a commensal inhabitant of the gut lumen, exerting no apparent effects on its host (Gasparich, 2010). The extremely high prevalence of *Spiroplasma* in *M. scabrinodis* might seem to suggest that, in this case, it is a faithfully transmitted intracellular symbiont rather than an

environmentally acquired gut commensal. However, social interactions and the overlapping of generations in insect societies enable the transmission of stable gut microbial communities from one generation to the next, and this in turn can select for the evolution of mutualistic gut microbial symbionts in social insects (Koch & Schmid-Hempel, 2011; Martinson et al., 2012; Engel & Moran, 2013). The biology of *Arsenophonus* is equally variable and whilst, unlike *Spiroplasma,* it appears to be more often restricted to an intracellular lifestyle (Nováková et al., 2009; Wilkes et al., 2011) exceptions certainly exist, including in social insects (Yañez et al., 2016).

To summarise, my results illustrate the importance of incorporating a geographic component into studies of symbiont communities. My initial focus on a single population revealed apparent negative interactions between different members of the *M. scabrinodis* symbiont community, and further sampling from multiple populations across the geographic range of the species revealed the presence of multiple strains of *Wolbachia* and *Spiroplasma* and evidence for a bimodal distribution of *Wolbachia* infection between populations.

6 Introgressive hybridisation accounts for mitochondrial divergence and patterns of *Wolbachia* infection in pharaoh ants

6.1 Abstract

Heritable symbionts are extremely common in animals. Many are capable of moving between species, and the subsequent selective sweep of symbionts can significantly alter the genetic structure of host populations. This is particularly true of host mtDNA, as heritable symbionts are in linkage disequilibrium with host mitochondria. When an infected species hybridises with an uninfected species, this linkage between host mtDNA and a symbiont can lead to the introgression of both the symbiont and the associated mtDNA haplotype whilst having little or no influence on host nuclear DNA. Here, I show that each of two genetically distinct strains of *Wolbachia* co-occur with one of two highly divergent mitochondrial haplogroups in the ant *Monomorium pharaonis*. This implies shared evolutionary histories of mitochondria and *Wolbachia*. Furthermore, variation in symbiont and

mitochondrial haplotype is not reflected in host nuclear genotypes, clearly establishing that the two mitochondrial haplogroups are not evidence of cryptic speciation. Taken together, this body of evidence strongly implies that at least one strain of *Wolbachia,* together with a heterospecific mitochondrial variant, was acquired by introgressive hybridisation by *M. pharaonis*. My results illustrate the importance of sequencing nuclear loci in addition to mtDNA, and add to the weight of empirical evidence suggesting that heritable symbionts are at least partially responsible for the prevalence of mitochondrial introgression across animals.

6.2 Introduction

Heritable bacterial symbionts are extremely common in animals, and interact with their hosts in variety of important ways (Dale & Moran, 2006; Duron et al., 2008; Jaenike, 2012). Two general categories of bacterial symbiont are commonly recognised; primary (or obligate) symbionts and secondary (or facultative) symbionts (Moran et al., 2008). Primary symbionts are indispensable to their hosts, and are generally found in species with nutritionally unbalanced diets where they provide essential nutritional benefits. For instance, *Buchnera* provides its aphid

hosts with amino acids that cannot be acquired from a diet of plant sap (Douglas, 1998; Shigenobu et al., 2000) and *Wigglesworthia* synthesises essential cofactors for blood-feeding tsetse flies (Akman et al., 2002; Dale & Moran, 2006). In contrast, there exists a broad range of secondary symbionts that can subtly alter or detrimentally manipulate the biology of their hosts. Some of these secondary symbionts are facultative mutualists that increase host fitness under certain ecological conditions. For instance, the aphid *Acyrthosiphon pisum*, which is an obligate host of the aforementioned *Buchnera*, can also play host to a number of other heritable bacteria that provide resistance to pathogens (Scarborough et al., 2005), parasitoids (Oliver et al., 2003, 2005) and heat stress (Montllor et al., 2002). However, many of the most successful and widespread secondary symbionts are not beneficial, and these include so-called 'reproductive parasites' spread through animal populations by manipulating the reproductive biology of their hosts for their own ends. Since most heritable bacteria are strictly maternally transmitted, males are an evolutionary dead end from their perspective (Engelstädter & Hurst, 2009a). As such, some reproductive parasites have evolved the ability to distort host sex ratios in various ways, which serves to redirect host investment away from males and towards female hosts, or enhances the fitness of females (Werren et al., 2008; Hurst & Frost, 2015). In a phenomenon called cytoplasmic incompatibility, other reproductive parasites reduce the fecundity of uninfected

females when they mate with an infected male. Infected females are immune to this effect, and the relative reduction in the fitness of uninfected females causes the infection to spread through the host population (Engelstädter & Hurst, 2009a).

Primary symbionts exhibit remarkably faithful co-divergence with their hosts, in some cases over the course of hundreds of millions of years (Moran et al., 2008). In contrast, although secondary symbionts are generally maternally transmitted on ecological timescales, the discordance between host and symbiont phylogenies clearly illustrates the fact that these symbionts move between species and infect novel hosts on evolutionary timescales (O'Neill et al., 1992; Russell et al., 2003). The invasion and spread of a secondary symbiont in a novel host can significantly alter the genetic structure of host populations, particularly with respect to mitochondrial DNA (mtDNA). Both heritable symbionts and mitochondria are generally maternally transmitted, and so are in linkage disequilibrium (Hurst & Jiggins, 2005). Consequently, when a secondary symbiont spreads through a naïve host population, the associated mtDNA haplotype may also spread via genetic hitchhiking. This hitchhiking effect can even lead to the simultaneous transfer of a secondary symbiont and mtDNA from one species to another following intraspecific hybridisation (Jiggins, 2003; Raychoudhury et al., 2009). Whilst many of the most popular and intuitive species concepts implicitly or explicitly assume that species boundaries are impermeable to genetic exchange

(Donoghue, 1985; Noor, 2002; Mallet, 2008) the prevalence and significance of interspecific hybridisation in plants has long been recognised (Baack & Rieseberg, 2007; Schwenk et al., 2008) and the ever increasing use of molecular markers in evolutionary biology suggests that hybridisation and subsequent introgression of genetic material from other species is not uncommon in animals (Dowling & Secor, 1997; Mallet, 2005; Abbott et al., 2013). Mitochondrial DNA appears to be particularly prone to introgression relative to nuclear DNA (Funk & Omland, 2003; Gompert et al., 2008), and instances of mitochondrial introgression have been recorded across a variety of animal taxa (Sullivan et al., 2004; Melo-Ferreira et al., 2005; Linnen & Farrell, 2007; McGuire et al., 2007). Whilst there are a number of plausible explanations for the prevalence of mitochondrial introgression, linkage disequilibrium between mitochondria and secondary symbionts appears to explain this phenomenon in a number of cases (Jiggins, 2003; Linnen & Farrell, 2007; Gompert et al., 2008; Raychoudhury et al., 2009; Xiao et al., 2012). Hybridisation between a male of an uninfected species and a female of a related species infected with a secondary symbiont will produce an infected hybrid, and subsequent backcrossing of the hybrid with the previously uninfected species, coupled with a selective sweep of the symbiont, will lead to the introgression of heterospecific mtDNA with little to no impact on host nuclear DNA (Hurst & Jiggins, 2005; Raychoudhury et al., 2009).

The widespread invasive ant *Monomorium pharaonis* is infected with two strains of the symbiont *Wolbachia* (Schmidt, 2010; Pontieri et al., 2016). These two strains do not appear to co-occur within individuals or colonies, and are genetically divergent (Schmidt, 2010) which suggests that they were acquired by independent evolutionary events. In addition, a previous study identified two distinct, highly divergent mitochondrial haplogroups within *M. pharaonis* (Frouz et al., 2009). Sequences from the two mitochondrial haplogroups differ by up to 12.7%, a degree of divergence which exceeds that normally seen within a species (Hebert et al., 2003; Xiao et al., 2012). This implies one of two possibilities. Firstly, *M. pharaonis* as currently recognised may actually consist of two cryptic species, corresponding to each of the two mitochondrial haplogroups (Frouz et al., 2009). In this case, mitochondrial genetic variation would likely be reflected in genetic variation at nuclear loci. Alternatively, the observed mitochondrial genetic variation may be the result of an introgression event with a related species, in which case we might expect to see markedly reduced levels of genetic variation at nuclear loci in comparison to mitochondrial genes, and no relationship between mitochondrial and nuclear genetic variation. If such an introgression event has occurred, the spread of the divergent mitochondrial haplotype may have been facilitated by a selective sweep of one of the two *Wolbachia* strains found in *M. pharaonis*, in which case we would also expect to see an association between

mtDNA haplotypes and *Wolbachia* infection. An introgression event in *M. pharaonis* could thus account for both the presence of non-co-occurring *Wolbachia* strains and the striking intra-specific mitochondrial divergence found within species.

Here, I aim to shed light on the evolutionary history of pharaoh ants and their *Wolbachia* symbionts by analysing the relationship between host nuclear, host mitochondrial and symbiont genetic variation. In particular, I aim to clarify whether the observed variation in mtDNA is related to infection with *Wolbachia*, and whether or not the intraspecific divergence in mtDNA is reflected in nuclear genetic variation.

6.3 Materials and methods

M. pharaonis workers were sampled from 12 colonies. Workers from three colonies were sampled directly from the field, whilst workers from the remaining 9 colonies were sampled from colonies maintained in the laboratory for a number of years. Of the laboratory lineages, three were inbred lines established from field collections, and three were hybrids of inbred lines produced as part of a crossing program (Pontieri et al., 2016). These hybrid colonies were of known ancestry with a single maternal lineage that was distinct from the other colonies used in this study, and

so they are not pseudoreplicates with respect to cytoplasmic ancestry. Finally, three colonies are likely hybrids between field collected lineages, but are of unknown ancestry. Details of the origin of colonies are listed in Table 2. Samples were stored in 100% ethanol prior to genetic analysis. DNA was extracted from 3-5 workers per colony by heating whole ants in a mixture 100 μl of 5% Chelex® solution and 5 μl of 10 mg/ml Proteinase K at 60°C for 60 min and then heating at 99°C for 15 min. The product was then centrifuged at 4680 rpm for 20 min and the supernatant was pipetted off.

Colony	**Samples Taken From**	**Inbred Lineage or Hybrid?**	**Maternal Lineage**	**Origin of Maternal Lineage**
Esher1	Field Collection	N/A	Esher1	UK
Res1	Field Collection	N/A	Res1	UK
Res2	Field Collection	N/A	Res2	UK
Thai22	Laboratory Colony	Inbred	Thai22	Chanthaburi, Thailand
U4	Laboratory Colony	Inbred	U4	Penang, Malaysia
U11	Laboratory Colony	Inbred	U11	Texas, USA
102	Laboratory Colony	Hybrid	U2	Texas, USA
H27	Laboratory Colony	Hybrid	U7	Warsaw, Poland
5021	Laboratory Colony	Hybrid	GH4	Fete, Ghana
A1	Laboratory Colony	Unknown	Unknown	Unknown
S1	Laboratory Colony	Unknown	Unknown	UK
L1	Laboratory Colony	Unknown	Unknown	UK

Table 2 *Details of* M. pharaonis *samples used in this study*

All sampled individuals were subsequently screened for *Wolbachia* infection by diagnostic PCR, using the *Wolbachia* specific primers coxA_F and coxA_R to amplify the bacterial copy of the gene *cytochrome* c *oxidase subunit 1,* hereafter *coxA* (Baldo et al., 2006) and a fragment of the mitochondrial gene *cytochrome* c *oxidase subunit 1,* hereafter *CO1*, was amplified as a host control, using the primers LCO1490 and HCO2198 (Folmer et al., 1994). In addition, one individual per lineage was selected for further genetic analysis, consisting of the amplification and sequencing of fragments of one *Wolbachia* gene (*coxA*), six nuclear genes (the protein coding genes *EF1αF1, EF1αF2, Wingless* and *Abdominal-A,* and the ribosomal genes *18s* and *28s*) and one mitochondrial gene (*CO1*). Finally, two representative individuals, each infected by one of the two *Wolbachia* strains present in *M. pharaonis,* were selected for multi-locus sequence typing (MLST) to determine the phylogenetic position of the two strains of *Wolbachia* infecting *M. pharaonis* (Baldo et al., 2006). PCR reactions were performed using 30 μl reaction mixtures, consisting of 14.4 μl molecular grade H_2O, 6 μl Promega GoTaq Flexi green buffer, 3.75 μl $MgCl_2$, 1.5 μl dNTP's (2.5 mM each), 0.6 μl each of the forward and reverse primer (10 μM), 0.15 μl Promega GoTaq DNA Polymerase and 3 μl DNA extract. Infected *Acromyrmex octospinosus* individuals were used as positive controls, and molecular grade H_2O was used for negative controls. PCR reactions consisted of 95°C for 2 min followed by 35 cycles of 95°C for 30 s, 50-64°C for 30 s

and 72°C for 1 min, and finally 72°C for 7 min. PCR products were run on 1% agarose gels, and visualised under UV light. The remaining PCR product was purified using a Qiaquick PCR purification kit, as per the manufacturer's instructions. Purified DNA was then sent to Eurofins Genomics for sequencing.

Nuclear, *CO1*, and *coxA* sequences were aligned using MUSCLE and genetic distances were calculated using MEGA7 (Kumar et al., 2016). A maximum likelihood tree of *CO1* sequences was constructed using MEGA7 based on the Tamura-Nei nucleotide substitution model with invariant sites, and a maximum likelihood tree of *EF1αF2* was constructed based on the Kimura 2-parameter model. *CO1* and *EF1αF2* sequences from *Monomorium floricola* retrieved from Genbank were used as outgroups.

Sequences of the five MLST genes were aligned in MUSCLE along with sequences from 22 strains of supergroup A *Wolbachia* and four strains of supergroup B *Wolbachia*. ClonalFrame 1.1 was then used to construct a Bayesian phylogeny that accounts for recombination events between strains (Didelot & Falush, 2006). The program was run three times with 250,000 generations for burnin and 250,000 post burnin generations. The convergence of the three runs, which began at different random configurations, was assessed using methods described in Gelman & Rubin, (1992) and implemented in ClonalFrame; Gelman and Rubin statistics for θ, R, v, δ and the TMRCA were all <1.2, indicating

convergence of independent runs. Post-burnin trees from all three runs were combined to build a majority-rule consensus tree.

6.4 Results

Screening of pharaoh ant colonies by diagnostic PCR revealed infection by *Wolbachia* in 7/12 colonies. In all infected colonies, all sampled workers were infected with *Wolbachia*. Sequencing of a *357bp* fragment of *coxA* revealed that two distinct strains of *Wolbachia* were present with no evidence of co-infection by multiple strains. A Bayesian phylogeny based on MLST genes confirmed this finding (see Fig 6.1).

Sequencing of a 604bp fragment of the *M. pharaonis CO1* gene recovered three mitochondrial haplotypes, which fell into two highly distinct haplogroups that differ from one another by at least 10.4% in terms of genetic distance. In contrast, very little genetic variation was observed at sequenced nuclear loci. No variation was detected in *EF1αF1, Wingless* and *Abdominal-A, 18s* or *28s,* and only a single polymorphic base-pair was identified in the *EF1αF2* sequences generated. Furthermore, variation at this position was not clearly related to a particular mitochondrial haplogroup (see Fig 6.2).

Despite the absence of any relationship between host nuclear and mitochondrial genetic variation, each of the mitochondrial haplogroups was clearly associated with a specific strain of *Wolbachia*. All individuals with mitochondria from haplogroup A were either infected with the *Wolbachia* strain *w*PharA_1 or were uninfected, whilst all individuals with mitochondria from haplogroup B were infected with the *Wolbachia* strain *w*PharA_2 (see Fig. 6.3).

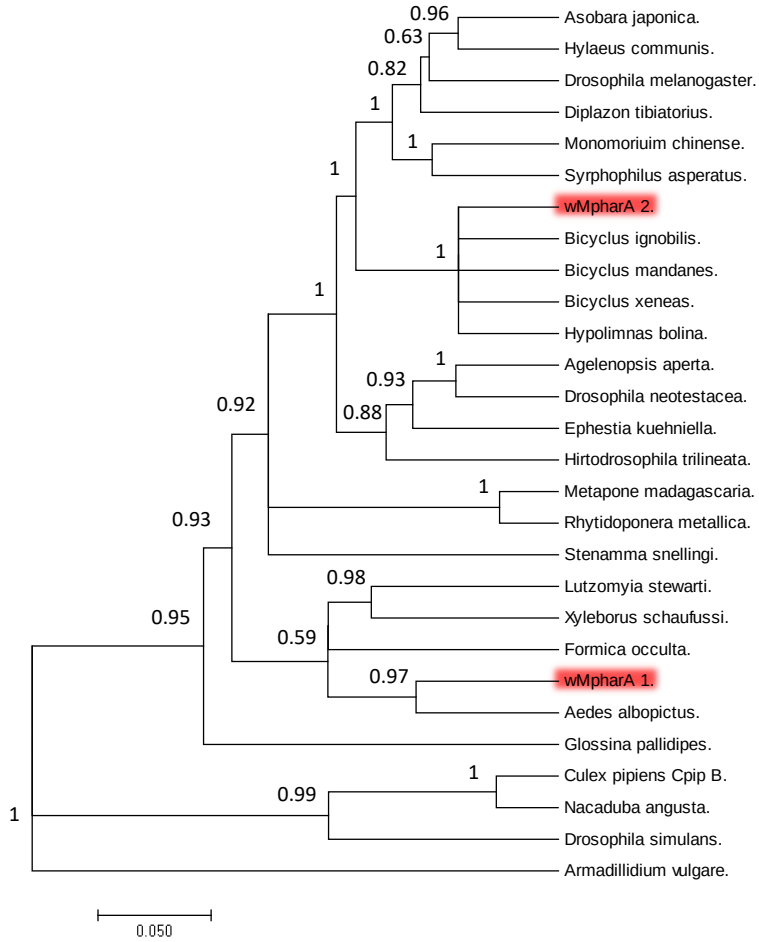

Figure 6.1 *A Bayesian phylogeny of* Wolbachia *strains infecting* M. pharaonis *(highlighted in red) and other arthropod species produced using ClonalFrame. Numbers adjacent to nodes correspond to Bayesian posterior probabilities. The scale bar represents the number of substitutions per site.*

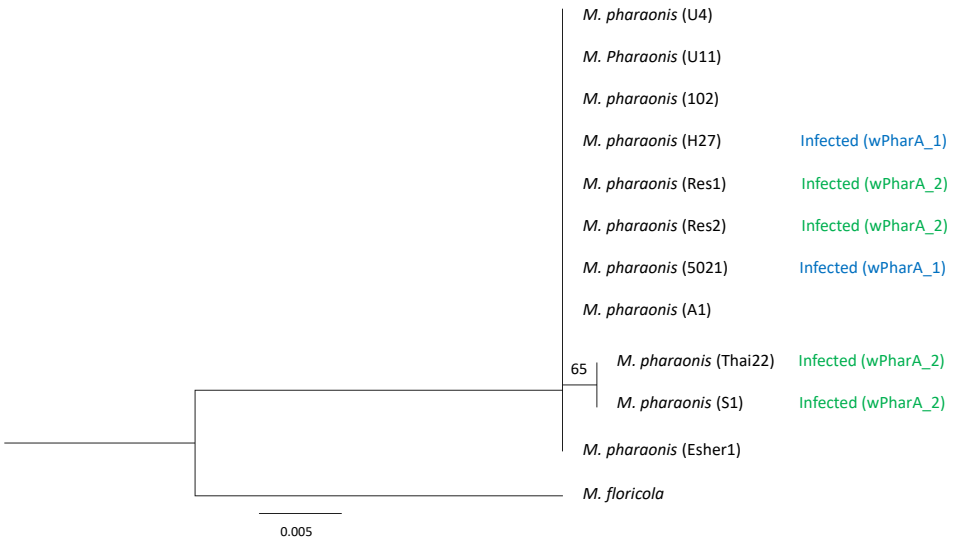

Figure 6.2 *A maximum likelihood phylogeny based on* EF1αF2 *sequences obtained in this study. The scale bar indicates the number of substitutions per site. Infection status is shown to the right of the phylogeny. Bootstrap values are shown next to nodes in the tree and were calculated using 5000 replicates. The sample L1 is not included as* EF1αF2 *could not be successfully sequenced.*

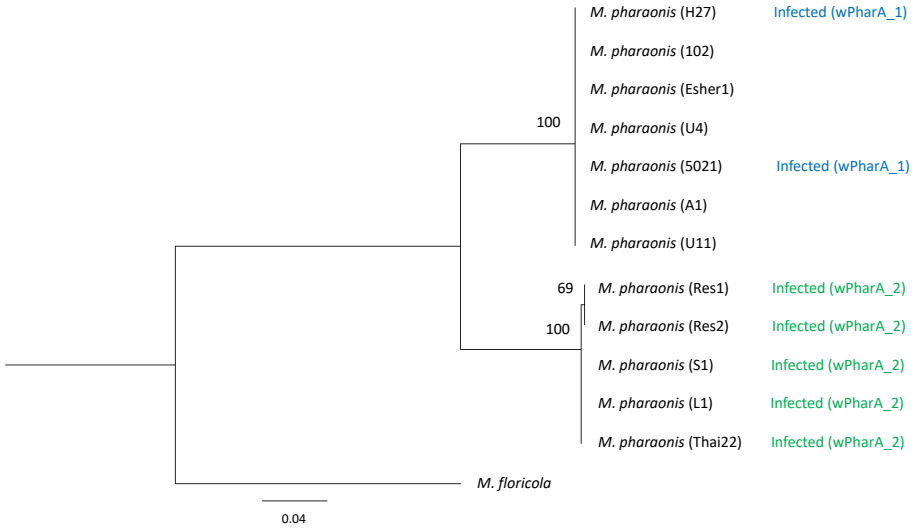

Figure 6.3 *A maximum likelihood phylogeny based on CO1 sequences obtained in this study. The scale bar indicates the number of substitutions per site. Infection status is shown to the right of the phylogeny. Bootstrap values are shown next to nodes in the tree and were calculated using 5000 replicates.*

6.5 Discussion

Previous studies have demonstrated significant mitochondrial sequence divergence within *M. pharaonis*, and my findings corroborate these earlier results. The three mitochondrial haplotypes I recovered clearly fall into two highly distinct haplogroups, and the level of genetic divergence between the two haplogroups is in line with that normally observed between species rather within species (Hebert et al., 2003; Xiao et al., 2012). Two simple explanations could account for this observation; either *M. pharaonis* is a complex of two cryptic species, or a second mitochondrial haplotype was introgressed into *M. pharaonis* following interspecific hybridisation.

My findings regarding nuclear genetic variation in *M. pharaonis* clearly support the latter hypothesis. By combining sequencing of mitochondrial and nuclear genes, I uncovered substantial mito-nuclear discordance within this species, as the striking divergence in mitochondrial DNA was not at all reflected in nuclear DNA. Whilst mitochondrial genes generally evolve more quickly than nuclear genes, my findings comfortably exceed the greatest observed magnitude of differences in nucleotide substitution rates between mitochondrial and nuclear protein-coding genes (Oliveira et al., 2008). Furthermore, what little variation that exists in the sequences of the nuclear genes I obtained does not correlate with variation in mitochondrial DNA. As such, reproductive isolation coupled with

differences in the rate of evolution of mitochondrial and nuclear DNA cannot explain the mito-nuclear discordance observed in *M. pharaonis*. Instead, my data imply that the mitochondria of a related species was introgressed into *M. pharaonis* following intraspecific hybridisation, leading to the spread of heterospecific mtDNA without substantially affecting host nuclear genetic variation.

Such an introgression event would also account for the clear relationship between *Wolbachia* infection and host mitochondrial haplotype. All sampled individuals that were uninfected, as well as all those infected with the *Wolbachia* strain *w*PharA_1, possessed mitochondria from haplogroup A, whilst all those infected with strain *w*PharA_2 harboured mitochondria from haplogroup B. This implies that *Wolbachia* and mitochondria have shared evolutionary histories in *M. pharaonis*, and linkage disequilibrium between *Wolbachia* and mitochondria provides a parsimonious explanation for this relationship.

The fact that some individuals possessing mitochondria from haplogroup A were infected with *w*PharA_1 and others were free from infection, whilst all individuals with mitochondria from haplogroup B were infected with *w*PharA_2, suggests that the *w*PharA_2 strain was the subject of the introgression event. Barring the loss of *Wolbachia* from imperfect maternal transmission or environmental stressors, an introgression event should perfectly link mitochondrial and symbiont haplotypes, as appears to be the case for haplogroup

B and *w*PharA_2. In contrast, if a strain of *Wolbachia* is acquired by horizontal transmission *sensu stricto* (i.e. through ecological interactions between unrelated species), both infected and uninfected individuals may share a similar or even identical mitochondrial haplotype, as with haplogroup A and *w*PharA_1. However, it should be noted that the fact that exactly the same mitochondrial haplotype is shared by many of the uninfected individuals, as well as those infected with the strain *w*PharA_1, suggests that this strain was either acquired very recently, or has been subsequently lost from some lineages. It may be possible to identify the source of *w*PharA_2 and haplogroup B by examining the hypothesised close relatives of pharaoh ants, *M. longi* and *M. wroughtoni* (Bolton, 1987) although it is possible that the original source of *w*PharA_2 and haplogroup B subsequently became extinct and will be impossible to identify. Although I hypothesise that the introgression of mtDNA and *Wolbachia* has occurred in pharaoh ants due to selection on *Wolbachia*, little is known about the biology of either strain of *Wolbachia* that infects this species. However, in a recent study, Pontieri et al., (2016) examined colony growth and sexual productivity following an artificial selection experiment in *M. pharaonis*. Their findings suggested that *Wolbachia* does not cause mating incompatibilities in pharaoh ants but may induce female-biased sex ratios. This effect may have been sufficient to lead to a selective sweep of introgressed *Wolbachia* in pharaoh ants.

Although some colonies sampled in this study originated as hybrids of inbred lineages, this is of little practical consequence to the interpretation of my findings. Firstly, whilst the hybrid origin of colonies will break down any association between nuclear and cytoplasmic genetic variation, a major finding of this study was the remarkable lack of nuclear genetic variation despite substantial divergence in mtDNA; in other words, there is no association for hybridisation to break down. Secondly, *Wolbachia* and mitochondria are both inherited clonally from the female parent, and so hybridisation will not break the association between mitochondrial genetic variation and *Wolbachia*.

In conclusion, my findings clearly illustrate the problems inherent with relying solely on mtDNA as a means of phylogenetic inference (Hurst & Jiggins, 2005; Galtier et al., 2009). However, sequencing of mtDNA alongside nuclear DNA and/or symbiont genes is clearly of value, providing fundamental insights into the nature of reproductive isolation, adaptive evolution in mitochondria, the spread of secondary symbionts, and historical demographic processes (Toews & Brelsford, 2012) and I anticipate that there will be a continuing shift towards the use of mtDNA as a marker because of, rather than in spite of, its unique biology. Furthermore, my results add to the weight of empirical evidence suggesting that heritable symbionts are at least partially responsible for the prevalence of mitochondrial introgression across animals.

7 Dispersal limitation predicts the incidence of *Wolbachia* across ants

7.1 Abstract

The endosymbiont *Wolbachia* is perhaps the greatest panzootic in the history of life on Earth, yet remarkably little is known regarding the factors that determine its incidence across species. One possibility is that *Wolbachia* more easily invades species with smaller effective population sizes, due to the increased strength of genetic drift. This should enable strains that induce mating incompatibilities to more easily cross the threshold prevalence above which they spread to either fixation or a stable equilibrium prevalence. Here, I provide empirical support for this hypothesis by analysing the relationship between dispersal limitation (as a proxy for effective population size) and the incidence of *Wolbachia* across 254 species of ants. I show that species in which the dispersal of reproductive females is limited are almost twice as likely to be infected with *Wolbachia* as species whose reproductive ecology is consistent with significant dispersal of females, and that this relationship persists after controlling for host phylogeny. I suggest that low

effective population sizes, in this case resulting from limited dispersal, may be an important factor in determining how easily *Wolbachia* becomes successfully established in a novel host, as it has done countless times.

7.2 Introduction

Wolbachia is a ubiquitous bacterial symbiont of arthropods and filarial nematodes, infamous for its ability to manipulate the reproduction of its hosts in several ways (Werren et al., 2008; Weinert et al., 2015). *Wolbachia* is maternally transmitted, and consequently a number of these reproductive manipulations, including male-killing, the feminization of genetic males, and the induction of parthenogenesis result in the distortion of host sex-ratios in favour of female offspring (Hurst & Frost, 2015). However, the most common effect of *Wolbachia* appears to be the induction of mating incompatibilities (Werren et al., 2008; Engelstädter & Hurst, 2009a). In this situation, *Wolbachia* causes uninfected females that mate with *Wolbachia*-infected males to suffer from a reduced egg viability. This reduces the fitness of uninfected females relative to infected females, ultimately causing the infection to spread through the population (Engelstädter & Hurst,

2009a). These strains of *Wolbachia* are said to cause cytoplasmic incompatibility (CI).

Early deterministic models concerning the spread of CI-inducing *Wolbachia* demonstrated that their spread is subject to positive frequency-dependent selection (Jansen et al., 2008; Engelstädter & Telschow, 2009). When the infection is rare in a population, there is little risk of mating with an infected male, and so the infection is unlikely to benefit its host. Conversely, when the infection is common, the risk of mating with an infected male is high, and so the CI strain is likely to benefit female hosts by removing the risk of a dramatic reduction in fecundity caused by mating incompatibilities (Engelstädter & Telschow, 2009). Furthermore, due to this positive frequency-dependent selection, CI strains that display imperfect maternal transmission or impose a fecundity cost upon their host will fail to spread unless their prevalence in a population is above a critical threshold (Werren & O'Niell, 1997; Jansen et al., 2008). When the infection is at a low prevalence, the small benefits conferred by avoidance of mating incompatibilities is outweighed by fecundity costs and imperfect maternal transmission, and so the infection is lost from the population. At a high prevalence of infection, the frequency-dependent benefit of infection more than compensates for any fecundity cost and/or imperfect transmission, causing the bacteria to spread to fixation or to a high equilibrium

prevalence within the population (Caspari & Watson, 1959; Hoffmann et al., 1990; Engelstädter & Telschow, 2009).

These basic epidemiological principles are highly relevant to the consideration of how *Wolbachia* comes to establish itself in a new species. It is well recognised that *Wolbachia* is frequently transmitted between unrelated species on evolutionary timescales, as evidenced by the remarkably low degree of co-cladogenesis between *Wolbachia* and its hosts (Werren & O'Niell, 1997; Vavre et al., 1999), either as a result of horizontal transmission (e.g. via parasitoid vectoring or shared food resources) or hybrid introgression (Heath et al., 1999; Raychoudhury et al., 2009; Stahlhut et al., 2010; Ahmed et al., 2015). However, when a CI-inducing strain of *Wolbachia* is first transferred from one species to another, its initial prevalence in the newly infected species is likely to be very low. Thus, if there is any fecundity cost associated with infection, or any inefficiency in maternal transmission, the infection prevalence will almost certainly be below the invasion threshold, and so will be unlikely to spread in the new host species (Egas et al., 2002; Jansen et al., 2008).

One solution to this apparent paradox is the presence of substantial genetic drift within populations (Stouthamer et al., 1999). Stochastic demographic processes may allow low prevalence infections to drift above the invasion threshold, from where they can subsequently spread via positive selection (Jansen

et al., 2008). Accordingly, stochastic models incorporating variable population sizes and population structure (i.e. metapopulations) demonstrate that small population sizes and structured populations, both of which result in increased genetic drift, facilitate the invasion of CI-inducing *Wolbachia* (Egas et al., 2002; Jansen et al., 2008; Reuter et al., 2008). Unfortunately, there is little empirical data to corroborate the predictions of these theoretical models regarding the importance of genetic drift to the spread of CI-inducing *Wolbachia*.

One taxonomic group that may be useful to test these predictions is the ants (Hymenoptera: Formicidae). In the majority of ant species, alate (wing-bearing) queens mate in swarms, disperse by flight, and found new colonies independently. This is known as independent colony foundation (ICF). However, in some species, new colonies are instead founded by the fragmentation of existing colonies and the subsequent dispersal of queens and workers on foot (known as dependent colony foundation, hereafter DCF) reducing gene flow within populations (Peeters & Molet, 2010; Cronin et al., 2013). Other aspects of the reproductive ecology of ants can also strongly influence queen dispersal (see Fig. 7.1). Whilst the majority of ant species have a single queen per colony (monogyny), some species are polygynous, with multiple fertile queens in some or all colonies (Hölldobler & Wilson, 1977; Keller, 1995; Hughes et al., 2008). This situation can arise via primary polygyny, when multiple queens found a new colony together and peacefully coexist, but is

much more commonly due to secondary polygyny, in which established colonies readopt related queens (Bourke & Franks, 1995; Trunzer et al., 1998; Kellner et al., 2007). Secondary polygyny is often associated with DCF but this is not exclusively the case (Peeters & Molet, 2010). For instance, dorylomorph species (commonly known as army ants) exclusively practice DCF, but are mostly monogynous, whilst some ant species practice secondary polygyny but generally only found new colonies independently (Rettenmeyer & Watkins II, 1978; Heinze & Foitzik, 2009; Steinmeyer et al., 2012; Cronin et al., 2013). Finally, the morphology of queens can have a substantial impact on their dispersal ability. In some species, queens possess either short, non-functional wings (brachypterous queens) or have lost their wings all together (ergatoid queens), and such queens must disperse on foot (Peeters, 2012). In other species, the queen caste either coexists with, or has been entirely replaced by, workers called gamergates which are capable of mating and sexually reproducing. These gamergates, being workers, lack wings and so cannot disperse aerially (Peeters, 2012). Again, the presence of these derived queen morphologies often, but not exclusively, co-occurs with DCF; for instance, in some species of ants in the genera *Pogonomyrmex* and *Cardiocondyla*, ergatoid queens disperse on foot yet found colonies independently (Schrempf & Heinze, 2007; Johnson, 2010).

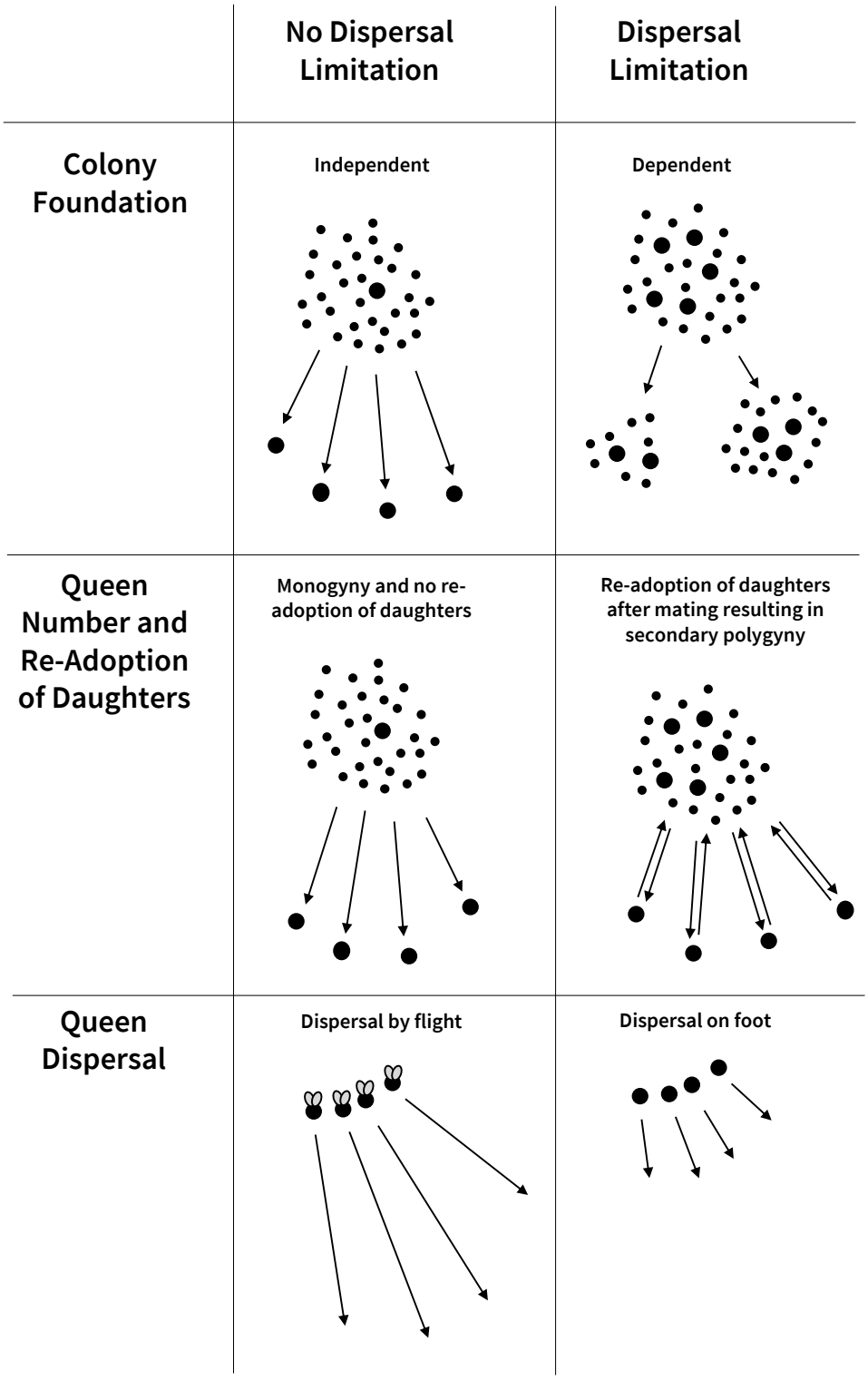

Figure 7.1 *Various life-history traits pertaining to reproduction heavily influence the dispersal ability of ant queens. Whilst these dispersal-limiting traits often co-occur, this is not always the case. Large black circles represent queens, small black circles represent workers, and arrows represent dispersal.*

These derived, dispersal-limiting life-history traits have convergently evolved multiple times in ants, permitting comparative analyses of their causes and consequences (Peeters, 2012; Cronin et al., 2013). In this study, I make use of the extensive literature on the incidence of *Wolbachia* in ants and combine this with surveys of the literature concerning the reproductive ecology of ants to examine the association between dispersal limitation (as a proxy for the strength of genetic drift) and the incidence of *Wolbachia.* This relationship has been examined previously by a few authors, with results of borderline significance (Wenseleers et al., 1998; Russell, 2012; Tsoi, 2013), but here I improve upon these analyses by incorporating a considerably larger dataset, including multiple dispersal-related life-history traits, and controlling for host phylogeny in my analysis.

7.3 Materials and methods

I compiled literature records of *Wolbachia* infection in ants, including only data obtained by screening for *Wolbachia* using standard diagnostic PCR methods; other methods, such as long PCR, are more sensitive but prone to the detection of low-level environmental contaminants that are not necessarily biologically significant (Hilgenboecker et al., 2008). I then surveyed the literature for data

relating to the reproductive ecology of these species, searching specifically for data relating to three main variables: a) mode of colony foundation, b) the presence or absence of secondary polygyny, and c) the morphology of queens. I then categorised each species for which sufficient data were available into one of two categories. The first category consisted of species where queens found colonies independently, colonies are monogynous, and queens have functional wings and disperse by flight before or after mating. The second category consisted of species that exhibited one or more of the following life-history traits that result in reduced female dispersal: a) the utilisation of DCF, b) the presence of secondary polygyny, and/or c) the presence of brachypterous or ergatoid queens, gamergates, or otherwise the dispersal of queens without flight. Socially parasitic species that invade colonies of other ant species were considered to utilise ICF (following (Peeters & Molet, 2010)), as were queens that found colonies by pleometrosis (i.e. foundress associations), as these means of colony foundation should have no *a priori* effect on the dispersal ability of queens. Similarly, species that exhibit primary polygyny, or secondary polygyny with exclusive adoption of unrelated queens, were included in the first category, as this should not influence the dispersal ability of queens, unlike the readoption of queens by their maternal colony. All records of polygyny were considered to represent secondary polygyny and the adoption of related queens unless there was evidence to believe this was

not the case, such as population genetic or behavioural data, or the presence of primary polygyny in closely related taxa. In total, I acquired data on both reproductive ecology and *Wolbachia* infection status for 254 ant species (see Appendices).

Generally speaking, related species cannot be treated as independent data points, because closely related species are more likely to share traits due to descent than distantly related species (Felsenstein, 1985). As such, the infection status and dispersal category of each species was mapped onto a phylogeny constructed from 18 published trees (see Appendices) in Mesquite v3.04 (Maddison & Maddison, 2017), to allow the implementation of a phylogenetically controlled analysis between these variables. This phylogeny contained a number of polytomies, and so to permit further analysis, all polytomies were randomly resolved 100 times, resulting in 100 fully resolved trees. All branch lengths were set to 1 and then Grafen-transformed with $\rho = 0.5$ (Grafen, 1989; Midford et al., 2011). I then analysed the relationship between infection status and dispersal limitation using Pagel's test of correlated evolution, as implemented in Mesquite (Pagel, 1994) and the analysis was run with 20 iterations and 2000 additional simulations.

7.4 Results and Discussion

My review of the literature found that 99/194 (51%) of species in which the dispersal of reproductive females is limited were infected with *Wolbachia*, whilst only 17/60 (28%) of species in which females found colonies independently, are not readopted, and disperse by flight were infected (see Fig. 7.2). This difference was shown to be highly significant in a phylogenetically controlled analysis (difference in maximum likelihoods = 9.02, p = 0.001; see Fig. 7.3). Furthermore, this relationship was not an artefact of sampling bias between the two dispersal categories, as there was no marked difference in the number of individuals screened for *Wolbachia* infection between dispersal-limited and non-limited species (Poisson regression, $\chi^2 = 0.807$, df = 252, p = 0.369).

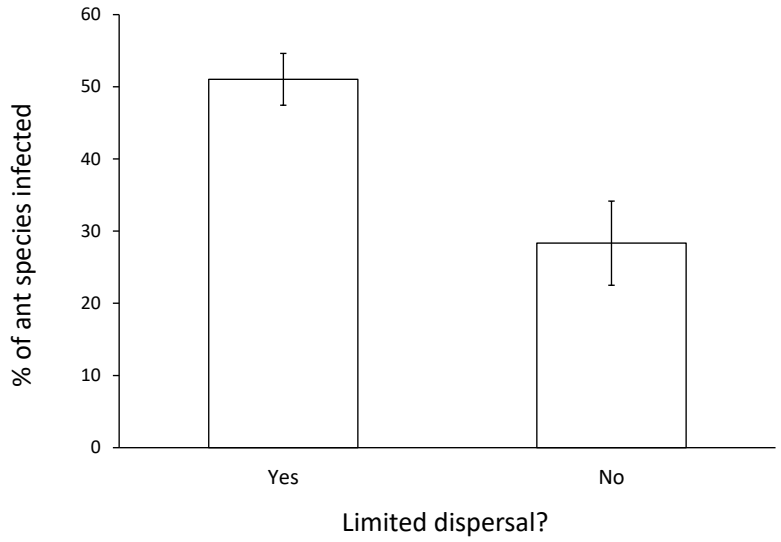

Figure 7.2 *The percentage of dispersal-limited and non-limited ant species infected with* Wolbachia ± SE. *Dispersal-limited species are significantly more likely to be infected with* Wolbachia *than species in which reproductive females can disperse widely (p = 0.001)*

My results provide strong evidence in support of the hypothesis that host life-history is an important determinant of the incidence of *Wolbachia* across taxa. In particular, the positive association between traits that result in limited dispersal of reproductives and infection with *Wolbachia* suggests that host population genetic structure may influence the ability of symbionts to establish themselves in an uninfected species. Numerous reports exist of CI-inducing *Wolbachia* that negatively affect proxies of host fitness such as longevity and fecundity (Hoffmann et al., 1990; Fleury et al., 2000; Perrot-Minnot et al., 2002; Fytrou et al., 2006; Ros & Breeuwer, 2009; Vasquez et al., 2011; Joshi et al., 2014; Zug & Hammerstein, 2015) and/or are imperfectly transmitted (Hoffmann et al., 1990; Werren, 1997a; Johanowicz & Hoy, 1999; Kittayapong et al., 2002). Both theoretical and empirical data show that such strains will fail to spread from low initial frequencies (Caspari & Watson, 1959; Hoffmann et al., 1990; Xi et al., 2005; Engelstädter & Telschow, 2009). However, stochastic demographic processes, exacerbated by population viscosity or substructure, may allow costly or imperfectly transmitted strains of CI-inducing *Wolbachia* to initially spread by drift up to a frequency sufficient for them to subsequently spread via positive selection (Jansen et al., 2008; Reuter et al., 2008). In the case of ants, life-history traits that limit the dispersal of reproductives

may significantly increase population viscosity and thus enable the spread of CI-inducing *Wolbachia* under a wider range of parameter conditions.

My findings regarding the significance of the relationship between dispersal limitation and *Wolbachia* infection contrast somewhat with those of previous analyses. Wenseleers *et al.*, (1998) and Russell (2012) found only marginally significant support for the relationship between mode of colony foundation and *Wolbachia* infection. However, both analyses used substantially smaller datasets than my analysis, which accounts for the considerable difference in the statistical significance of my findings despite the fact that the difference in *Wolbachia* incidence across categories is broadly comparable between this and the aforementioned studies. Tsoi, (2013) found no difference in the incidence of *Wolbachia* between ant species with alate and ergatoid queens, but this dataset was heavily biased towards species with alate queens. As well as my use of a larger dataset, I improved on these analyses by simultaneously considering multiple causes of dispersal limitation that do not necessarily overlap, and by controlling for the phylogenetic relationships between species in this dataset.

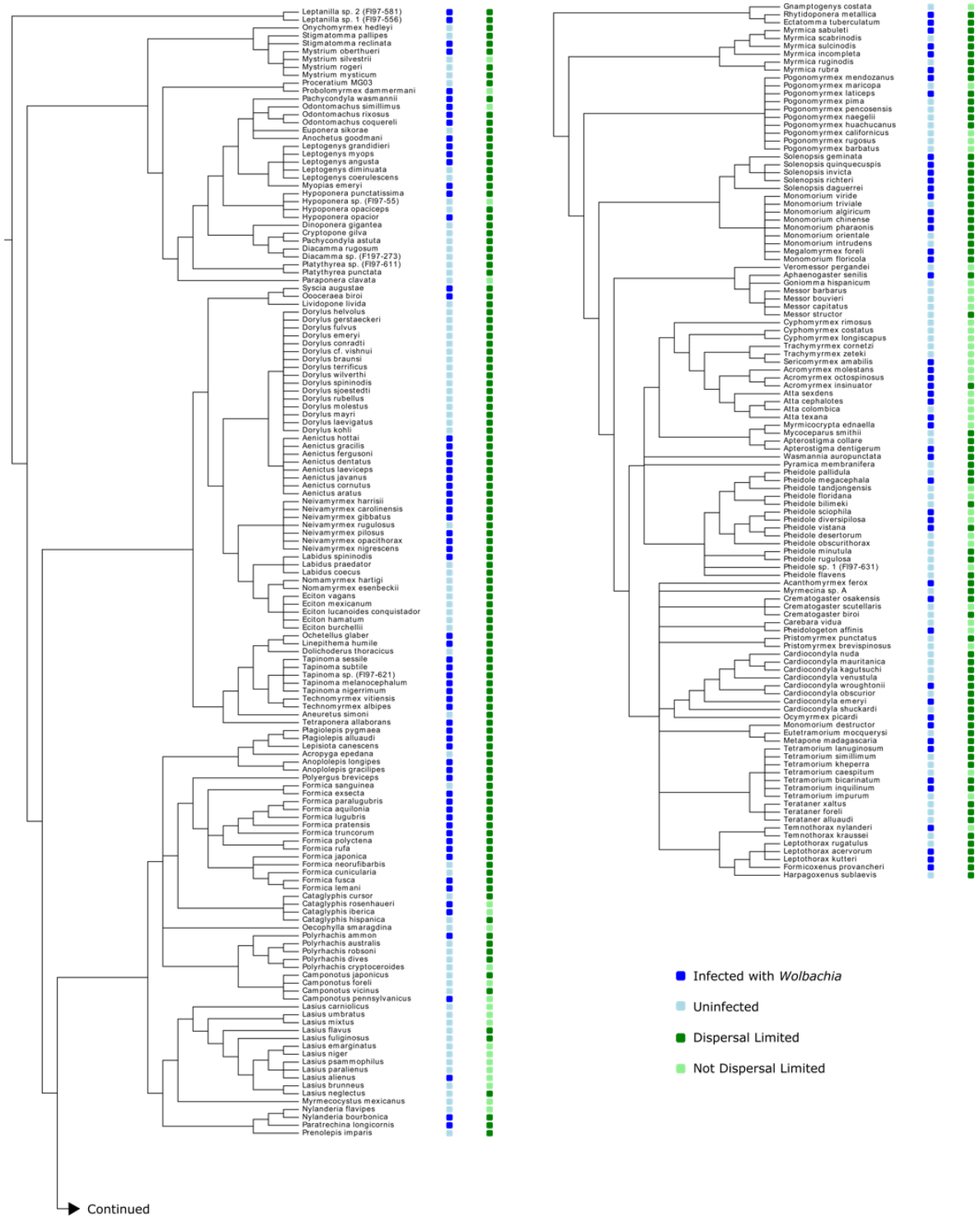

Figure 7.3 *The 254 species included in this analysis, with the phylogenetic relationships between species to the left of binomial names and infection status and dispersal category to the right*

Stochastic demographic processes are not the only way in which CI-inducing *Wolbachia* can spread from a low initial prevalence. Accompanying sex-ratio distortion or context dependent fitness benefits (e.g. protection from natural enemies) have also been proffered as mechanisms that could counterbalance selection against costly or inefficiently transmitted CI strains during the initial stages of infection (Egas et al., 2002; Fenton et al., 2011). For instance, the recent spread of *w*Ri in eastern Australia appears to have occurred in multiple locations from low frequency introductions, probably because the infection increases host fitness (Kriesner et al., 2013). However, it seems unlikely that these factors are sufficient to account for all cases of the spread of CI-inducing *Wolbachia*, especially given that the importance of context-dependent fitness benefits (e.g. protection from natural enemies) in natural populations remains unclear (Zug & Hammerstein, 2015). Furthermore, modelling suggests that, as with beneficial nuclear alleles, fitness enhancing *Wolbachia* will be stochastically lost from large populations when introduced at low frequency in the majority of cases, and so population viscosity or substructure may also increase the ease with which beneficial strains of *Wolbachia* invade a new species (Jansen et al., 2008).

Despite the strength of the relationship between limited dispersal and *Wolbachia* infection in ants, there are clear genus-level discrepancies in this pattern. For instance, a number of army ant genera which exclusively practice DCF,

including the genera with the largest colonies, *Dorylus* and *Eciton,* appear to be free from *Wolbachia*. It may be the case that army ant queens which spend their entire life, even during mating, sheltered within a colony of up to millions of workers, are never put at risk of exposure to infection with *Wolbachia*. In contrast, *Wolbachia* appears to be common in leaf-cutting ants, despite the fact that most species found colonies independently, do not re-adopt related queens, and disperse by flight (Van Borm et al., 2001; Frost et al., 2010; Russell, 2012). *Wolbachia* have been shown to occur extracellularly in the guts of *Acromyrmex* leaf-cutting ants, as well as intracellularly in a variety of other tissues, and it has been suggested that they may act as mutualists in *Acromyrmex spp.* (Andersen et al., 2012; Frost et al., 2014; Sapountzis et al., 2015). Such mutualistic effects may explain how *Wolbachia* has successfully invaded a number of leaf-cutting ant species despite the substantial dispersal of reproductives in this group.

In conclusion, my findings show that dispersal limitation is strongly associated with the incidence of *Wolbachia* in ants. Whilst a number of theoretical models have suggested that the strength of genetic drift, as a function of population size or structure, may be an important determinant of the ability of CI-inducing *Wolbachia* to invade a naïve population, my results provide empirical evidence in support of this hypothesis. The effective population sizes of hosts may

be a crucial factor determining the incidence of *Wolbachia* and account, in part, for its astonishing success.

8 General conclusions

In this thesis, I sought to address two broad issues regarding the biology of *Wolbachia*. Firstly, I aimed to improve the understanding of the phenotypic effects of *Wolbachia* in social insects, which is extremely limited at present. Secondly, I aimed to use ants as a system to better understand various aspects of the movement of *Wolbachia* between species. To conclude this thesis, I will discuss both of these goals in terms of my success in addressing them and the significance of my findings.

8.1 The phenotypic effects of *Wolbachia* in social insects

Despite the fact that the incidence of *Wolbachia* in social insects appears to be equivalent to that of solitary insects, very little is known about how *Wolbachia* influences the biology of social insects. This is probably not because of a lack of interest in the matter; rather, the significant difficulties inherent in breeding most social insects under laboratory conditions makes the creation and culture of infected and cured lines impossible for most species of social insects. Instead, I

chose to focus my initial attention on collecting large numbers of colonies from populations where *Wolbachia* is present but has not spread to fixation, and combining colony censuses with antibiotic treatment experiments in order to understand whether infection affects host fitness, colony sex-ratios, and/or causes mating incompatabilities.

In Chapter 2, I employed this approach with the ant species *Temnothorax crassispinus*. However, whilst *Wolbachia* was not found to infect all individuals within colonies, no unequivocally uninfected colonies were identified, precluding a clear assessment of the colony-level effects of infection. Nonetheless, I identified a weak but significant positive correlation between colony-productivity, as measured by the production of sexual offspring, and the prevalence of *Wolbachia* within the worker caste. Interestingly, this result contrasts with the apparent effect of *Wolbachia* in the ant *Formica truncorum,* where a negative correlation between the production of sexual offspring and the prevalence of *Wolbachia* was identified (Wenseleers et al., 2002).

Making the best of this approach clearly requires the identification of a species in which *Wolbachia* is present or absent at the level of the colony. In Chapter 3, I first showed that the common Palaearctic ant *Myrmica scabrinodis* fits this description; in approximately 80% of colonies all individuals appear to be infected, whilst the remaining 20% of colonies are entirely free from infection. However, the

subsequent collection and censusing of colonies did not reveal any significant differences between infected and uninfected colonies in terms of sex-ratio or a range of productivity-related measures. Whilst this may have been partly due to the difficulty of detecting small effects in the face of substantial natural varation in these measures, there certainly appeared to be no marked effect of infection on colony sex-ratios.

The collection of sympatric infected and uninfected colonies also allows antibiotic treatment experiments to be conducted on infected colonies with a proper control. Were only infected colonies available, it would be impossible to say whether any observed effects of antibiotics were a result of their interaction with *Wolbachia,* other constituents of the host microbiome, mitochondria, or a combination thereof. In the case of *M. scabrinodis*, I observed that, whilst antibiotics substantially reduced the production of new larvae across queenright sub-colonies, this effect was not specific to infected sub-colonies, but also occurred to a similar magnitude in uninfected sub-colonies. I interpret this as evidence that *Wolbachia* does not cause strong female-mortality type mating incompatibilities in *M. scabrinodis.* If this strain of *Wolbachia* caused mating-incompatibilities, antibiotic treatments will likely cause infected queens to produce uninfected eggs, and if these queens have mated with an infected male, a proportion of these eggs will fail to hatch. The resulting shortage of eggs would lead to a reduced production of

larvae in infected colonies, however this effect was not observed in my experiments.

The advantage of this experimental approach was its simplicitly, but it would certainly be possible to build upon this work in order to provide further support for this interpretation of the results. The assumption that antibiotics inhibit transmission of *Wolbachia* to eggs would certainly be worth testing through qPCR assays of *Wolbachia* density in eggs. Furthermore, providing antibiotics to colonies infected with CI-inducing *Wolbachia* should also affect secondary sex-ratios, given that fertilised (and thus female-destined) eggs will either die (female mortality type CI) or develop into males (male-development-type CI). In future, secondary sex-ratios could be assessed morphologically or genetically to provide futher support for this interpretation.

In Chapters 2 and 3, I implicitly assumed that the phenotypic effects of *Wolbachia* in social insects mirror those observed in solitary insects. Of course, social insects differ considerably from their solitary counterparts in many aspects of their development and life-history, and so it would be not be surprising to find effects of *Wolbachia* that are unique to social insects. In Chapter 4, I discussed the possibility that maternally transmitted symbionts such as *Wolbachia* may evolve to bias the development of social insects, causing female worker-destined larvae to preferentially develop into reproductives. Whilst this possibility has been

suggested before (Bourke & Ratnieks, 1999; Wenseleers, 2001), I provide an in depth discussion of this scenario, outlining three distinct reasons why maternally transmitted symbionts might evolve to bias the development of social insect hosts. I also provide suggestions for experimental approaches to identifying caste biasing symbionts in social insects.

Finally, in Chapter 5 I showed that the effects of *Wolbachia* can extend to other bacterial symbionts; *M. scabrinodis* workers infected with *Wolbachia* were significantly less likely to be infected with both *Spiroplasma* and *Arsenophonus*. This may occur as a result of interference competition between symbionts or because of selection on particular combinations of symbionts (Goto et al., 2006; Vautrin & Vavre, 2009; Ferrari & Vavre, 2011). More sensitive qPCR assays of bacterial density would help determine whether *Wolbachia* affects the density of different symbionts within host tissues as well as their presence or absence, as might be expected if such negative correlations occur as a result of intereference competition. Furthermore, whilst I attempted to assess the phenotypic effects of *Wolbachia* in Chapter 3, I collected no data concerning the effects of *Spiroplasma* or *Arsenophonus* on *M. scabrinodis*. Future work assessing the phenotypic effects of these symbionts would thus help in determining the evolutionary importance of these negative correlations between *Wolbachia, Spiroplasma* and *Arsenophonus*.

It could be argued that the negative correlations between *Wolbachia* and other symbionts undermine confidence in the findings of Chapter 3 regarding the effects of antibiotic treatment on larval production. Uninfected colonies were used as controls based on the assumption that the only difference between infected and uninfected colonies was the presence or absence of *Wolbachia*, but data from Chapter 5 show that differences in the frequency of other symbionts may occur in relation to the presence or absence of *Wolbachia.* However, it is important to note that whilst the negative correlations between *Wolbachia* and both *Spiroplasma* and *Arsenophonus* are statistically significant, the actual magnitude of the difference in their prevalence between infected and uninfected colonies is relatively small. For instance, whilst almost 100% of individuals free from *Wolbachia* are infected with *Spiroplasma,* roughly 80% of *Wolbachia* infected individuals were still infected with *Spiroplasma.* The results of the antibiotic treatment experiments are thus best explained through a *Wolbachia* mediated effect.

8.2 The movement of *Wolbachia* between species

Wolbachia is remarkably widespread, both in terms of the number of species it infects, and the taxonomic breadth of its hosts. A mixture of phylogenetic evidence

and some observational and experimental data clearly indiciate that the prevalence of *Wolbachia* and the diversity of its hosts are accounted for by the transmission of *Wolbachia* between species, either by hybrid introgression or horizontal transmission (Werren et al., 1995, 2008; Huigens et al., 2004; Raychoudhury et al., 2009). The second main goal of this thesis was to use ants as a system to better understand the movement of *Wolbachia* between species.

In Chapter 5, I strain-typed *Wolbachia* infections from across the range of *Myrmica scabrinodis*. Single populations generally harboured a single strain of *Wolbachia* or were uninfected, but across the range of the species I identified three phylogenetically distinct strains of *Wolbachia* that were likely acquired independently. Studies based on one or a small number of populations may thus underestimate the frequency of transmission events of *Wolbachia*. It is entirely possible that the screening of additional populations and the use of less conserved molecular markers might reveal additional strains of *Wolbachia* in *M. scabrinodis.*

In Chapter 6 I attempted to uncover how *Wolbachia* was acquired by the ant *Monomorium pharaonis*. Previous studies have shown that this species is infected with two strains of *Wolbachia* (Schmidt, 2010), and two genetically distinct mitochondrial haplotypes have also been isolated from this species (Frouz et al., 2009). I demonstrated that one of two mitochondrial haplotypes is exclusively associated with one strain of *Wolbachia,* whilst the second mitochondrial haplotype

is associated either with individuals infected by the second strain of *Wolbachia* or individuals free from infection. This linkage disequilirium between *Wolbachia* and mitochondria in *M. pharaonis* strongly suggests that one of the two strains of *Wolbachia* was acquired by hybrid introgression. In the past, *Wolbachia* has attracted considerable attention as a potential agent of speciation through its ability to cause reproductive isolation between populations. However, a growing number of studies have provided genetic evidence for the natural introgression of *Wolbachia* between related species; perhaps then, *Wolbachia* is just as important in breaking down boundaries between species by driving heterospecific mitochondrial DNA, and possibly also nuclear genes, into other species.

However, separate from the issue of how *Wolbachia* crosses the boundary between two species is the matter of how it succeeds in spreading following its establishment in a novel host. During its initial establishment, the frequency of infected hosts is likely to be extremely low, but strains of *Wolbachia* that cause mating incompatibilities are thought to be selected against at low frequencies if the infection carries a physiological cost or is imperfectly transmitted from mothers to offspring (Egas et al., 2002; Jansen et al., 2008). Theoretical work suggests that factors reducing host effective population size, such as population subdivision or population viscosity, may negate this effect and allow costly CI strains to spread from low initial frequencies (Stouthamer et al., 1999; Egas et al., 2002; Jansen et al.,

2008; Reuter et al., 2008). In Chapter 7, I showed that morphological and life-history traits which enhance population viscosity are significantly related to the incidence of *Wolbachia* in ants, providing support for this theory. Further evidence for the role of host effective population size in the spread of *Wolbachia* could be obtained by assessing the relationship between dispersal limitation and the incidence of *Wolbachia* in other taxa. Loss of flight has occurred many times across insects (Wagner & Liebherr, 1992), and broader comparative studies would help establish the generality of my findings. Additionally, experimental studies involving the manipulation of host population size and migration rates and the introduction of CI-inducing *Wolbachia* into laboratory or field populations would provide another useful perspective on this theory.

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10 Appendices

10.1 Supplementary references for Chapter 7

10.1.1 *Wolbachia* incidence

Data regarding the incidence of *Wolbachia* in ants were acquired from the following publications:

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