The evolutionary ecology of host-parasite interactions between *Drosophila* and *Spiroplasma*

Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of Doctor in Philosophy by Katherine Jane Hutchence

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Abstract

Arthropods commonly harbour maternally inherited endosymbionts which have a range of effects on their hosts. Phylogenetic evidence indicates the importance of occasional horizontal transmission in the establishment of new host-symbiont combinations. These events represent both a widened host range for the symbiont and a macromutation event for the host, thus influencing both host and symbiont evolution. In this thesis, I utilise the bacterial symbiont *Spiroplasma* and the fruit fly host *Drosophila melanogaster* to investigate the factors that are important in the establishment of infections in new host species. On examining two novel non-male-killing *Spiroplasma* strains in *D. melanogaster*, higher vertical transmission efficiency was found in the strain of *Spiroplasma* more closely related to the strain found natively in *D. melanogaster* than in a more distantly related strain and a cost of infection was observed in both cases. Symbiont vertical transmission efficiency did not increase on repeated passage, indicating that this trait may not be an initial target of selection. Transmission, timing and completeness of male-killing in *D. melanogaster* were compared between a male-killing *Spiroplasma* found naturally in the species, and a closely related strain transinfected from *D. nebulosa*. The native strain showed more efficient vertical transmission than the introduced strain, but there was no difference in male-killing ability between the two strains. On examining the above novel and native *Spiroplasma* infections in *D. melanogaster* using microarray technology, no up-regulation in host immune responses was observed in any of the *Spiroplasma* infections investigated. Hosts carrying the *Spiroplasma* strain that showed the weakest vertical transmission showed no significant disturbance to gene expression compared to uninfected controls, indicating that the poor performance of *Spiroplasma* is not due to a host response. A survey of *Drosophila* species from biodiverse regions found 43 of 412 individuals sampled to be infected with *Spiroplasma* (10.44%). Infected individuals represented 4 species groups (*saltans, melanogaster, willistoni, cardini*) and the first known case of *Spiroplasma* infection in the *saltans* group. This thesis ends with an assessment of the factors determining success and failure of novel infections. I argue that horizontal transmission is not only most successful when hosts are closely related but also where strains are more closely related to any resident strain. Future work should test the robustness and generality of this hypothesis.

Chapter 1

Introduction

1.1 The evolutionary ecology of inherited symbionts

Observations in plant genetics by Carl Correns from as far back as 1909 indicated the presence of traits in organisms that were inherited solely through the cytoplasm (Correns, 1909). For 70 years, cytoplasmic traits were acknowledged in the genetic literature, but beyond presenting interesting mutations (e.g. petite mutations in yeast, variegation in plants (Correns, 1909, Ferguson and Vonborstel, 1992)) were largely ignored by evolutionary ecologists (but see Birky, (1978)). In contrast, the last 20 years have seen an explosion of interest in cytoplasmically inherited traits, particularly from scientists working on arthropod biology and ecology. This increase in interest has followed the recognition that cytoplasmically inherited traits are not simply encoded in chloroplast or mitochondrial DNA, but also in microbial symbionts that are inherited in the same way as chloroplasts and mitochondria, that these symbionts are common and alter host biology in a number of ways.

Historically, the observation that arthropods carried maternally inherited microbes came from isolated examples in the literature. Evidence of inherited microbe presence was of two types. The first found the causes of interesting phenotypes to be maternally inherited. Examples include the discovery of distortion of the sex ratio by maternally inherited agents in woodlice (Vandel, 1941), flies (Malogolowkin, 1958, Cavalcanti *et al.*, 1958, Magni, 1952) and ladybirds (Lus, 1947) and the discovery of maternally inherited compatibility types in mosquitoes (Laven, 1951). The second type of study was more directed, and derived from the observations of insect morphologists. These workers identified organs carrying large numbers of bacteria within the body of insects. The most significant contribution here is the detailed descriptions of symbioses made by Paul Buchner in his seminal text 'Endosymbioses of animals with plant microorganisms', in which he documented the anatomy and biodiversity of insect-microbe interactions (Buchner, 1965).

The observations of interesting maternally inherited phenotypes and interesting anatomy in insects containing microbes continued until the early 1990s. At this point, the invention of the PCR process revolutionized our understanding of the frequency and biodiversity of these symbionts. Inherited microbes are commonly very difficult or impossible to culture. Finding an inherited microbe previously required either detailed microscopy or investigation of the causal nature of a phenotype uncovered by chance in the laboratory. PCR allowed the detection of symbionts with relative ease from samples for which there was no information other than which arthropod species it belonged to. An early finding was the presence of *Wolbachia* in 16% of species (Werren *et al.*, 1995a). PCR also allowed the taxonomic affiliation of microbes to be ascertained, through the sequence of 16S rRNA genes (Woese, 1989). Prior to this, the taxonomy of inherited microbes was very poorly resolved, and based on morphological features (e.g. presence inside or outside a vacuole) that were later revealed to be analogous rather than homologous traits, and thus unhelpful in identifying the relationship between microbes. DNA sequence based systematics has revealed symbionts to be biodiverse.

In this introduction, I will first establish why heritable symbionts are an important aspect of the biology of arthropods. I will then argue that aside from maternal inheritance, one particular aspect of the interaction between host and symbiont makes them of particular interest in terms of our understanding of arthropod evolutionary ecology: this is the importance of horizontal transmission of symbiont infection in establishing new symbioses. A central tenet driving this thesis is that this process is important both at the level of the host that becomes infected (a new symbiont-encoded trait evolves as a macromutation) and for the success of a symbiont (the number of host species a microbe infects is in part associated with its success in horizontal transmission). I then describe the *Drosophila-Spiroplasma* interaction which lends itself to study of the factors causing success and failure of new infections, before outlining the specific objectives of my thesis.

1.2 The importance of heritable endosymbionts in the biology of arthropods

Endosymbionts show a diverse range of phenotypes that affect host ecology and evolution. The relationship between heritable bacteria and their hosts varies considerably, from mutualism to highly specialised parasitism. Much of this can be explained by the mode of transmission of the organism. Where the transmission pattern of a parasite is vertical, it will share the same interests as the host. It is advantageous for both if the host produces as many offspring as possible, increasing the genetic continuity of both host and symbiont. However, heritable bacteria are transmitted only maternally and are unable to transmit onward if they find themselves in a male. Their interests therefore lie only in the production of daughters by the host. This is a source of conflict with the host and can result in reproductive parasitism: the manipulation of host reproduction towards the production or survival of infected females alone (Cosmides and Tooby, 1981).

Maternally inherited symbioses can therefore be classified into three kinds along a mutualism-parasitism spectrum. First, there are obligate symbioses, where the primary symbiont is required for host survival and/or fertility. Second, there are secondary symbioses, where the symbiont is not required for host function, but is beneficial under certain ecological circumstances. Third, there are secondary symbioses where the symbiont is not required for host function, but propagates through manipulation of host reproduction towards the production or survival of infected daughters. The symbionts in these cases are termed reproductive parasites.

Cases of obligate symbiosis are summarised in Table 1.1. This class of symbiosis has been most intensively studied in aphids (*Aphidoidea*), which harbour the maternally inherited endosymbiont *Buchnera aphidicola*. The host and bacteria have co-evolved together for a substantial period of their evolutionary history (>200Ma) and co-cladogenesis of several aphid families with their corresponding *Buchnera* has been revealed by phylogenetic analysis (Munson *et al.*, 1991). Both the host and symbiont have become dependent on the other for

their continued survival. The bacteria provide aphids with improved nutrition in the form of essential amino acids and possibly vitamins lacking in a phloem diet (Douglas, 1989, Douglas and Prosser, 1992). Aphids suffer stunted growth, sterility and premature death after treatment with antibiotics (Houk, 1987). This relationship is fundamental to the ecology of the host, as the symbiont has opened up new niches for the host to exploit, affecting its geographical range and potentially its biodiversity.

Buchnera lives in specialised cells within the host body which provide it with metabolites and protection. Over time it has lost the faculties needed to achieve independent life: the relevant genes have been disabled and the genome size has been reduced (Wernegreen, 2005). This can be seen when comparing the genome of a commensal bacterium with frequent horizontal transmission such as *E. coli* K-12, with a genome size of 4.37MB, to that of *Buchnera*, which is a mere 640KB (Shigenobu *et al.*, 2000). *Buchnera* bacteria are completely dependent upon their host for survival and have lost the capacity to horizontally transfer.

Cases in which the bacterial symbionts may not be fully obligate or mutualistic but still provide the host with an advantage under certain ecological circumstances are summarized in Table 1.2. The advantage produced by the symbiont commonly comes in the form of symbiont-mediated resistance to pathogens, parasites, or even predators. For example, *Regiella* symbionts of aphids provide their host with resistance to the fungal pathogen *Pandora neoaphidis* (Scarborough *et al.*, 2005) and symbiont-encoded resistance to fungi has also been observed in the crustaceans *Palaemon macrodactylus* and *Homoarus americanus* (Gilturnes *et al.*, 1989, Gilturnes and Fenical, 1992). Symbiont-encoded resistance to parasitic wasp attack is also probably widespread. A non-male-killing *Spiroplasma* infection of *Drosophila hydei* provides the host with defence against parasitoid wasps (Xie *et al.*, 2010) and two facultative symbionts protect their pea aphid host from attack by two parasitoid species (Oliver *et al.*, 2003, Oliver *et al.*, 2005, Ferrari *et al.*, 2004). Recent work has also revealed *Spiroplasma*-mediated resistance to parasitisation by *Howardula* nematodes in *Drosophila neotestacea* (Jaenike *et al.*, 2010). Perhaps most remarkably, symbionts may also produce protection against predation: *Pseudomonas* endosymbionts in *Paederus* beetles produce the toxin pederin that deters wolf spiders from predating the beetle in its larval stage (Kellner, 1999, Kellner, 2001, Kellner, 2002, Piel, 2002, Haine, 2008).

The above two sets of symbiosis represent cases where the spread of a microbe is driven by enhancing the fitness of its host. As noted previously, heritable bacteria were first discovered as sex ratio distorting organisms and it is for their reproductive parasitism phenotypes that they are most well known. Because these organisms are maternally inherited, males represent an evolutionary 'dead end'. To the symbiont, it is thus more advantageous for their host to produce as many daughters as possible. To this end heritable bacteria employ a wide array of strategies to maximise the production of females, at the disadvantage of the host, resulting in antagonistic co-evolution. A summary of the biodiversity of reproductive parasites and their phenotypes can be found in Table 1.3.

Many examples of reproductive parasitism can be seen in the single inherited microbe *Wolbachia*. A common adaptation is that of male-killing, where the male offspring of an infected female are exterminated at the embryonic stage (Hurst, 2003). This distorts the sex ratio of an infected population in favour of females and in some cases, such as the South Pacific butterfly *Hypolimnas bolina*, has reached such extremes as a 100:1 population sex ratio (Charlat *et al*., 2005). *Wolbachia* is also capable of inducing cytoplasmic incompatibility, which occurs when an infected male mates with an uninfected female and cannot produce viable offspring due to the effect of the infection in the male. It is speculated that this can drive reproductive isolation and thereby lead to speciation (Bordenstein *et al*., 2001). *Wolbachia* can also induce parthenogenesis in some species (Stouthamer *et al*., 1993) and feminisation in others (Negri *et al*., 2006). By inducing sex ratio distortion, these microbes alter the evolutionary ecology of reproduction (Charlat *et al*., 2007b, Jiggins *et al*., 2000b, Moreau and Rigaud, 2003), may drive sex determination system evolution (Rigaud, 1997) and engender strong selection on the host to suppress their action. The combination of mortality with sex ratio distortion makes them amongst the strongest drivers of natural selection in natural populations (Charlat *et al*., 2007a).

The above studies present a strong case that inherited microbes are an important feature of the species that carry them, either as obligate partners, facultative partners, or as inherited parasites. A full appraisal of the importance of inherited microbes also requires comment as

to the proportion of species that are infected with these microbes and the diversity of the microbes that are found. In terms of the latter issue, inherited microbes are very diverse. Inherited bacterial symbiont diversity is given in Table 1.1-1.3. In summary, microbes that are vertically transmitted in insects derive from diverse and distant bacterial groups. The Enterobacteriacae (a subgroup of the gamma-proteobacteria) provides very many examples of bacteria that have evolved from pathogen or commensal to being an inherited symbiont. However, inherited symbionts have also emerged from free living or non-inherited relatives on more than one or more occasions in the alpha-proteobacteria, the beta-proteobacteria, the Bacteroidetes or Flavobacteria group and the highly diverse genus *Spiroplasma*. Eukaryotes have also evolved to be inherited symbionts of arthropods, notably members of the Microspora (Terry *et al.*, 2004) and fungi (Gibson and Hunter, 2010).

The frequency with which insects are infected with symbionts is not known, but can be estimated from survey data. Perhaps the best known is *Wolbachia pipientis*, which has been found in screens to infect around 16-20% of all insect species worldwide, as well as 40% of all mite and spider species and many terrestrial isopods and filarial nematodes (Werren *et al.*, 1995a, Engelstadter, 2007). Recent studies are revealing other less well known inherited bacteria such as *Cardinium*, *Spiroplasma*, *Rickettsia* and *Arsenophonus*, to be common. For example, a survey by Duron *et al.*, (2008) sampled a wide range of arthropod species in Western Europe and found 32.4% of species to be infected by inherited bacteria. 22.8% were infected with *Wolbachia*, 6.6% with *Spiroplasma ixodetis*, 4.4% *Arsenophonus* and 4.4% *Cardinium* (N. B. these figures exceed 32.4% as some species are infected with more than one symbiont species). The above screens vary in the intensity of investigation. In surveys where 16-20% of species sampled were found to be infected with *Wolbachia* one or few individuals had been sampled as the token for a species. The survey of Duron *et al* utilized 10-20 individuals per species and produced an average estimate of 22.4% individuals infected per species. It is clear that if inherited microbes infect a fraction of the population, then the total number of species infected will be underestimated if just one or a few individuals are taken to represent the species (Jiggins *et al.*, 2001). Hilgenboecker *et al* (2008) noted this and suggested that the best realistic estimate of *Wolbachia* incidence is 60% of species, but with many species carrying infections at low prevalence.

The above surveys have been conducted for the better known inherited bacteria; however other bacteria are known to have evolved to be heritable. There are many cases of microbes which are secondary or primary symbionts that are not present in a wide range of insects, but are locally common. *Arsenophonus nasoniae*, for instance, is not globally common but is present in 30% of chalcid wasp species in the filth fly community (Duron *et al.*, 2010). Members of the genus *Spiroplasma* are very widespread in insects and individual records indicate they are quite commonly inherited infections. However, because not all *Spiroplasma* are inherited (Whitcomb, 1980), screen results simply cannot reveal the incidence of inherited infections in this genus: this can only be revealed by following the results of a screen with a detailed study to determine the presence of vertical transmission, thus the current estimate of the number of species infected with inherited bacteria is likely to be an underestimate. It is likely the majority of insect species are infected with inherited microbes.

Table 1.1: The biodiversity, distribution and phenotypes of primary symbionts of insects (as defined by the administration of antibiotics rendering the host unviable or infertile, and/or showing co-cladogenesis with host).

Bacterium & class	**Distribution**	**Phenotype(s)**	**Reference**
Buchnera aphidicola, γ–proteobacteria	Most aphids	Synthesis of essential amino acids	(Baumann, 2005)
Wigglesworthia, γ–proteobacteria	Tsetse flies	Synthesis of B vitamins	(Aksoy, 1995)
Blattabacterium, Flavobacteria	All Termites, cockroaches	Nitrogen metabolism	(Lo *et al.*, 2003)
Wolbachia (clades C and D), α–proteobacteria	Most filarial nematodes	Unknown	(Bandi *et al.*, 1998)
Blochmannia, γ–proteobacteria	*Camponotus* ants	Unknown	(Degnan *et al.*, 2004)
Unnamed (known as SOPE), γ–proteobacteria	*Sitophilus* weevil	Unknown	(Heddi *et al.*, 1999)
Baumannia, γ–proteobacteria	Sharpshooters	Unknown	(Baumann, 2005)
Tremblaya, β–proteobacteria	Mealybugs	Unknown	(Baumann, 2005)
Carsonella, γ–proteobacteria	Psyllids	Unknown	(Baumann, 2005)
Portiera, γ–proteobacteria	Whiteflies	Unknown	(Baumann, 2005)
Reisia, γ–proteobacteria	Lice	Synthesis of B vitamins	(Allen *et al.*, 2007)

Table 1.2: The biodiversity, distribution and phenotypes of inherited secondary symbionts that are facultatively beneficial (as defined by absence of reproductive parasitism, but viable uninfected host and lack of co-cladogenesis).

Bacterium & class	**Distribution**	**Phenotype(s)**	**References**
Wolbachia, α-proteobacteria	*Drosophila*	Resistance to RNA virus attack	(Hedges *et al.*, 2008, Teixeira *et al.*, 2008)
Sodalis glossinidus, γ–proteobacteria	Tsetse flies	Unknown; protects against trypanosome infection	(Toh *et al.*, 2006)
Rickettsia sp. α–proteobacteria	Aphids, others	Unknown	(Chen *et al.*, 1996)
Hamiltonella defensa, γ–proteobacteria	Aphids	Resistance to parasitoid infection	(Oliver *et al.*, 2003, Oliver *et al.*, 2005, Ferrari *et al.*, 2004)
Regiella insecticola, γ–proteobacteria	Aphids	Resistance to fungal pathogens	(Ferrari *et al.*, 2004, Scarborough *et al.*, 2005)
Serratia symbiotica, γ–proteobacteria	Aphids	Resistance to parasitoid infection	(Oliver *et al.*, 2003, Oliver *et al.*, 2005, Ferrari *et al.*, 2004)
Arsenophonus, γ–proteobacteria	5% of arthropods	Unknown; correlation of infection frequency with parasitisation observed. Common, but not required, in blood sucking triatomine bugs, suggest B vitamin role.	(Hansen *et al.*, 2007, Dale and Moran, 2006)
Spiroplasma ixodetis, Mollicutes	Aphids, psyllids	Unknown	(Fukatsu *et al.*, 2001)
Spiroplasma poulsoni strain, Mollicutes	*Drosophila hydei, Drosophila neotestacea*	Resistance to parasitoid and nematode infection	(Jaenike *et al.*, 2010, Xie *et al.*, 2010)

Table 1.3: The biodiversity, distribution and phenotypes of inherited symbionts displaying reproductive parasitic phenotypes.

Bacterium & class	Distribution	Phenotype(s)	References
Wolbachia pipientis, α–proteobacteria	>20% arthropods	CI, Feminization, Parthenogenesis induction, Male-killing	(Engelstadter and Hurst, 2009a)
Cardinium hertigii Flavobacteria/Bacteroidetes	5% arthropods	CI, Feminization, parthenogenesis induction	(Hunter, 2006)
Rickettsia, α–proteobacteria	Wide variety of arthropods	Parthenogenesis induction, Male-killing	(Perlman *et al.*, 2006)
Arsenophonus nasoniae, γ–proteobacteria	*Nasonia vitripennis* (Hymenoptera, Pteromalidae)	Male-killing	(Skinner, 1985)
Spiroplasma ixodetis, Mollicutes	Ladybirds, butterflies	Male-killing	(Hurst, 2003, Hurst *et al.*, 1999b, Jiggins *et al.*, 2000a)
Spiroplasma poulsonii, Mollicutes	Variety of *Drosophila* species	Male-killing	(Hurst, 2003, Williamson *et al.*, 1999)
Flavobacteria/Bacteroidetes (unnamed)	Ladybirds, butterflies	Male-killing	(Hurst *et al.*, 1999a)
Microspora	Shrimps	Feminization, late male-killing	(Agnew, 2003)
Unnamed Gammaproteobacteria	*Cheiolemenes sexmaculatus* Ladybirds	Male-Killing	(Majerus and Majerus, 2010)

1.3 The importance of horizontal transmission in the evolutionary ecology of symbiont-host interactions

The above argues that heritable symbionts are very important aspects of insect evolutionary ecology, both as parasites and partners. It also explains the conditions under which heritable symbionts spread within species, and persist in populations despite imperfect transmission efficiency. They are either directly advantageous to their host, and so the infected host number increases by selective advantage, or they manipulate host reproduction to increase their own transmission. Less understood are the conditions that produce the establishment of new infections between species. As mentioned above, heritable endosymbionts are prolific. In contrast to their frequency is the short tenure of secondary symbionts within particular host species. Despite being maternally inherited, co-speciation of symbiont and host is rarely seen outside of primary symbioses. A selection of studies demonstrating lack of co-cladogenesis is summarised in Table 1.4.

Table 1.4: A selection of studies where symbiont and host groups were found to not show co-cladogenesis, inferring horizontal transfer of infection.

Arthropod group	Microbe	Observation	Reference
Fig wasps	*Wolbachia*	*Wolbachia* very common, but very rarely shared by closely related species	(Shoemaker *et al.*, 2002)
Leaf miner community	*Wolbachia*	*Wolbachia* strains from the same host genus were not closely related	(West *et al.*, 1998)
31 insect species, 1 isopod	*Wolbachia*	Single strains of *Wolbachia* found in many disparate taxa	(Werren *et al.*, 1995b)
6 insect species	*Wolbachia*	Closely related *Wolbachia* strains found in distantly related hosts	(Oneill *et al.*, 1992)
9 *Drosophila* species	*Spiroplasma*	Multiple introductions of *Spiroplasma* strains found in *Drosophila* hosts	(Haselkorn *et al.*, 2009)

Thus, whilst symbionts are characterised by maternal transmission on a population biological timescale, horizontal transmission does occur and is a key element of heritable endosymbiont biology over evolutionary timescales. Horizontal transmission rates are thus the determinant of the frequency of symbiont infections amongst species. Observations of an absence of co-cladogenesis inspired experimental studies investigating whether ecologically realistic exposure of an uninfected member of one species to an infected member of another resulted in transmission of infection between the species (summarised in Table 1.5). Rigaud and Juchault, (1995), for instance, demonstrated that close contact between wounded woodlice individuals can result in *Wolbachia* transfer via the haemolymph. Many invertebrates, including woodlice, live in dense aggregations where close contact is likely. Later studies have demonstrated wounding to be common in this species and is speculated to be an overlooked phenomenon in arthropods (Plaistow *et al.*, 2003). In the case of parasitoid wasp hosts, who lay their eggs within the offspring of a fly as a food source for their larvae, transfer of *Wolbachia* (Huigens *et al.*, 2004, Huigens *et al.*, 2000) and *Arsenophonus* (Duron *et al.*, 2010, Skinner, 1985) has been shown to be possible from infected to uninfected wasp larvae when the same food source is shared, or in one case from a *Wolbachia* infected *Drosophila* host to an uninfected parasitoid wasp (Heath *et al.*, 1999). Endosymbionts can also be transmitted sexually as has been demonstrated in the pea aphid (*Acyrthosiphon pisum*) (Moran and Dunbar, 2006). Ectoparasitic mites have been demonstrated experimentally to be potential vectors, transferring male-killing *Spiroplasma* both within and between *Drosophila* species (Jaenike *et al.*, 2007).

Table 1.5: A review of empirical studies which examine the presence or absence of different routes of horizontal transmission for maternally inherited bacteria.

Route of transfer	Host species	Bacterium	Inter or intra-specific?	Notes	References
Shared larval host	*Trichogramma*	*Wolbachia*, causing parthenogenesis	Both	Intraspecific transmission with high fidelity. Interspecific transmission at lower rates, and infection was not transmitted beyond the third generation.	(Huigens *et al.*, 2004, Huigens *et al.*, 2000)
Shared larval host	*Nasonia vitripennis*	*Arsenophonus*, causing male-killing	Both	Larval host sharing relatively common in nature, and can occur with other species. High success of horizontal transmission in the laboratory both intra- and inter-specific.	(Duron *et al.*, 2010, Skinner, 1985)
Vectored by ectoparasitic mite, *Macrocheles*	*Drosophila nebulosa & willistoni*	*Spiroplasma* causing male-killing	Both	Transmission efficiency in new host species was low & MK efficiency was low	(Jaenike *et al.*, 2007)
Sexually transmitted from paternal to maternal line	Pea aphid *Acyrthosiphon pisum*	Candidatus *Regiella insecticola*, *Hamiltonella defensa*, *Serratia symbiotica*	Intra	Double infection possible by this mechanism. Speculated that mating between species would produce occasional inter-specific transmission. Infection stable.	(Moran and Dunbar, 2006)
Contact between wounded individuals	*Armadillidium vulgare*	*Wolbachia*	Intra	Dense aggregations and wounding common, so likely natural mechanism of transfer in wild	(Rigaud and Juchault, 1995)
Host-parasitoid	*Drosophila simulans - Leptopilina boulardi*	*Wolbachia* causing CI	Inter	Very rare event, and did not persist beyond the second generation.	(Heath *et al.*, 1999)

The rate at which symbionts transfer between host species varies depending on the symbiont species and in particular the type of symbiosis, i.e. whether they are obligate and beneficial, facultatively beneficial, reproductive parasites, or pathogenic (see Figure 1.1). Obligate beneficial symbionts (primary symbionts required for host function) have lost their ability to transmit horizontally, which can be seen in that the symbiont phylogeny is concordant with that of the host (Figure 1.1a). Secondary symbionts, which may be facultatively beneficial or reproductive parasites, vary in the rate at which they spread laterally. Aphid secondary symbionts (Oliver *et al.*, 2010), and the reproductive parasite *Arsenophonus nasoniae* (Duron *et al.*, 2010) transfer commonly in nature (Figure 1.1c), whereas other secondary symbionts, such as *Wolbachia* and *Cardinium*, show intermediate rates of lateral transfer (Figure 1.1b) (Baldo *et al.*, 2008, Russell *et al.*, 2009).

The movement of a symbiont from one host species into another can have dramatic effects on the new host species. In some cases, phenotype simply transfers. Here, the symbiont in the new host imbues this host with the same phenotype observed in its source. A known sex-ratio distorting microsporidian in the crustacean *Gammarus duebeni* caused feminisation of male offspring after intraspecific transfer to previously uninfected female hosts (Dunn and Rigaud, 1998) and parthenogenesis was found to be induced on transfer of *Wolbachia* from infected into uninfected *Trichogramma kaykai* hosts (Huigens *et al.*, 2000). Cytoplasmic incompatibility caused by *Wolbachia* has been transferred intraspecifically by artificial infection of *Drosophila simulans* eggs (Boyle *et al.*, 1993) and interspecifically from *D. simulans* to *D. mauritania* (Giordano *et al.*, 1995) and from *D. simulans* to *D. serrata*, although the latter also produced some negative fitness effects in its new host (Clancy and Hoffmann, 1997).

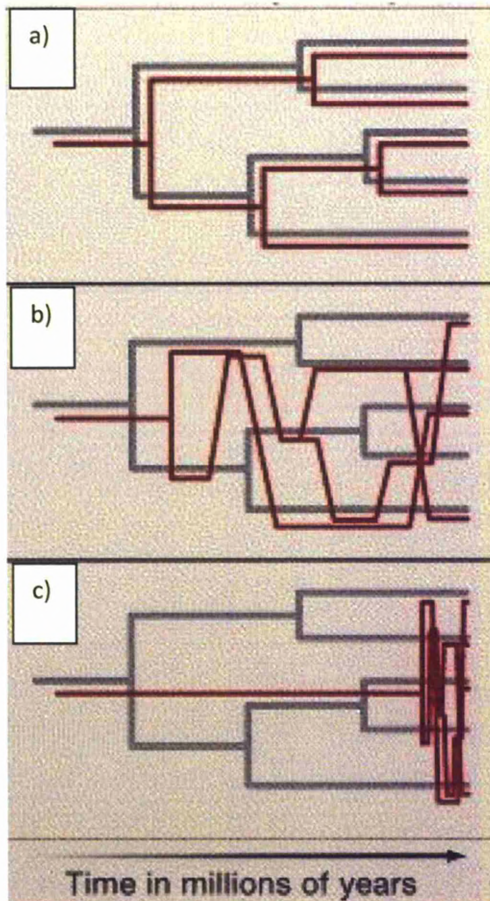

Figure 1.1: An illustration of the extent of concordance between host (grey line) and symbiont (red line) phylogenies over evolutionary time:

a) Co-cladogenesis, where a host and symbiont have co-evolved developing an obligate beneficial relationship. This is exemplified by *Buchnera*-aphid interaction.

b) Partially discordant phylogeny, where a symbiont is able to occasionally transmit horizontally and establish in a new species. This is typical of *Wolbachia*-insect interactions

c) Completely discordant phylogeny, where a symbiont moves frequently between host species. This is typical of *A. nasoniae* in chalcid wasps, and secondary symbionts of aphids.

Figure adapted from Dale and Moran (2006).

In other cases, the phenotype retains the same quality as in the original host, but the strength of the phenotype is different. For instance, transfer of *Wolbachia* strain wMel from *D. melanogaster* (ancestral host) to *D. simulans* is associated with a strengthening of the CI phenotype from weak (10% of progeny killed in incompatible crosses) to strong (>99% of progeny killed) (Boyle *et al.*, 1993). Interspecific transfer of traits has also been shown on the artificial transfer of a male-killing *Spiroplasma* from the coccinellid beetle *Adalia bipunctata*, which induced successful male-killing in recipient hosts of the same genus and imperfect male-killing in hosts in more distantly related recipients (Tinsley and Majerus, 2007).

There are also cases where the phenotype exhibited by the symbiont shows a change in quality when it is moved to a new host. Movement of *Wolbachia* strains causing CI in *Ephestia cautella* into *Ephestia kuehniella* was associated with the emergence of male-killing (Sasaki *et al.*, 2002). This is mirrored in the emergence of male-killing when CI *Wolbachia* strains are moved from *D. recens* into its sibling species, *D. subquinaria* (Jaenike, 2007). These cases are likely associated with escape from evolved suppression of male-killing (Hornett *et al.*, 2006). In the natural host, male-killer suppression has evolved and the sex ratio distorting phenotype is no longer seen. It then emerges in the new host, which has not previously evolved to suppress the male-killer.

The ability of symbionts to move into new host species potentially alters the evolutionary ecology of adaptation. Lateral transfer is a form of mutation in that it is a heritable genetic change, but it is dissimilar in form from the mutations usually seen in studies of eukaryotic evolution (Oliver *et al.*, 2010). A new symbiont infection represents a complex assemblage of genes that arrives in its new host as one package and may have already been providing, for example, natural enemy defence in its previous host species. This trait of defence will have evolved gradually in the symbiont over time. However in a lateral transfer event substantial protection of a particular host can arise instantaneously. Thus, the nature of the phenotype that is transferred and the magnitude of its selective advantage are likely to make it different from mutation as we usually think of it. An additional difference is in the mutation rate, usually defined as the chances of a mutation occurring in an individual or gene on reproduction, for lateral transfer the mutation rate is the rate at which an individual lacking a particular symbiont is infected by one through lateral transmission. This is not to say, as claimed by some (Goldenfeld and Woese, 2007), that laterally transferred traits such as those encoded by symbionts are non-Darwinian, only that it can be noted that the mutation rate and the distribution of selective coefficient are distinct in these cases and therefore determine a different pattern and tempo of evolution through natural selection (Hurst and Hutchence, 2010).

When a beneficial phenotype, or a phenotype that promotes the production of daughters over sons, is retained in new host species it will promote the maintenance and spread of that symbiont in a novel host population. However, in order to spread through a new population, a symbiont must also have successful vertical transmission to the next and subsequent generations. The ability of a symbiont to thrive when it finds itself in a new host will dictate its ability to spread. If the symbiont is able to spread, its equilibrium prevalence in a given host population is then determined again by vertical transmission efficiency, any impact on host fecundity and fertility and any reproductive parasitic phenotype it induces.

Novel symbionts commonly show differences in vertical transmission efficiency and cost from native infections. Studies of the transmission and phenotype of transinfected male-killing *Spiroplasma* from the two spot ladybird, *A. bipunctata*, to a variety of hosts demonstrated pathology of symbionts in some cases (*C. 7-punctata* rendered sterile following transinfection) and weakened transmission of infection in others (Tinsley and Majerus, 2007). Cost of infection, specifically a reduction in host fecundity and longevity, following artificial transfer has also been suggested for *Spiroplasma* infection transferred from *D. hydei* to *D. melanogaster* (Kageyama *et al., 2006)*. Weak transmission but not pathology is widely noted for *Wolbachia* (e.g. Clancy and Hoffmann (1997). Weak transmission and/or cost of infection has been noted for some aphid secondary symbionts in novel host species (Russell and Moran, 2005).

Past studies have inferred that the genetic distance between the native host and the recipient host has a large effect on the success of new infections. Clustering of *Wolbachia* phylogenies within insect genera indicate that these symbionts are more likely to move horizontally within a host genus than between distantly related hosts (Jiggins *et al.*, 2002, West *et al.*, 1998). Experimental results have been mixed (see Table 1.6). Where infection is successful, it is commonly the case that the donor and recipient hosts are closely related and the chance of success declines as the phylogenetic distance between the two hosts increases. This has been demonstrated by the transinfection of *Spiroplasma* in ladybird beetles where within-genus transfers

were successful, but across-genus infections resulted in imperfect male-killing, a phenomenon that would quickly result in the loss of the new infection over few generations (Tinsley and Majerus, 2007). Artificial transfer of *Spiroplasma poulsoni* from its native *Drosophila hydei* host into *Drosophila melanogaster*, a fly from a different subgroup, created an infection that was lost after three generations (Kageyama *et al.*, 2006). In both these cases new infections caused detrimental effects to the fitness of their new hosts, a result that would prevent symbiont spread. A further study by Rigaud and Juchault (1995) found successful spread of *Wolbachia* between woodlice of the same genus, but on transfer to a new genus infection was unable to transmit to the next generation (Rigaud and Juchault, 1995).

Despite this evidence, there are some striking exceptions to the rule of genetic distance influencing symbiont spread, for example the successful transfer of *Wolbachia* from the mosquito *Aedes albopictus* to *Drosophila simulans* (Braig *et al.*, 1994) and a lack of success on transferring *Wolbachia* from *Drosophila simulans* into *Drosophila serrata*, two species that share the same genus (Clancy and Hoffmann, 1997). It is clear from these studies that much remains to be uncovered about the mechanics of symbiont horizontal transfer.

Table 1.6: A summary of the results of artificial infection experiments with *Wolbachia* and other symbionts.

Donor	Recipient	Success or failure	Notes	Ref.
Aedes albopictus (mosquito)	*D. simulans*	Success	Transinfected *Wolbachia* function well in new host, CI phenotype transferred.	(Braig *et al.*, 1994)
D. mauritiana *D. simulans*	*D. simulans* *D. mauritiana*	Success	The strength of *Wolbachia* CI phenotype did not change in the recipient host.	(Giordano *et al.*, 1995)
D. melanogaster	*D. simulans*	Success	Higher CI efficiency in recipient host than natural host.	(Poinsot *et al.*, 1998)
D. simulans	*D. yakuba* *D. teissieii* *D. santomea*	Success	All recipient hosts showed a higher CI efficiency than the natural host.	(Zabalou *et al.*, 2004a)
D. simulans	*D. serrata*	Failure	Transinfected *Wolbachia* showed low transmission efficiency, lack of compatibility	(Clancy and Hoffmann, 1997)
Rhagoletis cerasi (cherry fruit fly)	*Ceratitis capitata* (medfly)	Success	Complete CI induced in recipient host.	(Zabalou *et al.*, 2004b)
D. simulans	*D. melanogaster*	Success	Transinfected lines with high bacterial titre expressed CI	(Boyle *et al.*, 1993)
Armadillidium vulgare	*Armadillidium nasatum*	Some success	Microinjection between species of same genera feminised males and caused sex ratio distortion in subsequent female offspring.	(Rigaud and Juchault, 1995)
Chaetophiloscia elongata	*Armadillidium vulgare*		Transinfection between genera produced infected mothers but no vertical transmission.	
Anisosticta novemdecimpunctata	*Adalia bipunctata*	Some success	*Spiroplasma ixodetis* was successfully transferred to beetles of a different genus to the donor but male-killing was imperfect and host showed reduced fitness	(Tinsley and Majerus, 2006)

Despite much study in this field, the factors that determine success or failure of new inherited endosymbiont infections are little known. When infections are moved into a novel host species neither the bacteria nor the host has co-evolved together. The physiological and genetic environment presented by a new host is likely be alien to a symbiont and prevent an infection establishing, especially where phylogenetic distance is great between donor and recipient. In cases where infection success appears to defy this explanation it may be that some important condition that allows compatibility is present in both donor and recipient by chance. There is evidence from past work by Hurst *et al* (2003) that it is the reaction of the symbiont to its new host environment, rather than an immune response elicited by the host, that is the cause of failure, in this case for *Spiroplasma* in new *Drosophila* hosts (Hurst *et al.*, 2003). It is clear that more extensive studies are required.

It is also important to understand the ability of endosymbionts to adapt to their new host and how rapidly this is able to occur. Rapid adaptation would allow infections to spread effectively, and an inability to adapt would limit new host availability or result in failure. Adaptation would include the ability to thrive within a new host and continue any traits that promote symbiont spread, such as beneficial effects to the host or reproductive parasitism. It has been seen in past work that horizontal transfer can cause pathology in the new host and decrease host fitness (Tinsley and Majerus, 2006, Kageyama *et al.*, 2006), a result that will also impair the spread of infection. However, it has also been observed that symbionts can evolve reduced induced pathology over just 20 generations in a new host (e.g. Carrington *et al* (2010), McGraw *et al* (2002)).

1.4 Study system

In order to investigate the establishment of new host-symbiont interactions a model host organism is required in which host response to infection can be assessed. *Drosophila melanogaster* is a widely used laboratory model species which has the advantage of being extensively studied and it certainly represents the best understood insect species. Because of our knowledge of the *Drosophila melanogaster* genome it is possible to use highly specialised microarray technology and established databases such as FlyBase to examine biological questions in greater depth, which gives this organism an advantage above other insect taxa, and indeed over other *Drosophila* species.

Wolbachia and *Spiroplasma* are both naturally present heritable infections in *D. melanogaster* and are the only two known to infect this species (Mateos *et al.*, 2006). To investigate the sources of variation and results of horizontal transfer of symbionts it is essential to utilise a symbiont that can be transferred relatively easily between hosts. Both *Wolbachia* and *Spiroplasma* have been shown to be artificially transferrable. *Spiroplasma* has the advantage of being an easier microbe in which to create transinfections, as demonstrated by greater experimental success than *Wolbachia* transfer. In addition there are a range of different strains of *Spiroplasma* recognised in *Drosophila* hosts, and a growing number of host species known to be infected (Watts *et al.*, 2009, Haselkorn, 2010) which allows scope for creating diverse novel infections. The only criterion for which *Wolbachia* makes a better study organism in *Drosophila* is that this bacterium is itself better studied, with genome sequences available for two *Wolbachia* symbionts from *Drosophila*, and no genome sequence for any *Spiroplasma* (Wu *et al.*, 2004, Klasson *et al.*, 2009).

Spiroplasma are phylogenetically gram-positive bacteria that are helical, motile and lacking a cell wall (Gasparich *et al.*, 2004). They are obligate host-associated bacteria with a wide range of hosts including insects, crustaceans, arachnids and plants (Gasparich *et al.*, 2004). They have a diverse array of effects on their hosts

ranging from reproductive parasitism (Counce and Poulson, 1962, Williamson and Poulson, 1979, Williamson *et al.*, 1999) to facultatively beneficial (Xie *et al.*, 2010, Jaenike *et al.*, 2010). *Spiroplasma* can be directly pathogenic in host species where vertical transmission is less important (Clark *et al.*, 1985, Mouches *et al.*, 1984). This effect has been recorded in honey bees (Clark, 1977, Mouches *et al.*, 1984), crabs (Wang *et al.*, 2005) and shrimp (Nunan *et al.*, 2005). *Spiroplasma* are also the cause of corn-stunt disease (*S. kunkelii*) and citrus stubborn disease (*S. citri*) in plants, which are both vectored by leaf-hopper insects (Jordan *et al.*, 1989, Whitcomb *et al.*, 1986). Within insects the majority of *Spiroplasma* strains show vertical transmission through the female line and it is these *Spiroplasma*, as inherited endosymbionts of insects, which are to be the focus of this thesis. Despite being characterised by maternal transmission there is growing evidence of occasional horizontal transmission events that carry infections from one species to another (Haselkorn *et al.*, 2009, Jaenike *et al.*, 2007).

The presence of *Spiroplasma* in *Drosophila* was first recorded in the late 1950s as a sex-ratio distorting agent of *D. willistoni* (Malogolowkin and Poulson, 1957), a trait that was shown to be artificially transferrable by the movement of haemolymph intraspecifically between infected and uninfected *D.willistoni* (Malogolowkin *et al.*, 1959) and interspecifically from *D. willistoni* to *D. melanogaster* (Sakaguchi and Poulson, 1960, Sakaguchi and Poulson, 1963). Since this time further artificial transfer experiments have shown that *Spiroplasma* can be transferred from *D. hydei* to *D. melanogaster* (Kageyama *et al.*, 2006) and that *Spiroplasma* can be vectored by ectoparasitic mites from *D. nebulosa* to *D. melanogaster* (Jaenike *et al.*, 2007). All of the above transfer experiments have shown limited success as new infections were quickly lost from recipient populations after few generations.

The presence of naturally occurring heritable *Spiroplasma* in *D. melanogaster* was first discovered in Brazil, where sex ratio distortion was found in natural populations (Montenegro *et al.*, 2000). The bacterium was found in 2.3% of the population, and was later identified as a *Spiroplasma* strain (known as MSRO) very closely related to *Spiroplasma poulsonii*, found in the *willistoni* group (known as NSRO) (Montenegro

et al., 2005). This is especially interesting as *D. melanogaster* are an Old World species of fruit fly native to Africa, whereas the *willistoni* group are native to the Americas (New World) and therefore separated in evolutionarily time by many millions of years (despite now being sympatric due to widened distribution of *D. melanogaster*). This indicates a relatively recent horizontal transmission event of *Spiroplasma* from *willistoni* group flies into *melanogaster* and suggests that *Spiroplasma* can move into new hosts and establish there in natural situations, despite low success within the laboratory.

Spiroplasma poulsoni as originally described is known as a male killer (Williamson *et al.*, 1999). However in 1979 non-male-killing *Spiroplasma* were found in around 45.9% of *D. hydei* in Japan, and 27 years later the infection is still prevalent (65.9%) (Ota *et al.*, 1979b, Kageyama *et al.*, 2006). This non-male-killing strain is closely related to the *Spiroplasma* from *D. willistoni* (WSRO), *D. nebulosa* (NSRO) and *D. melanogaster* (MSRO), which all show the male-killing phenotype. Within the time of this study the same strain of non-male-killing *Spiroplasma* in *D. hydei* was found to confer a benefit to its host in the form of defence against the parasitoid wasp *Leptopilina heterotoma* (Xie *et al.*, 2010). The above studies demonstrate that *Spiroplasma* in *Drosophila* are able to cause a range of phenotypes in their hosts and it can thus be presumed they use different mechanisms to drive their own spread, factors useful to this study.

An additional benefit of using *Spiroplasma* in this model is that *Spiroplasma* are widespread. In two general endosymbiont screens of arthropods Goodacre *et al* (2006) recorded *Spiroplasma* presence in 23 of 122 spider species tested and Duron *et al.*, (2008) found *Spiroplasma ixodetis* relatives in 9 of 136 arthropod species sampled across Western Europe. Within the genus *Drosophila*, Mateos *et al.*, (2006) screened lines from stock centres for the presence of *Spiroplasma* infection, recording 3 of 225 species to be infected. Subsequent to this work and in parallel with the work in this thesis Watts *et al.*, (2009) sampled 19 wild *Drosophila* species from North and Central America, finding *Spiroplasma* infection in 7 of these species. The prevalence of *Spiroplasma* make it a biologically interesting infection to work

with and in terms of practicality the latter studies indicate the availability of a variety of accessible infections with which to investigate new host-symbiont relationships (see Table 1.7 for summary).

Table 1.7: A summary of the *Drosophila* species known to be naturally infected with *Spiroplasma* to date, including *Spiroplasma* clade and phenotypic effects to the host.

***Drosophila* host**	***Spiroplasma* clade**	**Phenotype**	**References**
D. willistoni	*S. poulsoni*	Male-killing	(Williamson *et al.*, 1999, Malogolowkin and Poulson, 1957)
D. hydei	*S. poulsoni* and *S. citri*	Resistance to parasitoid attack, non-male-killing	(Ota et al., 1979b, Kageyama *et al.*, 2006, Xie *et al.*, 2010, Mateos *et al.*, 2006, Watts *et al.*, 2009)
D. melanogaster	*S. poulsoni*	Male-killing	(Montenegro *et al.*, 2000)
D. nebulosa	*S. poulsoni*	Male-killing	(Bentley *et al.*, 2007, Williamson *et al*, 1999)
D. aldrichi	*S. citri*	Non-male-killing	(Mateos *et al.*, 2006)
D. mojavensis	*S. citri*	Non-male-killing	(Mateos *et al.*, 2006)
D. simulans	*S. poulsoni*	Non-male-killing	(Watts *et al.*, 2009)
D. wheeleri	*S. citri*	Non-male-killing	(Watts *et al.*, 2009)
D. tenebrosa	*S. tenebrosa*	Non-male-killing	(Watts *et al.*, 2009)

1.5 Outline of thesis

This thesis is concerned with understanding the conditions permitting the establishment of new infections of *Spiroplasma* bacteria in *Drosophila* hosts. The thesis will examine this in terms of both phenotype of *Spiroplasma* in novel hosts and in terms of host gene expression in the presence of natural and introduced symbiont infections. The major aims of the thesis are twofold. First, to examine the hypothesis that infections closely related to a resident strain establish with higher transmission efficiency and lower cost than strains more distantly related. The logic here is that if the ability to prosper is a function of symbiont genotype, then strains that are more closely related to resident strains are more likely to prosper, so long as divergence between strains in their ability to colonize hosts is not rapid. The second major aim is to analyse whether changes in host gene expression in the presence of a symbiont play any role in determining whether symbionts prosper or are unsuccessful. Two hypotheses can be drawn for symbiont success and failure. First, success and failure may be purely environmental and the symbiont may simply not be suited to the new host environment. Second, success and failure may be associated with the reaction (or lack of it) by the host to the presence of a novel symbiont. Induction of immune system activation in a novel host would represent one possible means by which this could occur.

Chapter two begins with the artificial infection of *Drosophila melanogaster* with two *Spiroplasma* strains not native to this host with the aim of generating novel infections for study in this thesis, such that the causes of success and failure of new infections can be investigated. Working on the hypothesis that the infection most closely related to the natural *D. melanogaster* infection will show better symbiont performance, the novel infections in *D. melanogaster* can be characterised in terms of transmission efficiency, sex ratio distortion and fitness cost to the host. In addition, I investigate whether the property of transmission efficiency evolves over time, with the symbiont becoming adapted to its new host over multiple passages.

Chapter three examines two male-killing *Spiroplasma* infections, one native to *D. melanogaster* and the other artificially transinfected into *D. melanogaster* from *D. nebulosa* four years previously. Prior to this thesis, the majority of studies of *Spiroplasma* in *Drosophila* were conducted on the *D. nebulosa* strain maintained within *D. melanogaster* and the properties of this interaction have been examined extensively. However, it may in fact represent a strain that is not perfectly adapted to *D. melanogaster*. I compared the transmission efficiency, timing and completeness of male-killing for the natural and introduced *Spiroplasma* male-killing infections. This investigation will also allow us to gauge whether past work utilizing the transinfected infection gives a valid comparison to a natural *Spiroplasma-Drosophila* association, or whether it provides a view of an infection that is maladapted by virtue of being transinfected.

Chapter four follows on from the knowledge that many horizontal transmission events fail due to poor symbiont performance and utilises microarray technology to investigate whether this is associated with alteration in host gene expression, such as an active immune response, or whether it occurs independently of host reaction. The *Spiroplasma* infections in *D. melanogaster* outlined in Chapters 2 and 3 are compared in this assay. The central question in this chapter is whether infections that perform poorly following transinfection do so because of a host response to novel infections.

Chapter five is concerned with *Spiroplasma* prevalence and diversity in the wild. Previous studies have examined biodiversity in either full screens of laboratory maintained lines, or smaller screens of field collected flies. In this chapter, wild *Drosophila* in regions of high biodiversity are sampled at random in order to gain an accurate representation of the species composition found *in situ* and these are screened for *Spiroplasma* infection. This chapter aims to understand how commonly *Spiroplasma* infections occur in *Drosophila* and to obtain new isolates of *Spiroplasma* in *Drosophila* whose properties can be further studied. This motivation is driven by the tractability of *Drosophila* as a system of study and its ubiquitous use as a model species.

I conclude this thesis in **Chapter 6** with a synthesis and discussion of the wider implications of the findings presented in previous chapters. I argue that, in addition to the observation that infections move more easily between related hosts, infection also moves more easily into host species that carry similar existing infections. This implies that there are intrinsic properties of symbionts, conserved over relatively long periods of evolutionary time, that permit invasion of particular host species.

Chapter 2

Behaviour of new *Spiroplasma*-host associations

2.1 Abstract

Maternally inherited endosymbionts are found in numerous arthropod species. The frequency of endosymbiont infection amongst insects is partly a function of their ability to spread through a new host species following a lateral transfer event. This is determined by vertical transmission efficiency between generations in the new host, the direct fitness effects of infection and any phenotype of the infection that alters host sex ratio. In this chapter I report on these properties for two *Spiroplasma* strains transinfected into *D. melanogaster*, a species that hosts its own strain of *Spiroplasma*. The study had three motivations: First, to generate novel infections in *D. melanogaster* for study later in the thesis, such that the causes of success and failure of novel infections could be investigated. Second, to characterize the novel infections in *D. melanogaster* in terms of transmission efficiency, cost of infection and sex ratio distortion, with the hypothesis that the infection more closely related to the natural infection would show higher performance. Third, to investigate if the property of transmission efficiency would evolve over multiple passages through their novel host, such that poorly adapted strains would come to be better adapted in a short space of time, permitting invasion. With respect to these aims, two *Spiroplasma* strains, one from *D. hydei* and one from *D. mojavensis* hosts, were successfully introduced to *D. melanogaster*. Transmission efficiency was higher (82.78%) in the *Spiroplasma* originating from *D. hydei* than that from *D. mojavensis* (28.35%). A cost of infection was observed in reduced fertility in both treatments and smaller body size in the treatment with the *D. mojavensis* infection. These results are consistent with the hypothesis that the infection more closely related to the natural infection would show higher performance, however further work is needed for this to be conclusive. Finally, it was observed that transmission efficiency did not increase with repeated passage, indicating that this trait may not be an initial target of selection. It is suggested that novel strains may require ecologically contingent benefits to spread through natural populations.

2.2 Introduction

Many arthropod species harbour maternally inherited endosymbionts (Duron *et al.*, 2008, Gasparich, 2002). These organisms have various associations with their hosts including obligate beneficial mutualism (Ferrari *et al.*, 2004, Baumann, 2005, Allen *et al.*, 2007), non-obligate symbiosis that may provide a secondary benefit to the host (Scarborough *et al.*, 2005, Oliver *et al.*, 2005, Haine, 2008, Toh *et al.*, 2006, Hansen *et al.*, 2007) and reproductive parasitism (Engelstadter and Hurst, 2007, Bandi *et al.*, 2001, Charlat *et al.*, 2005, Dyson and Hurst, 2004, Bentley *et al.*, 2007). As a result, maternally inherited endosymbionts play a key role in host ecology and drive host evolution both as partners and antagonists.

Although characterised by maternal inheritance, the establishment of new symbioses most usually follows rare events of horizontal transmission. *Spiroplasma* bacteria have long been known to represent some of the most easily transmissible symbionts experimentally, with studies in the 1950s-60s demonstrating how these infections could be transferred following microinjection of small quantities of haemolymph from an infected individual to an uninfected one (Malogolowkin *et al.*, 1959, Sakaguchi and Poulson, 1963, Sakaguchi and Poulson, 1960, Malogolowkin and Poulson, 1957). Experimental study has further demonstrated that sharing of ectoparasitic mites can result in transfer of *Spiroplasma* from infected *D. nebulosa* into *D. melanogaster* (Jaenike *et al.*, 2007).

The importance of horizontal transfer of symbionts between species can be seen in the biodiversity and phylogenetic relatedness of *Spiroplasma* strains in *Drosophila*. Multiple introductions of five distinct *Spiroplasma* haplotypes were found in an endosymbiont phylogeny from nine *Drosophila* species (Haselkorn *et al.*, 2009). A recent event of horizontal transmission is indicated by the close relatedness of the *Spiroplasma* strains NSRO (found in the New World species *D. willistoni*) and MSRO (found in *D. melanogaster*, an Old World species) (Pool *et al.*, 2006). In

addition, *Spiroplasma* are widespread in *Drosophila* (Mateos *et al.*, 2006, Watts *et al.*, 2009), indicating an ability to move between species at some level. This ease of movement is probably at least in part associated with the presence of *Spiroplasma* in the haemolymph (Sakaguchi and Poulson, 1961), such that any haemolymph transfer can result in transinfection.

The above studies demonstrate that maternally inherited endosymbionts can spread between species in the natural environment. However, artificial transinfection studies suggest the conditions for spread may be limited. Horizontal transmission of the well-studied endosymbiont *Wolbachia* is often unsuccessful due to poor transmission efficiency (e.g. Clancy and Hoffmann (1997); see Engelstadter and Hurst (2009b) for review). Early transinfection studies of *Spiroplasma* reported that transinfection was possible, but the strains transferred were often 'unstable' and lost (Malogolowkin and Poulson, 1957, Malogolowkin *et al.*, 1959, Sakaguchi and Poulson, 1963, Sakaguchi and Poulson, 1960). This instability was recently anecdotally found by Kageyama *et al* (2006) who transferred the *Spiroplasma poulsoni* strain from its natural host *D. hydei*, into *D. melanogaster* and found it was lost in *D. melanogaster* culture within three generations. They further noted the transinfection was pathogenic (though no data were presented on this point) (Kageyama *et al.*, 2006). Further to this, Tinsley & Majerus (2007) demonstrated that transinfections of *Spiroplasma ixodelis* from *Adalia bipunctata* were less successful when the new host species was evolutionarily distant from *A. bipunctata*. *Spiroplasma* were poorly transmitted in these more distant hosts and found reduced fecundity and survivorship (Tinsley and Majerus, 2007). Thus, it is clear that *Spiroplasma* infections moved to new host species may transmit poorly or cause pathology. The above studies show that the properties of new *Spiroplasma*-host interactions are variable, with some laterally transferred strains simply not able to spread through natural populations.

This thesis is concerned with the process of lateral transfer and the limitations to the spread of inherited microbes in novel host species as a critical delimiter of the incidence of inherited symbiont infections in insect communities. This chapter has three main aims:

i) To establish two novel *Spiroplasma* infections in *D. melanogaster* to allow comparison of how *D. melanogaster* as a host responds to native and novel infections (see Chapter 4).

ii) To establish the transmission efficiency, cost of infection and sex ratio distortion activity of novel infections, both to allow comparison of host response to infections with different properties (Chapter 4) and to test the hypothesis that those strains that perform better in *D. melanogaster* will be those most closely related to the natural infection present in *D. melanogaster*.

iii) To establish whether *Spiroplasma* transmission efficiency is a sufficiently changeable trait that it would evolve during the course of passage in the laboratory. It is to be expected that many infections will perform sub-optimally in novel hosts, and here I sought to investigate if transmission efficiency would improve rapidly enough that a poorly inherited strain could establish in a host, notwithstanding its initial imperfect vertical transmission.

2.3 Materials and Methods

Recipient fly strain: Uninfected *Drosophila melanogaster* were standard Canton S (CS) strain, carrying *Wolbachia*, hereafter termed CS-.

Donor fly strains: *Spiroplasma* infected *Drosophila hydei* (*Spiroplasma* strain TEN 104-106 haplotype 1, hereafter termed HY1) and *Drosophila mojavensis* (*Spiroplasma* strain QUIN 903-28, hereafter MOJ) were as described in Mateos *et al*., (2006). The former of these (HY1) falls as an out-group to *S. poulsonii*, the infection found naturally in *D. melanogaster*. It is clearly monophyletic with this group, but is distinct on the sequence of both 16S rRNA genes and other housekeeping genes. The latter is somewhat more distantly related, being a member of the *S. citri* group (Mateos *et al*., 2006, Watts *et al*., 2009).

Transinfection of *Spiroplasma* into *D. melanogaster*: Microinjection was performed using pulled capillary needles attached to heavy paraffin oil filled fine tubing fixed to a Hamilton syringe (see Figure 2.1). A quantity of 0.1-0.2μl haemolymph was drawn from the thorax of the infected donor host by capillary action (see Figure 2.2) and microinjected into the abdomen of virgin female CS *D. melanogaster* aged less than 24 hours post eclosion (see Figure 2.3). The young age of flies makes it easier to penetrate the recipient fly cuticle without damage. Injected flies were aged for 14 days then out-bred with CS males to control for genetic background. The presence of infection was then tested in the next generation through PCR assay (see below). This procedure formed two treatments; *D. melanogaster* artificially infected with *Spiroplasma* from *D. hydei* (CS+HY1) and *D. melanogaster* artificially infected with *Spiroplasma* from *D. mojavensis* (CS+MOJ).

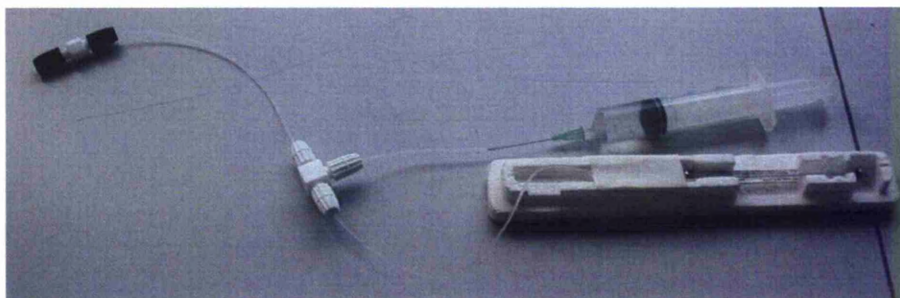

Figure 2.1: Apparatus used for microinjection.

Figure 2.2: Direction and location of needle insertion for extracting haemolymph.

Figure 2.3: Direction and location of needle insertion for injecting haemolymph.

PCR assay for *Spiroplasma* presence: In order to assess *Spiroplasma* infection status individual mothers were macerated in a 50µl 5% v/v Chelex 100 solution (Biorad) and 1µl Proteinase K added and the mix incubated at 37 °C overnight. Samples were then heated to 95°C for 10 min to denature the Proteinase K and centrifuged for 1 min at 13,000g (Walsh *et al.*, 1991). The DNA in the supernatant was used for PCR amplifications with the *Spiroplasma* specific primers SpoulF (5′-GCT TAA CTC CAG TTC GCC-3′) and SpoulR (5′-CCT GTC TCA ATG TTA ACC TC-3') as in Montenegro *et al.*, (2005). The PCR cycling conditions were an initial denature of 2 minutes at 94°C, followed 30 cycles of 15 seconds at 94°C, 1 minute annealing at 55°C and 40 seconds at 72°C. DNA extraction viability was ascertained via PCR with the general insect primers HCO(5'-TAA ACT TCA GGG TGA CCA AAA ATC A-3') and LCO (5'-GGT CAA CAA ATC ATA AAG ATA TTG G-3') as in Folmer, (1994), such that DNA negative templates could be excluded from analysis of transmission efficiency. PCR cycling conditions were an initial denature of 1 minute 30 seconds at 94°C, followed by 35 cycles of 15 seconds at 93°C, 1 minute annealing at 47°C and 1 minute at 72°C.

Maintenance of *Spiroplasma* infections and measurement of transmission efficiency on continuous passage: Maintenance of *Spiroplasma* infection is summarised in Figure 2.4. Virgin females were collected from infected mothers each generation and aged for 8-10 days. For CS+MOJ, 30 females from a variety of infected mothers (always six or more where possible) were crossed individually to CS- males. For CS+HY1, 25 females from at least five infected mothers were crossed individually to CS- males. Breeding females were then allowed to oviposit for five days on standard corn-meal agar fly media before being culled. Following culling, the mothers were screened for *Spiroplasma* using PCR as described above, and uninfected lineages discarded. The maintenance procedure was then iterated by collecting virgin flies from the infected lineages.

This maintenance regime provided ongoing measures of transmission efficiency over passage, material in which fitness parameters could be measured (below) and flies in which investigations of host gene expression could be ascertained (see Chapter 4). Transmission efficiency for the previous generation was ascertained by calculating the percentage of infected females from each infected mother in the parental generation, transmission efficiency then estimated as the mean percentage of progeny infected for each family per generation. This estimate then incorporates female variation in transmission efficiency. Infection was maintained for over 60 host generations in each case, which additionally permitted investigation of whether the property of transmission efficiency evolved during laboratory passage.

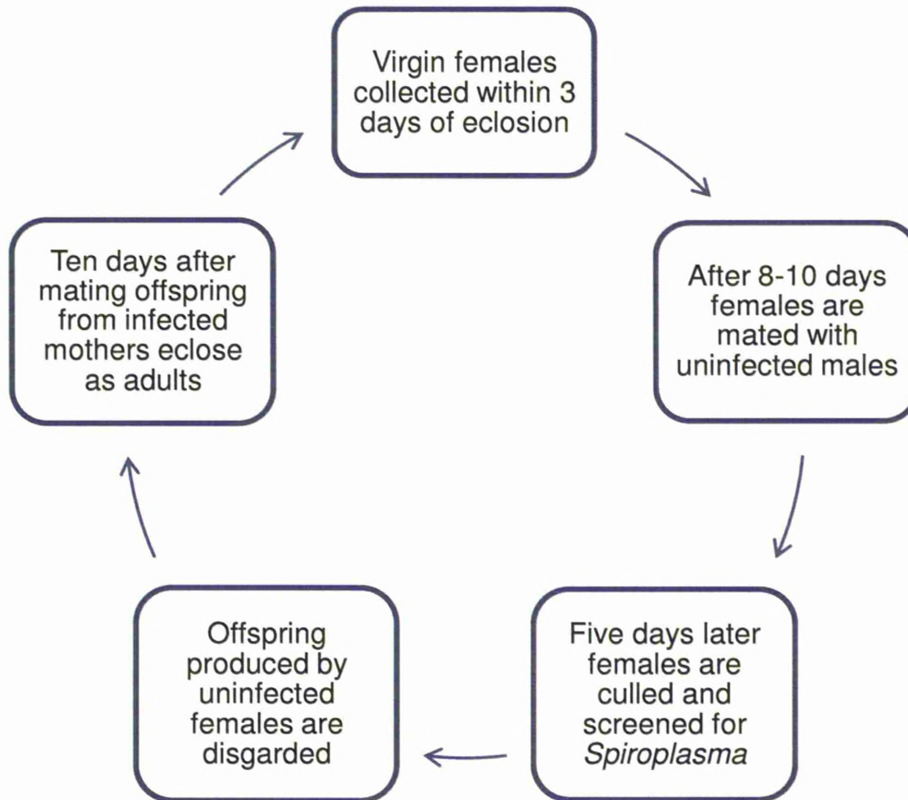

Figure 2.4: Flow-diagram describing the maintenance schedule for infected fruit fly treatments, CS+HY1 and CS+MOJ, in order to best preserve *Spiroplasma* infection.

Measures of the direct effects of infection with novel *Spiroplasma* strains on *D. melanogaster* fitness: Two fitness measures were taken; the number of offspring produced over a four day period as a measure of fertility, and wing area as a measure of body size (Robertson and Reeve, 1952, Reeve and Robertson, 1953). The former of these is a direct measure of female performance and the latter allows insight into fitness effects that accrue during larval development, as wing area is fixed upon eclosion. These measurements were taken at generation 21 (they were originally attempted unsuccessfully at generation 6).

To this end, first instar larvae were taken from infected and uninfected female *D. melanogaster* raised in a controlled environment at a controlled density. For the CS+HY1 comparison ten vials were seeded with 25 first instar larvae from CS+HY1 infected flies and 25 uninfected CS- combined to control for competition differences

that may arise between infected treatments (as CS+MOJ produce many uninfected offspring which may be better/poorer competitors than infected larvae). For the CS+MOJ comparison 40 vials were seeded with 50 CS+MOJ infected larvae each, the larger number to account for low *Spiroplasma* transmission rate between generations. Ten vials were seeded with 50 uninfected CS- larvae each to form an uninfected control.

On eclosion virgin females were collected, aged for three days and each female mated with two CS- males (two males were used to ensure mating success). 100 females of both CS+HY1 and CS- were established in this fashion and 400 females for CS+MOJ (to allow for poor transmission of infection). Breeding females were turned over into new vials every day for five days. The offspring in these vials were allowed to fully eclose and all adult male and female progeny were counted. After the five days, breeding females were isolated, wings collected, and the body screened for *Spiroplasma* infection as previously described. Wings were mounted on slides using Aquatex mounting medium (Merck) and images taken with a microscope mounted camera. The images were analysed using Image J 1.40g public domain software (Wayne Rasband). Measurements of wing length and width (see Figure 2.5) were taken and a graticule measure used to convert pixel area to area in mm^2.

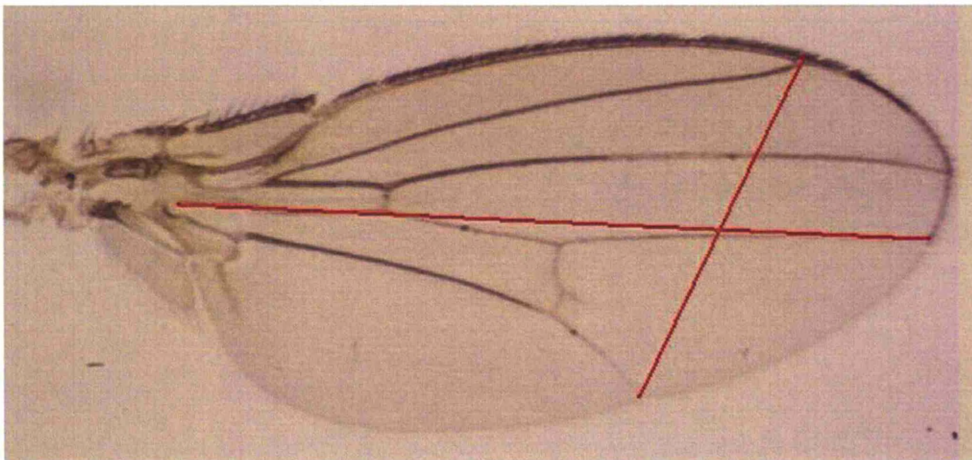

Figure 2.5: location of landmarks for wing length and width measurements taken to indicate wing area.

Within infected treatments (CS+HY1 and CS+MOJ) *Spiroplasma*-negative females were discarded from analysis. For fertility data, 31 mothers were selected per treatment, these including only individuals that had survived the five day breeding period. Analysis of fertility data excluded counts for day 1 (day 1 lays produced a disproportionate number of the eggs produced overall by each female as these were eggs stored during the prolonged period of virginity prior to the experiment. In order to measure continuous ability to produce progeny, data from this day were excluded *post hoc*). For wing size data 70 individuals with undamaged wings were selected per treatment.

2.4 Results

Transinfection of *Spiroplasma* into *D. melanogaster* and transmission efficiency on continual passage: Both the *Spiroplasma* strain from *D. mojavensis* and that from *D. hydei* were successfully established in *D. melanogaster* following haemolymph transfer, forming CS+MOJ and CS+HY1 treatments respectively. These treatments were maintained for 60 generations via artificial selection for progeny from infected females, as described in the methods. Variation in transmission efficiency between infections and over time is given in Figure 2.6. Percentage efficiency was calculated for both treatments between generations 16-60 as there were no gaps in the data for either treatment during this time and both treatments were maintained synchronously. Over this period, a mean of 82.78% of F1 daughters from CS+HY1 infected females and a mean of 28.35% of F1 daughters from CS+MOJ infected females were found to be themselves infected. It should be noted that stochasticity within infection over time is an expected product of the sampling regime, because of the relatively small number of foundress mothers being used to establish the next generation. A linear regression found no evidence for a change in transmission efficiency over time in either the CS+HY1 infection ($F = 0.64$; 1, 54 d. f; $p = 0.428$) or the CS+MOJ infection ($F = 0.08$; 1, 56 d. f; $p = 0.781$).

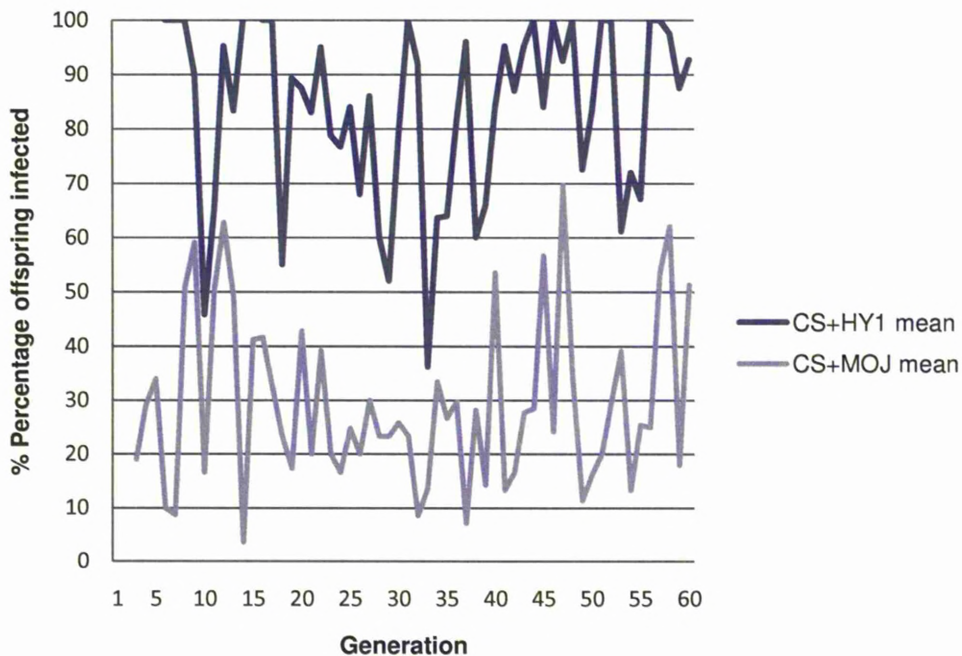

Figure 2.6: The transmission efficiencies of two *Spiroplasma* strains from *D. hydei* (CS+HY1) and *D. mojavensis* (CS+MOJ) in *D. melanogaster* over passage. Gaps in the data are where infection status was unable to be assayed.

Direct effects of novel *Spiroplasma* infection on *D. melanogaster* fitness measures obtained at generation 21: Fertility measured over a four day period was found to be significantly lower in *Spiroplasma* infected flies (CS+HY1 and CS+MOJ treatments) than in uninfected *D. melanogaster* (Kruskal-Wallis, H = 26.19, 2 d. f, p <0.001, General Linear Model, F = 15.28, 2 d. f, p <0.001) (see Figure 2.7). CS+MOJ females had a significantly smaller body size (as indicated by wing area) than either CS+HY1 or CS- flies (One-Way ANOVA, F = 45.25; 1, 209 d. f, p <0.001) and there was no significant difference in body size between CS+HY1 and CS- (see Figure 2.8).

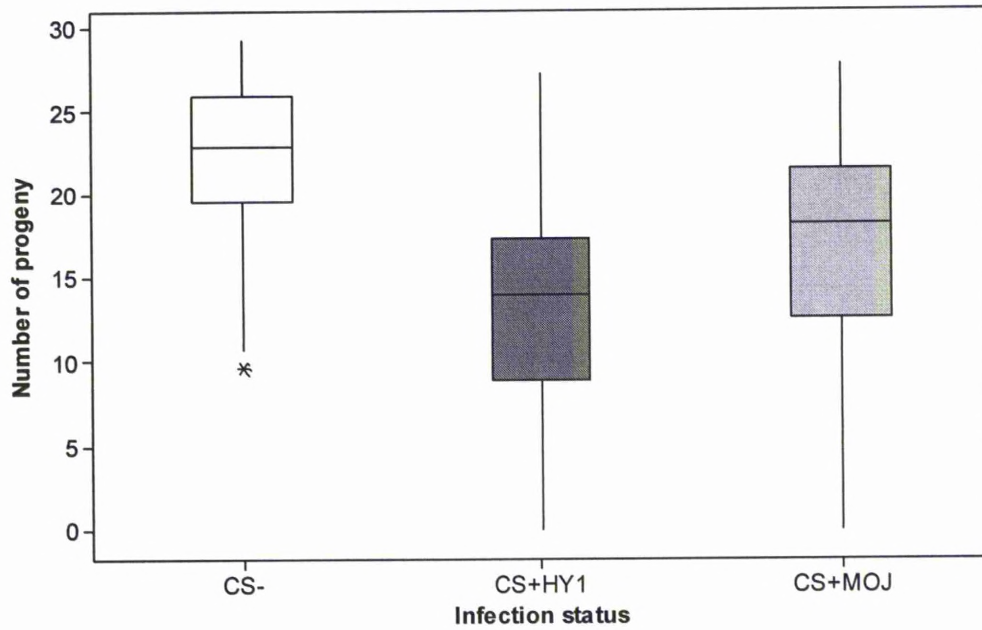

Figure 2.7: Fertility of *D. melanogaster* females over four days: Treatments were either uninfected (CS-, unfilled), or infected with *Spiroplasma* strains from *D. hydei* (CS+HY1, dark grey) or *D. mojavensis* (CS+MOJ, light grey). A significant difference was found between infected and uninfected flies (Kruskal-Wallis, H = 26.19, d. f = 2, p = 0.000, General Linear Model, F = 15.28, d. f = 2, p <0.001). Whiskers represent range of data, boxed area is inter-quartile range, and horizontal line is the median.

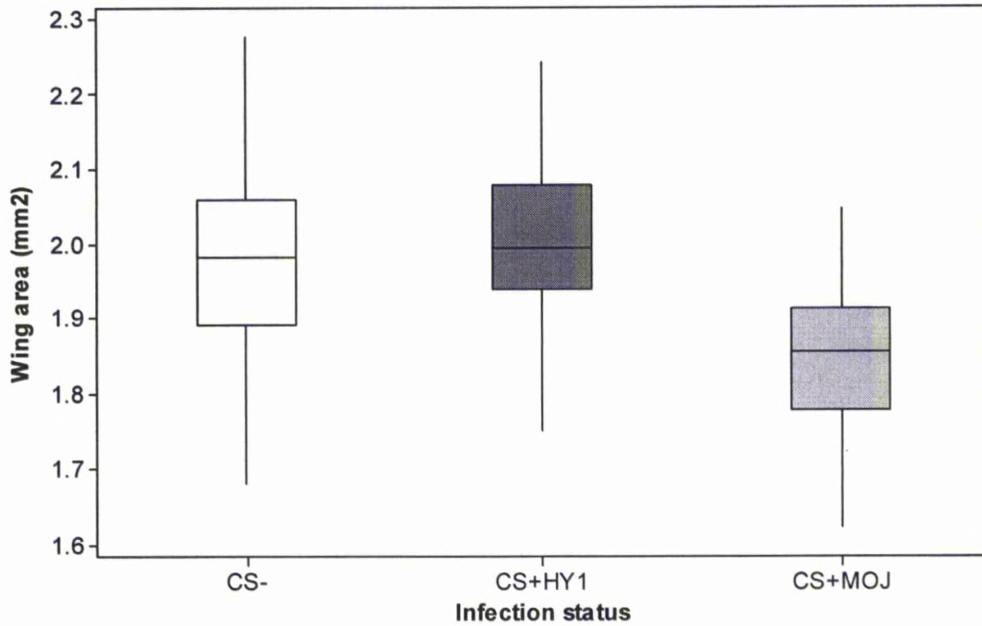

Figure 2.8: Relative wing area (mm^2) of female *D. melanogaster* of different infection status: Treatments were either uninfected (CS-, unfilled), or infected with *Spiroplasma* strains from *D. hydei* (CS+HY1, dark grey) or *D. mojavensis* (CS+MOJ, light grey). CS+MOJ flies have a significantly smaller wing area that CS+HY1 and CS- treatments (One-Way ANOVA, $F = 45.25$, 1, 209 d. f, $p = 0.000$). Whiskers represent range of data, boxed area is inter-quartile range, and horizontal line is the median.

Analysis of progeny sex-ratio found no significant difference between treatments (One-Way ANOVA, $F = 0.88$; 1, 89 d. f, $p = 0.419$) and no evidence of distortion away from 1:1 (see Figure 2.9). Anecdotally it can be noted that survivorship differed between treatments, with 98% of uninfected CS- mothers surviving throughout the five day laying period, compared to 75% of CS+HY1 mothers (there were too few appropriate CS+MOJ individuals to make a good comparison in this case as only those mothers with progeny from all five days were counted, due to large numbers in this treatment).

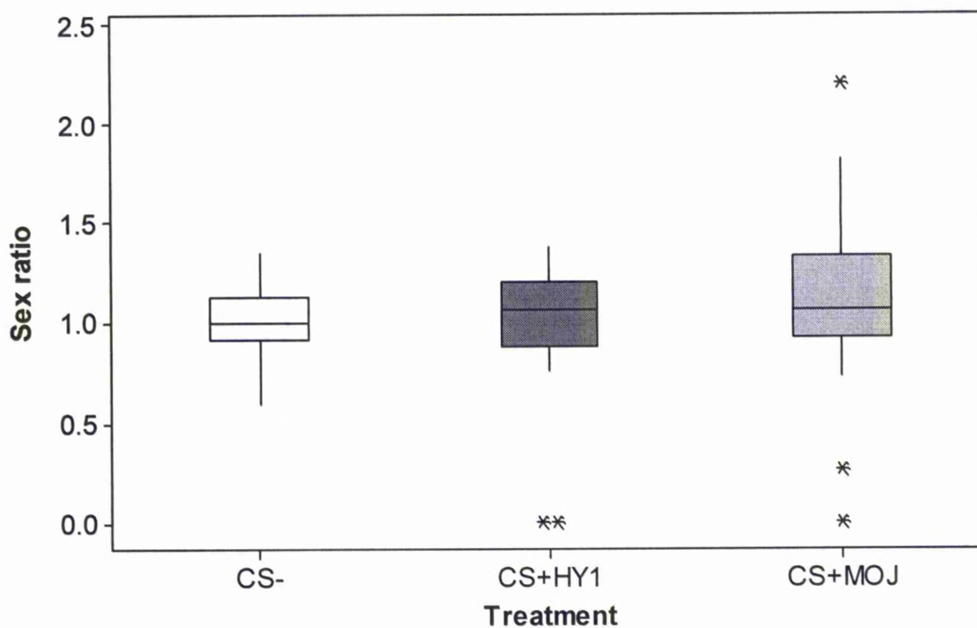

Figure 2.9: Sex ratios of progeny of female *D. melanogaster* of different infection status: Treatments were either uninfected (CS-, unfilled), or infected with *Spiroplasma* strains from *D. hydei* (CS+HY1, dark grey) or *D. mojavensis* (CS+MOJ, light grey). Sex ratios did not significantly deviate from 1:1 and there was no significant difference between treatments (One-Way ANOVA, $F = 0.88$; 1, 89 d. f, $p = 0.419$). Whiskers represent range of data, boxed area is inter-quartile range, horizontal line is the median and asterisks represent outliers.

2.5 Discussion

Native *Spiroplasma* bacteria from *D. hydei* and *D. mojavensis* hosts were successfully transferred into recipient *D. melanogaster* forming two heritable infection treatments, CS+HY1 and CS+MOJ respectively. This demonstrates that in principle *Spiroplasma* infection can transmit horizontally between species, a result that is concordant with patterns of spread observed in the literature (Watts *et al.*, 2009, Haselkorn *et al.*, 2009, Mateos *et al.*, 2006). However, the transmission efficiency of these two treatments was imperfect. The maintenance regime devised for the infections (ageing before reproduction to allow building of bacterial titre and removing uninfected mothers post hoc) ensured that the infection was continually inherited and allowed its properties and evolution to be studied both in this chapter and later in the thesis. Thus, the breeding regime overcame the issues of infection maintenance experienced in similar transinfections by Kageyama *et al* (2006) and allowed detailed study of the properties of infections that do not transmit well in their novel host.

The two infections both demonstrated a combination of low transmission efficiency and fitness reduction to their host that makes it clear that, should these infections transfer naturally into *D. melanogaster*, the infection would be unlikely to persist in the population. This is particularly pronounced for the infection from *D. mojavensis* (CS+MOJ), which transmitted to just 28.35% of an infected female's progeny over 44 generations of study. The *D. hydei* infection (CS+HY1) whilst performing better still shows both cost of infection (significant loss in fertility) combined with inefficient transmission that makes infection spread unlikely. For this infection to spread would require a very strong secondary benefit to the host. It has been demonstrated that this infection can establish natural enemy resistance in its native host, *D. hydei* (Xie *et al.*, 2010). If this resistance was also present in the transinfected *D. melanogaster* individuals and if natural enemy pressure was very high, there is the possibility of maintenance of infection at least in the short term. However, long term endosymbiont success would require either a persistent natural

enemy pressure, or for the infection to quickly evolve a lower virulence and higher transmission efficiency in its new host.

Following bacterial passage over time allows some insight to be gained into the likelihood that endosymbionts will evolve towards optimal transmission and virulence within a new host. Such evolution is known in *Wolbachia*, where the strain *w*Ri present in *Drosophila simulans* has evolved from being costly to mildly beneficial over 20 years in the field (Weeks *et al.*, 2007) and also in 20 generations following transinfection in the laboratory (Carrington *et al.*, 2010). Two things are notable in our data. First, transmission efficiency did not alter during 60 generations for either infection. Within the infection maintenance regime there is scope to allow better transmitting strains to be selected. However, no evidence of improved transmission was seen during the experiment. Second, the fitness experiment was conducted at generation 21, well into the time when an infection would need to have evolved reduced virulence, yet it was still pathological, causing reduced fertility in infected treatments. Unfortunately, there is no baseline virulence known in these strains against which to test the hypothesis of virulence evolution. Severe pathology was noted by Kageyama *et al.*, (2006) in the three generations immediately subsequent to transinfection of HY1 into *D. melanogaster*. This contrasts with the moderate virulence seen in my data, suggesting virulence evolution. However, for this conclusion to be reached, a comparison of virulence over time for particular infections needs to be conducted.

It is clear from this study and from previous studies of *Spiroplasma* in ladybirds (Tinsley and Majerus, 2007) that a major factor in determining whether a new infection succeeds or fails is if it produces a fitness reduction in its new host. The combination of these two results suggests that this pathology is a general issue affecting *Spiroplasma* lateral transfer success. Pathology has not been recorded following transinfection of *Wolbachia*, but is thought to occur when aphid secondary symbionts are moved to a novel host (Chen *et al.*, 2000, Russell and Moran, 2005). If it is a property of particular bacteria, then this may limit the lateral transfer ability of those bacteria and thus their incidence in insect communities. It is interesting to

question why pathology occurs at all. An intuitive view of interspecific transmission is that the infection would fail to thrive in its novel host and attain lower titre, meaning that it would be less costly in physiological terms for the host. It is possible that *Spiroplasma* loses regulation of titre in new hosts and over replication causes a cost. Alternatively, infections may have unexpected and deleterious interactions with host physiology, with either similar or lower titre to that found in the native host. Understanding these issues will require accurate establishment of titre, and detailed pathological observation.

What makes a symbiont likely to succeed in a novel host? Previous work had focussed on the degree to which hosts are related, with symbionts generally transmitting less well and causing pathology in hosts more distantly related to their source (Tinsley and Majerus, 2006). It can also be conjectured that strains of symbiont more closely related to the native strain will prosper. This hypothesis is based on the idea that similar symbiont properties are found where recent ancestry is shared, such that a symbiont closely related to one found naturally in a particular host is likely to also suit that host environment. In the study outlined, the infection most closely related to that native to *D. melanogaster* performed better in *D. melanogaster* than the more distant one (CS+HY1 produced an average of 82.78% infected offspring and CS+MOJ an average of 28.35% infected offspring). It can be speculated that the difference in transmission efficiencies observed between treatments is related to their phylogenetic distance from the native *Spiroplasma* infection found in *D. melanogaster*. These data represent consistency with the above hypothesis but are not a clear test. Further work would require many more *Spiroplasma* strains to be isolated and tested by transferring into *D. melanogaster* hosts. The recent acquisitions of *Spiroplasma* in *Drosophila* (Watts *et al.*, 2009) mean this is now possible.

In conclusion, the above findings demonstrate that *Spiroplasma* can be transferred horizontally into *Drosophila melanogaster*, but whether the new infection is successful depends on a number of factors. It is possible that the phylogenetic relatedness of the introduced bacteria to the native host infection may have an impact

on a new host-parasite relationship. A closer relatedness may mean that bacteria are better fitted to the new host environment, whereas a greater distance means the host environment is more alien and bacteria are less able to thrive. A more determining factor as to whether a new infection succeeds or fails is if it produces a fitness reduction in its new host. This study has demonstrated that novel *Spiroplasma* infections can negatively affect host fitness through body size, fertility and longevity. In nature such detrimental effects to the host would prevent the spread of new infections and the new endosymbiont could only increase in a population if it provided a very strong secondary benefit to the host.

Chapter 3

Phenotype and transmission efficiency of artificial and natural male-killing *Spiroplasma* infections in *Drosophila melanogaster*

3.1 Abstract

Drosophila melanogaster carries a male-killing *Spiroplasma* infection that is very closely related to strains that infect members of the *willistoni* clade. The strain NSRO, derived from *D. nebulosa*, has been used extensively for study of aspects of *Spiroplasma*-host interaction in the novel host *D. melanogaster*. However, differences in life history between *D. nebulosa* and *D. melanogaster* raise the possibility that strains from *D. nebulosa* will not have the same characteristics in *D. melanogaster* as native strains that have coevolved with *D. melanogaster*. In this chapter, I determine if NSRO has similar or different properties from the naturally infecting strains in terms of transmission efficiency and quality of male-killing. Native infections were observed to have stronger transmission efficiency than introduced NSRO infections during the early phases of host reproduction, but not during late reproduction. The quality of male-killing (its timing and intensity) did not differ between infection classes. Interestingly, strains transinfected into *D. melanogaster* 40 years and 4 years prior to this study did not differ in properties, suggesting selection is slow to improve transmission efficiency. I conclude that the strain NSRO does differ from the native strain in some characteristics, but is broadly similar with respect to male-killing strength. As a precautionary measure, it is proposed that future work seeking to reveal the nature of coevolved *Spiroplasma*-*Drosophila* interactions use the native strain.

3.2 Introduction

The genus *Spiroplasma* is a group of predominantly maternally inherited bacteria known to infect numerous arthropod and plant species (Duron *et al.*, 2008, Gasparich *et al.*, 2004). They have a diverse array of effects on their hosts, ranging from beneficial (Jaenike *et al.*, 2010, Xie *et al.*, 2010) to parasitic (Duron *et al.*, 2008, Hurst *et al.*, 1999b, Tinsley and Majerus, 2006, Majerus *et al.*, 1999), and thus dramatically influence host ecology and evolution. Due to their mode of transmission through the maternal line, male hosts are an evolutionary dead end and as a result a variety of *Spiroplasma* have evolved a male-killing phenotype, causing infected female hosts to only produce daughters. Male-killing infections have been observed in ladybirds (Hurst *et al.*, 1999b, Tinsley and Majerus, 2006, Majerus *et al.*, 1999), butterflies (Jiggins *et al.*, 2000a) and a range of *Drosophila* flies, including *D. melanogaster* (Pool *et al.*, 2006, Montenegro *et al.*, 2005).

Spiroplasma infections with a male-killing phenotype were first observed in members of the *Drosophila willistoni* group in the late 1950s and early 1960s (Malogolowkin and Poulson, 1957, Poulson and Sakaguchi, 1960b, Poulson and Sakaguchi, 1961b). Forty years later, male-killing was discovered in *D. melanogaster* sympatric with *willistoni* group flies (Montenegro *et al.*, 2005) and then later in flies from Uganda (Pool *et al.*, 2006). The agent of male-killing was again revealed to be a *Spiroplasma*, and molecular systematic data indicated that the *Spiroplasma* infections from *willistoni* group flies and *D. melanogaster* were very similar, with no differences detectable in either 16S rRNA gene sequence, or across the housekeeping genes *spoT*, *p58* and *fru* (Montenegro *et al.*, 2005). Given the presence of *Spiroplasma* in a variety of members of the *willistoni* clade, it can be suggested that the infection transferred laterally from a member of this clade into *D. melanogaster* in the recent past. Since this time, the potential for ectoparasitic mites to produce this interspecific transfer has been demonstrated experimentally (Jaenike *et al.*, 2007).

Spiroplasma infections in *D. melanogaster* and *D. nebulosa* naturally cause early male-killing, with males dying at the embryo stage (Counce and Poulson, 1962, Bentley *et al.*, 2007). However, past studies have reported incomplete male-killing (with some male offspring surviving to mature larval or pupal stages) when mothers lay at a young age (Counce and Poulson, 1966, Anbutsu and Fukatsu, 2003). More recently, a study by Kageyama *et al.* (2007) demonstrated that *Spiroplasma* induces late male-killing in the offspring of young mothers, with male death occurring during the larval, pupal and early adult phases. They suggested that bacterial titre may influence not only the presence or absence of male-killing, but also whether its timing is early or late. Newly emerging female *Drosophila* infected with *Spiroplasma* have a low endosymbiont density that increases with age (Counce and Poulson, 1966, Anbutsu and Fukatsu, 2003), leading to the hypothesis that the late male-killing observed in these studies may be due to low titre.

The reports of late male-killing and incomplete male-killing to date derive from artificial infections, more specifically infections that have been taken from a member of the *willistoni* group and placed in *D. melanogaster* (Ikeda, 1965, Sakaguchi and Poulson, 1963, Sakaguchi and Poulson, 1960, Kageyama *et al.*, 2007). This raises the possibility that incomplete male-killing and late male-killing are the product of a bacterium that is poorly adapted to its new host. One particularly important aspect of adaptation of *Spiroplasma* may include the timing of proliferation compared to the life history of its host. The life history of different species of *Drosophila* in which *Spiroplasma* are found is quite variable. For instance, female *D. hydei* become reproductively mature at 3 days post eclosion (Markow and O'Grady, 2006), which is likely to select for a *Spiroplasma* whose ability to transmit is optimized at 3 days after eclosion from the pupa. For a *Spiroplasma* placed into *D. melanogaster*, whose females become sexually mature 8 hours after eclosion (personal observation) this timing could produce poor transmission during early reproduction.

A consequence of this logic is that transinfected strains that have been placed in a more rapidly developing host should perform more poorly than the native strain in terms of transmission and strength of phenotype during the early period of host reproduction. In this chapter I compare the properties of a male-killing *Spiroplasma* from *D. nebulosa* placed into *D. melanogaster* with the properties of the native male-killing *Spiroplasma*. As discussed above, these two *Spiroplasma* strains are very closely related. However, their hosts have subtly different life history. *Drosophila nebulosa* takes 13-14 days to develop from egg to adult at 25°C, compared to 10 days for *D. melanogaster* (personal observations from rearing). *Drosophila nebulosa* females take 48 hours to reach reproductive maturity at this temperature, compared to 8 hours for *D. melanogaster* females. If *Spiroplasma* strains are adapted to their host life history, we would expect the strain that is native in *D. melanogaster* to have higher male-killing efficiency and higher transmission efficiency, in particular early in the reproduction of its host.

I therefore determined whether the transmission, timing and completeness of male-killing varies between; a) male-killing *Spiroplasma* found naturally in *D. melanogaster*, and b) male-killing *Spiroplasma* naturally isolated from *D. nebulosa* and subsequently transinfected into *D. melanogaster*. The study will first of all inform as to whether the strains have diverged in properties associated with adaptation to their particular hosts (in particular transmission efficiency, male killing efficiency and timing of male death). Further to this, the study will also allow us to gauge whether past work on *Spiroplasma-Drosophila* interactions provides a valid view of a natural *Spiroplasma-Drosophila* association, or whether it provides a view of an infection that is maladapted by virtue of being recently transinfected. This past work includes inferences on *Spiroplasma* life history strategies (Anbutsu and Fukatsu, 2003), on male-killing mechanisms (Kageyama *et al.*, 2007, Veneti *et al.*, 2005), and on interaction with host immune system (Anbutsu and Fukatsu, 2010), all of which should be interpreted differently if the infection is in fact not well adapted to its host.

3.3 Materials and methods

Materials: Flies used in this experiment were Canton-S strain *Drosophila melanogaster* that were *Wolbachia* positive. Flies in treatments A and B flies carried two natural strains of male-killing *Spiroplasma* (MSRO) from *D. melanogaster* collected in Brazil placed on a CS background (see (Montenegro *et al.*, 2000, Montenegro *et al.*, 2005) for details of lines). Flies in treatments C and D carried transinfected strains of male-killing *Spiroplasma* (NSRO) originally from *Drosophila nebulosa*. One line carries a strain collected from *D. nebulosa* in 2003 (described in Bentley *et al.*, (2007)), and transinfected into *D. melanogaster* CS in 2006. The other line carries the strain NSRO, collected initially in the 1960s and maintained in *D. melanogaster* Oregon-R since this time. This is the strain that has been extensively characterized in studies of *Drosophila-Spiroplasma* interactions (e.g. Anbutsu and Fukatsu (2010), Kageyama *et al* (2007), Anbutsu and Fukatsu (2003)). The infection was transinfected into CS *D. melanogaster* at the same time as the newer NSRO infection (see Table 3.1).

Table 3.1: *Spiroplasma* strains used in experimental treatment groups, all placed on a *D. melanogaster* CS background.

Treatment	**Spiroplasma strain**	**Origin**
A	Red 82 (MSRO)	Naturally present
B	Red 42 (MSRO)	Naturally present
C	m/g (NSRO)	Artificial infection, present in *D. melanogaster* CS 4 years prior to experiment
D	m/o (NSRO)	Artificial infection, present in *D. melanogaster* in the 1960s, and placed into *D. melanogaster* CS 4 years before this experiment.

Spiroplasma titre and transmission efficiency are known to be affected by female host age, and that these effects may be passed on maternally, such that low titre in a female is associated with low titre in her daughters and onward (Anbutsu and Fukatsu, 2003). In order to control against the effect of previous maintenance regimes, the flies in this experiment were maintained on a strict parallel schedule in the year prior to the study, with females always being 10-14 days old at the point of reproduction.

Investigating variation in the timing of male death and transmission efficiency between *Spiroplasma* infections in *D. melanogaster*: One generation prior to the experiment, virgin females from each treatment group were collected within 24 hours of eclosion to adult and crossed to CS males. This early cross was made to ensure *Spiroplasma* titre was not elevated as an artefact of many generations of late reproduction. Virgin female offspring from these crosses were then collected and individually mated to FM7i/Y males from FM7i stock in order to determine offspring sex. The FM7i chromosome expresses GFP from four hours into development, such that female eggs/larvae from the above cross will fluoresce green, whilst males (that carry the Y chromosome from their father) do not fluoresce. Eggs were then collected from individual females at days 2-3 post emergence, days 5-6, days 9-10 and days 13-14 on grape juice laying plates, allowing a break in between laying times. Following this schedule, individual females were collected and tested for *Spiroplasma* presence using PCR assay as described in Chapter 2. In addition to the infected treatment groups, a control of uninfected Canton-S virgins was crossed with Fm7i/Y males in parallel to ensure the FM7i chromosome did not itself cause sex-biased viability differences.

For each oviposition time point, the rate and time of death of male progeny for each mother was categorised as in Table 3.2. Initially, the sex ratio at the first larval instar (L1) was scored (24 hours after egg lay). If no male larvae were present, then the female was categorised as having complete early male-killing at that maternal age. If male larvae were present, then they were picked into *Drosophila* media vials and sex ratio was scored two days following eclosion as adults. If no males were present at

adulthood, male-killing was classified as being complete late male-killing at that maternal age. If males were present as viable adults the brood for that maternal age was classified as either incomplete male-killing (if the sex ratio deviated from 1:1 sex ratio) or no male-killing (if there was no deviation from 1:1 sex ratio). Using this methodology, a profile of male-killing intensity with age was created for each mother and this profile compared across mothers from each treatment group.

During the experiment, female flies were allowed to oviposit on standard media in the time periods between focal egg lays. Five F1 progeny from each of these lays were collected and tested for *Spiroplasma* presence and DNA template quality as described in Chapter 2. This allowed measurement and comparison of the transmission efficiency of the different infections at varying maternal ages.

Table 3.2: The categories of male-killing efficiency recorded for all experimental mothers.

Male-killing category	**Definition**
Early male-killing	No male larvae hatch from eggs
Late male-killing	Some male larvae present but no male adults
Incomplete male-killing	A few males reach adulthood
No male-killing	Normal 1:1 sex ratio is produced at offspring eclosion

3.4 Results

Transmission efficiency of the native *Spiroplasma* infections was higher than *Spiroplasma* infections introduced into *D. melanogaster* from *D. nebulosa* during early reproduction (days 2-3: Table 3.3), but equivalent during late reproduction (days 13-14: Table 3.4). Statistical analysis indicated transmission efficiency was heterogeneous between infections during early reproduction (contingency table comparing ratio of infected and uninfected daughters amongst infection classes: χ^2=32.6, 3 d.f., $p<0.01$), but there was no evidence to reject homogeneity amongst infections during late reproduction (χ^2=1.69, 3 d.f., N.S.). In the case of early reproduction, it is clear that the *Spiroplasma* strains native to *D. melanogaster* have higher transmission efficiency than the strains introduced into *D. melanogaster* from *D. nebulosa*.

Within infection analysis showed a significant change in transmission efficiency with maternal host age in the novel *Spiroplasma* infection treatments (m/g: χ^2= 4.84, 1 d.f., $p<0.05$. m/o: χ^2=5.80, 1 d.f., $p<0.025$) and no significant change in transmission efficiency between maternal host ages in the native infection treatments (RED 85: χ^2= 0.0, 1 d.f., NS; RED42: χ^2=0.01, 1 d.f, NS).

Table 3.3: Early transmission efficiency (egg lay days 2-3) of different strains of *Spiroplasma* in CS flies. 95% Confidence intervals calculated through iteration of binomial sampling distributions in Minitab. Sample size (Number of sib-ships, total number of daughters tested) in parentheses.

Infection	**Proportion of daughters infected (n)**	**Binomial CI**
RED85 (native)	100% (16, 80)	0.95 - 1.00
RED42 (native)	94% (20, 100)	0.89 - 0.975
m/g (transinfected, 4 years)	80% (16, 80)	0.71 - 0.88
m/o (transinfected, 40 years)	74% (18, 90)	0.65 - 0.835

Table 3.4: Late transmission efficiency (egg lay days 13-14) of different strains of *Spiroplasma* in CS flies. Confidence intervals calculated through iteration of binomial sampling distributions in Minitab. Sample size (Number of sib-ships, total number of daughters tested) in parentheses.

Infection	**Proportion of daughters infected (n)**	**Binomial CI**
RED85 (native)	100% (6, 24)	0.86 - 1.00
RED42 (native)	94% (10, 49)	0.84 - 0.985
m/g (transinfected, 4 years)	97% (7, 31)	0.835 - 0.999
m/o (transinfected, 40 years)	96% (6, 26)	0.81 - 0.999

I then examined whether the nature of male-killing varied between native and introduced infections in flies of different age. Control crosses involving uninfected CS flies produced a 1:1 ratio of male-female first instar larvae and adults at all ages, indicating that the FM7i/GFP chromosome used for sexing did not itself produce sex-biased mortality (see Table 3.5).

Table 3.5: The sex ratio produced by CS uninfected control females over time, where CS females were crossed to FM7i/Y males with the result that female F1 progeny express GFP. Number of crosses from which data were summed given in parentheses below laying date.

	Days 3-4 (n=15)	**Days 5-6 (n=16)**	**Days 9-10 (n=17)**	**Days 13-14 (n=11)**
Sex ratio at L1	143m:140f	136m: 128f	166m: 194f	48m: 58f
Sex ratio at Adult	111m: 110f	118m: 99f	115m: 144f	47f: 52f

Having established that the FM7i chromosome did not produce sex biased mortality, I then compared the quality of male-killing between flies of different infection status at different ages. A small fraction of flies from infected lines exhibited no male-killing. *Post hoc* analysis by PCR assay demonstrated these were uninfected with *Spiroplasma*. In accord with the results with respect to transmission efficiency above, these flies were from transinfected lines alone. In the remaining flies (in which infection was present and did transmit to at least some progeny), there is an overall improvement in male-killing efficiency with increased host age in all treatments, in line with previous findings of Kageyama *et al.*, (2007) (see Figure 3.1). There was a progressive decrease in the fraction of broods in which either incomplete or late male-killing was observed, and statistical analysis rejected the hypothesis that the proportion of broods demonstrating early male-killing was homogeneous between sampling times (χ^2=12.43, 3 d.f. $p<0.01$). It is notable that by days 13-14 all infected females produce complete early male-killing.

I then analysed the data to determine whether infections differed in properties within a given time period. During early reproduction, the null hypothesis of no effect of infection strain on the frequency of early male-killing was rejected (Fisher exact test comparison across four infection classes: $p=0.047$). Heterogeneity was here associated with a single infection, RED42, in which complete early male-killing was found in all flies. However, when infections were partitioned into native and introduced, there was no evidence to reject the hypothesis that native infections had a higher frequency of early male-killing than late male-killing infections (Fisher exact test comparison between native and introduced infections: $p=0.51$ NS). At days 5-6 and days 9-10 of reproduction, there was no evidence of heterogeneity in the rate of early male-killing (Fisher exact test comparison across four infection classes: $p=0.73$ at days 5-6, $p=0.23$ at days 9-10). At days 13-14 all infected flies exhibited early male-killing irrespective of strain of infecting *Spiroplasma*.

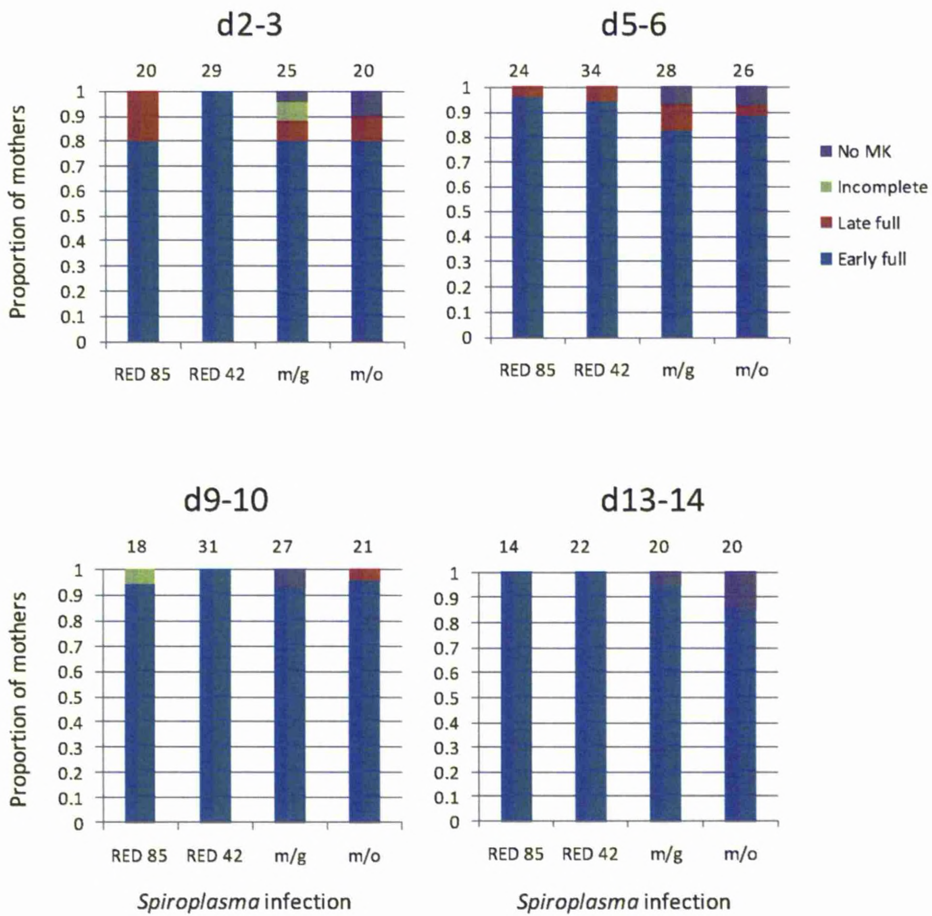

Figure 3.1: Male-killing efficiency of four *Spiroplasma* infections in *D. melanogaster*, two native to the host (Red85 and Red42) and two transinfected from *D. nebulosa* (m/g and m/o), with increasing host age at egg lay (d= days post eclosion). Number of mothers for which data were obtained at each time period is indicated above the bars.

3.5 Discussion

D. melanogaster either naturally infected with a native strain of *Spiroplasma*, or artificially infected with a strain taken from *D. nebulosa*, showed different characteristics in symbiont transmission efficiency, but similar characteristics with respect to male killing quality where infection was present. The native strain showed consistently high efficiency in transmitting to the next generation independent of host age at egg lay, whereas the novel infection treatments showed lower transmission efficiency to offspring produced at a host female age of 2-3 days, which improved to high efficiency with a maternal age of 13-14 days.

Notwithstanding differences in the transmission of the infection (which is measured adult to adult), there were no differences apparent in male-killing timing and efficiency. Male-killing did increase in efficiency with age, with early male-killing being the rule in late reproducing flies and present only in a sizeable majority of the offspring of young flies. However, whilst the data analysis is not powerful (late or incomplete male-killing is seen only in a fraction of young flies), there was no difference in timing or efficiency between native and introduced infections.

Overall, therefore, native strains are better adapted to their host in terms of early transmission efficiency, but the strains do not differ significantly in their ability to kill male hosts. It is notable that *D. nebulosa*, the source of the novel infections used in this study, has a different life history to *D. melanogaster*, the new host in this study. The egg to adult development time for *D. nebulosa* is 13-14 days and females reach sexual maturity 2 days after eclosion. It is likely that the native *Spiroplasma* infection is adapted to reach optimum bacterial titre over this 13+ day period in *D. nebulosa* order to increase its own transmission. The egg to adult development time in *D. melanogaster* is 10 days, with an eight hour period following this in which females reach reproductive maturity. Therefore an infection adapted to the longer development time of *D. nebulosa* that finds itself in a *D. melanogaster* host could be

expected to display sub-optimal characteristics such as lower transmission efficiency when the host reproduces at an earlier stage.

One observation of further note is that the two introduced infections (m/g and m/o) showed very parallel profiles in terms of transmission efficiency. This parallel profile exists despite one of these infections having been transferred into *D. melanogaster* in the recent past (four years before this study, 80 generations before the experiment) and the other infection having been transferred in over 40 years ago (more than 700 generations before the experiment). The similarity of transmission efficiency of these two infections reinforces the conclusion made in Chapter 2 that transmission efficiency is not a rapidly evolving trait. This conclusion is, of course, tempered by the knowledge that the two infections are not identical (both derive from *D. nebulosa*, but not the same *D. nebulosa* line).

Finally, the data allow comment to be made about the utility of past studies on *Spiroplasma-Drosophila* interactions based on the introduced infection NSRO. These studies are likely to be sound in terms of male-killer biology (e.g. Veneti *et al.*, (2005)), because the quality of male-killing exhibited by the two strains is broadly parallel. However, transmission efficiency of native and introduced infections does differ. This may reflect underlying differences in bacterial titre and in interaction with the host. Whilst the infections are broadly comparable, precaution would indicate that it is better to use a native strain rather than an introduced one if we seek to understand coevolved *Spiroplasma*-host interactions.

Chapter 4

How do insects react to novel inherited symbionts? A microarray analysis of *Drosophila melanogaster* response to the presence of natural and introduced *Spiroplasma* (adapted from Hutchence, K.J. *et al.* 2011 *Molecular Ecology* in press).

4.1 Abstract

Maternally inherited endosymbionts are found in numerous insect species and have various effects on host ecology. New symbioses are most commonly established following lateral transfer of an existing symbiont from one host species to another. Laboratory study has demonstrated that symbionts commonly perform poorly in novel hosts, with weak vertical transmission and maladaptive pathogenicity being observed in the generations following transfer. This poor performance likely limits symbiont occurrence. In this chapter, microarray technology is used to test whether poor symbiont performance observed following one year of vertical transmission through a new host is associated with alteration in host gene expression, or whether it occurs independently of this. I utilize the *Drosophila melanogaster–Spiroplasma* interaction and test the response of the host in the presence of both natural *Spiroplasma* infections and novel *Spiroplasma* infections transinfected previously from other host species. None of the *Spiroplasma* infections investigated produced up-regulation in host haemolymph/fat body based immune responses and the hypothesis that failure to thrive was associated with immune up-regulation was therefore rejected. One new infection was associated with a down-regulation of genes associated with egg-production compared to uninfected controls, indicative of damage to the host. The *Spiroplasma* infection that showed the weakest vertical transmission showed no significant disturbance to host gene expression compared to uninfected controls. It is concluded that the failure of *Spiroplasma* in novel host species is associated either with causing harm to their new hosts, or through a failure to thrive in the new host that occurs independently of host responses to infection.

4.2 Introduction

Many arthropod species harbour maternally inherited endosymbionts (Duron, 2008, Gasparich, 2002). These organisms have various associations with their hosts including obligate beneficial mutualism (Ferrari *et al.*, 2004, Baumann, 2005, Allen *et al.*, 2007), non-obligate symbiosis that may provide a secondary benefit to the host (Scarborough *et al.*, 2005, Oliver *et al.*, 2005, Haine, 2008, Toh *et al.*, 2006, Hansen *et al.*, 2007) and reproductive parasitism (Engelstadter and Hurst, 2007, Bandi *et al.*, 2001, Charlat *et al.*, 2005, Dyson and Hurst, 2004, Bentley *et al.*, 2007). As a result, maternally inherited endosymbionts are thought to drive many aspects of host ecology and evolution.

The factors determining the frequency with which insects are infected with symbionts are not well understood. The incongruence of host and symbiont phylogenies in many cases indicates that new host-symbiont combinations follow from lateral transfer, the movement of a symbiotic microbe from one species to another. Introduction of new infections through lateral transfer has been observed occasionally in the laboratory, for instance following transfer of ectoparasitic mites that act as 'shared needles', moving haemolymph from one species to another (Jaenike *et al.*, 2007). Following lateral transfer to a single individual, the establishment of these novel infections within a host species requires the infection to show good vertical transmission and cause little pathology in their new host species. However, it is known that symbionts in new host-symbiont interactions that do initially colonise the host successfully and can vertically transmit commonly produce either pathology in their host in subsequent generations or fail to transmit to a significant proportion of a female host's progeny (Kageyama *et al.*, 2006, Tinsley and Majerus, 2007). Thus, the ability of a symbiont to transmit vertically and thrive in a new host species represents an important constraint on the spread of new symbionts through host populations.

Whilst it is known that poor performance in new symbiont-host combinations will limit symbiont presence, the reasons for this poor performance are not well established. Comparison of the results of a variety of different experiments led Engelstaedter and Hurst (2007) to note that poor performance of the symbiont was most commonly associated with a high genetic distance between host species. However, the physiological basis of this trend is not clear. Three non-mutually exclusive hypotheses may explain poor performance of symbionts in novel hosts. First, the behaviour of the symbiont in a host species to which it is not adapted may perturb host physiological systems, causing a cost to the host. This would be most obviously evidenced in stress responses of the host, but may additionally be recognised in reproductive processes. Second, a symbiont in a novel host may induce the standing defences that protect against foreign microorganisms. It is certainly true that the importance of microbial symbionts in the lives of insects contrasts with a literature on their formidable innate immune defence against microbes (see Rolff and Reynolds (2009) for review). In this case, we would expect to see host immune responses up-regulated in the presence of symbionts that perform poorly (although it is notable that immune responses may also regulate beneficial symbioses (Nakabachi *et al.*, 2005). Finally, poor performance could be associated with a generalized failure of symbionts to thrive in host environments that are distinct from their native host, independently of any effect they induce in the host. In this case, gene expression of the host in the presence of poorly performing symbionts is expected to be closer to uninfected control hosts than gene expression of the host in the presence of well-adapted symbionts.

One of the most common symbionts in arthropods are members of the genus *Spiroplasma*. These wall-less bacteria are found in a wide range of arthropod hosts, and unlike many other inherited microbes, are found free in the haemolymph (Williamson and Poulson, 1979). Despite being found in the haemolymph, the *Spiroplasma* infection NSRO does not induce host immune system activity (Hurst *et al.*, 2003, Anbutsu and Fukatsu, 2010). This is probably associated with a lack of immune elicitors in *Spiroplasma*, arising from the absence of a peptidoglycan cell wall (Gasparich, 2002). However, *Spiroplasma* can be reduced in titre through ectopic activation of immunity (Hurst *et al.*, 2003, Anbutsu and Fukatsu, 2010).

Inherited *Spiroplasma* infections are also quite widespread in the genus *Drosophila*, including a natural infection (termed MSRO) in tropical populations of *D. melanogaster* (Montenegro *et al.*, 2005, Pool *et al.*, 2006). The power of *Drosophila* genetics, associated with the variety of biodiverse *Spiroplasma* infections in different members of the genus *Drosophila* (Haselkorn *et al.*, 2009), makes this an excellent place in which to investigate the nature of host-symbiont interactions (e.g. (Veneti *et al.*, 2005).

The study presented here uses the power of microarray approaches to investigate the causes of success and failure of symbionts in the generations following initial transinfection. I established lines of *D. melanogaster* carrying three different *Spiroplasma* strains that do not naturally reside in this species and allowed these infections to vertically transmit through *D. melanogaster* for a number of generations. These *Spiroplasma* infections showed varying vertical transmission efficiency in *D. melanogaster*, with one showing high vertical transmission efficiency, one medium, and one being poorly transmitted between generations. Host gene expression was then compared to ascertain the degree to which poor *Spiroplasma* performance is associated with induction of host systems, either of stress or defence.

4.3 Materials and methods

Insects and bacteria used: *Spiroplasma poulsonii s.l.* infection in natural populations of *Drosophila melanogaster* were originally recorded as being found on a *Wolbachia* positive genetic background (Montenegro *et al.*, 2005). The gene expression of *D. melanogaster* strain CS+*Wolbachia* positive (hereby described solely as CS) was therefore used as the baseline fly strain for analysis of *Spiroplasma* effects on host gene expression.

The effect of four different *Spiroplasma* infections on host gene expression was measured on this background (Table 4.1). Infections varied from the *S. poulsonii s.l.* infection found natively in *D. melanogaster* (MSRO), an *S. poulsonii s.l* naturally found in *D. nebulosa* as described in Chapter 3 (NSRO, very closely related to MSRO genetically, and shows excellent transmission in *D. melanogaster*), a strain from *D. hydei* as described in Chapter 2 (HY1, monophyletic with *S. poulsonii* but is genetically distinct from it and transmits less well in *D. melanogaster* than the native infection) and a *Spiroplasma* strain from *D. mojavensis* as described in Chapter 2 (MOJ, quite distantly related to infection natively found in *D. melanogaster*, and has very poor transmission efficiency in *D. melanogaster*).

These infections were all placed on the CS background 12-18 months prior to the experiment through intra-abdominal injection of haemolymph from the source species into young CS virgin female flies, as described in Chapter 2. They were then maintained in the transinfected CS line through vertical transmission with selection for infection via PCR assay. The transinfected CS lines were maintained genetically homogeneous through mating to males from the source CS lines each generation.

Table 4.1: Infection status of *D. melanogaster* strains used in microarray comparison in order of that least expected to provoke an immune response at top, through the most expected to provoke an immune response at bottom. Vertical transmission efficiency data from Chapters 2 and 3.

Treatment:	**Infection status and type:**	**Vertical transmission efficiency of *Spiroplasma***
CS	Uninfected with *Spiroplasma*	
CS+MSRO	As above, with natural *D. melanogaster* infection of *Spiroplasma*, transinfected to CS 16 months prior to the experiment.	>98%
CS+NSRO	NSRO was first identified as a strain capable of infecting *D. melanogaster* in 1960. The strain under study was that isolated from *D. nebulosa* described in Bentley et al., 2007 and transinfected to CS 16 months prior to the experiment.	>95%
CS+HY1	As above, transinfected with *D. hydei* strain TEN 104-106, haplotype 1, 12 months prior to the experiment.	c. 82.8%
CS+MOJ	As above, transinfected with *D. mojavensis* strain QUIN 903-28, 12 months prior to the experiment.	c. 28.4%
CS+pathogen	CS as above recently exposed to septic shock in the form of pricking with heat killed *E.coli* K12	

In each *Spiroplasma* infected fly strain used in this comparison, there was 12-18 months between establishment of the transinfected lines and their use in the experiment, to ensure infection titre had stabilized in the lines. It is notable that the performance of the strains determined above did not alter over the period of passage

prior to the experiment (see Chapter 2). The precaution of passage before analysis prevents any initial reaction to the act of injection itself being evident in gene expression pattern. The experiments thus do not represent a test of response to injected *Spiroplasma*, but to vertically transmitted symbiotic infections established in the recent past.

In addition to analysis of gene expression in these transinfected fly lines, gene expression was analysed in the CS non-manipulated control, and for a 'septic shock' positive control. This septic shock treatment was performed to ensure the microarray was sensitive to induced immune responses. Septic shock was performed through exposure of CS flies to heat-killed *Escherichia coli* through pricking 6 hours before cull as described in Hurst *et al.*, (2003).

Insect rearing for microarray: In order to accurately compare the effects of various infections on the gene expression of *D. melanogaster* all other differences between individuals and strains needed to be eliminated. Genetic differences between strains were avoided through the use of a standard CS strain as described above, which were prevented from diverging in the laboratory via mating strain females to males from the CS base from which they were derived. Differences associated with culture conditions were minimized through rigorous and concurrent rearing of flies of each type. For each fly strain a sample of virgin females were crossed with CS males. 25 first instar larvae were collected from each strain and used to seed vials in order to maintain a controlled density of growing larvae, these being placed in 10ml of standard corn-meal agar fly media in a CT room maintained at 25°C with a cycle of 12 hours light: 12 hours dark. 15 vials were set up for CS and CS+HY1, 7 vials were set up for CS+MSRO and CS+NSRO and 68 vials were set up for CS+MOJ. The number of vials seeded per treatment reflected the transmission efficiency of that infection and the ease of collecting virgin females, with CS+MOJ being the least efficient and therefore requiring higher numbers to ensure enough infected flies can be obtained for the experiment and MSRO and NSRO being the most efficiently transmitted, with infected individuals producing adult females only. These vials were allowed to develop to adulthood and on eclosion virgin females were collected.

Virgin females were allowed to age for 10 days. On the tenth day, 20 CS females were subject to a septic shock in the morning. All females flies were then culled in the afternoon via snap freezing and bodies stored in TRIzol (Invitrogen) at -80°C prior to RNA preparation. Numbers stored were as follows: CS = 20 individuals, CS+MSRO = 18 individuals, CS+NSRO = 21 individuals, CS+HY1 = 49 individuals, CS+MOJ = 107 individuals, CS+pathogen positive control = 14 individuals (6 individuals died through septic shock). These numbers again reflected the likelihood of infection through vertical transmission, with CS+MOJ being poorly transmitted, and thus requiring more females to be collected to create a subsample of infected individuals.

Molecular preparation for microarray: Extractions of both DNA and RNA were taken from individual whole flies using the TRIzol method. Individual flies were collected into TRIzol and homogenized. Following phase separation DNA was removed from the interface between organic and aqueous layers and RNA was removed in the aqueous layer and stored at -80. DNA was then promptly prepared for all individuals following the manufacturer's instructions, and *Spiroplasma* infection status tested using PCR assay as described in Montenegro *et al.*, (2005). This step was to ensure that uninfected flies from *Spiroplasma* infected lines, generated through inefficient transmission, could be eliminated such that all flies where gene expression was measured were known to be *Spiroplasma* positive. Eight *Spiroplasma*-positive individuals per treatment were then chosen and their RNA individually extracted and purified from the preserved aqueous stage and RNA concentration estimated using NanoDrop® ND-1000 Spectrophotometer.

Reverse transcription of 5µg RNA was performed using anchored oligo dT (Sigma) and Superscript III followed by second strand synthesis with Second Strand Buffer (Invitrogen), DNA Polymerase I (Invitrogen), RNaseH (New England Biolabs) and *E. coli* Ligase (GE Healthcare). The resultant ds DNA was then purified using G50 columns and DNA concentration estimated using NanoDrop® ND-1000 Spectrophotometer. 500ng ds DNA was then labelled as biological dye-swap replicates using the BioPrime DNA labelling Kit (Invitrogen) in the presence of

fluorescently labelled Cy3- or Cy5-dCTP (GE Healthcase). Samples were co-hybridised to long oligonucleotide microarrays (INDAC *Drosophila melanogaster* 14.5K long oligo array - GEO platform accession GPL5135) for 16 hours at 51°C using a GeneTac hybridisation station (Digilab Genomic Solutions Ins). Post hybridisation washes were performed according to slide manufacturer's (Full Moon Biosystems) recommendation. Arrays were scanned using the GenePix 400B dual laser scanner (Axon Instruments) at 5μm resolution and individually optimised PMT gain settings. Intensity values for each probe were extracted using Dapple (Buhler *et al.*, 2000). Detailed protocols for array spotting, labelling, hybridisation washes and scanning are available at http://www.flychip.org.uk/protocols/.

Microarray design: Gene expressions for eight individual female flies from each of the six treatments (as Table 4.1) were analysed. Every treatment was compared to a central reference pool of 48 uninfected CS flies using the array; each treatment group was compared to every other treatment group using statistical analysis.

Bioinformatics and statistics: Spot intensities were normalised within and between arrays using variance stabilisation (Huber *et al.*, 2002) in the vsn package in R/Bioconductor. The magnitude and significance of treatment effects for each spot intensity were estimated using linear models in the Limma package in R/Bioconductor (http://www.r-project.org and http://www.bioconductor.org). False discovery rates (FDRs) were calculated using the Benjamini-Hochberg method (Hochberg and Benjamini, 1990). Clusters of co-expressed transcripts, i.e. genes that exhibited a similar pattern of differential transcription among treatments, were identified using k-means clustering on genes that exhibited differential expression among treatments (Wit and McClure, 2004, Evans *et al.*, 2008). The k-means clustering procedure was run 100 times and the most robust set of clusters picked for further analysis, where robustness was defined as the extent to which the same genes appeared in the same clusters over replicate k-means runs. Over-representation of Gene Ontology (GO) terms within clusters was determined by hypergeometric tests (Allison *et al.*, 2006, Evans *et al.*, 2008).

4.4 Results

This study aimed to identify differences in gene expression patterns between six *D. melanogaster* treatment groups each of a different infection status (see Table 4.1). A total of 1174 probes were found to differ in expression among treatment groups, i.e. to exhibit an absolute log fold change of > 0.5 and an FDR of > 0.5. These 1174 probes were then grouped into one of 12 clusters using k-means clustering such that genes with a similar pattern of expression were grouped into the same cluster (see Table 4.2). For a complete list of genes falling in these clusters please see Appendix, Table A1. In total 33 GO terms were identified within the biological process ontology as significantly associated with one or more k-means clusters. The biological processes in which these gene expression changes are likely to impact are summarised in a heat map of GO terms that are overrepresented in each cluster (Figure 4.1).

Table 4.2: Results of k-means clustering using 12 centres, showing number of probes and robustness for each cluster.

Cluster	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
No. probes	11	16	17	19	32	61	85	99	131	164	182	357
Robustness (%)	98	92	97	99	92	89	85	97	97	94	78	73

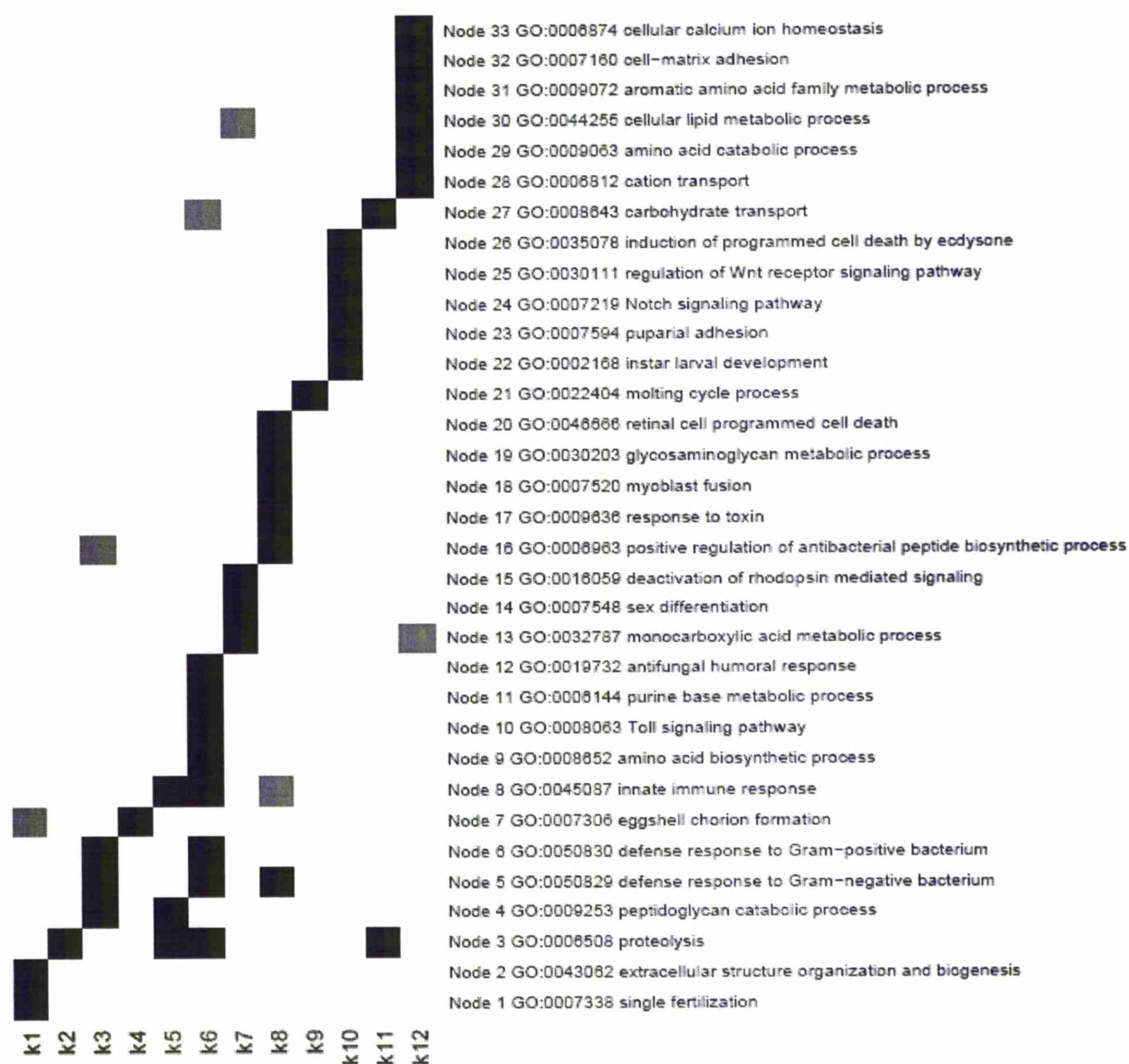

Figure 4.1: A heatmap showing the gene ontology terms in the biological process hierarchy for which significant over-representation of probes was found within one or more of the 12 k-means clusters (k1 - k12). Black areas indicate significance at $P < 0.01$, grey areas with significance at $P < 0.05$.

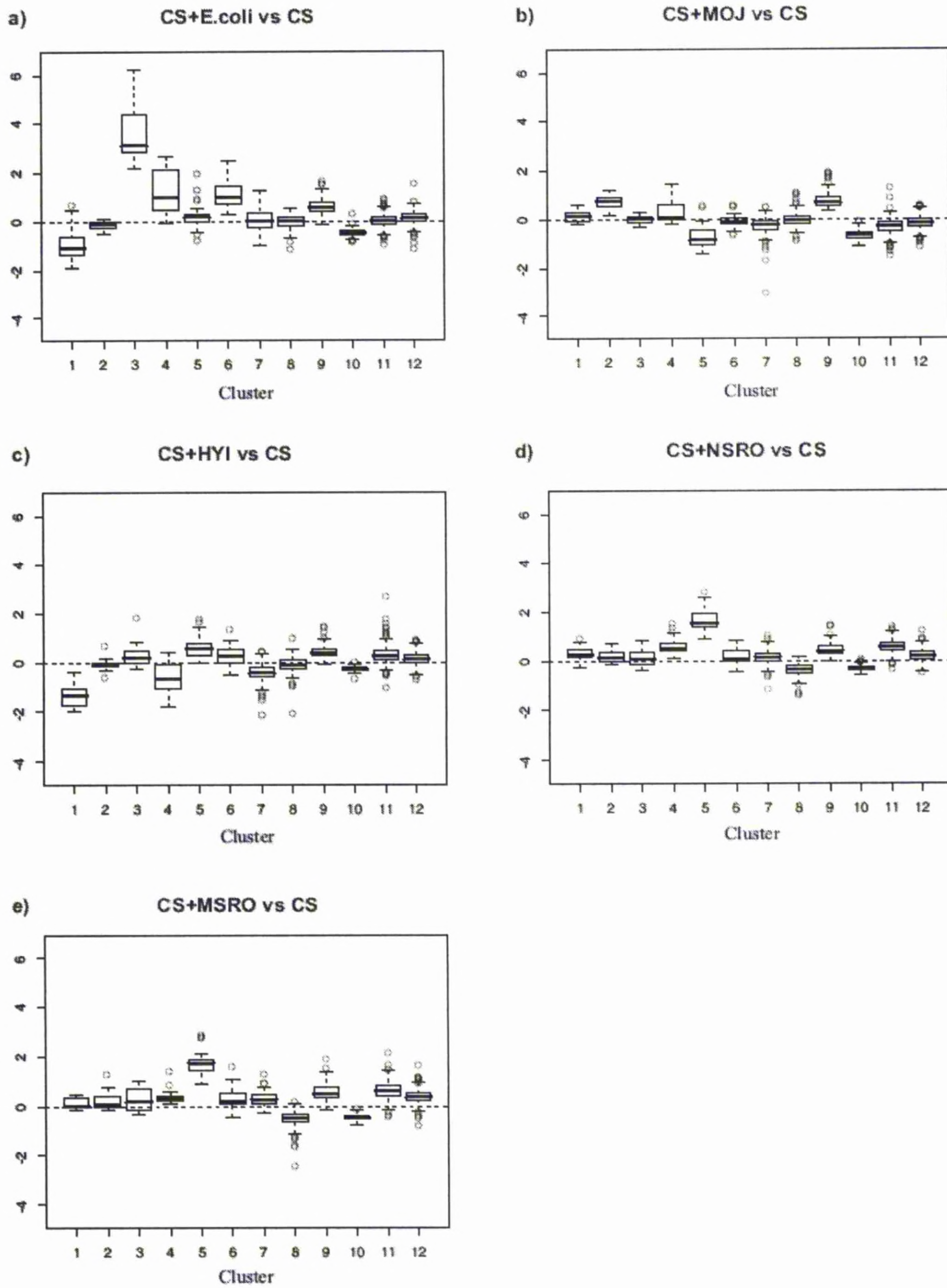

Figure 4.2: Boxplots of the log_2 fold changes of the five treatment fly strains compared to the control of uninfected CS for each of the 12 clusters.

Boxplots of the log_2 fold changes of the five treatment fly strains compared to uninfected CS flies for each of the 12 clusters are given in Figure 4.2 for each infection type. These boxplots clearly show that host gene expression patterns differ between flies of different infection status. The first notable feature is that expression in the positive control septic shock treatment (CS+pathogen) shows a very different profile to all symbiont infections, with an 8-fold up-regulation of gene cluster 3 (mean log_2 change in expression level = 3) and a 2-fold up-regulation of cluster 6. These responses, which reflect immune response to Gram-positive or Gram-negative bacteria and innate immunity or antifungal immunity respectively confirm the sensitivity of the array to detect immune activation and were not observed in any other fly treatment groups. A 3-fold down-regulation is also seen in cluster 1 for the septic shock treatment. This cluster of genes is associated with fertilisation and egg production.

Flies infected with the natural *Spiroplasma* infection MSRO, and those carrying the very closely related *Spiroplasma* NSRO, showed markedly similar changes in gene expression in comparison to the CS control. Individuals carrying these infections showed a 3 to 4-fold up-regulation of gene cluster 5, a pattern exhibited to a lesser degree by flies carrying the HY1 infection, but not observed in other treatment groups used in the comparison. A detailed analysis of response in this cluster is given in Table 4.3. Genes in this cluster are associated with non-specific immunity, particularly in the form of proteases and antimicrobials secreted in the gut.

Flies carrying the *Spiroplama* strains HY1 and MOJ (which are more distantly related to the native infection and perform more poorly in *D. melanogaster*), showed less perturbation of gene expression than flies carrying the native infections. Flies infected with the HY1 infection showed a 2-3 fold down-regulation of gene cluster 1 (associated with fertilisation and egg production) in comparison to other symbiont infections, but in common with septic shock flies. Finally, flies infected with the *Spiroplasma* strain from *D. mojavensis* (CS+MOJ), which has very poor vertical transmission in *D. melanogaster*, had no obvious perturbation in gene expression in comparison to uninfected controls.

Table 4.3: Cluster 5 genes and log_2 fold change (FC) and adjusted p-values in MSRO and NSRO treatment groups compared to uninfected CS fly controls.

FlyBaseID	FlyBase Gene	MSRO vs CS logFC	MSRO vs CS adjusted p-value	NSRO vs CS logFC	NSRO vs CS adjusted p-value
FBgn0034295		1.39	1.59E-03	1.24	1.46E-02
FBgn0038973		1.67	1.25E-03	1.25	3.97E-02
FBgn0035176		2.01	5.50E-04	1.58	1.78E-02
FBgn0033788		1.74	5.90E-05	1.52	6.98E-04
FBgn0004426	LysC	2.73	2.28E-04	2.52	1.71E-03
FBgn0002570	LvpH	1.52	3.72E-03	1.46	1.59E-02
FBgn0039342		1.46	5.12E-03	1.65	4.28E-03
FBgn0050360		1.33	4.40E-03	1.37	9.10E-03
FBgn0039330		2.13	9.77E-05	1.48	1.17E-02
FBgn0023197	Jon74E	2.80	1.95E-04	2.32	4.12E-03
FBgn0033327	PGRP-SC1b	1.56	9.71E-03	2.04	2.46E-03
FBgn0035664	Jon65Aiv	1.77	4.40E-03	1.90	6.37E-03
FBgn0040060	yip7	0.87	5.71E-02	1.14	3.44E-02
FBgn0034664		1.08	1.25E-03	0.92	1.66E-02
FBgn0039471		1.73	1.65E-05	1.55	2.16E-04
FBgn0004425	LysB	2.77	7.23E-05	2.61	3.31E-04
FBgn0033297		1.46	9.44E-04	1.40	4.17E-03
FBgn0036738		1.61	4.53E-04	1.72	6.06E-04
FBgn0034663		1.60	1.40E-03	1.47	9.04E-03
FBgn0040885		1.11	5.65E-03	1.21	7.85E-03
FBgn0043575	PGRP-SC2	1.08	8.68E-03	1.30	4.39E-03
FBgn0036766		1.78	2.69E-04	1.77	7.52E-04
FBgn0031654	Jon25Bii	1.76	5.08E-03	1.94	5.58E-03
FBgn0010425	epsilonTry	2.11	2.07E-04	2.00	8.73E-04
FBgn0032049		1.74	1.58E-03	1.89	1.93E-03
FBgn0003358	Jon99Ci	1.81	1.12E-03	1.51	1.80E-02
FBgn0010357	betaTry	1.80	1.02E-03	1.81	2.72E-03
FBgn0050160		1.47	2.89E-04	1.44	8.91E-04
FBgn0010359	gammaTry	1.97	2.07E-04	2.13	2.12E-04
FBgn0034296		1.65	1.18E-04	1.30	4.22E-03
FBgn0004430	LysS	2.86	1.15E-05	2.81	6.47E-05
FBgn0033296		1.74	1.56E-04	1.45	2.96E-03

4.5 Discussion

In this study, *Drosophila* gene expression was analysed in the presence of an array of symbiotic *Spiroplasma* strains, ranging from a strain naturally occurring in *D. melanogaster*, to strains more phylogenetically distant from this natural *D. melanogaster* strain and that infect other species of *Drosophila*. Associated with this variation in relatedness is variation in performance of the *Spiroplasma*, with the distantly related strain MOJ being poorly transmitted in *D. melanogaster* and the strain from *D. hydei* causing apparent pathology. My aim was to investigate the degree to which *Spiroplasma* success (in terms of pathology, vertical transmission efficiency) is reflected in differences in patterns of host gene expression from those seen in control flies without *Spiroplasma* symbionts.

Microarray analyses show that the pattern of host gene expression differs between fly strains of varying infection status. Whilst the positive control septic shock did register on the microarray, response in this cluster of genes was not seen in any other *D. melanogaster* strains used in the comparison, showing that inherited *Spiroplasma* infection does not elicit a septic immune response in its host (note, this analysis is of inherited *Spiroplasma* infection, and does not comment on any presence/absence of septic immune response at the point of introduction). The absence of such a response from the natural infection (MSRO) and its close relative (NSRO) is unsurprising, and concordant with the more limited previous survey of gene expression which suggested that native *Spiroplasma* bacteria are able to remain undetected by the host despite being exposed to the immune system (Hurst *et al.*, 2003).

More pertinently, the data allow us to reject the hypothesis that the two strains of *Spiroplasma* that perform poorly (MOJ and HY1) do so because of up-regulation in the septic immune system. *Spiroplasma* strains which are inherited can go undetected by the immune system in a wide range of *Drosophila* species, even ones they have not previously encountered. Being unseen by host immune systems during symbiosis is thus probably not a coevolved property of particular *Spiroplasma* symbioses, but is

rather a general property of *Spiroplasma*. The Mollicutes, of which *Spiroplasma* represents one genus, do not possess peptidoglycan cell walls. They thus generically lack one of the key elicitors of innate immune responses. This does suggest a need to repeat this work on other microbial symbionts in order to establish whether lack of induced immunity in response to new symbionts is a particular property of *Spiroplasma* symbionts or a general property of symbionts.

In Chapter 2 it was demonstrated that *Spiroplasma* in *D. hydei* (HY1) has a significant impact on host fecundity when present in *D. melanogaster*, a finding that has been previously noted anecdotally by Kageyama *et al.*, (2006). Evidence corroborating this hypothesis is seen in the significant down-regulation observed in a cluster of genes (cluster 1) involved in egg production and fertilisation (an expression pattern that is also seen in the CS+pathogen control). This is consistent with the notion that *Spiroplasma* occasionally causes pathogenicity in novel hosts (Tinsley and Majerus, 2007) and lower productivity (Kageyama *et al.*, 2006), adding a further factor potentially limiting the horizontal spread of *Spiroplasma* infection in the wild.

CS+MSRO and CS+NSRO represent flies infected with the natural *Spiroplasma* infection in *D. melanogaster* and flies infected with the closest relative of that infection, which is found in *D. nebulosa*. Identity of sequence across three housekeeping genes implies these *Spiroplasma* strains are very closely related (Montenegro *et al.*, 2005). One group of host genes (cluster 5) is significantly up-regulated in both of these strains and to a lesser extent in CS+HY1. The exact function of this cluster of genes is unclear. Where information is known, cluster 5 genes are associated with host gut function and immune challenge (Lemaitre, 2000, De Gregorio *et al.*, 2001, Werner *et al.*, 2000, Carlson and Hogness, 1985, Mellroth *et al.*, 2003, FlyBase *et al.*, 2004) (see Table 4.3). However, it is not clear whether expression of these genes will affect *Spiroplasma*. One of the more obviously 'anti-microbial' genes, PGRP-SC1B is known to be gut acting (Mellroth *et al.*, 2003), and acts to breakdown peptidoglycan, a component of bacterial cell walls (Steiner, 2004). Because peptidoglycan is absent in *Spiroplasma*, it is unlikely that this gene product

will affect *Spiroplasma* function. It is possible that these differences in gene expression reflect a difference in feeding behaviour of the fly associated with *Spiroplasma* infection, for example increased or decreased appetite.

Across the strains of *Spiroplasma* examined here, a correlation can be seen between the phylogenetic relatedness of strains and patterns of host gene disturbance. Those *Spiroplasma* that are most closely related behave most similarly, for example both the native *D. melanogaster* strain (CS+MSRO) and its closest relative from *D. nebulosa* (CS+NSRO) provoke all but identical patterns of host gene expression. CS+MOJ, the most distant relative, causes no disturbance to host expression, and CS+HY1 provokes a mild response that falls somewhere between that of CS+MSRO & CS+NSRO and the CS+MOJ lines. Whilst the general conclusion that perturbation of gene expression declines with genetic distance of the strain from the native requires more intense evaluation, patterns of host gene expression do mirror the *Spiroplasma* phylogeny and this pattern is consistent despite the low number of strains compared.

The lack of alteration of gene expression in flies infected with the *Spiroplasma* strain from *D. mojavensis* indicates that the failure of this infection to thrive in the host is not associated with any form of host response to infection. Rather, the infection simply cannot tolerate the foreign environment that is *D. melanogaster*. In ecological terms, the host species is beyond the fundamental niche of the bacterium (Hutchinson, 1957). The aspects of host physiology and or biology that make hosts suitable and unsuitable lie undetermined, but are not associated with induced host responses to infection.

In conclusion, activation of the immune system *per se* is unlikely to be the reason novel infections show poor vertical transmission (with the caveat that it is impossible to comment on any role of the immune system on first inoculation of the infection). Lateral transfer of *Spiroplasma* bacteria in the wild is likely to be limited by bacterial inability to rapidly adapt to a new host environment, with a failure to thrive leading

to low transmission efficiency and low bacterial titre, quickly resulting in loss from a population. In addition, this study is consistent with the idea that horizontal transmission events may result in unexpected host pathology, and this disadvantageous effect on a new host will also prevent *Spiroplasma* spread. Finally, whether a host is hostile to *Spiroplasma* growth is somewhat unpredictable – the *Spiroplasma* in *D. nebulosa* appears naturally adapted to *D. melanogaster*, despite the host species being phylogenetically quite distant.

Chapter 5

Spiroplasma prevalence in *Drosophila* species: a snapshot in Panama and the Caribbean

5.1 Abstract

Spiroplasma are maternally inherited endosymbionts found widely in arthropods. They have a diverse range of effects on their hosts ranging from reproductive parasitism to beneficial mutualism. It is one of only two known heritable endosymbionts of *Drosophila* species. To date, extensive screening has found *Spiroplasma* infection in 19 of over 200 *Drosophila* species sampled. Previous screens have been based on samples taken from stock centre culture or have been biased towards proband species, both approaches being flawed in their detection of the frequency of *Spiroplasma* infections. Here, *Drosophila* species were sampled directly from biodiverse wild regions to represent the natural species composition in each locality and screened from *Spiroplasma* infection. Forty three of the 412 individuals sampled in this survey were found to carry *Spiroplasma* (10.44%). Infected individuals represented 4 species groups (*saltans, melanogaster, willistoni, cardini*). The record in the saltans group is the first known case of *Spiroplasma* infection in this group. Infected individuals were found at all collection sites, but the highest proportion was found in Dominica where 27 of 160 individuals were positive for *Spiroplasma* (16.88%). This survey gives a preliminary indication of the presence of *Spiroplasma* infection in the natural composition of *Drosophila* species groups found within sample site communities, but further work is needed to expand on this screen and to draw more precise conclusions.

5.2 Introduction

Spiroplasma are bacteria derived from the Mollicute division and are defined by being helical, motile and lacking a cell wall (Gasparich *et al.*, 2004). They are obligate host-associated bacteria with a wide range of hosts including insects, crustaceans, arachnids and plants (Gasparich *et al.*, 2004). The majority of *Spiroplasma* strains in insects show vertical transmission through the female line, although there is growing evidence of occasional horizontal transmission events that carry infections from one species to another (Haselkorn *et al.*, 2009, Jaenike *et al.*, 2007). Their interactions with insects are diverse. Particular *Spiroplasma* have been recently revealed to be mutualistic and beneficial, for example producing tolerance to natural enemy attack in *Drosophila* (Jaenike *et al.*, 2010, Xie *et al.*, 2010). Previously, they were recorded as exhibiting reproductive parasitism via embryonic male-killing in ladybirds, butterflies and flies (Hurst *et al.*, 1999b, Majerus *et al.*, 1999, Tinsley and Majerus, 2006, Jiggins *et al.*, 2000a).

Vertical transmission may be less important for *Spiroplasma* in other host species, and they can be directly pathogenic (Clark *et al.*, 1985, Mouches *et al.*, 1984). Thus, infection is of commercial importance in honey bees (Clark, 1977, Mouches *et al.*, 1984), crabs (Wang *et al.*, 2005) and shrimp (Nunan *et al.*, 2005). Finally, *Spiroplasma* can represent plant disease agents vectored by arthropods. They are the cause of corn-stunt disease (*S. kunkelii*) and citrus stubborn disease (*S. citri*) in plants, which are both vectored by leaf-hopper insects. Although harmful to plants these *Spiroplasma* do not appear to harm their vectors and have been suggested to confer a benefit to their host in the form of cold tolerance (Ebbert and Nault, 2001, Ebbert and Nault, 1994). As a result of these diverse effects, *Spiroplasma* are of great importance to the ecology their hosts, with the potential to dramatically influence and drive host evolution.

There is a general appreciation that *Spiroplasma* are common associates of insects. Previously, this derived from accumulating individual records of *Spiroplasma* infection in the literature. Recently, there have been more focussed screens of wild collected individuals to estimate the incidence of *Spiroplasma* in nature. In the first general endosymbiont screen to include *Spiroplasma*, Goodacre *et al.*, (2006) recorded *Spiroplasma* presence in 23 of 122 spider species tested. In a study of endosymbiont occurrence in arthropods across Western Europe, Duron *et al.*, (2008) found infections from the *Spiroplasma ixodetis* clade in 9 of the 136 arthropod species sampled. These two studies both indicate *Spiroplasma* infections are common. However, the former suffers from being taxonomically narrow with respect to hosts sampled (especially as spiders are now known to show higher incidence of *Spiroplasma* than other arthropods (Duron *et al.*, 2008)). The latter study suffers from the shortcoming that it examines a subset of *Spiroplasma* diversity, that present in the *ixodetis* clade only.

Spiroplasma have long been known to be natural endosymbionts of *Drosophila* species, one of only two heritable endosymbionts infecting *Drosophila*, the other being the more intensively studied *Wolbachia* (Mateos *et al.*, 2006). These infections were examined extensively as the causative agent of maternally inherited male-killing in *willistoni* group flies during the 1960s (Poulson and Sakaguchi, 1961a, Counce and Poulson, 1961, Poulson, 1968, Poulson and Sakaguchi, 1960a). In the 1970s, it was recognised that *Drosophila hydei* carried non-male-killing *Spiroplasma* infection (Williamson and Poulson, 1979, Ota *et al.*, 1979a), an observation later confirmed by molecular systematic analysis (Kageyama *et al.*, 2006). This strain is now known to produce resistance to parasitoid natural enemies (Xie *et al.*, 2010). *Drosophila melanogaster* itself was demonstrated as carrying male-killing *Spiroplasma* in Brazil (Montenegro *et al.*, 2005) and Uganda (Pool *et al.*, 2006). Three members of the *tripunctata* radiation were later demonstrated to carry male-killing *Spiroplasma* (Montenegro *et al.*, 2006).

The brief review above contains many examples of *Spiroplasma* infection, where discovery of a *Spiroplasma* commonly followed isolation of *Drosophila* lines with

interesting maternally inherited phenotypes. However, PCR screening as employed by Duron *et al.*, (2008) and Goodacre *et al.*, (2006), allow a more accurate insight into how commonly *Spiroplasma* infection occurs in nature. In 2006, Mateos *et al.*, screened *Drosophila* lines from stock centres for the presence of *Spiroplasma* infection, recording 3 of 225 species to be infected (Mateos *et al.*, 2006). However, this study notably recorded species as negative for infection that are known to have infection from other studies (e.g. *D. melanogaster*, *D. willistoni*). It is clear that this study suffers from a false negative bias and underestimates incidence. False negatives in this case are likely associated with loss of infection in stock centre culture. This loss is inevitable for male-killing strains and likely for strains that provide a benefit in terms of natural enemy resistance, as stock centre cultures lack natural enemy pressure.

Subsequent to this work and in parallel with the work in this chapter, Watts *et al* sampled 19 wild *Drosophila* species from North and Central America finding *Spiroplasma* infection in 7 of these species (Watts *et al.*, 2009). This study has the merit of using natural material and therefore avoiding problems of loss in culture. However, it is narrow in its remit and the estimate is biased by the presence of proband species (species known to be infected prior to the study, for example *D. mojavensis* and *D. hydei*) within the sample.

This chapter has two aims. First, to understand how commonly *Spiroplasma* infections occur in *Drosophila* and add to the current body of work formed by previous studies. Second, to obtain new isolates of *Spiroplasma* in *Drosophila* whose properties can be further studied. This motivation is driven by the tractability of *Drosophila* as a system of study for *Spiroplasma*-insect interaction (as demonstrated in this thesis) and its ubiquitous use as a model species. I aimed to sample *Drosophila* species that represent the wild population composition found *in situ* and screen these for *Spiroplasma* infection. I have chosen tropical regions with high biodiversity as sample sites so as to gain a wide range of host species, and because it is known that *Spiroplasma* is likely to be found in these regions.

5.3 Materials and methods

***Drosophila* collection:** Flies were collected from various field sites across Panama and the Caribbean islands of Dominica, Grenada and Carriacou (see Figure 5.1 for sampling locations). Sampling locations were chosen to be as far from human habitation as circumstances allowed in order to avoid bias of samples with cosmopolitan *Drosophila* species (e.g. *D. melanogaster*, *D. hydei* and *D. simulans*) and to maximise biodiversity. Coastal areas were also avoided where possible due to their low *Drosophila* biodiversity. Trapping was via mixed fruit baits in bucket traps left for 2-4 days to allow flies to accumulate before collecting specimens. Bucket traps comprised of a sealable plastic container with small holes drilled in the sides to allow flies to enter, attracted by the bait. Collection was achieved by swift removal of the container lid and sweep netting the resulting swarm of flies which were then stored in 100% ethanol.

Figure 5.1: Collection localities for *Drosophila* specimens. Grey highlighted areas are: A = La Fortuna, B = Bocas del Toro, C = Barro Colorado Island, D = Dominica, E = Grenada and Carriacou.

Analysis of *Drosophila* biodiversity: In tropical regions, the trapping methods outlined above produced highly biodiverse *Drosophila* collections. Tropical *Drosophila* collections may contain upwards of 100 species. In many cases, accurate species identification cannot be made in the field. Many taxa, for instance, are only separable on the basis of male genitalia (e.g. *D. simulans* and *D. melanogaster*) and members of recent radiations (such as the *willistoni* clade) can only be resolved with behavioural assays or molecular systematics. Most species identification problems can be resolved with intense work on cultured material and expert advice. However, the process of culture itself acts as a sieve (a subset of species do not thrive in standard medium, also *Spiroplasma* infections can be lost through laboratory culture as mentioned previously). In addition, during the period of the project the movement of live *Drosophila* into the EU was prohibited and permit and courier companies stopped accepting *Drosophila*. This made it impossible to move live material collected.

In order to circumvent these issues, a broader approach to fly biodiversity was taken in which mitochondrial DNA barcodes were used in identification (Hebert et al., 2004). This gave the advantage that species information could be derived from specimens preserved in 100% ethanol that could also be used in molecular assays for *Spiroplasma* presence, as opposed to preservation for taxonomic identification which requires 70% ethanol to avoid desiccating the specimens but does not preserve DNA. The disadvantage to this method of identification is that one DNA barcode does not necessarily represent only one species, a phenomena known from studies of *Lepidoptera,* and also in the genus *Drosophila* (Hurst and Jiggins, 2005, Elias *et al.*, 2007). For this reason and to maximise accuracy in the data DNA barcodes were used to identify specimens to the level of species group in order to gain an account of host biodiversity.

DNA was extracted from individual flies via the Wizard® SV 96 Genomic DNA Purification System according to manufacturer's instructions. To ascertain *Drosophila* species all individuals were initially screened using the general insect primers HCO and LCO as described in Folmer, (1994) and Chapter 2, which amplify

640 bp of the cytochrome oxidase I gene of mitochondria. Product was cleaned of primer and unincorporated nucleotides using an ExoSAP digest and a cycle sequencing reaction was set up using the forward primer HCO. Sequencing product was then precipitated using sodium acetate and resuspended in HiDi formamide before running on an ABI sequencer. Sequencer output was selected by eye using BioEdit software and run through a nucleotide BLAST (Basic Local Alignment Search Tool) (Altschul *et al.*, 1990) analysis via the National Centre for Biotechnology Information (NCBI). Biodiversity of *Drosophila* collected is assessed to species group level only to ensure accuracy in the data where BLAST surveys did not give a unanimous result.

Analysis of *Spiroplasma* presence: All individuals were screened for general *Spiroplasma* presence using primers 23F (5' –CTC AGG ATG AAC GCT GGC GGC AT- 3') and TKSS (5' -TAG CCG TGG CTT TCT GGT AA- 3') as described in (Haselkorn *et al.*, 2009) using the DNA extracted as above. PCR cycling conditions were an initial denature of 3 min 94 °C, followed by cycles of 30 seconds at 94 °C, 45 seconds annealing at 65 °C, 45 seconds at 72 °C; annealing temperature was lowered 1.0 °C per cycle for 17 cycles, then kept for 20 cycles at 48 °C. Cycle sequencing reactions were performed using the forward primer 23F, otherwise all clean up, sequencing and analysis is as above.

5.4 Results

In total 412 individuals were sampled, representing 8 species groups (see Table 5.1). Forty three of the 412 individuals sampled were found to be infected with *Spiroplasma* (10.44%). Infected individuals represented 4 species groups (*saltans*, *melanogaster*, *willistoni* and *cardini*) with 10 infected individuals of unknown species grouping. Infected individuals were found at every collection site (see Table 5.2) with the highest proportion found in Dominica. The *Spiroplasma* infections found here represent strains from two host associations; *D. hydei* and *D. ananassae*, plus 4 unknown *Spiroplasma* infections (see Table 5.3).

Table 5.1: *Drosophila* species groups collected, number of individuals screened and infection status.

Species group	No. individuals sampled	No. individuals with *Spiroplasma* infection
saltans	95	12
melanogaster	156	15
willistoni	56	5
tripunctata	1	0
repleta	9	0
cardini	5	1
quinaria	1	0
virilis	1	0
Unknown	88	10

Table 5.2: *Spiroplasma* infection status partitioned by locations where infected individuals were collected.

Collection site	**No. Individuals sampled**	**No. *Spiroplasma* infected**	***Drosophila* species groups found**
La Fortuna, Panama	20	2	1 *willistoni*, 1 unknown
Bocas del Toro, Panama	64	3	2 *melanogaster*, 1 unknown
BCI, Panama	13	1	1 *melanogaster*
Dominica	160	27	11 *saltans*, 9 *melanogaster*, 3 *willistoni*, 4 unknown
Grenada and Carriacou	129	6	1 *melanogaster*, 1 *cardini*, 1 *saltans*, 1 *willistoni*, 2 unknown
Unknown	26	3	2 *melanogaster*, 1 unknown

Table 5.3 *Spiroplasma* diversity in collections.

***Spiroplasma* strain**	**No. infected individuals**	**Host species group**	**Collection site**
From *D. hydei*	37	Various	Various
From *D. ananassae*	2	*melanogaster*	BCI and Bocas del Toro
Unknown	4	2 unknown, 1 *melanogaster*, 1 *saltans*	2 Bocas del Toro, 1 Grenada, 1 Dominica

5.5 Discussion

Spiroplasma infection was found in 43 of the 412 individuals sampled in this survey (10.44%) and 4 of the 8 species groups represented (*saltans*, *melanogaster*, *willistoni* and *cardini*), plus 10 infected individuals of unknown species group. This is this first time *Spiroplasma* infection has been documented in the *saltans* group. One *saltans* species, *D. prosaltans*, collected in Brazil was recorded as exhibiting a maternally inherited sex-ratio distorting phenotype in the 1950's (Cavalcanti *et al.*, 1958, Cavalcanti *et al.*, 1957). This was put down to a complex interaction between nuclear genes and cytoplasmic factors, but the observations of the time exactly describe characteristics now known from maternally inherited endosymbiont infection that can have its phenotype suppressed by nuclear genes. It can be speculated that the production of all female broods in this species may be due to *Spiroplasma* infection, especially given the high proportion of sampled individuals found to be infected in the *saltans* group (12 infected individuals from 95 flies sampled, 12.63%) in this screen.

Infected individuals were found at all collection sites, but the highest proportion was found in Dominica where 27 of 160 individuals were positive for *Spiroplasma* (16.88%). The apparent high prevalence of *Spiroplasma* in Dominica may be due to a number of possible causes. First, there is likely to be an influence of sampling error due to varying numbers of individuals being collected at different sites and the sample sizes being relatively small. The highest number of individuals was collected from Dominica (160 individuals), however the number collected from Grenada and Carriacou was also large (129), but showed infection considerably less commonly (in only 6 individuals). Thus, whilst sampling error may be part of the source of variation, it is unlikely to be the complete source. A second possible explanation is in ecological variability. Recent studies have shown that some *Spiroplasma* in *Drosophila* provide resistance to natural enemies (Jaenike *et al.*, 2010, Xie *et al.*, 2010). Thus, varying levels of infection indicated in this study at different sites may mirror a geographic mosaic of the presence of natural enemies. A number of species groups have been shown to be infected in one locality, which suggests the presence

of a generalist enemy (most likely a nematode or parasitoid wasp) able to infect many species of *Drosophila*. No definite conclusions can be drawn on this subject from this screen alone. Further work would involve the collection of adult flies in given localities and the production of larvae from these adults to use as bait to attract natural enemies. Larval baits could then be screened for parasitoids and the parent flies screened for *Spiroplasma* infection to determine whether there is a correlation between the prevalence of the two.

The frequency of infection of *Drosophila* in this study is relatively high. However, the results are consistent with previous data from well studied groups. Notably, *Spiroplasma* presence in past non-molecular surveys of *willistoni* group flies estimate that 3-5% of individuals carry *Spiroplasma* (Williamson & Poulson, 1979), compared to 8% in this study, two estimates that are within sampling error of each other. It is likely that it is the underexplored *saltans* group flies that produce the somewhat high estimate of *Spiroplasma* presence in this screen and this group clearly deserves further study as a hotspot.

In regard to the reliability of data presented here, I believe the findings of this preliminary survey to be dependable, but with two caveats. Firstly, the genus *Spiroplasma* covers a wide range of organisms associated with insects, including some gut associated strains that are either non-heritable or environmental, for instance *S. citri* in fruit (Bove, 1997). Thus a sample that is *Spiroplasma* positive cannot definitively be ascribed to an inherited infection of the fly. A second issue is that flies collected in the field from each trap were stored collectively in alcohol before processing in the laboratory for sequence analysis. This has the potential to cause contamination, and there are some signs of this in a low level of contamination in the sequence traces. However, previous study has demonstrated that there is no reason to believe that this storage method causes an increase in the estimate of the incidence of intracellular bacteria within a sample and between individuals (Duplouy *et al.*, 2009). In addition, the occurrence of infection found in this study was spread intermittently throughout the samples collected, with no one storage vial having 100% infection, or an especially high number of infected individuals, which is the

pattern that would be expected if contamination had occurred. I am therefore confident that infection results are not a product of sample contamination.

In conclusion, *Spiroplasma* is present in several localities across Panama and on the islands of Dominica, Grenada, and Carriacou. It is present in four *Drosophila* species groups including *melanogaster*, *willistoni*, *cardini* and *saltans*, the last of which is the first recorded case of *Spiroplasma* infection in this group. This survey gives a preliminary indication of the presence of *Spiroplasma* infection in the natural composition of *Drosophila* species groups found within sample site communities, but further work is needed to expand on this screen and to draw more precise conclusions.

Chapter 6

Discussion

6.1 Synthesis

Lateral transfer of symbiont infections represents a major but poorly understood process. From the symbiont perspective the rate of lateral transfer, alongside the longevity of infection within a host species, determines the number of species infected by the symbiont. It represents a major process from the host perspective as the transfer of a symbiont into the new host often results in a macromutation; a mutational event of large phenotypic effect. Lateral transfer of inherited symbionts represents a poorly understood process, especially in that it is known to occur very commonly (as indicated from comparison of host and symbiont phylogenies). Whilst experimental research has indicated a number of potential mechanistic means by which transfer occurs, the factors defining whether transfer occurs remain poorly understood.

One feature of lateral transfer that has been suggested is that it is most likely to succeed where novel and native hosts are most closely related. These data derive from the results of the success and failure of artificial transinfection events, particularly of *Wolbachia*. In my thesis, I examined the success and failure of *Spiroplasma* symbionts in *Drosophila*. In Chapters 2 & 3 I examined a previously unexplored hypothesis, that lateral transfer into a new host will be most successful for strains of *Spiroplasma* that are more closely related to the strain found natively in that host species. Aside the native symbiont infection of *D. melanogaster*, the other hosts of *Spiroplasma* used were from different *Drosophila* species groups and thus hosts that are evolutionary distant from *D. melanogaster*; *D. hydei*, *D. nebulosa* and *D. mojavensis*. The performance of these transinfected strains that was found is in accord with the hypothesis that strains most closely related to the native strain

perform most well, although a wider experiment utilising a greater number and range of novel infections is needed to substantiate this hypothesis. The transmission efficiency gained by the strain from *D. nebulosa* is higher than that gained by the *D. hydei* infection and this is higher than that gained by the strain from *D. mojavensis*. This performance mirrors the genetic distance of the strains from the native infection: *Spiroplasma* from *D. nebulosa* is very closely related to the native strain of *D. melanogaster* (MSRO), *Spiroplasma* from *D. hydei* is distinct, but monophyletic with the native strain and that from *D. mojavensis* is more distantly related.

Further corroboration of this hypothesis is required as there are only three data points, which are too few to draw a complete conclusion although they do support the idea well. At the point this thesis began the strains of infected fly utilised in Chapters 2 and 3 were the only ones known and available. During the course of the thesis further strains have been identified (Watts *et al.*, 2009) and the data in Chapter 5 indicate that there is a diverse pool of *Spiroplasma* infections in wild *Drosophila*. Analysis of these strains should allow a more complete test of the above hypothesis. It would also be worthwhile to conduct reciprocal tests in different host species. In my thesis, I have examined performance in *D. melanogaster*. For many of the measures taken, performance in other host species could be conducted. This would be useful as it would make clear if the pattern was general, or specific to *D. melanogaster*.

Beyond testing the hypothesis that symbionts more related to native infections perform best in that host, the results from Chapters 2 & 3 also indicate that *Spiroplasma* in novel hosts can cause pathology. This result confirms the anecodotal observation of Kageyama *et al.* (2006) and mirrors the findings of Tinsley and Majerus (2007) for *Spiroplasma* in ladybird beetles. For *Wolbachia*, it is accepted that inefficiency of transmission is the main cause of failure for novel infections. For *Spiroplasma*, it is clearly the case that both inefficient transmission and pathology may cause failure of new infections to spread. An important area of research with respect to lateral transfer of *Spiroplasma* is the extent to which any phenotype is expressed in new hosts. The *Spiroplasma* infection in *D. hydei* was recently observed

to protect its host against attack by parasitic wasps (Xie *et al.*, 2010). The presence or absence of protection in novel hosts will be very important in determining whether *Spiroplasma* succeeds in new hosts. Laboratory transinfections usually result in infections that do not have perfect vertical transmission and may cause pathology. Without a form of drive, such as providing beneficial effects to the host or reproductive parasitism, these infections will be lost from the population. In Chapter 3 male-killing was observed to transfer as a fully functional trait (in that the infection showed the same male-killing properties in the novel host as the native infection). A key experimental question is the extent to which symbiont-mediated protection is transferable. Mechanistically it can be asked whether transferred symbiont-mediated protection would work against the same parasite in a different host. Ecologically, it would be important to know whether the new host is under the same natural enemy pressure as the donor host. The outcome of transinfection requires some study beyond the confines of the laboratory, as it is the natural environment (with natural enemies and environmental pressures) in which success is actually important.

The results of Chapters 2 and 3 also suggest that the fit of the symbiont to the host will need to be good at the onset of the new interaction. None of the three transinfected strains showed improvement in transmission efficiency over multiple passages in the laboratory. In other novel parasite-host combinations, for instance Myxoma virus in rabbits (Fenner, 1965), rapid evolution of the pathogen has been observed following transfer to the new host, resulting in a better fit of the parasite to that host. In the case of the myxoma virus this involved reduction in pathogenicity, maintaining an infective host for a longer duration and thus increasing myxoma fitness. Past studies of *Wolbachia* have indicated the potential for rapid evolution following transfer to a new host (e.g. McGraw *et al.*, (2002) Carrington *et al.*,(2010) and loss of infective ability in a native host following multiple passages in cell culture (McMeniman *et al.*, 2008). However, these studies examined virulence rather than transmission efficiency and might be criticized for being abnormal (in the case of myxoma, evolution following transfer to a new host was for the strain 'popcorn', which shows maladaptive virulence, for *Wolbachia*, cell culture passage is an unnatural environmental change). However, evolution of symbiont virulence in natural populations is known to occur in relatively short time frames (100

generations) (Weeks *et al.*, 2007). The results of my thesis indicate that transmission efficiency, a key parameter in determining if new infections persist, did not evolve during passage. There is some suggestion in my data that pathogenicity is more labile. The pathogenicity of the HY1 infection that I measured 40 generations after transfer into a new host was lower than anecdotally reported by Kageyama *et al.*, (2006) immediately following transinfection. However, this conjecture clearly needs detailed future study.

In Chapter 4 I examined whether host gene expression varied between symbiont infections, in particular by using microarray technology to examine if there were obvious host causes of the failure of infections to transmit, or obvious host consequences of being infected, expressed at the gene regulation level. The hypothesis that flies infected with novel *Spiroplasma* strains would react to them, resulting in inefficient transmission or pathology, was rejected. The strain of infection that caused least disturbance in host gene expression, that from *D. mojavensis*, was also the least well transmitted. There was no obvious up-regulation in immune defences against any infection. The native infection and the closely related infection from *D. nebulosa* both caused up-regulation in certain elements of gut proteolysis, and potentially gut acting anti-microbial factors. However, these are unlikely to be causes of the variation in performance, more likely they represent changes to nutritional state and feeding behaviour of hosts. Overall, the microarray analysis did not support host response to infection as a cause of variation in *Spiroplasma* performance. The host is more akin to an abiotic environment for the *Spiroplasma*, and *Spiroplasma* will be poorly adapted to some hosts as environments. Whilst it is the case that all *Drosophila* appear similar to us, it is clearly not the case that they are similar environments for *Spiroplasma*. Further, their fit to different *Drosophila* environments is not a product of different host gene expression responses to different *Spiroplasma*. It seems to be simply a matter of whether the *Spiroplasma* is able to thrive in that host environment.

What is not clear is whether this conclusion is specific to *Spiroplasma* or general to symbionts. *Spiroplasma* are unusual bacteria, possessing a different genetic code (Citti *et al.*, 1992) and being wall-less (Gasparich, 2002). The *Spiroplasma* genetic code has two rather than three stop codons (Citti *et al.*, 1992) and this may prevent expression of *Spiroplasma* genes in other species (because they may pre-terminate) and may prevent expression of genes from other species in *Spiroplasma* (as they may not terminate appropriately). The possession of a different genetic code may be a barrier to the transfer of genetic material to and from other symbionts and make *Spiroplasma* biologically distinct from them. It is notable that genetic material, in the form of phage virulence elements and surface proteins, are known to exchange within the symbiome (Darby *et al.*, 2010) and it is likely that *Spiroplasma* are not part of this extended symbiont gene pool.

The wall-less nature of *Spiroplasma* may also be important in making them distinct from other symbionts. Cell walls are the classic elicitors of immune function. Because they lack cell walls, it is likely that *Spiroplasma* never interact with immune systems, even in novel hosts. This may contrast with other symbionts that are walled, and whose walled structures may be immunogenic in hosts with which they have not coevolved. For these reasons, it would be worthwhile to test whether the findings of this thesis with respect to *Spiroplasma* were universal. The pea aphid (*A. pisum*) may be a good place in which to conduct this study. A completed genome (Richards *et al.*, 2010) will allow microarray approaches. The aphid community then presents a wide variety of symbionts that can be transferred into the pea aphid with ease (Russell *et al.*, 2003) and to which the reaction of the pea aphid can be ascertained. These strains also vary in their transmission and pathogenicity properties (Russell and Moran, 2005). Thus, links between host gene expression and symbiont properties could be made in the aphid system, with diverse gamma-proteobacterial symbionts. Usefully, *Spiroplasma* also occur in aphids occasionally (Fukatsu *et al.*, 2001) and this would allow direct comparison between reaction to different infections.

6.2 Final Perspective: What determines the success of symbionts in novel hosts and their prevalence in insect communities?

This thesis has examined the results of the transinfection of symbionts into a novel host species. The study has been primarily viewed from the symbiont perspective and has examined the transmission efficiency gained by the symbiont and the mechanistic causes of success and failure, including costs to the host and host response to infection. The novel *Spiroplasma* infections generated in Chapter 2 would have been unlikely to succeed in spreading through natural populations due to their low transmission efficiency. Notwithstanding this, symbiont infections in insects in the natural world are relatively common, as reinforced by the findings of Chapter 5. These two observations appear mutually incompatible but they are conclusions not confined to the studies in this thesis alone. This apparent contradiction is also true of *Wolbachia*, where laboratory transinfection frequently does not succeed, and gamma-proteobacterial symbionts of aphids, which do not thrive in novel hosts (Russell and Moran, 2005). Nevertheless, both are widespread symbionts in nature.

The contrast seen in the widespread nature of inherited endosymbionts coupled with the difficulties found in artificially producing new symbioses is most likely resolved by the sheer number of combinations of lateral transfer in the wild. Whilst it may be the case that transinfections to new host species are unlikely to succeed, if there are many new host species being naturally exposed to symbionts, then it is likely that some combinations will be compatible enough to allow new symbioses to establish and lateral transfer rates are then appreciable on an evolutionary timescale. In this thesis, infection in *D. nebulosa* performs fairly well in *D. melanogaster*. This compatibility between *Spiroplasma* and host was not predictable, save in the knowledge that *D. melanogaster* is known to have a similar, closely related, *Spiroplasma* infection. If only 1% of lateral transfer events to novel species are successful, transinfection will still occur at evolutionary relevant rates if many host species are being exposed. Even if the vast majority of infections fail to persist, the

few that do will be sufficient to explain the incidence in natural populations. Following this logic, symbionts with different incidence will vary in their ability to succeed following lateral transfer. It may be that the success of *Wolbachia* (compared to other symbionts) is associated with its tolerance of a breadth of host species.

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Appendix

Table A1: Gene identities and FlyBase addresses of the 1174 genes represented in each of the 12 k-means clusters, see Chapter 4.

Cluster	ID address	Flybase gene
1	FBgn0003979	Vm26Aa
1	FBgn0038395	
1	FBgn0000427	Dec-01
1	FBgn0003980	Vm26Ab
1	FBgn0000644	Fcp3C
1	FBgn0025834	
1	FBgn0000427	Dec-01
1	FBgn0003983	Vm34Ca
1	FBgn0052798	
1	FBgn0038611	
1	FBgn0014076	Vm32E
2	FBgn0030777	
2	FBgn0052504	
2	FBgn0032037	
2	FBgn0031471	
2	FBgn0015521	oho23B
2	FBgn0037222	
2	FBgn0037146	
2	FBgn0053288	
2	FBgn0030883	
2	FBgn0035343	
2	FBgn0023417	AP-2
2	FBgn0028883	
2	FBgn0037296	
2	FBgn0038539	Atg8b
2	FBgn0029907	Atx-1
2	FBgn0051246	
3	FBgn0004240	Dpt
3	FBgn0041581	AttB
3	FBgn0034881	
3	FBgn0043578	PGRP-SB1
3	FBgn0034329	IM1
3	FBgn0000277	CecA2

3	FBgn0034328	IM23
3	FBgn0010385	Def
3	FBgn0035806	PGRP-SD
3	FBgn0052185	
3	FBgn0025583	IM2
3	FBgn0028537	
3	FBgn0041579	AttC
3	FBgn0028396	TotA
3	FBgn0034407	DptB
3	FBgn0034511	
3	FBgn0040734	
4	FBgn0000360	Cp38
4	FBgn0037170	Trxr-2
4	FBgn0038469	
4	FBgn0000355	Cp15
4	FBgn0032127	
4	FBgn0041252	Femcoat
4	FBgn0000357	Cp18
4	FBgn0032789	
4	FBgn0035768	
4	FBgn0029568	
4	FBgn0030043	
4	FBgn0029697	
4	FBgn0032788	
4	FBgn0000356	Cp16
4	FBgn0052774	
4	FBgn0000359	Cp36
4	FBgn0041709	yellow-g
4	FBgn0035328	yellow-g2
4	FBgn0014466	Cp7Fc
5	FBgn0034295	
5	FBgn0038973	
5	FBgn0035176	
5	FBgn0033788	
5	FBgn0004426	LysC
5	FBgn0002570	LvpH
5	FBgn0039342	
5	FBgn0050360	
5	FBgn0039330	
5	FBgn0023197	Jon74E
5	FBgn0033327	PGRP-SC1b
5	FBgn0035664	Jon65Aiv

5	FBgn0040060	yip7
5	FBgn0034664	
5	FBgn0039471	
5	FBgn0004425	LysB
5	FBgn0033297	
5	FBgn0036738	
5	FBgn0034663	
5	FBgn0040885	
5	FBgn0043575	PGRP-SC2
5	FBgn0036766	
5	FBgn0031654	Jon25Bii
5	FBgn0010425	epsilonTry
5	FBgn0032049	
5	FBgn0003358	Jon99Ci
5	FBgn0010357	betaTry
5	FBgn0050160	
5	FBgn0010359	gammaTry
5	FBgn0034296	
5	FBgn0004430	LysS
5	FBgn0033296	
6	FBgn0031432	Cyp309a1
6	FBgn0034512	
6	FBgn0000279	CecC
6	FBgn0031726	
6	FBgn0038530	AttD
6	FBgn0052695	
6	FBgn0034364	
6	FBgn0003162	Pu
6	FBgn0052282	dro4
6	FBgn0040972	
6	FBgn0036876	
6	FBgn0035501	
6	FBgn0034647	
6	FBgn0031913	
6	FBgn0036203	
6	FBgn0039666	
6	FBgn0041182	TepII
6	FBgn0040735	
6	FBgn0010381	Drs
6	FBgn0039102	
6	FBgn0040582	
6	FBgn0035348	

6	FBgn0038465	Irc
6	FBgn0044810	TotX
6	FBgn0037396	
6	FBgn0027584	
6	FBgn0034330	
6	FBgn0040736	IM3
6	FBgn0051704	
6	FBgn0027550	
6	FBgn0010222	Nmdmc
6	FBgn0038346	
6	FBgn0031560	
6	FBgn0034470	Obp56d
6	FBgn0040653	IM4
6	FBgn0041180	TepIV
6	FBgn0003961	Uro
6	FBgn0033875	
6	FBgn0030262	Vago
6	FBgn0034871	
6	FBgn0003162	Pu
6	FBgn0002930	nec
6	FBgn0035977	PGRP-LF
6	FBgn0031561	
6	FBgn0032835	
6	FBgn0032773	
6	FBgn0015010	Ag5r
6	FBgn0011695	PebIII
6	FBgn0050098	
6	FBgn0032472	
6	FBgn0038074	
6	FBgn0038299	
6	FBgn0034382	
6	FBgn0033830	
6	FBgn0002997	ome
6	FBgn0011296	l(2)efl
6	FBgn0037724	Fst
6	FBgn0038930	
6	FBgn0040104	lectin-24A
6	FBgn0032167	
6	FBgn0030925	
7	FBgn0038147	
7	FBgn0001208	Hn
7	FBgn0052306	

7	FBgn0036715	Cad74A
7	FBgn0014000	Os9
7	FBgn0027571	
7	FBgn0031563	
7	FBgn0029765	
7	FBgn0024293	Spn43Ab
7	FBgn0002719	Men
7	FBgn0051928	
7	FBgn0035694	
7	FBgn0040827	
7	FBgn0039031	
7	FBgn0013949	Ela
7	FBgn0028526	
7	FBgn0003507	srp
7	FBgn0015570	alpha-Est2
7	FBgn0040609	
7	FBgn0002940	ninaE
7	FBgn0025390	
7	FBgn0040813	Nplp2
7	FBgn0033250	
7	FBgn0033366	Ance-4
7	FBgn0032322	
7	FBgn0014031	Spat
7	FBgn0000406	Cyt-b5-r
7	FBgn0029696	
7	FBgn0036262	
7	FBgn0034200	
7	FBgn0031417	
7	FBgn0032726	
7	FBgn0043783	
7	FBgn0031754	
7	FBgn0026188	ps
7	FBgn0001090	bnb
7	FBgn0034140	
7	FBgn0034394	
7	FBgn0037763	
7	FBgn0029990	
7	FBgn0013307	Odc1
7	FBgn0037547	
7	FBgn0030438	
7	FBgn0040732	
7	FBgn0029966	

7	FBgn0030160	
7	FBgn0030157	
7	FBgn0052602	
7	FBgn0000121	Arr2
7	FBgn0033820	
7	FBgn0028433	Ggamma30A
7	FBgn0025454	Cyp6g1
7	FBgn0053493	
7	FBgn0010019	Cyp4g1
7	FBgn0036115	
7	FBgn0038445	
7	FBgn0033065	Cyp6w1
7	FBgn0042201	Nplp3
7	FBgn0040398	EG:103E12.2
7	FBgn0052210	
7	FBgn0036938	
7	FBgn0030041	
7	FBgn0040074	retinin
7	FBgn0038194	Cyp6d5
7	FBgn0033820	
7	FBgn0029172	Fad2
7	FBgn0003067	Pepck
7	FBgn0031111	Obp19c
7	FBgn0039682	Obp99c
7	FBgn0016724	RfaBp
7	FBgn0051926	
7	FBgn0004045	Yp1
7	FBgn0050019	
7	FBgn0031305	Iris
7	FBgn0037405	
7	FBgn0037405	
7	FBgn0005391	Yp2
7	FBgn0051226	
7	FBgn0031845	
7	FBgn0004047	Yp3
7	FBgn0023479	Tequila
7	FBgn0040606	
7	FBgn0003250	Rh4
7	FBgn0032055	
7	FBgn0004623	Gbeta76C
8	FBgn0031511	
8	FBgn0052364	

8	FBgn0034075	Asph
8	FBgn0038012	
8	FBgn0004606	zfh1
8	FBgn0039672	
8	FBgn0052318	
8	FBgn0035412	
8	FBgn0030073	
8	FBgn0013548	l(2)dtl
8	FBgn0004133	blow
8	FBgn0036291	
8	FBgn0034399	
8	FBgn0051681	
8	FBgn0028915	
8	FBgn0030051	
8	FBgn0042627	v(2)k05816
8	FBgn0035833	
8	FBgn0033027	TpnC4
8	FBgn0039560	
8	FBgn0030829	
8	FBgn0032885	
8	FBgn0050438	
8	FBgn0035976	PGRP-LC
8	FBgn0015569	alpha-Est10
8	FBgn0051354	Hsp70Bbb
8	FBgn0000473	Cyp6a2
8	FBgn0011832	Ser12
8	FBgn0034816	
8	FBgn0033584	
8	FBgn0019972	Ice
8	FBgn0016054	phr6-4
8	FBgn0032998	
8	FBgn0033194	
8	FBgn0040650	
8	FBgn0005633	fln
8	FBgn0039356	
8	FBgn0050008	
8	FBgn0052021	
8	FBgn0003978	vls
8	FBgn0030660	
8	FBgn0021967	Pdsw
8	FBgn0001987	Gli
8	FBgn0042186	

8	FBgn0020506	Amyrel
8	FBgn0052041	
8	FBgn0029875	
8	FBgn0031049	Sec61gamma
8	FBgn0038437	
8	FBgn0032938	
8	FBgn0033836	
8	FBgn0015558	tty
8	FBgn0035160	
8	FBgn0005616	msl-2
8	FBgn0035151	
8	FBgn0003285	rst
8	FBgn0031483	
8	FBgn0031031	
8	FBgn0046685	Wsck
8	FBgn0037473	
8	FBgn0013276	Hsp70Ab
8	FBgn0031536	
8	FBgn0033903	
8	FBgn0050059	
8	FBgn0039268	
8	FBgn0032943	Tsp39D
8	FBgn0038627	
8	FBgn0038928	BG4
8	FBgn0026402	NiPp1
8	FBgn0031025	
8	FBgn0069969	
8	FBgn0015781	P5cr
8	FBgn0034931	
8	FBgn0052243	
8	FBgn0052650	
8	FBgn0014906	
8	FBgn0038857	
8	FBgn0039000	
8	FBgn0030187	Ipod
8	FBgn0050437	
8	FBgn0034335	GstE1
8	FBgn0051339	
8	FBgn0044510	
8	FBgn0034004	Fs
8	FBgn0030583	
8	FBgn0038828	

8	FBgn0033765	nemy
8	FBgn0039114	Lsd-1
8	FBgn0029939	
8	FBgn0039294	Cad96Cb
8	FBgn0039170	
8	FBgn0010039	GstD3
8	FBgn0027936	vih
8	FBgn0041717	
8	FBgn0034788	
8	FBgn0031519	
8	FBgn0031313	
8	FBgn0027583	
8	FBgn0031018	
9	FBgn0000564	Eh
9	FBgn0069934	
9	FBgn0052821	
9	FBgn0032636	
9	FBgn0058115	
9	FBgn0052700	
9	FBgn0050298	
9	FBgn0033696	Cyp6g2
9	FBgn0053267	
9	FBgn0053269	
9	FBgn0053347	
9	FBgn0032658	
9	FBgn0058128	
9	FBgn0031742	
9	FBgn0038291	
9	FBgn0010294	ng2
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9	FBgn0035654	
9	FBgn0035928	
9	FBgn0032523	
9	FBgn0069955	
9	FBgn0030524	
9	FBgn0058212	
9	FBgn0069914	
9	FBgn0058295	
9	FBgn0003149	Prm
9	FBgn0028981	
9	FBgn0030042	
9	FBgn0032126	

9	FBgn0058043	
9	FBgn0036158	
9	FBgn0030483	
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12	FBgn0000592	Est-6
12	FBgn0034354	
12	FBgn0051999	
12	FBgn0026315	Ugt35a
12	FBgn0034810	
12	FBgn0032069	
12	FBgn0001114	Glt
12	FBgn0027835	Dp1
12	FBgn0031908	
12	FBgn0039914	mav
12	FBgn0050002	
12	FBgn0037288	
12	FBgn0000635	Fas2
12	FBgn0013342	n-syb
12	FBgn0026721	fat-spondin
12	FBgn0033268	Obp44a
12	FBgn0033710	
12	FBgn0034269	
12	FBgn0040600	
12	FBgn0027542	
12	FBgn0020611	Vha44
12	FBgn0015766	Msr-110
12	FBgn0036783	CheA75a
12	FBgn0022774	Oat

12	FBgn0030148	
12	FBgn0069973	
12	FBgn0010387	Dbi
12	FBgn0052181	
12	FBgn0001145	Gs2
12	FBgn0036948	
12	FBgn0050085	