Evolution of cytoplasmic genetic variation: studying Wolbachia from a mitochondrial perspective

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Evolution of cytoplasmic genetic variation: studying Wolbachia from a mitochondrial perspective

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A thesis submitted for the degree of Doctor of Philosophy in the Faculty of Science

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In loving memory of my mother, Lucia Ospina,
to whom I owe all that is good and right in my life

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Similarly to other cytoplasmic genetic elements, intracellular endosymbionts of animals are passed to the next generation of hosts through the egg. This mode of transmission is key to understanding the evolution of these symbiotic associations. For instance, maternal inheritance creates a sexual asymmetry, as genetic variants that are advantageous to females would persist even when harmful to males. Wolbachia are well known for using such sexual asymmetry to their advantage: by actively reducing the reproductive chances of non-infective hosts, they often increase the relative fitness of infected matrilines. However, so long as these genetic elements prevent the normal transmission of host genetic material to the next generation, there would be antagonistic selective pressure over the reproductive phenotype from the host. This means that Wolbachia-related parasitic phenotypes, similarly to sex-antagonist mitochondrial mutations, would be likely attenuated by compensatory adaptation.

Despite being often perceived as parasites, emerging evidence on beneficial effects of Wolbachia offers new perspectives on the nature of Wolbachia-host interactions. Similarly to the current understanding on the maintenance of functional mitochondrial genetic variation, Wolbachia that pose advantages contingent to the host nuclear background and the environment may be selectively favoured and maintained at equilibrium, even in the face of attenuation of reproductive manipulation phenotypes.

This thesis explores various aspects of the interaction between cytoplasmic (namely Wolbachia and mitochondria) and nuclear genomes using Drosophila flies as models. Additionally to their well-characterised mitochondrial genomes, Drosophila can serve as host to a variety of Wolbachia infections with markedly distinct phenotypes, which provides the opportunity to test hypothesis regarding host adaptation related to phenotypic effects of cytoplasmic variants. Three major conclusions can be drawn from this thesis: Firstly, phenotypes related to cytoplasmic genetic variants can be highly contingent on the sex, physiological state and nuclear background of the host, as well as the environment. Secondly, adaptation of the host genome to cytoplasmic genetic variants is linked to their effects on the organismal fitness. Finally, beneficial effects of cytoplasmic variants that are contingent on the host genetic background and the environment may explain their polymorphism in natural populations.

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Abstract

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Table of contents

X

List of figures

List of tables

Overview of thesis chapters

The overall goal of this thesis is to provide insights on the impact of functional cytoplasmic genetic variation on the phenotype, ecology and evolution of host populations. Specifically, this thesis aims to expand on the knowledge of the evolution of cytoplasmic genetic variation from a perspective of onset and resolution of cyto-nuclear conflicts, with an emphasis on the evolutionary trajectories of Wolbachia symbioses. The null hypotheses tested in this work are: 1) cytoplasmic variants have equal effects on organisms regardless of the host genetic backgrounds, physiology and environment, and 2) the phenotypic effects of functional cytoplasmic genetic variants do not constitute a selective force on the nuclear genome of organisms. The first alternate hypothesis is that cytoplasmic variants have differential impacts on the host phenotype depending on the host genetic background, the physiology and the environment. The second alternate hypothesis is that adaptation of the host genome to cytoplasmic variants depends on the impact of such variants on the organismal phenotype.

This thesis is organised into six chapters. Chapters 1 to 5 have been conceived as standalone manuscripts. Chapter 1 is a review that establishes the theoretical framework for the following four chapters. Chapters 2 to 5 are research chapters, each presented as a separate unit comprised of an abstract, introduction, methods and discussion. By the time of submission, these chapters were manuscripts in preparation (Chapters 1 and 5), accepted (Chapter 3) or published (Chapters 2 and 4). Finally, Chapter 6 briefly summarises the main findings of this thesis and considers plausible future directions to expand on this work.

The first chapter of this thesis presents an introduction to Wolbachia as intracellular heritable endosymbionts that coevolve with their host. The review starts with an outline of the characteristics of Wolbachia thought to contribute to their extraordinary evolutionary success. The chapter then focuses on the ecology and evolution of Wolbachia-host interactions, with special emphasis on the onset and resolution of conflicts of interests between partners and the possible evolutionary trajectories of the symbioses. The chapter concludes by summarising some important parallels between Wolbachia and mitochondria, including their evolutionary histories, their impact on organismal fitness and the significance of cytoplasmic genetic variability in natural populations.

Chapters 2 (Correa and Ballard, 2012) and 3 (Correa and Ballard, 2014) evaluated the effects of laboratory adaptation, host age and environmental challenges on gonadal Wolbachia density of *Drosophila simulans*. Chapter 2, which focused on a strain of Wolbachia known to cause a strong reproductive manipulation (*w*Ha), showed that variability of symbiont titres decreased after few generations of laboratory rearing. The study also found patterns of sexual antagonism of Wolbachia density regulation, as titres in testes appeared to be less regulated than those in ovaries. Using a very similar methodology, Chapter 3 examined the density dynamics of an association with a weak reproductive manipulator (*w*Ma). Despite the fact that the association *w*Ma-Drosophila is thought to be older that *w*Ha-Drosophila, symbiont density in *w*Ma infected flies appeared to vary more than in *w*Ha flies. These findings suggests that regulation of Wolbachia density, rather than the product of coadaptation over time, may be a

consequence of selection on symbiont transmission fidelity, which would be strong when symbionts exert potent manipulation phenotypes.

In Chapter 4 (Correa et al., 2012), the focus of the thesis switches from Wolbachia adaptation to the organismal response to mitochondrial genetic variants. The specific goal of Chapter 4 was to determine if a small set of non-synonymous mtDNA mutations affected mitochondrial bioenergetics of *Drosophila melanogaster* at different ages. Results indicated that mutation loads that cause mild mitochondrial malfunction may elicit compensatory mechanisms to maintain energy homeostasis in young flies, with a fitness cost later in life. In the context of this thesis, Chapter 4 addresses important issues on the effects of cytoplasmic (mtDNA) genetic variation on the organismal phenotype. Such concepts as well as some methodological aspects of this piece of research laid the foundation for Chapter 5.

The final research chapter of this thesis, Chapter 5, studied the phenotypic and bioenergetic effects of co-inherited Wolbachia and mitochondrial variants in *D. simulans*. A highlight of this study is that the effects of Wolbachia and mitochondria are assessed in nuclear backgrounds that are native and foreign to these cytoplasmic elements. The results of this study suggest a rather novel role of Wolbachia as a phenotypic rescuer of mildly deleterious mito-nuclear genetic combinations. This exciting implication should direct future studies to investigate the role of Wolbachia-mitochondria synergism in the evolutionary ecology of the host.

Chapter 1

(Introduction)

Wolbachia associations with insects: winning or losing against a master manipulator

1.1 Introduction

With the publication of *On the origins of mitosing cells* in 1967, Lynn Sagan revived the long-standing but unpopular idea that essential components of eukaryotic cells such as mitochondria and plastids are derived from bacteria that some ancestral cell had engulfed (Sagan, 1967). Besides providing a robust explanation of how modern eukaryotic cells came to be, Sagan's idea showed that evolutionary change may occur with the acquisition of genomes through endosymbiosis; the cohabitation of nonrelated partners where one of them lives in the body of the other. The discovery of organelle DNA as a carrier of essential information for correct organelle function not only proved Sagan's predictions correct, but also showed that the genetic information of modern eukaryotic cells is compartmentalised into cytoplasmic and nuclear partitions (Mounolou and Lacroute, 2005). This implies that the highly coordinated work of cytoplasmic and nuclear genomes required for the assembly of the major energy-producing molecular machineries of eukaryotes is the product of two billion years of cyto-nuclear coevolution (Rand et al., 2004).

Such organelles are not the only examples of genomes of disparate origin that coevolve with the host genome inside the cell. Intracellular endosymbioses have occurred repeatedly in nature and are found in a large variety of animals, plants and other life forms. These organisms are known to play essential roles in aspects of their host ecology such as nutrition, reproduction, and pathogen resistance. It was once assumed that, because of the symbiont reliance on host reproduction for transmission, associations with endosymbiotic bacteria evolved toward beneficial symbioses.

Although there are many mutualistic heritable symbionts, there are also those that appear to behave selfishly, favouring their transmission at the cost of the host nuclear genomes. Paramount among these so-called 'reproductive parasites' is *Wolbachia pipientis* (hereafter Wolbachia), a heterogeneous group of intracellular bacteria of insect and other invertebrates.

Wolbachia are maternally inherited endosymbionts that have intrigued biologists since their discovery in 1924 (Hertig and Wolbach, 1924), and are among the most common life forms on earth. Being mostly non-essential from the host perspective, Wolbachia rely on their capacity to alter the host phenotype in order to spread (Moran et al., 2008). Such phenotypic alterations generally result in the reduction of reproductive chances of non-infective individuals, giving a relative advantage to individuals that pass the symbiont to their offspring. Maternal transmission implies that such manipulations, although beneficial for the infected matrilines, are harmful for the paternal lineages, clearly exemplifying the conflicts of interest that arise between cytoplasmic and nuclear genomes (Hurst, 1992; Rand et al., 2004). Such sexual asymmetry in favour of females is similar in principle to that between mitochondrial and nuclear genomes, where mitochondrial DNA (mtDNA) mutations can be maintained when favourable in females, even if they are harmful in males (Gemmell et al., 2004). Wolbachia-host associations therefore provide an opportunity to explore the coevolutionary processes that concur with intracellular lifestyles.

The aim of this review is to discuss the expanding body of evidence that highlight Wolbachia as a powerful source of evolutionary innovation for many invertebrates

(Duron and Hurst, 2013), with special attention to the roles Wolbachia play beyond reproductive manipulations. The first section summarises the aspects intrinsic to Wolbachia biology that contribute to their evolutionary success across and within host species. The second section explores how the adaptation of the host to Wolbachia infection influences the evolutionary trajectories of the symbiosis. The final section of this review explores interesting parallels in the evolution of Wolbachia and the most successful animal endosymbiont on earth, the mitochondria.

1.2 Wolbachia: The biology of a master manipulator

Wolbachia bacteria are a clear example of evolutionary success (Merçot and Poinsot, 2009). Estimates suggest that they infect more than 65% of all insect species (Hilgenboecker et al., 2008), but they are also widespread and common in other invertebrates such as molluscs, arachnids, crustaceans and nematodes (Werren et al., 2008). The often discordant phylogenies between Wolbachia and their hosts (O'Neill, et al., 1992; Heath et al., 1999, Vavre et al., 1999; Werren and Windsor, 2000) reveal extensive Wolbachia horizontal transmission over evolutionary time, which offers an explanation for the wide range of Wolbachia infections found among terrestrial invertebrates (Werren and Windsor, 2000). Nonetheless, lateral Wolbachia transmissions appear to be rare at ecological timescales. Therefore, the ample distribution and maintenance of Wolbachia infections in insects is likely to be a product of evolutionary processes that occur along very different timescales: in the longer term, Wolbachia retain the capacity to establish new infections through lateral transmission, while in the shorter term they maximise the reproductive fitness of infective matrilines.

This section summarises the aspects of the biology of Wolbachia that may contribute to their success in spreading both horizontally and vertically.

1.2.1 Wolbachia across the species barrier: Horizontal transmission

Wolbachia evolved from an ancient clade of alphaproteobacteria that comprises other obligatory intracellular organisms, such as *Rickettsia*, *Ehrlichia*, *Anaplasma*, and *Midichloria* (Weinert et al., 2009), and even the extinct ancestor of the modern mitochondria (Thrash et al., 2011). Wolbachia are a monophyletic group composed of at least eight different supergroups (A-H), where C and D are exclusively nematode symbionts, and supergroups A and B comprise the majority of arthropod infections. Although the lack of suitable outgroups prevented a satisfactory resolution of their phylogeny (Lo et al., 2007), estimates employing the base substitution rates of the *ftzZ* and 16S genes of super groups A, B, C and D suggest that their separation may have occurred approximately 100 million years ago (Werren et al., 1995). Considering that the split between nematodes and arthropods is approximately five times longer, lateral transmission between host phyla or independent acquisition of infection from a third party are a plausible origins for Wolbachia-invertebrate symbioses (Bandi et al., 1998).

From an evolutionary perspective, lateral transmission of Wolbachia is a frequent and ongoing process (Duron and Hurst, 2013). The key pieces of evidence in this regard are: lack of congruence between host and symbiont phylogenies (O'Neill et al., 1992; Stouthamer et al., 1999), closely related Wolbachia present in taxonomically unrelated hosts (Baldo et al., 2006b; Raychoudhury et al., 2009) and disparate Wolbachia types

present in the same host (Vavre et al., 1999). The astonishingly widespread distribution of Wolbachia across insects and other invertebrates highlights the importance of determining how Wolbachia crosses the species barrier.

1.2.1.1 Wolbachia: from one species to another

Although the processes involved in the establishment of new infections through lateral transmission are not thoroughly characterised, there is compelling evidence that suggests such events are frequent. First, Wolbachia are able to survive in extracellular environments for extended time periods. Rasgon and colleagues (2006) observed that, after a week of maintenance on a cell-free media, Wolbachia from *Aedes albopictus* lived and retained the capacity to invade cells and establish stable infections. Such ability would enhance the chance of the bacteria to colonise cells from an extracellular environment. Second, stable infections are routinely established in the laboratory through microinjections between different host taxa (Braig et al., 1994), which requires Wolbachia to successfully colonise a new female's germline from surrounding somatic tissues. In fact, Wolbachia's intrinsic ability to target and colonise stem cell niches in the ovary has been well demonstrated in Drosophila (Frydman et al., 2006; see section 1.2.2.1). Third, closely related Wolbachia infecting unrelated hosts species with intimate ecological contact have been observed, which suggests ecological routes for the symbiont to be laterally transmitted. Parasitoid wasps, whose larval development occurs within the body of other insects, have been observed to be especially susceptible to Wolbachia infection. European parasitoid wasps display double, and even triple, Wolbachia infections of bacterial types that closely resemble those of the insects they parasitise (Vavre et al., 1999). Another route of Wolbachia lateral transfer has been

described in species of terrestrial isopods, where haemolymph contact between infected and non-infected individuals is sufficient to transfer the bacteria between species (Rigaud and Juchault, 1995).

1.2.1.2 Wolbachia genomics and the ability to retain an infectious capacity

Intracellular lifestyles and maternal inheritance mean that bacterial endosymbionts are subject to environmental and population dynamics that drastically differ from those of a free-living organism. First, there is a reduction of the effective population size, owing to the constraints of living within host cells and tissues. Second, strong bottlenecks occur during the passage of the symbiont from mother to offspring. Third, there is a much-reduced opportunity of horizontal gene transmission due to low intracellular bacterial diversity. Finally, there are relaxed constraints on gene function due to the metabolite-rich environment of the cytoplasm (Reviewed in Toft and Andersson, 2010). These conditions not only reduce genetic variability, but also lower the efficiency of purifying selection to eliminate slightly deleterious mutations. Increased chances of fixation of such mutations (Muller's ratchet) may ultimately lead to loss of gene function and genomic size reduction (Moran, 1996; Dale and Moran, 2006). Indeed, ancient obligate symbioses almost invariably show massive genome reduction, often retaining between 10% and 20% of the genes of their free-living counterparts (Moran, 2003; Dale and Moran, 2006). Their genomes also appear to be purged of pseudogenes, phage sequences and other mobile genetic elements, which led to extraordinary genetic stability (Tamas et al., 2002). Evidence suggests that in these ancient genomic associations, the symbiont has surrendered control of their genetic functions to the host, even in aspects related to DNA replication and gene expression (Moran et al., 2008).

Despite a history of adaptation to an intracellular lifestyle of tens of millions of years, Wolbachia seem to maintain autonomy in processes that involve host immune evasion and establishment of stable de novo infections. This reflects an important conundrum in the evolutionary history of these organisms: how can Wolbachia be 'generalists in host use' (Baldo et al., 2006b) despite the erosive genomic processes that come with host restriction? The analysis of Wolbachia genomes has provided important clues in this regard. Although their small genome (1-1.7 Mbp) agrees with a reductive trend (Werren et al., 2008), Wolbachia genomes comprise large segments of mobile and repetitive DNA, not a common trait among ancient vertically transmitted endosymbionts. The acquisition and preservation of these repetitive and mobile elements is hypothesised to play crucial roles in the evolution of Wolbachia (Wu et al., 2004). The following is a brief outline of how these genomic traits may contribute to Wolbachia adaptation.

Insertion sequences are exceedingly abundant in Wolbachia but almost completely absent from other intracellular obligate symbionts. Moran and Plague (2004) propose that during the early stages of adaptation to an intracellular lifestyle, reduced purifying selection fails to purge slightly deleterious insertions of mobile elements in the genome, which increases the chances of inactivation of functional genes and contributes to the processes of genome erosion. Although in most cases insertion events are predicted to be deleterious, they may induce adaptive evolutionary change by inactivating metabolically redundant genes. This creates regions of homology through the bacterial chromosomes for intra-chromosomal recombination, changing patterns of gene expression by carrying promoters to other regions in the chromosome.

Other important mobile elements found in the genome of Wolbachia are bacteriophage sequences. Although bacteriophages are a significant force of genomic evolution in free-living bacteria, they are notably absent in the genomes of ancient obligate endosymbionts (Bordenstein and Reznikoff, 2005). In contrast, complete and truncated bacteriophage sequences have been described in many Wolbachia types (WO phages) and in some instances, functional phages have been observed to undergo lytic cycle (Masui et al., 2001). The base composition of WO phage DNA is similar to that of the Wolbachia chromosome, which suggests that such particles have been associated with Wolbachia for a long time (Masui et al., 2000). Furthermore, evidence suggests that there has been exchange of viral DNA between distant Wolbachia lineages, and that some phage regions undergo fast rates of recombination. Bordenstein and Wernegreen (2004) propose that the phage-mediated exchange of DNA between bacterial strains within the same intracellular environment constitutes an important source of genomic instability and could drive significant evolutionary change in Wolbachia genomes.

All the necessary machinery for homologous recombination is present in the genome of *w*Mel, the Wolbachia strain of *Drosophila melanogaster* (Wu et al., 2004). Either the result of functional recombination machinery or a consequence of the multiple mobile elements in the genome, *w*Mel has been shown to display extensive intra and intergenic recombination (Baldo et al., 2006a). This capacity to recombine likely constitutes a key mechanism for adaptation within an arthropod host, as advantageous alleles that arise through recombination could be rapidly fixed and spread through horizontal transmission. Moreover, horizontal gene transfer between distinct Wolbachia types also appears to be frequent (Baldo et al., 2005, 2006a; Duplouy et al., 2013). Such process

may give rise to advantageous genetic variants and reduce the accumulation of mildly deleterious mutations due to Muller's ratchet (Raychoudhury et al., 2009).

The maintenance of the global Wolbachia pandemic depends on the rates of acquisition and loss of infection within species, relative to the horizontal transmission (Werren et al., 2008). Plausibly, those Wolbachia lineages that readily establish new infections and recombine with other types have a greater chance to counteract the erosive effects of intracellular lifestyles than those confined to a single host population for extended periods of time. The peculiar host-generalist lifestyle of Wolbachia may result from a balance between vertical transmission, host switching and recombination.

1.2.2 Wolbachia adaptations to vertical transmission

From the host perspective, endosymbionts can be categorised as obligate (primary), which are necessary for host survival and reproduction, or facultative (secondary), which are non-essential. As opposed to primary symbionts, facultative endosymbionts such as most Wolbachia in insects are capable of engineering their own mechanisms of transmission to the next generation of hosts. Because they are generally not fixed in host populations, facultative symbionts also need to confer a reproductive advantage to the transmitting hosts in order to spread (Moran et al., 2008). Perhaps the most fascinating aspect of the biology of Wolbachia is the variety of phenotypes they exert upon their host in order to spread. Wolbachia have been found to be anything from essential endosymbionts to parasites that effectively kill non-infective individuals. This versatility undoubtedly reflects a long evolutionary history of adaptation to intracellular

lifestyle. Although many aspects of the biology of Wolbachia related to vertical transmission remain elusive, recent cytological, genetic and genomic studies have provided insight into the mechanisms that allow Wolbachia to be maintained in host populations as stable infections.

1.2.2.1 The egg as the route to the next generation

The passage of symbionts through the egg cytoplasm is the primary mode of Wolbachia transmission. Accurate transmission involves correct symbiont replication, avoidance of host defences and precise distribution of bacteria in the germline of the host cells (Moran et al., 2008). Some of what is known about the mechanisms of Wolbachia cell division comes from the study of filarial nematodes, as bacterial replication offers potential targets for pharmacologic treatment against filariasis (Taylor et al., 2005). Ultrastuructural analyses of the worm *Dirofilaria immitis* suggest two mechanisms of Wolbachia cell division: binary fission (common to bacillary forms) and a more complex *Chlamydia*-like cycle, during which Wolbachia is pleomorphic (Kozek, 2005). This cycle is thought to increase the survival potential of the microorganism by producing more progeny than binary fission and operating in synchrony with the worm development (Kozek, 2005). The analysis of Wolbachia genomes has also offered clues to the molecular mechanisms of cell division. Exploration of the genome of Wolbachia in *Brugia malayi* (*w*Bm) revealed the presence of core *fts* genes, which are essential for cytokinesis. These included the highly conserved *ftsZ*, whose translated product possesses the key residues and secondary structure required for correct enzymatic function (Li et al., 2011). The rapidly increasing Wolbachia genomes available from

multiple host species will further contribute to the understanding of Wolbachia cell division and propagation.

The maintenance of adequate microbe titres within the intracellular environment also requires Wolbachia to effectively escape the immune system of the host. This ability is not negligible as insects are capable of mounting sophisticated humoral and cellular immune responses against intruders (Siozios et al., 2008). Experiments on *Drosophila simulans* showed that Wolbachia do not activate the production of gram-negative specific antimicrobial peptides, but when infected animals were challenged with *Escherichia coli,* another gram-negative bacterium, they displayed a normal ability to mount an immune response. This indicates that Wolbachia in flies neither induce nor suppress humoral immune responses (Bourtzis et al., 2000). In addition, electron microscopy studies have revealed that Wolbachia reside within vacuoles of host origin in the cytoplasm (Cho et al., 2011). These vacuoles are likely phagosome-like structures that, under normal circumstances, are involved in the processes of degradation of intracellular particles through lysosome fusion and phagolysosome formation. The extent at which Wolbachia interact with and modifies this vacuole to avoid degradation is unknown, but it is plausible that they secrete effector molecules that manipulate the host intracellular environment.

Maternal transmission means that Wolbachia not only have to survive and multiply within the host, but also needs to effectively infect the female germline. Wolbachia are known to preferentially localise within specific regions of the oocyte during oogenesis (Ferree et al., 2005; Frydman et al., 2006). Recent studies show that Wolbachia accesses

the cytoplasm of the forming egg by two main routes. The first route is the direct passage of symbionts from the progenitor germline stem cells, which in some cases become infected during the embryonary development of the gonads. When infected germline stem cells divide, the differentiating daughter cells that ultimately form developed oocytes contain Wolbachia (Serbus et al., 2008). The second route is through stem-cell tissue tropism, where Wolbachia targets and colonises two groups of stem cell niches in the ovary, the germline stem cell niche at the anterior end of the ovariole and the somatic stem cell niche at the germarium. Evidence suggests that transmission of Wolbachia from stem cell niches into the developing oocyte is conserved in Drosophila and may even be the most prevalent mechanism of egg colonisation by Wolbachia (Frydman et al., 2006; Toomey et al., 2013).

It has been observed that the distribution of Wolbachia from early embryogenesis and until late gastrulation is established during late oogenesis. In *D. simulans*, factors intrinsic to different Wolbachia strains determine their embryonic localisation, which can be posterior, anterior, or cortical. Posterior and anterior localisations resemble those of the axis-specification mRNAs *oskar* and *bicoid*, and as such, bacteria may rely on microtubule-based machinery for their mobilisation through the embryo (Veneti et al., 2004). Intriguingly, only the posterior localisation pattern directly targets Wolbachia to the site of germ cell formation (germ plasm), which indicates that strains with a different localisation pattern must rely on other mechanisms to reach embryonic germ cells, such as the previously described stem cell niche tropism. This has important evolutionary implications as such mechanisms would allow both the passage of bacteria

from mother to offspring and the establishment of stable infection of symbionts transmitted horizontally (Toomey et al., 2013).

1.2.2.2 Reproductive manipulations and other phenotypic effects of Wolbachia

Despite Wolbachia's adaptations to reach the egg, symbiont transmission fidelity is often imperfect, especially in natural populations (Turelli et al., 1992; Turelli and Hoffmann, 1995). Unless there are mechanisms in which Wolbachia can increase the fitness of the matriline they infect, the persistence of imperfectly transmitted infection is difficult to explain (Hoffmann et al., 1998).

One strategy would be to establish a mutualistic association with the host, as increase in host fitness would aid the symbiont spread. Although mutualistic Wolbachia are not rare, many Wolbachia utilise another strategy: to induce parasitic phenotypes that selectively eliminate members of their host population that do not transmit the infection. These phenotypes, generally regarded as reproductive manipulations, have their basis in Wolbachia maternal transmission. Four reproductive manipulation phenotypes are described as caused by Wolbachia: cytoplasmic incompatibility (CI), male killing (MK), parthenogenesis induction (PI) and feminisation.

CI arrests the embryonic development of crosses between infected males and females that do not harbour the same Wolbachia type. Evidence suggests that Wolbachia modify the sperm of infected males and subsequently rescue the modification when present in the egg. This favours infected females but reduces the fitness of infected males. MK-inducing Wolbachia kills the sons of infected females, giving a competitive advantage

to the female larvae over their brothers. PI turns haploid males into diploid females in haplo-diploid species. Finally, feminisation turns genetic males into females by Wolbachia-related hormonal abnormalities.

This variety of reproductive phenotypes is atypical compared to other microbial reproductive manipulators (Werren et al., 2008). Nevertheless, different manipulations often share important similarities at the cytological level. Specifically, CI, MK and PI appear to alter cell division during embryonic development. CI-induced embryonic mortality is due to the delayed entry of the male pronucleous into the first embryonic mitotic division, which results in paternal delayed nuclear envelope breakdown, semi-condensed chromatin in metaphase and chromosome bridges during telophase (Tram and Sullivan, 2002). These anomalies are plausibly the consequence of delayed activity of the key cell cycle kinase Cdk 1, brought about by the modifications inflicted by Wolbachia on the sperm DNA during spermatogenesis (Tram and Sullivan, 2002).

Despite considerable work, very little is known about the processes involved in sperm modification or their subsequent rescue in the egg. Analyses of Wolbachia genomes, however, have rendered some important clues on the matter. A genomic trait of Wolbachia that has received considerable attention is their unusually high number of regions coding for ankyrin (ANK) domains (Iturbe-Ormaetxe et al., 2005). This is relevant as proteins with ANK domains often participate in cell signalling, regulation of gene expression and cytoskeleton integrity in eukaryotic cells, leading to the hypothesis that ANK sequences may be involved in Wolbachia-host interactions (Wu et al., 2004; Iturbe-Ormaetxe et al., 2005). Of particular interest is the fact that in Drosophila,

closely related Wolbachia strains that are incompatible to each other differ in the structure and expression of their ANK proteins (Iturbe-Ormaetxe et al., 2005). Moreover, in the mosquito *Culex pipiens* the only Wolbachia gene identified so far that displays sex specific expression corresponds to an ANK protein that curiously co-expresses with a WO phage gene (Duron et al., 2007). These observations not only reveal the strong link between ANK repeats and the mechanisms of symbiont-host interactions, but also suggest that their variation and evolution may be influenced by rearrangements mediated by mobile elements. Another piece of evidence on CI mechanisms comes from the mosquito *C. pipiens*, where Wolbachia seem to be implicated on the regulation of host cell cycle genes. Specifically, the Drosophila homolog *grauzone* in *C. pipiens*, a regulator of female meiosis, appear to be over-expressed in CI-Wolbachia infected animals, and the levels of up-regulation differed among incompatible lines (Pinto et al., 2013). The increasing availability of annotated Wolbachia genomic sequences and the ability to test the effect of Wolbachia genes by transfection into their host nuclear DNA will prove useful in the discovery of Wolbachia genes involved in reproductive manipulation genotypes.

The mechanisms by which Wolbachia induce sex distortion are yet to be understood. Similarly to CI-Wolbachia, PI-Wolbachia also induce cell cycle abnormalities. This reproductive phenotype has only been observed in animals with haplo-diploid sex determination, specifically, those in which unfertilised (haploid) eggs produce males and diploid (fertilised) eggs produce females (arrhenotoky). In the parasitoid wasp *Leptopilina clavipes*, infected wasps have normal meiosis but diploidy is restored by an unresolved anaphase in the first mitotic division, which turns would-be males into

parthenogenic females (Pannebakker et al., 2004). Finally, MK-Wolbachia in *Drosophila bifasciata* induces abnormalities at different times during early development of the male embryo. In these flies, defective remodelling and segregation of chromatin and chromosome bridging resulted in male embryos dying before eclosion. Although not thoroughly elucidated, these phenotypes are speculated to occur in relation to the X chromosome, due to its involvement in sex determination (Riparbelli et al., 2012).

Although less studied, beneficial Wolbachia are not negligible. For example, Wolbachia symbioses are essential for disease-causing filarial nematodes such as *Onchocerca volvulus* and *B. malayi*. Beneficial effects of endosymbiont bacteria are not surprising, as they possess many metabolic and biosynthetic capabilities that animals lack (Moran et al., 2008). For example, recent evidence suggests different Wolbachia types are involved in the provisioning of purines, pyrimidines (Brownlie et al., 2007), vitamin B (Hosokawa et al., 2010), and heme groups (Brownlie et al., 2009) to the host, as well as their involvement in iron metabolism and protection against natural enemies (Hedges et al., 2008; Brownlie and Johnson, 2009). These Wolbachia-host interactions are the subject of a later section in this review.

1.3 Wolbachia-host coevolution

It was once believed that parasitic and mutualist associations evolved in opposite ways. Classic theories stated that, while host-parasite adaptation typically followed evolutionary arms races, mutualist associations evolved in ways that enhanced the fitness of both partners (Reviewed in Sachs et al., 2011). Other models, however,

suggest that mutualism can be more accurately understood as reciprocal exploitations of partners that nonetheless result in net benefits to each partner (Herre et al., 1999). This opened the possibility that the evolutionary mechanisms underlying parasitism and mutualist were more similar than previously thought.

Ewald (1987) proposed that endosymbionts evolve along a parasitism-mutualism continuum depending on their fitness gains, and emphasised that the mode of symbiont transmission was a key determinant of parasitic or mutualistic evolutionary trajectories. Ewald predicted that the more intimately associated host and symbionts are, the stronger their tendency to evolve towards beneficial symbiosis and mutualism. Further development of Ewald's concepts led to a more elaborate conflict-of-interest perspective, which states that the stability of symbiotic associations depends on how well the reproductive interests of host and symbiont converge. Vertical transmission and lack of free-living stages of endosymbionts are recognised as powerful factors aligning the partners' needs and promoting long-term stability (Herre et al., 1999). This has led to the longstanding prediction that vertically transmitted endosymbionts evolve to become benign and establish stable mutualistic associations with their host (Weeks et al., 2007). This is not only logically appealing but has received important support from theoretical, ecological and molecular evidence. However, it does not account for the fact that Wolbachia infections are often lost from insect hosts before co-speciation can occur (Kremer et al., 2009). Therefore, a key question relating to Wolbachia evolution is: what are the evolutionary trajectories of Wolbachia symbiosis within a single host species? This section is dedicated to exploring this question.

1.3.1 Conflicts of interests between intimate associated partners

Parasitic Wolbachia have been described as selfish genetic elements: heritable units that spread despite the adverse effects they may cause on other genes of the organism they inhabit (Hurst and Werren, 2001). As any other cytoplasmic gene, Wolbachia are in conflict with the host nuclear genome over control of sex ratio. This is the case as sex biases in favour of females would increase the propagation of the cytoplasmic element in spite of nuclear autosomal genes (Hurst, 1992). Indeed, Wolbachia and other selfish entities can, and do, spread by altering the host sex ratio (Charlat et al., 2003). Feminisation, MK and PI are common sex ratio distorting phenotypes that effectively increase the frequency of infection in host populations (Hurst, 1992). Although not sex distorters *per se*, CI-Wolbachia are also in conflict with the nuclear genes, as infected males have reduced chances to produce viable offspring while infection in females protects their eggs from CI-induced mortality (Rand et al., 2004).

Any organismal fitness reduction caused by the disproportionate spread of selfish genetic elements has negative effects on unlinked genomic regions. Selection on those genomic regions would therefore favour the spread of suppressors or modifiers capable of counteracting the effect of the selfish elements (Werren, 2011). For Wolbachia, this would mean that host adaptation to infection would tend to resist or counteract the effects of reproductive modifications. Following, I present some examples of the dynamics of reproductive manipulations and how host adaptation may counteract their parasitic effects.

1.3.1.1 Host adaptation to sex distorter Wolbachia

MK, feminisation and PI result in an increase in the number of females in a population relative to males. The spread of these sex-distorting Wolbachia means that male individuals become increasingly rare, raising their reproductive success (Charlat et al., 2003). Therefore, unlinked nuclear genes that eliminate the infection or repress its parasitic effects would increase in frequency to restore the sex ratio to unity (Hurst, 1992). Of the three sex-distorting phenotypes, MK is perhaps the most harmful to host populations. Not only do host populations bear the costs of both mortality and failure to produce males, but the fitness compensation for female progeny is also low (Engelstädter and Hurst, 2009). Therefore, selection on MK suppressors would be predicted to be strong. A study by Hornett and colleagues (2009) on the butterfly *Hypolimnas bolina* clearly exemplify these dynamics in nature. Indo-Pacific populations of *H. bolina* are infected with the Wolbachia *w*Bol1 strain. In Polynesian populations, this Wolbachia induces MK, but the same strain in Southeast Asian *H. bolina* is present in both sexes and infected females produce a 1:1 sex ratio. Crosses between the two populations demonstrated the presence of a dominant suppressor of the MK phenotype in insects from Thailand and Philippines that was absent from the populations of Polynesia. The authors estimated a very rapid spread of the suppressor gene from past populations, and suggested that MK will disappear from the Wolbachia-*H. bolin*a association in the near future.

When the prevalence of Wolbachia in a host population is high, selection may favour the evolution of new sex determination systems to counteract the deleterious effects of sex-distorting Wolbachia. A notorious example of such adaptive change is presented by

the woodlouse *Armadillium vulgare* (O'Neill, et al., 1997). Normally, sex determination in these crustaceans is dictated by female heterogametic chromosomes (ZZ males and ZW females). In some populations, feminising Wolbachia induced ZZ individuals to develop into females, leading to infection spread and female sex bias. Host genotypes that prevent Wolbachia transmission or suppress feminisation, thus producing males, would have a high reproductive success and would tend to restore sex ratio balance. Fascinatingly, this seems to have resulted in the switch from the W chromosome to Wolbachia as sex determining factor in *A. vulgare*. Evidence suggests that sex determining Wolbachia may occur in other species besides *A. vulgare* (Charlat et al., 2003).

The genomic conflicts between PI-Wolbachia and the host genome, as well as their resolution, have particularly remarkable evolutionary consequences for species with haplo-diploid sex determination. As mentioned in section 1.2.2.2, sex determination in these organisms depends on the ploidy of the embryo; fertilised, diploid eggs hatch into diploid females while unfertilised eggs hatch into haploid males. Wolbachia infection turns unfertilised eggs into diploids, which would then hatch into females. This means that infected females produce daughters from both fertilisation and parthenogenesis. Stouthamer and colleagues (2010) proposed that Wolbachia sex bias creates a selective advantage on females with 'functional virginity alleles', that is, alleles that decrease the rates of fertilisation in order to favour male offspring. As these alleles spread and become fixed in the population, the capacity for sexual reproduction of the species is lost, ultimately rendering the host dependent on Wolbachia for reproduction. This model of evolution agrees with the observation that many wasp species that have fixed

Wolbachia infections are completely parthenogenic, and that antibiotically cured females are no longer capable of sexual reproduction (Russell and Stouthamer, 2010).

1.3.1.2 Host adaptation to cytoplasmic incompatibility inducing-Wolbachia

In its simplest form, CI-Wolbachia induce embryonic mortality in crosses between infected males and uninfected females. In other words, CI-Wolbachia 'utilise males to make uninfected females unviable' (Charlat et al., 2003). CI-Wolbachia pose opposite effects on each sex; infected females rescue their eggs from CI-inducing mortality, while infected males produce modified sperm that limit their successful matings with uninfected females. This also implies that costs and benefits are influenced by infection prevalence: at low prevalence, infection is a greater cost for males than it is a benefit to females; at high prevalence, infection is greatly advantageous for females and represents little cost to males (Turelli, 1994). Unlike sex-distorting Wolbachia, high prevalence of CI-Wolbachia produces little conflict with the host genome, as both females and males can readily transmit their genes to the next generation. Moreover, as resistance to infection is expected to be selected against, females are said to become addicted to CI-Wolbachia (Koehncke et al., 2009).

These models, although well supported by empirical data, cannot account for the recurrent losses of Wolbachia that are inferred from the incongruence of symbiont-insect phylogenies. It has been proposed that the processes that result in Wolbachia extinction are likely related to the decline in CI penetrance (Engelstädter and Hurst, 2009), that is, when the number of eggs that hatch from incompatible crosses increase. A popular hypothesis suggests that it is the bacterium that loses the capacity to induce

CI (Werren, 1997). This hypothesis assumes that the abilities of Wolbachia to induce CI (*mod*) and to rescue eggs from embryonic mortality (*resc*) are independent. In populations with high infection prevalence, the ability to modify sperm would be no longer necessary. As a consequence, *mod*- variants may arise and spread, as they would be fully compatible but do not invest in costly *mod*+ phenotypes. As the *mod*- variants spread, the ability to induce CI may be lost from the population and with time the endosymbiont may go extinct (Hurst and Mcvean, 1996).

It has also been proposed that attenuation of CI phenotypes is mediated by the spread of male-specific CI modifiers (Turelli, 1994; Koehncke et al., 2009). Using population genetics modelling, Koehncke and colleagues (2009) concluded that male-specific CI modifiers spread from fixed infections, even when the modifier bears a fitness cost to males. They also showed that the presence of an initial weak male-specific CI modifier eases the spread of subsequent modifiers, which may lead to the gradual elimination of CI. This theoretical finding has important implications for the evolutionary fate of Wolbachia; if symbioses break down after CI attenuation, the frequency of CI-suppressors would decline, once again making populations susceptible to CI-Wolbachia. This suggests that symbioses with CI-Wolbachia, rather than stable, could be better understood as cycles of infection spread and loss.

1.3.2 Conflict aftermath: Cooperation, addiction, and extinction

As long as there is conflict between Wolbachia and their host, evolution of resistance mechanisms is expected (Charlat et al., 2003). If adaptation of the host to Wolbachia

infection leads to elimination of harmful manipulation phenotypes, does that mean that Wolbachia are inexorably destined to reach extinction? Are there any other factors besides reproductive manipulations that result in favourable selection of infected individuals? Recent findings indicate that Wolbachia may play important roles in host physiology, suggesting ecologically contingent benefits of infection (Engelstädter and Hurst, 2009; Duron and Hurst, 2013). Therefore, the importance of Wolbachia on the ecology and evolution of their hosts may go far beyond their ability to induce reproductive manipulations. This section is dedicated to summarising these recent findings, and considers their implications in the evolutionary trajectories of the symbioses.

1.3.2.1 Wolbachia as obligate mutualists

Beneficial effects of Wolbachia symbiosis are particularly noticeable in organisms that depend on the symbiont for survival and/or reproduction. While Wolbachia are facultative in the vast majority of arthropod hosts, in filarial nematodes they are essential. Antibiotic elimination of Wolbachia results in infertility, inhibition of embryogenesis, arrested adult growth and death of the worm (Taylor et al., 2005). Nematode Wolbachia display characteristics typical of other ancient primary endosymbionts: the distribution of Wolbachia in the body of the worms is highly specific, the host and symbiont phylogenies are congruent and genome reduction and elimination of repetitive regions relative to facultative Wolbachia. Analysis of the genome of Wolbachia *w*Bm of *B. malayi* revealed the retention of metabolic pathways for the synthesis of riboflavin, flavin adenine dinucleotide, heme, and nucleotides,

which are predicted to be the main metabolic contribution to the worm (Foster et al., 2005).

Obligate Wolbachia symbioses in insects less common. Two examples that have caught the attention of evolutionary biologists involve the bedbug *Cimex lectularius* (Hosokawa et al., 2010) and the parasitic wasp *Asobara tabida* (Dedeine et al., 2001). In the bedbug *C. lectularius*, Wolbachia fulfils a role of dietary provisioner of vitamin B. Reduction or elimination of Wolbachia through antibiotic treatment rendered animals incapable of producing normally developing eggs. Dietary supplementation with vitamin B in these animals restored normal egg hatching. This association has characteristics of both primary and secondary endosymbioses, which suggest it is recent relative to other primary symbioses. Like other primary symbionts, Wolbachia in *C. lectularius* preferentially localises in bacteriomes and female gonads. The genomic size of this Wolbachia strain, however, resembles that of facultative Wolbachia. Although the molecular interactions that maintain the stability of this association are apparent, the evolutionary processes that gave rise to it are less clear. The F supergroup of Wolbachia, in which this particular Wolbachia falls into, includes symbionts of nematodes and termites that do not induce reproductive phenotypes; therefore it is difficult to attribute Wolbachia spread to manipulations. It is speculated that the acquisition of Wolbachia may have influenced the evolution of the feeding habits of these animals (Hosokawa et al., 2010). Indeed, obligate endosymbionts seem to be very common among animals that feed on diets that are poor in specific essential nutrients, such as sap (Baumann and Moran, 1997).

As opposed to coadaptive processes (gradual, beneficial change of both parts of the symbioses) shaping the evolution of Wolbachia-nematode and Wolbachia-bedbug associations, the obligate association with *A. tabida* is speculated to be the result of Wolbachia 'hacking' into a very fundamental process aspect of the wasp biology during a coevolutionary arm race (Aanen and Hoekstra, 2007). In insects, apoptosis is essential during the process of egg maturation, as it removes depleted nurse cells after they have transferred their cytoplasmic content to the oocyte (Cavaliere et al., 1998). In *A. tabida,* Wolbachia is necessary for normal progression of oogenesis, as aposymbiotic females show disproportionate apoptosis of nurse cells earlier than required, causing the abortion of egg development (Pannebakker et al., 2007). It has been hypothesised that during the early stages of the symbiosis, parasitic Wolbachia caused some degree of apoptotic inhibition that was harmful for gametogenesis. As a mechanism to recover ovarian functionality, the host may have responded by up-regulating apoptosis to a new level of functionality that compensated for the inhibitory effects of the symbiont. The spread and fixation of such a compensatory mechanism would have rendered the wasps incapable of producing their own eggs without Wolbachia. The evolutionary implications of these findings are crucial, as they show that obligate symbiosis may quickly evolve from parasitic associations through the spread of compensatory mutations, and that the gradual beneficial adaptation of coevolving partners is not strictly necessary for symbioses to become obligate.

1.3.2.2 Environment-dependent beneficial effects of Wolbachia

Because of their drastic effects on the host biology, reproductive manipulations of facultative Wolbachia have been a major research focus. This, to some extent, has

diverted attention from other phenotypic effects that may also be of great importance on the evolution of these symbioses (Iturbe-Ormaetxe and O'Neill, 2007). The last two decades of Wolbachia research have led to the discovery of multiple symbioses with largely or completely suppressed reproductive modifications that nonetheless appear at equilibrium (Iturbe-Ormaetxe and O'Neill, 2007). Models predict that, in order to persist, these associations require infected females to have a fecundity advantage that is independent from infection prevalence. In other words, these Wolbachia must confer fitness benefits to their host (O'Neill et al., 1997).

Any fitness benefit conferred by facultative symbionts is likely highly dependent on the environment and variable among host genotypes. If symbionts were uniformly beneficial, they would be expected to be fixed (Moran et al., 2008). The symbiosis between *D. melanogaster* and *w*Mel has been the subject of extensive analysis and has provided unparalleled insight into the phenotypic effects of infection other than reproductive modifications. The *w*Mel-*D. melanogaster* symbiosis is geographically widespread and its incompatibility phenotypes are weak or absent. Hoffmann and colleagues (1998) investigated the dynamics of *w*Mel in the field and found that the levels of CI were even weaker than in the laboratory, and maternal inheritance was imperfect. The observed stability of infection frequency in some populations led the authors to conclude that *w*Mel likely provided fitness benefits to the flies, but such effects were not yet apparent. Another study by Fry and Rand (2002) evaluated the effects of *w*Mel infection in laboratory maintained *D. melanogaster*, and found that fly lifespan was positively affected by infection, such effect being highly dependent on the nuclear background of the flies.

Sequencing of the *w*Mel genome, the first available for Wolbachia, provided substantial indirect evidence for the plausible metabolic roles of *w*Mel in flies. The presence of riboflavin and heme synthesis pathways (Wu et al., 2004), as well as evidence of positive selection in some of these genes (Brownlie et al., 2007), suggest that Wolbachia may provision the fly with these cofactors or some of their intermediates. Wolbachia may also represent an additional source of nucleotides, which could be beneficial in processes where high DNA replication is involved (Brownlie et al., 2007). Following up on their genomic analyses of *w*Mel, Brownlie and colleagues (2009) also found direct evidence on the beneficial role of Wolbachia in the iron metabolism of *D. melanogaster*. The authors investigated the effects of infection on flies exposed to different levels of dietary iron (Brownlie et al., 2009). Although Wolbachia appeared to have no effect on flies fed standard laboratory diets, flies raised on diets with a deficit or excess of iron greatly benefitted from the infection. Based on the seemingly low iron content of flies in nature, the authors concluded that Wolbachia contribution to the host iron metabolism is very likely an ecologically relevant trait.

Another fascinating effect of *w*Mel is its capacity to confer protection against RNA viruses (Hedges et al., 2008; Teixeira et al., 2008). The presence of Wolbachia in fly tissues has been linked to a reduction in Drosophila C virus titres of up to 10 000-fold relative to uninfected flies, and correlates with extended lifespan. Moreover, *w*Mel-related resistance is also effective against other RNA viruses and over different host genetic backgrounds (Teixeira et al., 2008). Importantly, new theoretical approaches suggest that protection against pathogens constitutes an important force driving the spread of facultative heritable symbionts (Reviewed in Haine 2007). Models predict that

wherever a virulent, horizontally transmitted pathogen infects a host population, a protective vertically transmitted endosymbiont would spread and greatly reduce the frequency of parasitised hosts at equilibrium. These three-way interactions would lead to the persistence of both pathogen and endosymbiont in the host population, but not to the fixation of the endosymbiont (Lively et al., 2005; Brownlie and Johnson, 2009). Empirical evidence on these symbiont-mediated protection dynamics is expanding. For example, Jaenike and colleagues (2010) documented a rapid spread of the vertically-transmitted *Spiroplasma* bacterium in American populations of *Drosophila neotestacea* and how such spread could be linked to their protective role against sterilising parasitic nematodes. Therefore, Wolbachia-mediated protection against pathogens constitutes a very plausible mechanism of invasion of host populations besides or in addition to reproductive modifications (Fenton et al., 2011).

The expanding evidence on environment-dependent Wolbachia benefits allows for speculation of other evolutionary trajectories of Wolbachia beside fixation and extinction. Depending on the extent of host dependence on the microbe, Wolbachia could also stably persist as facultative symbionts that fulfil important but sporadic host needs. A recent study showed that the facultative symbioses between arbustal mycorrhizal fungi (Glomeromycota) and the bacterial *Ca. Glomeribacter gigasporarum* have been evolutionary stable for 400 million years, almost twice the estimated time of the ancient obligate Buchnera-aphid association. Mondo and colleagues (2012) suggest that such stability is due to a balance between vertical transmission, recombination and host switching. Sporadic horizontal transmission and recombination may counteract the erosive effects of genetic drift observed in non-recombining genomes (see section

1.2.1.2), allowing organisms to maintain their infective capacity. Symbionts whose beneficial roles are restricted to particular environments would have an advantage if they retain the ability colonise new hosts. The authors of this study speculate that high environmental variability influences the symbiosis to be 'locked' in a stable facultative state.

The many similarities between the Glomeribacter-Glomeromycota model and Wolbachia (sporadic horizontal transmission, recombination, environment dependent advantages) give some support to the hypothesis that Wolbachia could be stably maintained in host populations as facultative symbionts. The commonly observed lack of symbiont-host co-speciation, however, argues against Wolbachia symbioses being maintained for extended periods of evolutionary time. Plausibly, the immense environmental variability to which Wolbachia-arthropod associations are exposed means that there would be many situations where symbionts would not be necessary or would simply be too costly to maintain, therefore driving local symbiont extinctions. Indeed, population genomic analyses of Wolbachia in *D. melanogaster* indicate that this infection was globally established once some 8 000 years ago and has been lost from multiple populations worldwide (Richardson et al., 2012).

If Wolbachia play important ecological roles in their hosts, it is plausible that the local extinction of a given variant occurs through the replacement with new Wolbachia strains. An excellent example of this was presented by Kriesner and colleagues (2013) on the Australian coastal populations of *D. simulans*. Historically, Australian *D. simulans* harboured the non-CI inducing Wolbachia strain *w*Au, at frequencies lower

than 0.3. At some point after 1994, the strong CI-inducer *w*Ri was introduced to the continent. Between 2004 and 2012, *w*Ri dramatically spread across the east coast completely replacing *w*Au and establishing itself at frequencies of over 0.9. A very important remark of this study was their suggestion that historical and recent invasions of *w*Au and *w*Ri respectively cannot be fully explained by CI dynamics and must have been driven by fitness benefits conferred by Wolbachia, implying that Wolbachia may fill some ecological role in these populations. Interestingly, both *w*Au and *w*Ri display antiviral protection phenotypes similar to those of *w*Mel in *D. melanogaster* (Osborne et al., 2009). Whether this is the particular beneficial trait that allowed the spread of Wolbachia in Australian population of *D. simulans* remains unknown.

Our understanding of the phenotypic effects of Wolbachia infections is expanding. We now know that there are Wolbachia-related phenotypes that are apparent under specific environmental circumstances and depend on the host genotypes. The study of Wolbachia infections in multiple environments and on carefully controlled host genetic backgrounds will continue to prove useful to our understanding of Wolbachia-host interactions.

1.4 Wolbachia, mitochondria and their interactions with the host and the environment

As described in the previous section, Wolbachia induced phenotypes are often the result of complex interactions between the genotypes of both partners and the environment (Mouton et al., 2007). Interestingly, the current understanding of non-neutral

mitochondrial genetic variations offers a very similar scenario, in which mtDNA mutants may have unequal fitness depending on the nuclear genetic background of the organism and the environment (Ballard and Pichaud, 2014). This section explores some of the evolutionary and ecological parallels between Wolbachia and mtDNA variants, and their implications in host adaptive processes.

1.4.1 Mitochondria and Wolbachia origins

Although the idea of a prokaryotic origin of mitochondria can be traced to 1890 (Kutschera and Niklas, 2005), it was not until the discovery and sequencing of mtDNA that their bacterial ancestry became obvious. The retention of ribosomal RNA coding sequences, a universal trait of modern mitochondrial genomes (Gray, 2012), allowed mtDNA lineages to be traced to a single origin from an ancestral alphaproteobacterium of the order Rickettsiales (Ferla et al., 2013). Thus, mitochondria and Wolbachia not only share their lifestyles and mode of propagation but also their ancestry.

The adaptive processes that resulted in obligate intracellular lifestyles of mitochondria and Wolbachia, as well as their impact on host evolution, are arguably very different. A recent and relatively well-supported hypothesis on the origin of mitochondria states that their alphaproteobacterium ancestor (protomitochondrion) fused to or was engulfed by a highly complex archaeobacterium (instead of a basal amitochondrial eukaryote as classically believed). Under this ‘symbiogenic’ hypothesis, the evolutionary novelty of eukaryotic cells emerged after or as a consequence of the establishment of this symbiosis (Koonin, 2010; Gray, 2012). Interestingly, such a scenario provides a

plausible selective factor for the evolution of eukaryotic cellular compartmentalisation. Hypothetically, the exposure of the archaeal genome to the DNA and translation products of the protomitochondrion may have negatively impacted the host gene expression, for which the separation of transcription and translation might have been adaptive (Koonin, 2010). Once in a compartmentalised cell, it has been argued that the conflicts of interest between cytoplasmic and nuclear genomes facilitated the evolution of other characteristic traits of eukaryotic cells such as sex, anisogamy and uniparental inheritance of cytoplasmic genes (Hurst, 1992; Law and Hutson, 1992). Although arguments against this symbiogenic hypothesis are yet to be addressed, the concept of the origin of modern eukaryotes being triggered by processes of conflict resolution between interacting genomes is intriguing.

The origin of Wolbachia is less clear. Their position within the Rickettsiales makes it reasonable to assume they originated from an ancestor with an intracellular lifestyle (Comandatore et al., 2013), more than100 million years ago. At present, it has not been possible to elucidate what was the first invertebrate lineage Wolbachia associated with, nor the nature of such association (mutualistic or parasitic). Considering their exclusive distribution within terrestrial invertebrates, it is plausible that Wolbachia emerged as symbionts of modern invertebrate forms. By the time the first Wolbachia associations appeared, invertebrate hosts had probably taken up most of the essential metabolic capacities from the protomitochondrion, which may have constrained the evolutionary novelty that the more derived Wolbachia alphaproteobacteria could have brought upon their hosts. Nonetheless, Wolbachia do confer novel physiologic abilities upon their

hosts, enabling them to explore otherwise inadequate environments (Wernegreen, 2004; Duron and Hurst, 2013).

1.4.2 Genomic reduction and lateral gene transfer

Mitochondrial genomes are under the same population constrains of obligate endosymbionts (Burger and Lang, 2003; see section 1.3.2.3); consequently, they share many genomic traits such as extreme size reduction, genomic stability and lack of repeat sequences. As a facultative symbiont, Wolbachia is said to possess a genome 'in transition' (Moran et al., 2008). Knowledge on the current processes of genomic evolution in organisms like Wolbachia may contribute to the understanding of the early phases of mitochondrial evolution.

Genomic size reduction is perhaps the most consistent genomic consequence of endosymbiosis (Burger and Lang, 2003), which in mitochondria occurred through gene loss and transference to the nucleus (Rand et al., 2004). During eukaryotic evolution, such lateral gene transfer (LGT) was substantial, which partially accounts for the fact that nuclear genes now encode the vast majority of the mitochondrial proteome. In fact, the role of LGT goes beyond organelle function, and genes that formerly belonged to the protomitochondrion are now involved in other cellular processes (Gray, 2012).

Genomic reduction has occurred in Wolbachia, though to a lesser extent than in other ancient intracellular endosymbionts (Toft and Andersson, 2010). Interestingly, recent studies have shown that LGT also occurs from Wolbachia to host chromosomes.

Wolbachia DNA fragments have been found in the genomes of various insect and nematode species. Hotopp and colleagues (2007) found traces of Wolbachia DNA fragments in six of 21 published genomes of various invertebrate taxa. Moreover, a recent study found that some transferred genes from *w*Bm into the *B. malayi* genome seem to be transcribed in a way that is stage-specific during the development of the worm (Ioannidis et al., 2013). These results suggest that Wolbachia may indeed represent an important source of genomic innovation for their hosts. Considering the vast abundance of past and present Wolbachia associations, the evolutionary consequences of this phenomenon can be considerable.

1.4.3 Cyto-nuclear interactions and compensatory mutations

How similar are the processes of host adaptation to Wolbachia infection and mitochondrial genetic variants? It was once thought that genetic variation of mitochondrial genes was selectively neutral (Ballard and Kreitman, 1995). Such an assumption, however, has been weakened by increasing evidence on the differential fitness of mtDNA variants in host populations (Ballard and Whitlock, 2004; Ballard and Rand, 2005; Dowling et al., 2008).

It is now apparent that the coordinate assembly of mitochondrial and nuclear encoded proteins is necessary for the correct function the electron transport system. This intricate molecular machinery produces up to 90% of cellular energy through oxidative phosphorylation. Therefore, mutations in the mtDNA that result in protein conformational changes would be predicted to impact oxidative phosphorylation

function, with consequences for cell energy homeostasis and overall organismal fitness (Ballard and Melvin, 2010; Horan et al., 2013). Because mtDNA is subjected to the effects of Muller's ratchet (Lynch and Blanchard, 1998; Rand et al., 2004), it has been suggested that selection favours responses in the nuclear genome that compensate for mildly deleterious mtDNA mutations to restore metabolic function (Dowling et al., 2008). The benefits of such compensatory mechanisms, limited to individuals with the mitochondrial mutation, are equivalent in principle to those that arise in response to negative effects of Wolbachia infection.

A simple prediction from this 'compensatory mutation hypothesis' is that experimentally changing the native nuclear background of the cytoplasmic genetic elements (either Wolbachia or mitochondria) would result in the disruption of the compensatory phenotypes. For mtDNA, the disruption of mito-nuclear coadapted complexes would result in decreased host fitness. For CI-Wolbachia, it may result in increased CI levels of naive versus adapted hosts. Experimental evidence supports these predictions. Laboratory crosses of the intertidal copepod *Tigriopus californicus* showed that third generation interpopulation hybrids displayed reduced fitness, subsequently restored by maternal (but not paternal) backcrosses. Moreover, lowered levels of ATP synthesis in the hybrid animals were restored by the re-introgression of the maternal nuclear background in these animals. These results clearly indicate the involvement of mito-nuclear interactions in ATP production and organismal fitness (Ellison and Burton, 2008). For Wolbachia, Poinsot and colleagues (1998) showed that the *w*Mel induced weak CI in their native host *D. melanogaster*, but displayed very high levels of

embryonic mortality upon transference to a naïve *D. simulans* background through microinjection.

Compensatory mutations are predicted to have important macroevolutionary consequences for their host. The *T. californicus* case exemplifies how mtDNA divergence between populations can drive differentiation in associated parts of the nuclear genome, which could lead to hybrid breakdown (Ellison and Burton, 2008). A recent study shows that Wolbachia variants may also drive reproductive isolation. Sympatric semispecies of the Neotropical *Drosophila paulistorum* require infection of semispecies-specific Wolbachia for viability. These obligate-mutualist Wolbachia are present at very low densities in host reproductive tissues. In semispecific hybrids, however, Wolbachia over-replication seems to cause pathogenic phenotypes such as embryonic inviability and male sterility, indicating semispecies-specific compensatory mechanisms against Wolbachia pathogenicity (Miller et al., 2010).

1.4.4 Sexual antagonism and mother's curse

Similarly to Wolbachia, the maternal inheritance of mtDNA may represent an important asymmetry between the sexes, which may lead to significant fitness differences between males and females that harbour the same mtDNA type. The mother's curse hypothesis suggests that natural selection would fail to purge cytoplasmic genetic variants that are deleterious for males, provided they do not negatively affect female fitness (Gemmell, et al., 2004). The deleterious effects of such mutants would be confined to male specific traits, and therefore very likely expressed as some degree of male infertility. A recent

study examined the transcriptome of isogenic *D. melanogaster* flies with variable mtDNA and standardised nuclear background. The results of this study indicated striking effects of mtDNA mutations on the expression of male-specific genes (those related to testis and accessory gland function). Female-specific genes and those with identical function for both sexes were, however, only moderately affected by the mutation load. Compensatory effects that encompass widespread control of the nuclear transcriptome likely cause the discrepancy observed between the sexes (Innocenti et al., 2011). A follow-up study on these and other similarly constructed fly lines tested the effect of mtDNA mutations on sperm competitiveness, and found that male fertility indeed varies *in vivo* across mtDNA variants (Yee et al., 2013).

The asymmetric fitness effects produced by reproductive manipulations are discussed in length in sections 1.2.2.2 and 1.3.1. Aside from reproductive manipulations, Wolbachia has also been linked with reduction of sperm production (Snook et al., 2000) and sperm competitive ability (de Crespigny and Wedell, 2006) in *D. simulans* males. In CI-inducing Wolbachia, lower sperm competitiveness may undermine the effects of the reproductive manipulations and alter Wolbachia spread dynamics.

1.4.5 Cross-talk, trade-offs, and adaptation to the local environment

Life history traits are often negatively associated with each other (Zera and Harshman, 2001). When the fitness cost of a beneficial trait results in a detrimental change in another trait, it is said that a trade-off exists (Stearns, 1989). Understanding trade-offs is essential for the study of evolutionary processes, as they provide an explanation for the

common occurrence of variability in life history traits (Zera and Harshman, 2001). Mutations that alter mito-nuclear molecular interactions may result in physiological trade-offs (Ballard and Pichaud, 2014). This is because the essential process of mitochondrial ATP synthesis produces by-products (in the form of reactive oxygen species) that lead to cellular oxidative damage, whose accumulated damaging effects are hypothesised to be causative of ageing. Using *D. simulans* as a model organism, Ballard and Melvin (2011) found that reduced activity of the cytochrome oxidase complex due to a mutated nuclear encoded subunit resulted in flies having significantly higher reproduction at early life but reduced lifespan. The study showed that mutant flies up-regulated mitochondrial function as a whole in an attempt to compensate for suboptimal cytochrome oxidase complex activity, which resulted in efficient ATP synthesis but higher production of reactive oxygen species. The authors hypothesise that a retrograde response from a mildly dysfunctional electron transport system may lie at the heart of such a compensatory mechanism.

It has been argued that mutations that affect mitochondrial function could be positively selected if they provide benefits in specific environments. Pichaud and colleagues (2012) studied the mitochondrial performance of *D. simulans* with *si*II and *si*III mitochondrial types and found that *si*II-harbouring flies had a higher catalytic capacity, which may provide advantages in terms of intensity of aerobic activity, endurance, or both. Another study on similar fly cohorts showed that *si*II flies recovered faster from cold coma, while *si*III were better at withstanding starvation, which may indicate that the improved catalytic capacity of *si*II flies comes at a cost (Ballard et al., 2007). Flies harbouring *si*III mitochondria are commonly found in sympatry with those harbouring

*si*II, with no indication of restricted gene flow between them (Ballard et al., 2002). This may be an indication that mtDNA genetic variation at the population level can occur if there are differential selective advantages for each mitochondrial type.

For Wolbachia-host associations, perhaps the most notorious trade-off is that between transmission fidelity and pathogenicity of infection, mediated by bacterial titres (McGraw et al., 2002). High Wolbachia titres may guarantee the transmission of microbes through the egg, but may result in pathogenicity for the host. Low symbiont density, on the other hand, may reduce the fitness costs of infection, albeit at a reduction in the number of infected host individuals in the next generation. If there is a fitness advantage for infected females, as in the case of those harbouring CI-Wolbachia, host and symbiont may act on the regulation of microbe titres to optimise the trade-off between the two parameters (Mouton et al., 2007). An exciting observation suggests a molecular mechanism by which Drosophila may modulate Wolbachia titres. Serbus and colleagues (2008) showed that the mRNP complex (mRNA and protein) formed by the transcript of the *grk* gene and its protein-binding partners has a cumulative, dose-sensitive impact on Wolbachia titre during oogenesis. The authors propose a model of interaction in which *grk* mRNP increases Wolbachia titres, which leads to a feed-back response that disrupts the function of the *grk* mRNP components, completing the regulatory loop. The involvement of cross-talk mechanisms in the regulation of symbiont/organelle function in response to environmental situations could represent an important hallmark in the long-term maintenance of stable cyto-nuclear associations. How widespread this mechanism is across Wolbachia-host symbioses is yet to be examined.

Wolbachia titres vary with environmental and physiological factors such as larval crowding (Wiwatanaratanabutr and Kittayapong, 2009), developmental and adult temperature (Mouton et al., 2006, 2007; Bordenstein and Bordenstein, 2011), and age (Unckless et al., 2009; Tortosa et al., 2010). In some scenarios, such over-replication could be interpreted as the destabilisation of the symbiosis where Wolbachia takes advantage of the host (McGraw et al., 2002; Rio et al., 2006). For example, the pathogenic *w*MelPop strain of Wolbachia is known to over-replicate in somatic tissues of *D. melanogaster*, producing abnormal fly phenotypes and shortening lifespan, which appear to be exacerbated at high temperatures (Min and Benzer, 1997; McGraw et al., 2002). In most cases, however, it is difficult to determine if Wolbachia titre is actively modulated in situations where higher bacterial densities are beneficial for the symbiosis. Osborne and colleagues (2012), for example, found important correlations between Wolbachia titres and antiviral protection for some naturally occurring Wolbachia variants in *D. simulans*. Wolbachia variants known occur at low densities failed to confer antiviral protection.

1.5 Concluding remarks

Wolbachia are a group of intracellular, maternally inherited bacteria with an impressive history of adaptation to intracellular lifestyles. Unlike other ancient obligate symbionts, Wolbachia are ‘generalists in host use’. This means that instead of adapting to a single host lineage, Wolbachia evolved ways to jump across host species and establish relatively stable associations in which they are maintained through vertical transmission. With every new established association, Wolbachia are in conflict with

nuclear genes, often conferring a disproportionate advantage to infected females to allow infection to spread. The reproductive manipulations induced by Wolbachia, perhaps the clearest representations of conflicts between cytoplasmic and nuclear genomes, are thought to be the general mechanism by which Wolbachia spread through host populations.

Recent evidence, however, challenges the view of Wolbachia as a ruthless manipulator of the host reproductive biology and suggests an evolutionary scenario where Wolbachia-host interactions are driven by the onset and resolution of conflicts of interest. Here, I have highlighted a view in which reproductive manipulations are only transient phenotypes, attenuated as the host adapts to infection. After attenuation of reproductive phenotypes, the stability of the symbioses would rely on the physiological advantages Wolbachia may confer upon their host. The availability of multiple sequenced Wolbachia genomes has been an important step forward in the understanding of Wolbachia-induced phenotypes, as they reveal a whole biochemical and genetic repertoire from which the host can metabolically benefit from the infection. The recent discovery of various Wolbachia-induced beneficial phenotypes supports this line of thought.

Compensatory mutations (or genetic modifiers) are a hallmark of the adaptation of the host genome to Wolbachia and other cytoplasmic genetic elements in conflict, mitochondria among them. The evolutionary trajectories of Wolbachia symbioses after attenuation of reproductive manipulations would depend on the physiological benefits of infection, which for facultative symbionts are likely to be dependent on the

environment. Here, I have also presented evidence that supports the view of environment-dependent facultative mutualism as a stable evolutionary outcome of Wolbachia infections beside extinction and obligate symbioses. Finally, our current understanding of the biology of mitochondria and Wolbachia unravels remarkable parallels in the way they interact with the nuclear genome. For example, we highlighted how Wolbachia and mtDNA polymorphisms may be maintained at equilibrium in host populations when they confer environment-specific fitness advantages. Great insights into both the Wolbachia and mitochondrial research fields can be revealed if these fields are considered to be overlapping, rather than independent from each other.

Chapter 2

Wolbachia gonadal density in female and male Drosophila vary with laboratory adaptation and respond differently to physiological and environmental challenges

Abstract

In symbiotic associations such as those between Wolbachia and insects, the within-host symbiont density plays an important role in the maintenance of the infection in natural populations, as it relates to transmission fidelity and the pathogenicity of the symbiont. Within-host density is speculated to be the result of complex interactions between the bacterial genotype, the host genotype and the environment, which may account for the substantial variation in Wolbachia titres among wild collected individuals compared to laboratory lines. Using quantitative PCR, we screened the Wolbachia gonadal density of individuals from 50 isofemale *Drosophila simulans* lines raised in standard conditions for at least three generations after collection from the wild. Although these newly collected lines displayed significant variation of ovarian Wolbachia titres, such variation was lost by F_{19}. Assaying these flies at different ages and under different environmental conditions indicated that symbiont titres in female gonads were not affected by the conditions tested here. However, male gonads were consistently affected by these treatments in a line-specific way. We propose that the differences in Wolbachia densities among ovaries of F_4 flies are the consequence of large differences in the field-collected females caused by the variable environment, and carried over for at least four generations. In addition, we provide evidence of sex-specific dynamics of Wolbachia in gonads of females and males. In combination, our results support the view of sex-specific Wolbachia evolutionary interactions for males and females, which has been predicted by theory and observed experimentally.

2.1 Introduction

Symbiotic associations between vertically transmitted microbes and arthropods are common (Hilgenboecker et al., 2008; Moran et al., 2008). The intracellular, maternally transmitted Wolbachia is perhaps the best studied of these symbiotic microbes, as it has the capacity to induce reproductive modifications in their host, such as cytoplasmic incompatibility (CI), male killing or parthenogenesis. Wolbachia infection ultimately increases the number of infected females in a population regardless of possible detrimental effects to the host (Turelli, 1994; Poinsot and Merçot, 1997). The density of Wolbachia within the arthropod host has been recognised as a critical factor in these associations, as it affects both transmission fidelity and pathogenicity of infection (McGraw et al., 2002). Several studies suggest that, apart from its role in transmission efficiency and virulence, the within-host density of Wolbachia also correlates positively with the strength of the reproductive modifications. Therefore, the density dynamics within individuals may govern the prevalence of infection in host populations (Reviewed in Jaenike, 2009).

The regulation of Wolbachia density is hypothesised to be the result of highly complex interactions involving the host and symbiont genotypes, as well as the environment (Mouton et al., 2007). The capacity to replicate of some Wolbachia strains appears in some instances to be independent from genetic background of the host they inhabit. For example, the parasitic strain *w*MelPop found in laboratory-reared *Drosophila melanogaster* often displays an over-replicative phenotype when artificially transferred into other insect species such as *D. simulans* (McGraw et al., 2002) and *Aedes*

albopictus (Suh et al., 2009). Nonetheless, many studies show clear evidence of the participation of both host and symbiont genotypes in the regulation of the within-individual Wolbachia titres. For example, in multiply Wolbachia infected wasps, the specific density of each strain remains constant independently of the presence of others, suggesting regulatory mechanisms in the host are specific to the microbe strain (Mouton et al., 2003). Similarly, Ikeda and colleagues (2003) artificially exchanged naturally occurring Wolbachia strains between the moths *Ephestia kuehniella* and *Cadra cautella* and found that the host genetic background strongly influenced the proliferation of each Wolbachia strain. Environmental and host physiological factors have also been shown to influence symbiont titres as well as the strength of the reproductive modifications induced by Wolbachia. The effects of a variety of factors including age (Clarke et al., 2002; Unckless et al., 2009; Tortosa et al., 2010), larval crowding (Wiwatanaratanabutr and Kittayapong, 2009), developmental and adult temperature (Mouton et al., 2006, 2007; Bordenstein and Bordenstein, 2011), dietary antibiotics (Clancy and Hoffmann, 1998) and the presence of insecticide resistance genes (Berticat et al., 2002) have been shown to impact Wolbachia titres in several host species. Although the causative mechanisms of such effects are not known, it is speculated that they affect the balance of the symbiotic relationship, perhaps by altering the within-host availability of nutrients, increasing the ability of the bacteria to replicate or altering the ability of the host to control the symbiont (Mouton et al., 2006).

In addition to understanding the host, Wolbachia and environmental contributions to the regulation of within-individual symbiont titres, the study of sex-specific Wolbachia dynamics may shed further light on the evolutionary processes that regulate these

interactions. Given its maternal transmission, Wolbachia have been predicted to evolve toward mutualism with the maternal host lineage, as their reproductive success is directly related to that of their female hosts (Herre et al., 1999). Infected females with CI-inducing Wolbachia have a reproductive advantage associated with the infection, as they produce viable offspring regardless of the infection status of the male (Friberg et al., 2011). Therefore, selection on both female host and symbiont is expected to converge towards improved transmission and low pathogenicity in females. Contrary to females, the CI-inducing Wolbachia infection in males represents a fitness compromise, as successful matings would occur only with infected females. In response, male genotypes may evolve modifiers that ultimately reduce the strength of the reproductive manipulation and overcome the fitness cost associated with the infection (Koehncke et al., 2009). Additionally, males represent an evolutionary dead end for the symbiont, and similar to mitochondria, symbiont-host adaptations that are advantageous for females might not result in adaptive advantages for males; this being more evident for sexually dimorphic traits (Innocenti et al., 2011). Evidence of such sexually-antagonistic evolution has been observed in that natural populations of *Aedes albopictus,* in which males lose their Wolbachia *w*AlbA infection very early in their adult life, while in females it is maintained throughout their lifespan (Dutton and Sinkins, 2004; Tortosa et al., 2010).

Two approaches are usually used to investigate the regulation of within-host Wolbachia density: the exchange of Wolbachia strains between genetically distinct hosts (usually by microinjection) and the comparison of distinct Wolbachia strains within the same host species. An alternative strategy that has the potential to shed light on infection

prevalence and plausibly, the strength of reproductive alterations in natural populations (Jaenike, 2009) is to screen a population for differences in Wolbachia titres among individuals or families. Despite this, only a very limited number of studies have been dedicated to explore this latter strategy. Unckless and colleagues (2009) screened more than 2 000 wild collected *Drosophila inubila* females for Wolbachia density in ovaries and found around 20 000-fold difference between the least and the most heavily infected individuals. Similar results were obtained for the mosquito *Ae. albopictus*, with the levels of Wolbachia infection in F_1 offspring of wild caught animals varying by approximately 180 000-fold (Ahantarig et al., 2008). Whether these remarkable differences in Wolbachia titres are heritable has yet to be explored.

In this study, we investigated the possible heritability of intrapopulation Wolbachia titres by measuring the ovarian Wolbachia density in individuals from 50 isofemale lines of *Drosophila simulans* four generations after field collection and repeated the measurement for a subset of those lines after15 generations of laboratory rearing. We concomitantly studied Wolbachia densities in male gonads to investigate if symbiont titre dynamics differ between the sexes. We selected the *D. simulans* population from Hawaii because this fly population is exclusively infected by the strong-CI inducing *w*Ha strain of Wolbachia (James and Ballard, 2000). This strain displays very high transmission fidelity, infection frequencies near fixation (Rousset et al., 1992; Turelli and Hoffmann, 1995; James and Ballard, 2000; Ballard, 2004) as well as no apparent fitness costs or benefits to the female host compared to uninfected individuals (Poinsot and Merçot, 1997). We subsequently examined the effects of age, temperature, immune challenge and diet on gonadal titres of females and males in two different isofemale

lines in order to explore the environmental effects of infection levels among different fly lines.

Overall, our results show that gonadal Wolbachia density is (i) highly variable among newly collected females, but such variation is lost during the process of laboratory adaptation, (ii) do not correlate between females and males from the same isofemale line, and (iii) is very unresponsive to environmental treatments in ovaries while densities in testes are affected in a line specific manner. Our study highlights the importance of considering the effects of laboratory rearing on the study of Wolbachia population dynamics and that sex-specific dynamics of Wolbachia titres may provide clues to understanding the evolutionary processes of this symbiont in nature.

2.2 Materials and methods

2.2.1 Drosophila collection and rearing

A total of 91 female *D. simulans* were collected in July 2009 from Honolulu, Hawaii. Flies were placed individually in food-containing vials and allowed to lay eggs. As per Australian Department of Agriculture (DAAF) regulation, F_1 flies derived from captured females were kept in a quarantine facility in Canberra. Their living offspring spring was received at laboratory in Sydney. As a consequence, the time between collection and experimentation was extended. Once in the laboratory the species status of *D. simulans* was assessed by morphological observation of F_2 males. Confirmed *D.simulans* lines were maintained in discrete generations at low densities on standard

treacle-semolina-yeast-agar media (with Methyl Paraben 0.4% as antifungal agent) at 23 °C at 50% humidity and under a 12 h light-dark cycle.

2.2.2 Wolbachia infection frequency and genetic variation

Founder females and four to five F_1 individuals of each line were stored in Gentra Puregene® cell lysis solution (Gentra Systems Inc., Minneapolis, MN, USA) for Wolbachia infection determination by polymerase chain reaction (PCR). Genomic DNA (gDNA) was extracted from whole flies using the Gentra Puregene® Cell Kit (Gentra Systems Inc., Minneapolis, MN, USA) following the Isolation from Solid Tissue protocol. PCR was performed using primers amplifying the *wsp* gene following Zhou et al. (1998). Infection was corroborated by amplification of a second, independently extracted DNA sample. Amplification of the COI barcoding region using universal mitochondrial primers (Folmer et al., 1994) was used to validate DNA quality. The number of infected and uninfected lines in this study was compared against previously reported data for this symbiont-host population (Rousset et al., 1992; Turelli and Hoffmann, 1995; James and Ballard, 2000).

We determined the Wolbachia strain and genetic variation in the population by sequencing a 549 bp region of the *wsp* gene following Zhou et al. (1998) and an additional 439 bp of the cell cycle-gene *ftsZ* following Baldo et al. (2006). These two loci are commonly used to determine the Wolbachia strain infecting arthropod hosts as they have shown to have highly variable regions (Werren et al., 1995; Zhou et al., 1998; Baldo et al., 2006). Sequencing was carried out for a subset of 20 lines, which were

determined to be Wolbachia infected. Chromatograms were visualised and edited using Sequencer 5.1 (Gene Codes, Ann Arbor, MI, USA).

2.2.3 Wolbachia density determination

Using quantitative PCR (qPCR), we determined Wolbachia density in the gonads of 15-day-old individuals from cultures three generations after field capture of founder female (F_4) flies and after being raised in standard laboratory conditions (Hercus and Hoffmann, 2000). We analysed gonads because of the transovarial nature of Wolbachia transmission and the association of Wolbachia with reproductive phenotypes when present in testes (Dobson et al., 1999). Fifteen-day-old individuals were tested as flies often mate in the field when they are two to three weeks old (Turelli and Hoffmann, 1995). F_4 individuals were assayed to minimise the potential for laboratory adaptation (Frankham and Loebel, 1992), and will be henceforth termed 'newly collected'.

Four to six flies of each sex were taken from each of 50 randomly chosen isofemale lines. Ovaries and testes were dissected in Drosophila ringer solution following the procedure of McDonald and Montell (2005). DNA was extracted from individual dissections as in section 2.2.2, and analysed by qPCR to obtain relative ratios of Wolbachia compared to a Drosophila single copy nuclear gene. For Wolbachia, primers were designed to amplify an 85 bp fragment of the *gatB* gene specific to the strain infecting this fly population (qgatBF: 5'-TTA TTG TTT GAT GTC GCT TTG GG and qgatBR: 5'-TCA GGC TCA GGG AAG TAT CT). To quantify a single copy Drosophila gene, primers were designed to amplify a 135 bp region of the *rpII215* gene

which encodes the large RNA polymerase II subunit, (qrpII215F: 5'-AGG CGT TTG AGT GGT TGG and qrpII215R: 5'-TGG AAG GTG TTC AGT GTC ATC). *In situ* hybridisation studies have shown that *rpII215* is present in a single copy in the genome of a variety of organisms including Drosophila (Aoyagi and Wassarman, 2000).

Each 10 µl reaction contained 5 µl of KAPA SYBR® FAST Universal qPCR Kit (Kapa biosystems, Boston, MA, USA), 4 ng gDNA, 0.2 µM of each forward and reverse primer and 0.2 µl ROX as per manufacturer directions. Reactions were performed in duplicates or triplicates using a Stratagene Mx3000 qPCR instrument (Agilent Technologies, Santa Clara, CA, USA). The qPCR protocol consisted of one initial denaturation step of 30 s at 95 °C, followed by 40 cycles of 3 s at 95 °C and 30 s at 60 °C. Melting curve analysis was performed after each run to confirm the specificity of the amplicons and the absence of primer dimers or contamination. The amplification efficiency of each gene was calculated by constructing a standard curve using serial dilutions of gDNA from infected flies. Copy number of the Wolbachia *gatB* gene relative to the Drosophila *rpII215* gene was calculated using qbasePLUS software (Biogazelle, Zulte, Belgium). For statistical analyses, individual normalised Wolbachia counts were square root transformed (Sokal and Rohlf, 1995).

To maintain clarity, isofemale lines were ranked and named according to increasing Wolbachia density in ovaries after the F_4 screening, from the lowest (HW01) to the highest (HW50) density line. The ranking was based on ovarian density because the vertical transmission of Wolbachia through the egg may allow heavily Wolbachia infected ovaries to pass their high Wolbachia loads onto the next generation (Ikeda et

al., 2003). Although present in testes, Wolbachia is absent from mature sperm (Binnington and Hoffmann, 1989) and therefore it is unlikely to influence the level of infection of individuals in subsequent generations (Hoffmann et al., 1986). To test if the symbiont density measurements from the two gonadal tissues differed, nested ANOVA using sex as the main factor and line the as the nested factor was performed using JMP© v5 (2007 SAS Institute, Cary, NC, USA). Spearman correlation analysis using Microsoft Excel was employed to test the correlation between ovarian and testicular densities of flies from the same isofemale line.

In order to determine if the Wolbachia densities found in F_4 flies were maintained in the laboratory over time, the 10 isofemale lines that showed lowest (HW01-05) and highest (HW46-50) Wolbachia densities in female gonads were re-tested at generation 19. We compared Wolbachia densities between generations (F_4 and F_{19}), between the groups harbouring the lowest (HW01-05) and the highest (HW46-50) density lines and the interaction of these two factors using a two-way ANOVA with JMP© v5. The factor 'line' was included as a nested effect within group.

2.2.4 Genetic variation at host mitochondrial and nuclear loci

We explored the genetic variation in the host to test whether there is any evidence of population genetic subdivision associated with the observed differences in Wolbachia density. The lines described as having 'low' (HW01-05) and 'high' (HW46-50) Wolbachia density were selected for this analysis. Genetic diversity at two Drosophila mitochondrial loci was determined. Due to their maternal coinheritance, Wolbachia CI-

driven selective sweeps may result in hitchhiking of the associated mitochondrial haplotype (Turelli and Hoffmann, 1991; Raychoudhury et al., 2010). Genetic variation in the mitochondrial genome was investigated by sequencing 646 bp of the COI barcoding region and a 503 bp fragment spanning from $tRNA_{Lys}$ to the beginning of the *ATP8* gene. These regions were selected because of their high variation, observed in published whole mitochondrial sequences of *D. simulans* (Ballard 2000a). The barcode region was amplified using the universal primers LCO1490 and HCO2198 (Folmer et al., 1994). The second fragment was amplified using the primers 3735+ and 4273-, previously used for Drosophila whole mitochondrial genome sequencing (Ballard 2000a). Conditions for mitochondrial primer sets were: initial denaturation at 95 °C for 2 min; 35 cycles at 95 °C for 30 s, 55 °C for 30 s, 72 °C for 45 s; and final extension at 72 °C for 5 min. PCR amplicons were directly sequenced as described above.

We determined the genetic variation at two nuclear encoded loci that have been shown to evolve in a model consistent with a strictly neutral equilibrium in other populations and species (Wells, 1996; Ballard 2000b). Compilation of these data is expected to enable estimates of nucleotide diversity as well as to allow future comparisons of genetic parameters of infected populations with distinct strains and at different points in the wave of Wolbachia infection (Turelli and Hoffmann, 1999). Amplification of a 469 bp fragment from intron 1 of the alcohol dehydrogenase-related gene (*Adhr*), and a 467 bp fragment from intron 2 of the glycerol 3-phosphate dehydrogenase (*Gdph*) were obtained. Primer pairs used for amplification were adhrF (5'-AAA CAG AAG TCG TGG AAA GC) and adhrR (5'-TTC TTC ATT TCT TCC CTT GC) for the *Adhr* locus and gpdhF (5'-TAC ATG CTC CAC AGG CAC TC) and gpdhR (5'-GAC ACG TAT

TTC GCT CCA CA) for *Gpdh.* The PCR cycling conditions for the nuclear primer sets were the same as for the mitochondrial primer sets. PCR products were cloned into the vector pJET1.2 and sequenced using the CloneJET^{TM} PCR Cloning Kit (Fermentas, Maryland, USA) according to the manufacturer's protocol. A single representative sequence was randomly chosen per isofemale line.

Sequences were exported to DNASP 5.10 (Rozas and Rozas, 1999) and nucleotide diversity (π) and the neutral parameter (θ) based on the total number of polymorphisms and segregating sites were calculated. Both π and θ were calculated based on the total number of mutations, excluding sites with gaps and/or missing data. Tajima's *D* was calculated to test whether this population violated a strictly neutral equilibrium model of mutation (Tajima, 1989).

2.2.5 Physiological and environmental effects on Wolbachia density

We examined whether Wolbachia densities in the gonads of adult Drosophila were influenced by adult age and three environmental variables likely to be important in nature. We chose adult flies to allow comparison between treatments, avoiding possible confounding effects during development (Hartenstein, 1993).

To produce flies for these assays, F_{20} flies from the HW01 and HW50 lines were placed in cages containing solid oviposition resources (4% agar, 10% molasses) supplemented with dry yeast paste. Eggs were collected following the procedure of Clancy and Kennington (2001) and placed at a density of approximately 200 eggs per bottle on

standard *treacle-semolina-yeast-agar* media. Rearing conditions were identical for all treatments. F_{21} adult flies were collected within six hours of eclosion. With the exception of zero age flies, adults were placed unsorted into population cages at a density of 100 flies per cage and randomly allocated to one of the treatments. Flies were allowed to mate for two days, sorted by sex and placed in new cages. Unless otherwise stated adults flies were maintained in these cages on *treacle-semolina-yeast-agar* media until they reached the appropriate age for testing. Following treatment, flies were frozen at -20 °C and Wolbachia density estimated as described above. The statistical significance of the physiological and environmental conditions and the effects of line were investigated using a two-way ANOVA in JMP© v5 for each assay.

2.2.5.1 Age

While it is not known how long Drosophila live in nature (Turelli, 2010), Wolbachia density in gonads and whole organisms have been reported to change with age (Binnington and Hoffmann, 1989; Bressac and Rousset, 1993). We quantified Wolbachia density in gonads in flies maintained at 23 °C at 0, 5, 10, 15, 20 and 30 days of age.

2.2.5.2 Immune Challenge

Flies are likely exposed to pathogens and able to elicit an immune response in nature (Govind, 2008). To assess if the cost of eliciting an immune response (McKean and Nunney, 2001) is reflected in Wolbachia density in flies, 11 day-old flies maintained at 23 °C were immunologically challenged by pricking with *Escherichia coli* TOP10 following Lemaitre et al. (1997). Flies were then returned to population cages for 4 days

and maintained at 23 °C to give them sufficient time to mount an immune response (McKean and Nunney, 2001), then harvested by freezing at day 15. Two control treatments were included: flies pricked with a sterile needle only (no *E. coli*) and non-pricked flies.

2.2.5.3 Temperature

In nature, ectotherms such as Drosophila are exposed to diurnal and seasonal temperature fluctuations (Dillon et al., 2007). Temperature has been shown to have a significant effect on Wolbachia-induced CI in Drosophila; as decreasing Wolbachia densities at high temperatures have been reported (Hoffmann et al., 1990). Moreover, it has been reported in some organisms that Wolbachia density is temperature specific (Mouton et al., 2006; Tortosa et al., 2010). To test whether adults maintained at different temperatures show differences in Wolbachia density, flies were kept at 17, 23 and 27 °C until they reached an age equivalent to 15 days at 23 °C. Age as calculated by degree-days (de Jong and van der Have, 2009) was 22 days at 17 °C and 11 days at 27 °C.

2.2.5.4 Diet

In addition to the general effect nutrition has upon fly fecundity and longevity (Lee et al., 2008), correlations between nutrition and Wolbachia CI levels have been reported (Clancy and Hoffmann, 1998). Here, we varied the nutrition content of the food on which adult flies were maintained to determine its effect on Wolbachia density. Flies in cages maintained at 23 °C were supplied diets containing 0.1, 0.5, 1 and 2 times the

standard amount of fresh yeast and treacle of the standard *treacle-semolina-yeast-agar* media for 15 days, then collected and frozen for further analyses.

2.3 Results

2.3.1 Wolbachia infection frequency and genetic variation

In this study, 93.4% of the isofemale lines collected were infected with Wolbachia. Previous reports on wild populations of *w*Ha infected *D. simulans* collected from Hawaii have shown levels of infection between 98 to 100% (Rousset et al., 1992; Turelli and Hoffmann, 1995; James and Ballard, 2000; Ballard, 2004). The chi-square test of independence (χ^2) showed that the proportion of infected captured females found here (85 of the 91) differed significantly from that compiled from previous studies, as determined by a Yate's adjusted chi-square test (177 of 178; $\chi^2_{adj} = 6.427$, $p < 0.01$). One possible explanation for the lower frequency of infection is that the Wolbachia strain infecting flies in Hawaii has changed over time. To investigate this hypothesis we sequenced two Wolbachia loci. The *wsp* and *ftsZ* sequences obtained from 20 isofemale lines unambiguously identified *w*Ha as the Wolbachia strain infecting this fly population (James and Ballard, 2000). The sequences are identical to those previously reported (GenBank ID: AF020068 and AY508998.1, respectively). No nucleotide polymorphisms were found in sequences obtained from the 'high' and 'low' density lines.

2.3.2 Wolbachia density determination

Quantitative PCR performed on F_4 flies revealed variable Wolbachia densities among lines. The number of Wolbachia genomes per Drosophila genome between the least and the most heavily infected individuals varied 23.89-fold in ovaries and 32.58-fold in testes. Wolbachia densities of female and male gonads and across the 50 isofemale lines are presented in Fig. 2.1. Nested ANOVA showed that Wolbachia densities differed significantly between ovaries and testes ($F_{1,98} = 483.99$, $p < 0.001$) and among lines ($F_{1,413} = 65.16$, $p < 0.001$). Additionally, Wolbachia densities in testes did not correlate with those of ovaries as shown by the Spearman correlation analysis (testes density = 1.96 ovary density + 0.07; $R^2 = 0.0378$, $p = 0.18$; Appendix 2.1). All subsequent statistical analyses of gonadal Wolbachia density were performed independently for females and males.

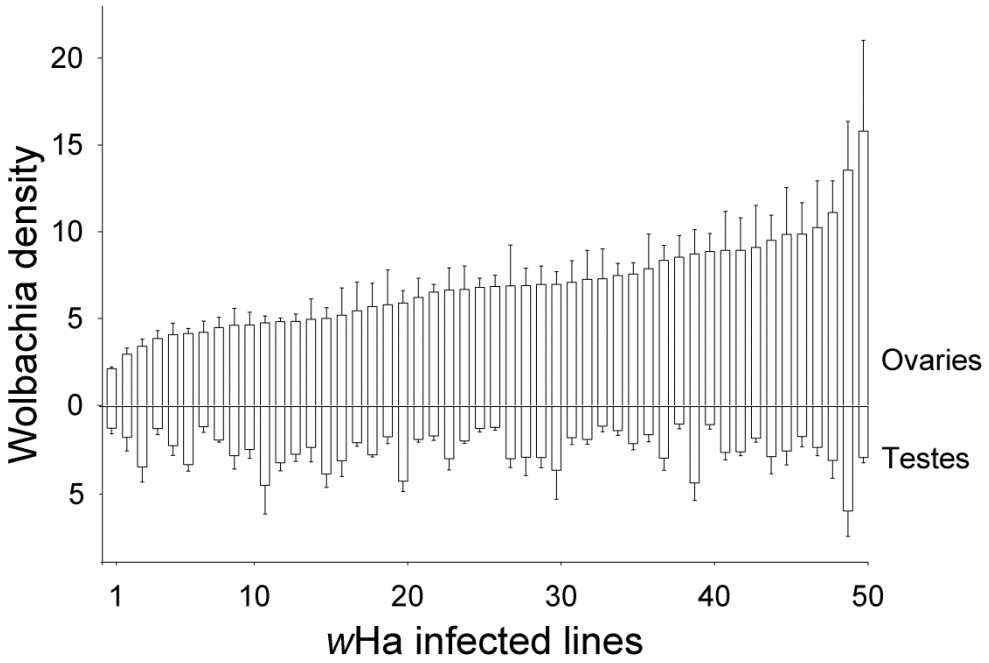

Fig. 2.1 Gonadal Wolbachia densities of *Drosophila simulans* individuals from 50 isofemale lines, four generations after field collection. Ovarian density of Wolbachia in F_4 flies after field collection differs between sexes and among lines. The ranking was based on ovarian density because transmission of Wolbachia through the egg may influence Wolbachia abundance of the offspring. Bars represent mean normalised Wolbachia density per line ± SEM.

For ovarian Wolbachia titres, two groups containing the five least infected (HW01-HW05) and the five most infected (HW46-HW50) lines at F_4 were selected for further analysis (10 lines in total). Statistical analysis of the F_4 data from ovaries confirmed that the two groups differed significantly from each other ($F_{2,40} = 39.64$, $p < 0.001$) and the homogeneity of the lines within the 'low' and 'high' density groups ($F_{8,50} = 7.35$, $p = 0.66$). In F_{19}, the overall variation among individuals was significantly lower (6.77-fold difference between the least and most heavily infected individuals). In addition, the titre differences of lines from the 'low' and 'high' ovarian density groups were not maintained by the 19th generation. The 'low' ovarian densities lines showed a significant Wolbachia titre increase, from a mean group density value of 3.26 in F_4 to 5.64 in F_{19} ($F_{1,42} = 49.48$, $p < 0.001$), while lines of the high-density group showed a titre reduction, from 12.38 in F_4 to 4.82 ($F_{1,45} = 34.62$, $p < 0.001$). The average group density of these 'low' and 'high' groups did not statistically differ from each other at F_{19} ($F_{1,55} = 2.37$, $p = 0.13$; Fig. 2.2). Two-way ANOVA of F_4 an F_{19} flies showed that density is significantly influenced by generation ($F_{1,106} = 9.63$, $p < 0.001$), density group ($F_{1,95} = 44.57$, $p < 0.001$) and their interaction ($F_{1,106} = 59.33$, $p < 0.001$), but the lines within density group were not significantly different from each other ($F_{8,95} = 0.87$, $p = 0.59$).

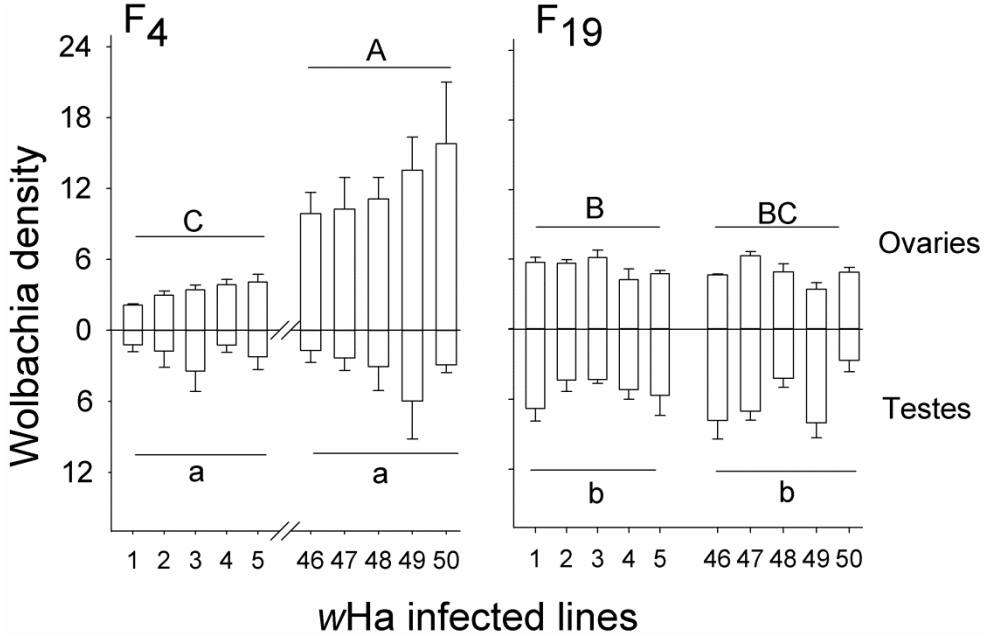

Fig. 2.2 Gonadal Wolbachia density in *Drosophila simulans* at F_4 and F_{19} after field collection. The variation of Wolbachia ovarian density observed for a subset of F_4 flies is lost by generation 19. Density in testes increased from F_4 to F_{19}. Bars represent mean normalised Wolbachia density per treatment ± SEM. Bars not connected by the same letter vary significantly according to Tukey's HSD test with $Q_{females} = 2.62$, $Q_{males} = 2.63$ and $\alpha = 0.05$.

Testicular symbiont density among the above selected lines showed that there were no differences between the 'low' and 'high' density groups at F_4 ($F_{2,27} = 3.36$, $p = 0.08$), but the lines within these groups differed ($F_{8,36} = 2.81$, $p = 0.02$). Analysis of F_{19} flies showed no variation among groups ($F_{1,42} = 0.57$, $p = 0.45$) but identified differences within groups ($F_{8,51} = 2.79$, $p = 0.01$). Between generations, there was an overall increase of 50% in Wolbachia density in testes from F_4 to F_{19} (Fig. 2.2). Two-way ANOVA showed that densities in testes were influenced by generation ($F_{1,88} = 36.77$, $p < 0.001$) and differed significantly among lines within groups ($F_{16,88} = 2.64$, $p < 0.01$); however, no differences were found among density groups ($F_{1,88} = 2.88$, $p = 0.09$), nor due to the interaction between generation and group ($F_{1,88} = 0.40$, $p = 0.52$).

2.3.3 Genetic variation at host mitochondrial and nuclear loci

To test whether there was genetic subdivision associated with Wolbachia infection we explored host genetic variation in two regions of mtDNA and at two autosomal loci. The groups of lines described as having 'low' and 'high' Wolbachia density at F_4 were included. No variation was detected in the mtDNA. The two regions of mtDNA yielded a concatenated mtDNA dataset of 1149 bp without variation. The sequence was identical to previously reported sequences of *D. simulans* from Hawaii (GenBank ID: AF200835.1).

The nuclear encoded *Adhr* and *Gpdh* sequences were 469 and 467 bp in length respectively (GenBank ID: JX4552200-JX455219). There were high levels of variation at both loci examined but the variation did not differ between the sets of flies

harbouring the lowest and highest densities of Wolbachia at F_4 (Appendix 2.2). As a consequence the results from the two sets of lines were pooled. The *Adhr* locus exhibited a total of 13 polymorphic sites, with nucleotide diversity (π) of 9.30×10^{-3} and neutral parameter (θ) of 9.61×10^{-3}. At the *Gpdh* locus 15 polymorphic sites were detected ($\pi = 12.99 \times 10^{-3}$ and $\theta = 11.52 \times 10^{-3}$). Tajima's *D* test supports the hypothesis that these nuclear loci evolve in a manner that does not depart significantly from a strictly neutral model of mutation (*Adhr*: $D = 0.61$; *Gpdh*: $D = -0.15$).

2.3.4 Physiological and environmental effects on Wolbachia density

Overall, the treatments tested here showed that ovarian density of Wolbachia titres was constant between 5 and 30 days of adult age and were not affected by any of the environmental treatments. Symbiont titres in testes, on the other hand, increased with age and, except for diet, were affected by the treatments in a line specific manner.

2.3.4.1 Age

Wolbachia density may change with age and this may influence the transmission dynamics and the proportion of uninfected flies in a population (Binnington and Hoffmann, 1989; Bressac and Rousset, 1993; Hurst et al., 2001). In F_{21} flies from the HW01 and HW50 lines there was an overall increase in Wolbachia density with age for both ovaries and testes (Fig. 2.3). Densities in ovaries showed an increase in Wolbachia over the first 5 days post eclosion, after which it remained constant until the end of the assay (day 30). In contrast, density in testes gradually increased with age. For both females and males, two-way ANOVA showed a significant effect of age on Wolbachia

densities ($F_{5,69} = 9.06$, $p < 0.001$ and $F_{5,68} = 13.29$, $p < 0.001$, for ovaries and testes, respectively). An age by line interaction was significant for testes ($F_{5,68} = 3.52$, $p < 0.01$) but not for ovaries ($F_{5,69} = 0.84$, $p = 0.52$). Line effects were not significant in either sex (ovaries: $F_{1,69} = 0.37$, $p = 0.55$; testes $F_{1,68} = 0.68$, $p = 0.41$).

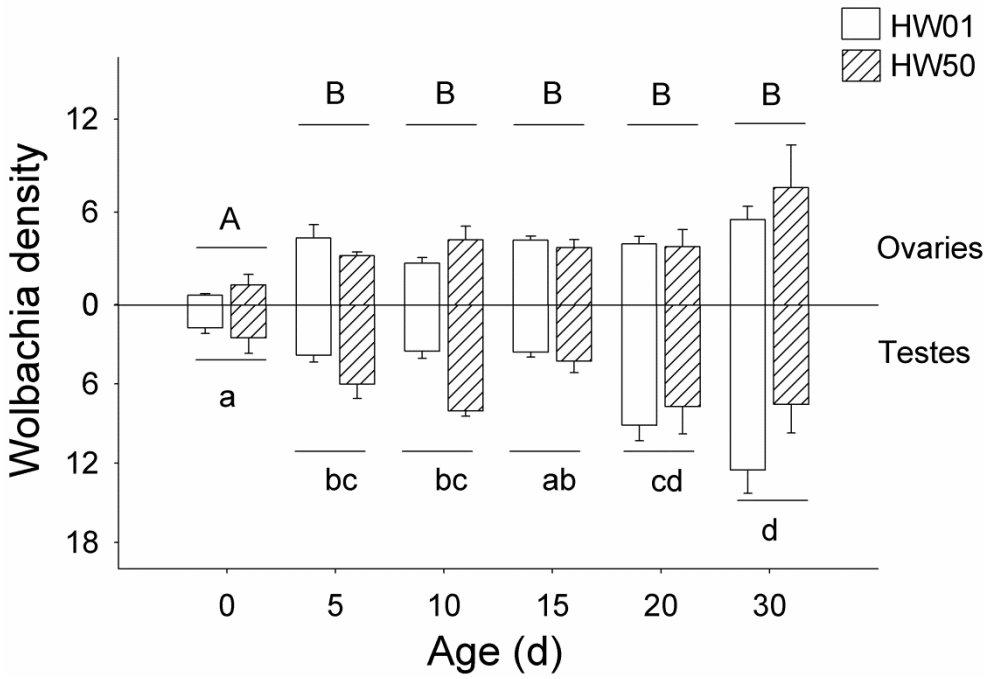

Fig. 2.3 Wolbachia density in fly gonads increase with age. Wolbachia densities in testes increased as the fly ages. Ovaries show an increase in density within the first 5 days of life after which it remains constant. Bars represent mean normalised Wolbachia density per treatment ± SEM. Bars not connected by the same letter vary significantly according to Tukey's HSD test with $Q_{females} = 2.94$, $Q_{males} = 2.94$ and $\alpha = 0.05$.

2.3.4.2 Immune challenge

In flies, the immune response to infection has been shown to be energetically costly, and a trade-off exists between immune ability and other components of fitness (McKean and Nunney, 2001). In this study, immune challenge with *E. coli* showed no effects in Wolbachia density in ovaries, but a line specific effect in testes. In ovaries, ANOVA showed no significant effect of immune challenge ($F_{2,33} = 0.91$, $p = 0.41$), line ($F_{1,33} = 1.18$, $p = 0.26$) or on immune challenge by line interaction ($F_{2,33} = 0.05$, $p = 0.41$). In testes, there was a significant increase in Wolbachia density in gonadal tissue of *E. coli* pricked males of line HW01 only (Fig. 2.4). ANOVA showed a significant interaction between immune challenge and line ($F_{2,34} = 7.34$, $p < 0.01$). There was no overall treatment effect ($F_{2,34} = 3.67$, $p = 0.04$), nor an effect of line ($F_{1,34} = 3.35$, $p = 0.08$).

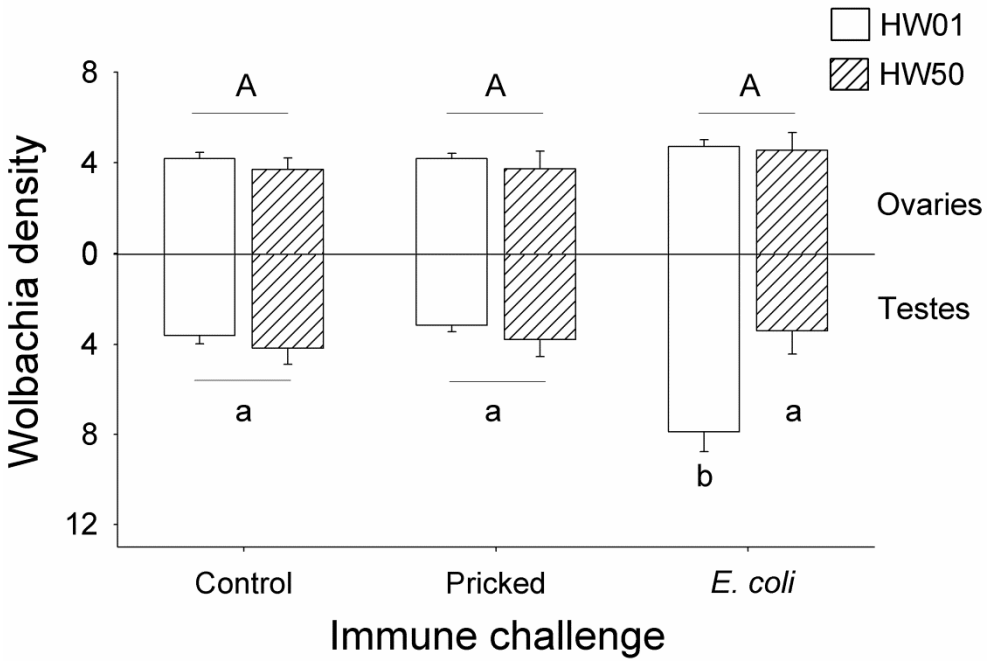

Fig. 2.4 Effect of immune challenge on Wolbachia density is sex and line specific. Wolbachia densities in testes of line HW01 is significantly higher when flies are pricked with *E.coli*. No other effect of treatment was observed in males of the second line or in females. Bars represent mean normalised Wolbachia density per treatment ± SEM. Bars not connected by the same letter vary significantly according to Tukey's HSD test with $Q_{females} = 3.05$, $Q_{males} = 3.04$ and $\alpha = 0.05$.

2.3.4.3 Temperature

The effects of temperature on Wolbachia density have been previously reported for different hosts and Wolbachia strains; however, such studies focused on the effect of developmental temperature on Wolbachia infection (Clancy and Hoffmann, 1998; Mouton et al., 2006; Wiwatanaratanabutr and Kittayapong, 2009). The temperature at which adult females were maintained did not influence Wolbachia densities in ovaries ($F_{2,33} = 2.54$, $p = 0.38$), nor did the different lines ($F_{2,33} = 1.92$, $p = 0.17$) or any interaction effect between line and temperature ($F_{1,33} = 0.81$, $p = 0.10$). In testes, line HW50 maintained at 17 °C showed low Wolbachia density when compared to the other treatments (Fig. 2.5). ANOVA on testes showed a significant effect of temperature ($F_{2,30} = 5.14$, $p < 0.01$), line ($F_{1,30} = 8.78$, $p < 0.01$) and a temperature by line interaction ($F_{2,33} = 5.66$, $p < 0.03$; Fig. 2.5) on Wolbachia densities.

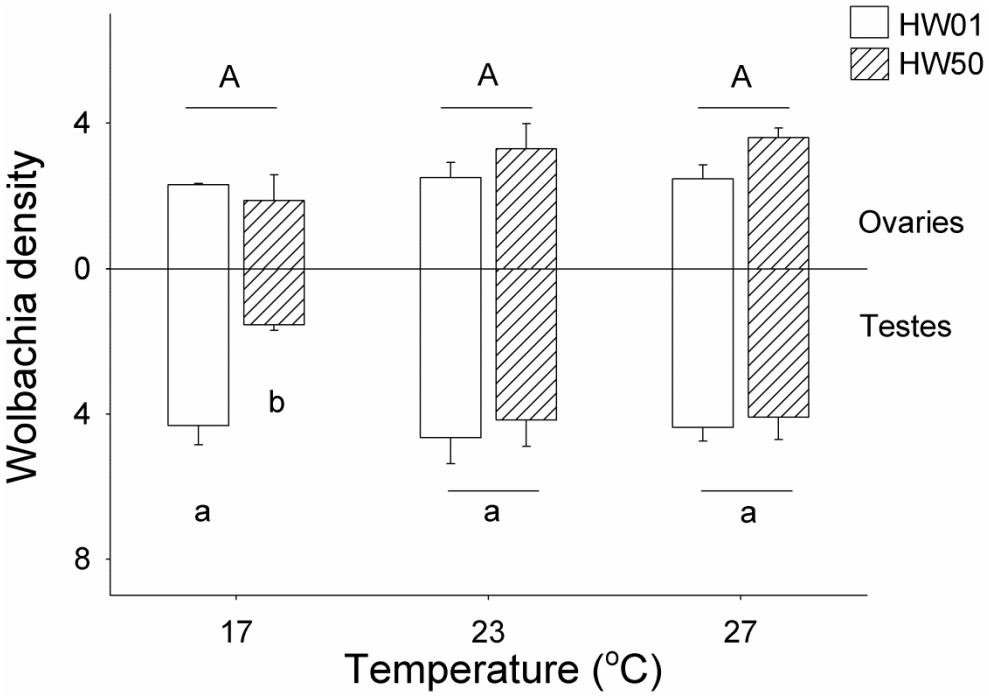

Fig. 2.5 Effect of temperature on Wolbachia density is sex and line specific. Wolbachia densities in testes of line HW50 is significantly lower when adult flies are maintained at 17 °C. No other effect of treatment was observed in males of the other line or in females. Bars represent mean normalised Wolbachia density per treatment ± SEM. Bars not connected by the same letter vary significantly according to Tukey's HSD test with $Q_{females} = 3.05$, $Q_{males} = 2.05$ and $\alpha = 0.05$.

2.3.4.4 Diet

In Drosophila, variation of the protein and carbohydrate dietary content has a significant effect on the lifespan and fecundity of Drosophila (Lee et al., 2008), and it has been suggested to affect the strength of Wolbachia associated phenotypes such as CI (Clancy and Hoffmann, 1998). We observed that adult Drosophila fed diets containing 0.1, 0.5, 1 and 2 times standard amounts of yeast and sugar did not show differences in either ovarian or testicular Wolbachia densities. No differences in Wolbachia density could be attributed to diet (ovaries: $F_{3,45} = 0.16$, $p = 0.69$; testes: $F_{3,45} = 1.92$, $p = 0.14$), the lines (ovaries $F_{1,45} = 2.46$, $p = 0.08$; testes: $F_{1,45} = 1.99$, $p = 0.17$) or to any line-specific effect of diet (ovaries: $F_{3,45} = 0.67$, $p = 0.58$; testes: $F_{3,45} = 0.04$, $p = 0.99$; Appendix 2.3).

2.4 Discussion

The within-host symbiont density is a key factor influencing the prevalence of Wolbachia in insect populations, as it affects transmission fidelity, pathogenicity and severity of reproductive manipulations (Jaenike, 2009). A number of studies have reported strikingly high variation in Wolbachia densities among individuals from natural populations (Ahantarig et al., 2008; Unckless et al., 2009; Tortosa et al., 2010). Such variation is likely to be caused by complex interactions involving the symbiont genotype, the host genotype, and the environment (Mouton et al., 2006, 2007).

We found a 24-fold difference in ovarian Wolbachia titres among individuals of recently collected isofemale fly lines, even after being raised in a controlled environment for at least two generations. Assuming the population we tested was solely

infected by the *w*Ha strain of Wolbachia (as suggested by the sequencing of the *wsp* and *ftsZ* genes and lack of mtDNA variation) our observations are plausibly the result of differences in Wolbachia density regulation intrinsic to the specific fly lines, environmental effects, or a combination of the two. The effect of host heterogeneity is supported by the fact that the population sampled here has a relatively high level of genetic diversity (Ballard, 2000b), and this may facilitate the individuals to respond differently to physiological or environmental stressors (Booy et al., 2000), in this case, Wolbachia infection. Alternatively, it could be argued that the effects seen in F_4 flies are a reflection of larger differences in the wild caught mothers caused by the field environment and carried over for at least four generations.

To explore the genetic and environmental scenarios, we chose the lines whose females displayed the lowest (HW01-HW05) and highest (HW46-HW50) bacterial loads in the ovaries at F_4, to be re-assayed 15 generations later. Our results showed that Wolbachia titres of the 'low' and 'high' ovarian density lines were not maintained; specifically, the densities of the 'high' lines decreased and while those of the 'low' lines increased, stabilising to the same level in F_{19}. These observations support the hypothesis that titre variation among F_4 flies is a reflection of larger differences in nature that stabilised after several generations in a constant environment. Several lines of evidence support this view. First, 20 000-fold ovarian density variation was found among individuals of wild collected *D. inubila* (Unckless et al, 2009), which suggests that the variation of this trait in the wild could be higher than that observed here. Second, experiments on *Ae. albopictus* have shown that the F_1 of wild collected animals showed nearly 100 000-fold variation among individuals even after being reared under controlled conditions

(Ahantarig et al., 2008), while established laboratory lines displayed much reduced variation in comparison (Dutton and Sinkins, 2004). Third, Dutton and Sinkins (2004) demonstrated that rearing of *Ae. albopictus* females for six generations in high crowding and high nutrient conditions significantly increased the Wolbachia density in females compared to controls, suggesting intergenerational cumulative effects. An alternate hypothesis is that laboratory adaptation caused the changes we observed. Adaptation to captivity and laboratory conditions involves an increase of mean fitness to the new environment through genetic change (Frankham and Loebel, 1992). Such genetic change can be very substantial, and research suggests that captive populations may undergo major genome-wide selective sweeps in less than 50 generations (Montgomery et al., 2010). We suggest this is unlikely because genetic drift might (by chance) cause some lines to become less fit. Further, it has been shown that additive genetic variance governing fitness traits do not change substantially in Drosophila after 29 generations of laboratory rearing (Matos et al., 2000).

The environmental and physiological treatments explored in this study showed no effect on gonadal Wolbachia density in females. Therefore, the causative factors for density variation in F_4 ovaries remain unclear. Given that Wolbachia titres in ovaries seem to be determined during the larval stages (Bandi et al., 1999), it is possible that the pre-adult environment, rather than the one of the adult, is more relevant in determining Wolbachia loads in female gonads. Indeed, the effects of larval crowding and larval temperature have been reported to affect adult Wolbachia density (Dutton and Sinkins, 2004; Wiwatanaratanabutr and Kittayapong, 2009). In addition, high levels of Wolbachia infection may be caused by cumulative effects of numerous multiplicative

acting factors (Unckless et al., 2009) and therefore, the variation of a single environmental variable at a time on a single generation might not be enough to recreate the differences observed at F_4. It is also plausible that other aspects of the fly ecology not explored here, such as the likely change of the fly's digestive tract microbiota due to the laboratory diet (Sharon et al., 2010) may have influenced the change of Wolbachia titres between F_4 and F_{19} flies. Further studies exploring the causes of variation among female flies would be necessary to elucidate these possibilities.

In combination, our results support the view of sex-specific Wolbachia evolutionary interactions for males and females, which has been predicted by theory (Koehncke et al., 2009) and observed experimentally (Tortosa et al., 2010). The over-replication of Wolbachia in host tissues could be interpreted as a representation of bacterial pathogenicity (Berticat et al., 2002; McGraw et al., 2002). Environmental or physiological factors that negatively affect the organismal fitness could compromise their ability to regulate bacterial replication, therefore resulting in an increase in Wolbachia abundance (McGraw et al., 2002). If Wolbachia were better adapted to females than to males, it could be predicted that titres in sexually dimorphic tissues (such as gonads) would be influenced to a greater extent by environmental stressors or sub-optimal conditions in males. We consistently observed that titres in testes were more strongly affected than in ovaries by the laboratory treatments. In particular, the effects age and immune challenge on testicular Wolbachia titres support the above prediction. The results also suggest that the testicular susceptibility to Wolbachia over-replication vary between lines, and may therefore imply the presence of genetic variants within this population with different susceptibility to Wolbachia proliferation.

These results support a view in which harmful, male-specific Wolbachia traits may occur in addition to and independently from CI, in a way comparable to the mother's curse hypothesis of mitochondrial-induced, male-specific effects (Gemmell et al., 2004). Indeed, it has been shown that Wolbachia in testes can negatively affect male fertility and sperm counts in Drosophila (Snook et al., 2000). Such effects may further reduce the chances of infected males to pass their genes onto the next generation, and therefore, the exclusion of Wolbachia from testes may be favoured as males adapt to infection. This may have important consequences for the evolution of CI-Wolbachia symbioses. It has been hypothesised that resistance to CI would not occur if all individuals in a population are infected (Turelli, 1994), therefore allowing for an stable maintainance of infection at fixation. Exclusion of Wolbachia from testes as compensation for harmful fertility effects could result in lowered CI strength even when CI no longer poses fitness cost to the male (at fixation). Koehncke and colleagues (2009) propose that the rise of alleles that slightly attenuate CI (for example, the exclusion of Wolbachia from testes), favour the subsequent invasion of other CI repressors, therefore predicting that host adaptation to Wolbachia infection would generally result in the loss of CI.

We have identified at least three limitations in this study. Firstly, we only measured Wolbachia densities in adult gonads. Although this methodology allowed us to find significant differences among treatments and sexes, the inclusion of other tissues and life stages could test the hypotheses proposed in this work. Secondly, we chose to limit our environmental tests to adult flies, but we acknowledge that the larval environment could have an important effect on Wolbachia density. Finally, our observations are

based solely on a strong CI inducing Wolbachia strain that appears to be nearly fixed in natural populations of *D. simulans*. Understanding the evolutionary dynamics of infection in natural populations would be improved by the inclusion of another Wolbachia strain that differs in its reproductive manipulations and infection frequency.

Here, we provide evidence of the loss of variation of Wolbachia titres during the process of laboratory adaptation in *D. simulans* and proposed that natural Wolbachia density can be maintained to an extent for at least four generations. We also provide evidence of sex-specific dynamics of Wolbachia in female and male gonads under different conditions, which supports the view of Wolbachia evolving antagonistically for females and males. Measuring the within-host Wolbachia density is perhaps the most direct way to assess the symbiont-host evolutionary dynamics, as the level of infection can shed light on the pathogenicity and heritability of the infection. Discovering the main factors governing the variation of symbiont titres in natural populations would therefore contribute to our understanding the evolution of reproductive parasites and their persistence in host populations.

Chapter 3

What can symbiont titres tell us about coevolution of Wolbachia and their host?

Abstract

There is a long-standing prediction that associations with vertically transmitted symbionts evolve towards maximisation of host reproductive success, eventually leading to mutualist symbiosis and coadaptation. Under this scenario, the regulation of symbiont titres in host tissues would be expected to be greater when partners have coevolved for a long time than when they have recently met. *Wolbachia pipientis*, a common vertically transmitted symbiont of invertebrates, often has the capacity to spread through the host population without being beneficial to the hosts, by means of reducing the hatch rate in crosses between uninfected females and infected males. This manipulation, namely cytoplasmic incompatibility (CI), may exert strong selection on the accuracy of infection transmission from mother to offspring, and therefore, on regulation of symbiont titres in the ova. Here, we examined the symbiont density dynamics in gonads of *Drosophila simulans* infected with the *w*Ma strain of Wolbachia, known to cause mild CI and likely to be the oldest Wolbachia infection known to this fly species. Further, we compared these results with those obtained for the more recent association between *D. simulans* and the potent CI-inducer *w*Ha. We aimed to determine if the regulation of Wolbachia density in fly gonads is greater in the older association, as would be predicted solely by gradual coadaptation, or if the selection exerted by CI on reproductive fitness could also play a role, therefore showing tighter regulation on flies with the stronger CI-inducing strain. We observed that Wolbachia density in gonads of *w*Ma infected flies changed with laboratory adaptation and were disturbed by environmental challenges, which contrasted with the stability of ovarian *w*Ha density to the same treatments. Our observations are in line with the prediction that

selection on reproductive fitness influences the evolution symbiont density regulation in Drosophila, and may provide insights into the evolutionary processes involved in the maintenance or loss of Wolbachia.

3.1 Introduction

Symbiotic associations between bacterial microbes and animals occur in every ecological niche. Theoretically, associations with vertically transmitted symbionts are thought to coadapt towards obligate symbiosis (Ewald, 1987; Yamamura, 1993). This concept is supported by the observation that many heritable endobacteria are capable of satisfying the metabolic needs of the host they inhabit (Sachs et al., 2011). If true, it may be hypothesised that associations established for longer periods of time would be more stable than recent associations. Interestingly, *Wolbachia pipientis*, the most common endosymbiont of arthropods, often appears to be maintained in populations without conferring any apparent physiological benefit to the bearer. Furthermore, Wolbachia and their arthropod hosts very rarely display phylogenetic concordance (Werren et al., 1995; Baldo et al., 2006), indicating that infections are often extinct from the host population before co-speciation can occur. Together, these observations suggest alternative outcomes to the evolution of Wolbachia-host associations besides coadaptation towards mutual dependence. Furthermore, the stability of some Wolbachia associations may not necessarily correspond to the length of the symbiosis.

It has been hypothesised that the coevolution of Wolbachia and their arthropod host may be driven by conflicts of interests, switching from mutually beneficial to exploitative depending on the environment (Herre et al., 1999; Wernegreen, 2004). A common approach to study the evolutionary mechanisms that underpin such relationships of cooperation or conflict is to quantify the relative density of endosymbionts in host tissues (Dale and Moran, 2006). This approach is relevant as the

abundance of endosymbionts in host tissues represents a trade-off: high bacterial density may improve heritability of the symbiont, but excessive replication could represent a fitness cost to the host (McGraw et al., 2002; Mouton et al., 2004; Dale and Moran, 2006). Generally, obligate symbioses display fine-tuned control over symbiont titres, implying cooperative mechanisms of host growth and symbiont replication. However, associations where symbionts are non-essential often display symbiont titres that are variable (Dale and Moran, 2006) and prone to be disturbed by changes in the physiological state of the host (Rio et al., 2006). If the evolution of symbiont density regulation was driven solely by gradual coadaptive processes, old associations would be predicted to display tight control over symbiont replication, while less rigid control would be expected in more recent associations.

A notable characteristic of Wolbachia that may influence the way it adapts is its capacity to manipulate the host's reproduction to increase the number of infected females, allowing the infection to spread throughout the host population. Perhaps the best studied of such modifications is cytoplasmic incompatibility (CI), in which crosses between infected males and uninfected females render no or few offspring. Because of the sexual bias of this incompatibility, selection on infection maintenance differs between females and males. In females, CI-inducing Wolbachia allows the production of viable offspring with any male of the population and, therefore, resistance to infection would be selected against. Males are an evolutionary dead end to the symbiont and experience a fitness reduction when infected. Uninfected males or those whose CI-inducing phenotype is attenuated would have an advantage (Turelli, 1994). Importantly, it has been postulated that this sexual antagonism of Wolbachia adaptation could be the

cause of symbiont extinction, mediated by increased male resistance to CI (Koehncke et al., 2009).

In Correa and Ballard (2012) we studied the dynamics of gonadal Wolbachia titres of female and male *Drosophila simulans* with laboratory adaptation, at different fly ages and after environmental perturbations. In that study, we used a Hawaiian population infected with the Wolbachia strain *w*Ha. This strain displays transmission fidelity and infection frequency near 100%, is a very strong CI inducer (Rousset et al., 1992a; Turelli and Hoffmann, 1995; James and Ballard, 2000; Ballard, 2004) and poses no observable fitness effects on the female host (Poinsot and Merçot, 1997). We observed that gonadal titre variation among individuals decreased with laboratory adaptation and that titres in testes were more prone to be altered by age and environmental disturbances than in ovaries. Here, we use the same methodology to explore the density dynamics of a weak CI-inducer present in a population of *D. simulans* from east Africa. This Wolbachia strain, *w*Ma, has been identified in flies from Madagascar, La Réunion Island and Kenya. There are at least four reported differences between the *w*Ma and *w*Ha infected fly populations. First, *w*Ma infected *D. simulans* are polymorphic in the level of infection (usually less than 50%) and the strength of CI, ranging from 0 to 50% unhatched eggs compared to compatible crosses (James and Ballard, 2000; Merçot and Charlat, 2004). Second, the *w*Ma strain is known to be stochastically lost from populations (Dean, 2006) while *w*Ha loss occurs rarely (Poinsot et al., 2000). Third, evidence from mitochondrial DNA phylogeography and CI studies indicate that *w*Ma infection is older in this fly species than is *w*Ha (Rousset and Solignac, 1995; Ballard, 2004). Finally, because *D. simulans* is thought to be endemic to east Africa (Lachaise et

al., 1988), flies harbouring the *w*Ma infection in Kenya are expected to be genetically more variable than those with the *w*Ha infection in Hawaii.

In this study we examined the symbiont density dynamics of the *w*Ma strain in recently collected and laboratory-adapted fly lines, and obtained symbiont density data in ageing and environmentally challenged flies. We qualitatively compared these results with those reported in Correa and Ballard (2012) for the *w*Ha infected population. Our goals were to determine whether: 1) laboratory adaptation and environmental perturbations affect the patterns of symbiont density in the two sexes equally, and 2) Wolbachia density dynamics reflect patterns of symbiont-host adaptation related to CI strength and age of the association. We discuss our results contemplating the possible roles that CI, age of the association, sexual antagonism and symbiont effects on host fitness play in the host-symbiont coevolution and the possible evolutionary outcomes of these associations.

3.2 Materials and methods

3.2.1 Drosophila collection and rearing

A total of 153 females were collected from Nairobi, Kenya in March, 2011. Females morphologically identified in the field as *D. simulans* were allowed to lay eggs individually in vials. Isofemale lines were transferred through quarantine in Melbourne, Australia and then to the laboratory in Sydney. In the laboratory, the observation of male genitalia of F_2 flies corroborated the species as *D. simulans* in each line. Lines

were maintained in discrete generations at low densities on standard *treacle-semolina-yeast-agar media* at 23 °C, 50% humidity and under a 12 h light-dark cycle.

3.2.2 Wolbachia infection frequency and genetic variation

Wolbachia infection was assessed by PCR for each isofemale line. Briefly, two samples of three to four F_2 individuals were collected and their genomic DNA (gDNA) extracted using the Gentra Puregene® Cell Kit (Gentra Systems Inc., Minneapolis, USA), following the Isolation from Solid Tissue protocol. PCR was performed in duplicate using primers for the *wsp* gene following Zhou et al. (1998). Amplification of the COI barcoding region (Folmer et al., 1994) was performed in parallel as a control of DNA quality. Lines showing positive amplification for both the *wsp* and the COI fragments in both duplicates were considered infected. Conversely, lines were considered uninfected if there was positive amplification of the COI fragment but negative for the *wsp* for both DNA extractions. Populations of *D. simulans* from Kenya have been described to harbour two Wolbachia types, *w*Ma and *w*Ri (Rousset et al., 1992b; Dean et al., 2003). We sequenced the *wsp* fragment and an additional *ftsZ* region to determine the Wolbachia strain infecting each *wsp* positive line and to detect possible polymorphism among the bacterial population.

3.2.3 Wolbachia density determination

Following the procedure of Correa and Ballard (2012), we used quantitative PCR (qPCR) to measure Wolbachia density in gonads of 15 day-old individuals, three

generations after field capture (F_4). For these experiments, flies that harboured the *w*Ma infection were selected. Briefly, four to six males and females were sampled from all infected lines and their gonads dissected in Drosophila ringer solution. DNA was extracted from individual dissections and qPCR was used to obtain relative ratios of Wolbachia genomes compared with a Drosophila single copy nuclear gene. For Wolbachia, primers were designed to amplify a 105 bp fragment of the *gatB* gene specific to the strain infecting this fly population (qgatB2F: 5'-GCG TTA CAT CGG TTC ATG TGA TGG and qgatB2R: 5'-CAC AAC GAG TGC CAA ATG TGC T). The primer pair qrpII215F and qrpII215R (Correa et al., 2012) was used to quantify the host (*D. simulans*) single copy large subunit of the RNA polymerase II (*rpII215*). Reaction conditions for both primer pairs followed Correa and Ballard (2012). For statistical analyses, individual normalised Wolbachia counts were log transformed to fit a normal distribution (Sokal and Rohlf, 1995).

Isofemale lines were ranked and named according to increasing ovarian Wolbachia density after the F_4 screening, from the lowest (KY01) to the highest (KY35) density line. To test if the symbiont density measurements from ovaries and testes differed, a nested ANOVA using line nested within sex was performed using JMP© v5 (SAS Institute, Cary, USA). Spearman correlation analysis using Microsoft Excel was employed to test the correlation between ovarian and testicular densities of flies from the same isofemale line.

To investigate if line specific differences in ovarian symbiont titres were maintained in laboratory conditions, gonadal densities of Wolbachia were measured at generation F_{19}

for 10 lines that showed lowest and highest densities at F_4. Throughout the study every effort was made to ensure flies were raised under identical conditions. To account for the spontaneous Wolbachia loss of this particular fly population under laboratory conditions, the selection of lines to be tested at F_{19} followed a two-step process. First, we preselected the 10 lines with lowest (KY01-KY10) and the 10 lines with highest (KY26-35) Wolbachia infection at F_4. Second, these preselected lines were monitored each generation and those that lost the infection or were partially infected at F_{18} were excluded. For both high and low density groups, five of the 10 preselected retained 100% levels of infection at F_{18}. Wolbachia/host genome ratios were then obtained for each of the infected lines at F_{19}. A two-way ANOVA using JMP© v5 was used to compare generations (F_4 and F_{19}), low and high density groups and their interaction. The factor 'line' was included as a nested effect within group. To follow-up the trends observed between F_4 and F_{19}, additional data for a subset of low and high density lines at F_{22} was included for both *w*Ma (KY01, KY09, KY29 and KY35) and *w*Ha (HW01, HW02, HW49 and HW50) infected flies from Correa and Ballard (2012).

Additional *ad hoc t*-tests were conducted to determine if the symbiont density average significantly differs between F_4 and F_{19} flies. Alpha error level was set at $p < 0.001$ as correction for multiple comparisons. Additionally, O'Brien tests (O'Brien, 1981) were performed to compare variances. For comparative purposes, these analyses were also performed on the datasets of the *w*Ha-infected flies from Correa and Ballard (2012). Due to the considerable time gap between the measurements performed on Hawaiian and Kenyan flies, direct statistical comparisons between these datasets were not performed.

3.2.4 Genetic variation at host mitochondrial and nuclear loci

We assayed the host genetic variation to test whether there was any evidence of population genetic subdivision in this African population of *D. simulans,* associated with the observed differences in Wolbachia density at F_4 or Wolbachia loss at F_{18}. Following Correa and Ballard (2012) two regions of the mitochondrial genome (578 bp fragment of the COI barcoding region and a 509 bp fragment spanning from $tRNA_{Lys}$ to the beginning of the *ATP8*) and two regions of the nuclear genome (a 483 bp fragment from the intron 1 of the alcohol dehydrogenase-related gene (*Adhr*), and a 469 bp fragment from intron 2 of the glycerol 3-phosphate dehydrogenase (*Gdph*)) were sequenced.

Nucleotide diversity (π) and the neutral parameter (θ) on the total number of polymorphisms and segregating sites were calculated for the five lines with the lowest (KY01-KY05) and the highest (KY26-KY35) densities at F_4. Both π and θ were calculated based on the total number of mutations, excluding sites with gaps and/or missing data. Tajima's *D* was calculated to test whether this population violated a strictly neutral equilibrium model of mutation (Tajima, 1989). Within the low and high density Wolbachia groups there were no observable differences in the genetic variation in the flies that lost infection from those in which it was maintained.

3.2.5 Physiological and environmental effects on Wolbachia density

Following Correa and Ballard (2012) we examined whether Wolbachia densities in gonads of adult *D. simulans* from Kenya were influenced by adult age, diet, temperature and immune challenge. These assays were selected because as they represent ecologically relevant variables shown to affect Wolbachia density in the laboratory (Clancy and Hoffmann, 1998; Mouton et al., 2006; Rio et al., 2006; Tortosa et al., 2010; Correa and Ballard, 2012). The specific lines tested were KY01 and KY35 because they maintained Wolbachia infection and they represent the span of symbiont density at F_4. Experiments were performed in F_{21} individuals.

Briefly, for testing the effects of adult age, 0 day-old flies were collected within 12 h of eclosion. For the remaining age treatments, flies were collected from population cages and frozen at 5, 10, 15, 20 and 30 days post-eclosion.

Diet effects were measured by providing adult flies with variations of the standard *treacle-semolina-yeast-agar* diet. Food fed to adult flies contained 0.1, 0.5, 1 (control) and 2 times the amount of fresh yeast and treacle. Adult flies were fed on these diets until they reach 15 days of age, when they were frozen for later testing.

Temperature effects were measured in flies maintained at 17, 23 and 27 °C throughout their adult life. In an effort to measure the effect of temperature without the bias of differential developmental rates, collections were made at 22, 15 and 11 days for flies maintained at 17, 23 and 27 °C respectively. These times were chosen as they represent

an equivalent physiological age throughout the treatments, as determined by day-degrees (de Jong and van der Have, 2009).

For the immune challenge test, 11 day-old flies were needle-pricked with a concentrated suspension of TOP10 *Escherichia coli*. Controls included sterile needle-pricked and non-pricked flies. Individuals treated thus were then returned to population cages for four days to allow time to mount an immune response (McKean and Nunney, 2001) before being collected at 15 days of age and frozen.

After the above mentioned treatments were conducted, flies dissected and Wolbachia density in gonads was estimated by qPCR. The statistical significance of age and environmental conditions and the possible effects of line were investigated using a two-way ANOVA in JMP© v5 for each assay. Linear regression was performed on continuous factors that showed statistical significance.

3.3 Results

3.3.1 Wolbachia infection frequency and genetic variation

From the total of 153 females collected from Nairobi, 103 *D. simulans* cultures were established. Of these, 41 were infected with the *w*Ma strain and none with the *w*Ri strain of Wolbachia. No lines were polymorphic for Wolbachia infection. Sequence of the *wsp* and *ftsZ* genes were identical to those reported for the *w*Ma strain of Wolbachia in

NCBI (GenBank AF020069.1 and AY509001.1 respectively). No polymorphisms were found at either the *wsp* or the *ftsZ* Wolbachia fragments.

3.3.2 Wolbachia density determination

At generation four, 35 of the initial 41 Wolbachia positive lines remained infected. Quantitative PCR performed on fly gonads from these lines revealed variable Wolbachia density (Appendix 3.1). Relative Wolbachia titres varied by 11.42-fold in ovaries and by 118.33-fold in testes. The data displayed a log-normal distribution, and was log-transformed for inclusion in ANOVA models. Comparing Wolbachia titres among gonadal tissues with line as nested factor revealed a significant difference between ovaries and testes ($F_{1,69} = 53.04$, $p < 0.001$) and among lines ($F_{1,278} = 2.87$, $p < 0.001$). There was no significant correlation between ovarian and testicular density among isofemale lines (testes density = 1.68 ovary density + 2.9; $R^2 = 0.02$, $p = 0.38$). From the 35 lines determined to be infected at F_4, 10 lines that harboured low (KY01, 02, 03, 06 and 09) and high (KY26, 28, 29, 31 and 35) ovarian density were selected for quantification at F_{19}. This sampling strategy was used to investigate if the process of laboratory adaptation resulted in decreased variability in symbiont titres among isofemale lines. In addition, we investigated if the average gonadal density differed between F_4 to F_{19} flies. Results for density in ovaries are presented first, followed by those for testes.

At F_4, Wolbachia density in ovaries from the low and the high-density groups differed. The F_4 low-density group had an average of 2.64 ± 1.16 Wolbachia genomes per fly

genome while the high density group had 5.32 ± 0.32 (Fig. 3.1). In F_{19} flies, the low density group showed a mean Wolbachia density of 5.58 ± 0.33 and the high density group a density of to 5.35 ± 0.36 (Fig. 3.1), indicating that by F_{19} there was an overall increase of Wolbachia density of the population, bringing it to the level of the high density group at F_4. Two-way ANOVA showed a significant influence of generation ($F_{1,112} = 16.12$, $p < 0.001$), density group ($F_{1,101} = 32.52$, $p < 0.001$) and their interaction ($F_{1,112} = 34.01$, $p < 0.001$). The significant effect of line within density group ($F_{8,112} = 0.87$, $p < 0.001$) was lost when KY35 was excluded ($F_{8,77} = 1.58$, $p = 0.15$), but the significant effects of other factors remained (generation: $F_{1,97} = 24.58$, $p < 0.001$; group: $F_{1,87} = 20.43$, $p < 0.001$; generation by group: $F_{1,97} = 35.10$, $p < 0.001$). Further comparisons between F_4, F_{19} and an additional F_{22} dataset obtained under similar conditions for a subset of Kenyan and Hawaiian flies showed that the trend of density increase continued beyond F_{19} for *w*Ma infected flies while in *w*Ha infected flies Wolbachia density was maintained to similar levels (Appendix 3.2).

For testes, the general trend was for Wolbachia density to increase over time in the laboratory. At F_4, mean testicular Wolbachia density was 2.12 ± 0.38 for the low-density group and 4.32 ± 0.75 for the high density group (Fig. 3.1). At F_{19}, mean testicular Wolbachia density was 13.52 ± 1.59 for the low-density group and 15.00 ± 2.24 for the high density group (Fig. 3.1). Two-way ANOVA showed that densities in testes were strongly influenced by generation ($F_{1,98} = 60.82$, $p < 0.001$), but not by density group ($F_{1,86} = 2.87$, $p = 0.09$), nor their interaction ($F_{1,86} = 2.97$, $p = 0.09$). Lines within groups did not differ from each other ($F_{9,98} = 0.48$, $p = 0.96$). As in females, the comparison of F_4, F_{19} and F_{22} density in testes of *w*Ma infected flies showed further

increase of symbiont density later in the process of laboratory adaptation while the density of F_{22} *w*Ha infected males was similar to that of F_{19} (Appendix 3.2).

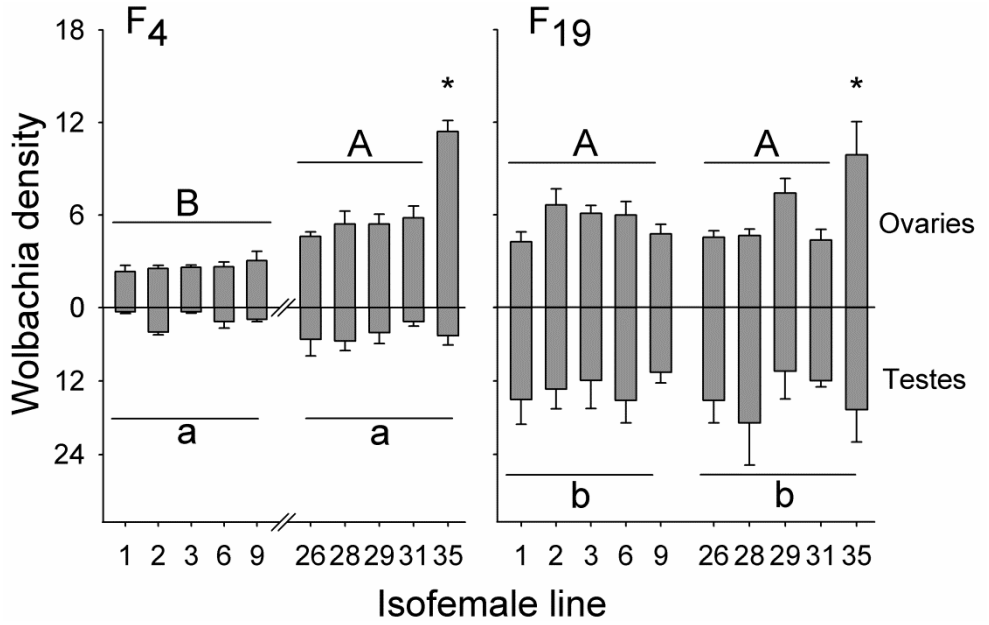

Fig. 3.1 Differences in gonadal Wolbachia density of F_4 fly lines are not maintained after 15 generations of rearing in the laboratory. Bars represent mean relative Wolbachia density per treatment ± SEM. Bars not connected by the same letter vary significantly according to Tukey's HSD test with $Q_{females} = 2.62$, $Q_{males} = 2.63$ and $\alpha = 0.05$. Ovarian density of Line KY35 differs significantly from the others and was excluded from the Tukey's test.

3.3.2.1 Wolbachia density comparison between strains

We hypothesised that, if Wolbachia and host evolve to become obligate mutualists, the older *w*Ma-Drosophila association should reflect more stable symbiont titres than the more recent *w*Ha/Drosophila association. Here we qualitatively compare the results collected in this study with the data presented by Correa and Ballard (2012).

Ad-hoc t-tests comparing Wolbachia density in the ovaries of F_4 and F_{19} *w*Ma infected flies showed that mean density significantly differed between generations (F_4 = 3.98 ± 1.09, F_{19} = 5.56 ± 1.05; $t_{1, 112}$ = 3.47, $p < 0.001$). In contrast, *w*Ha-infection across generations was more stable (F_4 = 5.80 ± 1.11, F_{19} = 5.19 ± 1.03, $t_{1, 58}$ = 0.98, p = 0.33, Fig. 3.2). Variability, on the other hand, was the same between *w*Ma generations ($F_{1, 112}$ = 1.39, p = 0.24) but significantly decreased in *w*Ha-infected flies with laboratory rearing ($F_{1, 100}$ = 33.39, $p < 0.001$).

As expected, *t*-tests comparing the Wolbachia densities in F_4 and F_{19} infected testes showed significant differences, increasing from F_4 to F_{19}, for both *w*Ma (F_4 = 2.36 ± 2.71, F_{19} = 10.67 ± 2.28; $t_{1, 97}$ = 8.18, $p < 0.0001$) and *w*Ha-infected males (F_4 = 2.30 ± 2.02, F_{19} = 5.20 ± 1.62; $t_{1, 77}$ = 6.75, $p < 0.001$; Fig. 3.2). Variability did not differ between F_4 and F_{19} flies for either *w*Ma ($F_{1, 97}$ = 1.59, p = 0.21) or *w*Ha infected males ($F_{1, 77}$ = 0.04, p = 0.83).

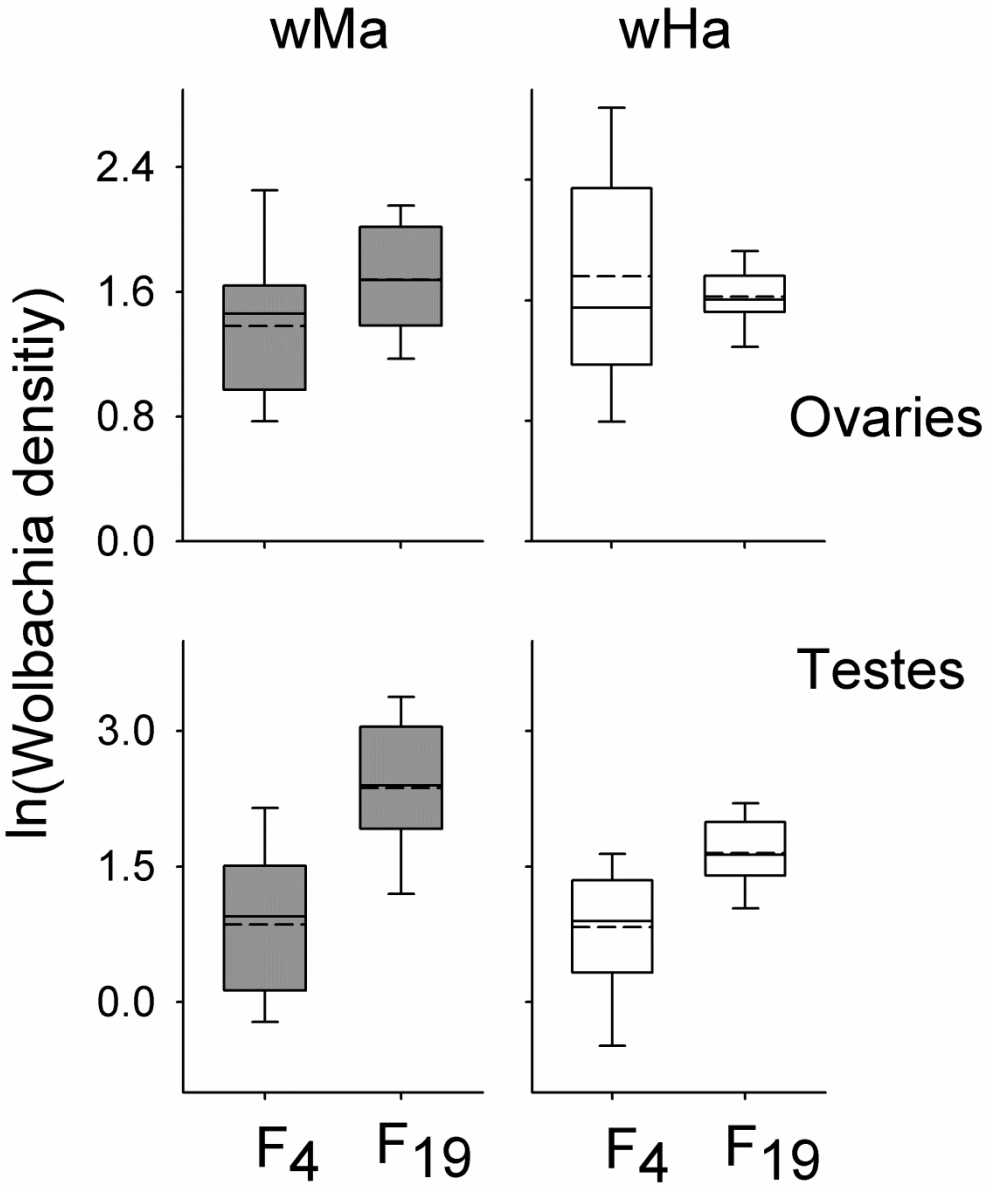

Fig. 3.2 Box plots of mean (dashed lines) and median (solid lines) log-transformed Wolbachia density in gonads of *D. simulans* at F_4 and F_{19}. The 25 (bar below median) and 75 (above median) percentiles are indicated. Grey and white boxes represent *w*Ma and *w*Ha infected flies respectively.

3.3.3 Genetic variation at host mitochondrial and nuclear loci

In the *w*Ma infected flies we explored host genetic variation in two mtDNA regions and at intronic segments of two autosomal loci. No variation was found across the two mitochondrial regions. Sequence was identical to those previously reported for *D. simulans* from La Reunion island harbouring an *si*III mtDNA haplotype (GenBank ID: AF020069.1).

Analysis of the nuclear *Adhr* (KM016467- KM016476) and *Gpdh* (KM016477-KM016477) sequences for this fly population showed high levels of variation at both loci. Such variation is similar for the two Wolbachia density groups considered (Appendix 3.3). Therefore, calculation of genetic diversity indices and the neutral parameter included the whole Kenya dataset for each locus. The *Adhr* locus exhibited a total of 21 polymorphic sites, with nucleotide diversity (π) of 12.75×10^{-3} and neutral parameter (θ) of 9.61×10^{-3}. At the *Gpdh* locus 23 polymorphic sites were detected ($\pi = 15.09 \times 10^{-3}$ and $\theta = 18.11 \times 10^{3}$). Tajima's *D* test supports the hypothesis that these nuclear loci evolve in a manner that does not depart significantly from a strictly neutral model of mutation (*Adhr*: $D = -0.87$; *Gpdh*: $D = -0.87$).

3.3.4 Physiological and environmental effects on Wolbachia density

Gonadal Wolbachia *w*Ma titres in females and males were affected by age and the environmental treatments tested here. In general, Wolbachia titres in testes were

affected to a greater extent than those in ovaries. Moreover, Wolbachia density seemed to correlate positively with age and negatively with food concentration.

3.3.4.1 Age

In ovaries, Wolbachia density increased dramatically between 0 and 5 days of age. This was followed by more modest increases until 30 days of age. Two-way ANOVA with age as a continuous factor, showed a significant age effect on Wolbachia densities ($F_{1,78}$ = 27.28, $p < 0.001$). There was no significant effect of line ($F_{1,63}$ = 1.30, p = 0.30) nor an age by line interaction ($F_{1,63}$ = 0.43, p = 0.52). As both the KY01 and the KY35 lines did not significantly differ from each other, data was collapsed and linear regressions performed using age as the independent variable and Wolbachia density as the dependent one. Significant positive linear correlation was found between Wolbachia density and age ($Density_{Ova}$ = 0.27 (Age) + 4.47; R^2 = 0. 42, $p < 0.001$; Fig. 3.3).

Testes showed a much more dramatic increase in Wolbachia abundance, with density at age 30 almost doubling that of 15 day-old flies (Fig. 3.3). Two-way ANOVA with age as a continuous variable, showed a significant age effect on Wolbachia densities ($F_{1,78}$ = 179.15, $p < 0.001$). There was no significant effect of line, nor an age by line interaction (line: $F_{1,63}$ = 0.20, p = 0.65, age by line: $F_{1,63}$ = 0.06, p = 0.80). Again, both lines tested did not significantly differ from each other and data was collapsed. A linear regression shows a positive linear correlation between Wolbachia density and age ($Density_{Test}$ = 2.99 (Age) - 2.94 +; R^2 = 0.77, $p < 0.001$; Fig. 3.3).

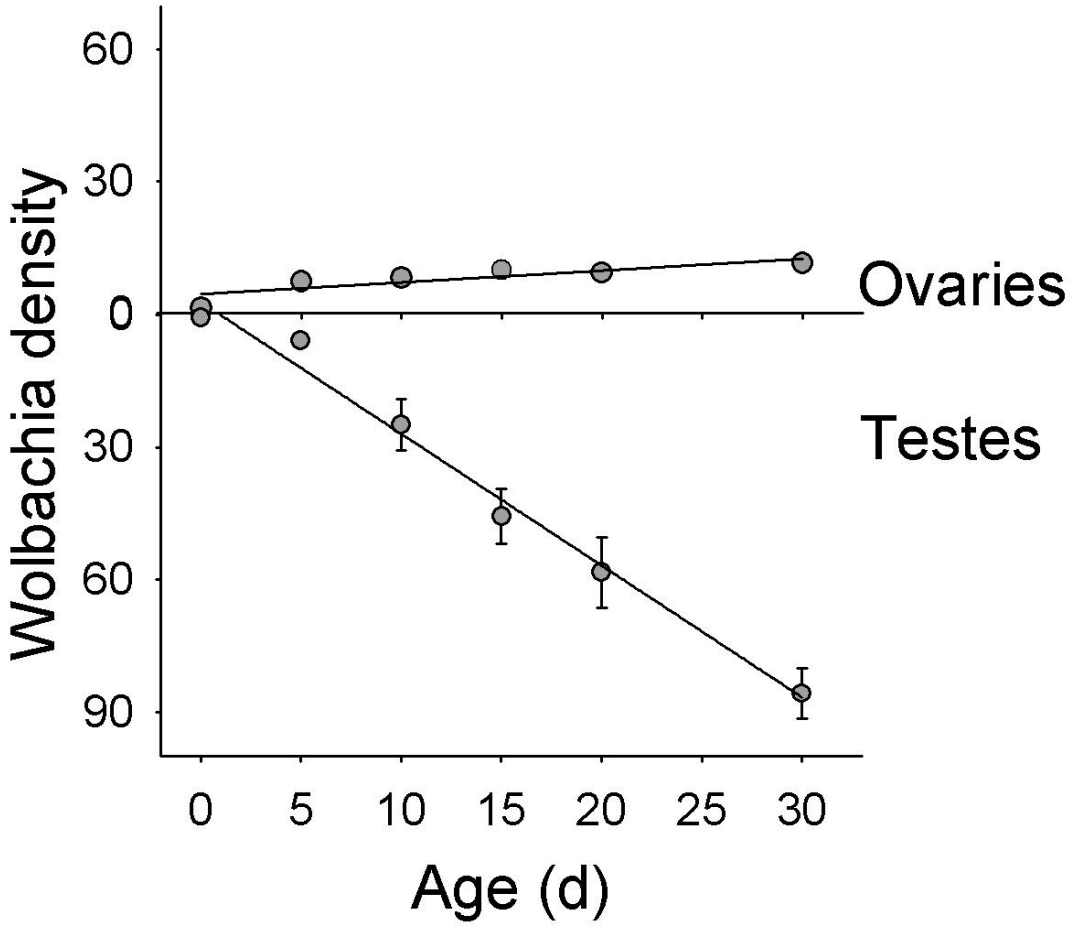

Fig. 3.3 Wolbachia *w*Ma density in gonads of Kenyan flies increase with fly age, such increase being more evident in testes. Circles represent mean normalised Wolbachia density pooled for the KY01 and KY35 ± SEM. Linear regression is significant for both ovaries ($Density_{Ova}$ = 0.27 age + 4.47, +; R^2 = 0. 42, $p < 0.001$) and testes ($Density_{Test}$ = 2.9862541 (Age) - 2.943927 +; R^2 = 0. 77, $p < 0.001$).

3.3.4.2 Diet

Wolbachia density in *w*Ma infected flies decreased with increasing nutrient concentration in the diet in both gonadal tissues (Fig. 3.4). ANOVA using food concentration as continuous variable, showed a significant effect of diet on ovarian titres ($F_{1,52}$ = 12.01, $p < 0.01$) but there were no significant line ($F_{1,52}$ = 0.67, p = 0.42) or line by diet effects ($F_{1,52}$ = 1.12, p = 0.28). Linear regression on ovarian data confirmed a significant negative correlation between food concentration and symbiont abundance (Density = 14.48 - 3.13 (Diet); R^2 = 0. 22, $p < 0.001$; Fig. 3.4).

For testes, ANOVA showed a significant effect of diet ($F_{1,47}$ = 4.50, $p < 0.05$) but symbiont abundance differed among the two lines ($F_{1,37}$ = 6.04, $p < 0.05$). There was no significant line by diet interaction effect ($F_{1,47}$ = 0.05, p = 0.70). Because testicular data differs among lines, regression between food concentration and density was performed independently for KY01 and KY35. For KY01, correlation was significant (Density = 63.94 - 14.46 (Diet); R^2 = 0. 25, $p < 0.05$; Fig. 3.4). For KY35, Wolbachia density tended to decrease with increasing nutrient concentration in food, but the linear regression was not significant (Density = 45.28 - 6.26 (Diet); R^2 = 0.03, p = 0.41). Overall, this suggests that Wolbachia density in KY01 testes was more sensitive to nutrient concentration than that in KY35 testes.

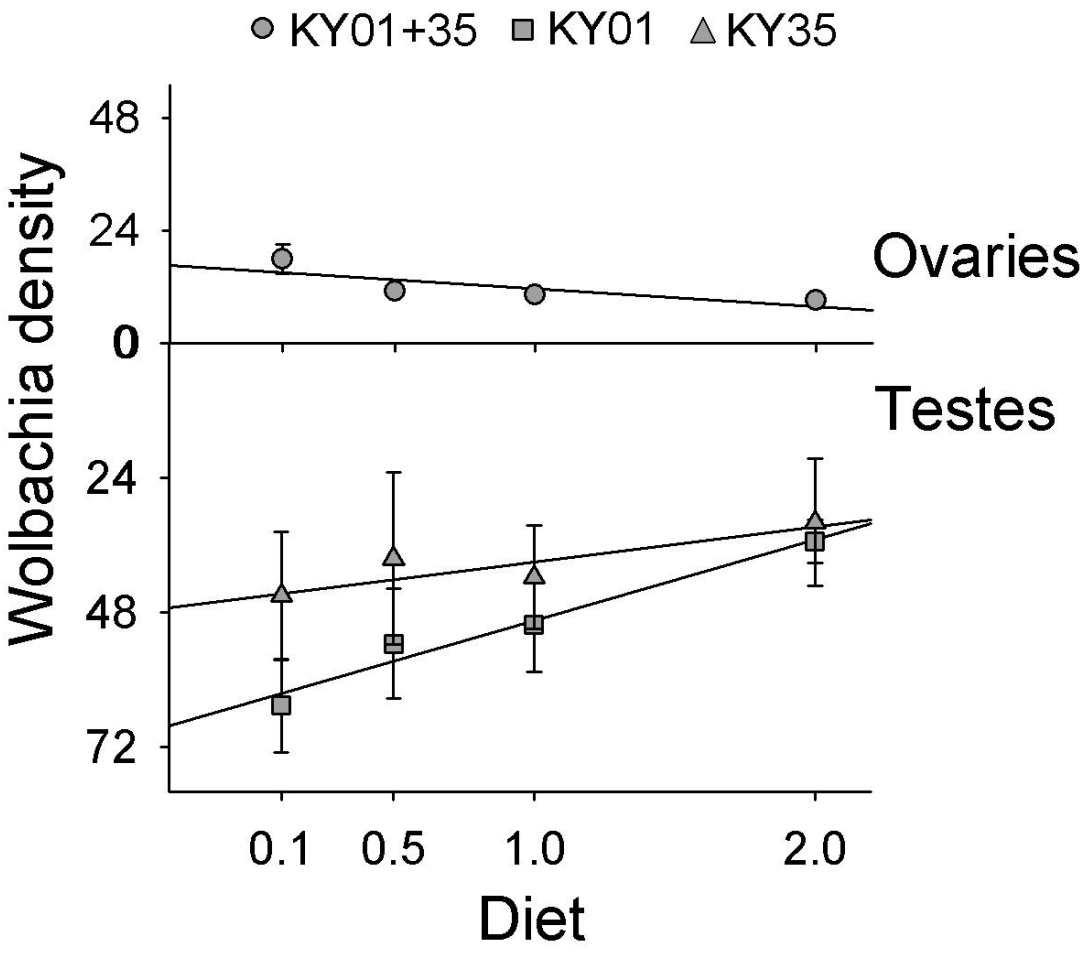

Fig. 3.4 In *w*Ma infected flies from Kenya, Wolbachia gonadal density tends to increase with dilution of food. Wolbachia consistently decreases with food concentration in ovaries, while in testes the trend appears to be line-specific. Linear regression shows significance correlation for ovaries (pooled lines; $Density_{Ova} = 14.48 - 3.13$ (Diet); $R^2 = 0.22$, $p < 0.001$) and for males only in the KY01 line ($Density_{Test} = 63.94 - 14.46$ (Diet); $R^2 = 0.25$, $p = 0.013$).

3.3.4.3 Temperature

In an attempt to compare the effect of temperature in a standardised physiological age, Wolbachia gonadal density of flies maintained at 17, 23 and 27 °C was measured at different chronological ages. Nonetheless, it is difficult to completely rule out the effect of these chronological differences, especially given the strong effect of age observed in Fig. 3.3. Flies raised on these conditions, however, displayed significant differences in Wolbachia gonadal density (Fig. 3.5), with a trend contrasted with that of ageing. In ovaries, density was lower when adult flies were maintained at 27 °C. ANOVA including each treatment as a discrete factor showed a significant effect in ovarian Wolbachia titres ($F_{1,36} = 5.78$, $p < 0.01$). No significant effect of line ($F_{1,36} = 0.05$, $p = 0.84$) or line by temperature interaction (ovaries: $F_{1,36} = 0.06$, $p = 0.94$) was observed.

In testes, Wolbachia density was lower at both 17 and 27 °C treatments compared to the controls at 23 °C (Fig. 3.5). ANOVA using temperature as a discrete factor showed it significantly affected testes ($F_{1,33} = 10.95$, $p < 0.001$), however, no significant effect of line ($F_{1,33} = 1.88$, $p = 0.18$) or line by temperature interaction ($F_{1,33} = 1.19$, $p = 0.32$) was detected.

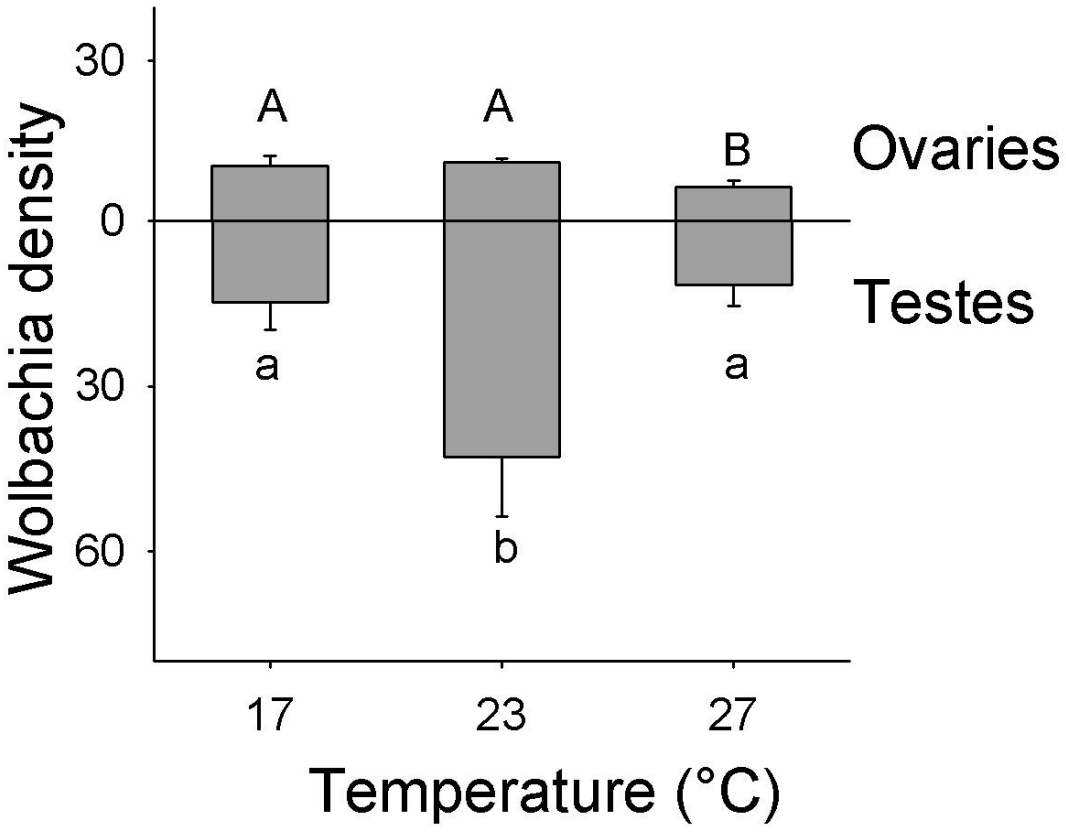

Fig. 3.5 Wolbachia density in ovaries and testes is affected by temperature treatments. Bars represent mean normalised Wolbachia density pooled for the KY01 and KY35 ± SEM. Bars not connected by the same letter vary significantly according to Tukey's HSD test.

3.3.4.4 Immune challenge

Wolbachia density in ovaries and testes was not significantly affected by *E. coli* challenge. ANOVA showed no significant effect of the main treatments (ovaries: $F_{1,34} = 1.35$, $p = 0.27$; testes: $F_{1,33} = 1.64$, $p = 0.21$; Appendix 3.4). Also, there were no significant line effects (ovaries: $F_{1,34} = 2.99$, $p = 0.09$; testes: $F_{1,33} = 2.81$, $p = 0.10$) or line by treatment interactions (ovaries: $F_{1,34} = 0.87$, $p = 0.43$; testes: $F_{1,33} = 0.48$, $p = 0.62$; Appendix 3.4).

3.4 Discussion

In symbiotic associations, maternal transmission and lack of free-living stages of endosymbionts align the interests of the partners, as their reproductive success depends on the fitness of the infected female hosts. For such systems, classic coevolutionary theory states that coadaptation or reciprocal selection gradually couples the metabolic and reproductive interests of the partners, resulting in a state where neither can live without the other (Yamamura, 1993), therefore implying that facultative associations are only transitory (Mondo et al., 2012). Wolbachia, however, deviates from this prediction as it rarely persists long enough in their host lineages to cospeciate. If gradual coadaptive processes were solely responsible of shaping the evolution of Wolbachia density regulation in Drosophila, it could be hypothesised that the older *w*Ma-Drosophila association would display greater control of Wolbachia titres than in the more recent *w*Ha-Drosophila. Alternatively, other processes such as selection on reproductive fitness could also influence the ability of the host to regulate symbiont density. In this case, the weak CI and low infection frequency characteristic of the *w*Ma

strain could result in lower selective pressure over transmission fidelity, and therefore *w*Ma flies would display lower control over symbiont density than flies with the strong CI-inducing *w*Ha. The results we present here, in conjunction with those of Correa and Ballard (2012), support this alternative hypothesis and may indicate that in some cases, the coevolution of Wolbachia/host may also be driven by processes other than coadaptation towards cooperative partnerships.

We start by considering Wolbachia density dynamics in ovaries. First, we observed that the significant differences among *w*Ma-*D. simulans* at F_4 were not maintained after generations of laboratory rearing (Fig. 3.1), similarly to what was observed for *w*Ha-*D. simulans* (Correa and Ballard, 2012). We hypothesised that line-specific differences at F_4 could be a reflection of larger differences among the wild collected founder females, lost with consecutive generations of rearing in a controlled environment (Dutton and Sinkins, 2004; Ahantarig et al., 2008). Although differences among isofemale lines were lost, the variability of ovarian titres of the *w*Ma-Drosophila was the same for F_4 and F_{19} flies, contrasting with the much narrower variability of *w*Ha-Drosophila at F_{19} compared to F_4 (Fig. 3.2). Because Wolbachia titres results from complex symbiont-host-environment interactions, we speculate that reduced environmental heterogeneity would lead to decreased symbiont titre variability if there is selection operating to maintain symbiont titres within an optimum range. If true, our observations could indicate that selection on Wolbachia density regulation is stronger in *w*Ha infected flies than it is in *w*Ma infected flies, due to the fitness cost of incompatibility in uninfected Hawaiian females. It is plausible that the variability of *w*Ma-Drosophila density after laboratory adaptation is due to polymorphism in the traits

involved in symbiont density regulation, a likely scenario given the low infection frequency of *w*Ma in nature and the high nuclear genetic variability of the Kenyan versus the Hawaiian fly populations.

In this study we observed that symbiont density in ovaries of Kenyan flies tended to increase with generations of laboratory rearing (Fig. 3.1, Appendix 3.2). This result contrasts with the stability of the mean *w*Ha ovarian density after an equal number of laboratory generations (Correa and Ballard 2012; Fig. 3.2). Such density increase, however, may be explained by the effects of laboratory rearing on CI and infection frequency. Because fly cultures were maintained in discrete generations, matings only occur among young flies, plausibly causing CI to be stronger than in the wild (Reynolds and Hoffmann, 2002). Increased titres of ovarian *w*Ma could be the result of rapid adaptation to maximise transmission fidelity in cultures, where uninfected females are at disadvantage (Friberg et al., 2012). Reduction of symbiont titres has also been observed in ovaries of *D. simulans* transinfected with the pathogenic, CI-inducing *w*MelPop strain (McGraw et al., 2002). Together, these observations suggest that CI and high frequency of infected individuals are strong selective forces towards an optimisation of symbiont titres, maximising transmission fidelity while minimising costs of symbiont over-replication.

Wolbachia density in ovaries of Kenyan lines increased with age and was affected by diet and temperature. Again these results contrast with the stability of *w*Ha infected females to the same treatments (Correa and Ballard, 2012). These observations may have at least two plausible explanations. First, coadaptation may have led to the

emergence of traits in the fly that actively modulate symbiont densities to adjust to the metabolic needs of the host. However, active modulation of a beneficial symbiont would likely lead to increased symbiont heritability, which is not observed in *w*Ma infected flies. Second, changes in *w*Ma titres may be due to a lack of adaptive mechanisms to maintain symbiont densities within optimum levels, rendering the symbiosis susceptible to Wolbachia over-replication or clearance. This second scenario is plausible if there is low selective pressure towards perfect transmission fidelity, which could be the case if CI is weak and frequency of infection is low. This argument is supported by Correa and Ballard (2012) who observed that *w*Ha flies exhibit stable ovarian titres after environmental challenges. More generally, populations infected by low or non CI-inducer Wolbachia display lower levels of maternal transmission than those harbouring strong CI inducers (James and Ballard, 2000). This second scenario, however, does not consider the effect of possible fitness benefits from Wolbachia. Such fitness benefits have been proposed as the mechanism of maintenance of Wolbachia when CI is low or non-existent (O'Neill et al., 1997).

Mondo and colleagues (2012) proposed that facultative associations involving non-essential vertically transmitted endosymbionts can be evolutionary stable when hosts' costs and benefits from infection fluctuate with shifting environmental pressures. Consequently, it could be argued that, besides low CI, there is weak selection for transmission fidelity in the *w*Ma-Drosophila because benefits from symbioses occur under specific environmental or physiological circumstances. Plausibly, nutrition may be important as we observe that *w*Ma titres increased when food was diluted. Further,

genomic analyses indicate that facultative Wolbachia in Drosophila may play an important role in dietary provisioning (Brownlie et al., 2009).

The analysis of Wolbachia titres in male gonads provides further insight on how selection may operate on the mechanisms of symbiont density regulation. As males represent an evolutionary dead end for the symbiont, adaptive mechanisms that regulate symbiont density are expected to be weaker in males than females, especially in sexually dimorphic tissues (Innocenti et al., 2011). This is expected to result in more conspicuous effects in testes than ovaries, as seen in this study (Figs. 3.2-3.4). Similar patterns of Wolbachia sex-specific density dynamics have previously been observed. Rio et al. (2006) showed conspicuous male-specific over-replication of parasitic Wolbachia in offspring of immune-challenged tsetse flies. Also, *Aedes albopictus* displays markedly different Wolbachia titres in females and males, associated with a reduced expression of CI in their populations (Tortosa et al., 2010). Recent research suggests that the sexual bias of Wolbachia-host associations plays an important role in determining long-term evolutionary fate of the symbioses. Because infection in males results in incompatible mates with the uninfected fraction of the female population, males would benefit from developing resistance to the CI phenotype. Koehnke and colleagues (2009) argue that the emergence and quick spread of male-specific genetic modifiers that decrease the strength of CI may be the evasive cause of Wolbachia extinction from natural populations.

Throughout this study, we interpret our results as differential regulation exerted by the fly, resulting from their adaptation to the infection. Nevertheless, the question of how

much of the observed variation in titres is attributable to variation of the Wolbachia population remains unanswered. Indeed, past studies have argued that the variability of symbiont effects on host fitness can be caused by differences between closely related Wolbachia variants within a host population (Weeks et al., 2007). Some of our observations here, however, suggest homogeneity of the Wolbachia genotype within the two fly populations studied. First, the absence of variation among mtDNA sequences may be suggestive of a recent sweep of Wolbachia *w*Ma in Kenyan flies (but see Dean et al. 2003). Second, if Wolbachia titre variation among isofemale lines were caused by differences among closely related strains, line-specific density patterns would have been retained throughout generations of laboratory rearing. We did not observe such pattern in *w*Ma-Drosophila nor *w*Ha-Drosophila (Correa and Ballard, 2012). Third, the lack of variation in *wsp* and *ftsZ* sequences recovered from Hawaiian and Kenyan flies could be indicative of highly similar if not identical Wolbachia strains across each population. Nonetheless, sequence analysis of these loci likely insufficient to assess genetic variability of the bacteria, as Wolbachia functional variability has been linked to genomic rearrangements at large scale (Woolfit et al., 2013) and not to mutational change on specific genes (Riegler et al., 2005). In contrast to symbiont variability, the autosomal genetic variation of these host populations is relatively high (Ballard, 2000) advocating for adaptive processes in the host rather than in the symbiont.

We recognise at least three important limitations of this study. First, results from datasets obtained generations apart may have been influenced by seasonal and/or microenvironmental factors. In the case of generational comparisons within this study, the trend of increasing *w*Ma titres at three different time points supports the hypothesis

that laboratory adaptation plays an important role on altering symbiont titres. In the case of comparisons between this study and Correa and Ballard (2012) we limited our analysis to a qualitative comparison. While every effort was made to conduct the studies under identical conditions, they were done two years apart due to the logistical difficulties of collecting *w*Ma flies from Africa and *w*Ha flies from Hawaii, respectively. A second limitation is that we restricted our analysis to quantification of Wolbachia titres in the wild-type host genetic background to investigate Wolbachia-host adaptive processes. These observations add to our understanding of symbiont/host interactions, however, direct tests on the fitness consequences of Wolbachia in multiple host genetic backgrounds are necessary to unambiguously corroborate the hypothesis raised here. Finally, our results are based on a comparison of a single-older and a single-younger Wolbachia strain infecting the same host species. Further comparisons between associations of known different ages are necessary to test the generality of our predictions.

Overall, our analysis of *w*Ma titres in gonads of Kenyan flies showed that this older Wolbachia/Drosophila association does not display a tighter control of symbiont density than the more recent *w*Ha-Drosophila association (Correa and Ballard, 2012), which would have been predicted if traits of symbiont regulation evolved solely from gradual, reciprocal adaptation towards mutualism. Based on our observations, we postulate that the selection exerted by CI and infection frequency on transmission fidelity may also play an important role shaping the evolution of symbiont regulation traits. We speculate that in lengthily associations, reduced CI strength brought about by the selection of male CI modifiers (Koenhke et al., 2009) could lower the selective pressure on

transmission fidelity, ultimately resulting in lax symbiont density regulation. Under this scenario it could be further speculated that the persistence of nonessential, low CI-inducing Wolbachia in host populations may rely on a fitness benefit caused by the symbiont under particular environmental or physiological circumstances as has been shown for other non-essential endosymbionts (Mondo et al., 2012).

Chapter 4

Mitochondrial DNA variants influence mitochondrial bioenergetics in *Drosophila melanogaster*

Abstract

The influence of mitochondrial DNA (mtDNA) mutations on human disease has been extensively studied, but the impact of mutations within the adaptive range is debated. We studied males from lines of *Drosophila melanogaster* that have a standardised nuclear genome but different mtDNA, at two ages. We measured mitochondrial respiration on permeabilised muscle fibres, hydrogen peroxide production of isolated mitochondria and mtDNA copy number of whole individuals. Our results show that a small set of naturally occurring mtDNA mutations can have a significant influence on mitochondrial bioenergetics that may change as the organism ages.

4.1 Introduction

The influence of mtDNA mutations in organismal fitness and human disease is well documented (Dowling et al., 2008; Tucker et al., 2011; Wallace, 2010). The effects of specific mtDNA mutations within the adaptive range, however, remain controversial. The goal of this study is to link naturally occurring mtDNA variation with differences in mitochondrial bioenergetics in *Drosophila melanogaster*.

The extent at which mitochondrial bioenergetics are influenced by mtDNA variation has been studied in a variety of organisms including *Drosophila simulans* (Katewa and Ballard, 2007; Melvin and Ballard, 2006; Pichaud et al., 2011; Pichaud et al., 2012), mice (Moreno-Loshuertos et al., 2006), and humans (Amo and Brand, 2007; Amo et al., 2008). In *D. simulans*, studies comparing the bioenergetic outcomes of two sympatric mitochondrial haplotypes have shown that they differ in respiration and electron transport (Katewa and Ballard, 2007; Pichaud et al., 2011; Pichaud et al., 2012). A limitation of these *D. simulans* studies is that the high interhaplotypic divergence (Dean and Ballard, 2005) makes it difficult to determine the bioenergetic consequences of specific mutations. Mouse cell lines harbouring different mtDNA genomes in a homogeneous nuclear genetic background showed significantly different performance in oxidative phosphorylation (OXPHOS) as well as reactive oxygen species (ROS) production (Moreno-Loshuertos et al., 2006). Again, the large number of differences between the mtDNA genomes in these cell lines decreases the power to detect the bioenergetic consequences of specific mutations. Wallace and colleagues (2010) proposed that human mtDNA haplogroups A, C, D and X had decreased coupling

efficiency and increased heat production that were both beneficial for humans moving out of Africa into colder climates. Amo and Brand (2007) elegantly tested this hypothesis in ρ^0 cells but found that mitochondria from Arctic haplogroups had similar or even greater coupling efficiency than mitochondria from tropical haplogroups.

We measured mitochondrial respiration in permeabilised fibres (defined as the rate of oxygen consumption of muscle mitochondria) using an Oxygraph-2k respirometer. This approach enables the analysis of mitochondria in their normal intracellular assembly (Horan et al., 2012) and has been shown to be relevant in Drosophila (Pichaud et al., 2011). Specifically, we quantified state 2', state 3, cytochrome *c* respiration, and uncoupling respiration using complex I substrates. State 2' respiration is a resting state of non-phosphorlylating respiration and measures the degree to which the movement of hydrogen ions from the mitochondrial intermembrane space across the inner membrane and to the matrix is uncoupled from the phosphorylation of ADP to ATP (Gnaiger, 2009). State 3 respiration measures the rate at which oxygen is consumed during phosphorylation of ADP to ATP. Cytochrome *c* respiration is generally used as a test of intactness of outer mitochondrial inner membrane (Kuznetsov et al., 2008). Uncoupling respiration indicates the maximum rate of respiration in the mitochondria when uncoupled from ATP turnover (Amo et al., 2008); differences in this parameter indicate variation at the level of substrate oxidation (Brand and Nicholls, 2011). These measurements allowed the calculation of Respiratory Control Ratio (RCR, state 3/state 2') and the Uncoupling Control Ratio (UCR, uncoupling respiration/state 3). RCR is considered a general test of mitochondrial dysfunction (Brand and Nicholls, 2011). UCR enables determination of whether the phosphorylation system (ATPase and

transport of ADP/ATP by adenine nucleotide translocase) exerts a limitation over the mitochondrial respiration. If UCR is close to 1.0, it shows the phosphorylation system can support the maximum respiration rates observed in state 3.

The efficiency of OXPHOS is mechanistically influenced by loss of electrons in complexes I and III that contribute to the formation of potentially harmful ROS that damage both the mitochondrial and cellular membranes, DNA and proteins (Kidd, 2000; Kidd, 2005). ROS is also produced from glycerol-3 phosphate dehydrogenase (Miwa et al., 2003). Further, the gradual accumulation of damage to mtDNA creates a positive feedback loop leading to a decline in function of mitochondria-encoded protein complexes and an increase in residence time of the electrons on sites capable of mediating one-electron reductions of oxygen (O_2) to yield superoxide ($O_2^{\bullet -}$) and hydrogen peroxide (H_2O_2). We measured the rate of H_2O_2 production in the presence and the absence of rotenone. In the presence of rotenone, electron transfer from complex I to ubiquinone is restricted causing a maximum rate of superoxide production from complex I. In the absence of rotenone, electron flow from complex I to complex III is unimpaired and H_2O_2 production from the whole electron transport system (ETS) can be assessed (Miwa et al., 2003). H_2O_2 production rate was measured in isolated mitochondria and not on permeabilised fibres. ROS production in permeabilised fibres may be biased by the artificial increase in high oxygen pressure, required to prevent restricted oxygen diffusion trough the tissue (Aragones et al., 2008; Boushel et al., 2007; Gnaiger, 2003; Gnaiger, 2009; Pesta and Gnaiger, 2012).

Finally, we quantified mtDNA copy number to investigate the hypothesis that flies harbouring mildly deleterious mtDNA mutations compensate for bioenergetic dysfunction (Bai and Wong, 2005; Ballard and Melvin, 2010; Ballard and Melvin, 2011). Investigating the control of mtDNA copy number has elucidated many of the factors involved in the replication of the mitochondrial genome (Lee and Wei, 2000; Passos et al., 2007). However, the mechanisms involved in the regulation of this process remain obscure (Moraes, 2001). In human mitochondrial diseases most patients with mtDNA deletions demonstrate compensatory mtDNA over-replication (Bai and Wong, 2005).

We tested males from a set of *D. melanogaster* lines with distinct mtDNA genomes in a standard w^{1118} isogenic nuclear background (Clancy, 2008). The homogeneity of the nuclear genome of these flies ensures that any difference in mitochondrial bioenergetics is likely to be caused by differences in mtDNA. Males were chosen because the 'mothers curse' hypothesis predicts that mitochondrial dysfunction is greater in males than females (Gemmell et al., 2004), and have been used to examine male specific-effects in flies (Innocenti et al., 2011). Previous studies using these Drosophila lines found that males harbouring mtDNA derived from Japan are the shortest lived (Clancy, 2008) have the slowest decline in male influence on fecundity over time, as well as having the lowest starvation resistance and lowest lipid proportion (Aw et al., 2011). There are three mutations in Japan flies that may cause bioenergetic effects. The first mutation is located in position 182 of ND2 in complex I, which results in an amino acid change from Histidine to Tyrosine. This replacement causes a change in the polarity of the residue and may affect the structure of complex I. The second mutation occurs in

$tRNA^{Ser}_{AGY}$ which has an A to C transversion at the wobble position in the anticodon. This mutation may affect transcriptional accuracy and efficiency (Akashi, 1994). The third mutation is a T to C transition occurring at position 121 of the 12S rRNA gene, and may destabilise the structure of the 12S rRNA. We show that a small set of differences in the mtDNA of flies from Japan have a significant effect on mitochondrial bioenergetics.

4.2 Materials and methods

4.2.1 Flies and husbandry

Fly lines used in this study (Alstonville, Dahomey, Japan and w^{1118}) were constructed by Clancy (2008). The whole coding mtDNA genome of these lines (excluding the AT-rich region) is known, including protein (Clancy, 2008) tRNA and rRNA coding regions (Aw et al., 2011).

To ensure homogeneity of the nuclear background, each line was backcrossed to the inbred w^{1118} line for five generations prior experimentation and tested within three generations. For two generations before each study, and during all experiments, the density of flies in cages was strictly controlled. Flies produced for all experiments followed Aw et al. (2011) with the exception that in this study flies were aged 11-12 days and 25-26 days. Experimental flies were kept at 23 °C with 50% humidity and under a 12 h light-dark cycle.

4.2.2 Mitochondrial respiration assays on permeabilised fibres

We used permeabilised muscle fibres from thorax for functional assays on ETS complexes. Preparation of Drosophila fibre bundles has been previously optimised for mitochondrial respiration experiments using the Oxygraph-2k respirometer (Oroboros Instruments, Innsbruck, Austria) (Pichaud et al., 2011; Pichaud et al., 2012). Following Pichaud et al. (2011), flight muscle fibres from two Drosophila thoraces were permeabilised in BIOPS relaxing solution complemented with 63 µg/mL saponin. Thorax dissections and fibre permeabilisations were conducted on ice. After permeabilisation, muscle bundles were immediately transferred into the respirometer chambers, filled with air-saturated mitochondrial respiration medium containing 115 mM KCl, 10 mM KH_2PO_4, 2 mM $MgCl_2$, 3 mM HEPES, 1 mM EGTA, 0.2% BSA, pH 7.2 (Pichaud et al., 2011; Pichaud et al., 2012).

Respiration rates of permeabilised fibres were measured at 25 °C. Data acquisition and analysis were performed using the software DatLab (Oroboros Instruments, Innsbruck, Austria). The oxygen electrodes of the respirometer were calibrated with air-saturated respiration medium at the experimental temperature before each run. Additional instrumental zero oxygen calibrations using sodium dithionite were also carried out routinely. O_2 solubility for medium respiration was calculated for 25 °C according to Rasmussen and Rasmussen (2003).

To enable oxygen diffusion in permeabilised fibres, oxygen was injected into the chambers (Aragones et al., 2008; Boushel et al., 2007; Gnaiger, 2003; Gnaiger, 2009).

Mitochondrial respiration was then measured after transferring the fibres to respiration medium supplemented with 10 mM pyruvate, and 10 mM L-proline (State 2' respiration). Respiration rates were measured after the addition of the following chemicals: (i) ADP 5 mM (complex I state 3 respiration, CI); (ii) cytochrome *c* from equine heart 10 µM (an index functional integrity of the outer mitochondrial membrane, CIc); (iii) carbonyl cyanide-*p*-trifluoromethoxyphenylhydrazone (FCCP) 0.5-4 µM (uncoupler, CIc+u) and (iv) antimycin A 2.5 µM (inhibitor of complex III, residual oxygen consumption). The concentration of the each substrate was chosen to be saturating and was optimized previously for this approach (Pichaud et al., 2011). This protocol allowed the calculation of RCR for complex I (RCR, CI/state 2'), cytochrome *c* effect (CIc/CI) and UCR (CIcu/CIc). Results for respiration rates were expressed in pmol of O_2 consumed per second per mg of dry fibres, and were corrected by subtracting the residual oxygen consumption (after inhibition of complex III). Samples displaying CIc/C ratios higher than 1.1 were discarded as integrity of mitochondria in the preparation could be compromised (Kuznetsov et al., 2008).

For each experiment, the results from two preparations (one per chamber) were averaged for analysis. Data from four to seven experiments were collected per treatment (5, 6, 7 and 5 aged 11-12 days from Alstonville, Japan, Dahomey and w^{1118} flies respectively and 5, 5, 5 and 4 aged 25-26 days from the same lines). The dataset was analysed using ANOVA with JMP© v5 (2007 SAS Institute, Cary, NC, USA) after exclusion of outliers and log-transformation when data were not normally distributed.

4.2.3 Isolation of intact mitochondria and ROS production

Intact mitochondria were isolated from male *D. melanogaster* aged 11-12 days and 25-26 days. The procedure was adapted from Melvin and Ballard (2006). Briefly, 50 fly thoraces were dissected on ice and placed in 500 µL of ice-cold mitochondrial isolation buffer (154 mM KCl, 1 mM EDTA, pH 8.0). The thoraces were homogenised by 80 strokes up and down using sterile kontes pellet pestles (Kimble Chase, Vineland, NJ, USA). The homogenate was diluted with 200 µL of mitochondrial isolation buffer and filtered through a 1-cc syringe (BD, Frankling Lakes, NJ, USA) with 2 cm^2 of gause pad (Johnson & Johnson, New Brunswick, NJ, USA) compacted in the tip. The homogenate was centrifuged at 1500 x *g* for 8 min at 4 ^oC. The pellet was gently washed with 200 µL of ice-cold mitochondrial isolation buffer 1 to 2 times to remove the lipids. The final mitochondrial pellet was re-suspended in 40 µL of mitochondrial isolation buffer. This procedure yielded approximately 12 µg/µL of mitochondrial protein as determined by a Bio-Rad D_c protein assay kit (Richmond, CA, USA). Three mitochondrial extractions were prepared for each line-age combination.

The quality of the mitochondrial isolations was assessed by measuring the respiratory control ratio (RCR) in a Clark electrode (Rank Brothers, Cambridge, UK). To be consistent with the RCR measured on permeabilised fibres, we calculated the RCR by dividing the state 3 by state 2' respiration rates (Gnaiger, 2003). Extracted mitochondria with RCR values above 3 are considered of good quality when measured in Clark electrodes (Estabrook, 1967). Briefly, 3 mL of mitochondrial respiration medium containing 5mM of pyruvate and 5mM of L-proline and 120 µg of mitochondrial

protein were added to the Clark electrode incubation chamber at 25 ^{o}C. Oxygen consumption was measured before and after addition of ADP (0.25 mM final concentration). These concentrations were optimised for mitochondrial respiration assessment using isolated mitochondria with the Clark electrode (Melvin and Ballard, 2006) and therefore differed from those used for permeabilised fibres.

The rate of H_2O_2 production in mitochondrial complex I was measured after stimulation with pyruvate (5 mM) and L-proline (5 mM), with and without rotenone for each mitochondrial preparation. H_2O_2 was quantified using Amplex® Red H_2O_2/Peroxide assay kit (Life Technologies^{TM}, Eugene, OR, USA). H_2O_2 reacts with Amplex® Red in the presence of horseradish peroxidase to form the oxidative product resofurin, which has maximum absorbance at 560 nm (Zhou et al., 1997). H_2O_2 assays were performed in 96-well microtiter plates, with each well containing 5 µg of mitochondrial protein, 5mM L-proline, 5mM sodium pyruvate, 20 U/mL superoxide dismutase, Amplex® Red reagent as per manufacturer's instructions and mitochondrial respiration medium to make up a final volume of 100 µL. Superoxide dismutase was added to transform all $O_2^{\bullet-}$ molecules into H_2O_2. Rotenone (2 µM) was added to prevent the transfer of electrons from complex I to ubiquinone, causing a maximum rate of $O_2^{\bullet-}$ production from complex I (Miwa et al., 2003). Standards of H_2O_2, mitochondria with rotenone, control lacking mitochondria, control lacking substrates and control lacking horseradish peroxidase were included. The measurements were conducted at 25 ^{o}C within 4 h of mitochondrial extraction to ensure minimal mitochondrial degradation. Results were recorded every 2 min for 1 h using a SpectraMax Plus spectrophotometer and SoftMax Pro software (Molecular Devices Corp., Sunnyvale, CA, USA). The amount of ROS

production in each well was expressed as nmol of H_2O_2 production per minute per mg of mitochondrial protein. ROS production of each mitochondrial preparation was calculated as mean H_2O_2 production using Microsoft Excel. JMP© v5 software was used for statistical analyses.

4.2.4 Mitochondrial DNA density

The relative number of mtDNA genomes per diploid nuclear genome for the line/age treatments assayed here was determined by quantitative PCR (qPCR). Genomic DNA (gDNA) was extracted from six whole males of each line and age group using a Gentra Puregene® Cell Kit (Gentra Systems Inc., Minneapolis, MN, USA). The mtDNA was quantified by amplifying a 113 bp region of the mitochondrial large ribosomal RNA gene (lrRNA, CR34094) using primers q13259F (5'- TCG TCC AAC CAT TCA TTC C) and q13371R (5'- ATA AAG TCT AAC CTG CCC ACT GA). No nuclear copy of lrRNA was found in *D. melanogaster* (FlyBase version 2011_07, released July 21, 2011). Nuclear genomic DNA was quantified by amplifying a 135 bp region of the single-copy nuclear gene that encodes the 215 kDa subunit of RNA polymerase II (rpII215; CG1554) using primers qrpII215F (5'-AGG CGT TTG AGT GGT TGG) and qrpII215R (5'-TGG AAG GTG TTC AGT GTC ATC). *In situ* hybridisation studies have shown that a single copy of rpII215 is present in the Drosophila genome (Aoyagi and Wassarman, 2000).

Quantitative PCR reactions were performed in duplicate using the KAPA® SYBR FAST Universal qPCR Kit (Kapa biosystems, Boston, MA, USA) and a Stratgene Mx3000P

QPCR instrument (Agilent Technologies, Santa Clara, CA, USA). Each 10 µl reaction contained 5 µl of 2× KAPA® SYBR FAST master mix, 10 ng DNA, 0.2 µM of each forward and reverse primer and 0.2 µl 50 × ROX reference Dye Low. The thermal cycling protocol consisted of a 30 s denaturation step at 95 °C, followed by 40 cycles of 3 s at 95 °C and 30 s at 60 °C. A dissociation curve analysis was performed following the PCR protocol to confirm specificity of the primers and absence of primer dimers or contamination. The amplification efficiency of each gene was calculated by constructing a standard curve using serial dilutions of gDNA.

Copy number of the mtDNA genome relative to the nuclear genome was calculated using qbase^{PLUS} software (Biogazelle, Zwijnaarde, BE) as $2 \times (E_{gDNA}{}^{Ct(gDNA)} / E_{mtDNA}{}^{Ct(mtDNA)})$ where E is the primer pair specific amplification efficiency. Results for six flies were averaged to obtain relative mtDNA copy number for each line and time point. ANOVA was performed to test for the effects of line, age and line × age interaction on mitochondrial density using JMP© v5 software.

4.3 Results

4.3.1 Mitochondrial respiration on permeabilised fibres

RCRs and the effect of cytochrome *c* (Table 4.1) were used as indexes of mitochondrial functional integrity (Pichaud et al., 2011; Pichaud et al., 2012). Two-way ANOVA comparing log-transformed RCR values among treatments showed no effects of line ($F_{3,32} = 0.68$, $p = 0.57$), age ($F_{1,32} = 0.96$, $p = 0.33$) or their interaction ($F_{3,32} = 0.09$, $p = 0.96$). Cytochrome *c* effect measured as the ratio of O_2 consumption before (CI) and

after (CIc) cytochrome *c* injection, showed minimum effect on most preparations (Table 4.1). One-sample *t* test corroborated that CIc/CI were not significantly different from 1 ($t_{41} = 0.796$, $p = 0.43$).

Japan showed the lowest state 3 respiration and Dahomey the highest at 11-12 days of age (Fig. 4.1). Phosphorylating (state 3) respiration using complex I substrates was compared among lines and between ages. A two-way ANOVA showed no significant differences among lines ($F_{3,32} = 2.58$, $p = 0.078$), ages ($F_{1,32} = 0.01$, $p = 0.92$) or their interaction ($F_{3,32} = 1.51$, $p = 0.23$). However, there was a significant differences among lines at 11-12 days of age ($F_{3,32} = 5.06$, $p < 0.05$). Analyses of line specific changes in state 3 respiration rates showed that Japan displayed an increase of 18%, while Dahomey decreased by 12%. Rates in Alstonville and w^{1118} were constant and changed less than 5%.

No significant differences were found between State 2' respiration rates or UCR values. State 2' respiration, mainly due to proton leak, was measured in presence of complex I substrates and in the absence of ADP. ANOVA showed that state 2' respirations did not differ among treatments, and there were no effects of fly line ($F_{3,32} = 0.98$, $p = 0.41$), age ($F_{1,32} = 0.73$, $p = 0.40$), nor their interaction ($F_{3,32} = 0.21$, $p = 0.88$, Appendix 4.1). Respiration rates were compared before and after the addition of FCCP to determine if the ATP synthase and the adenine nucleotide translocase exerted a limitation on the mitochondrial respiration. No differences among UCR values were found between lines ($F_{3,32} = 2.18$, $p = 0.11$), ages ($F_{1,32} = 1.60$, $p = 0.21$) or their interaction ($F_{3,32} = 1.15$, $p = 0.3$, Appendix 4.2).

Table 4.1 Average respiratory control ratio (RCR) and cytochrome *c* effect in permeabilised fibres of males from four lines of Drosophila at two different ages (± SEM).

Age (days)	Line	RCR	Cytochrome *c* effect
11-12	ALST	20.46 ± 6.93	0.97 ± 0.03
	DAH	16.61 ± 3.22	0.99 ± 0.01
	JAP	14.26 ± 1.73	1.06 ± 0.02
	w^{1118}	13.20 ± 1.14	1 ± 0.03
25-26	ALST	20.76 ± 3.20	0.99 ± 0.01
	DAH	17.07 ± 2.88	0.98 ± 0.04
	JAP	18.09 ± 4.22	1.02 ± 0.02
	w^{1118}	16.38 ± 0.57	0.98 ± 0.01

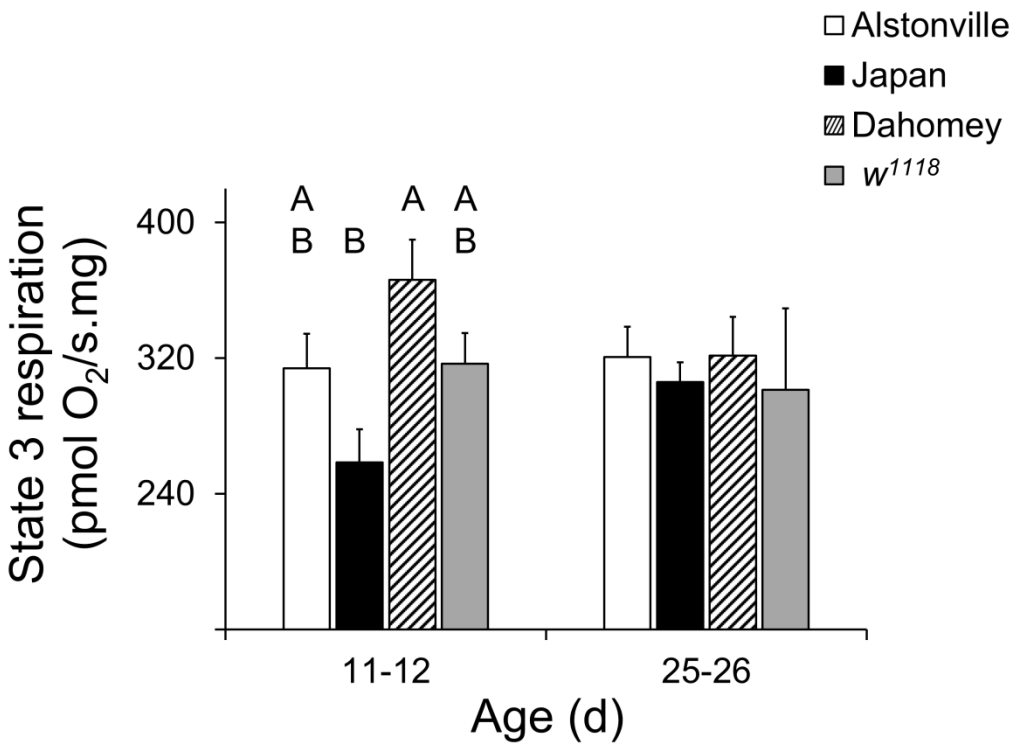

Fig. 4.1 Complex I phosphorylating respiration rate (state 3; CI) of flight muscle mitochondria form permeabilised fibres of Drosophila males with distinct mtDNA but standardised nuclear DNA. Fly lines used were Alstonville, Japan, Dahomey and w^{1118} at two different ages. Respiration rates (expressed in O_2 pmol/s.mg of fibres) differ between fly lines of 11-12 days of age but not among 25-26 flies. Bars represent mean phosphorylating respiration ± SEM. Letters above bars indicate significant differences between lines as determined by Tukey's test with Q = 3.15 and $\alpha = 0.05$.

4.3.2 Mitochondrial ROS production

Mitochondrial preparations were of high quality. RCR measured with the Clark electrode was always above 3.0 indicating high quality preparations (Melvin and Ballard, 2006). The mean RCR of the mitochondrial preparations was 6.64 ± 0.62 (SEM) for 11-12 day-old flies and 6.98 ± 0.17 for 25-26 day-old flies. Differences among RCR measures with the Clark electrode and the Oxygraph-2k respirometer were expected given the nature of the samples analysed (Kuznetsov et al., 2008).

In the presence of rotenone, electron transfer from complex I to ubiquinone is restricted causing a maximum rate of $O_2^{\bullet-}$ production from complex I (Miwa et al., 2003). Overall, 11-12 day-old flies have a lower maximum rate of H_2O_2 production than 25-26 day-old flies. In the presence of rotenone, the increase in maximum rate of mean H_2O_2 production rate with age was about 24% in Japan and w^{1118} and 9% for Alstonville and Dahomey (Fig. 4.2). ANOVA shows a significant effect of line ($F_{3, 40} = 7.68$, $p < 0.001$), age ($F_{1, 40} = 18.59$, $p < 0.001$) and line × age interaction ($F_{3, 40} = 2.912$, $p = 0.046$).

In the absence of rotenone, electron flow from complex I to complex III is not restricted. Therefore, the overall rate of $O_2^{\bullet-}$ formation by the ETS can be estimated. In all lines, 11-12 day-old flies have a lower mean H_2O_2 production rate than 25-26 day-old flies. The increase in mean H_2O_2 production rate with age was approximately 28% in Japan and w^{1118} and 15% for Alstonville and Dahomey (Appendix 4.3). ANOVA shows significant effects of age ($F_{1, 40} = 46.98$, $p < 0.001$) and a fly line × age interaction ($F_{3, 40} = 2.99$, $p = 0.04$) but no effect of fly line ($F_{3, 40} = 2.02$, $p = 0.13$).

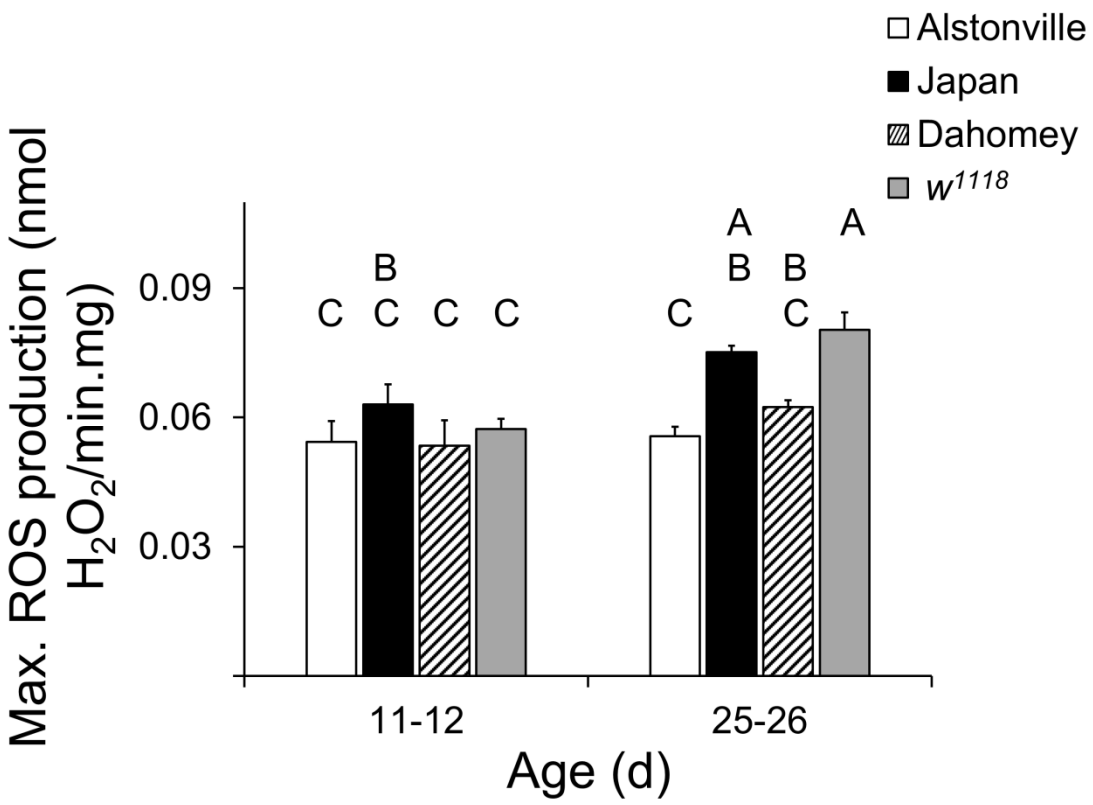

Fig. 4.2 Maximum rate of H_2O_2 production of isolated mitochondria (expressed as H_2O_2 nmol.min^{-1}.mg^{-1}of protein) of Drosophila males with distinct mtDNA but standardised nuclear DNA. Fly lines used were Alstonville, Japan, Dahomey and *w*^{1118} at two different ages. Maximum rate of H_2O_2 production rate increases with age. Japan and *w*^{1118} line have the highest maximum rate of mean H_2O_2 production compared to the other lines. Bars represent mean H_2O_2 production rate ± SEM. Letters above bars indicates significant differences between lines as determine by Tukey's test with Q = 3.20 and α = 0.05.

4.3.3 MtDNA density

We inferred mitochondrial density from the number of mtDNA copies per diploid nuclear genome. All lines displayed an increase of mtDNA density with age. Among them, Japan had highest number of mtDNA copies and the highest increase in density with age (46.9%). Dahomey displayed a similar increase of mtDNA density (46%), followed by Alstonville (30.8%) and w^{1118} (13.2%). Compared to the other three lines, mtDNA copy number in the Japan was 10.8% greater at 11-12 days and 21.0% at 25-26 days (Fig. 4.3). ANOVA showed significant effects of fly line ($F_{3, 38} = 5.1$, $p < 0.01$) and age ($F_{1, 38} = 35.1$, $p < 0.001$) but no effect of fly line by age interaction ($F_{3, 38} = 2.1$, $p = 0.1$).

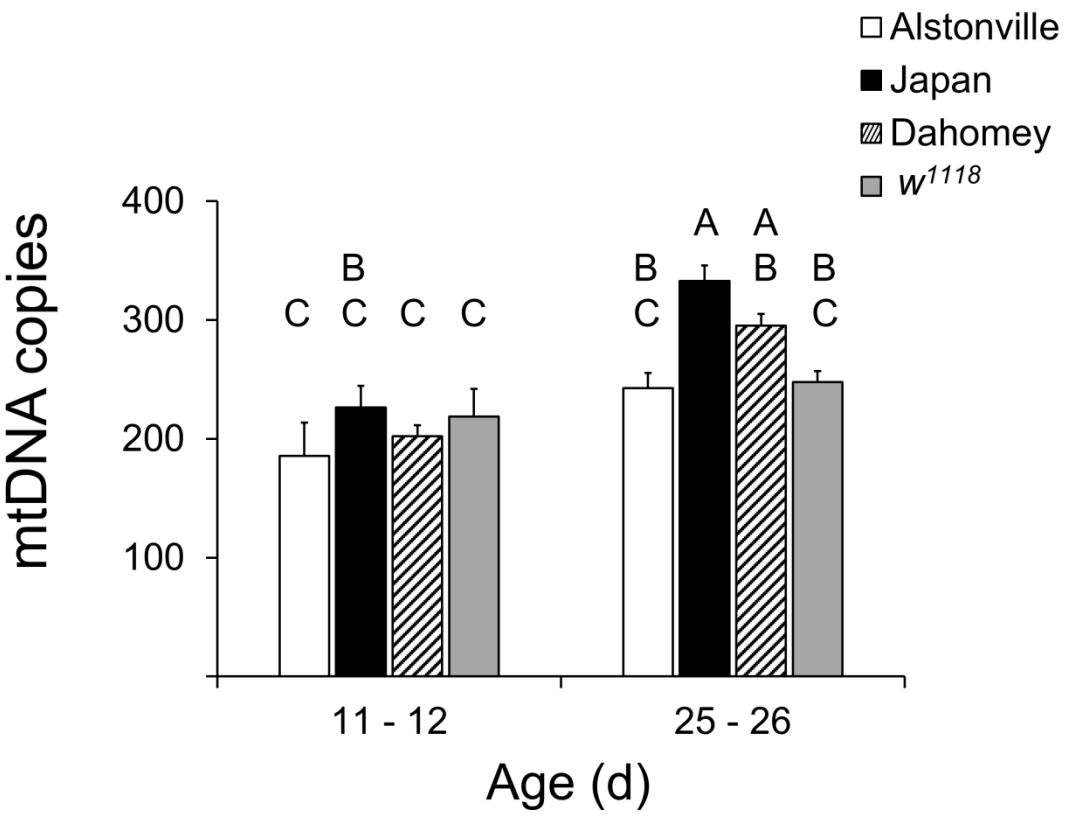

Fig. 4.3 Mitochondrial DNA copy number per nuclear DNA genome whole individuals of Drosophila males with distinct mtDNA but standardised nuclear DNA. Fly lines used were Alstonville, Japan, Dahomey and w^{1118} at two different ages. MtDNA copy number increases with age in all lines and is greatest in Japan. Bars represent mean mtDNA copy number relative to nuclear DNA ± SEM. Letters above the bars indicate significant differences between lines as determined by Tukey's HSD test with Q = 3.2 and $\alpha = 0.05$

4.4 Discussion

The results presented in this study show that a small set of naturally occurring mtDNA mutations can have a significant influence on mitochondrial bioenergetics as an organism ages. This has important implications for the study of mitochondrial dysfunction in males. Specifically, it suggests that mtDNA mutations within the adaptive range can influence mitochondrial bioenergetics and therefore affect a variety of physiological parameters, including physical activity, lipid storage and lifespan (Aw et al., 2011; Clancy, 2008). This study does not consider the influence of these mutations on the evolution of mtDNA as females were not included.

Of the four fly lines with distinct mtDNA variants studied here, Japan is the most phenotypically distinct. These flies have the shortest lifespan (Clancy, 2008) and are unique in terms of male influence on female fecundity, starvation resistance and lipid proportion (Aw et al., 2011). In this study, significant differences were also observed in Japan at the bioenergetic level. Japan displays the lowest phosphorylating respiration rate at 11-12 days and the greatest increase in rate to 25-26 days of age (18%). Japan also displays the highest rates of H_2O_2 production and mtDNA density at 11-12 days of age and both parameters increased with age by 28% and 46.9% respectively.

We hypothesise that the mitochondrial mutation load of Japan flies results in mild mitochondrial malfunction, which then elicits a compensatory response to maintain ATP homeostasis. Japan flies have low phosphorylating respiration rates and high rates of H_2O_2 production at 11-12 days of age. Increased ROS production induced by

dysfunctional mitochondria may then elicit chronic oxidative stress, which in term may enhance mtDNA replication and copy number. Low concentrations of ROS may stimulate an increase in mitochondrial mass and mtDNA copy number (Lee and Wei, 2000). Empirical studies have reported that nuclear encoded redox-responsive transcription factors such as nuclear respiratory factor 1 (NRF-1) and nuclear respiratory factor 2 (NRF-2), responsible for the regulation of mitochondrial biogenesis, increased under low concentration of ROS. The activation of NRF-1 and NRF-2 results in initiation of mtDNA replication and cell proliferation to compensate for the deficit in mtDNA copy number. The higher mtDNA copy number may result in more respiratory complexes (Passos et al., 2007) and this may represent a compensatory mechanism for reduced OXPHOS function (Ballard et al., 2010; Moreno-Loshuertos et al., 2006).

There are at least three mutations in Japan flies that may account for the observed bioenergetic effects (Aw et al., 2011; Clancy, 2008). The complex I ND2 mutation (Histidine to Tyrosine at position 182) may affect the structure and function of this complex. Deficiencies in complex I are the most common cause of OXPHOS dysfunction and mutations in its mitochondrial-encoded proteins have been related to metabolic syndromes in humans (Janssen et al., 2006). Exploratory studies have shown that in patients with autism there is significant impairment of complex I function, enhanced mitochondrial rate of H_2O_2 production and mtDNA over-replication (Giulivi et al., 2010). Moreover, it has been shown that mitochondrial mutations in ND2 may account for increased levels of ROS production (Mithani et al., 2008). This may indicate a possible link between the ND2 mutation in Japan and its bioenergetic traits. In addition to the change in ND2, Japan harbours mutations in $tRNA^{Ser}_{AGY}$ and 12S

rRNA. These mutations may destabilise the RNAs structure and affect accuracy and efficiency of the mitochondrial transcription and translation (Akashi, 1994), which could also account for the increase in mtDNA copies. Further, mutations that cause tRNA to be abnormally folded are pathology-causing and exacerbate mitochondrial biogenesis as a compensatory response in mice (Moreno-Loshuertos et al., 2011).

The w^{1118} flies also have a high rate of ROS production (Fig. 4.2). Plausibly, the substitution from the neutral and polar Asparagine to the acidic Aspartic acid in subunit III of complex IV (COIII) at residue 40 could cause an increase in residence time of electrons at complex III and all sites upstream in the ETS. Amino acid changes may influence proton pumping and alter subunit interactions within the complex IV quaternary structure. An Asparagine to the acidic Aspartic acid change has been shown to eliminate proton pumping activity in subunit 1 of the cytochrome *bo* ubiquinol oxidase of *Escherichia coli* (Thomas et al., 1993). Quaternary structure modelling of complex IV in Drosophila shows that residue 40 is in a 5-amino acid loop between two alpha helices (Ballard et al., 2010). At 4 Å distance only residues within COIII are in close contact. At 6 Å distance residue 40 interacts with one residue of Cox6A and two residues of Cox7A.

As with the previous study by Aw et al. (2011) that considered the organismal physiology of this set of flies there are at least three limitations of this study. First, the bioenergetics of the mtDNA mutations was examined in a single genetic background and mitochondrial-nuclear interactions were considered negligible. Testing each mtDNA type in distinct genetic backgrounds would test the generality of the results

obtained. A second limitation of this study is that mutation(s) in the A+T rich region and/or in chromosome 4 may influence the phenotypic traits measured. Finally, the study was conducted under one set of laboratory conditions and it is possible that small changes in these conditions may lead to different results. This is certainly plausible for flies harbouring Japan mtDNA, as the mutation at position 182 of ND2 in complex I, results in an amino acid change from Histidine to Tyrosine. Indeed, providing a diet consisting of nutrients that introduce electrons into the ETS via complexes II and III, therefore bypassing complex I, may be a clinically relevant method to experimentally test the influence of the complex I mutation on the lifespan of flies harbouring Japan mtDNA.

Chapter 5

Effects of Wolbachia infection in native and introgressed cyto-nuclear genotypes in *Drosophila simulans*

Abstract

Wolbachia are common bacterial heritable endosymbionts of insects, generally regarded as reproductive parasites that manipulate host reproduction in order to spread. Traditionally, it has been assumed that the spread and maintenance of Wolbachia in host populations largely depended on such reproductive manipulations. Recent studies, however, reveal that Wolbachia often confer diverse beneficial effects to their host physiology, which in many cases are conditional to the environment and the host genetic background. Physiological beneficial traits related to Wolbachia infection are likely essential for the maintenance of symbioses when manipulation phenotypes are weak or absent. In this study, we examined the effects of two Wolbachia strains –a weak and a strong reproductive manipulator- on life history traits and respiration of *Drosophila simulans*. Using backcrossing to alter the genetic background of flies, we investigated if infection-related phenotypes were linked to the host nuclear background, or were intrinsic to the symbiont type. Overall, beneficial traits related to infection were noted, but such effects were not limited to the genetic background that naturally occurs with each Wolbachia strain. These findings may have important implications for the understanding of the adaptive role of Wolbachia in natural populations.

5.1 Introduction

Symbioses between bacteria and animals are ubiquitous and constitute an important source of evolutionary innovation, as the incorporation of the endosymbionts' metabolic functions may award beneficial biochemical and physiological properties to the host (Feldhaar, 2011; Moran et al., 2008). Heritable symbionts that spend their entire lifecycles within a single host are often beneficial or even required for host reproduction and survival (Wernegreen, 2004). Theory suggests this to be the case as natural selection favours endosymbionts that improve the fitness of the transmitting female host, therefore increasing the endosymbionts' likelihood of survival and propagation.

Although obligate beneficial symbioses are common (Moran and Wernegreen, 2000), a number of maternally inherited endosymbionts appear to be maintained in host populations through parasitic phenotypes that manipulate the host reproduction. Among these are Wolbachia, a group of intracellular bacteria that are widespread among insects and other invertebrates (Werren et al., 1995). Many Wolbachia symbionts of insects are thought to spread by inducing cytoplasmic incompatibility (CI), a reproductive modification in which crosses between infected males and uninfected females have reduced hatch rates compared to other crosses. CI-Wolbachia are in conflict with the host nuclear genome as they represent a cost to the host paternal lineages (Charlat et al., 2003), and consequently, selection in males is predicted to favour CI attenuation (Koehncke et al., 2009). Indeed, variable levels of CI induction by Wolbachia are commonly observed in natural populations. Moreover, it has been suggested that weak

or absent CI is an indication of long coexistence of Wolbachia and their hosts (Solignac et al., 1994).

Theory predicts that in associations where CI is weak or absent, infection would be lost unless there are beneficial fitness traits associated with symbiont presence (Hoffmann et al., 1998; O'Neill et al., 1997). In recent years, there has been a sudden increase of studies reporting beneficial fitness effects of facultative Wolbachia (Engelstädter and Hurst, 2009; Iturbe-Ormaetxe and O'Neill, 2007). Nonetheless, such studies often describe a great deal of variation in such phenotypes, as they often depend on the genetic background of the hosts and on environmental factors (Fry et al., 2004; Reynolds and Hoffmann, 2002; Zhang et al., 2010). Interestingly, such effects seem not be limited to weak-CI inducing Wolbachia, but also occur in strains that produce strong manipulation genotypes (Kriesner et al., 2013; Weeks et al., 2007). A question that arises from this is: Are fitness benefits associated to Wolbachia the product of coadaptation between partners or are they intrinsic traits of the symbiont that nonetheless enhance the symbiosis? Investigating this question requires the comparison of Wolbachia effects between host genetic backgrounds naïve to the infection and those that naturally occur and coevolve with the symbiont.

Drosophila simulans is an ideal model for the study of Wolbachia-host interactions. First, this ubiquitous species is host to a variety of Wolbachia strains with markedly distinct levels of CI phenotypes, infection frequency and symbiont transmission fidelity (Ballard, 2004; Merçot and Charlat, 2004; Zhou et al., 1998). Moreover, *D. simulans* exhibits divergent mitochondrial DNA (mtDNA) haplogroups with a striking lack of

intrahaplogroup diversity, which evidences the powerful signature left by Wolbachia in the natural history of this species (Ballard, 2004). Two fly populations that differ in their Wolbachia associations are found in Hawaii and Kenya. Nearly all Hawaiian *D. simulans* individuals tested for infection harbour the strong CI inducer *w*Ha, which is transmitted from mother to offspring with high fidelity (Ballard, 2004; James and Ballard, 2000; Rousset and Solignac, 1995; Turelli and Hoffmann, 1995). In contrast, infected flies from Kenya, which are often less than 50% of the individuals in the population, carry the *w*Ma strain of Wolbachia. This Wolbachia type induces variable levels of CI and does not display high transmission fidelity (James and Ballard, 2000; Merçot and Charlat, 2004).

Phylogeograpic analyses and CI studies suggest that the *w*Ma infection is older in *D. simulans* (Ballard, 2004; Rousset and Solignac, 1995), and most likely finds its origin in Madagascar, from where the fly species is endemic (Ballard, 2004). Infection of *D. simulans* with Wolbachia *w*Ha is thought to be a more recent event, likely occurring in flies that harboured a derived *si*I haplotype (Poinsot et al., 2000). In addition, the variability of the nuclear genome of Kenyan flies is expected to be higher than that of Hawaiian flies. One reason for this is that Kenyan populations are closer to the hypothesised place of endemicity for *D. simulans* (Lachaise et al., 1988). Another reason is that the colonisation of the Hawaiian island by *D. simulans* implies that this fly population experienced founder effects due to low population density, most likely decreasing its genetic variability (Steiner et al., 1976). This is supported by sequences of intronic regions of the alcohol dehydrogenase-related (*Adhr*) and the glycerol phosphate dehydrogenase (*Gpdh*) loci in these flies, which showed higher genetic

diversity for Kenyan flies; π was estimated to be between 16 and 37% higher in Kenyan flies while θ was up to 57% higher (Correa and Ballard, 2014).

In this study, we investigated the effects of two *D. simulans* Wolbachia strains when maintained in their natural nuclear backgrounds, and when placed in foreign nuclear backgrounds through introgression. By comparing infection effects in flies with naturally occurring and foreign nuclear backgrounds, we aimed to detect signs of host-symbiont adaptation in naturally occurring cyto-nuclear combinations. Besides Wolbachia, another important feature of these flies is their divergent mtDNA type. Flies from Hawaii harbour an *si*I mitochondrial haplotype co-occurring with *w*Ha while *w*Ma infected Kenyan flies harbour an *si*III haplotype. Importantly, there are a number of fitness effects associated to these two mtDNA types (Ballard, 2004; De Stordeur, 1997) that need to be investigated in order to disentangle Wolbachia effects from mitochondrial effects. Because of their maternal inheritance, Wolbachia and mitochondria cannot be segregated using backcrossing. Consequently, the effects of cytoplasmic types (*si*III from Kenyan and *si*I from Hawaii) were also considered in our experimental design.

We measured oxygen consumption of flight muscle, feeding rate and starvation resistance in female and male flies. Egg laying and hatching were also measured. We hypothesised that flies are affected by Wolbachia infection, and that such effects differ with host nuclear background and sex. We predicted the effects of the low CI-inducing *w*Ma to be more benign than those of the strong CI-inducer *w*Ha. In addition, if coadaptation occurs, beneficial effects of Wolbachia would be limited to or at least

more conspicuous in flies with nuclear backgrounds native to the infection. Our results generally support the hypothesis of beneficial effects of low-CI inducing Wolbachia, however, such effects were not limited to nuclear backgrounds native to the Wolbachia type. We discuss these results in light of evolutionary mechanisms that allow Wolbachia-Drosophila symbioses to be maintained in nature.

5.2 Materials and methods

5.2.1 Fly lines

Isofemale fly lines from Hawaii and Kenya, collected 2010 (Correa and Ballard, 2012) and 2011 (Correa and Ballard, 2014), respectively, were used to establish focal fly lines. The aim of our crossing scheme was to obtain infected and uninfected fly lines with *si*I and *si*III cytoplasmic types (each associated to a different Wolbachia strain), both on homogenised nuclear backgrounds from Hawaii (HW) and Kenya (KY). This scheme consisted of three steps: i) generation of uninfected fly lines with inbred nuclear backgrounds through sibling-mating; ii) backcrossing of the original Wolbachia infected lines to inbred lines to generate infected lines with native and introgressed cyto-nuclear genotypes and iii) generation of uninfected control flies with introgressed cyto-nuclear genotypes through antibiotic treatment. This scheme was used separately and simultaneously on two sets of Hawaiian and Kenyan paired isofemale lines. We obtained a total of 16 fly lines, which consisted of two genetically independent replicates for each infection/cytoplasmic type/nuclear background combination (Fig. 5.1).

For the generation of homogenised nuclear backgrounds, a subset of flies from each of the four original isofemale lines were cured from Wolbachia infections using a standard tetracycline treatment protocol (Hoffmann et al., 1986) for two consecutive generations. Infection clearance was corroborated by PCR following Correa and Ballard (2014) and Wolbachia-cured lines were subjected to eight generations of sibling-mating to reduce nuclear genetic heterogeneity. Early during this inbreeding scheme, flies were fed on food that previously housed untreated males from the original stocks to allow reconstitution of the gut flora after antibiotic treatment. Four inbred lines with naturally occurring cyto-nuclear combinations and no Wolbachia were produced (Fig. 5.1; i).

Within each Hawaii-Kenya pair of lines, the two homogenised nuclear backgrounds generated in step (i) were backcrossed to the lines they originated from (native lines) and were also introgressed into the lines that harboured the cytoplasmic type from the other population (introgressed lines). To achieve this, single inbred, uninfected males (nuclear donors) were crossed with single virgin females from the original lines (recipients), following the scheme in Fig. 5.1 (ii). Virgin female progeny resulting from these crosses was mated again to a male from the nuclear donor line and the process was repeated consecutively for a total of eight generations. The resulting eight Wolbachia infected fly lines would theoretically harbour a nuclear background that is approximately 99.6% that of the inbred lines and the cytoplasmic type of the original lines.

Because Wolbachia and its co-occurring mitochondrial type cannot be separated through backcrossing, effects associated with disruption of mito-nuclear interactions

occur at the same time as those incurred by the Wolbachia infection. To disentangle Wolbachia effects from those caused by mitochondria and mito-nuclear interactions, a subset of flies from introgressed lines was tetracycline treated and their gut flora reconstituted as before. This rendered four additional uninfected lines with mitochondrial types foreign to their nuclear background and absent Wolbachia (Fig. 5.1; iii).

Wolbachia infection status, Wolbachia strain and mtDNA type were confirmed for all experimental fly lines using PCR amplification and sequencing. Briefly, total gDNA was extracted from three individuals of each fly line and amplification of the Wolbachia *ftsZ* gene was carried out. Amplification of the mitochondrial COI fragment was performed in parallel on all gDNA samples as a control for DNA quality. Amplified *ftsZ* and COI fragments were then sequenced and compared to published sequences of these regions in *D.simulans*. To verify that none of the fly lines of this study had been inadvertently contaminated throughout the crossing protocol, we also sequenced intronic regions of the Drosophila *Adhr* and *Gpdh* nuclear genes in all experimental lines, and compared them to those of the four nuclear donors in our crossing scheme (Nuclear donors, Fig. 5.1, i). Samples from these nuclear donor lines were obtained from flies stored soon after the inbreeding process was completed. DNA extraction, amplification and sequencing followed Correa and Ballard (2012). All 16 experimental fly lines harboured the expected infection status, cytoplasmic and nuclear genetic backgrounds (Appendix 5.1, Appendix 5.2)

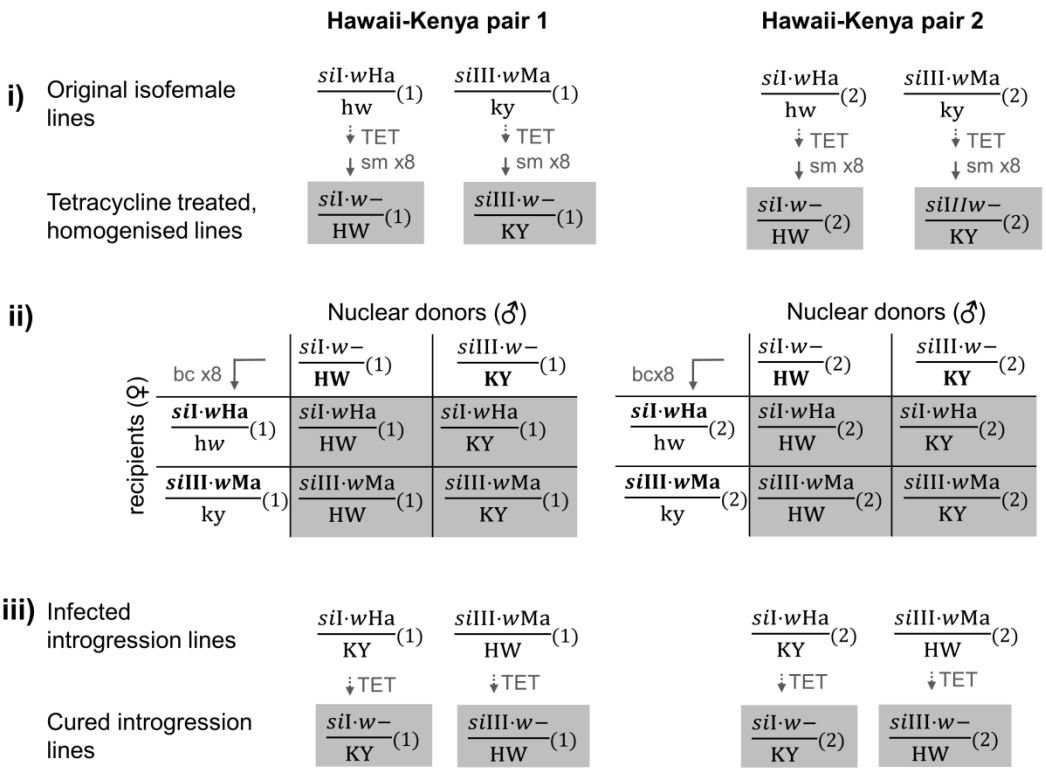

Fig. 5.1 Crossing strategy to obtain Wolbachia infected and uninfected fly lines with combined cyto-nuclear genotypes from Hawaii and Kenya. The genetic makeup of each line is noted: numerator shows cytoplasmic type (*si*I or *si*III) and Wolbachia infection (*w*Ha, *w*Ma or *w*-) and the denominator shows the nuclear background (hw or ky for the original lines or HW or KY after sibling mating). 'TET' represents two consecutive generation of tetracycline treatment, 'sm x 8' represents eight generations of sibling mating and 'bac x 8' represents eight generations backcrossing of the inbred nuclear background into the original lines.

5.2.2 Fly husbandry

Female and male flies from all 16 experimental lines were included in the experiments. Flies from each line were assayed in two experimental blocks that were produced from independent parental flies and separated in time. Prior breeding of experimental individuals, fly cultures were reared at controlled larval density and were maintained in a constant environment at 23 ± 1 °C, 50% relative humidity, 12 h light-dark daily cycle for at least two generations.

Parental flies younger than 14 days were released into cages with oviposition dishes (4% agar and 10% molasses with yeast paste on top) and allowed to lay eggs for 8 h. Egg collection followed Clancy and Kennington (2001). Eggs were placed on 40 mL of standard *treacle-semolina-yeast-agar* media, at a density of approximately 230 eggs per bottle. Upon eclosion, experimental flies were transferred to 1 L cages and maintained at the constant conditions described above. Solid adult diet (following the 1:12 protein to carbohydrate ratio diet in Pichaud et al. (2013)) was provided in vials fitted to the cages and replenished every two days.

Unless stated otherwise, experimental flies were allowed to mate for two days, sorted by sex and placed into new cages. A total of four cages were set up per line and sex for each experimental block, each cage containing between 90 and 120 flies. For most assays, flies were randomly collected from two of these cages by aspiration and allocated to the experimental treatments. Because some assays required measurements

to be taken over a span of several days, the age at which flies were started on each experiment varied, but all flies were tested before 14 days of age.

5.2.3 Oxygen consumption of fly thorax

We used permeabilised muscle fibres dissected from fly thoraces, which has been previously optimised for respiration measurements in the Oxygraph-2k respirometer (Pichaud et al., 2011). The use of permeabilised fibres allows the measurement of cellular oxygen consumption, while maintaining mitochondria and other intracellular components in their natural assembly (Horan et al., 2012). Therefore, this methodology is suitable for the analysis of tissues that harbour both Wolbachia and mitochondria within cells. PCR analyses indicated the presence of Wolbachia DNA in thoraces of Wolbachia infected experimental fly lines (Appendix 5.3).

We measured rates of oxygen consumption of flight muscle in 13-14 day-old flies. The procedure for fibre permeabilisation followed Pichaud et al. (2013). Briefly, thoraces from four to six flies (sourced in equal numbers from two cages to control for the effects of different housing) were dissected and fibres of the flight muscle loosened in BIOPS relaxing solution. Fibres were subsequently incubated in saponin-supplemented BIOPS (63 µg/mL) and washed in respiration medium. All permeabilisation steps were conducted on ice. Prior to each run, both respirometer chambers were calibrated with air-saturated respiration medium at 23 °C. After air calibration, the complex I linked substrates malate, pyruvate and proline were added to the medium to a final concentration of 10 mM each. Permeabilised fibres were blotted, split in two groups and

weighed using a Sartorius CP2P Electronic Micro Precision Balance (Sartorius, Gottingen, Germany), after which they were transferred into the two chambers of the Oxygraph-2k (Oroboros Instruments, Innsbruck, Austria). Chambers were then partially closed and oxygen was injected and allowed to reach a concentration of 600 nmol/mL, which facilitates correct O_2 diffusion through the tissue (Gnaiger, 2009). Chambers were then sealed completely to stop further gas exchange with the outside and signal acquisition was monitored.

Oxygen consumption per milligram of permeabilised muscle fibres was measured in the presence of substrates. Basal respiration rate (state 2') was obtained after stabilisation of the oxygen flux signal. Phosphorylating respiration (state 3) was obtained after the addition of ADP (5 mM). Respiratory control ratios (RCRs, state 3/state 2'), commonly reported as indicator of coupled electron transport system (ETS) to ATP synthesis (Gnaiger, 2009) were also calculated per sample. ADP injection was followed by cytochrome *c*, included to calculate an index of functional integrity of the outer mitochondrial membrane (cytochrome *c*/State 3) (Gnaiger and Kuznetsov, 2002). This index assesses the quality of the muscle preparations and preparations with an index higher than 1.1 were rejected. Injections that followed were *sn* glycerol-3-phosphate (20 mM) to monitor maximum respiration and the complex III inhibitor antimycin A (2.5 μM). Inhibition of complex III allowed measuring the residual oxygen consumption of the fibres that was unrelated to oxidative phosphorylation, to be subsequently subtracted from all previously measured rates. Measurements obtained from the two respirometer chambers were averaged for each run. Four to five replicated runs were performed per line, sex and experimental block.

5.2.4 Life-history traits

5.2.4.1 Feeding rate

Food consumption of flies was measured using a modified protocol from Ja et al.(2007). Briefly, Drosophila vials containing 5 mL of 1% agar were capped with foam plugs for adequate air circulation. These plugs were fitted with a single truncated pipette tip that held a 5 µL graduated capillary micropipette (VWR, Pennsylvania, USA) containing liquid diet with the same composition of that supplied to the population cages (P:C = 1:12), but without agar as solidifying agent.

Three 7 day-old randomly selected flies from two population cages were collected by aspiration, placed into capillary-fitted vials and allowed to acclimate to this feeding setting for 24 h. Food in micropipettes was replaced daily and intake was measured for the three flies in each vial for five consecutive days. Identical vials without flies were included to control for loss of food volume by evaporation. A total of six replicate vials were set up per line and sex in each block. Food consumption was calculated as the average volume of liquid food consumed per fly per day.

5.2.4.2 Starvation

For starvation resistance assays, we used a modified protocol from Kenny et al., (2008), supplying 1% agar as *ad libitum* water source. Briefly, two of the four cages per line, sex and experimental block were set aside for starvation measurements. Once flies reached 12-13 days of age, cages were inspected and dead flies removed, after which vials with adult diet were replaced with vials containing 5 mL of the 1% agar solution.

Dead flies were removed and counted every 8 h until all flies were dead. Starvation resistance was measured as the time passed for 50% and 90% of the flies in a population cage to die.

5.2.4.3 Egg laying and hatching

To measure oviposition, 2 day-old non-virgin females and males from each line were randomly taken from cages and placed as single couples into 15 mL plastic screw-capped tubes, which were previously truncated to fit a foam plug. Food was provided by topping the inside of the screw caps with 200 µL of solid 1:12 PC diet. This setting provided a food surface from which flies fed and lay eggs upon, that could be replaced with minimum disruption to the flies.

Individual fly pairs were allowed to acclimate to this setting for a day, after which lids were collected and replaced by fresh ones. From 4 days of age, eggs laid were counted and lids were returned back to the incubator for additional ~32 h after which the unhatched eggs were checked. Fecundity data is reported as the total number of eggs laid per fly over the 11-day period. Egg hatching was calculated as 'laid eggs – unhatched eggs/ laid eggs' over the same time period. A total of 10 replicated pairs of flies were set up per line and block. Replicates in which either female or male died during the experiment were excluded from analyses.

5.2.5 Statistical analysis

Prior inclusion into statistical models, datasets obtained for each assay were tested for outliers and their frequency distribution was examined. Subsequently, appropriate data transformations were conducted for their inclusion in analysis of variance (ANOVA). Statistical analyses were performed in two steps. First, to evaluate the effect of sex, nuclear and cytoplasmic genetic backgrounds on the variables measured in this study, we performed an ANOVA that included these variables as main effects as well as all their interaction terms. Because of the coinheritance with mitochondria, Wolbachia effects were tested as nested effect within the three main factors. This ANOVA included complete datasets for each experiment.

Two subsequent ANOVA models tested Wolbachia related effects separately for flies with native and introgressed cyto-nuclear genotypes. These models aimed to directly investigate Wolbachia related effects on different genotypes without the confounding effects of mito-nuclear interactions. In here, ANOVA included sex, Wolbachia infection and genotype as main effects and as well as their interaction terms. This two-step analysis was used on all datasets, with only minor adjustments for each assay. Measurements of oxygen consumption, feeding rate and starvation resistance included sex a main factor, while egg laying and hatching did not. Also, for feeding rate the factor 'day' was included as random effect. For all analyses, the effect of replicated fly lines was included as nested effect within other factors. Also, variation due to experimental blocks was included in the models as a random effect. Where significant effects were detected for interaction terms, post-hoc Tukey's Studentised HSD tests

were conducted. All statistical analyses were performed using JMP® 10 software (SAS Institute, Cary, IN, USA). ANOVAs were performed using the REML method for variance components of random effects. Significance was defined at $p < 0.01$ to reduce false discovery rate.

5.3 Results

5.3.1 Oxygen consumption of fly thorax

First, the quality of muscle preparations was determined by the addition of cytochrome *c* and the inspection of RCRs. Exogenous cytochrome *c* did not result in marked increase of oxygen consumption, indicating the integrity of the inner mitochondrial membrane of our preparations. Two one-sided *t*-tests performed on the ratio cytochrome *c* /state 3 confirmed it was between 1 ($t_{282} = 22.96$, $p < 0.001$) and 1.1 ($t_{282} = -16.68$, $p < 0.001$). In addition, RCRs were 20.46 ± 0.70 for female thorax preparations and 17.70 ± 0.58 for males, which further confirmed the suitability of our preparations for analysis.

Basal respiration rates were uniform across sexes, nuclear backgrounds and cytoplasmic types (Fig. 5.2, Table 5.1). Uninfected *si*III/HW flies showed approximately 60% higher basal respiration rates than *w*Ma infected flies with the same genotype. Testing the effects of infection separately on native and introgressed fly lines confirmed effects of infection were only significant for the introgressed *si*III/HW flies (Table 5.2).

As shown by ANOVA (Table 5.1), phosphorylating and maximum respiration rates differed between sexes; female muscle fibres consumed oxygen at higher rates than

male fibres. The significant effects of cytoplasmic type on respiration depended on the nuclear background of the fly (Table 5.1). Post-hoc Tukey's HSD test indicated that *si*I/HW flies had reduced oxygen consumption compared with *si*I/KW flies and flies with *si*III cytoplasmic types (Fig. 5.2). This statistical model, however, did not find significant differences between Wolbachia infected and uninfected flies with the same cyto-nuclear makeup.

Subsequent ANOVA conducted separately on native and introgressed flies showed that there were no significant effects of infection in flies with native cyto-nuclear combinations and that respiration rates were mostly affected by nuclear genotype and sex (Table 5.2). Conversely, flies with disrupted cyto-nuclear genotypes differed in their phosphorylating respiration rates when Wolbachia was present. Post-hoc Tukey's HSD test confirmed that the *si*III/HW flies consumed oxygen at lower rates when *w*Ma Wolbachia was present (Fig. 5.2).

Table 5.1 Summary of *F* statistics obtained with ANOVA models including Wolbachia infection (I) as a nested effect within sex (S), nuclear background (N) and cytoplasmic type (C).

	Main factors				Interaction terms				Nested effects		Random effects (% var)	
	Error df	Sex (S) *df* = 1	Nuclear background (N) *df* = 1	Cytoplasmic type (C) *df* = 1	S x N *df* = 1	S x C *df* = 1	N x C *df* = 1	S x N x C *df* = 1	Infection (I) *df* = 8	Line *df* = 16	Block	Day
O_2 consump.												
Basal	31.80	0.01	5.61*	1.35	0.52	0.01	0.14	0.07	4.97** (31.67)	1.26 (31.51)	8.24	
State 3	34.22	39.58***	24.18***	37.92***	4.00	0.01	16.84**	3.18	1.95 (34.64)	3.85** (33.87)	6.85	
Max	34.28	8.10**	21.08***	24.14***	2.49	0.01	5.9*	1.54	1.14 (34.22)	2.65** (34.16)	21.171	
Feeding rate	31.86	401.67***	0.8072	22.84***	11.02**	1.23	108.66***	6.15*	9.48*** (31.81)	20.74*** (31.77)	1.42	0.58
Starvation												
50%	33.01	935.99***	273.01***	0.14	347.62***	101.66***	7.70*	0.01	6.9*** (32.79)	6.29*** (32.58)	2.09	
10%	32.59	746.47**	75.67**	0.19	255.08**	103.11**	3.60	0.01	9.38*** (32.40)	5.90*** (32.22)	12.09	
Eggs												
Laid	15.94		2.39	3.83			6.14		4.27 (15.94)	1.47 (15.94)	15.74	
Hatched	16.44		4.34*	61.65***			23.28***		2.82 (16.35)	3.80 (16.29)	12.87	

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.0001$

Table 5.2 Summary of *F* statistics obtained with ANOVA models performed separately for native and introgressed Drosophila. Wolbachia infection (I), genotype (G) and sex (S) are tested as main effects.

		Main effects			**Interaction terms**				**Nested effect**	**Random (% var)**	
	Error df	S *df* = 1	Genotype (G) *df* = 1	I *df* = 1	S x G *df* = 1	S x I *df* = 1	G x I *df* = 1	S x G x I *df* = 8	Line	Block	Day
						Native cyto-nuclear genotypes					
O_2 consump.											
Basal	17.2	0.07	0.91	0.47	0.44	0.29	2.46	0.86	0.39 (16.92)	5.20	
State 3	17.3	27.82***	52.31***	0.11	1.85	0.02	0.21	0.43	4.49 (17.66)	8.97	
Max	16.9	5.60*	30.45**	1.16	0.74	0.07	0.24	0.04	2.60* (16.87)	31.64	
Feeding rate	16.02	153.34**	15.19**	0.86	9.23***	0.10	0.63	2.81	24.23*** (15.97)	1.20	4.33
Starvation											
50%	16.57	443.97**	140.11**	5.91*	394.29***	5.29*	10.16***	0.39	6.27*** (16.40)	0.00	
10%	16.07	721.17**	67.56**	14.88	666.58***	0.60	53.98***	1.04	9.17*** (15.87)	0.00	
Eggs											
Laid	7.99		0.10	0.01			5.53*		0.58 (7.99)	10.50	
Hatched	8.23		54.17***	5.44*			4.69		7.21** (8.14)		
						Introgressed cyto-nuclear genotypes					
O_2 consump.											
Basal	15.28	0.02	5.05*	13.52**	0.15	0.87	13.05**	3.24	1.80 (15.23)	10.82	
State 3	16.67	12.39**	0.98	9.19**	2.25	0.81	6.08*	2.40	2.96* (16.58)	4.00	
Max	16.65	2.75	0.17	5.57*	2.85	1.33	7.59*	0.59*	2.95* (16.54)	2.04	
Feeding rate	15.80	248.84***	7.33*	55.53***	2.35	0.18	0.38	12.36***	16.60*** (15.80)	1.86	1.69
Starvation											
50%	16.43	461.40***	131.14***	21.41***	35.89***	2.37	1.06	8.23	6.43*** (16.20)	5.42	
10%	16.22	256.40***	28.56***	13.49***	11.62***	4.13	1.74	7.60*	4.87*** (16.07)	26.34	
Eggs											
Laid	7.98		5.12	4.16			6.39*		2.08 (7.98)	21.09	
Hatched	8.24		10.21**	2.87			0.05		0.94 (8.18)		

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.0001$

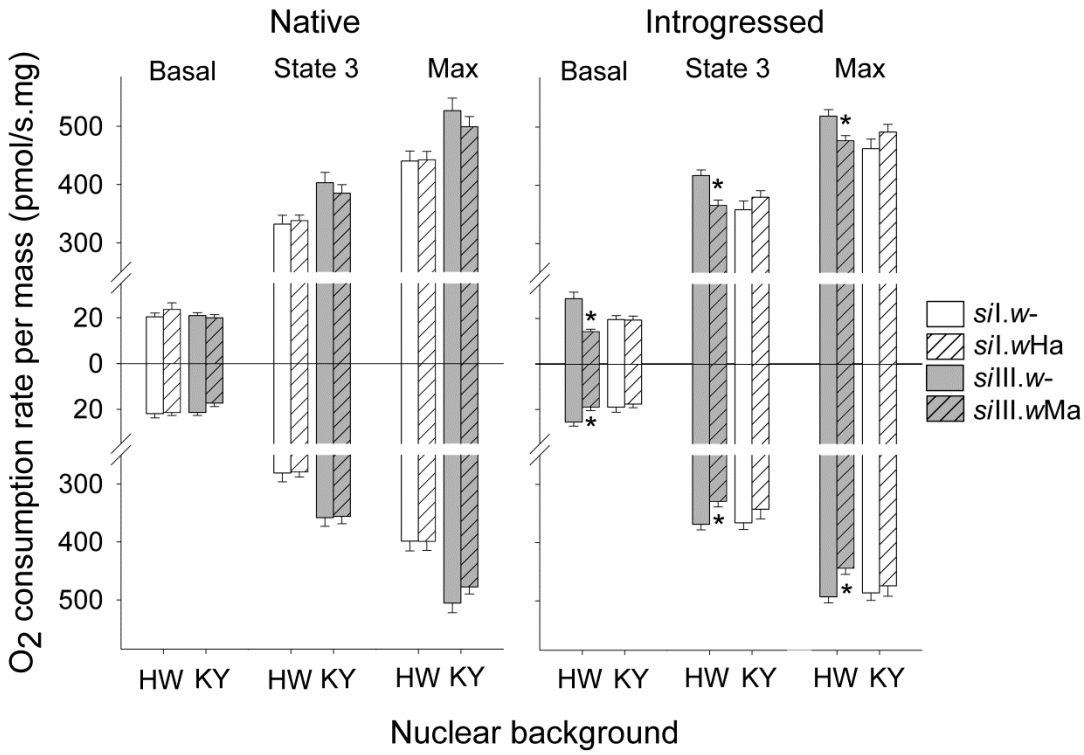

Fig. 5.2 Basal, state 3 (phosphorylating) and maximum oxygen consumption rates of flight muscle in female (upper chart) and male (lower chart) *Drosophila simulans*. Genotypes of the flies tested here consisted of combined nuclear (HW and KY) and cytoplasmic backgrounds (*si*I and *si*III) from Hawaii and Kenya. Tukey's *post hoc* test was employed to detect differences between infected and uninfected flies that shared the same cyto-nuclear genotype. Significant differences are noted by *. Bars represent means ± SEM. Significance was set at $p < 0.01$.

5.3.2 Life history traits

5.3.2.1 Feeding rate

The amount of food consumed by individual flies was significantly affected by sex, cytoplasmic type and Wolbachia infection. Interactions effects of nuclear background with sex (S x N) and with cytoplasmic type (N x C) were also significant according to ANOVA on the whole dataset (Table 5.1). In general, female flies consumed at least 30% more food than males. The effect of Wolbachia infection on flies with the same cyto-nuclear makeup was significant; infected flies consumed nearly 10% less food than uninfected flies.

Analysis of flies with native nuclear backgrounds showed a significant interaction effect between sex and genotype (S x G); Tukey HSD test further indicated that females with Kenyan genotypes ate more than those with Hawaiian genotypes, but such difference was not observed in males (Table 5.2). In flies with native cyto-nuclear genotypes, however, feeding rate did not differ by the presence of Wolbachia (Fig. 5.3). In contrast, the feeding rate of flies with disrupted cyto-nuclear genotypes varied significantly with Wolbachia infection, and such Wolbachia effects depended on the sex and the genotype of the flies. The effect of infection reducing amount of ingested food was higher in *si*III/HW males (31%), followed by *si*I/KY females (16%), *si*III/HW females (7%) and *si*I/KY males (6%) (Fig. 5.3).

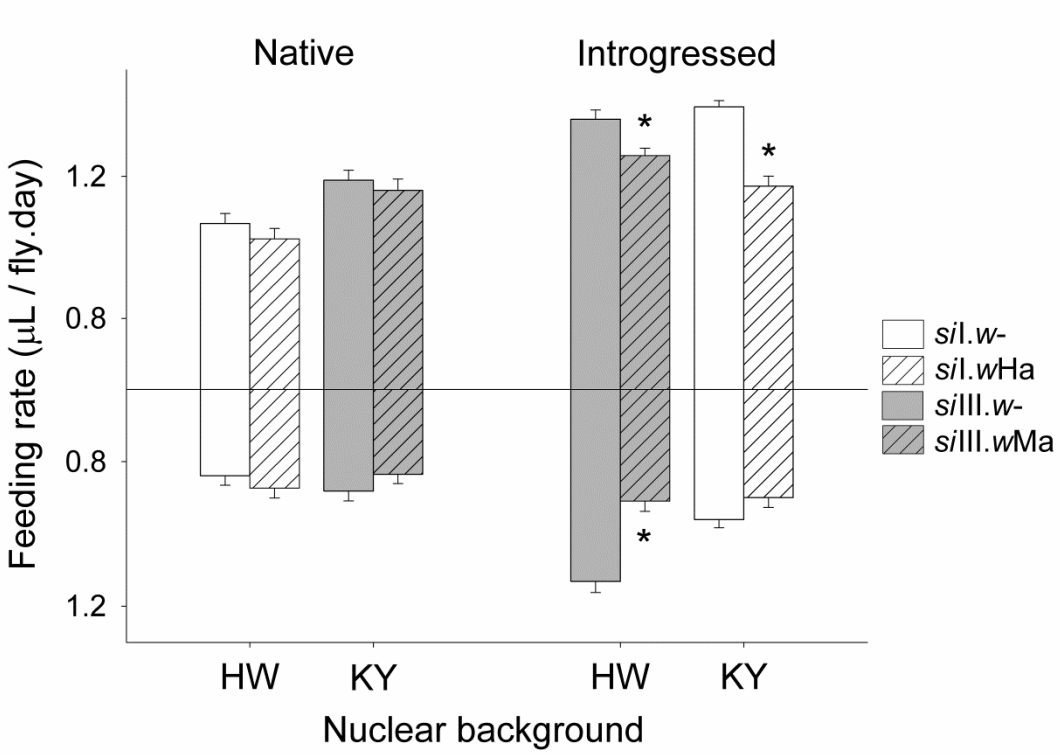

Fig. 5.3 Mean daily amount of food consumed by individual female (upper chart) and male (lower chart) *Drosophila simulans*. Genotypes of the flies consisted of combined nuclear (HW and KY) and cytoplasmic backgrounds (*si*I and *si*III) from Hawaii and Kenya. Tukey's *post hoc* test was used to detect differences between infected and uninfected flies that shared the same cyto-nuclear genotype. Significant differences are noted by *. Bars represent means ± SEM. Significance was set at $p < 0.01$.

5.3.2.2 Starvation

Starvation resistance, measured as the time elapsed for 50% and 90% of the flies in a population cage to die in absence of a food source, was largely determined by sex and nuclear background. The interaction between those two terms (S x N) as well as that between sex and cytoplasmic type (S x C) also showed to be significant (Table 5.1). In general, female flies lived approximately 85% longer than males. *Post hoc* Tukey's HSD test showed that females with HW genetic backgrounds lived the longest regardless of their cytoplasmic type, and were followed by *si*I/HW and *si*III/HW flies. Males, on the other hand, lived the longest when harbouring *si*III cytoplasmic types and *si*I/HW males were the shortest-lived. The effects of Wolbachia were also significant according to this ANOVA model; infected females and males lived longer (13% and 6%, respectively) than their uninfected counterparts.

The effect of Wolbachia in flies with native cyto-nuclear combinations depended on the genotype (Table 5.2); *post hoc* Tukey's HSD test indicated females with Kenyan genotypes lived longer when harbouring *w*Ma (Fig. 5.4). The effects of Wolbachia infection in flies with disrupted cyto-nuclear genotypes were more complex: in females, Tukey's HSD test showed starvation resistance to be highest in *si*III/HW flies regardless of their infection status, while infected *si*I/KY flies had increased starvation resistance compared to uninfected flies with the same genotype. In males, Tukey's test revealed that uninfected *si*I/KY flies had the lowest starvation resistance among males, and infected *si*III/HW males the highest (Fig. 5.4).

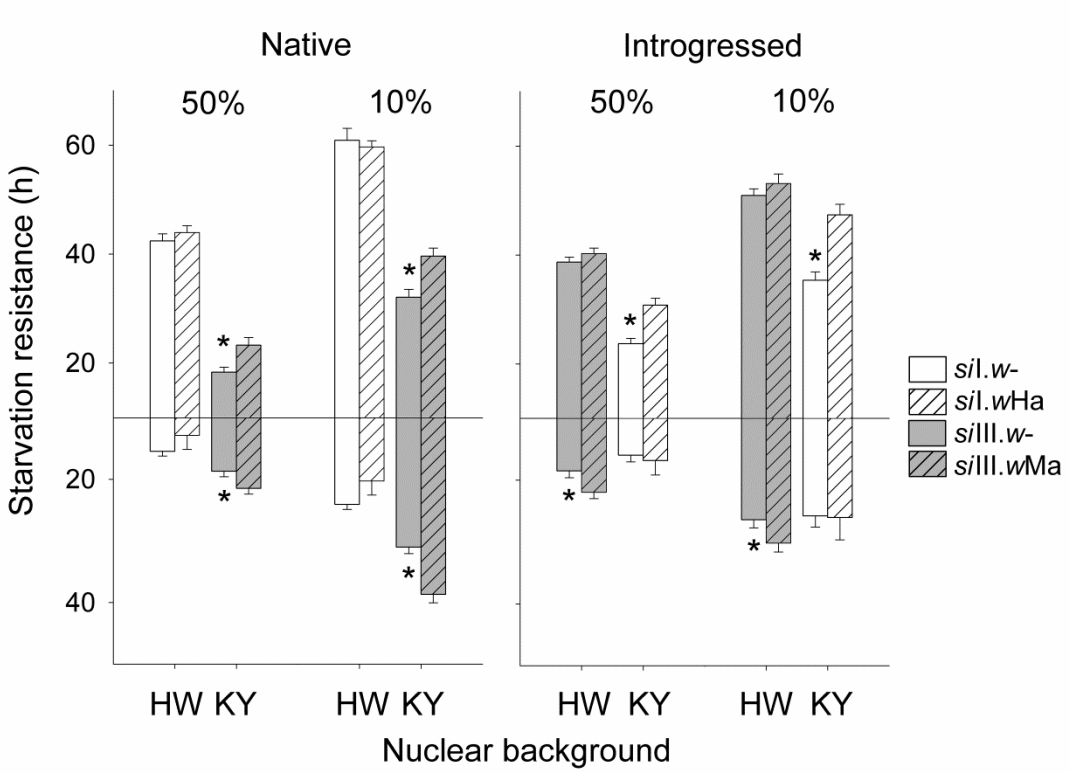

Fig. 5.4 Starvation resistance (50% and 10%) of female (upper chart) and male (lower chart) *Drosophila simulans*. Fly genotypes combined nuclear (HW and KY) and cytoplasmic (*si*I and *si*III) genetic backgrounds from Hawaii and Kenya. Tukey's *post hoc* test was used to detect differences between infected and uninfected flies that shared the same cyto-nuclear genotype. Significant differences are noted by *****. Bars represent means ± SEM. Significance was set at $p < 0.01$.

5.3.2.3 Egg laying and hatching

No significant effects of cytoplasmic type, nuclear backgrounds or Wolbachia infection on egg laying were found with ANOVA on the whole dataset at an alpha level of 0.01 (Table 5.1). However, some trends can be identified from the independent analysis of flies with native and introgressed nuclear backgrounds. First, infection in native flies appeared to differ for the two fly populations; Hawaiian flies seemed to lay fewer eggs when infected by *w*Ha, while Kenyan flies laid more eggs if *w*Ma was present (Fig. 5.5A). While ANOVA showed this interaction (N x I) could be significant at an alpha level of 0.05 (Table 5.2), *post hoc* Tukey's test did not statistically support such Wolbachia effects. In flies with introgressed cyto-nuclear genotypes, egg laying was similar across flies, except for the *si*III/HW uninfected flies that laid fewer eggs (approximately 20% less). Post-hoc Tukey's test confirmed the statistical significance of this observation ($0.01 < p < 0.05$).

Unlike egg laying, egg hatching varied across flies with different cyto-nuclear genetic backgrounds. ANOVA on the complete dataset showed significant effects of cytoplasmic type on egg hatching, such effects being largely influenced by the nuclear background of the fly (Table 5.1). Tukey's post-hoc test identified *si*I/HW flies as the ones with highest hatch rate, followed by those with KY nuclear backgrounds (regardless of their cytoplasmic type). The *si*III/HW flies displayed the lowest egg hatching proportion (Fig. 5.5B). No Wolbachia-related effects were found with ANOVA on the whole dataset (Table 5.1), nor by testing native and introgressed flies separately (Table 5.2).

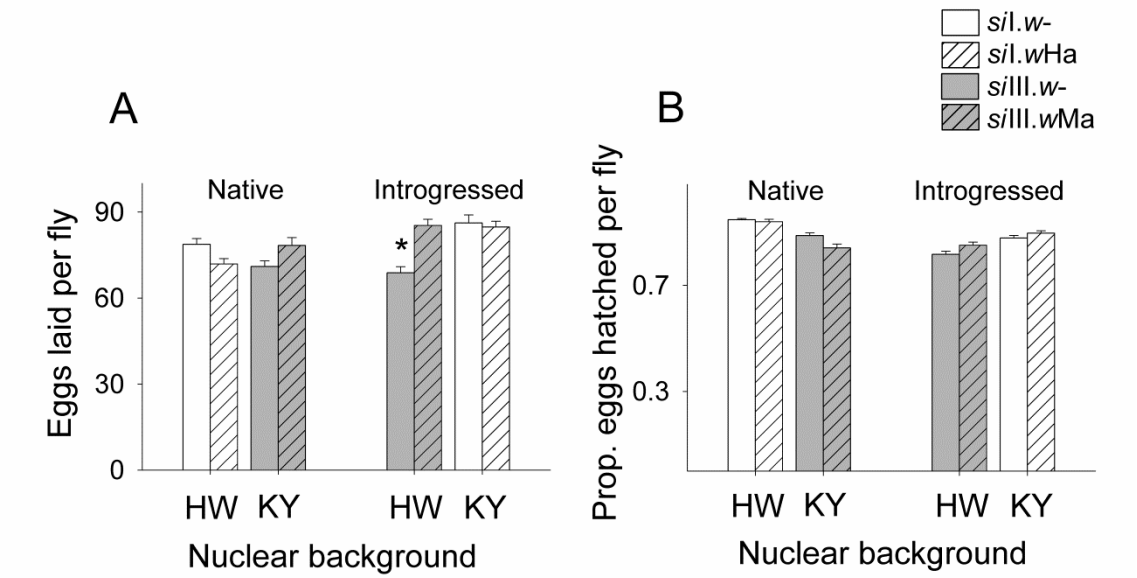

Fig. 5.5 Number of eggs laid (A) and proportion of eggs hatched (B) of *Drosophila simulans* with combined nuclear (HW and KY) and cytoplasmic (*si*I and *si*III) genetic backgrounds from Hawaii and Kenya. Tukey's post hoc test was used to detect differences between infected and uninfected flies that shared the same cyto-nuclear genotype. Significant differences are noted by *****. Bars represent means ± SEM. Significance was set at $p < 0.05$.

5.4 Discussion

Natural selection favours symbioses with maternally inherited bacteria when infected females have a fitness advantage over their non-infected counterparts. Such advantage may occur either directly by the gain of physiological benefits from infection or indirectly by reduction of reproductive success of non-infected females, as is the case with CI (Turelli, 1994). It has been hypothesised that Wolbachia with attenuated or absent CI phenotypes are more likely to display characteristics of mutualists than strong CI inducers, as infection permanence in host populations would rely on such traits (O'Neill et al., 1997). In this study we tested the hypothesis that benign effects of infection are more noticeable in associations with weak CI-inducing Wolbachia. Additionally, we tested if beneficial effects of Wolbachia are more likely to be detected in flies with nuclear backgrounds that occur naturally with a particular infection, as would be predicted by coadaptation. Our results show that Wolbachia infection significantly affects respiration rates and life history traits of the host, and such effects are highly dependent on the fly genotype. Although the prediction of beneficial traits associated with weak CI-inducing Wolbachia is supported here, the hypothesis that advantageous traits are more evident in individuals with genetic backgrounds 'adapted' to the infection is not strongly supported by our results.

The only direct beneficial effect of Wolbachia infection in its native genetic environment was found for the weak CI-inducing *w*Ma. Specifically, Kenyan flies revealed increased survival during starvation assays when harbouring *w*Ma. Starvation is recognised a major stress in natural populations of (Hoffmann and Parsons, 1991) and

levels of starvation tolerance may be directly affected by natural selection (Hoffmann et al., 2001a). Studies on the starvation resistance in *Drosophila melanogaster* showed that flies from Afrotropical populations displayed markedly higher tolerance to food deprivation than flies from other locations (Da Lage et al., 1990), which could suggest that starvation resistance is selectively advantageous for fly populations inhabiting that geographical region. Extrapolation of our results to natural population, however, needs to be taken with caution, as starvation resistance, as well as other stress related traits in Drosophila, changes with the process of laboratory adaptation (Hoffmann et al., 2001b). Indeed, previous assays on wild caught *D. simulans* from Kenya (Ballard et al., 2008) showed levels of starvation resistance that were double of those observed here. Nonetheless, our finding may still represent a relevant physiological effect of infection in flies, further supported by the fact that male flies with a foreign nuclear background also appeared to stand starvation better when infected by *w*Ma.

Intriguingly, effects of Wolbachia infection appeared to be more conspicuous in flies with disrupted cyto-nuclear genotypes. Flies with *si*III cytoplasmic types and nuclear backgrounds from Hawaii showed to be particularly affected by *w*Ma infection; infected flies had lower basal, phosphorylating and maximum respiration rates, consumed less food, had higher starvation resistance and laid more eggs than their uninfected counterparts. Flies with *si*I cytoplasmic types and Kenyan nuclear backgrounds also consumed less food and had increased starvation resistance when infected with *w*Ha. This may be the case for a number of reasons. A simple explanation would be that the effects of Wolbachia infection vary across different host nuclear backgrounds, and by altering the background of infected flies, effects of Wolbachia could become masked or

unmasked. Fry and Rand (2002), for example, found that life-extending effects of Wolbachia in *D. melanogaster* were more noticeable in hybrids from inbred stocks than in either of their parental fly strains, suggesting an interaction between Wolbachia and inbreeding levels of the host. The Wolbachia-host interaction effects observed here were significant for two independently generated replicate lines, which suggests that the underlying genetic cause of such interaction effects is better explained by the genetic differentiation between fly populations than by the effects of randomly placing Wolbachia in a novel genetic background.

A second hypothesis is that beneficial effects of Wolbachia may be more noticeable in flies with disrupted mito-nuclear genotypes, where it could act as a phenotypic 'rescuer'. Mitochondrial coadaptive theory predicts reduced performance of non-coadapted mito-nuclear genotypes (Edmands and Burton, 1999; Rand et al., 2006). In such genetic environments, the metabolic capacity of Wolbachia may aid to restore function of sub-optimal mito-nuclear phenotypes (Chen et al., 2012). Phenotypically, it has been shown that male flies with an *si*I mitochondrial type had poorer locomotor activity and survival when present on *si*II or *si*III-adapted nuclear backgrounds, and males with *si*III mtDNA developed slower on an *si*I-adapted nuclear environment (James and Ballard, 2003). Additionally, here we show that uninfected flies with disrupted cyto-nuclear interactions consumed more food than flies with undisrupted cyto-nuclear genetic makeups. Interestingly, infected flies with the same genotypes consumed less food. The study of respiration rates is particularly appropriate for the testing of this hypothesis, as the function of the mitochondrial ETS requires a highly coordinated expression of both nuclear and mitochondrial genes.

Here, we observed that phosphorylating and maximum respiration rates in *si*III/HW flies are significantly higher than in *si*I/HW flies and comparable to those of flies with native Kenyan genotypes. At first glance, these results point towards mtDNA as the main factor influencing ETS function in flies with Hawaiian nuclear backgrounds. Closer examination of basal respiration rates, however, showed that these flies consumed significantly more oxygen in absence of ADP, which may indirectly indicate that the efficiency of substrate utilisation is lower due to proton leak (Brand et al., 2005; Jastroch et al., 2010). Notably, *w*Ma infected flies with the same cyto-nuclear genotypes had basal respiration rates comparable to native *si*III flies. Phosphorylating and maximum respiration rates in these infected flies were also lower than in their uninfected counterparts. This indicates a possible role of Wolbachia assisting the process of host energy production by decreasing rates of proton leak. Measuring the kinetics of membrane potential in mitochondrial isolates could provide direct evidence on differences of proton leak between infected and uninfected cohorts (Katewa and Ballard, 2008).

These results align with the observations that Wolbachia infected *si*III/HW flies required less food and had higher starvation resistance, as reduced rates of proton leak would translate into more efficient substrate utilisation by the mitochondria. Increased egg laying of infected flies may also be a consequence of Wolbachia compensating for sub-optimal metabolic function (Fig 5.6). One mechanism in which Wolbachia could improve sub-optimal ETS function is by provisioning the host with heme groups (Brownlie et al., 2007), whose function as electron carrier in the cytochrome *c* molecule could ultimately facilitate the transport of electrons from complex III to complex IV in

ETS. Such mechanism appears of importance in the filarial nematode *Litomosoides sigmodontis*, which upon Wolbachia clearance was found to up-regulate heme-dependent respiratory chain complexes in an attempt to maintain metabolic homeostasis (Strübing et al., 2010).

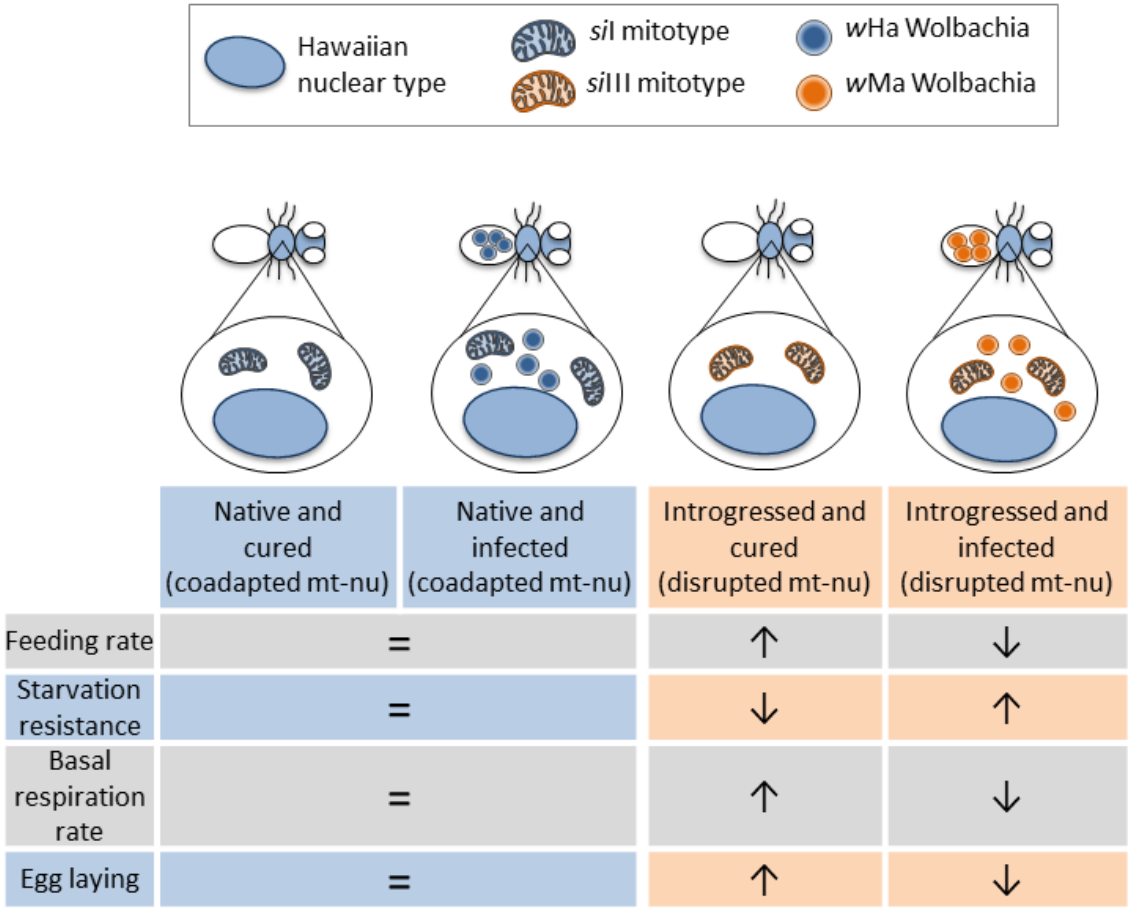

Fig. 5.6: Effects of varying Wolbachia and mitochondrial types in flies with Hawaiian nuclear backgrounds. In flies with the native *si*I mitochondrial type, *w*Ha infection does not appeat to significantly affect the measured traits. When naturally occurring mito-nuclear complexes were disrupted by introgression of the foreign *si*III cytoplasmic type, *w*Ma infected flies diplayed lowered feeding rate, increased resistance to starvation and lowered basal respiration rates than they cured counterparts. These results are compatible with a hypothesis of Wolbachia acting as a metabolic 'rescuer' for individuals with mildly deleterious cyto-nuclear genetic makeups.

Extending on this line of thought, it may be plausible that the co-occurrence of *w*Ma Wolbachia with *si*III mtDNA may, to some extent, counteract the effects of mildly deleterious *si*III-nuclear genotypes in natural populations. Flies with *si*III haplotypes are often found in sympatry with *si*II flies at a frequency of 40%, with one to two thirds of the *si*III flies infected by *w*Ma (Ballard, 2004). Comparisons of these two haplotypes have unambiguously shown that *si*II mitochondria have higher catalytic capacities than *si*III mitochondria (Pichaud et al., 2011), even when present in nuclear backgrounds of *si*III flies (Pichaud et al., 2012). In addition, relative fitness of *si*III mitochondria, assessed by haplotype fixation after microinjection in eggs, showed that *si*II injected mitochondria outcompete *si*III while the reverse does not occur (De Stordeur, 1997). Our results, may point to Wolbachia as a possible reason for the maintenance of the seemingly weaker *si*III mitochondria in sympatry with *si*II (James and Ballard, 2003). This prediction has some support in the observation that the competitive advantage of native *si*III flies is enhanced by the artificial introduction of *w*Ha (Dean, 2006). Testing this hypothesis would require measurement of life history and bioenergetic traits in infected and uninfected cohorts of *si*II and *si*III flies, and possibly, under different levels of environmental stress.

It is intriguing that *si*III/HW introgressed genotypes produced measurable changes in mitochondrial metabolism but the *si*I/KY did not. This is especially true considering that *si*I mitochondria often appears to have reduced fitness than *si*II or *si*III (De Stordeur, 1997). Due the lack of mtDNA diversity of Hawaiian flies (Ballard, 2004), it could be the case that Hawaiian nuclear backgrounds are highly adapted to *si*I

mitochondrial types, which may cause the effects of mito-nuclear disruption to be more severe. It is also plausible that the elevated genetic diversity of the Kenyan population (in both mitochondrial and nuclear genomes) results in Kenyan genetic backgrounds that are more tolerant to mtDNA diversity. It could be speculated that under more stringent environmental conditions, deleterious effects of the *si*I/Kenya genotypes may be expressed. A more in detailed analysis of mitochondrial respiration could also reveal slight changes in catalytic capacities of OXPHOS between introgressed and native flies. For example, Pichaud and colleagues (2012) detected significant differences in catalytic capacities of specific ETS complexes of *si*II and *si*III flies by using a protocol that allowed measuring the function of various of OXPHOS enzymes separately.

Our results support the idea that weak CI-inducing Wolbachia persist in natural populations as they convey beneficial characteristics to the host. Explaining the persistence of weak CI-inducers such as *w*Ma has represented an important challenge for theoretical models with CI as the main driver of Wolbachia invasion. Recent theoretical work has more accurately predicted the persistence of weak CI-inducing Wolbachia by including direct beneficial effects of infection to the host. Fenton and colleagues (2011) show that different levels of beneficial Wolbachia traits (specifically, the protection against natural enemies) could widen the conditions for Wolbachia invasion and greatly facilitate persistence of infection, even for 'poor' strains with weak CI and inefficient vertical transmission. The fact that the seemingly beneficial traits of *w*Ma infection were mostly found in flies with disrupted cyto-nuclear genotypes did not support our initial prediction of improved fitness in coevolved symbiont-host associations. A plausible explanation for this is that beneficial effects of Wolbachia are

difficult to detect in the benign conditions of the laboratory, as they often dependent on the host genetic background and the environment (Dean, 2006; Fry et al., 2004; Fry and Rand, 2002; Reynolds and Hoffmann, 2002). An example of this is presented by Brownlie and colleagues (2009) who demonstrate that *w*Mel, a well characterised low-CI inducing strain of *D. melanogaster*, plays an important role in iron metabolism in the fly. Such role is particularly important when dietary iron supplementation is poor, as would be the case in the field, but not in nutrient-rich laboratory diets. Strategies that may prove useful for testing the effects of Wolbachia as metabolic 'rescuer' include the examination of Wolbachia effects of under a range of nutritional stress (Lee et al., 2008) and the use of mutant Drosophila lines with deficient metabolic pathways.

There are a number of limitations to this study. First, the use of backcrossing as the strategy to replace the nuclear background of native flies may not be entirely effective in breaking host-symbiont interactions, which may lead to an underestimation of the effects of Wolbachia on foreign nuclear backgrounds. Despite this, significant interaction effects between nuclear background and infection were found across all experiments, which may indicate adequate levels introgression. Another limitation is that only two replicated fly lines were tested per infection/cytoplasmic/nuclear combinations. Given the importance of nuclear genetic variation on Wolbachia effects, the use of multiple replicated fly lines or flies with standard outbred backgrounds may be necessary to unambiguously characterise host-symbiont interaction effects.

The results of this study agree with the current notion that Wolbachia, though non-essential, may be beneficial and can confer advantages under certain circumstances

(Duron and Hurst, 2013). In this particular study, we found evidence that Wolbachia may be beneficial in flies with mildly deleterious cyto-nuclear phenotypes, which indicate a role of Wolbachia as metabolic rescuer. Moreover, beneficial effects appear intrinsic to the symbiont rather than the product of coadaptation. Here, we demonstrate the usefulness of *in-situ* bioenergetic techniques in the evaluation of Wolbachia effects in flies. The link between bioenergetic differences and life history traits found here indicates that Wolbachia may influence fundamental metabolic processes at the cellular and molecular level. This not only supports the notion that intracellular symbionts are an important source of evolutionary innovation, but also suggests that bioenergetics may provide a link between the genotype and the phenotype in Wolbachia symbiosis.

Chapter 6

General discussion

6.1 Introduction

This work examined host adaptation to Wolbachia infection and mtDNA variation from a 'conflict of interest' perspective. The aim of this thesis was to expand on the knowledge of the evolutionary trajectories of functional cytoplasmic genetic variants, with emphasis on Wolbachia-host associations. There are three major conclusions that can be drawn from this work. Firstly, phenotypes related to cytoplasmic genetic variants (understood as mtDNA functional variation and presence or absence of Wolbachia infection) are highly contingent on the sex, physiological state and nuclear background of the host, as well as the environment. Secondly, adaptation of the host genome to cytoplasmic genetic variants is linked to the effects that those variants exert upon the organismal fitness. Finally, maintenance of cytoplasmic genetic polymorphism in populations may be explained if such diversity poses beneficial effects contingent to host genetic background and the environment. This discussion focuses on how the research conducted here adds to the current understanding of the evolution of Wolbachia symbioses and how new paradigms on the evolution of variation at the level of mtDNA may aid to such understanding. Specifically, I discuss how the data presented in this thesis fits within the body of knowledge in support of three major predicted outcomes of evolutionary trajectories of Wolbachia-host associations: Obligate symbioses, extinction and stable facultative symbioses.

6.2 Coadaptation and the evolution towards obligate symbioses

Early predictions on the evolution of intracellular heritable symbionts suggest that lack of free living stages and vertical transmission of symbionts align the reproductive interests of symbiont and host, prompting coadaptation (defined as the gradual, coordinate and reciprocal changes of both partners in benefit the symbiosis) and eventually leading to obligate mutualism (Ewald, 1987; Yamamura, 1993). This receives support from the fact that many intracellular symbionts are indispensable for their hosts' survival and reproduction (Wernegreen, 2004). Although coadaptive processes may have been crucial shaping the evolution of obligate symbioses such as Buchnera-aphids or even in Wolbachia-nematode associations, evidence contradicts the generality of coadaptive processes leading towards obligate mutualism in Wolbachia-insect associations. This is perhaps most clearly illustrated by the general lack of co-cladogenesis found between Wolbachia and host phylogenies, evidence of recurrent infection losses over evolutionary time.

This thesis explored this problem from a different angle. If coadaptation were a general process shaping Wolbachia-host evolution, then facultative symbioses would only represent a transitory state that would evolve into obligate mutualism given enough time. This leads to the following testable prediction: partners associated for long periods of time would resemble obligate symbioses more closely than partners that have recently met. Chapters 2 and 3 of this thesis explored this prediction by measuring symbiont density in gonads of two Wolbachia-*Drosophila simulans* associations known to differ in their age, as well as the strength of CI phenotype and the frequency of

infection in natural populations. Chapter 2 explored the density dynamics of the *w*Ha infection in *D. simulans*. This Wolbachia strain is present in nearly all Hawaiian *D. simulans* and has been described to induce a potent CI phenotype. Chapter 3 measured the same parameters on flies from the same species infected with the weak CI inducer *w*Ma, hypothesised to be the oldest Wolbachia strain this fly and occurring in around a third of individuals from Kenyan fly populations. If coadaptation shaped the evolution of Wolbachia-hosts associations, then symbiont titres in the older *w*Ma-Drosophila association would be more tightly regulated than those of the *w*Ha-Drosophila association. Our results did not meet this prediction. In fact, Wolbachia density appeared to variable and prone to environmental disturbances in flies harbouring *w*Ma, while for *w*Ha infected flies titres varied less and were unaffected by environmental challenges.

These observations, rather than reflecting Wolbachia-fly coadaptive evolution, could be better explained as the product of selection on symbiont transmission fidelity in females due to CI. In host populations where almost all individuals are afflicted by strong CI-Wolbachia, females that do not inherit the infection would have little chances to reproduce. Therefore, selection would favour individuals that transmit the infection with high fidelity (Turelli, 1994). Females capable of doing so while minimising the cost on infection (for example, by controlling Wolbachia over-replication) would also have an advantage, which may result in widespread mechanisms of symbiont regulation in the population. On the other hand, selection on infection maintenance would not be as strong in females from populations harbouring weak CI-Wolbachia, which means that mechanisms of symbiont density regulation may not be as strongly selected as in

populations harbouring potent CI-inducers. This interpretation also agrees with the observation that populations infected by potent CI-Wolbachia often display high transmission fidelity.

Is it common that old Wolbachia association display weak CI and recent ones, potent CI? In other words, is the strength of reproductive manipulations something that changes during the evolutionary history of Wolbachia symbioses? The following section addresses this question.

6.3 Conflicts of interest, resolution… and extinction?

The coadaptive hypothesis presented in the previous section does not seem to take into account the selective sexual asymmetry that arises with symbiont maternal transmission; this is, maternally inherited symbionts (and more generally, cytoplasmic genetic elements) are selectively favoured when beneficial to infected females, even if they are harmful for males. Reproductive manipulators such as Wolbachia use this to their advantage; by actively reducing the reproductive chances of individuals that do not transmit the infection, the microbe increase the relative fitness of infected matrilines in order to spread. Wolbachia that induce reproductive manipulations fall into the definition of selfish genetic elements (SGEs), genetic units with traits that enhance their own transmission relative to the rest of the individual's genome (Hurst, 1992). SGEs are in conflict with the nuclear genome as their enhanced transmission decreases that of unlinked genomic regions. Such regions then experience antagonistic selective pressure

over the phenotype induced by the SGE, which lead to selection on genetic modifiers that counteract such phenotype (Werren, 2011).

Interestingly, conflicts caused by CI decrease when infection frequency approaches 1, as the chances of incompatible matings disappear if all individuals of a population are infected (Charlat et al., 2003). Nonetheless, there may be other Wolbachia-male conflicts that occur in addition to and independently from CI. Similarly to the mother's curse hypothesis on mitochondrial-induced male-specific defects (Gemmell et al., 2004), selection may fail to eliminate Wolbachia that negatively affect males, as they constitute an evolutionary dead end to the symbiont. This may render males ill-suited to regulate Wolbachia, especially in sexually dimorphic tissues such as gonads.

The results presented in Chapter 2 and 3 provide evidence in support of a mother's curse-like effect of Wolbachia infection. Results indicated that for both *w*Ha and *w*Ma associations, symbiont titres in testes were more variable and prone to change with age and environmental perturbations than those in ovaries, which can be interpreted as an inability of male gonadal tissues to regulate Wolbachia in comparison to ovaries. The presence of Wolbachia in testes may have negative effects on male fertility. For example, Snook and colleagues (2000) found that infected *D. simulans* males produced fewer sperm cysts than uninfected ones, with as many as ~3 000 less sperm being produced by the former in a 7 day period. Testing the generality of such observation may be necessary to corroborate this hypothesis.

As long as Wolbachia infection prevents the normal transmission of host genetic material to the next generation, genetic modifiers that attenuate their effect would be selectively favoured (Werren, 2011). Exclusion or reduction of Wolbachia titres in testes has been suggested as a host compensatory mechanism that reduces the levels of CI expression and counteracts harmful fertility effects of Wolbachia (Engelstädter and Hurst, 2009). This may have important consequences for the evolution of CI-Wolbachia symbiosis, as it may provide a plausible explanation as to why CI may not drive and/or maintain Wolbachia at fixation. Koehncke and colleagues (2009) propose that the rise of alleles that slightly attenuate CI (through the reduction of symbiont titres in testes, for example) favour the subsequent invasion of other CI repressors. This is because male resistance to CI is expected to spread if less than 100% of the individuals of a population are infected (Turelli, 1994). Koehncke and colleagues conclude that host adaptation to Wolbachia infection would generally result in the loss of CI.

Studies on the distribution of Wolbachia in testes have shown that the relationship between testicular Wolbachia titres and CI expression is a subtle one. For example, there is compelling evidence showing that the number of infected sperm cyst, rather than the overall Wolbachia density of testes, is a better predictor of CI levels in many associations (Veneti et al., 2003). Therefore, host mechanisms that target exclusion of Wolbachia from these regions may efficiently counteract CI. McGraw and colleagues (2001) showed that the over-replicating strain *w*MelPop failed to induce CI in their native *Drosophila melanogaster* background, but showed potent CI induction after transinfection into a naïve *D. simulans* background. Analysis of Wolbachia density and distribution in testes showed that the inability of *w*MelPop to induce CI in *D.*

melanogaster was likely caused by a failure of bacterial cells to invade sperm bundles inside cyst, an ability that they displayed in the naïve host. Moreover, this study showed that despite the absence of *w*MelPop in bundles of *D. melanogaster*, the overall density in testes was high, increased with hosts age and did not correlate with CI levels. This study offered some interesting observations. First, it showed that exclusion of Wolbachia from sperm bundles indeed constitutes an important host mechanism against CI, which may have evolved as an adaptation to the related, naturally occurring *w*Mel strain. Second, it showed that Wolbachia testicular density *per se* does not explain CI levels but may reveal other aspects of the behaviour of the infection, such as the tendency to over-replicate and the variation of symbiont effects depending on the physiological state of the host. These observations agree with the findings of Chapters 2 and 3 of this thesis.

Here, an interesting trend observed for both *w*Ha and *w*Ma infected testes was an increment of testicular Wolbachia density with laboratory adaptation, which was more marked for *w*Ma-infected flies. Although the research conducted here does not provide a direct explanation for this phenomenon, few reasons can be speculated. For instance, the relaxed conditions for flies raised in the laboratory (constant supply of nutrient-rich food, ease of mating effort, absence of natural enemies) may allow males to survive with Wolbachia loads higher that they could support in field conditions. Additionally, the maintenance of isofemale cultures at low numbers may have negatively impacted the genetic diversity of the fly lines, resulting in the loss of alleles implicated in density regulation, which could also account for the observation of line-specific density effects of environmental perturbations. The shifts in Wolbachia density and variability with

laboratory adaptation, however, point to Wolbachia and host interactions to be highly dynamic with changing environment.

The studies of Chapters 2 and 3 highlight the importance of considering the sexual antagonistic nature of host adaptation to Wolbachia infection and how it may drive the evolution of CI attenuation. Koehncke and colleagues (2009) propose that loss of CI ultimately drives Wolbachia to extinction in natural populations at evolutionary scales, which can be independently observed by comparing host and symbiont phylogenies. This, however, contrast with the observation of numerous Wolbachia infection that appear to have no phenotype to support their permanence in natural populations.

Models have predicted that for non or weak-CI inducing Wolbachia to be maintained in host populations, it is necessary for the bacteria to confer positive effects on host fitness that are independent from infection frequency (O'Neill et al., 1997). However, it was not until recently that physiological positive effects of Wolbachia have been consistently examined. Such studies add another layer of complexity to the study of the evolution of Wolbachia symbioses, offering a perspective about Wolbachia other than that of ruthless manipulators of the host reproductions. The following section expands on this.

6.4 Wolbachia as facultative beneficial symbiont

"The acquisition of the reproducing other, of the microbe and its genome, is no mere slideshow. […]The incorporation and integration of "foreign" genomes, bacterial and

other, led to significant, useful heritable variation. The acquiring of genomes has been central to the evolutionary process throughout the long and circuitous history of life."

– Lynn Margulis and Dorion Sagan, *Acquiring genomes*

The capacity of Wolbachia to spread in host populations despite their 'parasitic' nature has earned these organisms the interest of the scientific community as potential candidates for the control of insect populations and vector-borne human diseases. A very notable case is the research of Wolbachia-related technology for the control of transmission of dengue virus. Motivated by the discovery of a CI-inducing, life-shortening Wolbachia strain in laboratory *D. melanogaster*, Scott O'Neil and his group suspected that dengue virus transmission could be lowered if the lifespan of its vector, *Aedes aegypti,* could be somehow shortened by Wolbachia (Cook et al., 2008). The reasoning behind such approach was simple: mosquitoes infected with parasitic Wolbachia would invade natural host populations by reproductive modification, but would not live long enough to transmit the virus to humans (McMeniman et al., 2009). The research conducted to determine the feasibility of that bio-control strategy led to a serendipitous discovery: Wolbachia-infected individuals were in fact protected against viral infection (Hedges et al., 2008, Teixeira et al., 2008). This not only changed the course of this research but also revolutionised the views on Wolbachia, from parasites that exclusively rely on reproductive manipulations, to endosymbionts with parasitic traits that nonetheless may aid the host fitness.

During the past decade, the refinement of molecular biology and physiology techniques as well as the availability of whole Wolbachia genomic sequences has lead to a number

of important discoveries on positive phenotypic effects of Wolbachia infection. The roles of Wolbachia as a provisioner in times of nutritional stress (Brownlie et al., 2009) and as protection from natural enemies (Brownlie and Johnson, 2009; Teixeira et al., 2008) are prominent among them. Curiously, many past studies failed to find benefits or even found decreased fitness associated to infection (Reviewed in Harcombe and Hoffmann, 2004). This was probably the case as beneficial effects of Wolbachia may be undetectable when assayed at nutrient-rich, disease and predator-free standard laboratory conditions. Also, as discussed in the previous section, fitness effects of Wolbachia may only be expressed in particular host genetic background, which suggests that observations on limited numbers of inbred laboratory lines may be biased. Together, this highlights an important (and possibly overlooked) attribute of Wolbachia-induced phenotypes: their high contingence on the environment and host genetic background.

Wolbachia beneficial effects that depend on environmental conditions may provide an explanation for the maintenance of facultative symbioses with weak or nonexistent reproductive phenotypes. A clear example is provided by the *w*Mel-*D. melanogaster* association. Research on the dynamics of *w*Mel in natural populations by Hoffmann and colleagues (1998) showed that infection frequency were often stable despite weak CI and imperfect maternal transmission. Back then, the authors speculated that *w*Mel likely provided fitness benefits to the flies, but such effects were not apparent at the time. A decade later, it was found that *w*Mel significantly improved fitness of flies reared in iron restricted (or overloaded) diets. Notably, indirect evidence showed that dietary iron in the wild was similar to that of the iron-restricted diet (Brownlie et al., 2009). Moreover,

it has also been discovered that *w*Mel is a potent protective agent against naturally occurring, lethal viral infections in *D. melanogaster* (Teixeira et al., 2008).

Coincidentally or not, the current understanding of naturally occurring mtDNA diversity bears resemblance to Wolbachia infections, in the sense that mtDNA variants with environmentally or physiologically contingent benefits, or whose effects depend on the nuclear genetic background, may be maintained in natural populations (Ballard and Pichaud, 2014). Chapter 4, which is part of a larger body of research that includes other published work (Aw et al., 2011; Pichaud et al., 2013b), showed that an small set of functional mtDNA variants influenced aspects of *D. melanogaster* mitochondrial oxidative phosphorylation (OXPHOS) and metabolism (Correa et al., 2012), which were likely causal of observable differences life history traits in the adult fly (Aw et al., 2011). Two important highlights from these studies are: i) functional mutations may have measurable effects on the physiology and fitness of the organism; and, ii) the effect of such mutations differed with ageing and diet, which means that naturally occurring variants may have different adaptive values under various circumstances. Perhaps the major limitation of the research conducted in Chapter 4 was that experiments were conducted on single highly inbred nuclear background, which does not allow to directly determining if the observed traits are caused by mtDNA alone or by disruption of coevolved cyto-nuclear complexes.

Chapter 5 expanded upon this work, and included Wolbachia infection as another source of cytoplasmic genetic variation. A highlight of this study was the examination of cyto-nuclear interactions through ‘exchanging’ the nuclear genetic backgrounds of

two genetically distinct fly populations. Having its basis on the concepts explored in the previous three chapters, Chapter 5 investigated various aspects of cyto-nuclear interactions by testing the effects of cytoplasmic and nuclear genetic factors on life history traits and bioenergetics of *D. simulans* females and males. The working hypothesis was that disruption of cyto-nuclear interactions (from both Wolbachia and mitochondria) would negatively affect host fitness. The results of the study were in line with the hypothesis that Wolbachia, though non-essential, can be beneficial under particular circumstances. However, the data do not support the hypothesis Wolbachia-related beneficial phenotypes from host adaptation to the infection.

Wolbachia appeared to confer greater benefits to flies with mildly deleterious cyto-nuclear phenotypes (brought about through the disruption of coadapted mito-nuclear complexes), which could point to a role of Wolbachia in rescuing mildly deleterious mito-nuclear genotypes. This concept, although novel, has already been proposed elsewhere. Chen and colleagues (2012) found that phenotypic effects of the mutant nuclear gene tko^{25t} in *D. melanogaster* (a mitoribosomal protein gene that causes deficiency in OXPHOS) were suppressed by the cytoplasm of flies that contained or, more accurately, were adapted Wolbachia. Fly strains with a tko^{25t} nuclear background and a cytoplasmic type that previously harboured Wolbachia displayed rescued phenotypes, likely related to an increase in mtDNA copy number and mitochondrial biogenesis. The authors propose that the capacity of the cytoplasmic genetic background of these flies to rescue the defective tko^{25t} phenotype arose as a compensatory mechanism to withstand Wolbachia infection.

The results of Chapter 5, however, point to different mechanisms of compensation for mildly mitochondrial dysfunction. Uninfected flies with a mito-nuclear disrupted genotype showed abnormal respiration rates that were linked to shorted starvation resistance, higher dietary intake and decreased rate of egg laying. The presence of Wolbachia in flies with the same nuclear makeup seemed to rescue these slightly deleterious phenotypes, bringing them to a similar level to those of flies with coadapted mito-nuclear complexes. Rather than compensatory mutations to infection, these observations could be more easily explained as a result of an intrinsic ability of Wolbachia as a metabolic rescuer of slightly malfunctioning cyto-nuclear genotypes. Although the evidence here does not point directly to a cause for such 'rescue' phenotype, a plausible metabolic trait that could offer a link between Wolbachia and mitochondrial respiration is the capacity of the bacteria to synthesise heme groups. Few independent studies have suggested that Wolbachia may have important functions on their hosts as providers of these specific cofactors. In the nematode *Brugia malayi*, all the genes for the synthesis of heme are in the obligate Wolbachia but completely absent from the worm's nuclear genome. Moreover, Wolbachia clearance in the filarial nematode *Litomosoides sigmodontis* showed up-regulated heme-dependent respiratory chain complexes in an attempt to maintain metabolic homeostasis (Strübing et al., 2010). Analysis of the genome (Brownlie et al., 2007) and the phenotype of Wolbachia-infected Drosophila (Brownlie et al., 2009) also point to heme as a mechanism of beneficial interaction of symbiont and host at the biochemical level.

The role of Wolbachia as a metabolic rescuer may have important implications for the observed mitochondrial genetic variation in natural populations. It is plausible that in

the absence of reproductive manipulations, Wolbachia effects on energy homeostasis may contribute to the maintenance of mitochondrial genotypes that would otherwise be outcompeted by fitter ones. Future studies that investigate the effects of mitochondrial genetic variants in the fitness and evolvability of insect populations would greatly benefit to include Wolbachia as another source of functional cytoplasmic variability.

6.5 Future directions

The studies of this thesis investigated interactions between cytoplasmic genetic elements and the nuclear background and how the effect of such interaction may affect the organismal phenotype across different environments and as the organism ages. This thesis shows that the effects of cytoplasmic genetic variants on their host can be contingent to the host nuclear background and sex, as well as to environmental factors. Analyses of the effects of cytoplasmic variants would greatly benefit if a wide spectrum of environmental variation are considered. For example, great insight on the roles of Wolbachia or mitochondrial variants could be obtained if tested against the nutritional geometry framework proposed by Lee and colleagues (2008), or following the methodology of Pichaud et al (2013a) to test a thermal sensitivity through the biological range. Another approach that may result particularly useful to test a possible link between Wolbachia and cellular energy homeostasis is the use of Drosophila mutants with impaired OXPHOS function.

A link between Wolbachia and organismal bioenergetics opens a number of possibilities for the study of the evolution of these symbioses. The use of permeabilised insect fibres

for the measurement of oxygen consumption may constitute an important technical advance for the study of Wolbachia and host interactions. Further refinement of the technique may allow dissecting Wolbachia-related effects at the level of substrate utilisation, provisioning of metabolic intermediates, and efficiency of ATP synthesis (through ADP:O ratio, for example). Moreover, the increasing availability of annotated Wolbachia genomes may be important on the design of top-down approaches for the study of Wolbachia at the bioenergetic, physiologic and organismal level.

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Appendix

Appendix 1: List of publications

Appendix 1.1. List of publications included as part of the thesis

Correa, C., Aw, W., Melvin, R., Pichaud, N., Ballard, J.W.O. 2012. Mitochondrial DNA variants influence mitochondrial bioenergetics in *Drosophila melanogaster*. Mitochondrion 12, 459-464.

Correa, C. C., Ballard, J. W. O. 2012. Wolbachia gonadal density in female and male Drosophila vary with laboratory adaptation and respond differently to physiological and environmental challenges. Journal of Invertebrate Pathology 111, 197-204.

Correa, C., Ballard, J. W. O. 2014. What can symbiont titres tell us about co-evolution of wolbachia and their host? Journal of Invertebrate Pathology 118, 20-27.

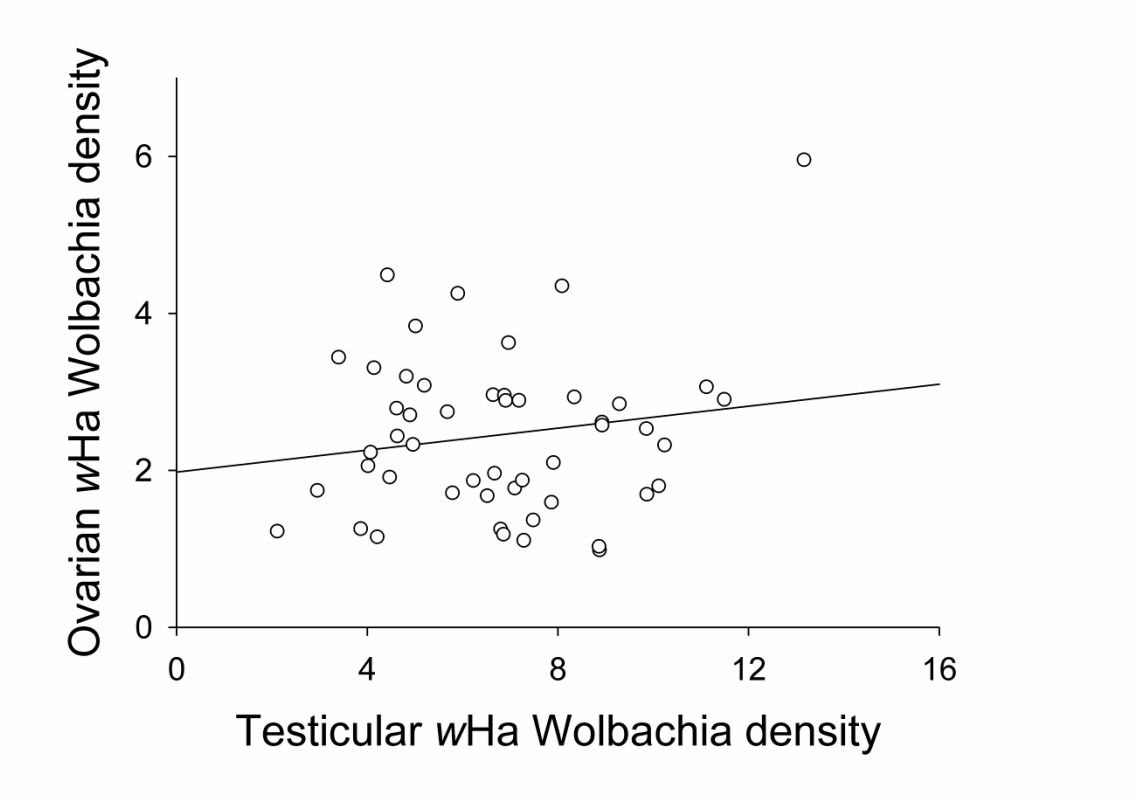
Appendix 1.2. List of co-authored publications

Aw, W., **Correa, C.C.,** Clancy, D., Ballard, J.W.O., 2011. Mitochondrial DNA variants in *Drosophila melanogaster* are expressed at the level of the organismal phenotype. Mitochondrion 11, 756-763.

Pichaud, N., Messmer, M., **Correa, C.C**., Ballard, J. W. O., 2013. Diet influences the intake target and mitochondrial functions of *Drosophila melanogaster* males. Mitochondrion 6, 817-822.

Appendix 2: Chapter 2 supplementary material

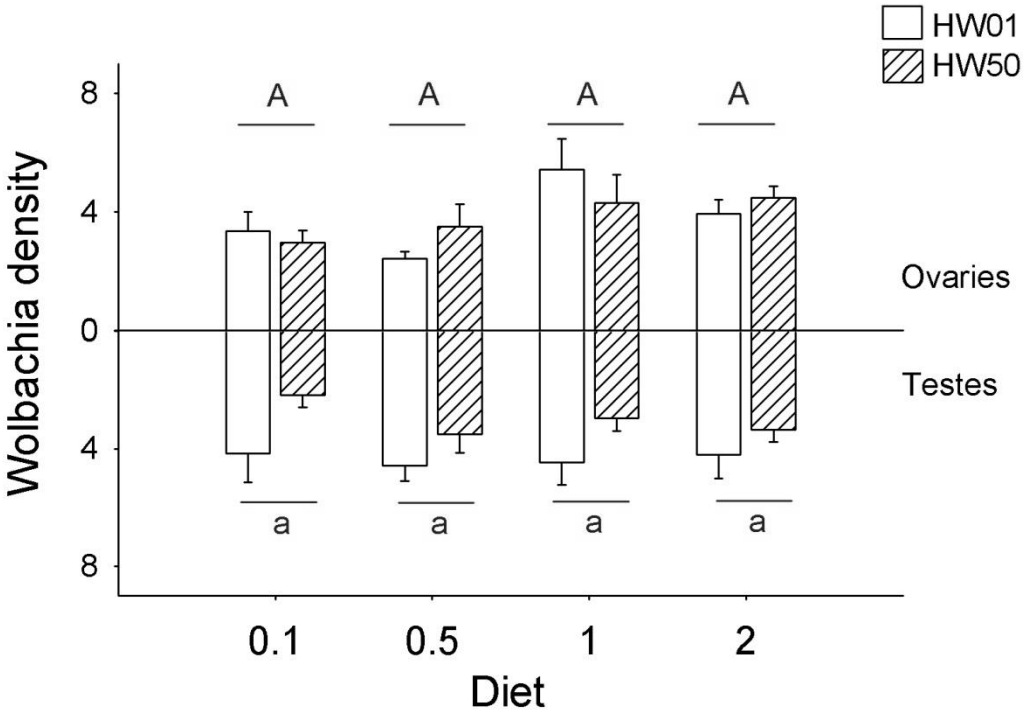
Appendix 2.1. Wolbachia density in ovaries does not correlate to that of testes from flies of the same isofemale line. Mean Wolbachia density per line vary in a different manner for ovaries and testes. No significant correlation was found using Spearman correlation analysis (testes density = 1.96 + 0.07; $R^2 = 0.0378$, $p = 0.18$).



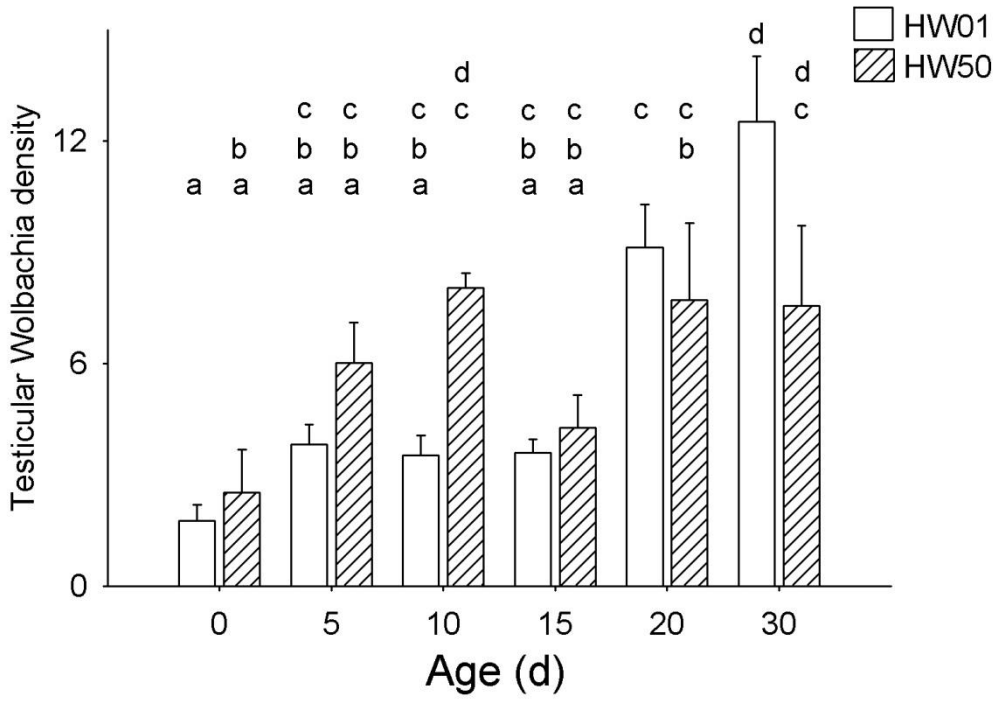
Appendix 2.2. Estimated nucleotide polymorphisms among ten isofemale lines of *Drosophila simulans* collected from Hawaii. Two fragments from the neutrally evolving nuclear genes *Adhr* (469 bp) and *Gpdh* (467 bp) genes were examined.

Density Group	*N*	Nucleotide diversity			
		π ($\times 10^{-3}$)		θ ($\times 10^{-3}$)	
		Adhr	*Gpdh*	*Adhr*	*Gpdh*
Low	5	9.21	12.12	10.04	13.51
High	5	9.17	15.95	8.00	14.48
Combined	10	9.30	12.99	9.61	11.52

Appendix 2.3. Differences in host diet are not reflected in Wolbachia density in gonads. Adult flies maintained in food containing 0.1, 0.5, 1 and 2 times the amount of sugar and yeast of the standard media do not show any significant difference in Wolbachia densities fly gonads. Bars represent mean normalised Wolbachia density per treatment ± SEM. Bars not connected by the same letter vary significantly according to Tukey's HSD test with $Q_{females} = 3.2$, $Q_{males} = 3.2$ and $\alpha = 0.05$

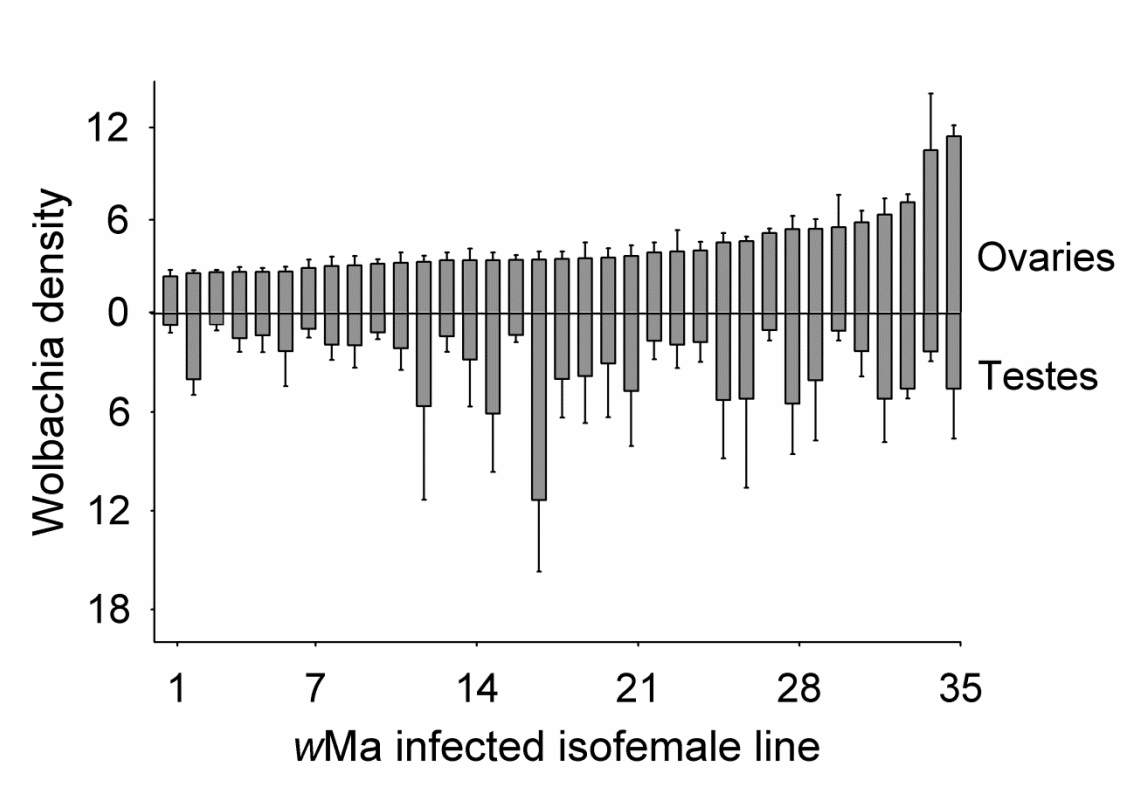


Appendix 2.4 Wolbachia density in fly testes increase with age for HW01 and HW50 fly lines. Bars represent mean normalised Wolbachia density per line and age ± SEM. Testicular Wolbachia density from HW01 and HW50 does not differ significantly in flies of the same age according to an *ad-hoc* Tukey's HSD test performed for all age/line treatments ($Q = 3.4$ and $\alpha = 0.05$).

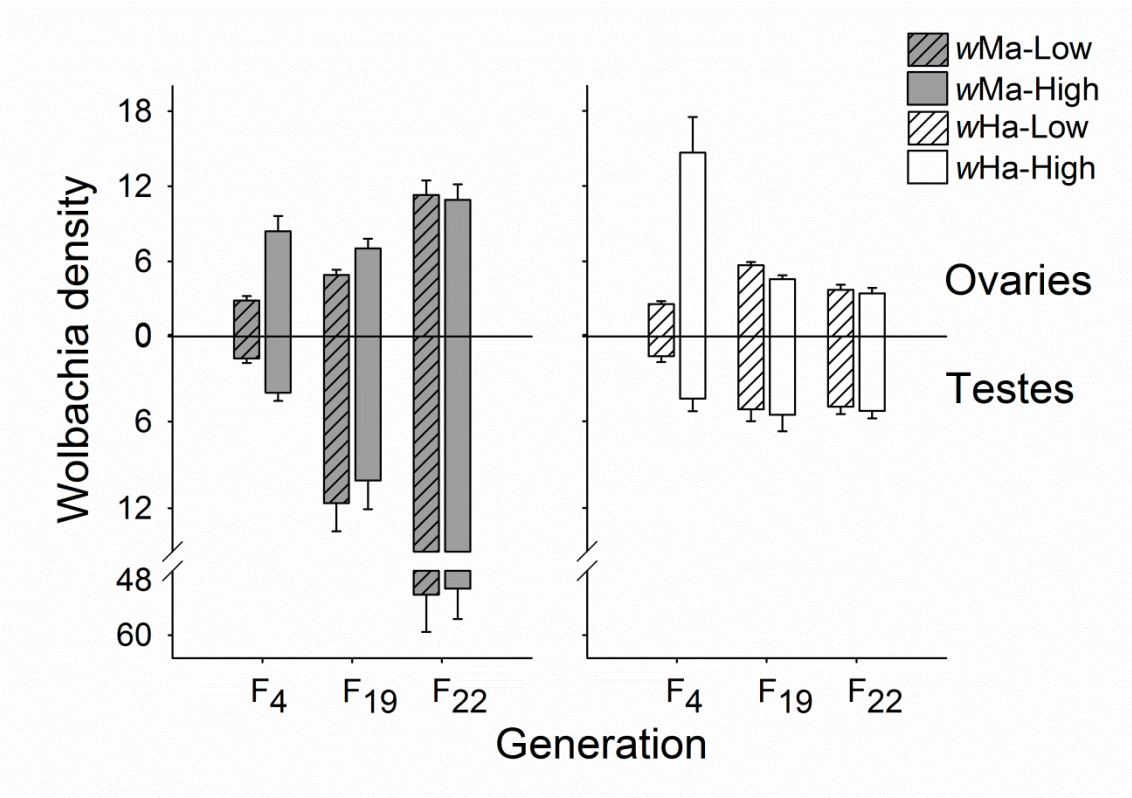


Appendix 3: Chapter 3 supplementary material

Appendix 3.1. Average gonadal Wolbachia densities in *Drosophila simulans* from 35 isofemale lines, four generations after collection. Ovarian density of Wolbachia four generation after field collection differs between sexes and among lines. Ranking and line designation were based on ovarian density. Bars represent mean Wolbachia density per line ± SEM.



Appendix 3.2. Wolbachia gonadal density continues to increase with laboratory adaptation after F_{19} for *w*Ma infected flies, but is maintained for *w*Ha infected flies. Bars represent mean relative Wolbachia density per treatment ± SEM.

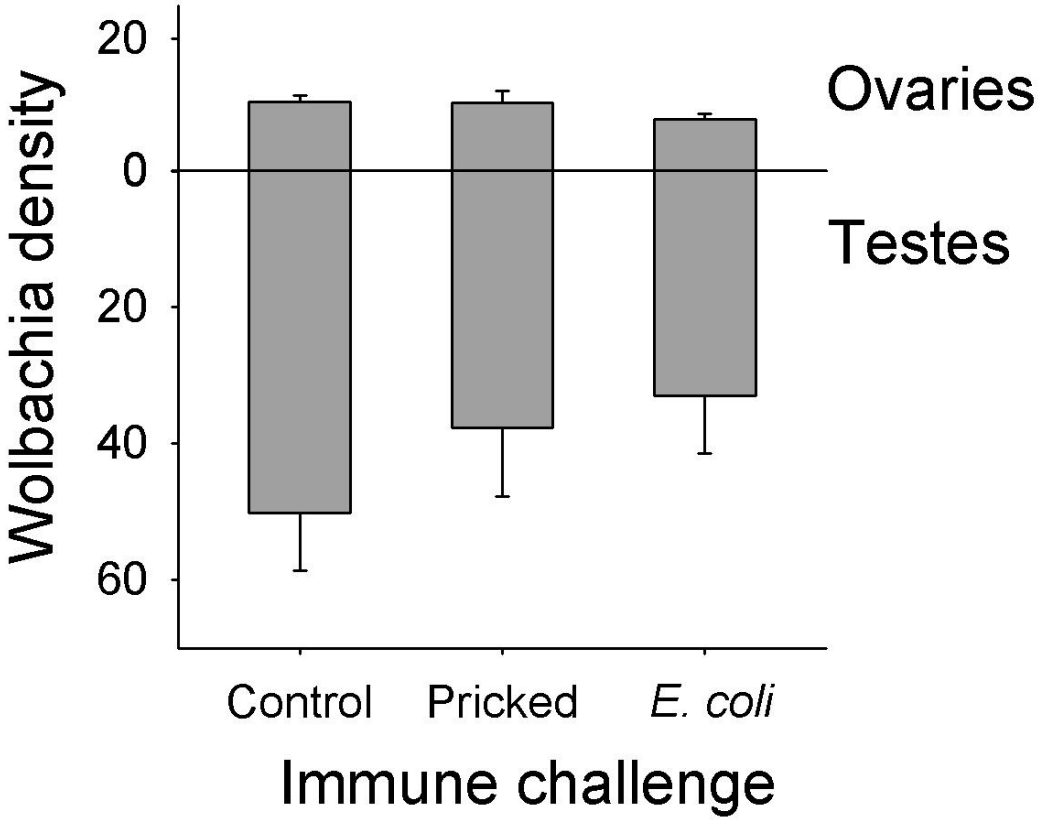


Appendix 3.3. Estimated nucleotide polymorphisms among ten Wolbachia infected isofemale lines of *Drosophila simulans* collected from Kenya and Hawaii. Two fragments from the nuclear genes *Adhr* and *Gpdh* were examined.

	Density Group	*N*	Nucleotide diversity			
			π ($\times 10^{-3}$)		θ ($\times 10^{-3}$)	
			Adhr	*Gpdh*	*Adhr*	*Gpdh*
Kenya	Low	5	13.92	16.96	14.15	19.12
	High	5	10.13	15.63	11.14	16.88
	Combined	10	12.75	15.09	9.61	18.11
Hawaii*	Low	5	9.21	12.12	10.04	13.51
	High	5	9.17	15.95	8.00	14.48
	Combined	10	9.30	12.99	9.61	11.52

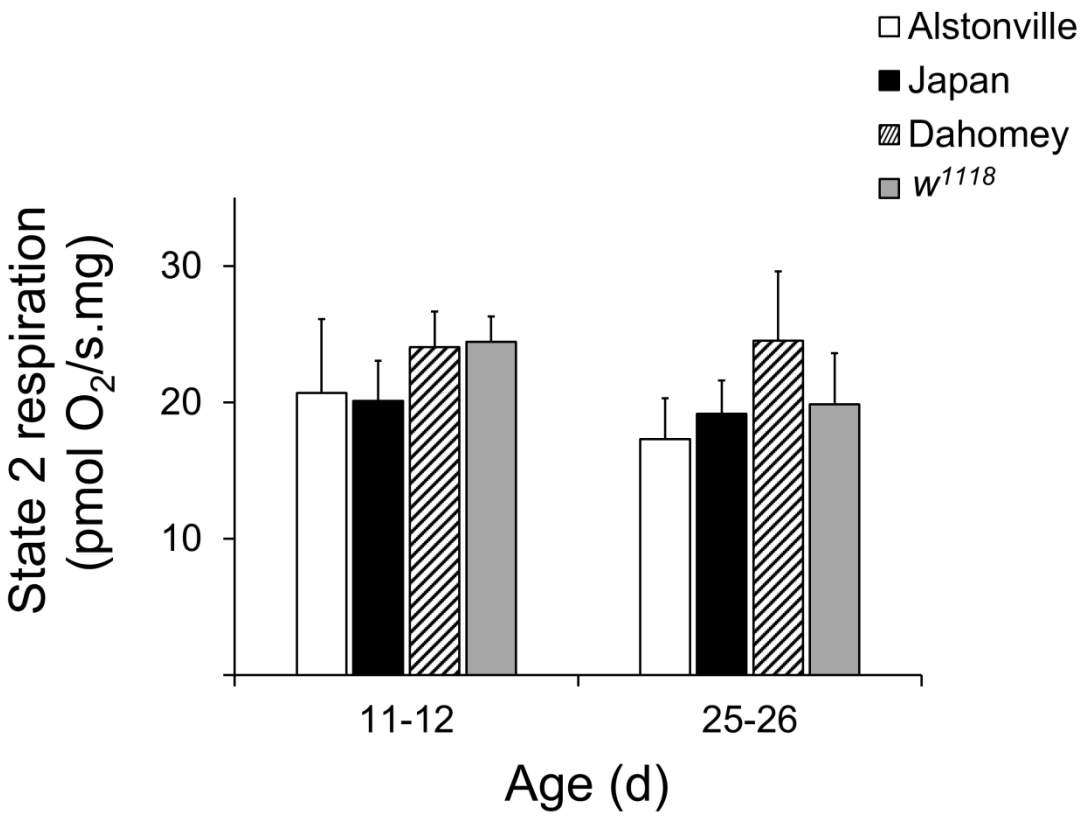
* Data obtained from Correa and Ballard (2012).

Appendix 3.4. Immune challenge with *E. coli* does not affect Wolbachia density. Bars represent mean normalised Wolbachia density pooled for the KY01 and KY35 ± SEM.

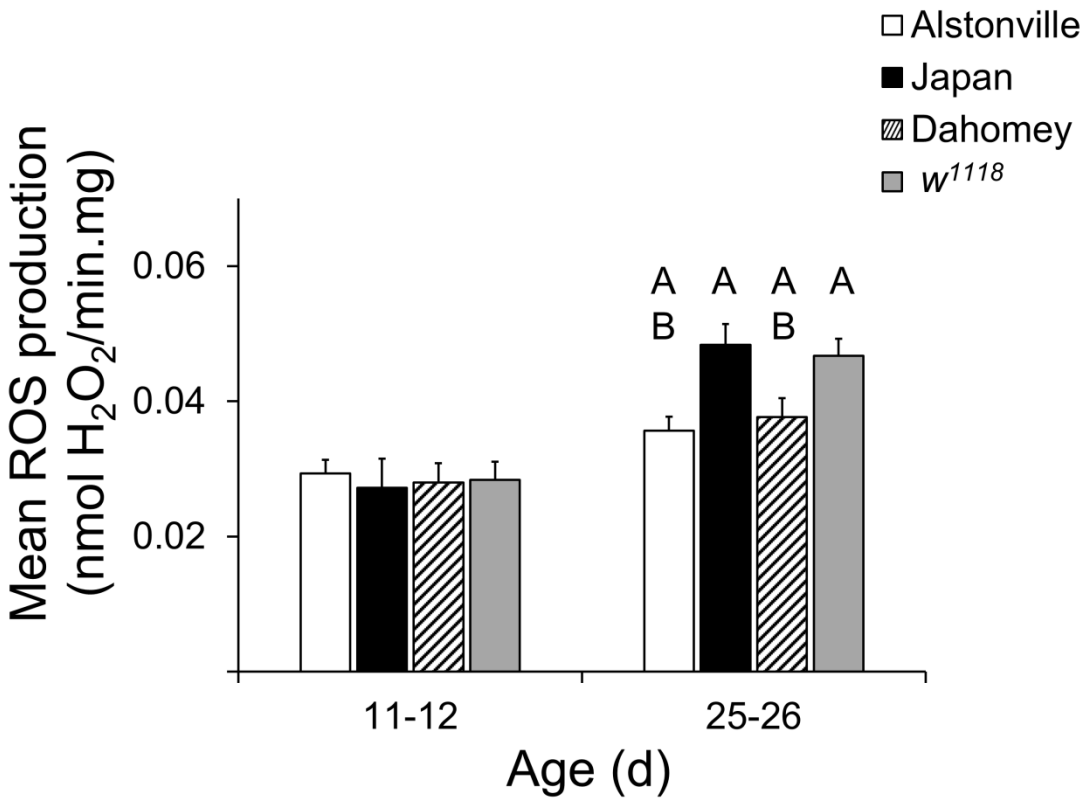


Appendix 4: Chapter 4 supplementary material

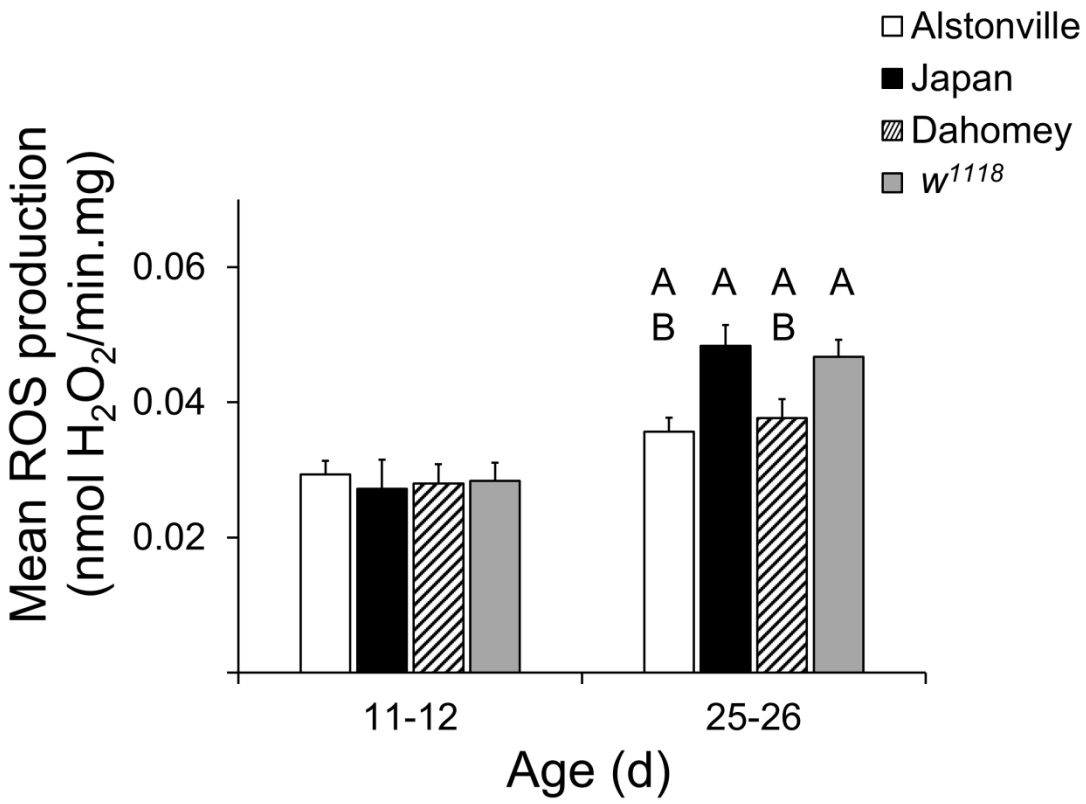
Appendix 4.1. Complex I proton leak-based respiration rate (state 2', expressed in O_2 pmol/s.mg of fibres) of flight muscle mitochondria form permeabilised fibres of Drosophila males with distinct mtDNA but standardised nuclear DNA. Fly lines used were Alstonville, Japan, Dahomey and w^{1118} at two different ages. Not significant differences in proton leak respiration were found among lines or between the two ages tested here. Bars represent mean respiration rate for ATP synthesis respiration ± SEM.



Appendix 4.2. Uncoupled Respiratory Control (UCR), calculated here as state CIc/ CIc+u of flight muscle of Drosophila males with distinct mtDNA but standardised nuclear DNA at two ages. UCR is close to one and does not differ among lines or between ages. Bars represent mean UCR ± SEM.



Appendix 4.3. Mean H_2O_2 production rate (expressed as H_2O_2 nmol/min.mgof protein) of isolated mitochondria of Drosophila males with distinct mtDNA but standardised nuclear DNA. Fly lines used were Alstonville, Japan, Dahomey and w^{1118} at two ages.Mean H_2O_2 production rate increases with age. Japan and w^{1118} have a higher mean H_2O_2 production compared to other lines. Bars represent mean H_2O_2 production ± SEM. Letters above bars indicate significant differences as determine by Tukey's test with a = 0.05 and Q = 3.20.



Appendix 5: Chapter 5 supplementary material

Appendix 5.1. Verification of Wolbachia infection, strain and mtDNA type of experimental fly lines. Positive *ftsZ* amplification was observed only in lines expected to be infected. Sequencing of *ftsZ* and COI amplicons confirmed that experimetnal lines harboured the predicted Wolbachia strain (when infected) and mtDNA type.

Original lines	Experimental lines	*ftsZ* amplification?	Wolbachia strain[a]	COI haplotype[b]
***si*I·*w*Ha** (1) hw	*si*I·*w*Ha (1) HW	Yes	*w*Ha	*si*I
	*si*I·*w*- (1) HW	No		*si*I
	*si*I·*w*Ha (1) KY	Yes	*w*Ha	*si*I
	*si*I·*w*Ha (1) KY	No		*si*I
***si*III·*w*Ma** (1) ky	*si*III·*w*Ma (1) KY	Yes	*w*Ma	*si*III
	*si*III·*w*- (1) KY	No		*si*III
	*si*III·*w*Ma (1) HW	Yes	*w*Ma	*si*III
	*si*III·*w*- (1) HW	No		*si*III
***si*I·*w*Ha** (2) hw	*si*I·*w*Ha (2) KY	Yes	*w*Ha	*si*I
	*si*I·*w*- (2) HW	No		*si*I
	*si*I·*w*Ha (2) KY	Yes	*w*Ha	*si*I
	*si*I·*w*Ha (2) KY	No		*si*I
***si*III·*w*Ma** (2) ky	*si*III·*w*Ma (2) KY	Yes	*w*Ma	*si*III
	*si*III·*w*- (2) KY	No		*si*III
	*si*III·*w*Ma (2) HW	Yes	*w*Ma	*si*III
	*si*III·*w*- (2) HW	No		*si*III

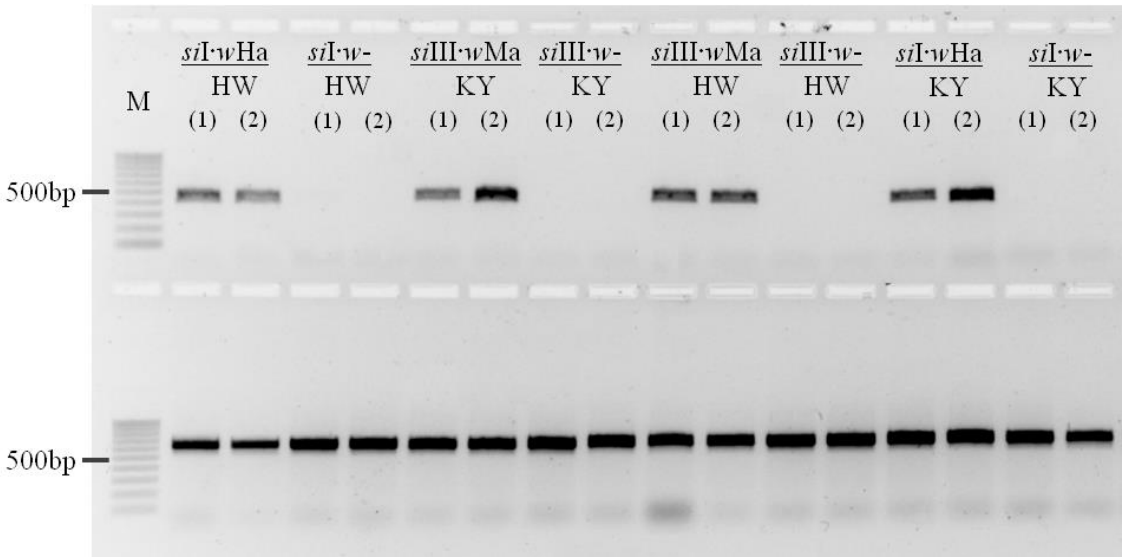
[a] Lines with cytoplasmic types derived from Hawaii and Kenya displayed *ftsZ* fragments matching published *w*Ha (GenBank ID: AY508998.1) and *w*Ma (GenBank ID: AY509001.1) sequences, respectively.

[b] Lines with cytoplasmic types derived from Hawaii and Kenya displayed COI fragments matching published mtDNA sequences of *si*I (GenBank ID: AF2008351) and *si*III (GenBank ID: AF020069.1) *D.simulans* mitochondrial types, respectively.

Appendix 5.2. Verification of the nuclear background of experimental fly lines. Diagnostic DNA polymorphisms were identified in *Adhr and Gpdh* intronic sequences of donor lines and sequences from the 16 experimental lines were examined on those sites. Results indicate successful introgression of nuclear DNA and lack of contamination.

		Adhr (469 bp)							*Gpdh* (467 bp)							
	Position	113	128	159	168	181	285	333	10	25	48	55	82	96	168	181
	Consensus	C	G	A	: :	C	C	T	T	C	GC	A	T	G	A	T
Exp. lines	siI•wHa (1) HW	A		G	AT			C								
	siI•w- (1) HW	A		G	AT			C								
	siIII•wMa (1) HW	A		G	AT			C								
	siI•w- (1) HW	A		G	AT			C								
Nuclear donor	*si*I•*w*- (1) **HW**	A		G	AT			C								
Exp. lines	*si*I•*w*Ha (2) HW															
	*si*I•*w*- (2) HW															
	*si*III•*w*Ma (2) HW															
	*si*III•*w*- (2) HW															
Nuclear donor	*si*I•*w*- (2) **HW**															
Exp. lines	*si*III•*w*Ma (1) KY						A								C	C
	*si*III•*w*- (1) KY						A								C	C
	*si*I•*w*Ha (1) KY						A								C	C
	*si*I•*w*- (1) KY						A								C	C
Nuclear donor	*si*III•*w*- (1) **KY**						A								C	C
Exp. lines	*si*III•*w*Ma (2) KY	C				T			A	T	AT	G	A	A		
	*si*III•*w*- (2) KY	C				T			A	T	AT	G	A	A		
	*si*I•*w*Ha (2) KY	C				T			A	T	AT	G	A	A		
	*si*I•*w*- (2) KY	C				T			A	T	AT	G	A	A		
Nuclear donor	*si*III•*w*- (2) **KY**	C				T			A	T	AT	G	A	A		

Appendix 5.3. Agarose gel showing positive amplification of the Wolbachia *ftsZ* fragment using DNA isolated from thoraces as template. Samples were included for each experimental fly line. Bands on the upper panel (~450 bp) correspond to *ftsZ* amplicons, while those in the lower panel (~650 bp) correspond to COI amplicons. Positive *ftsZ* amplification was observed only in lines expected to harbour Wolbachia. Positive COI amplification confirmed the quality of each DNA preparation.



Experimental procedures followed Correa and Ballard (2012). Amplicons were observed after electrophoresis on a 1.2% agarose gel stained with SYBR® Safe stain, including a 100 bp molecular marker (M) as size reference.