Genomic and environmental factors influence *Wolbachia-Drosophila* symbiosis

Ewa Anna Chrostek

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Declaration

I declare that this dissertation is the product of my own work and that it includes original results obtained between April 2011 and April 2014 in the laboratory of Dr. Luis Teixeira at the *Instituto Gulbenkian de Ciência.* Contributions of the collaborators are listed in the Author Contributions sections at the beginning and in the Acknowledgements sections at the end of each chapter. The work described here was funded by my PhD fellowship SFRH / BD / 51625 / 2011, the following FCT grants: PTDC/BIA-MIC/108327/2008, PTDC/SAU-MII/105655/2008 and the Wellcome Trust (http://www.wellcome.ac.uk) grant 094664/Z/10/Z.

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“When one tugs at a single thing in nature, he finds it attached to the rest of the world.”

- John Muir

Abstract

Intracellular, vertically transmitted bacteria form complex and intimate relationships with their hosts. *Wolbachia,* maternally transmitted α-proteobacteria, live within the cells of numerous arthropod species. *Wolbachia* are famous master manipulators of insect reproduction: to favour their own spread they can induce male killing, parthenogenesis or cytoplasmic incompatibility. *Wolbachia* can also protect various insects from pathogens, which makes them a promising tool for the control of vector-borne diseases. Mosquitoes with *Wolbachia* have already been released in the wild to eliminate dengue. Yet, how *Wolbachia* manipulate their hosts remains largely unknown.

This work aimed at understanding the interaction of *Wolbachia* with *Drosophila melanogaster*. We started by analysing a set of closely related variants of the *Wolbachia* strain present in *Drosophila melanogaster, w*Mel. We sequenced their genomes and made a phenotypic characterization in terms of *Wolbachia* titres, host longevity and host protection from viruses. We observed that the most protective variants reach higher *Wolbachia* densities and may have a cost to the host. Importantly, the phenotypes cluster *w*Mel variants into two groups that match two monophyletic groups. Comparison between genomes of *w*Mel-group and *w*MelCS-group enabled identification of differences potentially responsible for the phenotypes. Furthermore, analysis of the over-proliferative and life-shortening laboratory variant *w*MelPop and a very closely related *w*MelCS variant suggested that amplification of eight *Wolbachia* genes, called Octomom region, causes virulent phenotype. Subsequently, taking advantage of natural Octomom copy number variation between individual flies, we derived *w*MelPop lines with different Octomom copy numbers. We proved that the number of Octomom copies correlates with virulence: the more copies, the higher the

proliferation of *Wolbachia* and the sooner the flies die. This study provides evidence, that *Wolbachia* can evolve fast through gene amplification, despite a low nucleotide mutation rate and that the regulation of endosymbiont titres can be broken with a single genetic change in the symbiont. Finally, our results provide the first link between genes and phenotypes in *Wolbachia* endosymbionts.

Next, we compared antiviral protection between the natural, highly protective *w*Mel variant *w*MelCS_b and *w*Au transferred from *D. simulans* to *D. melanogaster*. We show that *w*Au protects against viruses better than *w*MelCS_b. Furthermore, *w*Au proliferates in this host at a higher rate, reaches higher titres and shortens the host lifespan when compared with *w*MelCS_b. We also show that the *w*Au *Wolbachia* strain, foreign to *Drosophila melanogaster*, does not induce a general activation of innate immune pathways. This is important, since protection by transinfected *Wolbachia* in mosquitoes has been proposed to occur due to endosymbiont induced immune priming.

Finally, we tested temperature dependence of antiviral protection provided by natural highly protective *Wolbachia w*MelCS_b in *Drosophila melanogaster*. We focused our analysis on pre- and post-infection temperature. We discovered that the pre-infection temperature is absolutely crucial for the protection, as flies raised at 18 °C are not protected by *Wolbachia*. The post infection temperature determines the overall virus induced mortality in flies with and without *Wolbachia* and is higher at 25 than at 18 °C. Post infection temperature can also, depending on the virus dose, affect the strength of protection. We concluded that antiviral protection is a temperature sensitive trait, absent under certain thermal conditions.

Altogether, our work provides important insight into the biology of *Wolbachia*, and its interaction with *Drosophila melanogaster*. It can guide future studies aiming at understanding *Wolbachia* mechanisms of action

and inform the present programmes deploying *Wolbachia* as a vector-borne disease control agent. Our data help understanding evolution of *Wolbachia* in nature and highlight the role of environment in the *Wolbachia*-*Drosophila* defensive symbiosis.

Sumário

As bactérias intracelulares transmitidas verticalmente estabelecem relações íntimas e complexas com os seus hospedeiros. *Wolbachia* são α-proteobactérias transmitidas maternalmente e que infectam uma grande variedade de artrópodes. A infecção com *Wolbachia* pode ter um forte impacto no seu hospedeiro. Em muitas espécies, *Wolbachia* alteram a biologia reprodutiva de seus hospedeiros, de modo a aumentar o *fitness* das fêmeas infectadas. *Wolbachia* podem também proteger os seus hospedeiros contra patógenos, tendo sido já introduzidos na natureza mosquitos artificialmente infectados com *Wolbachia* para eliminar o vírus dengue. Apesar da sua importância, a informação disponível sobre a interacção entre *Wolbachia* e os seus hospedeiros ao nível celular e molecular é ainda escassa. Neste trabalho, nós identificámos e caracterizámos vários factores envolvidos nesta interacção.

Em primeiro lugar, analisámos diferentes variedades de *Wolbachia* presentes em *Drosophila melanogaster* (*w*Mel). Os genomas destas *Wolbachia* foram sequenciados e procedemos a uma caracterização fenotípica, em termos de densidades de *Wolbachia*, longevidade e protecção do hospedeiro contra vírus. Observámos que as variedades mais protectoras são as que possuem densidades de *Wolbachia* mais elevadas e podem constituir um custo para o hospedeiro. Nós descobrimos que as variedades de *w*Mel se separam, baseadas nos fenótipos, em dois grupos que correspondem aos dois grupos monofiléticos de *w*Mel. Isso permitiu a identificação das diferenças genéticas entre estes grupos que deverão ser responsáveis pelas diferenças fenotípicas.

A análise do genoma de *w*MelPop, uma variante patogénica de *w*Mel próxima à variante *w*MelCS, sugeriu que a amplificação de uma região

genómica (Octomom) induz este fenótipo. A partir de moscas com número de cópias de Octomom variável, gerámos linhas de *w*MelPop com diferentes números de cópias de Octomom. Observámos que o número de cópias de Octomom está correlacionado com a virulência: um maior número de cópias corresponde a uma maior quantidade de *Wolbachia* e à morte prematura das moscas. Demonstrámos assim que a amplificação de Octomom é responsável pelo fenótipo patogénico da *w*MelPop. Este estudo fornece evidências de que *Wolbachia* pode evoluir rapidamente através de amplificação génica, apesar de possuir uma baixa taxa de mutação de nucleótidos e também que o controlo de níveis dos endosimbiontes pode ser quebrado com uma única alteração genética no simbionte. Por fim, os nossos resultados mostram a primeira ligação entre genes e fenótipos em *Wolbachia*.

Em seguida, comparámos a protecção antiviral fornecida pela variante natural de *w*Mel mais protectora (*w*MelCS_b) e pela variante *w*Au, transferida de *D. simulans* para *D. melanogaster*. A comparação directa dos fenótipos foi realizada em hospedeiros com a mesma base genética. Mostrámos que *w*Au confere maior protecção contra vírus do que *w*MelCS_b. Além disso, *w*Au prolifera neste hospedeiro a uma taxa maior, atingindo densidades maiores e encurtando o tempo de vida do hospedeiro, quando comparado com *w*MelCS_b. Mostrámos que a variante exógena de *Wolbachia, w*Au, não induz uma ativação geral da immunidade inata. Isto é particularmente importante uma vez que, em mosquitos transifectados com *Wolbachia*, foi proposto que a protecção a vírus é devida ao aumento da activação de imunidade inata.

Por fim, testamos a dependência da temperatura da proteção antiviral fornecida pela variante natural de *w*Mel mais protectora (*w*MelCS_b) em *Drosophila melanogaster*. A nossa análise incluiu diferentes temperaturas antes e após a infecção. Descobrimos que a temperatura antes da infecção é absolutamente crucial para a protecção: moscas

criadas a 18 °C não são protegidas por *Wolbachia*. A temperatura após a infecção determina a mortalidade induzida por vírus em moscas com e sem *Wolbachia* sendo maior a 25 do que a 18 °C. A temperatura após a infecção pode também afectar a protecção, dependendo da dose de vírus. Concluímos, que a protecção antiviral é sensível à temperatura, e pode mesmo estar ausente em determinadas condições térmicas.

No geral, o nosso trabalho fornece informações importantes sobre a biologia da *Wolbachia* e a sua interacção com *Drosophila melanogaster*. As nossas descobertas irão orientar futuros estudos focados na compreensão dos mecanismos de acção da *Wolbachia* e informar os actuais programas de utilização de *Wolbachia* como agente de controlo de doenças transmitidas por vectores. Os nossos dados ajudam também à compreensão da evolução de *Wolbachia* na natureza e destacam o papel de factores ambientais na simbiose defensiva entre *Wolbachia* e *Drosophila*.

List of Abbreviations

AMP	Antimicrobial peptide
APSE	*Acyrthosiphon pisum* secondary endosymbiont
CI	Cytoplasmic Incompatibility
DCV	*Drosophila* C virus
DNA	Deoxyribonucleic acid
dpi	Day post infection
FHV	Flock house virus
indel	Insertion-deletion polymorphism
IS	Insertion sequence
PAXS	Pea aphid X-type symbiont
RNA	Ribonucleic acid
SNP	Single nucleotide polymorphism
$TCID_{50}$	Median tissue culture infectious dose
*w*Mel	*Wolbachia* endosymbiont of *Drosophila melanogaster*
WSP	*Wolbachia* surface protein

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Chapter 1 – General Introduction

1. Symbiosis - "the living together of unlike organisms"

(Heinrich Anton de Bary, 1879)

No organism is an island: each one is influenced by, and in turn influences, other organisms and the environment. Direct, long-lasting relationship between organisms of two different species is called symbiosis (Paracer and Ahmadjian, 2000). Symbiosis implies that partners spend at least portion of their lives together and can be either beneficial to both partners (mutualism), beneficial to one and neutral to the other (commensalism) or beneficial to one and harmful to the other (parasitism). However, this gradation form mutualism to parasitism is not absolute and, depending on the circumstances, the presence of the same symbiotic partner can be either beneficial, neutral or detrimental.

Symbiotic relationships can also be categorized according to physical localisation of the organisms involved. We can discriminate between endo- and exosymbiosis (Das and Varma, 2009). Endosymbiosis requires from a symbiont to live within the tissues of its partner, either within or outside the cells, while exosymbiosis implies maintenance of a symbiont on the exterior of the body, including inner surface of digestive tract and the ducts of exocrine glands (Das and Varma, 2009).

Symbiosis is omnipresent and fundamental in biology and many symbiotic relationships are favoured by natural selection. Widely accepted endosymbiotic theory provides an example of an association that has started over 1.5 billion years ago. An ingestion of a free-living α-proteobacteria by the eukaryotic cell ancestor gave rise to mitochondria of all eukaryotes (reviewed in Lang *et al.*, 1999).

The main focus of this work, however, is the equally successful and fascinating case of the symbiosis between insects and microorganisms, especially the case of *Wolbachia* endosymbionts of *Drosophila*.

1.1. Microbes as insect symbionts

Bacteria are an ancient, diverse and ubiquitous group of organisms, playing essential roles in many ecosystems. As they live virtually everywhere their paths must have crossed with these of animals, including the largest phylum in the animal kingdom – arthropods, and their most numerous and diverse group - insects.

Insects' bodies form stable hospitable niches for microbes, while microbes may possess and easily gain useful, from an insect point of view, metabolic properties. Thus, many mutualistic associations evolved. The ability to host beneficial microorganisms is even thought to partially explain insects' successful colonization of many different habitats (Mandrioli, 2009).

Insect mutualistic symbioses can be divided into obligate, i.e., when the insect is unable to survive without its partner, or facultative, when the relationship is not essential for survival and reproduction of the insect. This is an insect-centric perspective only, as many insect symbionts, especially endosymbionts, are highly adapted to their hosts and symbiosis is obligatory for them in most of the cases.

In obligate associations one-hundred-percent efficient symbiont transmission from parents to offspring is the only way to ensure viability and fertility of the progeny. In contrast, vertical transmission of facultative endosymbionts may be imperfect. Consequently, to persist in populations facultative symbionts need to provide transmitting hosts with a selective advantage - increase either their survival or reproduction (Moran *et al.*, 2008). This can be achieved by mutualistic means, e.g. by nutrient provisioning and protection against natural enemies, or by hijacking host reproductive mechanisms to increase the production and success of infected offspring (Moran *et al.*, 2008). In agreement with the theory, insect associated microbes play diverse beneficial roles in physiology of

their hosts. They provision nutrients (e.g. Douglas, 1998; Baumann, 2005; Feldhaar and Gross, 2009; Sloan and Moran, 2012), affect development (e.g. Lee and Brey, 2013), stimulate maturation of hosts immune system (e.g. Weiss *et al.*, 2011) and affect reproduction (Stouthamer *et al.*, 1999; Bandi *et al.*, 2001; Werren *et al.*, 2008; Fast *et al.*, 2011; Duron and Hurst, 2013). Microbes can also protect their hosts against pathogens, predators or even stress engaging in, so called, defensive symbioses (e.g. Hedges *et al.*, 2008; Teixeira *et al.*, 2008; Oliver and Moran, 2009; White and Torres, 2009; Xie *et al.*, 2013).

1.2. Defensive symbionts of insects

Defensive symbiosis occurs when a microbe protects its host from infectious agents, other stressful stimuli or the consequences of their actions. Defensive symbionts are facultative for their hosts and can spread and persist in populations under the pathogen pressure due to the fitness advantage they confer. Importantly, despite substantial benefits of carrying defensive symbiont in the presence of enemies, these symbionts may constitute a metabolic burden or cause damage to the host in a long term. Therefore, they can be costly for the host.

Many symbionts of insects were shown to mediate interactions between their hosts and their hosts' natural enemies. For example, *Pseudomonas* symbiont of *Paederus* rove beetles produces a toxin, pederin (Kellner, 2001), that repels spiders, thus conferring protection against predation (Kellner and Dettner, 1996).

One of the most extensively studied examples of defensive symbiosis is a relationship between aphids and microorganisms associated with them. The pea aphids *Acyrthosiphon pisum* carry obligate nutritional symbionts, *Buchnera aphidicola*, but frequently also additional facultative maternally-transmitted secondary symbionts. These bacteria provide insects with

thermal tolerance (Montllor *et al.*, 2002; Russell and Moran, 2006) and resistance to natural threats, i.e. parasitoids. Parasitoids attack aphid nymphs and lay eggs within their tissues; as these eggs develop they kill the host. *Hamiltonella defensa* and *Serratia symbiotica*, two secondary symbionts of pea aphids, confer protection against *Aphidius ervi* parasitoid attack (Oliver *et al.*, 2003, 2006; Hansen *et al.*, 2012), and in the double endosymbiont carriers the effect is stronger (Oliver *et al.*, 2003, 2006). These symbionts impair the parasitoid larva development, therefore increasing the survival of the aphids. Additionally, *Hamiltonella*-infected aphids, parasitized as third instar nymphs, produce considerably more offspring than *Hamiltonella*-free controls (Oliver *et al.*, 2005), meaning that this symbionts increase fitness of the host also in terms of reproductive output. The *Hamiltonella*-aphid system was also used to check variation in the protection to parasitoids conferred by different symbiont genotypes (Oliver *et al.*, 2005). All tested bacterial strains were protective, but the fraction of aphids surviving parasitoid attack ranged from 19 to almost 100 %. This study included both: natural *Hamiltonella* symbionts of *A. pisum* and *A. caraccivora* symbiont transinfected to A. pisum (Oliver *et al.*, 2005). Curiously, some genes responsible for the *Hamiltonella*-conferred parasitoid resistance are encoded by *H. defensa* lysogenic bacteriophage (*A. pisum* secondary endosymbiont; APSE) and protection was suggested to occur through the action of phage-derived toxins (Oliver *et al.*, 2005, 2009; Moran *et al.*, 2005; Degnan and Moran, 2008). APSE also controls the *H. defensa*-aphid symbiosis, as its loss is associated with increased symbiont densities and severe deleterious effects on aphids' fitness (Weldon *et al*., 2013).

More recently, another facultative endosymbiont of aphids, *Regiella insecticola* (strain 5.15), has been shown to protect *Myzus persicae* and *Aphis fabae* aphids against *Aphidius colemani* parasitoids *(Vorburger et al.*, 2010) and *A. pisum* aphids from *A. ervi* parasitoids (Hansen *et al.*,

2012). As a different *R. insecticola* strain infecting *A. pisum*, LSR1, does not exhibit protective phenotype, Hansen *et al.* compared the genomes of the defensive and non-defensive *Regiella* (Hansen *et al.*, 2012). They detected many genes differentiating between the two strains, potentially responsible for the protective phenotypes. Mechanisms contributing to bacterial pathogenicity: toxins, secretion systems, parts of endotoxin biosynthetic pathway and a two-component signalling system are predicted to be functional only in the protective strain. Thus, the direct toxic effect of microbial components on attacking parasitoids is a plausible explanation of *R. insecticola*-conferred protection *(Hansen et al.*, 2012).

Another threat to aphid populations are deadly fungal infections and *Regiella* was proven protective against some pathogenic fungi (*Pandora neoaphidis* (Ferrari *et al.*, 2004; Scarborough *et al.*, 2005) and *Zoophthora occidentalis* (Parker *et al.*, 2013)), but not *Beauveria bassiana* (Parker *et al.*, 2013). Recently, it has been shown that protection against fungi in aphids can also be provided by other facultative, vertically transmitted bacteria: *Rickettsia, Rickettsiella and Spiroplasma* (Łukasik *et al.*, 2013).

Another symbiont, *Streptomyces philanthi*, living in the antennal glands of digger wasps, *Philanthus triangulum*, protects wasps' offspring against infections (Kaltenpoth *et al.*, 2005). Protection occurs due to the behaviour of female wasps that place antennal gland secretion, enriched in *Streptomyces* products, in brood cells. Treated cells are protected from fungal infestation and the treatment increases survival probability of the emerging larvae (Kaltenpoth *et al.*, 2005).

The phenomenon of defensive symbiosis has also been studied in *Drosophila*, where *Spiroplasma*, well-known reproductive parasites (male-killers), have recently been recognized as protective symbionts. These motile, cell wall-less, extracellular bacteria with a characteristic

helical morphology have been shown to decrease the size and transmission of a nematode parasite of *Drosophila neotestacea* and to rescue the fertility of nematode-parasitized flies normally sterilized by infection (Xie *et al.*, 2013). This strong protective effect has probably led to rapid continent-wide *Spiroplasma* spread in North American *D. neotestacea* populations in the last decades (Cockburn *et al.*, 2013). In other *Drosophila* species, *Drosophila hydei* and *Drosophila melanogaster, Spiroplasma* were shown to increase survival rate of flies attacked by parasitoid wasps (Xie *et al.*, 2010, 2013). Some of these protective *Spiroplasma* strains do not cause reproductive manipulations, apparently relying solely on the selective advantage of the defences they confer.

Last, but not the least, there is *Wolbachia*, a potent pathogen-blocking agent, that will be discussed in detail in the next chapters.

Bacterial-insect symbioses are prevalent in nature and defensive symbionts mentioned above constitute only a fraction of microbes associated with insects. Moreover, different microorganisms affect their hosts and each other simultaneously and constantly. Perceiving organisms as metaorganisms (Bell, 1998; Viagi *et al.*, 2012) constituted by different populations of prokaryotes, eukaryotes and viruses may be crucial for understanding ecology and evolution of symbiotic associations (Bosch and McFall-Ngai, 2011).

However, we still lack basic knowledge on how symbioses are regulated, what are the roles of many of the species and how homeostasis is maintained in these associations. Only integrated approaches, including genomics, functional analyses and ecological studies, can help to solve the most basic questions. Understanding relationships between insects and their microbiota can allow harnessing associations important for human health and economy and discovery of universal mechanisms operating in animal - bacterial interactions.

2. Wolbachia

Wolbachia are obligate, intracellular, maternally transmitted bacteria living in symbiosis with many invertebrates. *Wolbachia* form a monophyletic clade within α-proteobacteria and they were further taxonomically subdivided into supergroups (Werren *et al.*, 1995; Bandi *et al.*, 1998; Lo *et al.*, 2002, 2007; Rowley *et al.*, 2004; Casiraghi *et al.*, 2005). Currently, ten supergroups are described, with symbionts of arthropods belonging to the supergroups A, B, G, H, I and K, and symbionts of nematodes grouped in clades C, D and J. Supergroup F accommodates symbionts of both, arthropods and nematodes (Ros *et al.*, 2009).

Wolbachia were discovered 90 years ago in the mosquito *Culex pipiens* (Hertig and Wolbach, 1924), but only later identified as a causative agent of mating incompatibilities between uninfected females and infected males (Yen and Barr, 1973). As the presence of *Wolbachia* is extensively surveyed in wild arthropod populations*, Wolbachia* seem to be the most prevalent intracellular bacteria on the planet (Hilgenboecker *et al.*, 2008; Zug and Hammerstein, 2012). As it was mentioned before *Wolbachia* can also infect filarial nematodes, and these symbioses are extremely important for practical reasons. Filariae causing human diseases, including river blindness (onchocerciasis) and elephantiasis (lymphatic filariasis), depend on obligatory symbiosis with *Wolbachia* (Taylor and Hoerauf, 1999). Thus, antibiotics directed towards *Wolbachia* provide an effective antifilarial treatment (Hoerauf *et al.*, 2000). In contrast to the obligatory nature of nematode-bacteria relationships, most of the associations between *Wolbachia* and arthropods are facultative.

Wolbachia adopted two main strategies to persist or spread within hosts' populations. The first strategy involves reproductive manipulations. *Wolbachia* are famous master manipulators of arthropod reproduction,

acting to increase fitness of the symbiont-transmitting females at the expense of their evolutionary dead-end – males (Werren *et al.*, 2008). The most extensively studied manipulations include cytoplasmic incompatibility (CI) (Yen and Barr, 1973), male killing (Hurst *et al.*, 1999), feminization (Rousset *et al.*, 1992) and parthenogenesis (Stouthamer *et al.*, 1993) (Figure 2.1).

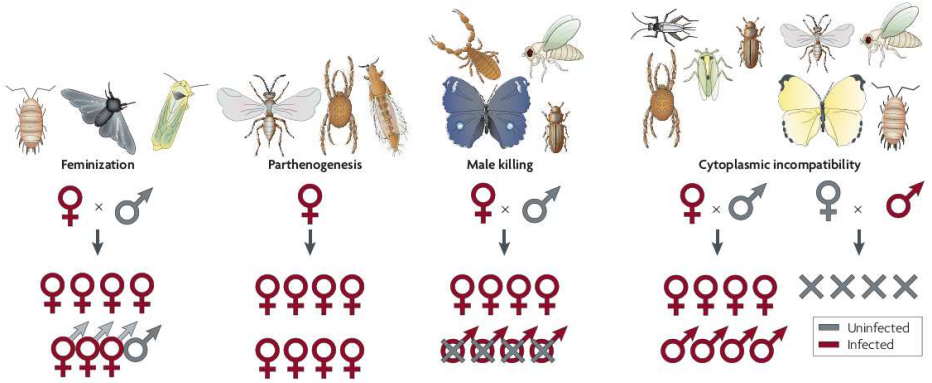

Figure 2.1. *Wolbachia*-induced reproductive manipulations.
Feminization results in genetic male embryos becoming females. Parthenogenesis induction eliminates males from reproduction. Male killing eliminates infected males to the advantage of surviving infected female siblings. Cytoplasmic incompatibility prevents infected males from successfully mating with females that lack the same *Wolbachia* types. Adapted by permission from Macmillan Publishers Ltd: Nature reviews. Microbiology (Werren *et al.*, 2008), copyright 2008.

Another strategy adopted by *Wolbachia* to thrive in arthropod communities is conferring infected individuals with fitness advantage. One of these is protection against pathogens, the phenotype identified initially in the *Wolbachia-Drosophila* symbiosis (Hedges *et al.*, 2008; Teixeira *et al.*, 2008). Also, some anti-parasitoid properties, along with other positive fitness effects, were associated with *Wolbachia* in whiteflies *Bemisa tabaci* (Xue *et al.*, 2012). Combined effects of

Wolbachia anti-apoptotic and pro-mitotic activity increase *Drosophila mauritiana* egg production four times (Fast *et al.*, 2011). However, it has been suggested that such a huge increase in reproductive output may actually be detrimental to the females (Zug and Hammerstein, 2014).

The interactions between *Wolbachia* and their hosts change over time. *Wolbachia* *w*Ri, which, using CI, invaded *D. simulans* populations in California was primarily associated with 15 % reduction in fly fecundity (under the laboratory conditions) (Weeks *et al.*, 2007). During the first 20 years this association has evolved and now infected females exhibit around 10 % fecundity advantage over uninfected females in the laboratory (Weeks *et al.*, 2007).

As *Wolbachia* affect hosts' biology, they must affect hosts' evolution. In populations strongly affected by reproductive manipulations, selection should favour suppressor genotypes (Werren and Beukeboom, 1998; Hurst and Werren, 2001; Hornett *et al.*, 2006). Co-evolution between *Wolbachia* and parasitic wasp, *Asobara tabida*, led to complete dependence of the insect on its endosymbiont. *Asobara* need *Wolbachia* for reproduction as ovaries of aposymbiotic females undergo extensive apoptosis (Pannebakker *et al.*, 2007). *Wolbachia* can also be a source of new biological properties. In bedbugs, *Cimex lectularius*, *Wolbachia* act as nutritional mutualists, enabling utilisation of vitamins B deficient diet - human blood (Hosokawa *et al.*, 2010).

Finally, due to the potential of *Wolbachia* to cause mating incompatibilities, infections with this endosymbiont were postulated to drive speciation (Werren, 1998; Bordenstein, 2003; Telschow *et al.*, 2005, 2007; Jaenike *et al.*, 2006; Bordenstein and Werren, 2007; Miller *et al.*, 2010). Reproductive isolation, prerequisite for speciation, was shown to be caused by *Wolbachia* in *Nasonia* species complex (Bordenstein and Werren, 2007). Moreover, *Wolbachia* cause pre- and post-mating isolation between *Drosophila paulistorum* semispecies

(Miller *et al.*, 2010). Therefore, these endosymbionts have potential to affect not only insects' life history traits, but also to cause evolutionary changes and alter history of species.

2.1. *Wolbachia* endosymbionts of *Drosophila melanogaster* - *w*Mel

Wolbachia endosymbionts of *Drosophila melanogaster* were identified at the end of XX century (O'Neill *et al.*, 1992; Rousset, Vautrin & Solignac, 1992; Holden *et al.*, 1993; Bourtzis *et al.*, 1994). Since then, riding on the never-passing wave of *Drosophila* popularity, they became one of the best studied *Wolbachia* endosymbionts of insects. As it was mentioned before, to spread and persist in populations, symbionts must provide a fitness advantage to the harbouring host or hijack host's reproductive functions to maximize their own spread. In the light of this theory, high prevalence of *Wolbachia* in natural populations of *Drosophila melanogaster* (Hoffmann *et al.*, 1994; Solignac *et al.*, 1994; Ilinsky and Zakharov, 2007; Verspoor and Haddrill, 2011; Fenton *et al.*, 2011) has been a mystery for a long time. Firstly, *Wolbachia* of *Drosophila melanogaster* cause only a weak and conditional cytoplasmic incompatibility (Hoffmann, 1988; Hoffmann *et al.*, 1994, 1998; Yamada *et al.*, 2007), so the spread through reproductive manipulations was unlikely. Also, no obvious fitness advantage could be attributed to the infection with this endosymbiont (Hoffmann *et al.*, 1998; Olsen *et al.*, 2001; Fry and Rand, 2002; Harcombe and Hoffmann, 2004; Fry *et al.*, 2004; Montenegro *et al.*, 2006). Fecundity, sperm competition, thorax length and lifespan effects were all small and dependent on host genetic background (Hoffmann *et al.*, 1998; Olsen *et al.*, 2001; Fry and Rand, 2002; Harcombe and Hoffmann, 2004; Fry *et al.*, 2004; Montenegro *et al.*, 2006). In 2008 *Wolbachia*-conferred protection against pathogens was discovered (Hedges *et al.*, 2008; Teixeira *et al.*, 2008) and proposed

as an explanation of high *Wolbachia* frequencies in the wild *Drosophila* populations (Teixeira *et al.*, 2008). It has been shown that *Drosophila* C virus (DCV) challenge of *Wolbachia*-carrying flies leads to lower viral loads and prolonged survival in comparison to *Wolbachia*-free flies (Figure 2.2) (Teixeira *et al.*, 2008). The presence of *Wolbachia* also increases the lifespan of Flock house virus (FHV) (Figure 2.2) and cricket paralysis virus infected *Drosophila melanogaster* (Hedges *et al.*, 2008; Teixeira *et al.*, 2008).

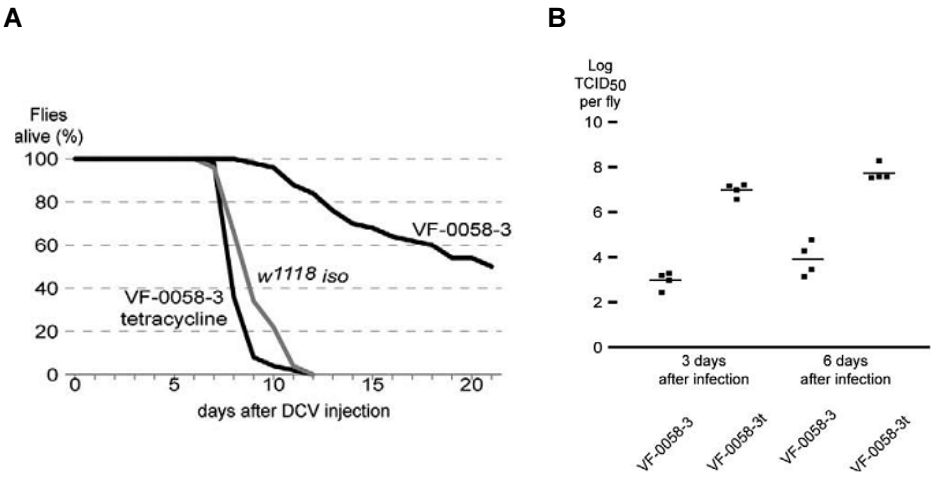

Figure 2.2. *Wolbachia* confers protection to *Drosophila* C virus.
A) Survival of *Wolbachia*-infected flies (VF-0058-3) and *Wolbachia*-free flies (VF-0058-3 tetracycline and w^{1118} *iso*) upon the infection with DCV. B) Virus titres in flies with and without *Wolbachia* 3 and 6 days post viral infection. Adapted from (Teixeira *et al.*, 2008).

Since 2008, native *Wolbachia* have been shown to protect their diverse hosts against many RNA viruses (e.g. Osborne *et al.*, 2009; Glaser and Meola, 2010; Unckless and Jaenike, 2011). Interestingly, fitness advantage by native *Wolbachia* was never detected upon other challenges, like DNA virus (Teixeira *et al.*, 2008), infectious bacteria

(Wong *et al.*, 2011; Rottschaefer and Lazzaro, 2012) and parasitoids (Xie *et al.*, 2013).

Wolbachia endosymbionts of *Drosophila melanogaster* were characterised molecularly using sequences of 16S rDNA, *ftsZ, dnaA,* and fast evolving *wsp* gene and designated as *w*Mel strain (Holden *et al.*, 1993; Bourtzis *et al.*, 1994; Werren *et al.*, 1995; Zhou *et al.*, 1998). Later, the genome of *w*Mel was sequenced and annotated (Wu *et al.*, 2004), which shed light on some of the *Wolbachia* metabolic properties and potential interactions with the host (described in detail in the Section 3).

Subsequently, *Wolbachia w*Mel strain was shown to be heterogeneous and different variants have been defined using polymorphisms between their genomes. Riegler *et al.* described five *Wolbachia w*Mel variants: *w*Mel, *w*Mel2, *w*Mel3, *w*MelCS and *w*MelCS2 (Figure 2.3) (Riegler *et al.*, 2005). It has also been reported that the frequencies of *Wolbachia* genotypes in wild *Drosophila melanogaster* populations changed during the 20th century (Riegler *et al.*, 2005; Nunes *et al.*, 2008). Once prevalent, *w*MelCS-like variants have been replaced by *w*Mel-like variants (Riegler *et al.*, 2005; Nunes *et al.*, 2008). More recent phylogenetic analysis provided additional evidence supporting the replacement and revealed that it has started before 20th century and remains incomplete (Richardson *et al.*, 2012). Moreover, it demonstrated clustering of *w*Mel variants into two monophyletic groups: *w*Mel-like and *w*MelCS-like (Richardson *et al.*, 2012). Yet, the shift in *w*Mel variant frequencies in nature remains unexplained, as phenotypic differences between *w*Mel- and *w*MelCS-like genotypes were never reported before.

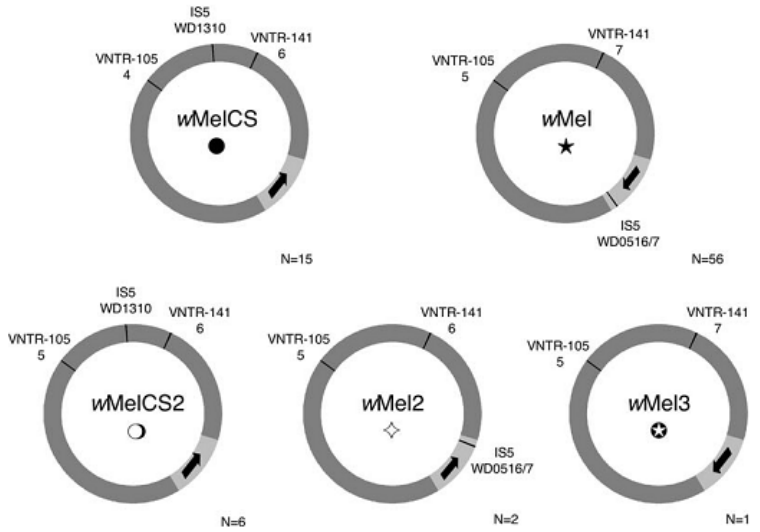

Figure 2.3. Chromosomal maps of five different *w*Mel variants.
The genotypes are differentiated by two variable number tandem repeat (VNTR) loci, two differential insertion sites of IS5, and a large chromosomal inversion. Reprinted from Current Biology, (Riegler *et al.*, 2005), copyright 2005, with permission from Elsevier.

Apart from antiviral protection, natural *w*Mel variants do not exert any obvious effect on the host. In contrast, *w*MelPop is a virulent variant, which was isolated form a laboratory *D. melanogaster* stock in a screen for mutations causing brain degeneration (Min and Benzer, 1997). This variant over-proliferates massively within host tissues, including brain, and shortens lifespan of the flies (Min and Benzer, 1997; McGraw *et al.*, 2002). Despite dramatic difference in phenotype between *w*MelPop and other *w*Mel variants, including closely related *w*MelCS, the genetic bases of *w*MelPop pathogenicity were unknown (Riegler *et al.*, 2005, 2012). *w*MelPop is unique among *Wolbachia*, as endosymbionts depend on their hosts for survival and their presence is usually associated with a low fitness cost. Thus, *w*MelPop is interesting due to its virulence, mysterious

pathogenicity basis, but also as good candidate for an arboviral disease control agent (discussed in the Sections 2.2 and 2.3).

2.2. Artificial *Wolbachia* – host associations

Wolbachia holds a promise for successful control of some arthropod-borne diseases. Firstly, *Wolbachia* can suppress populations of vectors: releases of males that mate but cannot produce offspring with local females may limit the total number of mosquitoes in the certain area (Zabalou *et al.*, 2004). Also, as cytoplasmic incompatibility facilitates *Wolbachia* spread among insects, it could potentially introduce pathogen-blocking transgenes into wild populations (Zabalou *et al.*, 2004). However, this scenario is less probable as currently there are no tools to manipulate *Wolbachia* genetically. Moreover, only old mosquitoes transmit diseases to humans, because for the transmission to occur:
(i) mosquito needs to feed on the infected individual,
(ii) virus has to travel from insect's gut to salivary glands,
(iii) mosquito needs to feed again.
Life-shortening effect of *Wolbachia w*MelPop could break the disease transmission cycle by eliminating older individuals before they have enough time to complete all these steps (Cook *et al.*, 2008). Finally, *Wolbachia* can eliminate the pathogens by making the mosquitoes more resistant (Hedges *et al.*, 2008; Teixeira *et al.*, 2008; Moreira *et al.*, 2009). All the reasons mentioned above have led to considerable efforts to establish protective or incompatible *Wolbachia* infections in natural vectors of human diseases. This was necessary as in some of these mosquitoes native *Wolbachia* infection is not protective while others are naturally uninfected.
New *Wolbachia*-host associations can be difficult to initiate. Dengue vector *Aedes aegyptii* is, up to now, thought to lack natural *Wolbachia*

infections. To introduce *w*MelPop to this mosquito species, a few years of serial passages in mosquito cell lines were necessary. During this time *Wolbachia* "got adapted" to the new mosquito intracellular environment, and this adapted strain is referred to as *w*MelPop-PGYP (McMeniman *et al.*, 2008). The *w*MelPop-PGYP adaptation has recently been described on the level of *Wolbachia* genome (Woolfit *et al.*, 2013), but it is not known which genomic changes (out of an insertion, two deletions, and two single nucleotide polymorphisms (SNPs)) are responsible for the success of the transfer.

Malaria transmitting mosquitoes of *Anopheles* genus were also thought to be *Wolbachia*-free (Hughes, Ren, *et al.*, 2011). Recently, not only stable *Wolbachia* infections in *Anopheles stephensi* were established (Bian, Joshi, *et al.*, 2013) but also *Wolbachia* sequences were found in samples from *Anopheles gambiae* natural populations (Baldini *et al.*, 2014). Thus, knowledge about *Wolbachia* prevalence in nature could help to optimize future transinfection strategies. Especially, because transinfections between closely related species can be achieved without additional procedures, as it was the case for *w*AlbB from *Aedes albopictus* transferred to *Ae. aegyptii* or *w*Au transferred between *D. simulans* and *D. melanogaster* (Xi *et al.*, 2005; Zabalou *et al.*, 2008; Bian *et al.*, 2010; Yamada *et al.*, 2011).

Heterologous *Wolbachia*, most extensively studied in mosquitoes, inhibit a broad range of parasites and pathogens, including dengue and chikungunya viruses, *Plasmodium* spp., and filarial nematodes (Kambris *et al.*, 2009; Moreira *et al.*, 2009; Bian *et al.*, 2010; Bian, Joshi, *et al.*, 2013; Hughes, Koga, *et al.*, 2011). In all these instances protection against pathogens and parasites has been associated with immune activation in *Wolbachia*-transinfected animals (Kambris *et al.*, 2009, 2010; Moreira *et al.*, 2009; Bian *et al.*, 2010; Hughes, Koga, *et al.*, 2011; Rancès *et al.*, 2012), that persisted in most of the cases (Kambris *et al.*,

2009, 2010; Moreira *et al.*, 2009; Bian *et al.*, 2010; Rancès *et al.*, 2012). Antiviral effect was also shown to be complemented by resistance to bacteria in *w*Mel and *w*MelPop transinfected *Aedes aegyptii* (Ye *et al.*, 2013). Transinfected mosquitoes may also exhibit cytoplasmic incompatibility (Blagrove *et al.*, 2012) and shorten lifespan (McMeniman *et al.*, 2009; Kambris *et al.*, 2009; Suh *et al.*, 2009). However, fitness cost associated with life-shortening symbionts can be severe, impeding mosquitoes spread in the wild.

Pathogen blocking capabilities of *Wolbachia* can change after the interspecies *Wolbachia* transfer. *w*AlbB does not limit the dengue titres in its native *Aedes albopictus* host (Mousson *et al.*, 2012) but transferred to *Ae. aegyptii* exhibits strong pathogen blocking (Bian *et al.*, 2010). As it was mentioned before, replacement of a native *Wolbachia* with a novel strain may arm the mosquito with a new layer of antiviral defences (Bian, Zhou, *et al.*, 2013).

Some other *Wolbachia*-associated phenotypes also arise only in a novel host (Zabalou *et al.*, 2008). Transinfected *Wolbachia* can become virulent (Bouchon *et al.*, 1998; McGraw *et al.*, 2002; Sasaki *et al.*, 2002), but the most severe effects were seen to fade away in the course of co-adaptation (McGraw *et al.*, 2002; McMeniman *et al.*, 2008). Interspecific host transfer may lead to generation of pathogenic *Wolbachia* that are unable to adapt as they kill the hosts before the hosts reproduce. This is the case for endosymbiont of *Armadillidium* transinfected to *Porcelio dilatatus*. Pathogenicity of *Wolbachia* in this isopod is associated with massive bacterial proliferation and necrosis of nervous tissues, probably leading to host paralysis and premature death (Bouchon *et al.*, 1998). These phenotypes resemble the phenotypes exerted by native but virulent *w*MelPop in *D. melanogaster* (Min and Benzer, 1997).

Wolbachia transfers between hosts were also performed to study other *Wolbachia*-associated phenotypes, including cytoplasmic incompatibility.

The work of Yamada *et al.*, 2011 was based on two closely related *Wolbachia* strains: *w*Mel (able to induce and rescue CI) and *w*Au (unable to induce or rescue CI). *w*Au is native to *Drosophila simulans* and to be used next to *w*Mel it was microinjected to *D. melanogaster* embryos. The authors aimed at restoring CI modifying and rescuing properties by expressing candidate *Wolbachia* effector proteins form *w*Mel (where CI operates) in *w*Au harbouring flies. They could neither induce CI-like mortality nor supress the CI-phenotype in the incompatible crosses, but the tool they generated, *w*Au in *D. melanogaster* background (Yamada *et al.*, 2011), serves for future research.

2.3. Mechanisms of *Wolbachia*-conferred antiviral protection

Although *Wolbachia*-conferred antiviral protection has been extensively studied in many systems the mechanisms of its action remain unknown. There are few main hypotheses aiming at explaining *Wolbachia*-conferred protection (Teixeira *et al.*, 2008). Firstly, *Wolbachia* could activate immune system of the host and *ipso facto* influence pathogens. *Wolbachia* could also down-regulate host's immune response to the pathogen to prevent self-damage. *Wolbachia* transinfection experiments described in the previous section provide numerous evidences that host immune system responds to newly introduced endosymbiont. In mosquitoes transinfected with *Wolbachia* the antiviral effect is associated with activation of host's immune system (Kambris *et al.*, 2009, 2010; Moreira *et al.*, 2009; Bian *et al.*, 2010; Blagrove *et al.*, 2012; Pan *et al.*, 2012; Rancès *et al.*, 2012). However, natural co-evolved *D. melanogaster* – *Wolbachia* associations are characterised by strong antiviral protection without immune upregulation (Bourtzis *et al.*, 2000; Wong *et al.*, 2011; Rancès *et al.*, 2012; Teixeira, 2012). This means that general immune priming cannot explain all cases of pathogen blocking. It

is either specific to the newly established *Wolbachia*-insect symbioses or it only enhances the action of immune priming independent of the main antiviral mechanism.

Many pathways involved in antiviral immunity in insects were tested for interactions with *Wolbachia*, under the assumption that *Wolbachia* confers protection using host's immune system. The main antiviral mechanism in *Drosophila* is the small interfering RNA pathway, which detects virus-derived double-stranded RNA and suppresses viral replication. The loss of small interfering RNA pathway function had no effect on *Wolbachia*-conferred antiviral protection (Hedges *et al.*, 2012). Other pathways required for response to virus in insects are Imd pathway (Costa *et al.*, 2009) and Toll pathway (Zambon *et al.*, 2005; Ferreira *et al.*, 2014). These pathways were initially described as mediating response to pathogenic bacteria and fungi, and their activation leads to upregulation of specific immune genes, including antimicrobial peptides (Lemaitre and Hoffmann, 2007). The same antimicrobial peptides are up-regulated by *Wolbachia* presence in all described cases of artificial transinfection and immune priming (Kambris *et al.*, 2009, 2010; Moreira *et al.*, 2009; Bian *et al.*, 2010; Blagrove *et al.*, 2012; Pan *et al.*, 2012; Rancès *et al.*, 2012). Moreover, Pan *et al.* have shown that some of these antimicrobial peptides, namely cecropin and defensin, reduce dengue virus load when overexpressed in the midgut and fat body of mosquitoes (Pan *et al.*, 2012). However, neither of these two pathways is required for *Wolbachia*-conferred antiviral protection in *Drosophila*, as mutants with *Wolbachia* consistently exhibit significantly lower virus titres than the mutants without *Wolbachia* (Rancès *et al.*, 2013). The last pathway known to mediate antiviral responses in *Drosophila*, JAK-STAT (Dostert *et al.*, 2005), remains to be tested.

Another possible explanation of *Wolbachia*-conferred protection would be a competition between *Wolbachia* and pathogens for limited resources

(Teixeira *et al.*, 2008). Several nutrients were proposed as limiting factors for both *Wolbachia* and virus growth. Iron is a promising candidate, as its availability restrains growth of many bacteria and *Wolbachia* has been shown to interfere with ferritin expression and iron metabolism in insects (Kremer *et al.*, 2009) and to increase iron-deprived females reproductive output (Brownlie *et al.*, 2009). Second potential growth limiting factor within the host is cholesterol, especially because insects do not synthetize it and rely solely on nutritional supplementation (Clark and Bloch, 1959). Cholesterol enrichment of fly diet has recently been shown to modulate the interaction of the *Wolbachia*-infected flies with virus (Caragata *et al.*, 2013). Caragata *et. al.* (2013) show that the higher the levels of cholesterol in the food, the higher the *Drosophila* C virus titres. Also, *w*MelPop infected flies fed on a high cholesterol diet had slightly lower *Wolbachia* densities. However, *Wolbachia w*MelCS densities were cholesterol independent (Caragata *et al.*, 2013). Along similar lines, *Wolbachia w*MelPop-PGYP was shown to decrease endogenous cholesterol levels in *A. aegyptii*, but cholesterol supplementation could not rescue impaired fecundity or egg viability (Caragata *et al.*, 2014). Overall, the role of cholesterol in *Drosophila*-*Wolbachia* and *Drosophila*-*Wolbachia*-virus interactions remains unclear.

Crucial for our understanding of *Wolbachia*-conferred protection was the link between higher *Wolbachia* density and stronger protection. This has already been demonstrated using different *Wolbachia* genotypes (the ones that grow to higher densities protect better) (Osborne *et al.*, 2009; Frentiu *et al.*, 2010), or using the same *Wolbachia*-infected host treated with gradient of antibiotics (Lu, Bian, *et al.*, 2012; Osborne *et al.*, 2012). Thus, it is feasible that the mechanisms that control *Wolbachia* densities within the host are the ones that ultimately control antiviral protection.

The control of *Wolbachia* densities is not well understood, but both, host and endosymbiont genotypes influence it (McGraw *et al.*, 2002; Veneti *et*

al., 2003; Mouton *et al.*, 2003, 2007; Kondo *et al.*, 2005). Autophagy, a conserved intracellular mechanism responsible for degradation of unnecessary or dysfunctional cellular components, has recently been shown to control *Wolbachia* numbers across all distinct symbiotic relationships, from worms to flies (Voronin *et al.*, 2012). Therefore, autophagy may control viral infection indirectly, *via* control of *Wolbachia* densities. Autophagy can also be pro- or antiviral (Kudchodkar and Levine, 2009). Therefore the interactions of *Wolbachia* with autophagic machinery may interfere with viral life cycle.

Another important cellular process manipulated by *Wolbachia* that could explain antiviral protection is apoptosis. *Wolbachia* is known to block apoptosis as endosymbiont removal from *Asobara tabida* causes massive apoptosis in the germline that sterilizes the wasp (Dedeine *et al.*, 2001). Also, antibiotic clearance of *Wolbachia* from *Brugia malayi* causes apoptosis across all tested developmental stages of nematode (Landmann *et al.*, 2011). On the other hand, viruses can manipulate apoptotic pathways, e.g. *Wolbachia*-sensitive Flock house virus (Teixeira *et al.*, 2008) induces it (Settles and Friesen, 2008). Therefore, antagonistic effect of *Wolbachia* on apoptosis in host-virus interaction could underlie protective phenotype.

In addition, it has been shown that *Wolbachia* regulate microRNA expression in the host, and by doing so regulate expression of the genes crucial for their own maintenance (Hussain *et al.*, 2011; Osei-Amo *et al.*, 2012). *Ae. aegypti* DNA methyltransferase gene (*AaDnmt2*) regulated by one of the *Wolbachia* induced microRNAs, aae-miR-2940 (Hussain *et al.*, 2011), has even been suggested to contribute to *Wolbachia*-conferred antiviral protection (Zhang *et al.*, 2013). Overexpression of *AaDnmt2* in mosquito cells reduces *Wolbachia* densities and promotes replication of dengue virus in the cells without *Wolbachia*. However, when aae-miR-2940 was specifically inhibited leading to the increase in *AaDnmt2*

expression, the cells with *Wolbachia* had only slightly higher and not statistically different virus titres than the cells treated with nonsense inhibitor. Therefore, the model in which aae-miR-2940 down-regulates the expression of *AaDnmt2*, allowing *Wolbachia* to reach its physiological densities and block dengue virus (Zhang *et al.*, 2013) does not hold. Moreover, the *Dnmt2* was not affected by *Drosophila* C virus or Flock house virus infection in a set of 19 *Wolbachia* strains transfected into *Drosophila simulans* common genetic background (Martinez *et al.*, 2014). Finally, no correlation between *Dnmt2* expression in each line and the protective ability of the corresponding *Wolbachia* was detected (Martinez *et al.*, 2014).

The first report on protection in *Drosophila melanogaster* provided evidence that *Wolbachia* can either increase survival decreasing pathogen burden (resistance), as was the case of *Drosophila* C virus, or increase survival without interfering with pathogen load (tolerance to infection), which was the case for Flock house virus (Teixeira *et al.*, 2008). This suggested, that the mechanism of protection might be different for different viruses. However, the recent study by Martinez *et al.* (2014) shows that protection against DCV is strongly genetically correlated with protection to FHV. Therefore, an action of a single mechanism of protection active against the two pathogens seems plausible (Martinez *et al.*, 2014).

Although the last few years brought us closer to understanding factors influencing *Wolbachia*-conferred protection, we do not know how general most of these findings are. Also, as the mechanism or mechanisms of protection remain unknown further efforts are necessary.

3. Genomic studies of obligate symbionts

Many obligate intracellular bacteria cannot be cultured or genetically manipulated, thus genomic approaches provide the only insight into their metabolism and interactions with the hosts. These approaches have already been deployed to generate hypotheses explaining reproductive manipulations of *Wolbachia* (Wu *et al.*, 2004; Sinkins *et al.*, 2005; Klasson *et al.*, 2008) and nutrient provisioning in many insect species (Akman *et al.*, 2002; van Ham *et al.*, 2003; Degnan *et al.*, 2005; McCutcheon and Moran, 2007; Nakabachi *et al.*, 2006). Our understanding of some defensive symbioses has also progressed recently, mainly due to the comparative genomic approaches (Hansen *et al.*, 2012).

Genomes of obligate intracellular bacteria evolve under very specific constraints. On one hand effective symbionts population sizes are small, because of restricted space within the host and limited number of hosts. Frequent bottlenecks and rare populations mixing, implicit in vertical transmission, allow accumulation of slightly deleterious mutations and loss of beneficial alleles due to drift (Moran, 1996). Stable and nutrient-rich environment removes the necessity to maintain many of the metabolism and stress response genes, which leads to genome reduction.

As all endosymbionts are descendants of free-living bacteria and many of them were acquired independently, today they find themselves at different stages of adaptation to the host (Figure 3.1) (Toft and Andersson, 2010).

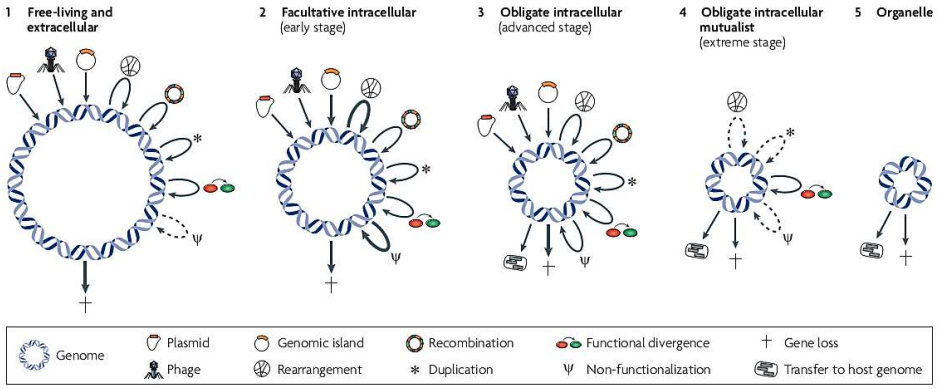

Figure 3.1. Stages of bacteria host-adaptation.

Arrows pointing to the genome indicate acquisition of genes through horizontal gene transfer. Arrows that loop back to the genome indicate changes within the genome. Arrows pointing away indicate gene loss. Influence of each of these events at the different stages is shown by the weight of the arrow. Adapted by permission from Macmillan Publishers Ltd: Nature Reviews: Genetics (Toft and Andersson, 2010), copyright 2010.

As depicted in Figure 3.1 endosymbiont genomes undergo not only gradual gene loss, but also changes in abundance of mobile elements, phages and phage-derived genes. Obligate endosymbiotic bacteria, which are mostly maintained in highly specialized host cells or organs – bacteriomes, have some of the smallest and most eroded genomes among known bacteria (Shigenobu *et al.*, 2000; Pérez-Brocal *et al.*, 2006; McCutcheon and Moran, 2007; Nakabachi *et al.*, 2006; Bennett and Moran, 2013). Accordingly, genomes of obligate mutualistic *Wolbachia* of nematodes are usually smaller than these of facultative endosymbionts of arthropods (Table 3.1).

Table 3.1. Genome sizes of the sequenced *Wolbachia* strains.

Wolbachia	**Host species**	**Phylum**	**Symbiosis type**	**Genome size [MB]**	**Reference**
*w*Mel	*Drosophila melanogaster*	*Arthropoda*	facultative	1.27	(Wu *et al.*, 2004)
*w*Ri	*Drosophila simulans*	*Arthropoda*	facultative	1.45	(Klasson *et al.*, 2009)
*w*Ha	*Drosophila simulans*	*Arthropoda*	facultative	1.30	(Ellegaard *et al.* 2013)
*w*No	*Drosophila simulans*	*Arthropoda*	facultative	1.30	(Ellegaard *et al.* 2013)
*w*Pip	*Culex pipiens*	*Arthropoda*	facultative	1.48	(Klasson *et al.*, 2008)
*w*Rec	*Drosophila recens*	*Arthropoda*	facultative	1.13	(Metcalf *et al.*, 2014)
*w*Cle	*Cimex lectularius*	*Athropoda*	obligate	1.25	(Nikoh *et al.*, 2014)
*w*Bm	*Brugia malayi*	*Nematoda*	obligate	1.08	(Foster *et al.*, 2005)
*w*Oo	*Onchocerca volvulus*	*Nematoda*	obligate	0.96	(Darby *et al.*, 2012)

On the other hand, the pressure for maximal adaptation to the hosts eliminates mutations leading to the loss of symbiont genes beneficial to the host (Canbäck *et al.*, 2004). Nutritional mutualist *Buchnera* was even shown to possess additional control over the copy number of genes useful for aphid by exporting them to plasmids (Rouhbakhsh *et al.*, 1997).

Of note, Figure 3.1 suggests that all endosymbionts are on the way to become organelles. This is not true, as extreme genome reduction may

also lead to symbiont replacement: acquisition of new symbiont with bigger genome and potentially more metabolic capabilities and loss of an ancient but reduced partner (Koga and Moran, 2014). Remarkably, genome can be completely lost from the cytoplasmic entities. This is the case for hydrogenosomes, highly derived mitochondria of *Trichomonas vaginalis* (Palmer, 1997), that still function because the genes responsible for their maintenance were transferred to the cell nucleus (Martin and Herrmann, 1998).

Wolbachia, just like other endosymbionts, have strongly reduced genomes. All are AT rich and lack many of the genes essential in free-living bacteria. As *Wolbachia* possess many amino acid transporters and catabolising genes, it was postulated that they use host-derived amino acids for energy production (Wu *et al.*, 2004; Foster *et al.*, 2005). Competition for amino acids between host and *Wolbachia* has recently been tested using *A. aegyptii* and its adapted *Wolbachia w*MelPop-PGYP (Caragata *et al.*, 2014). Mosquitoes with *w*MelPop-PGYP fed on sheep blood, supposedly lacking all essential amino acids, display significant fecundity and egg viability defects not observed in aposymbiotic animals. Supplementation of the sheep blood with tryptophan, valine, methionine, leucine or mix of the four only slightly increases fecundity but significantly improves egg viability (Caragata *et al.*, 2014). However, as even the egg viability rescue was not complete, it is possible that the lack of other components missing from the sheep blood impairs mosquitoes reproduction (Caragata *et al.*, 2014).

Facultative *Wolbachia* genomes harbour numerous mobile genetic elements, insertion sequences, as well as prophage and repeated regions (Wu *et al.*, 2004; Klasson *et al.*, 2008, 2009). These are the likely causes of many genomic rearrangements, including the ones identified between *w*Mel and *w*Pip and *w*Mel and *w*Bm, which lead to the lack of conservation of the general gene order between these *Wolbachia* strains

(Wu *et al.*, 2004; Foster *et al.*, 2005; Klasson *et al.*, 2008). Similar structure of the *H. defensa* genome suggests that plasticity and dynamic nature are properties of many endosymbiont genomes (Degnan *et al.*, 2009).

An evident peculiarity of *Wolbachia* genomes is an exceptionally high content of genes coding for proteins with ankyrin repeats. Ankyrin repeats are 33-residue sequence motifs abundant in eukaryotic proteins and they mediate protein-protein interactions. Ankyrin proteins are believed to have been acquired by bacteria from eukaryotes by horizontal gene transfer (Bork, 1993). As such, these proteins are candidate effector proteins of *Wolbachia*. *Wolbachia* strains encode different numbers of ankyrin proteins, from 23 in *w*Mel (Wu *et al.*, 2004) to 60 in *w*Pip (Klasson *et al.*, 2008). A type IV secretion system (TIVSS), also encoded in *Wolbachia* genomes, has been shown before to deliver *Legionella pneumophila* and *Coxiella burnetii* ankyrin domain-containing effectors into eukaryotic cells (Pan *et al.*, 2008). Altogether, this suggests that *Wolbachia* interact with the host using exported effector proteins.

Genomes are also able to provide insight into *Wolbachia* microevolution. Using 179 lines of *Drosophila melanogaster* from North America, Europe, and Africa, Richardson *et al.*, (2012) estimated the short-term evolutionary rate for *w*Mel *Wolbachia*. Based on the 3rd codon position *Wolbachia* substitution rate is 10 times lower than that of the *Drosophila* genome and more than 30 times lower than that of obligate aphid symbiont *Buchnera aphidicola* (Moran *et al.*, 2009). Richardson and colleagues explain this observation by the presence of the functional DNA repair pathways in *Wolbachia* (Wu *et al.*, 2004), but not in *Buchnera* (Moran and Mira, 2001). In contrast to *B. aphidicola*, no differences were detected in the estimated substitution rates for 1st, 2nd and 3rd codon position in *Wolbachia* (Moran *et al.*, 2009; Richardson *et al.*, 2012), indicating that the purifying selection acting strongly on

B. aphidicola is much weaker in the case of *w*Mel (Richardson *et al.*, 2012).

Recent shift from morphological and functional characterisation of bacteria to whole genome sequencing and molecular phylogenomics refined our perception of the biology of many symbionts, including *Wolbachia*. Increasingly abundant genomic data could be a starting point for molecular characterisation of host-endosymbiont interactions and discovery of function of novel genes. Finally, these data may help to understand evolutionary origin and history of the host-endosymbiont associations.

4. Temperature influence on insect-microbes interactions

Environment affects individual organisms and relationships between them. Conditions encountered by the host shape the niche within its body, impacting associated beneficial and pathogenic organisms. Therefore, numerous physical and biological factors were described as important for symbioses. These include population density (Dutton and Sinkins, 2004; Wiwatanaratanabutr and Kittayapong, 2009), nutrient availability (Brownlie *et al.*, 2009; Caragata *et al.*, 2013, 2014) and last but not the least, temperature (Reynolds *et al.*, 2003; Mouton *et al.*, 2006, 2007; Guruprasad *et al.*, 2011; Bordenstein and Bordenstein, 2011; Kusmintarsih, 2012; Lu, Zhang, *et al.*, 2012; Murdock *et al.*, 2014). It is also known that all these variables are interconnected, and can produce either synergistic or antagonistic effects (Triggs and Knell, 2012), which are additionally dependent on the genotypes of both partners (Stacey *et al.*, 2003; Oliver *et al.*, 2005). This should be kept in mind in the context of the temperature impact on *Wolbachia-Drosophila* symbiosis.

4.1. Thermal sensitivity of insects

Insects' body temperature is variable and dependent on ambient temperature. It can be sensed and regulated by producing heat (e.g. moths generate heat in the thorax prior to flight) or behaviour (i.e. looking for optimum and avoiding extremes) (Denlinger and Yocum, 1998).
Drosophila melanogaster develop between 12 and 32 °C (Ludwig and Cable, 1933; David and Clavel, 1966) and their thermal optimum depends on geographical origin of the population (Cohet *et al.*, 1980). Standard *Drosophila* laboratory maintenance involves housing flies at 25 °C or 18 °C. In our laboratory this results in 10 or 20 days development

from eggs to adults, respectively. Flies raised at these two temperatures differ in body size, with 18 °C raised flies being larger as a consequence of increased cell sizes (French *et al.*, 1998). The rate of metabolic processes is also lower in *Drosophila* maintained at 18 °C (Berrigan and Partridge, 1997).

Insect immune responses also depend on temperature. Many insects fight infections more efficiently at elevated temperatures (e.g. Kobayashi *et al.*, 1981; Carruthers *et al.*, 1992; Blanford *et al.*, 2000; Frid and Myers, 2002; Thomas and Blanford, 2003), while in others higher temperatures inhibit immune function (e.g. Thomas and Blanford, 2003; Bensadia *et al.*, 2006; Fels and Kaltz, 2006; Allen and Little, 2010; Karl *et al.*, 2011). *Drosophila melanogaster* infected with pathogenic bacteria survive longer at 17 °C than at 25 or 29 °C, which was shown to be an outcome of stronger immune response and poorer bacteria proliferation (Linder *et al.*, 2008). Again, temperature optimal for immune function is dependent on the origin of flies (Lazzaro *et al.*, 2008).

4.2. Temperature dependence of insect-endosymbiont interactions

Endosymbionts can be sensitive to temperature (Wernegreen, 2012). This is often explained by many slightly deleterious mutations that destabilize proteins structures (Moran, 1996). High heat shock protein expression, especially that of chaperonin GroEL, was suggested to be stabilizing degenerated proteins, compensating for these mutations in physiological range of conditions (Fares *et al.*, 2004; Wernegreen, 2012), but may be insufficient in the conditions of thermal stress.

Wolbachia densities, responsible for the strength of *Wolbachia*-induced phenotypes, show complex dependence on temperature. In some insect species they decrease with increased temperature, while in others they increase. Non-linear temperature dependence was also observed, with

the optimum at intermediate temperatures (Table 4.1.) (reviewed in Murdock *et al.*, 2014).

Table 4.1. Temperature influence on *Wolbachia* densities.

Adapted from Murdock *et al*., 2014.

System	Temperature	Result	Reference
Nasonia vitripennis (wasp)	18 °C, 25 °C, 30 °C	Densities were significantly lower at cooler and warmer temperatures, relative to wasps held at 25 °C.	(Bordenstein and Bordenstein, 2011)
Exorista sorbillans (Uzifly)	26 °C and 33 °C	*Wolbachia* were eliminated at 33 °C relative to *Wolbachia*-infected females at 26 °C.	(Guruprasad *et al.*, 2011)
Tetranychus urticae (spider mite)	19 °C, 22 °C, 25 °C, 28 °C, 31 °C	*Wolbachia* densities were highest in both males and females at 25 °C in the ZJ[a] mite strain and was highest in YC[a] female mites at 25 °C and in YC[a] males at 28 °C relative to mites reared at cooler and warmer temperatures.	(Lu, Zhang, *et al.*, 2012)
Leptopilina heterotoma (wasp)	20 °C and 26 °C	A7[a] females reared at 20 °C experienced significantly lower *Wolbachia* density, while *Wolbachia* densities in SF4[a] females remained unaffected by temperature.	(Mouton *et al.*, 2006, 2007)
Aedes albopictus (Asian tiger mosquito)	25 °C and 37 °C	Elevated temperature significantly decreased *Wolbachia* density in all stages in both males and females.	(Wiwatanaratanabutr and Kittayapong, 2009)
Aphytis melinus (wasp)	24 °C, 25 °C, 27.5 °C, 30 °C, and 32.5 °C	*Wolbachia* densities decreased in wasps reared at warmer temperatures (30 °C and 32.5 °C) relative to cooler temperatures.	(Vasquez *et al.*, 2011)

[a] ZJ, YC, A7 and SF4 refer to different genotypes of the spider mites and wasps.

Densities of other symbionts may also decrease at higher temperatures. In aphids, 4 hours at 39 °C can eliminate 97 % of the endosymbiont *Buchnera* (Montllor *et al.*, 2002). This has obvious negative effects on fitness of the host as *Buchnera* are obligate nutritional mutualists. However, secondary endosymbionts can provide aphids with protection against heat stress (Montllor *et al.*, 2002; Russell and Moran, 2006), not only by limiting the loss of *Buchnera* inhabited bacteriocytes (Montllor *et al.*, 2002), but probably by metabolic compensation for the loss of primary endosymbiont (Koga *et al.*, 2003). Moreover, Dunbar *et al.* reported that a single base deletion in the genome of *Buchnera* can completely change aphids thermal tolerance (Dunbar *et al.*, 2007). Therefore, thermal sensitivity of the symbiont can determine thermal sensitivity of the host.

4.3. Defensive symbiosis and temperature

Defensive symbioses should be affected by temperature in a more complex way, as apart from host and endosymbiont, pathogen, parasite or predator and its thermal tolerance have to be added to the equation.

Insect-pathogen encounter outcomes at any temperature depend on the previously discussed insect immune response and pathogen performance.

The temperature effect in *Anopheles stephensi* artificially transinfected with *Wolbachia* was explored in the context of protection against *Plasmodium* (Murdock *et al.*, 2014). While *Wolbachia* densities were directly correlated with temperature, anti-plasmodium effect was changing in a more unsystematic way and ranged from actual pathogen blocking, through no effect, to enhancement of the infection (Murdock *et al.*, 2014).

Variation in the symbiont-conferred protection has been studied more extensively in aphids subjected to parasitoids attack (Bensadia *et al.*, 2006; Guay *et al.*, 2009). In particular, elevated temperature can compromise *Hamiltonella defensa*-conferred protection to parasitoids (Bensadia *et al.*, 2006). Interestingly, protection remains strong in aphids co-infected with *H. defensa* and pea aphid X-type symbiont (PAXS) (Guay *et al.*, 2009). Therefore, the effect of temperature on defensive symbiosis depends on the presence of other symbionts (Guay *et al.*, 2009).

Importantly, effects of temperature on the protection should also be determined by the temperature impact on the mechanisms of that protection. Therefore, protection dependent on the phage encoded toxins or other effectors (Moran *et al.*, 2005; Degnan and Moran, 2008) must be related to their production rate and thermal stability (Oliver and Moran, 2009).

Temperature is a powerful force shaping symbioses. Environmental temperature may stabilize the insect-symbiont interactions, ensuring survival of both partners. Temperature changes can cause loss of mutualistic balance, triggering shifts to parasitism and host extinction, switches to novel partners or mutualism abandonment (Toby Kiers *et al.*, 2010). Finally, temperature can promote success of some host-symbiont associations over others (Dunbar *et al.*, 2007; Versace *et al.*, 2014). Therefore, *via* its influence on symbioses, temperature affects many ecosystems. Understanding the role of temperature in symbiotic associations may allow better management of insects of economical and medical importance. Finally, as temperature can destroy bacterial-animal homeostasis, it may serve as a tool to study the mechanisms ensuring homeostasis maintenance.

5. The aims of this thesis

First, we aimed at assessing if and how *Wolbachia* genotype affects *Wolbachia*-associated phenotypes in *Drosophila melanogaster*: We approached this question by:

a) testing variability in the antiviral protection and other phenotypes associated with natural variants of *Wolbachia* endosymbiont of *Drosophila melanogaster* *w*Mel; sequencing *Wolbachia* genomes to understand genetic bases underlying the differences between the variants.

b) identifying phenotypic differences between benign *w*MelCS_b and pathogenic *w*MelPop and, using genome sequences, uncovering genetic bases of *w*MelPop virulence.

c) assessing the antiviral effect and cost of highly protective *Wolbachia* *w*Au transferred to *Drosophila melanogaster* from *Drosophila simulans* and comparing it with natural endosymbiont of *Drosophila melanogaster.*

Finally, we intended to understand how temperature affects *Wolbachia*-conferred antiviral protection. We characterised the influence of temperature before and after viral infection on the antiviral effect of *w*MelCS_b.

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Chapter 2 - *Wolbachia* variants induce differential protection to viruses in *Drosophila melanogaster*: a phenotypic and phylogenomic analysis

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Supplementary tables are included in the Appendix I. Other supplementary material (Figures and Datasets) are published online, and are not included here, due to the file sizes and formats.

Author contributions: As stated in author contributions in the reference above I participated in all stages of the preparation of this manuscript. Together with Marta Marialva, Francis Jiggins and Luis Teixeira we conceived and designed the experiments. Most of the experiments on phenotypic characterisation of the variants, including replicates of the survival of the virus pricking experiments, longevity experiments, virus levels and *Wolbachia* growth quantifications, experiments with *w*MelPop and viruses and qPCR validation of Octomom amplification were performed by me. Marta Marialva performed the first survival experiments on *w*Mel variants and tetracycline treated controls infected with DCV and FHV. She also infected the variants for virus levels quantification and quantified *Wolbachia* in the young flies of all the variants. Sara Esteves performed a replicate of the longevity assays and quantified *w*MelPop levels along time. Julien Martinez extracted DNA from flies and prepared it for sequencing, and Lucy Weinert analysed the sequencing data and constructed the phylogenetic tree of *w*Mel variants. Together with Luis Teixeira and Francis Jiggins we analysed the data and wrote the manuscript.

Summary

Wolbachia are intracellular bacterial symbionts that are able to protect various insect hosts from viral infections. This tripartite interaction was initially described in *Drosophila melanogaster* carrying *w*Mel, its natural *Wolbachia* strain. *w*Mel has been shown to be genetically polymorphic and there has been a recent change in variant frequencies in natural populations. We have compared the antiviral protection conferred by different *w*Mel variants, their titres and influence on host longevity, in a genetically identical *D. melanogaster* host. The phenotypes cluster the variants into two groups - *w*MelCS-like and *w*Mel-like. *w*MelCS-like variants give stronger protection against *Drosophila* C virus and Flock house virus, reach higher titres and often shorten the host lifespan. We have sequenced and assembled the genomes of these *Wolbachia,* and shown that the two phenotypic groups are two monophyletic groups. We have also analysed a virulent and over-replicating variant, *w*MelPop, which protects *D. melanogaster* even better than the closely related *w*MelCS. We have found that a ~21kb region of the genome, encoding eight genes, is amplified seven times in *w*MelPop and may be the cause of its phenotypes. Our results indicate that the more protective *w*MelCS-like variants, which sometimes have a cost, were replaced by the less protective but more benign *w*Mel-like variants. This has resulted in a recent reduction in virus resistance in *D. melanogaster* in natural populations worldwide. Our work helps to understand the natural variation in *w*Mel and its evolutionary dynamics, and informs the use of *Wolbachia* in arthropod-borne disease control.

Introduction

Many arthropods are infected by bacterial secondary (facultative) symbionts (Moran *et al.*, 2008). These are vertically transmitted bacteria that are not essential for the host to survive or reproduce, but nonetheless can have important effects on their host's biology. The fitness of these secondary symbionts is directly linked to their host's fitness; their transmission through successive generations is dependent on the breeding success of their hosts. This close association and dependence is predicted to favour the evolution of mutualism (Axelrod and Hamilton, 1981). Nonetheless, the presence of replicating bacteria in the host is bound to have a cost. This fitness cost and imperfect vertical transmission would theoretically lead to elimination of vertically transmitted symbionts from host populations (Turelli, 1994; Hoffmann *et al.*, 1998). Specific phenotypes associated with secondary symbionts explain their maintenance. Some secondary symbionts are parasites and manipulate their host reproductive biology (Turelli, 1994; Engelstaedter and Hurst, 2009). Others are mutualists and confer a fitness advantage to their hosts (e.g. resistance to environmental stress or pathogens) (Jaenike, 2012). Genetic variability of the symbiont may impact all these associated phenotypes. Therefore, understanding this genotypic and phenotypic variability is essential to understand facultative symbionts population genetics.

In recent years it has become clear that symbionts can modulate the interactions between hosts and parasites in many taxa (Gil-Turnes *et al.*, 1989; Grivel *et al.*, 2001; Oliver *et al.*, 2003; Kaltenpoth *et al.*, 2005; Scarborough *et al.*, 2005; Barton *et al.*, 2007; Haine, 2008; Hedges *et al.*, 2008; Teixeira *et al.*, 2008; Xie *et al.*, 2010; Jaenike *et al.*, 2010; Weiss *et al.*, 2011; Cirimotich *et al.*, 2011; Littman and Pamer, 2011; Jaenike, 2012). Insects are no exception to this pattern, and secondary symbionts

can play a key role in protecting their hosts against infection or parasitism (Scarborough *et al.*, 2005; Haine, 2008; Hedges *et al.*, 2008; Teixeira *et al.*, 2008; Xie *et al.*, 2010; Jaenike *et al.*, 2010; Weiss *et al.*, 2011; Oliver *et al.*, 2003; Jaenike, 2012). Protection to pathogens may be the fitness advantage that enables these bacteria to invade insect populations. For example, the recent spread of *Spiroplasma* in North American populations of *Drosophila neotestacea* may be a consequence of the protection to nematode parasites conferred by these bacteria (Jaenike *et al.*, 2010). Also, the bacterium *Hamiltonella defensa* increases in frequency in aphid cage populations in the presence of parasitoid wasps, to which it provides protection, but decreases in the absence of it (Oliver *et al.*, 2008). Presence of a protective symbiont can, therefore, be treated as an heritable, albeit non-Mendelian, condition-dependent beneficial genetic change (Jaenike, 2012).

The intracellular α-proteobacteria *Wolbachia* protects *Drosophila melanogaster* against viral infections (Hedges *et al.*, 2008; Teixeira *et al.*, 2008). *Wolbachia* are estimated to infect 40% of arthropod species (Zug and Hammerstein, 2012) and are, therefore, some of the most common intracellular bacteria known. Their success may be related to their anti-viral protective effect on natural hosts (Hedges *et al.*, 2008; Teixeira *et al.*, 2008; Osborne *et al.*, 2009; Glaser and Meola, 2010), although this protection is not always observed (Osborne *et al.*, 2009; Bian *et al.*, 2010; Longdon *et al.*, 2012). Other mechanisms, many involving manipulation of the host reproduction, can also maintain *Wolbachia* in natural populations (Stouthamer *et al.*, 1999; Werren *et al.*, 2008). The most common manipulation is cytoplasmic incompatibility (CI), which renders the crosses between *Wolbachia* infected males and uninfected females sterile or with low viability, giving a relative fitness advantage to infected females. Nonetheless, even when *Wolbachia* can cause CI, a beneficial effect, like protection to viruses, may contribute to the invasion

of a new host (Fenton *et al.*, 2011).

Wolbachia-conferred protection against viruses is of particular interest because of potential applications in vector-borne disease control. Mosquitoes infected with *Wolbachia* can be more resistant to human arboviruses (Moreira *et al.*, 2009; Glaser and Meola, 2010; Bian *et al.*, 2010; Walker *et al.*, 2011; Blagrove *et al.*, 2012; van den Hurk *et al.*, 2012) and other human pathogens (Kambris *et al.*, 2009; Moreira *et al.*, 2009; Kambris *et al.*, 2010; Hughes *et al.*, 2011). A large effort is being made to use *Aedes aegypti* mosquitoes trans-infected with *Wolbachia* variants from *D. melanogaster* in limiting dengue virus transmission (Moreira *et al.*, 2009; Walker *et al.*, 2011). Pilot releases of these trans-infected mosquitoes have already been conducted successfully (Hoffmann *et al.*, 2011) and intervention in dengue endemic areas is planned (Walker, *et al.*, 2011).

There can be a great deal of genetic variation in how symbionts modulate host-pathogen interactions. Different *D. simulans* lines infected with different *Wolbachia* strains, for instance, show variation in the protection to viruses (Osborne *et al.*, 2009). The protection ranges from nearly complete to none, and the combinations showing higher protection have higher levels of the endosymbiont (Osborne *et al.*, 2009). While these *Wolbachia* strains are distantly related, other studies have found variation within populations of closely related symbionts. For example, *H. defensa* protects aphids from parasitoid wasps only when it carries a lysogenic bacteriophage (Oliver *et al.*, 2009). Understanding this genetic variation among symbionts may explain the frequency of different variants in natural populations and give insight into the mechanisms underlying the interactions.

In natural populations of *D. melanogaster* there has been a recent replacement of *Wolbachia* variants (Riegler *et al.*, 2005; Nunes *et al.*, 2008; Richardson *et al.*, 2012). *Wolbachia* is present in most natural

populations of *D. melanogaster,* although with variable frequencies of infection (Hoffmann, 1988; Holden *et al.*, 1993; Solignac *et al.*, 1994; Hoffmann *et al.*, 1994; Mateos *et al.*, 2006; Ilinsky and Zakharov, 2007; Nunes *et al.*, 2008; Verspoor and Haddrill, 2011; Richardson *et al.*, 2012; Ilinsky, 2013). Only a single strain, *w*Mel, is known to infect *D. melanogaster,* but several closely related genotypes of this strain - *w*Mel, *w*Mel2, *w*Mel3, *w*MelCS and *w*MelCS2 - were defined on the basis of polymorphic genetic markers (Riegler *et al.*, 2005). The frequencies of these genotypes in isolates from natural populations of *D. melanogaster* have changed during the 20th century. Early isolates have a high proportion of *w*MelCS type, while the *w*Mel genotype is predominant in late 20th century isolates (Riegler *et al.*, 2005). This *w*Mel genotype replacement was supported by the analysis of *Wolbachia* genotype-associated mitochondrial DNA haplotypes (Nunes *et al.*, 2008). More recently the genomes of 179 different *w*Mel variants and 290 associated and non-associated mitochondria were assembled (Richardson *et al.*, 2012). Their analysis showed that all *w*Mel variants come from a single infection event and the most recent ancestor of all *w*Mel and mitochondria dates to about 8,000 years ago (Richardson *et al.*, 2012). The low genetic diversity and excessive rare variants in *w*Mel, in the well sampled North American population of the *Drosophila* Genetic Reference Panel (Mackay *et al.*, 2012), are consistent with a recent sweep of *w*Mel variants (Richardson *et al.*, 2012). However, the *w*MelCS and *w*Mel types diverged several thousand years ago (Richardson *et al.*, 2012) and the sweep is incomplete, since there are still *w*MelCS variants in natural populations (Ilinsky and Zakharov, 2007; Nunes *et al.*, 2008; Richardson *et al.*, 2012; Ilinsky, 2013).

Phenotypic differences associated with different *w*Mel variants could explain why their frequencies have changed. CI, despite being weak in *D. melanogaster*, has been shown to vary in level in flies harbouring

different *w*Mel genotypes (Veneti *et al.*, 2003; Ilinsky and Zakharov, 2011). However, the contribution of host or symbiont genetic variation to these differences is not resolved in these studies. Overall clear phenotypic differences between natural *w*Mel variants are not known.

A *w*Mel variant that clearly induces a particular phenotype is *w*MelPop (Min and Benzer, 1997). This variant was isolated from a laboratory stock and it is pathogenic: it over-proliferates and shortens host lifespan (Min and Benzer, 1997; McGraw *et al.*, 2002; Reynolds *et al.*, 2003). In terms of genetic markers *w*MelPop is indistinguishable from *w*MelCS (Riegler *et al.*, 2012); however, no *w*MelCS variant with a similar phenotype has been isolated from the wild. Both *w*MelPop and *w*Mel genotype have been introduced into *Ae. aegypti* as a strategy to block dengue (Moreira *et al.*, 2009; Walker *et al.*, 2011), and they protect differently from viral infection (Walker *et al.*, 2011; van den Hurk *et al.*, 2012). Because of the potential field application and the pathogenicity of *w*MelPop, it is also important to understand in more detail the phenotypic and genomic differences between *w*MelPop and other variants.

Here we compare the antiviral protection conferred by different *w*Mel variants, in genetically identical *D. melanogaster* hosts. We show that *w*MelCS-like confer greater antiviral protection than *w*Mel-like variants, but have higher bacterial densities and can reduce the survival of the flies. Through the assembly of their genomes and phylogenetic analysis we reconstruct the relationship of the strains. We also investigate in detail the phenotypic differences between the closely related *w*MelCS and *w*MelPop and propose a genomic basis for them. This analysis strengthens the notion that susceptibility to infectious disease can rapidly evolve due to changes in symbionts found in the host population.

Materials and Methods

Fly strains and husbandry

D. melanogaster lines with *Wolbachia* are described in Table 1 (page 73). Lines with *Wolbachia* variants described in Riegler *et al*. (2005) were kindly provided by Markus Riegler and Scott O'Neill. *w*MelCS_b source and DrosDel w^{1118} isogenic background were described elsewhere (Ryder *et al.*, 2004; Teixeira *et al.*, 2008). *w*Mel variants were introduced in the DrosDel w^{1118} *iso* isogenic background by chromosomes replacement using a first and third double balancer line and a second chromosome balancer line. The crosses were performed with *Wolbachia*-infected females, ensuring endosymbiont transmission through the germline. The fourth chromosome was not isogenized. All the *Wolbachia* genotypes were confirmed by PCR, as described in Riegler *et al*. (2005) (data not shown).

The lines were cleaned of possible chronic viral infections as described elsewhere (Brun and Plus, 1978; Teixeira *et al.*, 2008).

In order to homogenize the gut microbiota, embryos from each line were sterilized with 2% sodium hypochlorite, followed by 70% ethanol and washed with sterile water. Embryos were placed in new food vials and 150 µl of a bacterial inoculum from a reference stock was added. The inoculum was produced by mixing 5 ml of sterile water with 2 g of food from 10 days old vials containing *VF-0058–3* flies (Teixeira *et al.*, 2008), and filtering it to remove eggs and larvae.

Tetracycline-treated lines were cleaned of *Wolbachia* infection by raising them for two generations in ready-mix dried food (Philip Harris) with 0.05 mg/ml of tetracycline hydrochloride (Sigma). Experiments were performed on lines that were raised without antibiotics for at least 6 generations.

Drosophila lines were maintained on standard cornmeal diet at a constant temperature of 25 °C. We focused the analysis on males under the assumption that *Wolbachia* levels would be more stable in these. *Wolbachia* is present in ovaries and the sizes of these vary greatly with mating status and physiology of the female.

Long-term survival analysis

To measure the lifespan of different fly lines 10 flies were placed per vial (without yeast) per replicate, at 25 °C. Vials were checked for survival and changed every 5 days.

The analysis of survival data was performed with the Cox proportional hazard mixed effect model. Fixed effects include genotype and repeat of the experiment while replicate vials within the same experiment were considered as a random effect. This method accounts for variation between vials of the same line in the same experiment and variation between replicates of the experiment. Model fitting was done using the coxme package in R (Team, 2012). Tukey´s test was applied for pairwise comparisons of Cox hazard ratios between all *w*Mel variants and DrosDel w^{1118} *iso.*

Virus production and infection

Viruses were produced and titrated as in Teixeira *et al*. (Teixeira *et al.*, 2008), with minor changes. DCV was titrated in Schneider's Line 2 (SL-2), while FHV was titrated in Schneider *Drosophila* line 2 (DL2).

For viral infections CO_2 anesthetized flies were pricked in the thorax. The 0.15 mm diameter needles used for infection (Austerlitz Insect Pins) were dipped into a virus solution diluted to the desired concentration in 50 mM Tris-HCl, pH 7.5. After the infection flies were kept in vials without yeast, 10 flies per vial. DCV infected flies were maintained at 18 °C, while FHV

infected flies were maintained at 25 °C. Vials were checked for survival daily and changed every 5 days. Unless otherwise stated, infection was performed on 3 to 6 days old flies. Survival analysis was done as above.

RNA extractions and cDNA synthesis

For each sample 10 flies were pooled and homogenized with a plastic pestle in 1 ml of Trizol Reagent (Invitrogen). RNA was extracted according to manufacturer's protocol and re-suspended in 50 µl of DEPC-treated water (Ambion). RNA concentrations were determined using NanoDrop ND-1000 Spectrophotometer. cDNA was prepared from 1 µg of total RNA using Random Primers and M-MLV Reverse Transcriptase (both Promega). Primers were allowed to bind to the template RNA for 5 min at 70 °C and the reaction proceeded to 25 °C for 10 min, 37 °C for 60 min and 80 °C for 10 min.

DNA extractions

For *Wolbachia* relative quantification, ten flies were used per replicate. DNA was extracted according to DrosDel protocol (http://www.drosdel.org.uk/molecular_methods.php) (Ryder *et al.*, 2004). For *w*Mel Octomom genes relative quantification, total DNA was extracted from replicates of ten flies using a standard phenol-chloroform protocol. The DNA concentrations were checked with NanoDrop ND-1000 Spectrophotometer.

Real-time quantitative PCR

The real-time qPCR reactions were carried out in 7900HT Fast Real-Time PCR System (Applied Biosystems) or CFX384 Real-Time PCR Detection System (BioRad). For each reaction in 384-well plate (Applied Biosystems or BioRad) we used 6 µl of iQ SYBR Green supermix (Bio

Rad), 0,5 µl of each primer solution at 3,6 µM and 5 µl of diluted DNA. Each plate contained three technical replicates of every sample for each set of primers. Primers used are described in Table S7.

The thermal cycling protocol for the amplification of Wolbachia genes was as follows: initial 50 °C for 2 min, denaturation for 10 min at 95 °C followed by 40 cycles of 30 s at 95 °C, 1 min at 59 °C and 30s s at 72 °C. Amplification of DCV and FHV was performed using the same conditions, except an annealing temperature of 56 °C. Melting curves were analysed to confirm specificity of amplified products. We obtained Ct values for manual threshold of 10 using the program SDS 2.4 or with Bio-Rad CFX Manager with default threshold settings.

Relative amounts were calculated by the Pfaffl Method (Michael W Pfaffl, 2001) using *Drosophila* Rpl32 as a reference gene for *wsp* and viruses and *wsp* as a reference for *Wolbachia* Octomom genes.

Kruskal-Wallis rank sum test (kruskal.test in R) was performed on *Wolbachia* and viruses quantification data to detect differences within all the lines. Pairwise comparison between all variants was performed with Wilcoxon rank sum test with Holm correction (pairwise.wilcox.test in R). Direct comparison between wMel-like and wMelCS-like variants was performed with a linear mixed-effects model fit by maximizing the restricted log-likelihood on the log of the values (lme in R). Time course analysis of *Wolbachia* titres was performed with a linear model fit (lm in R).

Cluster analysis and correlations

The data in Table S2 was used for the cluster analysis of *w*Mel variants (hclust in R). In each column the mean was subtracted from the data, for centering, and the result divided by the standard deviation, for scaling.

Complete linkage hierarchical clustering was performed on Euclidian distances between *w*Mel variants.
Correlations were calculated using Pearson's product moment correlation (cor.test in R).

Sequencing and genome assembly

The genome assembly of the *w*Mel variants was done with the invaluable help of Casey Bergman (University of Manchester).
For each fly line, 20 females were anaesthetized under CO_2 and washed in 50% bleach solution for 3 min. Females were then briefly washed in distilled water and dissected under a microscope. The two ovaries of the 20 females were pooled for DNA extraction. DNA was extracted using the Gentra Puregene DNA Purification kit according to the manufacturer's protocol, including an RNase A treatment. Yields of purified DNA ranged between 1.1 and 4.2 µg. Library preparation and sequencing were performed at the Eastern Sequence and Informatics Hub (Cambridge, UK). 75 bp paired-end libraries were prepared with an insert size of 300 bp and sequenced in one lane of HiSeq2000 (Illumina). Base calling was performed using the Offline Basecaller (version 1.9.3) from Illumina, and demultiplexing was handled by bespoke Eastern Sequence and Informatics Hub software. The reads are submitted to the Sequence Read Archive (accession number: ERP002662).
Forward and reverse fastq sequences were mapped individually to single database containing a mitochondrial reference sequence extracted from the *D. melanogaster* Release 5 genome sequence (chrU:5288528-5305749) and the *D. melanogaster Wolbachia* endosymbiont reference genome (GenBank ID: AE017196) and converted to paired end alignments using BWA version 0.5.9-r16 (Li and Durbin, 2009). BWA output was converted to SAM format and reads mapping to the

mitochondria or *Wolbachia* reference sequences were extracted and sorted using SAMtools version 0.1.18. Sorted BAM files were used for variant base calling followed by a standard SAMtools version 0.1.16 pileup pipeline (Li *et al.*, 2009). Individual strain consensus fastq sequences were generated using pileup2fq.pl with minimum and maximum read depths set to 10 and 100, respectively, and converted to fasta format using seqtk (https://github.com/lh3/seqtk). Individual reference-based fasta consensus sequence files were merged into multiple alignments from http://bergman.smith.man.ac.uk/data/wolbachia/DGRP_DPGP_Wolbachia_v1.tgz (Richardson *et al.*, 2012). Alignment columns that had an N in any strain (which can represent either a fully ambiguous character or a deletion relative to the reference) were then removed.

Fasta file of assembled sequences of *Wolbachia* variants and associated mitochondria are in Dataset S1 and S2, respectively (doi:10.1371/journal.pgen.1003896.s001 and doi:10.1371/journal.pgen.1003896.s002). Tables of variants for these *Wolbachia* and mitochondria together with data from *Wolbachia*-carrying strains described in Richardson *et al.* (2012) are in Dataset S3 and S4, respectively (doi:10.1371/journal.pgen.1003896.s003 and doi:10.1371/journal.pgen.1003896.s004).

Phylogenetic analysis

We produced a dated evolutionary history of *Wolbachia* using BEAST v1.7.2 (Drummond *et al.*, 2012). The *Wolbachia* and *Drosophila* mitochondrial phylogenies have been shown to be fully congruent (Richardson *et al.*, 2012), so they share the same evolutionary history. We therefore concatenated the *Wolbachia* variants alignments with their respective host *Drosophila* mitochondrial alignments, removing all indels.

We included the *Wolbachia* reference strain AE017196, even though no host *Drosophila* mitochondrial alignment exists, after checking that its inclusion made no qualitative difference to either the dates or topology. This alignment was then partitioned into eight different groups representing different categories of sites; first and second codon positions, third codon positions, noncoding RNA genes and intergenic sites (for both the *Wolbachia* and *Drosophila* mitochondria). Each partition had their own HKY+Γ model of evolution (Hasegawa *et al.*, 1985; Shapiro *et al.*, 2006) but linked to the same dated phylogeny and constant population size coalescent tree prior. In order to calibrate the molecular dating, we assigned a prior lognormal distribution of rate based on the *Drosophila* mutation rate (Haag-Liautard *et al.*, 2008; Richardson *et al.*, 2012) to third codon positions of the *Drosophila* mitochondria, sites that are less likely to be under purifying selection. Rates at all other site classes were given a prior of uniform distribution between 0 and 1, whereas priors on all other parameters were given default values as specified in BEAUti v1.7.2 (Drummond *et al.*, 2012).

Genetic polymorphism and predicted genes analyses

For single nucleotide polymorphism analysis a multiple alignment was built, with only the sequences of the *w*Mel variants analysed in this report, and alignment columns that had an N in any strain were removed. Variant sites were then extracted and mapped back to reference coordinates using custom R and PERL scripts (Dataset S5, doi:10.1371/journal.pgen.1003896.s005). Variants that differ between all *w*Mel-like and all *w*MelCS-like variants were identified and mapped to predicted genes or non-coding regions with Galaxy (Giardine *et al.*, 2005). Identification of synonymous or non-synonymous substitutions was performed with custom Python scripts.

To identify duplications and deletions that have led to copy number variation (CNVs), we examined depth of sequence coverage across the *Wolbachia* genome. To do this we partitioned the genome into non-overlapping 200bp bins and used the mean shift approach implemented in CNVnator (Abyzov *et al.*, 2011) to infer differences in copy number and identify break-points. The variants *w*Mel, *w*Mel3 and *w*MelCS2_a were not analysed as they had highly variable coverage. Analysis of regions containing duplications was aided by UCSC Genome Browser http://genome.ucsc.edu/ (Karolchik *et al.*, 2003; Schneider *et al.*, 2006).

We also used the program Pindel (Ye *et al.*, 2009) to search for 'split reads', which map to two different positions in the *Wolbachia* genome. To reduce artefacts we only retained structural variants where at least one strain had 10 or more supporting reads and where at least one strain had no supporting reads. As we know the phylogeny of these strains, we expect most true structural variants to be present in monophyletic clades (i.e. they have only arisen once). Out of 18 variants detected, 17 fulfilled this criterion, suggesting that our methods are robust.

Predicted protein domain analysis was based on the reference genome (Wu *et al.*, 2004) or using NCBI CD-search (Marchler-Bauer *et al.*, 2010)).

Results

Phylogenomic Analysis of *w*Mel Variants

To address the question of how genetic variability within the *Wolbachia* *w*Mel strain affects resistance to viruses, we analysed the five genotypes described by Riegler *et al.* on the basis of a small number of genetic markers (Riegler *et al.*, 2005). For the genotypes *w*Mel and *w*Mel3 we used one *D. melanogaster* line, while for *w*Mel2, *w*MelCS and *w*MelCS2 we used two lines for each genotype (Table 1).

Table 1. *w*Mel variants used.

Variant name	*Wolbachia* genotype	Stock name/ number	Reference
*w*Mel	*w*Mel	yw^{67C23}	(Riegler *et al.*, 2005)
*w*Mel3	*w*Mel3	Umea 94 / 103466	(Riegler *et al.*, 2005)
*w*Mel2_a	*w*Mel2	Amamioshima / E-10032	(Riegler *et al.*, 2005)
*w*Mel2_b	*w*Mel2	Amamioshima / E-10030	(Riegler *et al.*, 2005)
*w*MelCS_a	*w*MelCS	Canton S / CS	(Riegler *et al.*, 2005)
*w*MelCS_b	*w*MelCS	VF-0058-3	(Teixeira *et al.*, 2008)
*w*MelCS2_a	*w*MelCS2	Kurdamir / 103393	(Riegler *et al.*, 2005)
*w*MelCS2_b	*w*MelCS2	Anapa-79 / 103432	(Riegler *et al.*, 2005)
*w*MelPop	*w*MelCS	Popcorn / w^{1118}	(Riegler *et al.*, 2005)

Wolbachia genotypes are based on the diagnostic PCR assays described in Riegler *et al.* (Riegler *et al.*, 2005). Further information about origin of variant can be found in the indicated reference.

We will refer to each *w*Mel originating from a unique *D. melanogaster* line as a variant. In order to determine the phylogeny of these variants we sequenced and assembled their genomes and associated mitochondria. We sequenced 75bp paired-end libraries and mapped the reads to the *w*Mel reference genome (GenBank ID: AE017196) and to the

mitochondrial genome in *D. melanogaster* Release 5 genome sequence (chrU:5288528-5305749). This mapping strategy was previously used to assemble and analyse the genomes of 179 *Wolbachia* and 290 mitochondria (Richardson *et al.*, 2012). We produced a phylogenetic tree of the *w*Mel variants together with the 179 *Wolbachia* genomes described in Richardson *et al.* (2012) (Figure 1 and Figure S1 (doi:10.1371/journal.pgen.1003896.s006)).

The *w*Mel variant genome clusters together with the reference genome AE017196 in clade III. The only differences we found between them were five positions with an ambiguous call for the *w*Mel nucleotide. This indicates a good quality of the sequencing and assembly, since the reference genome was sequenced from this variant (Wu *et al.*, 2004). *w*Mel3 is also assigned to clade III and is the most closely related, out of all the genomes in the phylogenetic tree, to *w*Mel and AE017196. This *w*Mel3 variant is the only known variant with this genotype. The original *D. melanogaster* stock that had *w*Mel3 was probably related to the laboratory stock used for *Wolbachia w*Mel sequencing. The only genomic marker from Riegler *et al.* that distinguishes *w*Mel3 from *w*Mel is the absence of the IS5 (ISWip1) WD0516/7 (Riegler *et al.*, 2005). This seems to be a consequence of a very recent excision of this mobile element in the *w*Mel3 variant since it is present in the closely related *w*Mel variant and in *w*Mel2_a and *w*Mel2_b. Sanger sequencing of this region shows that this would be a precise excision of the transposon (data not shown).

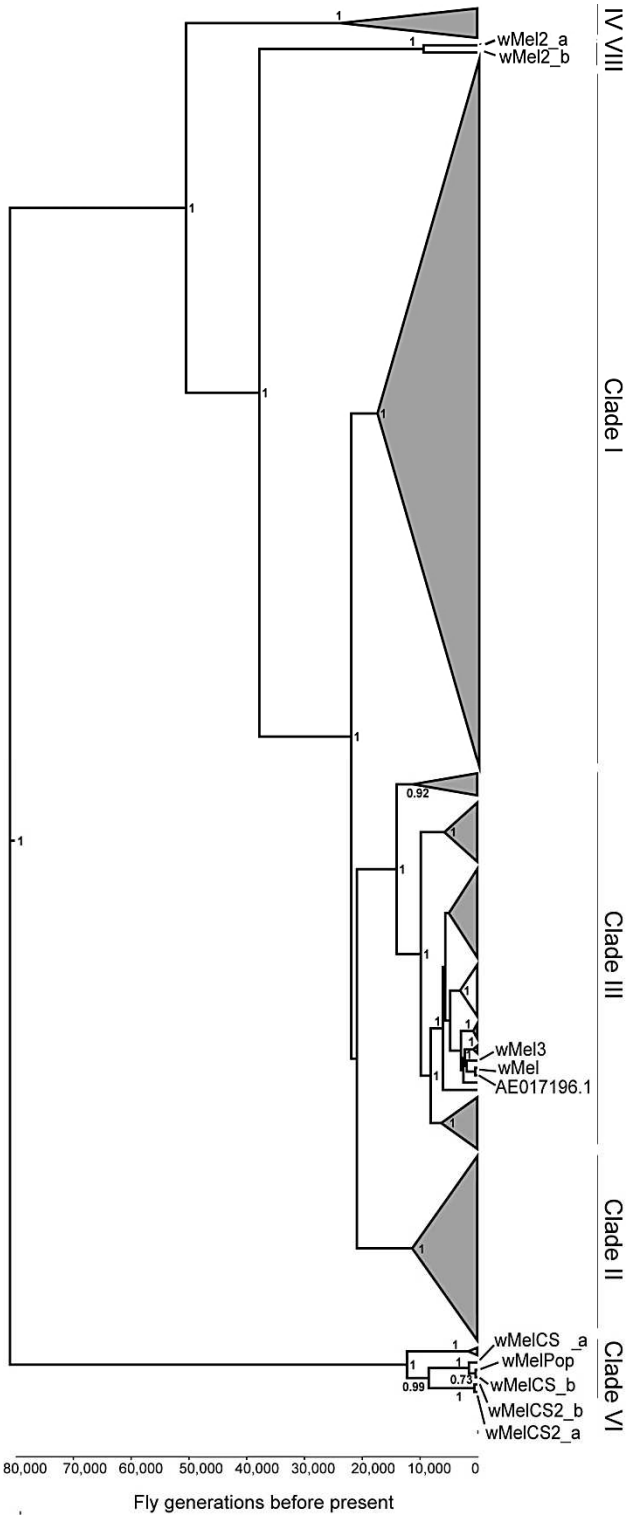

Figure 1. Phylogeny of *w*Mel variants.

Phylogenomic tree was reconstructed using the concatenated sequences of complete *Wolbachia* and mitochondrial genomes. The length of the branches reflects the estimated number of *Drosophila* generations, which was calibrated using the mitochondrial mutation rate. The node labels show posterior supports >0.5. The clades are named after Richardson *et al.* 2012.

*w*Mel2_a and *w*Mel2_b variants form the new major clade VIII. We estimate that the most recent common ancestor of this clade and clade III dates to 37,537 fly generations before present. The original flies carrying these two variants were captured in the Amami-oshima islands in Japan (Riegler *et al.*, 2005) and eight other lines with *w*Mel2 genotypes have origins in China, Thailand, Philippines and India (Nunes *et al.*, 2008). Therefore, clade VIII may be exclusive to Asian *D. melanogaster* populations.

*w*MelCS_a, *w*MelCS_b, *w*MelCS2_a and *w*MelCS2_b variants belong to clade VI and are relatively closely related. As expected from the genomic markers, *w*MelCS_a and *w*MelCS_b are more similar to each other than to *w*MelCS2_a and *w*MelCS2_b. Our data confirms that *w*MelCS-like variants belong to clade VI, as predicted by Richardson *et al.,* based on ISWip1 *in silico* mapping (Richardson *et al.*, 2012).

Variants of the genotypes *w*Mel, *w*Mel2 and *w*Mel3 are more closely related to each other than to variants of the *w*MelCS and *w*MelCS2 genotypes. We estimate that the most recent common ancestor of all these variants dates back to 80,000 fly generations before present and corresponds to the most recent common ancestor of all *w*Mel variants.

The laboratory variant *w*MelPop is indistinguishable from *w*MelCS, based on genomic markers (Riegler *et al.*, 2012). We have also sequenced and assembled its genome and found it to be closely related to *w*MelCS_b (Figure 1 and Figure S1 (doi:10.1371/journal.pgen.1003896.s006)).

*w*Mel Variants Provide Differing Levels of Protection to Viruses

To compare the phenotypic effects of the *w*Mel variants, we replaced the first, second and third chromosome of *Drosophila* lines carrying these variants with chromosomes of the DrosDel w^{1118} isogenic line (Ryder *et al.*, 2004), using balancer chromosomes. All lines were cleaned of

possible chronic viral infections, as previously described (Brun and Plus, 1978; Teixeira *et al.*, 2008). The microbiota associated with these lines, as well as the control *Wolbachia*-free DrosDel w^{1118} isogenic line (w^{1118} iso), are expected to be diverse and, presumably, eliminated by the virus cleaning procedure. To homogenize the microbiota associated with these lines, surface sterilized embryos of each line were raised in fly food containing an inoculum of *Drosophila*-associated microbiota from a reference stock.

We tested the mortality after *Drosophila C virus* (DCV) infection in the lines harbouring different *Wolbachia w*Mel variants (Figure 2A). DCV is a non-enveloped, positive sense single-stranded RNA virus of the Dicistroviridae family, that is a natural pathogen of *D. melanogaster* (Brun and Plus, 1978; Johnson and Christian, 1998). It has been shown before that *Wolbachia* gives strong resistance to this virus (Hedges *et al.*, 2008; Teixeira *et al.*, 2008). All lines with *Wolbachia* survive the DCV challenge better than the w^{1118} iso line without *Wolbachia*, demonstrating that all these *w*Mel variants confer protection to DCV. We have analysed the survival data of these infected lines with a Cox proportional hazard mixed effect model (Cox, 1972). This method determines the Cox hazard ratio for each line, which in this experiment is a measure of the risk of death of DCV-infected flies from each *Wolbachia* line relative to the risk of death of DCV-infected flies from the *Wolbachia*-free line (Figure 2B). A Tukey's test on Cox hazard ratios allows the comparison between all the lines and shows that the *Wolbachia* variants segregate into two groups (Figure 2B). The Cox hazard ratios of the *w*MelCS-like lines (*w*MelCS_a, *w*MelCS_b, *w*MelCS2_a and *w*MelCS2_b) are not significantly different from each other but are lower and significantly different from *w*Mel-like lines (*w*Mel, *w*Mel2_a, *w*Mel2_b, and *w*Mel3). Therefore, variants of clade VI (*w*MelCS-like) confer higher protection to DCV infection than variants of clades III and VIII (*w*Mel-like). There are still some statistically

significant differences in survival between lines of the *w*Mel-like group (Figure 2B).

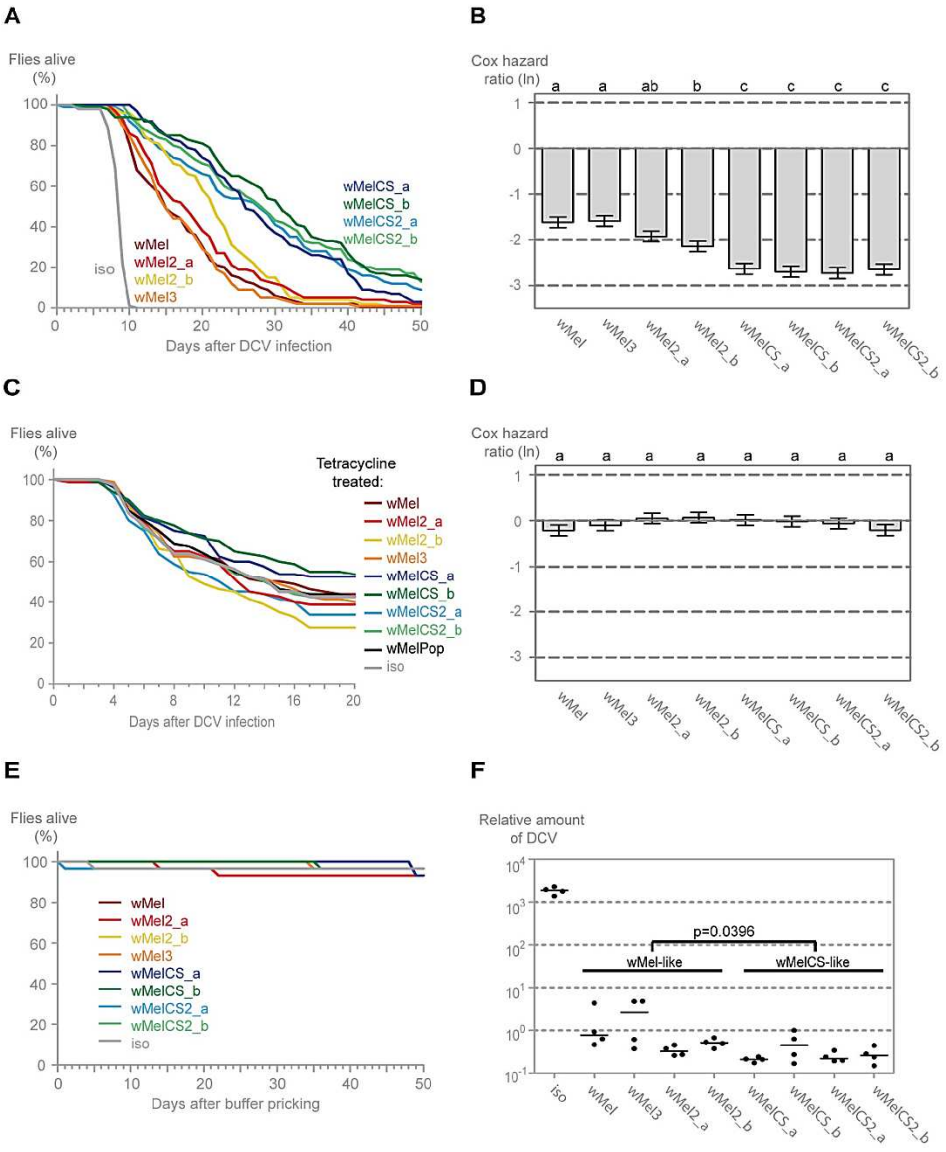

Figure 2. *Wolbachia* *w*Mel variants confer different protection to *Drosophila* C virus.

Continued on the next page.

Figure 2. (continued) (A) One hundred males of each *w*Mel variant line and w^{1118} *iso* were pricked with DCV ($10^{7.5}$ $TCID_{50}$/ml) and survival was followed daily. Two more replicates were performed with similar results.
(B) Cox hazard ratio of each *w*Mel variant line compared to w^{1118} *iso* when infected with DCV ($10^{7.5}$ $TCID_{50}$/ml). The natural logarithm of the Cox hazard ratio is shown. Error bars represent standard error. Letters refer to compact letter display of Tukey´s test of all pairwise comparisons. Analysis is based on three independent replicates (doses: 10^{7} $TCID_{50}$/ml, $10^{7.5}$ $TCID_{50}$/ml and 10^{9} $TCID_{50}$/ml), each with 100 flies per line, with 10 flies per vial. w^{1118} *iso* is assigned to group "d" in the compact letter display of Tukey´s test (not shown).
(C) Eighty males of each tetracycline treated line, derived from the *w*Mel variants lines and w^{1118} *iso,* were pricked with DCV ($10^{5.5}$ $TCID_{50}$/ml) and survival was followed daily. Two more replicates were performed with similar results.
(D) Cox hazard ratio of each tetracycline treated line, derived from the *w*Mel variants lines, compared to w^{1118} *iso* tetracycline treated line, when infected with DCV. Analysis is based on three independent replicates, one with 80 flies per line ($10^{5.5}$ $TCID_{50}$/ml) and two with 100 flies per line (one at 10^{7} $TCID_{50}$/ml and one at $10^{7.5}$ $TCID_{50}$/ml), with 10 flies per vial. w^{1118} *iso* tetracycline treated line is assigned to group "a" in the compact letter display of Tukey´s test (not shown).
(E) Thirty males of each *w*Mel variant line and w^{1118} *iso* were pricked with buffer and survival was followed daily.
(F) 3-6 days old males of each *w*Mel variant line and w^{1118} *iso* were pricked with DCV ($10^{7.5}$ $TCID_{50}$/ml) and collected 3 days later for RNA extraction and RT-qPCR. Relative amount of DCV was calculated using host Rpl32 gene expression as a reference and values are relative to median of *w*MelCS_b samples. Each point represents a replicate (ten males per replicate, four replicates per *Drosophila* line), and lines are medians of the replicates. DCV loads are two-fold higher in *w*Mel-like variants than in *w*MelCS-like variants (linear mixed-effect model, p=0.0396).

However, the statistical analysis does not allow a clear subdivision. In the timeframe of this experiment there is no significant difference in survival

between the lines pricked with buffer only (Figure 2E). Therefore, we conclude that the differences in survival upon viral challenge are due to variability in protection to viruses.

Wolbachia and mitochondria are both maternally inherited. Consequently, introducing *Wolbachia* variants into the same host genetic background implies co-inheritance of the mitochondria associated with them. To determine the influence mitochondria may have on the survival upon the viral infections we cured all the lines of *Wolbachia* by treating them with tetracycline and we re-homogenized the microbiota in the newly established lines. We have performed this infection with the same dose of DCV as for the *Wolbachia* lines and also with a lower dose in order to better reveal potential differences between lines (all *Wolbachia*-free lines are more susceptible to viral infection) (Figure 2C and 2D). We did not observe any statistically significant difference in survival between the tetracycline-treated lines after DCV infection. A direct comparison between *w*Mel and *w*MelCS-like derived lines also showed no significant difference (Tukey's test on the mixed effects Cox model fit of the survival data, p= 0.953). We conclude that the genetic variability in the mitochondria is not separating these lines regarding the susceptibility to DCV and the original segregation is due to *Wolbachia* variation. However, we cannot formally exclude the possibility of a *Wolbachia*-mitochondria genetic interaction.

To determine if the differences in survival between the two groups were due to differences in viral titres, we assessed the viral load in infected flies using real-time quantitative reverse transcription PCR (qRT-PCR) with DCV-specific primers (Deddouche *et al.*, 2008). We assayed titres 3 days post infection, since at this point there is already extensive viral replication but it is not yet at its maximum and there is still no lethality associated with infection (Teixeira *et al.*, 2008). All *w*Mel variants confer resistance to DCV, having on average 5000-fold less virus than control

(Figure 2F). The comparison of the virus titres between the *w*Mel-like and *w*MelCS-like groups shows a significant two-fold difference (linear mixed-effect model, p=0.040). The *Drosophila* lines with *w*MelCS-like variants have lower viral titres, in agreement with better survival after DCV infection.

To assess if the *w*Mel variants show differential protection against other viruses we analysed their interaction with Flock house virus (FHV).

This is also a non-enveloped positive sense single-stranded RNA virus. However, it belongs to the Nodaviridae family and it is not a natural pathogen of *D. melanogaster* (Dearing *et al.*, 1980; Ball and Johnson, 1998). We have shown before that *Wolbachia* protect *Drosophila* against FHV infections not by limiting the pathogen burden but by increasing survival under similar pathogen load; that is by increasing tolerance to this virus (Teixeira *et al.*, 2008). Consistently with the DCV results we observe that all variants give protection to FHV (Figure 3A). Moreover, the variants split into the same two *w*Mel and *w*MelCS-like groups, with the latter conferring greater protection (Tukey's test on the mixed effects Cox model fit of the survival data (Figure 3B)). There are, again, some statistically significant differences within the *w*Mel-like group but not between the same variants that show differences in survival after DCV infection.

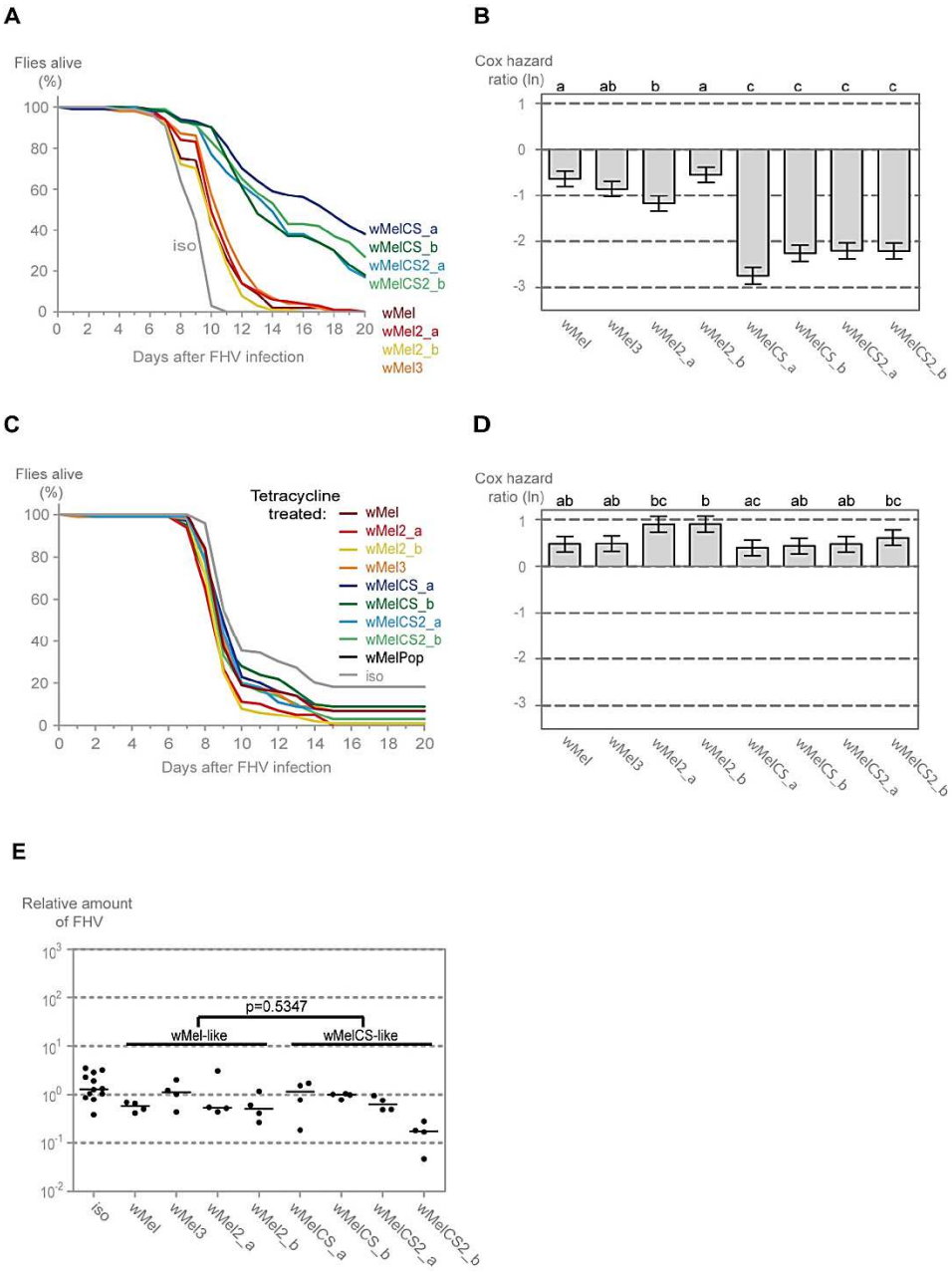

Figure 3. *Wolbachia wMel* variants confer different protection to Flock house virus.

(A) One hundred males of each *w*Mel variant line and w^{1118} *iso* were pricked with FHV (10^9 $TCID_{50}$/ml) and survival was followed daily. One more replicate was performed with similar results. Continued on the next page.

Figure 3. (continued) (B) Cox hazard ratio of each *w*Mel variant line compared to w^{1118} *iso* when infected with FHV (10^9 $TCID_{50}$/ml). The natural logarithm of the Cox hazard ratio is shown. Error bars represent standard error. Letters refer to compact letter display of Tukey´s test of all pairwise comparisons. Analysis is based on two independent replicates, one with 100 flies per line and one with 50 flies per line, with 10 flies per vial. w^{1118} *iso* is assigned to group "d" in the compact letter display of Tukey´s test (not shown).
(C) One hundred males of each tetracycline treated line, derived from the *w*Mel variants lines and w^{1118} *iso,* were pricked with FHV (10^8 $TCID_{50}$/ml) and survival was followed daily.
(D) Cox hazard ratio of each tetracycline treated line, derived from the *w*Mel variants lines, compared to w^{1118} *iso* tetracycline treated line, when infected with FHV (10^8 $TCID_{50}$/ml). Analysis is based on one replicate with 100 flies per line, 10 flies per vial. w^{1118} *iso* tetracycline treated line is assigned to group "a" in the compact letter display of Tukey´s test (not shown).
(E) 3-6 days old males of each *w*Mel variant line and w^{1118} *iso* were pricked with FHV (10^9 $TCID_{50}$/ml) and collected 3 days later for RNA extraction and RT-qPCR. Relative amount of FHV was calculated using host Rpl32 mRNA as a reference and values are relative to median of *w*MelCS_b samples. Each point represents a replicate (ten males per replicate, four replicates per *Drosophila* line), and lines are medians of the replicates. FHV loads are not significantly different between *w*Mel and *w*MelCS-like variants (linear mixed-effect model, p = 0.5347).

The survival of the tetracycline treated lines upon infection with a lower dose of FHV showed similar results to the DCV challenge (Figure 3C). Although there are some statistical differences between lines, there is no clear segregation between *w*Mel and *w*MelCS-like derived lines (Figure 3D) and a direct comparison between these groups showed no significant difference (Tukey's test on the mixed effects Cox model fit of the survival data, p=0.153). This shows that the difference in survival to FHV infection in the non-tetracycline treated lines is not solely due to

differences in mitochondria. The differences that we can still observe between lines may be a consequence of differences between mitochondria or due to incomplete isogenization or homogenization of the microbiota in these lines.

To test if there was also a difference in FHV titres between the two groups of *w*Mel variants we measured the levels of this virus three days after infection by qRT-PCR. The comparison of the virus titres between the *w*Mel-like and *w*MelCS-like groups shows no statistically significant difference (Figure 3E, linear mixed-effect model, p=0.535). Therefore the differences in survival between the two groups are due to differences in tolerance to FHV, not resistance.

The above results show that all tested *w*Mel variants confer protection to DCV and FHV. There is a differential protection that separates the *w*Mel variants into two groups. The *w*MelCS-like group lines, compared with the *w*Mel-like lines, have a better survival upon infection with both viruses, higher resistance to DCV, and higher tolerance to FHV.

Wolbachia Densities and Host Lifespan Are *w*Mel Variant Dependent

In order to characterize better the differences between *w*Mel variants and understand the basis of the differential protection, we analysed the titres of *Wolbachia* in the different lines. We determined by qPCR the levels of *Wolbachia* genomes relative to host genomes in males of two age groups: 3-4 and 6-7-day-old. (Figure 4A).

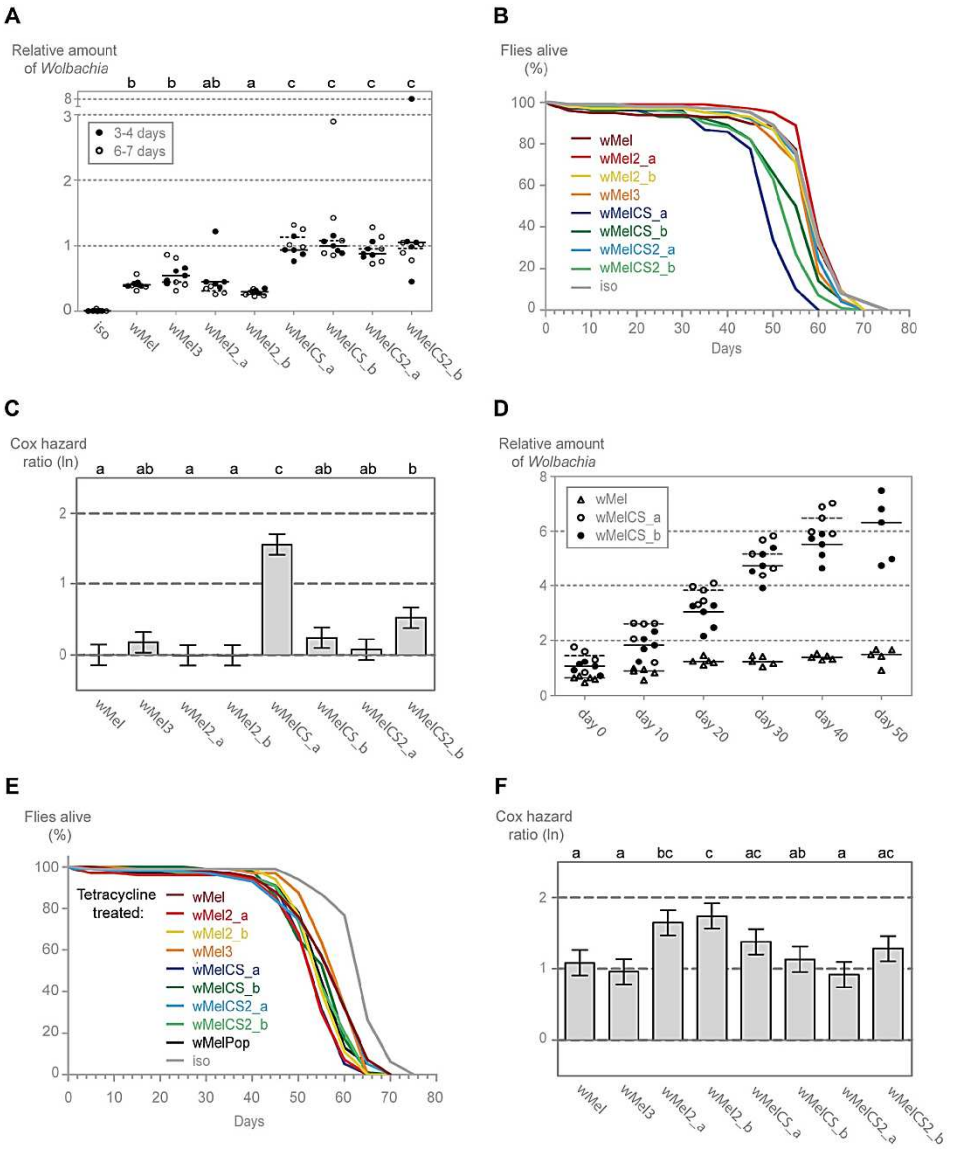

Figure 4. *Wolbachia* densities and *Drosophila* longevity are variant dependent.

(A) 3-4 and 6-7 days old males of each *w*Mel variant line and w^{1118} *iso* were collected for DNA extraction and qPCR. Relative amount of *Wolbachia* genomic DNA was calculated using host Rpl32 as a reference gene and values are relative to median of 3-4 days old *w*MelCS_b samples. Continued on the next page.

Figure 4. (continued) Each point represents a replicate (ten males per replicate, four replicates per *Drosophila* line), and lines are medians of the replicates. The compact letters display of pairwise Wilcoxon rank sum tests between variants is shown on the top.
(B) The survival of one hundred males of each *w*Mel variant line and w^{1118} *iso* was checked every five days. One more replicate was performed with similar results.
(C) Cox hazard ratio of each *w*Mel variant line compared to w^{1118} *iso*. The natural logarithm of the Cox hazard ratio is shown. Error bars represent standard error. Letters refer to compact letter display of Tukey´s test of all pairwise comparisons. Analysis is based on two independent replicates, each with 100 flies per line, with 10 flies per vial. w^{1118} *iso* is assigned to group "a" in the compact letter display of Tukey´s test (not shown).
(D) Males of *w*Mel, *w*MelCS_a and *w*MelCS_b lines were collected for DNA extraction and qPCR every 10 days. Day 0 corresponds to 3-6 days old flies, *w*MelCS_a were collected up to 40 days and *w*Mel and *w*MelCS_b up to 50 days. There are no further time points due to high mortality. Each point represents a replicate (ten males per replicate, five replicates per time point), and lines are medians of the replicates. Relative amount of *Wolbachia* genomic DNA was calculated using host Rpl32 as a reference gene and values are relative to median of samples of *w*MelCS_b at day zero.
(E) The survival of one hundred males of each tetracycline treated line derived from the *w*Mel variants lines and w^{1118} *iso* was checked every five days. The experiment was repeated once with similar results.
(F) Cox hazard ratio of each tetracycline treated line, derived from the *w*Mel variants lines, compared to w^{1118} *iso* tetracycline treated line. Analysis is based on two independent replicates, each with 100 flies per line, with 10 flies per vial. w^{1118} *iso* tetracycline treated line is assigned to group "d" in the compact letter display of Tukey´s test (not shown).

We observe that, for each variant, the titre of *Wolbachia* is very similar between the two age groups and that there is no tendency for higher or

lower titres at these two time points. However, lines with different *w*Mel variants vary in *Wolbachia* titres and can, once more, be separated into *w*MelCS and *w*Mel-like groups. A pairwise Wilcoxon rank sum test shows that *w*MelCS-like variants titres are not significantly different between them but different when compared to *w*Mel-like variants. *w*Mel-like variants show some differences between themselves but with no clear sub-groups. The median *Wolbachia* titre of *w*MelCS-like lines is 2.55 times higher than *w*Mel-like lines. These results show that *w*Mel titres are, at least partially, controlled by the symbiont genotype.

To determine if these differences in *Wolbachia* titres have any long-term effect on the *D. melanogaster,* we followed the long-term survival of these lines in the absence of any viral challenge (Figures 4B and 4C). The three lines with the shortest average lifespan are all infected with *w*MelCS-like variants. Of these, the *w*MelCS_a line has a significantly greater mortality rate compared to all other variants and w^{1118} *iso*, and *w*MelCS2_b has a statistically significant greater mortality rate than w^{1118} *iso* and three of the *w*Mel-like lines. Despite its shorter mean lifespan, when analysed as proportional hazards the *w*MelCS_b line is not significantly different from the control. Furthermore, when *w*MelCS and *w*Mel-like group survivals are directly compared the difference is not significant (Tukey's test on the mixed effects Cox model fit of the survival data, p=0.073). Therefore we can only state that some *w*MelCS-like variants have a deleterious effect on longevity. Nonetheless, these results exclude the hypothesis that *Drosophila* lines with *w*Mel-like *Wolbachia* succumb to viral infection faster due to a deleterious effect of the variants they are harbouring.

Prompted by these lifespan shortening effects, we investigated how the *Wolbachia* titres of *w*MelCS_a, *w*MelCS_b and *w*Mel change through the host life (Figure 4D). We observe that in these three variants *Wolbachia* levels increase with *Drosophila* age (this was not evident in the data set

of Figure 4A due to the small interval between the two age groups analysed). Based on the comparison of linear and log-linear models, a linear growth explains better these increases than an exponential one (Table S1). The titres at eclosion are not significantly different between the three variants in a multiple linear regression analysis (intercept *w*Mel: 0.722; intercept difference between *w*MelCS_a and *w*Mel: 0.416, p=0.090; intercept difference between *w*MelCS_b and *w*Mel: 0.222, p=0.328). However, while there is a significant increase in *w*Mel titres with the host age (slope *w*Mel: 0.017, p=0.003), the *w*MelCS_a and *w*MelCS_b growth rate is 6.5-8 times faster than *w*Mel (slope difference between *w*MelCS_a and wMel: 0.115, p<0.001; slope difference between *w*MelCS_b and wMel: 0.092, p<0.001). *w*MelCS_a also has a twenty percent faster growth than *w*MelCS_b (slope difference between *w*MelCS_a and *w*MelCS_b: 0.024, p=0.010). These results show that the two tested *w*MelCS-like variants have a higher growth rate than the *w*Mel-like variant tested. Moreover, *w*MelCS_a, the variant that shortens host lifespan, has the highest growth rate.

Finally, we analysed the lifespan of the tetracycline treated lines in order to assess the mitochondria contribution to the differences seen in the *w*Mel variants lines survival (Figure 4E and 4F). Although we see small differences between the lines they do not match the differences seen in the *w*Mel variant lines (e.g. the *w*MelCS_a line treated with tetracycline does not have the shortest lifespan) (Figure 4F). There is no difference in survival of *w*Mel and *w*MelCS-group derived lines (Tukey's test on the mixed effects Cox model fit of the survival data, p=0.615). We do observe, however, a statistically significant difference between these groups and w^{1118} iso derived line (p<0.001 for *w*Mel-group *vs* w^{1118} iso derived lines, p<0.001 for *w*MelCS-group *vs* w^{1118} iso derived lines). The w^{1118} iso line was subjected to the same tetracycline treatment and the difference in survival may be due to variation in mitochondria (see

(Clancy, 2008)).

Since the *w*MelCS-like variants have higher titres of *Wolbachia* and better protection to viruses we tested the correlation between *Wolbachia* titres and the survival upon viral infections, viral titres and long-term survival (data in Table S2). We found significant correlations between *Wolbachia* titres and survival upon DCV and FHV infection (Pearson's product moment correlation, p=0.034 and p=0.002 with Bonferroni correction, respectively), but not with the other phenotypes.

Given the recurrent phenotypic differences between the *w*Mel-like group and the *w*MelCS-like group, we tested if, overall, our data led to the clustering of *w*Mel variants into these two groups. To do this we analysed data of survival to viral infection, viral titres upon infections, long-term survival and *Wolbachia* titres together (Figure 5 and Table S2). A cluster analysis of the scaled values, based on Euclidian distances, shows that the *w*Mel variants phenotypes cluster them into a *w*Mel and a *w*MelCS-like group. This phenotypic clustering (Figure 5) has a phylogenetic basis (Figure 1) and the two groups correspond to the basal clade VI (*w*MelCS-like) and to variants of the more closely related clades III and VIII (*w*Mel-like).

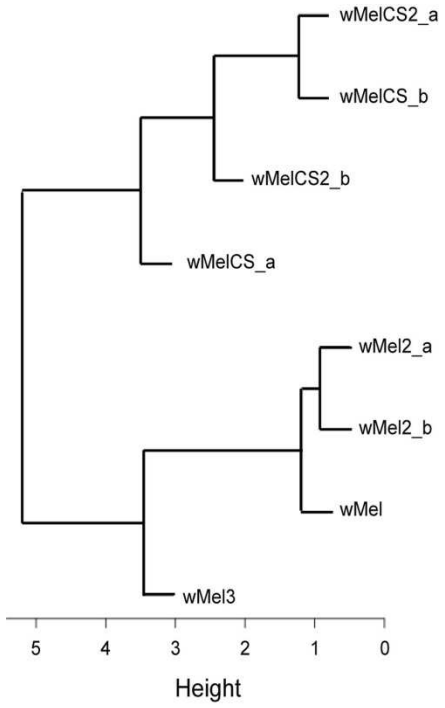

Figure 5. Phenotype-based cluster analysis of *w*Mel variants.
Cluster diagram of the *w*Mel variants based on the Euclidian distance of the scaled values of Cox hazard ratios of long-term survival, survival to FVH and DCV infections, FHV and DCV titres upon infection, and *Wolbachia* titres (Data in Table S2).

*Wolbachia w*MelPop Provides the Strongest Resistance against Viruses

The life-shortening *w*MelPop *Wolbachia* strain is known to over-proliferate in its native *D. melanogaster* host (Min and Benzer, 1997) and it has been shown to confer protection to DCV (Hedges *et al.*, 2008). Importantly, this variant has been transferred to *Aedes aegypti* where it also limits infection by several viruses, like dengue and Chikungunya, and the malaria parasite *Plasmodium gallinaceum* (Moreira *et al.*, 2009). *w*MelPop is indistinguishable from *w*MelCS based on genomic markers

(Riegler *et al.*, 2012), therefore we made a detailed comparison between *w*MelPop and *w*MelCS_b in the same conditions as for the other *w*Mel variants. However, this set of experiments was performed with 1-2 days old flies to minimize the variability due to different *Wolbachia* levels within the *w*MelPop sample or the *w*MelPop deleterious effect.

Upon challenge with DCV, young flies carrying *w*MelPop have 235-times lower viral loads than the flies with *w*MelCS_b (over 3000-fold less than w^{1118} iso) (Figure 6A, Mann-Whitney test, $p<0.001$).

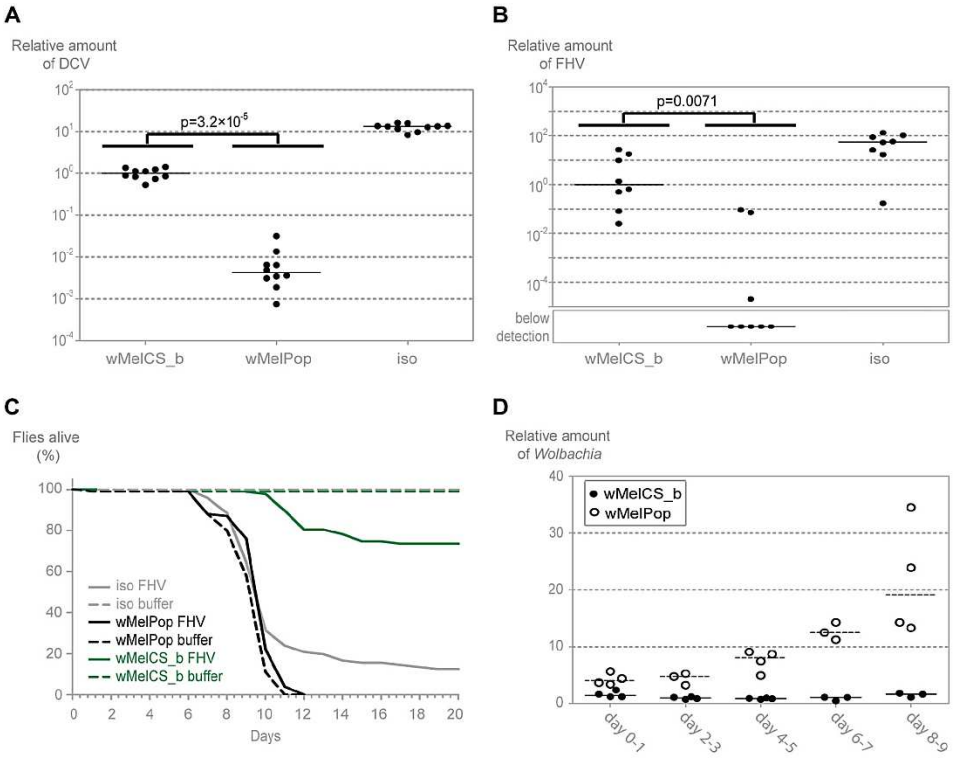

Figure 6. *w*MelPop confers the strongest antiviral protection.

(A) 1-2 days old males of the lines *w*MelPop, *w*MelCS_b, and w^{1118} *iso* were pricked with DCV (10^9 $TCID_{50}$/ml) and collected 3 days later for RNA extraction and RT-qPCR. Continued on the next page.

Figure 6. (continued) Relative amount of DCV was calculated using host Rpl32 expression as a reference and values are relative to median of *w*MelCS_b samples. Each point represents a replicate (ten males per replicate, ten replicates per *Drosophila* line), and lines are medians of the replicates. DCV titres are 235 times lower in *w*MelPop line than in *w*MelCS_b line (Mann-Whitney test, $p=3.2\times10^{-5}$).

(B) 1-2 days old males of the lines *w*MelCS_b, *w*MelPop, and w^{1118} *iso* were pricked with FHV (10^7 $TCID_{50}$/ml) and collected 3 days later for RNA extraction and RT-qPCR. Relative amount of FHV was calculated using host Rpl32 expression as a reference and values are relative to median of *w*MelCS_b samples. Each point represents a replicate (ten males per replicate, eight replicates per *Drosophila* line), and lines are medians of the replicates. FHV titres are lower in *w*MelPop line than in *w*MelCS_b line (Mann-Whitney test, p = 0.007).

(C) One hundred 1-2 days old males of the lines *w*MelCS_b, *w*MelPop, and w^{1118} *iso* were pricked with FHV (10^7 $TCID_{50}$/ml) or buffer, and the survival was followed daily.

(D) Males of *w*MelCS_b and *w*MelPop lines were collected for DNA extraction and qPCR every 2 days. Each point represents a replicate (ten males per replicate, three to four replicates per time point), and lines are medians of the replicates. Relative amount of *Wolbachia* genomic DNA was calculated using host Rpl32 as a reference gene and values are relative to median of samples of *w*MelCS_b at day 2-3.

*w*MelPop also has much lower titres of FHV three days post infection when compared with *w*MelCS_b (Figure 6B, Mann-Whitney test, p=0.007). In most of the *w*MelPop samples FHV titres were below the limit of detection of the qRT-PCR. Therefore the difference between the medians of *w*MelCS_b and *w*MelPop is not quantifiable but it is over ten thousand fold (over one million-fold when compared with w^{1118} iso). These results show that *w*MelPop gives stronger resistance to viruses

than the closely related *w*MelCS_b. These data also demonstrates that *Wolbachia* can confer strong resistance to FHV.

We tested *w*MelPop protection to viral infection in terms of survival upon infection with FHV (Figure 6C). Contrary to *w*MelCS_b, the presence of *w*MelPop does not increase survival of FHV infected flies (Tukey's test on the mixed effects Cox model fit, *w*MelCS_b *versus* w^{1118} iso lines infected with FHV, $p<0.001$; *w*MelPop *versus* w^{1118} iso lines infected with FHV, $p=0.229$). This is due to a very strong pathogenic effect of *w*MelPop, even in the absence of FHV; at 25 ºC all flies are dead by day 12 (*w*MelPop *versus* w^{1118} iso lines not infected with FHV, $p<0.001$). This pathogenic effect at 25 ºC has been reported before (Min and Benzer, 1997; McGraw *et al.*, 2002; Reynolds *et al.*, 2003; Osborne *et al.*, 2009) but seems stronger in our experiment. Nonetheless, FHV does not cause any mortality in the *w*MelPop line (*w*MelPop line infected and not infected with FHV, $p=0.816$), which is consistent with the strong resistance we observed. Therefore, although *w*MelPop confers strong resistance to FHV, it does not increase lifespan of an FHV infected host because it is very deleterious by itself.

Given the strong antiviral resistance and pathogenic effect we observe with *w*MelPop at 25 °C, we decided to measure how *Wolbachia* titres change with age in flies infected with *w*MelPop and *w*MelCS_b (Figure 6D). wMelPop growth is better explained by an exponential model of growth than a linear model (Table S1) with an estimated doubling time of 3.4 days. *Wolbachia* titres and growth rate are significantly higher in *w*MelPop (log-linear model, intercept difference between *w*MelPop and wMelCS_b: 0.904, $p<0.001$; slope difference: 0.224, $p<0.001$). At the day of our viral infection (1-2 days) *w*MelPop titres are 3 to 5 times higher than *w*MelCS_b titres.

Once again we observe that the *w*Mel variant with higher titres gives stronger protection to viruses. In *w*MelPop the exponential growth leads

to a much stronger resistance to DCV and FHV but severely reduces the host lifespan.

Genetic Basis of the Phenotypic Differences between *w*Mel Variants

Having identified phenotypic differences between *w*Mel variants we asked what their genetic bases were. To answer that we used the information from the sequence analysis and their assembled genomes. From the multiple alignments we extracted variant sites that were different between all *w*Mel-like and all *w*MelCS-like variants, in order to focus on common differences. We detected 108 single nucleotide polymorphisms (SNPs) between these two groups of variants, a tandem duplication and seven insertion-deletion polymorphisms (indels) (Tables 2, S3 and S4). 83 of the SNPs map to annotated *w*Mel genes (Wu *et al.*, 2004), of which 59 are non-synonymous substitutions. The 55 genes that differ in these 59 SNPs encode proteins with a wide variety of functions, based on predicted conserved domains (Table 2). This set contains a high number of genes coding ankyrin-repeat containing (ANK) proteins: WD0073, WD0292, WD0514, WD0636, WD0754, and WD0766.

Table 2. Coding non-synonymous SNPs between *w*Mel-like and *w*MelCS-like *Wolbachia* variants.

Gene name		Gene description[a]	Nucleotide			Amino acid		
			position	*w*Mel - like	*w*MelCS- like	position	*w*Mel- like	*w*MelCS -like
WD0019		transcription antitermination protein NusG, putative	18552	A	G	191	Q	R
WD0024	rpoBC	DNA-directed RNA polymerase, beta/beta' subunit	26870	G	A	2060	E	K
WD0033		Piwi/Argonaute/Zwille siRNA-binding domain[a]	36114	C	T	158	V	I
WD0036	prsA	ribose-phosphate pyrophosphokinase	39135	A	C	99	K	Q
WD0041			45207	A	G	12	M	T
WD0068		outer membrane protein TolC, putative	65076	A	G	122	N	S
WD0073		ankyrin repeat-containing protein	69287	A	G	298	T	A
WD0086	secD	protein-export membrane protein SecD	79898	G	A	91	T	I
WD0115		transposase, IS4 family	109211	T	G		STOP	E
WD0129		membrane protein CvpA, putative	118051	C	T	15	V	I
WD0130	ribE	riboflavin synthase, alpha subunit	118692	A	G	19	F	S
WD0131			119806	A	G	285	L	P
WD0190	mutS	DNA mismatch repair protein MutS	173865	C	T	50	G	R
WD0223		Rossmann-fold NAD(P)(+)-binding proteins; Bacterial NAD-glutamate dehydrogenase[a]	203561	C	T	1233	V	I
WD0223		Rossmann-fold NAD(P)(+)-binding proteins; Bacterial NAD-glutamate dehydrogenase[a]	204413	T	C	949	N	D
WD0262		RuvC_resolvase[a]	248476	G	A	108	A	T
WD0292		prophage LambdaW1, ankyrin repeat domain protein	273138	A	G	40	S	P
WD0363			347096	C	T	52	Q	STOP
WD0400		ABC transporter, HlyB/MsbA family, putative	381187	T	C	143	I	T
WD0427	atpB	ATP synthase F0F, A subunit	409057	A	G	139	E	G
WD0433	pccA	propionyl-CoA carboxylase, alpha subunit	414527	G	A	246	T	M
WD0443		OTU-like cysteine protease[a]	427731	C	T	119	R	C
WD0469		cytidine and deoxycytidylate deaminase family protein	452129	C	T	55	S	L
WD0513		RHS repeat-associated core domain[a]	505589	G	A	56	T	I
WD0514		ankyrin repeat-containing protein	506438	C	A	255	A	S
WD0530	pyrH	uridylate kinase	517457	C	T	37	A	T
WD0562		transposase, truncation	547769	C	T	62	E	K
WD0610		helicase, SNF2 family	591593	C	G	126	Q	H
WD0614		O-methyltransferase	598213	G	A	483	D	N
WD0636		ankyrin repeat-containing prophage LambdaW1	628654	G	T	124	A	E
WD0638		Phage tail protein[a]	630778	A	G	112	L	P
WD0639		prophage LambdaW5, baseplate assembly protein J	631303	T	C	201	M	V
WD0666	rplF	ribosomal protein L6	650962	C	T	23	S	N

Table 2. (continued)

Gene Name		Gene description[a]	Nucleotide			Amino acid		
			position	*w*Mel - like	*w*MelCS-like	position	*w*Mel-like	*w*MelCS -like
WD0754		ankyrin repeat-containing protein	728880	T	C	48	E	G
WD0758		glutaredoxin family protein	732864	C	G	18	G	A
WD0766		ankyrin repeat-containing protein	739409	T	G	139	L	W
WD0766		ankyrin repeat-containing protein	739559	T	C	189	I	T
WD0813	proS	prolyl-tRNA synthetase	780933	G	C	196	G	R
WD0814	acpS	holo-(acyl-carrier-protein) synthase	781622	A	G	4	S	G
WD0838			803009	G	A	41	V	I
WD0838			805011	G	A	709	C	Y
WD0839	uvrB	excinuclease ABC, subunit B	805888	G	C	524	Q	E
WD0867	purH	phosphoribosylaminoimidazolecarboxamide formyltransferase/IMP cyclohydrolase	838894	A	G	260	E	G
WD0898			864943	C	A	2	L	F
WD1029	aspC	aspartate aminotransferase	989918	C	G	24	A	G
WD1044		No annotation or conserved domains	1006175	G	A	33	G	D
WD1064	rpoH	heat shock sigma factor RpoH	1024202	A	G	42	N	D
WD1090	rpsA	ribosomal protein S1, putative	1048565	T	C	451	D	G
WD1137		PD-(D/E)XK nuclease family transposase[a]	1089274	T	C	6	I	V
WD1140		PD-(D/E)XK nuclease family transposase[a]	1091606	T	C	34	D	G
WD1200	priA	primosomal protein N	1147604	G	C	423	G	A
WD1216		sensor histidine kinase/response regulator	1164424	C	T	391	H	Y
WD1237	clpA	ATP-dependent Clp protease, ATP-binding subunit ClpA	1185021	G	A	667	A	T
WD1278			1220566	A	G	244	Y	C
WD1278			1220989	A	G	385	D	G
WD1292		ribonuclease, BN family	1232581	C	T	124	A	V
WD1297		lipolytic enzyme, GDSL family	1239247	C	T	181	R	H
WD1312		DsbA-like disulfide oxidoreductase	1253148	C	T	217	G	E
WD1318	infB	translation initiation factor IF-2	1260309	C	T	309	G	D

[a] When absent: conserved domains prediction by CD-search tool at NCBI [ref-s]

Marchler-Bauer A et al. (2013), *"CDD: conserved domains and protein three-dimensional structure."*, Nucleic Acids Res.41(D1)348-52.
Marchler-Bauer A et al. (2011), *"CDD: a Conserved Domain Database for the functional annotation of proteins."*, Nucleic Acids Res.39(D)225-9.
Marchler-Bauer A, Bryant SH (2004), *"CD-Search: protein domain annotations on the fly."*, Nucleic Acids Res.32(W)327-331.

In order to understand the basis of the strong phenotypic differences between the closely related *w*MelCS_b and *w*MelPop variants we have investigated the differences between their genomes. Previous studies

have not identified any genetic differences between *w*MelCS and *w*MelPop (Iturbe-Ormaetxe, Woolfit, *et al.*, 2011; Riegler *et al.*, 2012). From the genome sequence analysis we found only two SNPs unique to *w*MelPop, and six positions where there was an ambiguous call for the *w*MelPop nucleotide. We Sanger sequenced these regions in *w*MelCS_b and *w*MelPop, and found that only two synonymous SNPs were true differences between these variants (position 943,443, G>A, unique to *w*MelPop; position 858,287, T>C, unique to wMelCS_b). In our analysis of split sequencing reads, there were no indel polymorphisms unique to *w*MelPop that met our filtering criteria. Therefore we cannot identify any SNPs or small indels that could be clearly related to the phenotypic differences.

To identify other possible differences between *w*MelPop and *w*MelCS_b we analysed copy number variation in their genomes. We mapped the sequence reads to the *w*Mel reference genome and examined variation in the depth of coverage. In *w*MelPop there is a large increase in read depth in a ~21kB region. Using the mean shift approach implemented in CNVnator (Abyzov *et al.*, 2011) we estimated that this region has been amplified approximately five times (Figure 7A and Figure S2 (doi:10.1371/journal.pgen.1003896.s007) t test: $p<10^{-20}$; breakpoints: 486,601-507,800). Due to the extensive amplification, probable association with the over-proliferative phenotype, and containing eight predicted genes, we call it the Octomom region.

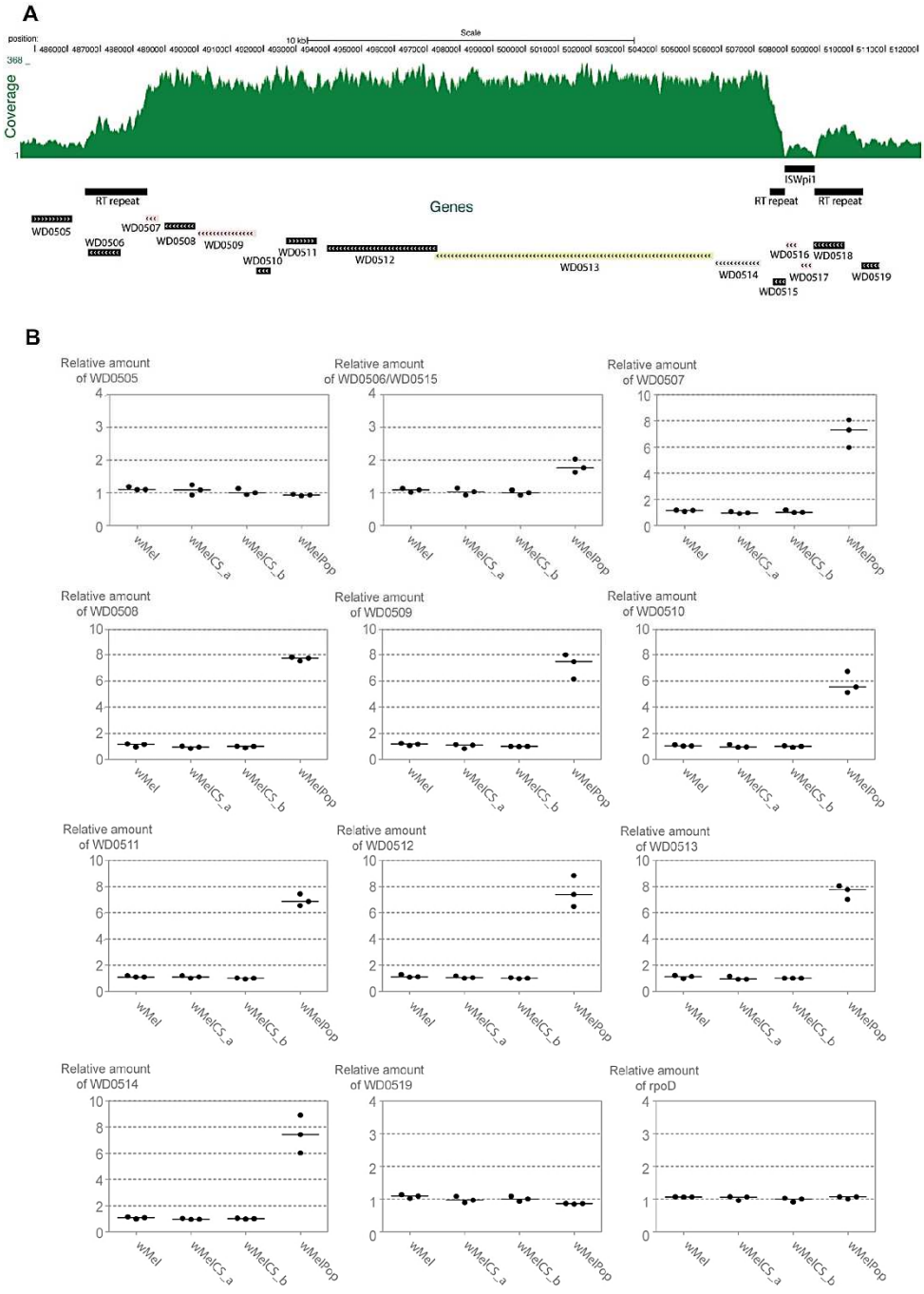

Figure 7. Genomic region amplified in *w*MelPop.

Continued on the next page.

Figure 7. (continued). (A) Depth of coverage of sequence reads of *w*MelPop mapped to *w*Mel reference genome (GenBank: AE017196) in region 484,564 to 512,000. Nucleotide positions, predicted genes, RT repeats and the ISWpi1 element in this region of *w*Mel are shown. The 5' RT repeat extends from 486,532 to 488,449 (1912bp). The 3' RT repeat in *w*Mel extends from 507,470 to 510,325 but is split in two parts due to the insertion of an ISWpi1 (IS5) transposon from 507,928 to 508,848. This ISWpi1 is not present in *w*MelPop or the closely related *w*MelCS_b. Figure modified from USCS genome browser (http://genome.ucsc.edu/) [121,122].
(B) Relative amounts of genomic copy number of Octomom genes (WD0506-14), genes adjacent to Octomom region (WD0505 and WD0519) and control gene *rpoD* in *w*Mel, *w*MelCS_a, *w*MelCS_b and *w*MelPop were calculated using *wsp* as a reference gene. Values are relative to median of *w*MelCS_b samples. Each point represents a replicate (ten males per replicate, three replicates per *Drosophila* line) and lines are medians of the replicates.

There are two repeated regions, with the same orientation, flanking the Octomom region (*RT repeat* in Figure 7A). The 5' repeat region contains WD0506, which is annotated as a pseudogene in the reference genome (GenBank: AE017196 (Wu *et al.*, 2004)), but it may encode a 329aa protein with a *reverse transcriptase (RT) with group II intron origin* domain. In the *w*Mel reference genome the 3' repeat region is split in two parts due to the insertion of an ISWpi1 (IS5) transposon (Figure 7A) (ISWpi1 is repeated 13 times in the *w*Mel genome (Cordaux *et al.*, 2008)). This ISWpi1 insertion, however, is absent in the *w*MelCS-like variants, including *w*MelPop. In fact presence/absence of this insertion is one of the genomic markers used to distinguish *w*Mel variants (IS5 WD0516/7) (Riegler *et al.*, 2005). Accordingly, in the coverage plot (Figure 7A) there is no coverage at the interface between the ISWpi1 and the RT repeat regions in *w*MelPop. Therefore, this region in *w*MelPop is 100% identical to the 5' RT repeat (confirmed, in the region of ISWpi1

insertion, by Sanger sequencing, data not shown). Two other 100% identical RT repeats occur in the genome, at positions 243,822-245,739 and 584,482-582,565 and a smaller 718bp sequence at positions 633,948-634,665, also 100% identical in its length.

The amplified region in *w*MelPop contains eight predicted genes between the RT repeats, WD0507-WD0514 (Figure 7A, Table S5). WD0507-11 encode proteins potentially involved in DNA replication, repair, recombination, transposition or transcription. The genes WD0512-14 have previously been shown to be an operon (Iturbe-Ormaetxe *et al.*, 2005). WD0513 protein has an Rhs domain and WD0514 encodes a ANK repeat protein, but the function of any of the three proteins encoded in this operon is unknown.

The Octomom region was first noticed because of its presence in the strain *w*Mel but absence in many other *Wolbachia* strains (Iturbe-Ormaetxe *et al.*, 2005). It has since been found that there are homologues of WD0512-14 in wPip (Klasson, Kambris, *et al.*, 2009; Woolfit *et al.*, 2009) and of WD0514 in several strains of *Wolbachia* supergroup A (Siozios *et al.*, 2013). We find orthologues of all the genes of the Octomom region, including the RT repeat, in the genome of wPip (GenBank: AM999887.1 (Klasson *et al.*, 2008)). In *w*Pip WD0507-10 orthologues have conserved synteny with *w*Mel. We also find WD0507-509 homologue syntenic blocks in the prophages WOVitA1 of *w*VitA (GenBank: HQ906662.1 (Kent *et al.*, 2011)) and WOVitB1 of *w*VitB (GenBank: HQ906666.1 (Kent *et al.*, 2011))) and in *w*AlbB (GenBank: CAGB01000117.1).

WD0512-3 and their *w*Pip homologue are also an interesting example of a horizontal gene transfer between *Wolbachia* and mosquitoes (Korochkina *et al.*, 2006; Klasson, Kambris, *et al.*, 2009; Woolfit *et al.*, 2009). Previously, their homologues have only been found in *Culicidae* (*Aedes*, *Anopheles* and *Culex*). We have also found homologues of

WD0513 in the recently sequenced genome of *Daphnia pulex* (GenBank: EFX66732.1 (Colbourne *et al.*, 2011)). DAPPUDRAFT_229333 and DAPPUDRAFT_300516 are 35% and 32% identical to this protein, respectively.

To confirm the depth of coverage results we performed qPCR to determine relative genomic copy numbers of the genes immediately adjacent and inside the Octomom region in *w*Mel, *w*MelCS_a, *w*MelCS_b and *w*MelPop (Figure 7B). All genes tested showed the same relative amount in *w*Mel, *w*MelCS_a, *w*MelCS_b. The genes immediately outside the Octomom region WD0505 and WD0519, as well as two other control genes located elsewhere in the genome, *rpoD* and *gmk,* show the same copy number in *w*MelPop and in the other *w*Mel variants (between 0.86 and 1.09 relative to *w*MelCS_b) (Figure 7B and data not shown). In contrast, in *w*MelPop the eight genes inside Octomom, WD0507-14, have estimated copy numbers between 5.54 and 7.78 times the levels of *w*MelCS_b (with a median of 7.42 times). These results confirm the extensive amplification detected by the depth of coverage analysis and show a 7-fold amplification of this region.

The results for WD0506/WD0515 (the qPCR primers amplify both) show 1.77 fold difference between *w*MelPop and *w*MelCS_b (Figure 7B). There are 4 identical copies of the amplified region in *w*Mel, *w*MelCS_a and *w*MelCS_b (in the 4 full RT repeats). If this region was also amplified 7 times in *w*MelPop we would expect 2.75 more copies in *w*MelPop than in *w*MelCS_b. The fold difference between the *w*MelPop and *w*MelCS_b is lower than expected but shows an amplification of this region and indicates that in *w*MelPop there are 3 more copies of this gene.

The only other large duplication in the *w*Mel variants detected using CNVnator were in *w*Mel2_a and *w*Mel2_b, where a large region corresponding to the phage WO-B has been duplicated (Figure S2 (doi:10.1371/journal.pgen.1003896.s007); t tests: $p<0.001$; breakpoints

in both: 569,001-634,000). This is a stable duplication since the most recent common ancestor of these lines dates to an estimated 9,252 host generations ago (Figure 1). Independent WO-B prophage amplifications have been shown before in *Wolbachia* strains; it is present in two copies in *w*Ri (Klasson, Westberg, *et al.*, 2009) and five copies in *w*Pip (Klasson *et al.*, 2008).

Discussion

We have found that genetically closely related variants of *Wolbachia* from *D. melanogaster* vary in the degree to which they protect their hosts against viral infection. The *Wolbachia* variants that provide the greatest protection have higher titres and often shorten the lifespan of their hosts (Table S6). Previous work has shown that in natural populations these highly protective *w*MelCS-like variants were recently largely replaced by less protective *w*Mel-like variants. The genome sequences of strains conferring different levels of protection have allowed us both to reconstruct the evolution of antiviral protection and identify candidate genes that may affect it.

Phylogeny and Genomics of *w*Mel Variants

Large-scale genome sequencing of *w*Mel variants from natural populations of *D. melanogaster* has previously identified two major monophyletic groups of *Wolbachia* (Richardson *et al.*, 2012). We were working with a set of *Wolbachia* variants that had been identified using a small number of genetic markers, so we sequenced the genomes of these variants and their associated mitochondria in order to determine where they fall on the phylogeny. We found that these variants belong to both major monophyletic groups, which diverged approximately 80,000 fly generations before present. This date corresponds to the most recent common ancestor of all *w*Mel variants in *D. melanogaster*.

We found that there are striking differences in the degree to which the strains from the different phylogenetic clades protect flies against viruses, with the less common *w*MelCS-like clade providing the stronger protection and having higher *Wolbachia* densities. This phylogenetic basis for the phenotypic differences confirms that genetic differences

between *w*MeI variants are responsible for the variation in symbiont titres and resistance to viruses. Therefore, we identified the common genetic differences between all the *w*MelCS-like variants and all the *w*Mel-like variants. We found eight indels and 108 SNPs that differ between them, with polymorphisms in the coding sequence of 58 proteins. This number is still too high in order to speculate on possible individual contributions to the phenotypic differences. Future experimental work will help to further reduce this finite number of candidate differences.

We have also compared the genome of the pathogenic variant *w*MelPop with the closely related *w*MelCS_b. The *w*MelPop genome has only two unique differences from the other strains – a single synonymous SNP and a 7-fold amplification of a ~21kb region which we named Octomom. The Octomom amplification is therefore the most probable cause of *w*MelPop pathogenicity and increased protection against viruses. In bacterial genomes copy number variation is very common and mostly involves unequal recombination between two direct sequence repeats, amplifying the region in between (Andersson and Hughes, 2009). The Octomom region in *w*MelCS is flanked by two identical 1912b direct repeats, which may provide the origin for the initial duplication in *w*MelPop. In bacteria and viruses gene amplifications have been shown to increase growth or virulence (Mekalanos, 1983; Kroll *et al.*, 1991; Mavingui *et al.*, 1998; Andersson and Hughes, 2009; Elde *et al.*, 2012). In the future it will be important to show functional data linking this amplification and the pathogenic phenotype of *w*MelPop.

The functions of the genes in the Octomom region are unknown. The WD0506-WD0511 proteins have predicted domains that are related to interactions with nucleic acids and could have a role in DNA replication, transcription or repair. Therefore, the amplification of these genes could have a direct effect on the replication of *Wolbachia*. WD0512-14 have homology to proteins or protein domains of eukaryotes and are,

consequently, candidate effector proteins of *Wolbachia*. Hypothetically they could mediate the pathogenicity through interaction with the host.

Key adaptive traits of bacterial pathogens and symbionts are often controlled by genes that are frequently gained and lost through evolution, which are collectively known as the 'accessory genome'. The Octomom region appears to fit this pattern as it is partially or totally absent in several *Wolbachia* strains (Salzberg *et al.*, 2005; Iturbe-Ormaetxe *et al.*, 2005; Ishmael *et al.*, 2009). WD0512-3 homologues have also been suggested to be amplified in *w*Sim (Ishmael *et al.*, 2009), although they are not present in its unassembled genome sequence (Salzberg *et al.*, 2005). Homologues of some Octomom genes in other strains have been described (Klasson, Kambris, *et al.*, 2009; Woolfit *et al.*, 2009; Ishmael *et al.*, 2009; Siozios *et al.*, 2013) and here we identify more in WOVitA1, WOVitB1, and *w*AlbB. Moreover, we detect orthologues of all the Octomom genes in *w*Pip, although not as one syntenic block. As the number of sequenced genomes of different *Wolbachia* strains increases it will be interesting to understand the evolutionary history of this region. In particular if there is horizontal gene transfer between strains, as suggested before (Iturbe-Ormaetxe *et al.*, 2005; Woolfit *et al.*, 2009). This would be compatible with our finding of some of these genes in prophage regions of WOVitA1 and WOVitB1.

The horizontal transfer of these genes may also occur between *Wolbachia* and their insect hosts, as two of the genes in the Octomom region, WD0512-3, are homologous to genes previously identified only in *Culicidae* mosquitoes (Korochkina *et al.*, 2006; Klasson, Kambris, *et al.*, 2009; Woolfit *et al.*, 2009). The direction of the horizontal gene transfer between mosquitoes and *Wolbachia* is not clear (Korochkina *et al.*, 2006; Klasson, Kambris, *et al.*, 2009; Woolfit *et al.*, 2009). In mosquitoes these homologues constitute a family of proteins termed salivary gland surface proteins (SGSs). There is evidence that *Ae. aegypti* aaSGS1 is a

receptor for malaria sporozoite in salivary glands (Korochkina *et al.*, 2006) and *An. gambiae* Sgs4 and Sgs5 are components of the saliva (King *et al.*, 2011). We have identified two other homologues of WD0513 in the crustacean *Daphnia pulex*. The number of sequenced crustaceans genomes is very low so we do not know how prevalent these genes are in crustaceans. However, the absence of homologues in any other sequenced insect opens the possibility that there was also horizontal gene transfer between *Daphnia*/Crustaceans and either mosquitoes or *Wolbachia.*

Phenotypes Associated with *w*Mel Variants

Symbionts could protect their hosts against infection either by limiting pathogen titres (resistance) or by reducing the harmful effects of those pathogens (tolerance) (Schneider and Ayres, 2008). We have previously reported that *Wolbachia* provides tolerance to FHV and resistance to DCV (Teixeira *et al.*, 2008). In this study we found similar FHV titres in lines with *w*MelCS-like and *w*Mel-like variants, despite the former having far lower mortality rates. This indicates that natural *w*Mel variants differ in how they modulate tolerance to FHV infection rather than resistance (although *w*MelPop confers strong resistance to FHV, see below). On the other hand, the levels of DCV change between the two groups, with *w*MelCS-like variants having a two-fold reduction in DCV titres when compared with *w*Mel-like variants. This difference is small, especially when compared to the 5,000-fold reduction in titres in relation to the control without *Wolbachia*, but is reflected in a substantial change in survival. Therefore, it is possible that there is also a tolerance component in the variants differential protection to DCV. However, with our data we cannot distinguish between these hypotheses since it is possible that even a small change in viral titre is sufficient to explain the better survival

(see also discussion in (Ayres and Schneider, 2008)). Nonetheless, induced tolerance to DCV has been shown before for the *Wolbachia* strain *w*Ri in *D. simulans* (Osborne *et al.*, 2009). Therefore, the interaction of *Wolbachia* with different viruses may always have components of resistance and tolerance modulation.

The more protective *w*MelCS-like variants reach 2.5 higher titres than *w*Mel-like variants in the first days after adult eclosion, and then continue to proliferate during the lifespan of their host. These results show that in *D. melanogaster* the control of *Wolbachia* levels is also dependent on the endosymbiont genotype. It has been shown before that the host genotype and *Wolbachia* strain can influence *Wolbachia* titres (McGraw *et al.*, 2002; Veneti *et al.*, 2003; Mouton *et al.*, 2003; Kondo *et al.*, 2005; Mouton *et al.*, 2007; Jaenike, 2009; Lu *et al.*, 2012). In *Leptopilina heterotoma* each strain's titre is even independent of the presence of the other strains (Mouton *et al.*, 2003). Different strains of *Wolbachia* also reach different levels in *D. simulans*, although the host nuclear genetic background has not been controlled in this study (Osborne *et al.*, 2009). Our results show that these differences are also seen between *Wolbachia* variants that are very closely related to each other (their most recent common ancestor is estimated to date to only 8,000 years or 80,000 fly generations before the present).

The positive correlation between *Wolbachia* titres and protection against viral infection suggests that this may be the cause of the greater protection provided by the *w*MelCS-like variants. It is important to note that the strains are not phylogenetically independent, so the association between protection to viruses and titres might have arisen independently in the ancestors of the *w*Mel-like and *w*MelCS-like groups. However, this seems unlikely, as a density effect has been previously reported in *Wolbachia*-mediated antiviral protection. *Wolbachia*-host combinations with higher titres of *Wolbachia* show higher protection (Osborne *et al.*,

2009; Frentiu *et al.*, 2010; Lu *et al.*, 2012) and decreasing levels of *Wolbachia* with antibiotic treatment lowers protection (Lu *et al.*, 2012, Osborne *et al.*, 2012). Correlations between titres and other *Wolbachia*-associated phenotypes have been shown before (e.g. with cytoplasmic incompatibility) (Bressac and Rousset, 1993; Boyle *et al.*, 1993; Breeuwer and Werren, 1993; Bourtzis *et al.*, 1996; Bordenstein *et al.*, 2006; Jaenike *et al.*, 2009; Unckless *et al.*, 2009). Therefore, the simplest hypothesis is that the differential protection to viruses of the *w*Mel variants is a consequence of their titres.

The localization of the protective symbionts and the pathogens could be an important factor to understand their interaction (Osborne *et al.*, 2009). *Wolbachia,* DCV and FHV have been shown to infect several tissues of *D. melanogaster* (Lautié-Harivel and Thomas-Orillard, 1990; Clark *et al.*, 2005; Dostert *et al.*, 2005; Galiana-Arnoux *et al.*, 2006; Eleftherianos *et al.*, 2011). Although the information on localizations is not necessarily exhaustive there are some tissues of overlap between *Wolbachia* and the two viruses where the interaction could occur. It will be important in the future to determine the tissue distribution of the different *Wolbachia* variants and how it contributes to the overall differences in titres. It will also be interesting to know if *Wolbachia* titres increase with host age is uniform between all the tissues. It has been previously shown that some *Wolbachia* strains grow at different rates in heads and ovaries (McGraw *et al.*, 2002).

We found that some of the most protective *w*Mel variants reduce the survival of their hosts, suggesting that there may be a trade-off between symbiont-mediated protection and other components of fitness. This cost could be either due to the metabolic cost of their replication or damage caused by their presence. The difference between the *w*Mel-like and *w*MelCS-like strains was less clear-cut for this trait. We observed that two *w*MelCS-like lines had significantly greater mortality rates. A third line

infected with *w*MelCS_b has previously been shown to have a shorter lifespan than the control (Teixeira *et al.*, 2008), although this is not significant in this report. The fourth *w*MelCS-like line did not show any detectable effect on lifespan. This variation could be due to the cost of *Wolbachia* infection being difficult to assess in normal laboratory conditions. This reduction in longevity may not directly affect the fitness of flies in the wild since probably not many flies live up to this late age and their fertility would be very low. However, the assay can be interpreted as a proxy for fitness costs associated with the *Wolbachia* variants, which are expressed in other unknown ways in the wild.

The phenotypes of the line carrying the laboratory variant *w*MelPop are consistent with the differences between natural variants. Our results are in agreement with previous reports that *w*MelPop can reach high titres and shorten lifespan (Min and Benzer, 1997; McGraw *et al.*, 2002; Reynolds *et al.*, 2003), as well as to give strong protection to viruses (Walker *et al.*, 2011; van den Hurk *et al.*, 2012). Here we directly compare this variant with *w*MelCS_b, its closest related variant, in their natural host. *w*MelPop is the variant that reaches higher levels in *D. melanogaster*, gives the strongest resistance to viruses, and most severely shortens the host lifespan at 25 ºC. The pathogenic effect was described before at 25 ºC (Min and Benzer, 1997; McGraw *et al.*, 2002; Reynolds *et al.*, 2003). Yet, this phenotype at 25 ºC seems to be stronger in our experimental conditions. This is probably related with the *w*MelPop exponential growth that we detect. We also observed that flies with *w*MelPop have very strong resistance to DCV and FHV. The strong resistance to FHV induced by *w*MelPop may indicate that there is no qualitative difference between the interference of *Wolbachia* with DCV and FHV. Again, it may be only a question of different degrees of resistance and tolerance to different viruses.

Evolution and Dynamics of *Wolbachia* in Populations

Analysis of *Drosophila* lines collected from the early 20th century to the present has indicated that natural selection has driven a recent and fast replacement of *w*MelCS-like variants by *w*Mel-like variants and their associated mitochondria (Riegler *et al.*, 2005; Nunes *et al.*, 2008). A more recent phylogenomic analysis of *Wolbachia* and mitochondria is consistent with a *w*Mel-like global replacement, although it indicates that this event is not complete and started before the 20th century (Richardson *et al.*, 2012). Overall, it is clear that there was a relatively recent and rapid replacement of *w*MelCS with *w*Mel-like variants at a worldwide level. Therefore, our results indicate that this has resulted in a recent and rapid decline in the level of antiviral protection that *Wolbachia* provides to *D. melanogaster* in the wild. Consequently, we can conclude that the driving force for this change in *w*Mel frequencies was not an increase in viral protection. On the other hand, the *w*MelCS-like variants that have higher titres and can have a cost, have been replaced with variants with lower titres and, most probably, lower cost to their hosts.

Our data suggests that the balance between benefit (protection to viruses) and cost may have shifted recently, resulting in selection favouring lower levels of protection. In the simplest scenario, the rate at which this replacement has occurred would allow us to easily estimate the net benefit that the low protection strain has had. There are however several complexities that could affect the dynamics of this replacement. First, if the viruses are predominantly transmitted within *D. melanogaster* populations rather than among different fly species, then the spread of a low protection strain might increase the viral prevalence (Fenton *et al.*, 2011). This might make the fitness of the low protection strain negatively frequency dependent, potentially stably maintaining both strains in the population. Second, the difference in the density of the high and low protection variants might affect other aspects of *Wolbachia* fitness, such

as its vertical transmission efficiency or the strength of cytoplasmic incompatibility (Jaenike 2009; Hoffmann *et al.*, 1990; Unckless *et al.*, 2009). These parameters can be experimentally measured and their effects explored with simple extensions to standard models.

In order to block transmission of dengue, the *w*Mel and *w*MelPop variants were recently introduced into the mosquito *Ae. aegypti* (Moreira *et al.*, 2009; Walker *et al.*, 2011). Our work in *D. melanogaster* is in agreement with the mosquito data showing that *w*MelPop confers both a higher protection to viruses and a higher fitness cost when compared to *w*Mel (Walker *et al.*, 2011; van den Hurk *et al.*, 2012; Hussain *et al.*, 2013). The deployment of these *Wolbachia* infected mosquitoes in the field has to take in consideration the trade-off between fitness costs which make it difficult to invade a population and protection to dengue. Our analysis indicates that *w*MelCS-like variants have an intermediate phenotype in terms of benefit and cost, and could be considered as an alternative.

Our data also indicate that if there is a strong selection for a mosquito-*Wolbachia* combination with lower fitness costs, this might result in lower protection to viruses. The dynamics of this selection may influence the success of this strategy to control dengue infection. In addition to the replacement of *w*MelCS-like variants with *w*Mel-like variants in *D. melanogaster*, rapid evolution of *Wolbachia* has been observed in natural populations of *D. simulans*, resulting in an increase in fertility of *Wolbachia* infected flies (Weeks *et al.*, 2007). Finally, if the Octomom region amplification is the basis of *w*MelPop higher titres and protection to viruses, it could have important consequences on its long-term maintenance in mosquito populations. Duplications in bacterial genomes can be very unstable due to homologous recombination (Andersson and Hughes, 2009). If loss of the duplication is frequent in a *w*MelPop

infected mosquito population, a rapid selection of a variant with low replication and low protection to viruses may be expected.

The differences in protection to viral infection with *w*Mel variants demonstrate that in order to understand *Wolbachia* protection to viruses in *D. melanogaster* one has to consider not only presence or absence of *Wolbachia* but also the genetic variability of the symbiont. Our results provide another example of how bacterial symbionts can cause rapid evolution in natural populations and control important traits. Furthermore, they illustrate how the ease with which genomes can be sequenced can provide clues to the molecular basis of these traits.

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Chapter 3 - Mutualism breakdown by amplification of *Wolbachia* genes

Supplementary Figures are included in the Appendix II.

Author contributions: All the experimental work presented in this chapter was performed by me. Together with Luis Teixeira we conceived and designed the experiments, analysed the data and wrote this chapter.

Summary

Microbial mutualists, particularly vertically transmitted endosymbionts, need to control their proliferation in order to minimize the cost to their hosts. Cytoplasmic, maternally inherited bacteria *Wolbachia* are the most common endosymbionts of insects, providing some of them with fitness benefits. In *Drosophila melanogaster Wolbachia w*MelPop is a unique virulent variant that proliferates massively in the hosts and shortens their lifespan. This variant is also important as it protects against viral infections and was already transinfected to mosquito vectors of human diseases to block dengue, chikungunya and malaria. Genetic bases of *w*MelPop virulence are unknown, but understanding them is crucial to predict *w*MelPop dynamics in the released mosquito populations. Here we show that amplification of a region containing eight *Wolbachia* genes, called Octomom, is responsible for *w*MelPop virulence. Using *Drosophila* lines selected for different Octomom copy numbers we demonstrate that the number of Octomom copies determines *Wolbachia* titres and the strength of the lethal phenotype. Octomom amplification is unstable and reversion of copy number to one reverts all the phenotypes. Our results provide a link between genotype and phenotype in *Wolbachia* and identify a genomic region regulating *Wolbachia* proliferation. We also prove that these bacteria can evolve rapidly despite a low nucleotide substitution rate. Our results show that transition from a mutualist to a pathogen may occur due to a single genomic change in the endosymbiont. This implies that there must be constant selection on endosymbionts to control their densities.

Introduction

Vertically transmitted bacterial endosymbionts are ubiquitous in arthropods, particularly in insects, and they range from mutualists to reproductive parasites (Moran *et al.*, 2008). Vertical transmission leads to dependence of the symbiont on the fitness of the host (Ewald, 1987; Lipsitch *et al.*, 1996). Therefore, it is advantageous for endosymbionts to control their own replication and minimize the cost to their hosts. *Wolbachia*, conceivably the most prevalent bacterial endosymbionts of insects (Hilgenboecker *et al.*, 2008; Zug and Hammerstein, 2012), are maternally transmitted and exhibit a range of phenotypes, including manipulation of host reproduction. Cytoplasmic incompatibility, parthenogenesis, male killing and feminization of genetic males bias host sex ratio in favour of females, maximizing *Wolbachia* spread in populations (Werren *et al.*, 2008). *Wolbachia* are also able to protect insects from pathogens, increasing their fitness upon infection (Hedges *et al.*, 2008; Teixeira *et al.*, 2008; Kambris *et al.*, 2009, 2010; Moreira *et al.*, 2009; Bian *et al.*, 2010; Hughes *et al.*, 2011). Antiviral protection provided by *Wolbachia* was initially described in naturally infected *Drosophila melanogaster* (Hedges *et al.*, 2008; Teixeira *et al.*, 2008), and later found to act in transinfected mosquito vectors of human diseases (Kambris *et al.*, 2009, 2010; Moreira *et al.*, 2009; Bian *et al.*, 2010; Hughes *et al.*, 2011).

*w*Mel is the *Wolbachia* strain infecting *Drosophila melanogaster*. In the laboratory conditions *w*Mel exerts a very weak mating incompatibility between infected males and uninfected females (Hoffmann *et al.*, 1994), most of the natural *w*Mel variants do not affect longevity at all, while the rest is associated with a small lifespan reduction late in life (Chrostek *et al.*, 2013), and all of them provide a very strong antiviral protection (Chrostek *et al.*, 2013). Therefore, *w*Mel does not seem to be a

reproductive parasite but a mutualist conferring protection to viruses (Hedges *et al.*, 2008; Teixeira *et al.*, 2008). Host protection is positively correlated with *Wolbachia* density: the higher the titres of *Wolbachia*, the higher the antiviral protection (Osborne *et al.*, 2009, 2012; Frentiu *et al.*, 2010; Lu *et al.*, 2012; Chrostek *et al.*, 2013, 2014). On the other hand, high endosymbiont densities can be costly in the absence of viral infection and *Wolbachia* variants conferring strong protection often shorten the lifespan of the flies (Min and Benzer, 1997; Chrostek *et al.*, 2013, 2014). There is thus a fine balance between density, benefit and cost to the host. Although natural variants of *w*Mel can be called mutualists, the laboratory *w*Mel variant *w*MelPop is pathogenic: it over-proliferates in the tissues and dramatically shortens the lifespan of infected flies (Min and Benzer, 1997; McGraw *et al.*, 2002; Reynolds *et al.*, 2003; Chrostek *et al.*, 2013). We have recently identified genetic differences between *w*MelPop and the closely related non-pathogenic variant *w*MelCS_b (Chrostek *et al.*, 2013). The *w*MelPop genome contains an amplification of a ~21kB region, named Octomom, which includes eight *Wolbachia* genes (WD0507 to WD0514) flanked by direct repeats. This amplification in *w*MelPop was also described by Woolfit and colleagues (Woolfit *et al.*, 2013). We have also found two synonymous SNPs between these two variants, one of which is unique to *w*MelPop (position 943,443, G>A), and the other unique to *w*MelCS_b (position 858,287, T>C; *w*MelPop is identical to other *w*Mel variants) (Chrostek *et al.*, 2013). The *w*MelPop unique SNP leads to synonymous substitution. Therefore, we hypothesized that Octomom region amplification is underlying *w*MelPop virulence.

Here we show that, in support of our original hypothesis, the Octomom region amplification is the cause of the *w*MelPop phenotypes: over-replication and pathogenicity.

Materials and Methods

Fly strains

D. melanogaster w^{1118} stock with *Wolbachia* *w*MelPop was provided by Markus Riegler and Scott O'Neill. *w*MelPop OPL stock was provided by William Sullivan and Laura Serbus. Both *w*MelPop stocks are derived from Min and Benzer original stock (Min and Benzer, 1997). DrosDel isogenic background (*iso*) flies with no *Wolbachia* and with *w*MelCS_b or *w*MelPop were described before (Ryder *et al.*, 2004; Teixeira *et al.*, 2008; Chrostek *et al.*, 2013).

DNA extractions

DNA was extracted from individual flies (*w*MelPop) or pools of ten flies (*w*MelCS_b controls in the selection experiments). Each fly or pool of flies was squashed in 250 µl of Tris HCl 0.1 M, EDTA 0.1 M, SDS 1 % (pH 9.0) and incubated 30 min at 70 °C. Next, 35 µl of 8 M CH_3CO_2K was added, samples were mixed by shaking and incubated for 30 min on ice. Samples were then centrifuged for 15 min at 13.000 rpm at 4 °C and the supernatant was diluted 100× for qPCR.

RNA extractions and cDNA synthesis

For each sample ten 3-6 days old flies were pooled and homogenized with a plastic pestle in 1 ml of Trizol Reagent (Invitrogen). RNA was extracted according to manufacturer's protocol and re-suspended in 50 µl of DEPC-treated water (Ambion). RNA concentrations were determined using *NanoDrop* ND-1000 Spectrophotometer. cDNA was prepared from 1 µg of total DNAse-treated RNA using Random Primers and M-MLV Reverse Transcriptase (all Promega). Primers were pre-incubated with template RNA for 5 min at 70 °C. Next, the enzyme was added and

reactions were placed at 25 °C for 10 min, 37 °C for 60 min and 80 °C for 10 min.

Real-time quantitative PCR

The real-time qPCR reactions were carried out in the CFX384 Real-Time PCR Detection System (BioRad) as described before (Chrostek *et al.*, 2013). Briefly, each of the reactions was performed with 6 µl iQ SYBR Green Supermix (Bio Rad), 0,5 µl of each primer (3,6 mM) and 5 µl of diluted DNA. We performed at least two technical replicates per biological sample for each set of primers. Primers sequences were described before (Chrostek *et al.*, 2013). The following thermal cycling protocol was applied: initial 50 °C for 2 min, denaturation for 10 min at 95 °C followed by 40 cycles of 30 s at 95 °C, 1 min at 59 °C and 30 s at 72 °C. Melting curves were examined to confirm specificity of amplified products. Ct values were obtained with Bio-Rad CFX Manager with default threshold settings. Ct values were subjected to quality check - samples with standard deviation between technical replicates exceeding 0.5 were discarded. Relative amounts of transcripts and genes were calculated by the Pfaffl Method (Pfaffl, 2001). To apply the method the efficiency of each of the primer pair was pre-determined in a separate experiment. For the Octomom expression data values were normalized to *gmk* expression. For the genomic Octomom copies values were normalized to the single copy *wsp* gene.

Sequencing of WD0514 – WD0507 junction

WD0514 – WD0507 junction was amplified using specific primers (Link_seq_1 and Link_seq_2) and Sanger sequencing was performed with these primers and the primers annealing inside the junction (Link_seq_3-7) by Source Bioscience Berlin, Germany. Primer sequences are listed in Table S1.

Selection experiments

Selection for high and low copy Octomom *w*MelPop lines in w^{1118} and *iso* backgrounds was initiated with females from a single vial of each background. For each background ten single females were separated into individual vials and allowed to lay eggs for five days before being sacrificed for WD0513 copy number determination. The offspring of the female with the highest and the lowest Octomom copy number was used to start the next generation. This general procedure was repeated at every generation of selection. Three replicates of high and low copy Octomom selection lines for each background were established at generation two. From that point on we selected one female/line/generation with the desired Octomom copy number (based on real-time qPCR). Female age for egg laying (0-2 days) and qPCR (5-7 days) was controlled from generation four and two for *iso* and w^{1118} lines, respectively. At generation seven of the w^{1118} lines we started to also select a one copy Octomom *w*MelPop. At this point we selected the WD0513 copy number closest to one for this selection regime, and the WD0513 copy number closest to two for the two Octomom copy lines.

From generation two to generation 13 of the w^{1118} selection and from generation two to generation 22 of the *iso* selection we were selecting from between six to ten females. From generation 15 of the w^{1118} selection and from generation 23 of the *iso* selection we were selecting from three females per line.

At generations 14 of w^{1118} lines and 18 of *iso* lines the selection was not performed.

Preparation of flies for phenotypic analyses

For phenotypic analyses of flies carrying *w*MelPop with different Octomom copy numbers single females were placed in vials, allowed to lay eggs for five days and sacrificed to determine WD0513 copy number.

The progeny of females with the specified Octomom copy numbers was selected for the phenotypic analyses. All lifespan assays were performed at 25 °C and 29 °C, the temperature regimes applied in the first report on *w*MelPop phenotypes (Min and Benzer, 1997).

In order to directly compare *w*MelPop with the full range of Octomom copy numbers, *w*MelCS_b and flies without *Wolbachia* we used hybrids between w^{1118} and *iso* genetic backgrounds (Tables S2 and S3). Females with desired *Wolbachia* status, which is transmitted to the next generation, were crossed with males from the other genetic background. Since females were used in the phenotypic analyses their genetic backgrounds are all equal and heterozygous between w^{1118} and *iso,* irrespective of the direction of the crosses. We used females with high Octomom copy number from both backgrounds to control for possible influence of the direction of the cross and maternal effects potentially associated with different backgrounds.

Lifespan and *Wolbachia* densities experiments

Females, whose mothers Octomom copy number was assessed by qPCR, were collected at eclosion (10 per tube), allowed to mate for 24 h (5 males per tube), separated from males and either checked for survival at 25 °C or 29 °C every day or kept at 25 °C and sacrificed at indicated timepoints for *Wolbachia* densities quantification. Females were maintained on a standard cornmeal diet without live yeast and passed to fresh vials every 3 days. The mothers of females used for phenotypic analyses were derived from selection lines at the generations indicated in Table S3.

Virus production and infection

Drosophila C virus was produced and titrated as described before (Teixeira *et al.*, 2008; Chrostek *et al.*, 2013). Infections were performed

by pricking 1-2 days old female flies with virus at 10^9 $TCID_{50}$/ml. After infection flies were kept in vials without live yeast, 10 flies per vial at 18 °C. It was shown before that wMelPop is not pathogenic to the flies at this temperature (Reynolds *et al.*, 2003). Flies were checked for survival daily and passed to fresh vials every 5 days.

Statistical analysis

Survival data were analysed by Cox proportional hazard mixed effect models. Octomom copy number was considered a fixed effect and replicate tube (containing 10 flies) within the same experiment was considered random. Model fitting was done using coxme package in R (Team, 2012). Tukey´s test was applied for pairwise comparisons of Cox hazard ratios between all *w*MelPop lines, *w*MelCS_b and flies without *Wolbachia.*

Analysis of growth curves of *w*MelPop lines with different Octomom copy number was performed with log-linear model fits (lm in R). The slopes of different fitted regression lines were compared and corrected for multiple comparisons (Bonferroni correction).

Spearman correlation between Octomom copy number and median time to death was performed in R (cor.test).

Western blot

Ten mated females from high and low *iso* selection lines, whose mothers were individually tested for Octomom copy number, were aged for 10 days before protein extraction. Flies without *Wolbachia* were used as negative control. Anti-WSP rabbit polyclonal antibody was provided by Bourtzis Kostas (Veneti *et al.*, 2003; Zabalou *et al.*, 2004) and pre-absorbed in fixed *Wolbachia*-free *D. melanogaster* embryos. Anti-beta-tubulin mouse monoclonal E7 antibody was acquired from Developmental Studies Hybridoma Bank (Chu and Klymkowsky, 1989).

Results

Currently *Wolbachia* cannot be genetically manipulated, which hinders functional studies on *Wolbachia* genes functions. However, bacterial amplified DNA sequences have been described before as unstable (Andersson and Hughes, 2009) leading us to test the hypothesis that natural variation in the Octomom copy number exists and causes distinct phenotypes. To detect Octomom copy number variation we tested several single females for the copy number of the Octomom gene WD0513 (Figure 1A).

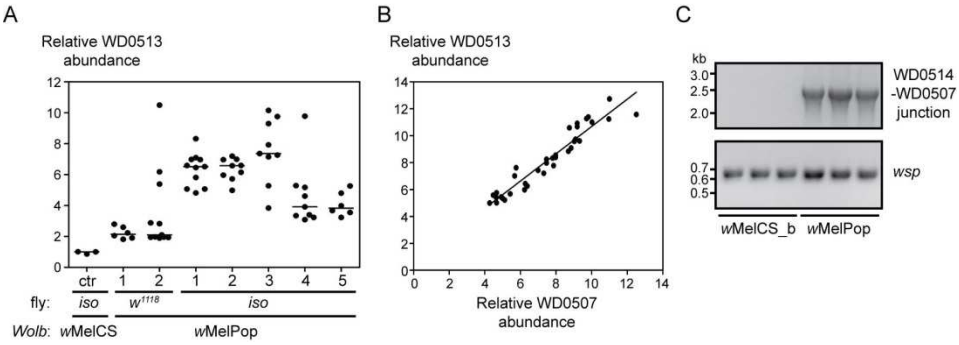

Figure 1. Individual *w*MelPop flies differ in Octomom copy numbers.
(A) WD0513 copy number variability in single females from two *w*MelPop stocks with w^{1118} and *iso* genetic backgrounds, relative to *wsp*. We tested two replicates of w^{1118} stock and five replicates of *iso* stock. *w*MelCS_b *iso* flies were used for copy number normalization. Lines are medians of the replicates. (B) Relation between WD0507 and WD0513 abundance in single *w*MelPop females. Each dot represents a female and the regression line is shown. The estimates for the fitted regression line are: slope = 1.036 ± 0.041, intercept = 0.182 ± 0. 204, R^2= 0.92. (C) PCR of the predicted WD0514-WD0507 junction in *w*MelPop flies. *w*MelCS_b was used as a negative control. PCR for *wsp* gene was used as a DNA quality control.

We analysed two fly stocks infected with *w*MelPop: w^{1118} derived from the original stock in the Benzer lab (Min and Benzer, 1997) and a DrosDel isogenic w^{1118} (*iso*) stock into which we introgressed *w*MelPop from the w^{1118} stock (Chrostek *et al.*, 2013). *w*MelCS_b samples were used as a reference for one WD0513 copy on the basis of the coverage analysis of our previous *Wolbachia* sequencing data (Chrostek *et al.*, 2013). All *w*MelPop samples analysed had at least a duplication of the Octomom region, with high variation in WD0513 copy number between individual females, ranging from two to ten copies. To check if the Octomom region is amplified as a unit we tested WD0507 and WD0513 copy number simultaneously in individual flies. The results confirm that in each fly the copy numbers of the two genes are the same (Figures 1B and S1). A common mechanism of gene amplification in bacteria leads to tandem duplications and formation of new junctions between units (Andersson and Hughes, 2009). We detected the presence of this new predicted WD0514-WD0507 junction by PCR and Sanger sequencing (Figures 1C and S2). These data show that the Octomom copy number is highly variable and the amplification is consistent with a tandem duplication.

To test Octomom amplification effect on *w*MelPop virulence we established *Drosophila* lines with different Octomom copy numbers. Individual females with the highest and the lowest Octomom copy number were selected throughout several generations in both w^{1118} and *iso* backgrounds (Figures 2 and S3).

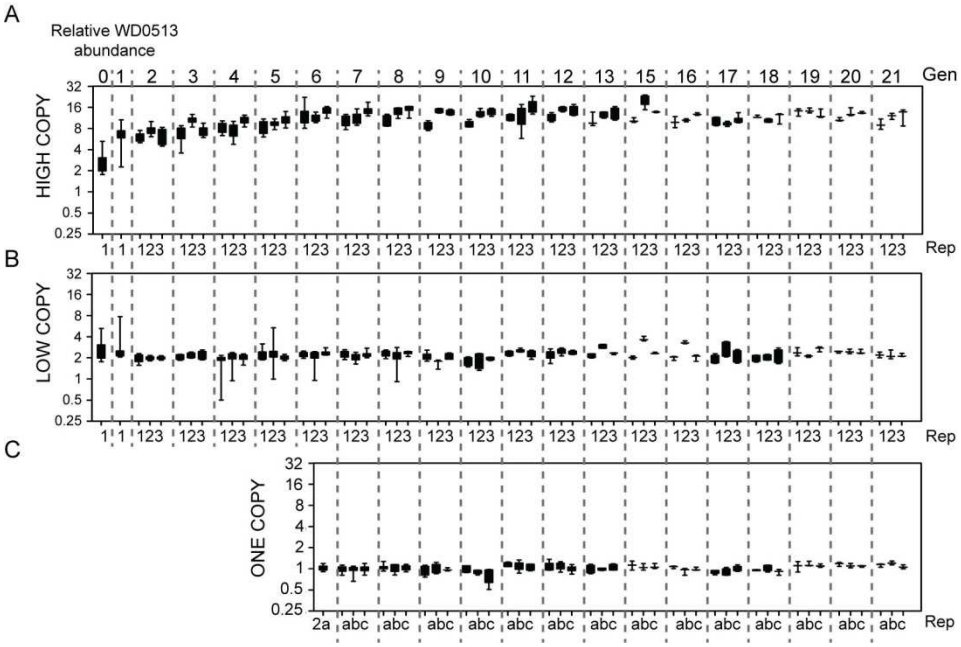

Figure 2. Octomom copy number is heritable and can be selected.
Selection for high (A), low (B) and one (C) WD0513 copy number *w*MelPop in w^{1118} flies. Selection was started with females coming from one vial (Generation zero). The female with the highest (A) or lowest (B) WD0513 abundance was always the founder of the next generation. At generation two both selection regimes were split into three replicate lines. At generation six we derived one copy line from the low copy selection line two that was subsequently split into three lines kept independently (C). From that point on, the low copy regime was maintained at two Octomom copies. The boxes extend from the 25th to 75th percentiles and whiskers include all the values. Dashed lines separate generations. Gen = generation, Rep = replicate.

Octomom copy number is heritable: high copy *Drosophila* mothers produce mostly high copy offspring while the inverse is observed for low copy mothers. In the course of selection for low Octomom copy number in w^{1118} background we recovered a *w*MelPop line with only a single copy of Octomom (Figure 2C). Therefore, from generation six onwards we

maintained three selection regimes: high, two and one Octomom copy number. The *w*MelPop unique synonymous SNP is present in all three selection lines, including the line with a single Octomom copy (Figure S4).

Taking advantage of the different selection lines we compared the phenotypes of *w*MelPop flies with different Octomom copy numbers. To perform these assays we used the progeny of females individually tested for Octomom copy number (Tables S2 and S3). As *Wolbachia* *w*MelCS_b was associated with *iso* fly genetic background and one Octomom copy line appeared only in w^{1118} background to directly compare the two we used hybrids between iso and w^{1118}. This way all flies had the same genetic background heterozygous between *iso* and w^{1118}. Two high copy *w*MelPop lines, each in different genetic background, were used to control for the maternal effects. We verified that our control works and there is no difference between the two high copy lines. We predicted that the higher the copy number the more severe the pathogenic phenotype and that the one Octomom copy line would be phenotypically identical to *w*MelCS_b. Survival data demonstrate that differences in Octomom copy number lead to differences in host longevity: the more Octomom copies, the earlier the flies die (Figures 3A and S5A-G). The line with one Octomom copy derived from *w*MelPop is indistinguishable from *w*MelCS_b and *Wolbachia*-free control (Figures 3A and S5E-G).

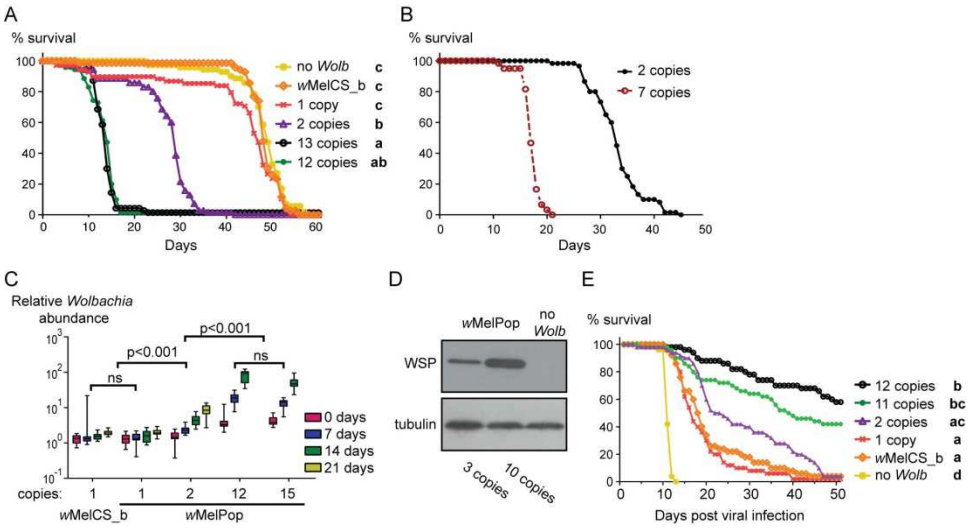

Figure 3. Octomom amplification determines *w*MelPop phenotypes.

(A) Lifespan of females with different *w*MelPop Octomom copy numbers, *w*MelCS_b and *Wolbachia*-free controls at 29 °C. Seventy females per line were analysed, flies are the progeny from crosses between *iso* and w^{1118} lines. Letters indicate groups of significantly different survival curves by Tukey's test of all pairwise comparisons of Cox hazard ratios. (B) Lifespan of females from the forward selection *iso* low copy line two (two Octomom copies) and matched reverse selection line (seven copies) at 25 °C. Mixed effects Cox model fit, $p<0.001$.

(C) Time-course of *Wolbachia* densities in females with different *w*MelPop Octomom copy numbers, starting at eclosion (day zero). Each bar represents *wsp* genomic levels in 16-20 single females (progeny from crosses between *iso* and w^{1118} lines). The boxes extend from the 25th to 75th percentiles and whiskers include all the values. Values are normalized to median of samples of *w*MelCS_b at day zero. Statistical analysis was performed using log-linear model and the *P-values* refer to comparisons of slopes (ns – non significant).

(D) Western blot with anti-WSP antibody of pools of ten 10 days old *iso* females with three or ten Octomom copies. *Drosophila* tubulin was used as a loading control. (E) Survival of females with different *w*MelPop Octomom copy numbers upon viral infection at 18 °C. Continued on the next page.

Figure 3. (continued) Fifty females per line were analysed; flies are the progeny from crosses between *iso* and w^{1118} lines. Letters indicate groups of significantly different survival curves by Tukey's test of all pairwise comparisons of Cox hazard ratios.

Even a single duplication of this region is enough to significantly shorten the host lifespan (median time to death is reduced by 39%) (Figures 3A and S5E-G). To further test the dependence of the phenotype on Octomom copy number we reversed the direction of the selection in selected *iso* lines (choosing the highest Octomom copy number females from the low copy lines and the lowest from the high copy lines, from generation 17 onwards) (Figure S6A), simultaneously maintaining the forward selection regime as controls (Figure S3). Comparison of the lifespan of females from forward and reverse selections confirmed that Octomom copy number determines *w*MelPop pathogenicity (Figures 3B S6B-D). Overall, Octomom copy number negatively correlates with longevity (Figure S7) and by manipulating it we can control *Wolbachia* virulence.

We next asked if *Wolbachia* growth is associated with Octomom copy number. We tested *Wolbachia* levels in flies with different Octomom copy numbers over time by real time quantitative PCR (Figure 3C). The higher the Octomom copy number the higher the density of *Wolbachia*. The levels differ at eclosion and the growth of *Wolbachia* is faster in flies with higher Octomom copy number. Both high copy lines have the same growth rates, which are higher than the growth rate of the two copies line. This in turn is higher than the one copy *w*MelPop and *w*MelCS_b, which have the same *Wolbachia* growth rates (Figure 3C). We confirmed this Octomom copy number effect on *Wolbachia* densities by comparing WSP *Wolbachia* protein abundance between flies harbouring *w*MelPop

with three and ten Octomom copies (Figure 3D). The flies with ten copies had more WSP protein than the flies with three Octomom copies.

The density of *Wolbachia* is known to be related with *Wolbachia*-conferred antiviral protection and *w*MelPop provides very strong protection (Osborne *et al.*, 2009, 2012; Frentiu *et al.*, 2010; Lu *et al.*, 2012; Chrostek *et al.*, 2013, 2014). The survival of virus infected flies confirmed that the higher the Octomom copy number, the stronger the antiviral protection (Figures 3E and S5H). As with pathogenicity and growth rate, one Octomom copy *w*MelPop is phenotypically identical to *w*MelCS_b in terms of antiviral protection.

We showed that *w*MelPop *Wolbachia* is genetically and, consequently, phenotypically unstable. We also observed that releasing our lines from the selection regime and maintaining them at 25 °C in crowded vials for five generations caused a decrease in the copy number of three out of four lines tested (Figure S8). The only line where the copy number did not change over the five generations started with two Octomom copies. Also, examination of another *w*MelPop stock originating from the Min and Benzer laboratory (Min and Benzer, 1997) did not show the expected life-shortening phenotypes and, accordingly, Octomom amplification (Figure S9A,B). Presumably, Octomom copy number reverted to one copy and the phenotype was lost in this stock.

Octomom amplification could promote *w*MelPop virulence in several ways. The most parsimonious explanation is, however, that Octomom genes are overexpressed and these cause the phenotype. To test that we checked the expression of Octomom genes, immediately adjacent genes and genes distant from the region by reverse transcription real-time qPCR. All Octomom genes were expressed to the higher extent in *w*MelPop than in *w*MelCS, but immediately adjacent genes were not misregulated (Figure S10).

Discussion

Our results identify the genetic basis of *Wolbachia* *w*MelPop virulence. By selecting for *Wolbachia* with different Octomom copy numbers we show a functional link between copy number and *w*MelPop phenotypes. The more copies of Octomom, the higher the densities of *Wolbachia*, the faster the hosts die, but the stronger the antiviral protection. Furthermore, all these phenotypes are reverted in the *w*MelPop line selected for one Octomom copy, establishing unequivocally that Octomom copy number drives these phenotypes. There are several lines of evidence that *Wolbachia* levels determine the strength of the *Wolbachia*-associated phenotypes (Osborne *et al.*, 2009, 2012; Frentiu *et al.*, 2010; Lu *et al.*, 2012; Chrostek *et al.*, 2013, 2014). Therefore, different replication capacity of *w*MelPop with distinct Octomom copy numbers is the likely cause of the differences in the other phenotypes.

Identification of the virulence determinant of *w*MelPop has been crucial due to *w*MelPop potential as vector borne disease control agent. *w*MelPop transinfected into arboviral vectors, like malaria-transmitting *Anopheles gambiae* (Jin *et al.*, 2009) or dengue vector *Aedes albopictus* (Suh *et al.*, 2009), would hopefully limit the spread of human pathogens. However, the unstable nature of the *w*MelPop pathogenicity should be taken into consideration while planning field interventions using *w*MelPop transinfected mosquitoes.

Interesting example of Octomom region evolution was recently observed by Woolfit and colleagues. They also identified Octomom amplification in the *D. melanogaster* *w*MelPop genome (Woolfit *et al.*, 2013) and a deletion of Octomom region in *w*MelPop mosquito adapted variant, *w*MelPop-PGYP. As *w*MelPop-PGYP retained a strong life-shortening effect in *Aedes aegypti*, while an *A. aegypti*-adapted *w*Mel variant was benign, the authors dismissed Octomom as responsible for the high

virulence of *w*MelPop also in *D. melanogaster*. We argue that the difference between *w*MelPop-PGYP and *w*Mel phenotypes in mosquitoes may be due to other genetic changes during their adaptation to a new host, some already described for *w*MelPop-PGYP (Woolfit *et al.*, 2013). Alternatively, it may be because these two variants belong to the two monophyletic groups of *Wolbachia* from *D. melanogaster: w*Mel group and *w*MelCS group (Richardson *et al.*, 2012; Chrostek *et al.*, 2013). *w*MelCS-like variants have been described as replicating faster than *w*Mel-like variants and sometimes shortening host lifespan (Chrostek *et al.*, 2013) and this difference may be exacerbated in mosquitoes.

Amplification of Octomom is in agreement with the common gene amplification by non-equal recombination in bacteria (Andersson and Hughes, 2009): i) it is flanked by direct repeats (see (Woolfit *et al.*, 2013; Chrostek *et al.*, 2013)), ii) it seems to amplify as a unit since different Octomom genes are equally amplified in the same fly (Figure 1B), iii) we confirmed the predicted novel joint point (Figure 1C and S2), and iv) the amplification is unstable. As Octomom genes are overexpressed and may cause the phenotype, functional analysis of Octomom-encoded proteins is required to better understand the *Wolbachia*-host interaction. These genes can either act on bacterial cell division or be responsible for the attenuation of the host's control over the symbiont (see discussion in (Chrostek *et al.*, 2013)). Interestingly, this region is a part of *Wolbachia* accessory genome since it is not present in all *Wolbachia* strains and shows signs of horizontal gene transfer (see (Iturbe-Ormaetxe *et al.*, 2005; Korochkina *et al.*, 2006; Klasson, Kambris, *et al.*, 2009; Woolfit *et al.*, 2009; Chrostek *et al.*, 2013)).

Vertically transmitted endosymbionts are subjected to different levels of selection. An increase in replication may confer a fitness advantage to the bacteria in intra-host competition but a disadvantage at the inter-hosts level, as it can have a high cost to the host and reduce symbiont

transmission. *w*MelPop was most probably isolated due to husbandry conditions buffering the cost of harbouring pathogenic bacteria and low population numbers increasing drift. Our results demonstrate that a single mutation (a duplication) can profoundly alter endosymbiont replication. Moreover, the degree of amplification and associated strength of the phenotypes can rapidly change and be fully reversible. Nucleotide mutation rate in *w*Mel is low (Richardson *et al.*, 2012) but repetitive sequences are frequent in the genome (Wu *et al.*, 2004), therefore gene amplification may be a common mechanism favouring rapid evolution (see also (Andersson and Hughes, 2009; Elde *et al.*, 2012)). Accordingly, gene amplifications in other *w*Mel variants (Chrostek *et al.*, 2013) and other *Wolbachia* strains (Klasson *et al.*, 2008; Klasson, Westberg, *et al.*, 2009) have previously been reported, although without any associated phenotypes. This conversion of a mutualist into a pathogen by a single genomic event suggests that virulent mutations in microbial symbionts may be frequent and constantly counter-selected. Therefore, symbiont titres may be at a labile equilibrium achieved in the course of co-evolution and to a large extent selected at the level of the symbiont.

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Chapter 4 - High anti-viral protection without immune upregulation after interspecies *Wolbachia* transfer

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Author contributions: As stated in author contributions in the reference above I participated in all stages of the preparation of this manuscript.
In particular, I performed all the survival experiments presented in this chapter, virus levels, *Wolbachia* growth and AMPs quantifications. Marta Marialva provided preliminary data on *w*Au survival upon virus infection used as replicates of the experiments analysed here and pricked the flies for flock house virus quantification. Ryuichi Yamada and Scott L. O'Neill provided the *D. melanogaster* stock transinfected with *w*Au. Together with Luis Teixeira we analysed the data and wrote the manuscript.

Summary

Wolbachia, endosymbionts that reside naturally in up to 40-70% of all insect species, are some of the most prevalent intracellular bacteria. Both *Wolbachia w*Au, naturally associated with *Drosophila simulans*, and *w*Mel, native to *Drosophila melanogaster*, have been previously described to protect their hosts against viral infections. *w*Mel transferred to *D. simulans* was also shown to have a strong antiviral effect. Here we directly compare one of the most protective *w*Mel variants and *w*Au in *D. melanogaster* in the same host genetic background. We conclude that *w*Au protects better against viral infections, it grows exponentially and significantly shortens the lifespan of *D. melanogaster*. However, there is no difference between *w*Mel and *w*Au in the expression of selected antimicrobial peptides. Therefore, neither the difference in anti-viral effect nor the life-shortening could be attributed to the immune stimulation by exogenous *Wolbachia.* Overall, we prove that stable transinfection with a highly protective *Wolbachia* is not necessarily associated with general immune activation.

Introduction

Wolbachia, intracellular bacteria inhabiting up to 40-70% of known insect species (Hilgenboecker *et al.*, 2008; Zug and Hammerstein, 2012), have been initially described as powerful manipulators of arthropods reproduction (Werren *et al.*, 2008). *Wolbachia* are maternally transmitted and, in some hosts, provide infected females with a relative fitness advantage by cytoplasmic incompatibility, male killing or other forms of reproductive manipulation. Recently, Wolbachia have been attracting widespread attention due to their ability to protect their hosts against viral infections. This phenomenon has been initially reported in *Drosophila melanogaster* carrying its natural *w*Mel *Wolbachia* strain (Hedges *et al.*, 2008; Teixeira *et al.*, 2008). Interestingly, antiviral protection was the first phenotype of *Wolbachia* discovered in *D. melanogaster* that could explain high prevalence of the symbiont in natural populations of fruit flies (Brun and Plus, 1978; Hoffmann *et al.*, 1994; Solignac *et al.*, 1994; Johnson and Christian, 1999; Ilinsky and Zakharov, 2007; Kapun *et al.*, 2010; Verspoor and Haddrill, 2011; Fenton *et al.*, 2011).

The ubiquity of *D. melanogaster* in research has placed *w*Mel *Wolbachia* strain among the most extensively studied insect symbionts. Based on the molecular markers it has been shown that *w*Mel strain consists of five polymorphic variants, namely: *w*Mel, *w*Mel2, *w*Mel3, *w*MelCS and *w*MelCS2 (Riegler *et al.*, 2005). Our previous work (Chrostek *et al.*, 2013) has placed these variants in the context of a recent *w*Mel phylogenetic analysis (Richardson *et al.*, 2012) and shown that they cluster into two monophyletic groups: *w*Mel-like and *w*MelCS-like. The *w*MelCS-like variants reach higher densities in the host and provide more antiviral protection than the *w*Mel-like variants. Moreover, some *w*MelCS-like variants shorten the lifespan of their hosts (Chrostek *et al.*, 2013),

including the extreme example of the pathogenic *w*MelPop (Min and Benzer, 1997).

*w*Au is a *Wolbachia* native to *D. simulans* that used to be present at low frequencies in Australia and does not induce cytoplasmic incompatibility [18,19]. Based on the analyses employing molecular markers different authors concluded that *w*Mel of *D. melanogaster* and *w*Au of *D. simulans* are closely related and both belong to the *Wolbachia* supergroup A (Zhou *et al.*, 1998; Charlat *et al.*, 2004; Iturbe-Ormaetxe *et al.*, 2005; Baldo *et al.*, 2006; Paraskevopoulos *et al.*, 2006).

*w*Au and one of the most protective *Wolbachia* *w*Mel variants - *w*MelCS_b, the two strains used in this study, have been previously described as protective against *Drosophila* C virus (DCV) and flock house virus (FHV) (Hedges *et al.*, 2008; Teixeira *et al.*, 2008; Osborne *et al.*, 2009, 2012). Moreover, *w*Mel has been previously transferred from *D. melanogaster* to *D. simulans* (Poinsot *et al.*, 1998) and protection in this new *Drosophila*-host association was similar to the protection provided by *w*Au in its natural host (Osborne *et al.*, 2009). However, different *Wolbachia* lines were studied in different *D. simulans* genetic backgrounds, preventing direct comparison of the protective abilities of *w*Au and *w*Mel.

This study compares the antiviral protection and other phenotypes provided by *w*MelCS_b and *w*Au in genetically identical *D. melanogaster* hosts. In mosquitoes recently transinfected with *Wolbachia* the antiviral effect is frequently associated with activation of the host immune system (Kambris *et al.*, 2009, 2010; Moreira *et al.*, 2009; Bian *et al.*, 2010; Blagrove *et al.*, 2012; Pan *et al.*, 2012; Rancès *et al.*, 2012), while in natural co-evolved *D. melanogaster* – *Wolbachia* associations antiviral protection is strong but expression of immune genes remains unchanged (Bourtzis *et al.*, 2000; Wong *et al.*, 2011; Rancès *et al.*, 2012; Teixeira,

2012). Therefore we also evaluated general activation of the fly immune system by *w*MelCS_b and *w*Au transinfected to *D. melanogaster*.

Materials and Methods

The data for iso and *w*MelCS_b in the Figures 1D, 1F, 2C are already published in Chrostek *et al.* 2013. All the remaining data, all statistical analysis and all conclusions are original.

Fly strains and husbandry

D. melanogaster with *w*MelCS_b, DrosDel w^{1118} *isogenic* flies and the matching controls without *Wolbachia* were described before (Ryder *et al.*, 2004; Teixeira *et al.*, 2008). *D. melanogaster* with *w*Au from *D. simulans* Coffes Harbour (CO) was described before (Yamada *et al.*, 2011). The 1st and 3rd chromosome of the *D. melanogaster* stock with *w*Au were replaced with DrosDel w^{1118} *isogenic* chromosomes using a first and third double balancer line. Next, a second chromosome balancer line was used to replace the 2nd chromosome. As both *Wolbachia* and mitochondria are maternally transmitted the *w*Au, *w*MelCS_b and *Wolbachia*-free iso control lines may have different mitochondria, despite having the same nuclear genetic background. Cleaning the stocks of possible chronic viral infection and gut flora homogenization were performed as in (Teixeira *et al.*, 2008; Chrostek *et al.*, 2013). *Drosophila* were maintained at a constant temperature of 25 °C on standard cornmeal diet. All the experiments were performed on 3-6 days old male flies.

Long-term survival analysis

The lifespan of different fly lines was tested at 25 °C, with 10 flies per vial, and analysed using Cox hazard models as previously reported (Chrostek *et al.*, 2013) with the coxme package in R (Team, 2010). We considered genotype and repeat of the experiment fixed and replicate vials within the same experiment random.

Virus production and infection

Viruses were produced, titrated and used to infect flies as before (Teixeira *et al.*, 2008; Chrostek *et al.*, 2013). Infections were performed on 3-6 days old flies. After the infections 10 flies per vial were kept on food without live yeast at 18 °C for DCV or at 25 °C for FHV. Survival was monitored daily and vials were changed every 5 days. Statistical analysis was performed the same way as for long-term survival data.

Nucleic acids extractions and real-time qPCR

DNA for the quantification of *Wolbachia* was extracted using standard phenol-chlorophorm protocol. RNA for assessment of viral titres and gene expression was extracted using Trizol (Invitrogen) with an additional DNAse treatment (Promega) of the AMPs RNA samples prior to cDNA synthesis. cDNA was prepared as described previously (Chrostek *et al.*, 2013). Real-time qPCR reactions were carried out in 7900HT Fast Real-Time PCR System (Applied Biosystems) with the iQ™ SYBR® Green supermix (Bio Rad). Each plate contained three technical replicates of every sample for each set of primers. Primers for *Wolbachia*, DCV and FHV were previously described (Chrostek *et al.*, 2013), while primers for AMPs are listed in Table 1.

Table 1. Primers used to detect AMPs in real-time quantitative PCR experiments.

Target	Forward primer sequence (5'-3')	Reverse primer sequence (5'-3')
Defensin	TATCGCTTTTGCTCTGCTTG	TGTGGTTCCAGTTCCACTTG
Diptericin	ACCGCAGTACCCACTCAATC	CCATATGGTCCTCCCAAGTG
Cecropin A1	CATCAGTCGCTCAGACCTCAC	TTCTTCAGCCACCCAGCTTC
Drosomycin	TACCAAGCTCCGTGAGAACC	CAGGGACCCTTGTATCTTCC

For the four antimicrobial peptides the thermal cycling protocol used was: 50°C for 2 min, 10 min at 95°C and 40 cycles of: 95°C for 30 sec, 59°C for 1 min and 72°C for 30 sec. This was followed by the generation of dissociation curve to verify the specificity of the reactions. Data was analysed in R (Team, 2010) using Wilcoxon rank sum test with Holm correction for FHV levels, DCV levels at each time point and AMPs levels. The increase of *Wolbachia* variants titre over time was analysed using a linear model (lm) in R (Team, 2010).

Results and discussion

*w*Au provides stronger antiviral protection than *w*MelCS_b in *D. melanogaster*

It was previously shown that *w*Au provides strong protection against viruses in its native *D. simulans* host (Osborne *et al.*, 2009). We have discovered that among *Wolbachia* endosymbionts of *Drosophila melanogaster* *w*MelCS_b is one of the most potent in viral interference (Chrostek *et al.*, 2013). In order to directly compare these two strains in *Drosophila melanogaster,* we used *Wolbachia*-infected lines in a genetically identical DrosDel w^{1118} isogenic background (Ryder *et al.*, 2004). *w*MelCS_b was naturally associated with this background while *w*Au was introduced from *D. simulans* to *D. melanogaster* (Yamada *et al.*, 2011) and subsequently placed in this background by chromosome replacement using balancers. A *Wolbachia*-free line, designated "*iso*", was used as a control in all experiments. All flies were virus-free and had homogenized gut microbiota (see Chrostek *et al.*, 2013).

To compare antiviral properties of *w*MelCS_b and *w*Au, we challenged the flies carrying the respective *Wolbachia* strains and *iso* controls with two viruses: DCV (Figure 1A), a natural pathogen of *Drosophila,* and FHV (Figure 1B), initially isolated from a coleopteran host, but now widely used in studies on dipteran immune response. We observed that *w*Au significantly prolongs the survival of the infected flies in comparison with both *iso* and *w*MelCS_b carrying flies (Figure 1A, S1A, 1B and S1B; Tukey's test on the mixed effects Cox model fit, *w*Au versus both, *w*MelCS_b and *iso*, for DCV: $p<0.001$; for FHV: $p<0.001$). This effect is almost completely abolished in tetracycline-treated flies derived from *Wolbachia*-positive stocks (Figures 1C, S1C, 1D and S1D; DCV infected *w*Au tet *vs iso* tet, $p=0.0774$ and *w*Au tet *vs* *w*MelCS_b tet, $p=0.0161$; FHV infected *w*Au tet *vs iso* tet, $p= 0.1147$ and *w*Au tet *vs* *w*MelCS_b tet,

p=0.8881). The difference between *w*Au tet and *w*MelCS_b tet is very small in the case of DCV infection (compare Figures S1A and S1C).

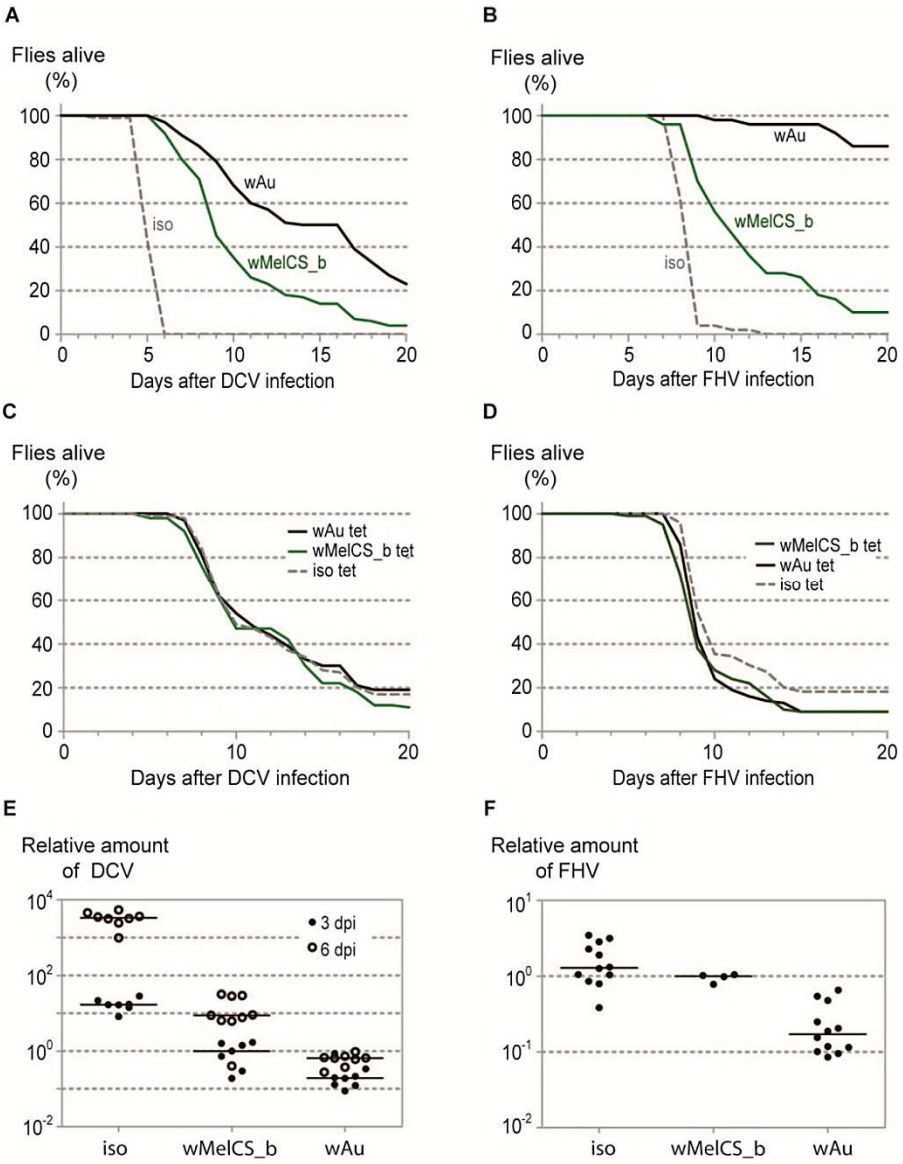

Figure 1. *w*Au provides more antiviral resistance than *w*MelCS_b in *D. melanogaster.*

Continued on the next page.

Figure 1. (continued) (A) One hundred *Wolbachia*-free *iso*, *w*Au and *w*MelCS_b infected male flies were pricked with DCV (10^9 $TCID_{50}$/ml) and survival was followed daily. This experiment was repeated twice and statistical analysis was performed on the data from all 3 repetitions (Figure S1A).
(B) Fifty *Wolbachia*-free *iso*, *w*Au and *w*MelCS_b infected male flies were pricked with FHV (10^8 $TCID_{50}$/ml) and survival was followed daily. This experiment was repeated and statistical analysis was performed on the data from both repetitions (Figure S1B).
(C) One hundred males from *w*Au, *w*MelCS_b and *iso* tetracycline-treated lines were pricked with DCV (10^7 $TCID_{50}$/ml) and survival was followed daily. This experiment was repeated and statistical analysis was performed on the data from both repetitions (Figure S1C).
(D) One hundred males from *w*Au, *w*MelCS_b and *iso* tetracycline-treated stocks were pricked with FHV (10^8 $TCID_{50}$/ml) and survival was followed daily. For data analysis see Figure S1D.
(E) *Wolbachia*-free *iso*, *w*Au and *w*MelCS_b carrying male flies were pricked with DCV (10^9 $TCID_{50}$/ml) and collected 3 and 6 days later for RNA extraction and RT-qPCR. Relative amounts of DCV were calculated using host Rpl32 mRNA as a reference and presented values are relative to median of *w*MelCS_b samples 3 dpi. Each point represents a replicate (ten males per replicate, 8 replicates per *Drosophila* line per time point), and lines are medians of the replicates. DCV loads are significantly different between the lines with *w*Au and *w*MelCS_b both 3 dpi (pairwise Wilcoxon rank sum test, p=0.03) and 6 dpi (pairwise Wilcoxon rank sum test, p<0.001).
(F) *Wolbachia*-free *iso*, *w*Au and *w*MelCS_b carrying male flies were pricked with FHV (10^9 $TCID_{50}$/ml) and collected 3 days post infection for RNA extraction and RT-qPCR. Relative amount of virus were calculated using host Rpl32 mRNA as a reference and presented values are relative to median of *w*MelCS_b samples. Each point represents a replicate (ten males per replicate), and lines are medians of the replicates. FHV loads are significantly lower in flies with *w*Au comparing to flies with *w*MelCS_b (pairwise Wilcoxon rank sum test, p=0.003).

The strong inhibition of virus-induced mortality in *w*Au carrying flies could be either due to the direct reduction of pathogen load (resistance) or due to neutralization of negative impact of the pathogen on the fly's health without direct influence on the virus titres (tolerance or resilience). To distinguish between these two possibilities we tested the levels of each virus in whole flies either 3 and 6 days post infection (dpi) for DCV or 3 dpi for FHV (Figures 1E and 1F). Consistent with previous reports both *Wolbachia* strains reduce the DCV load. However, this effect is much stronger for *w*Au, which is approximately 4.5 times more efficient 3 dpi (pairwise Wilcoxon rank sum test, p=0.03) and over 13 times more efficient 6 dpi (pairwise Wilcoxon rank sum test, p<0.001) in reducing the DCV titres than *w*MelCS_b. Flies carrying *w*Au have also 5.8 times less FHV 3 days after infection in comparison with *w*MelCS_b (pairwise Wilcoxon rank sum test, p=0.003). All these data allow us to conclude that *w*Au protects better against viral infections than one of the most protective *w*Mel variants and this can be, at least partially, explained by the reduction of the viral titres.

*w*Au reduces the lifespan of *D. melanogaster* and grows exponentially

We have previously reported the cost of antiviral protection in terms of reduced longevity for some *w*MelCS-like *Wolbachia* variants (Chrostek *et al.*, 2013). Here we have also tested the longevity of the *Wolbachia* infected flies in the absence of viral challenge (Figure 2A).

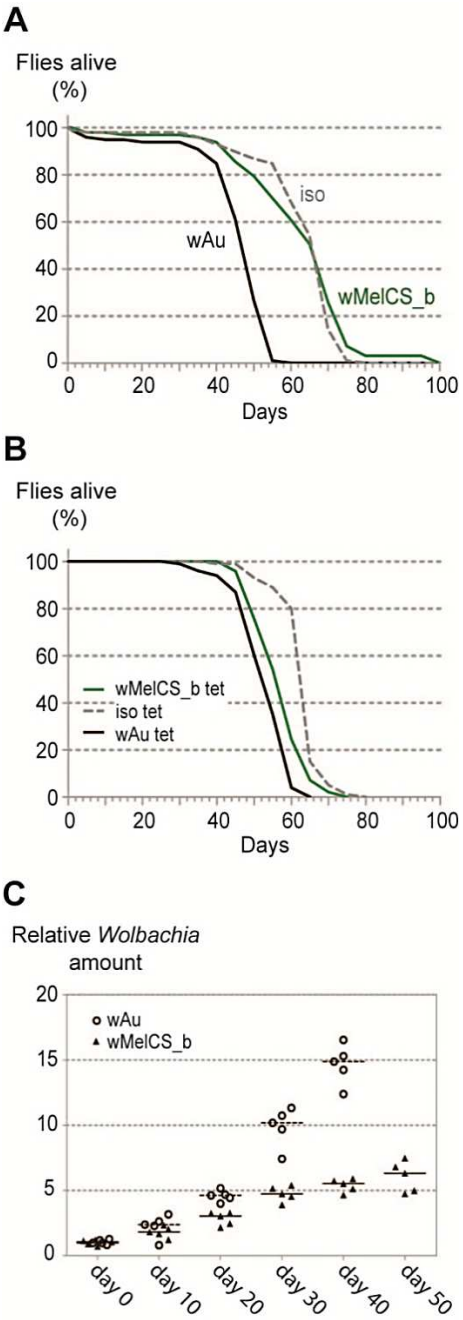

Figure 2. *w*Au shortens the lifespan of the flies and grows exponentially within the hosts.

(A) The survival of one hundred *Wolbachia*-free *iso*, *w*Au and *w*MelCS_b carrying male flies was checked every five days. The experiment was repeated once with comparable results and analysis was performed on both repetitions (Figure S1E).

(B) The survival of one hundred males derived from *iso*, *w*Au and *w*MelCS_b tetracycline-treated stocks was checked every five days. The experiment was repeated once with comparable results and analysis was performed on both repetitions (Figure S1F).

(C) qPCR on DNA isolated from males of *w*MelCS_b and *w*Au lines, collected every 10 days. Day 0 corresponds to 3-6 days old flies, after day 40 the *w*Au carrying flies were not collected due to the high mortality. Each point represents a sample (each sample consisted of ten males), and lines are medians of the samples. Relative amount of *Wolbachia* genomic DNA was calculated using host Rpl32 as a reference gene and all values are relative to median of samples of *w*MelCS_b at day 0.

We observed that *w*Au shortens the lifespan of flies by 20 days (31% difference in median time to death) in comparison with *w*MelCS_b

(Figure 2A, S1E; Tukey's test on the mixed effects Cox model fit, *w*Au versus *w*MelCS_b and *iso*, $p<0.001$) demonstrating that harbouring this protective endosymbiont is associated with a cost in the absence of infection. After elimination of *Wolbachia* from our fly stocks the flies derived from the *w*Au line also live shorter, but there is only a 5 days difference (9% in median time to death) between them and *w*MelCS_b derived flies (Figure 2B). Despite being smaller, this effect is also significant (Figure S1F; Tukey's test on the mixed effects Cox model fit, *w*Au tet versus *w*MelCS_b tet and *iso* tet, $p<0.001$). This difference and the one we observed for the DCV-infected tetracycline treated *w*Au and *w*MelCS_b lines may be due to differences in mitochondria between *w*Au and *w*MelCS_b fly stocks (see James and Ballard, 2003; Clancy, 2008) or to a mutation in the nuclear genetic background that could have arisen since the lines were separated. Given these results we cannot completely rule out an interaction between these possible mitochondrial or nuclear variation and *Wolbachia* as the cause of the differential phenotypes seen in the presence of *Wolbachia.*

The association between *Wolbachia* densities and the strength of antiviral-protection is well established. Various experimental approaches, i.e. treatment of *Wolbachia*-infected flies with increasing antibiotic concentrations or examining natural variation in endosymbiont density, have shown that the higher the *Wolbachia* density, the stronger the antiviral protection (Osborne *et al.*, 2009, 2012; Frentiu *et al.*, 2010; Lu *et al.*, 2012; Chrostek *et al.*, 2013). In order to assess if *w*Au titres were also higher than *w*MelCS_b titres, we tested the densities of these symbionts throughout their host's lifespan (Figure 2C). We observed that the *Wolbachia* densities at adult emergence are the same for both strains (log-linear model, intercept difference: 0.165027, $p=0.352$), but *w*Au grows much faster than *w*MelCS_b (slope difference between *w*Au and *w*MelCS_b: 0.046097, $p<0.001$). The exponential growth of the symbiont

may be the cause of the life-shortening, either by direct tissue damage or by constituting a significant metabolic burden compromising the insect's health. This is reminiscent of host life-shortening by the exponentially growing wMelPop strain (Min and Benzer, 1997; McGraw *et al.*, 2002; Chrostek *et al.*, 2013).

*w*Au does not stimulate *D. melanogaster* immune system despite recent transfer from *D. simulans*

Immune upregulation has been shown to occur after transfer of *Wolbachia* into a new insect species (Kambris *et al.*, 2009, 2010; Moreira *et al.*, 2009; Bian *et al.*, 2010; Blagrove *et al.*, 2012; Pan *et al.*, 2012; Rancès *et al.*, 2012). Stimulation of the insect immune system by *Wolbachia* is one of the proposed mechanisms explaining *Wolbachia*-mediated antiviral protection in mosquitoes (Kambris *et al.*, 2009, 2010; Moreira *et al.*, 2009; Pan *et al.*, 2012). On the other hand, chronic immune activation was also proven to be responsible for lifespan reduction in *Drosophila melanogaster* (Libert *et al.*, 2006).

To test if chronic immune activation could be responsible for the high antiviral protection and life-shortening by *w*Au we examined the expression of genes encoding antimicrobial peptides (AMPs). We chose AMPs that were previously shown to be highly induced by the presence of exogenous *Wolbachia* (Kambris *et al.*, 2009, 2010; Moreira *et al.*, 2009; Bian *et al.*, 2010; Blagrove *et al.*, 2012; Pan *et al.*, 2012; Rancès *et al.*, 2012), and that represent targets of the two main *Drosophila* immune pathways: Toll and Imd (Figure 3).

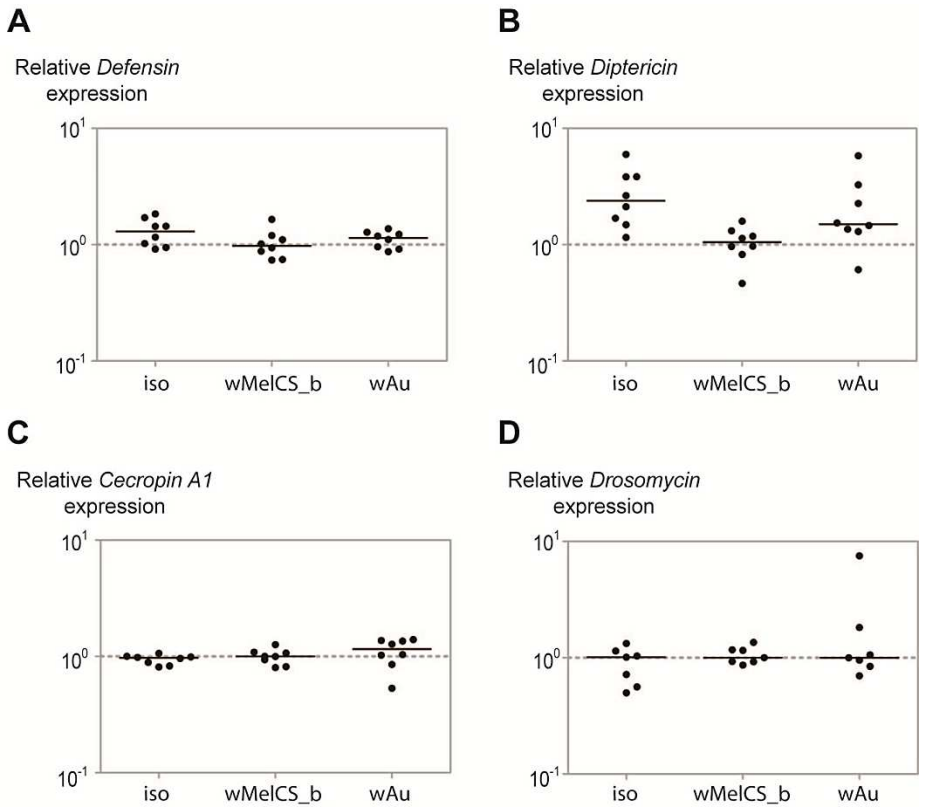

Figure 3. Expression of antimicrobial peptide genes in *Wolbachia*-free *iso*, *w*Au and *w*MelCS_b harbouring flies.

qRT-PCR on the RNA collected from 3-6 days old whole flies performed with the primers specific for *Defensin* (A), *Diptericin* (B) *Cecropin A1* (C) and *Drosomycin* (D). Relative expression of the host antimicrobial peptide genes was calculated using host Rpl32 as a reference. Values are relative to median of samples of *w*MelCS_b. The only statistically significant difference is in *Diptericin* gene expression between *iso* and *w*MelCS_b (pairwise Wilcoxon rank sum test, p=0.006).

Quantitative RT-PCR showed that there is no difference between *w*Mel, *w*Au and *iso* in the expression of *Defensin*, *Cecropin A1* and *Drosomycin* (Figure 3). There is also no significant difference between *w*MelCS_b and *w*Au in the expression of *Diptericin*. The lack of an induction of these

AMPs by *w*Au indicates that the Toll and Imd pathways are not activated in transinfected *Drosophila melanogaster*. As the expression of the four AMPs is the same in the *w*Au and the *w*MelCS_b infected flies, we could not attribute either the difference in antiviral effect or the lifespan-shortening to the immune activation by exogenous *Wolbachia.* The only statistically significant difference emerging from our analysis was in *Diptericin* gene expression between *iso* and *w*MelCS_b (p=0.006). However, this effect was not observed in the previous studies (Bourtzis *et al.*, 2000; Wong *et al.*, 2011; Rancès *et al.*, 2012; Teixeira, 2012) and the three other AMPs are not regulated by the presence of *w*MelCS_b.

Our findings add to previous reports on high AMPs expression not only after *Drosophila* - mosquitoes transfers (Kambris *et al.*, 2009, 2010; Moreira *et al.*, 2009; Bian *et al.*, 2010; Blagrove *et al.*, 2012; Pan *et al.*, 2012; Rancès *et al.*, 2012) but also on *Wolbachia* transferred within the same genus, i.e. *w*AlbB from *A. albopictus* to *A. aegyptii* (Bian *et al.*, 2010). The contrast between the effects of these transfers on immunity and lack of immune activation by *w*Au transferred to *D. melanogaster* could be explained in various ways. The first possible explanation may be the phylogenetic distances between the source and target host insect species; the most recent common ancestor of *A. albopictus* and *A. aegyptii* dates to 34–42 million years ago (Crochu *et al.*, 2004), while *D. melanogaster* and *D. simulans* diverged only 2.3 million years ago (Russo *et al.*, 1995). Therefore, *w*Au could be better pre-adapted to infect *D. melanogaster* inconspicuously. Another explanation is that *D. melanogaster* has co-evolved with *Wolbachia* while *A. aegyptii* natural populations are not infected with this endosymbiont. Thus, *D. melanogaster* may have evolved not to respond to *Wolbachia* infection. This may also explain why *A. albopictus* has a provisional or no immune response to *Wolbachia* somatic transient infection (Blagrove *et al.*, 2012).

Finally, *w*Au and *w*Mel might be so similar that the insect's immune system does not perceive *w*Au as foreign.

It would be interesting to know which genetic differences between the closely related *w*Au and *w*MelCS explain the different phenotypes. *w*Au genome is not sequenced, however, several differences between the genome of *w*Au and *w*MelCS are described. *w*Au lacks a 21.86 kb genomic region present in *w*MelCS, named Octomom, which includes genes from WD0506 to WD0518 (Iturbe-Ormaetxe *et al.*, 2005; Chrostek *et al.*, 2013). This fragment contains genes with domains homologous to eukaryotic proteins (putative *Wolbachia* effector proteins) and many proteins possibly involved in DNA repair and processing. The amplification of this region has been recently proposed to be responsible for the over-replicative phenotype of *w*MelPop *Wolbachia* variant (Chrostek *et al.*, 2013), although alternative explanations have been suggested (Woolfit *et al.*, 2013). There are also many other differences in the number or coding sequences of ankyrin repeat genes between *w*Mel strain genomes and *w*Au (Iturbe-Ormaetxe *et al.*, 2005; Siozios *et al.*, 2013) (see also (Chrostek *et al.*, 2013) and (Woolfit *et al.*, 2013) for sequence of *w*MelCS). All the above analyses were based on PCR amplification, gene sequencing and DNA hybridization and only the sequencing of the whole *w*Au genome would allow to complete the comparison.

Our study uses *w*Au and one variant of *Wolbachia* *w*Mel – *w*MelCS_b – in the same *D. melanogaster* genetic background and provides a direct comparison of the protective capabilities of the two strains. We conclude that *w*Au protects better against viral infections – it increases lifespan of virus-infected flies and significantly limits viral replication. Additionally, we have discovered that *w*Au grows exponentially within this host and significantly shortens its lifespan in the absence of viral infection, demonstrating that harbouring this protective endosymbiont is associated

with a fitness cost. Testing the expression of selected antimicrobial peptides showed that there is no difference between *w*Mel and *w*Au. Therefore, we could not attribute either the difference in anti-viral effect or the lifespan-shortening to the immune activation by exogenous *Wolbachia.* Our work provides evidence that interspecies *Wolbachia* transfer is not always associated with general immune up-regulation in the recipient host.

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Chapter 5 - Temperature dependence of *Wolbachia*-conferred antiviral protection

Supplementary Figure S1 is included in the Appendix IV.

Author contributions: The experimental work presented in this chapter was performed by me, except for the dose response survival experiment and infections of flies with different doses of DCV for virus titres and *Wolbachia* densities measurements, which were performed by Marta Marialva. All the data presented here were statistically analysed by Nelson Martins. This work was supervised by Luis Teixeira.

Summary

Defensive symbioses, where animals rely on their microbial partners for protection against natural enemies, are widespread in nature. Like all other organisms, hosts, symbionts and pathogens depend on the surrounding environment. Moreover, environmental factors may have a strong impact on these complex systems.

Endosymbiotic bacteria *Wolbachia* infect an array of insect species and provide some of them with antiviral protection. They are currently being tested in the field as a biological tool for the control of dengue virus. However, the mechanisms of protection and factors influencing it are largely unknown.

Using natural *Drosophila melanogaster* – *Wolbachia* association we tested antiviral protection at different thermal regimes. The protection is only observed at some conditions, while at others we cannot detect differences between flies with and without *Wolbachia*. Temperature before the viral infection is crucial for the protection, while the post-infection temperature determines the infection progression and outcome.

Our work shows that *Wolbachia*-conferred antiviral protection is temperature dependent, and that under certain conditions the protection is almost eliminated. This can lead to future comparative approaches determining the mechanism of *Wolbachia*-conferred antiviral protection. Also, the strong dependence of *Wolbachia*-conferred protection on environmental context should be taken into account by programs deploying *Wolbachia* as an antiviral agent in the field.

Introduction

Temperature is a powerful force shaping life on Earth. It affects all biological processes: enzymatic reactions, strength of molecules binding and membranes permeability. As a consequence, temperature influences development, physiology, behaviour and evolution of organisms and sets limits on their geographical distribution. Symbiotic associations, with their full complexity, also depend on temperature. A variation in the aphids thermal tolerance governed by the genome of *Buchnera*, their obligate symbiont (Dunbar *et al.*, 2007), provides a striking example.

Wolbachia are intracellular maternally-transmitted α-proteobacteria infecting many arthropod species (Hilgenboecker *et al.*, 2008; Zug and Hammerstein, 2012). They adopted an array of lifestyles and can exert a wide range of phenotypes, ranging from reproductive manipulations (Werren et al., 2008), through nutritional provisioning (Hosokawa *et al.*, 2010) to pathogen blocking (Hedges *et al.*, 2008; Teixeira *et al.*, 2008). *Wolbachia*-conferred protection against pathogens has now become one of the most promising approaches to control vector borne diseases (McGraw and O'Neill, 2013). Therefore, recognizing factors that may influence protection in nature has gained prime importance.

Temperature has been shown before to affect *Wolbachia*-insect interactions in many ways. *Wolbachia* densities are regulated by temperature (Mouton *et al.*, 2006, 2007; Bordenstein *et al.*, 2006; Lu, Zhang, *et al.*, 2012), and heat stress seems to reduce or completely eliminate the bacteria (Wiwatanaratanabutr and Kittayapong, 2009; Guruprasad *et al.*, 2011). Cytoplasmic incompatibility, vertical transmission and fitness of *Wolbachia*-associated insects are also temperature dependent (e.g. Hurst *et al.*, 2001; Reynolds *et al.*, 2003; Bordenstein *et al.*, 2006; Jia *et al.*, 2009; Lu, Zhang, *et al.*, 2012), most

probably due to the direct relationship with *Wolbachia* densities. Moreover, environmental temperature was shown to affect prevalence and frequencies of different *Wolbachia* genotypes in the laboratory populations of *D. melanogaster* (Versace *et al.*, 2014). This suggests that geographic distribution of endosymbionts of *D. melanogaster* can be explained by their relative fitness effects at varying thermal conditions (Versace *et al.*, 2014).

Finally, temperature influences *Wolbachia* and *Wolbachia*-induced *Plasmodium* blocking in somatically transinfected *Anopheles stephensi* (Murdock *et al.*, 2014). Even though *Wolbachia* densities were directly and strongly correlated with post-infection temperature, protection was not and *Wolbachia* either blocked, had no effect, or enhanced *Plasmodium* infection (Murdock *et al.*, 2014). The complex, non-linear effects observed by Murdock *et al.* (2014) are still the only report on the role of temperature in *Wolbachia*-induced pathogen blocking.

Here we tested how different assay conditions influence a natural *Wolbachia* – insect – pathogen relationship, in particular protective abilities of *Wolbachia*. We asked how different pre- and post-infection temperatures affect the *Wolbachia*-carrying *Drosophila melanogaster* response to *Drosophila* C virus (DCV). Knowing that *Wolbachia* densities determine the strength of antiviral protection (Osborne *et al.*, 2009, 2012; Frentiu *et al.*, 2010; Lu, Bian, *et al.*, 2012; Chrostek *et al.*, 2013, 2014; Martinez *et al.*, 2014) we also tested how *Wolbachia* endosymbiont of *D. melanogaster* responds to different thermal regimes and to the presence of viral infection.

Materials and Methods

Fly strains and husbandry

DrosDel w^{1118} *isogenic D. melanogaster* with *w*MelCS_b *Wolbachia* (*Wolb*+) and the matching controls without *Wolbachia* (*Wolb*-) were described elsewhere (Ryder *et al.*, 2004; Teixeira *et al.*, 2008; Chrostek *et al.*, 2013). Stocks were maintained at a constant temperature of 25 °C on standard cornmeal diet.

Virus infection experiments

DCV was produced, titrated and used to infect flies as before (Teixeira *et al.*, 2008; Chrostek *et al.*, 2013). Flies for experiments were raised at different temperatures: 12 females and 6 males were placed together in bottles with food for 4 days to produce offspring at either 25 °C, 18 °C or at fluctuating temperature (18 °C to 25 °C gradual increase during 12 h, and 25 °C to 18 °C decrease during the subsequent 12 h). After 10 days (25 °C), 15 days (fluctuating temperature) or 20 days (18 °C) the flies started to eclode. Three days later, 0-3 days old flies were collected from these bottles and placed in the vials, ten males per vial. Flies were aged for 3 more days at the same temperature. Afterwards, 3-6 days old flies were pricked intrathoracically with virus. After infection, flies were placed at either 25 °C, 18 °C or at fluctuating temperature. Survival was monitored daily and vials were changed every 5 days.

Nucleic acids extractions and real-time qPCR

DNA for the quantification of *Wolbachia* was extracted from pools of 10 flies using Drosdel protocol (http://www.drosdel.org.uk/molecular_methods.php) (Ryder *et al.*, 2004). RNA for assessment of viral titres was extracted using Trizol (Invitrogen). cDNA was prepared as described previously (Chrostek *et al.*, 2013).

Real-time qPCR reactions were carried out in 7900HT Fast Real-Time PCR System (Applied Biosystems) with the iQ™ SYBR® Green supermix (Bio Rad). Primers and thermal cycling protocols for *Wolbachia* and DCV were previously described (Chrostek *et al.*, 2013).

Statistical analysis

All the statistical analysis was performed in R (Team, 2012). Analysis of survival data was performed with the Cox proportional hazard mixed effect models. Fixed effects, depending on the experiment, included temperature, dose of DCV, and presence/absence of *Wolbachia,* while replicate vials within the same experiment were considered a random effect. Model fitting was done using the coxme package in R.

Log-transformed qPCR data were used to compare *Wolbachia* and DCV titres. Normality of the data was assessed with Shapiro-Wilks' normality test. The effects of temperature and *Wolbachia* on normal data (virus levels after infection with different doses of DCV and *Wolbachia* levels) were tested using general linear models, and marginal (least square) means were compared between the conditions of interest using the lsmeans package in R. The non-normal data on DCV levels across all thermal regimes were analysed with Kruskal Wallis and Wilcoxon rank sum tests. Correlation between logit-transformed survival proportions at day 8 and DCV titres was determined using Pearson's product moment correlation. DCV titres growth over time was analysed with censored Gaussian linear models with the survreg function in the survival package in R.

Results

Our standard DCV infection protocol includes housing flies at 25 °C before the infection and at 18 °C after the infection (Figure 1A) (Teixeira *et al.*, 2008). Relatively high doses of virus cause strong lethality in the flies without *Wolbachia*, while *Wolbachia*-carrying flies are protected (Teixeira *et al.*, 2008; Chrostek *et al.*, 2013, 2014).

To test how different infection temperatures affect *Wolbachia*-conferred protection to DCV, we used *Drosophila melanogaster* carrying one of its natural *Wolbachia* variants, *w*MelCS_b (*Wolb*+) and matching *Wolbachia*-free control (*Wolb*-) (Teixeira *et al.*, 2008; Chrostek *et al.*, 2013). The flies were raised from egg to adult at 25 °C, 0-3 days old adults were collected to the fresh vials and aged for 3 more days at the same temperature. *Wolb*+ and *Wolb*- 3-6 days old flies were challenged with serial dilutions of DCV and after the infection maintained at either 18 °C (Figures 1A and 1B) or at 25 °C (Figures 1C and 1D). The serial virus dilutions were used to control for the possible differential virus infectivity at different temperatures, i.e. to control that the effects on protection are not due to poorer virus infectivity at one of the temperatures.

As expected, *Wolbachia*-free flies die earlier than the *Wolbachia*-harbouring flies after DCV infection (Cox hazard ratio between *Wolb*+ and *Wolb*- flies = -1.84±1.89, $|z|=9.76$, $p<0.001$). On average, virus induced mortality is higher at 25 °C than at 18 °C (compare 1B and 1D for a single dose, mean Cox hazard ratio between 18 °C and 25 °C = -1.88±0.16, $|z|=11.7$, $p<0.001$), indicating that temperature either speeds up viral replication or weakens fly defence mechanisms. The lethality of flies without *Wolbachia* is also delayed at 18 °C (mean Cox hazard ratio between 18 ºC and 25 ºC of Wolb- flies =-1.33±0.19, $|z|=7.22$, $p<0.001$). This shows that temperature by itself influences the progression of DCV infection.

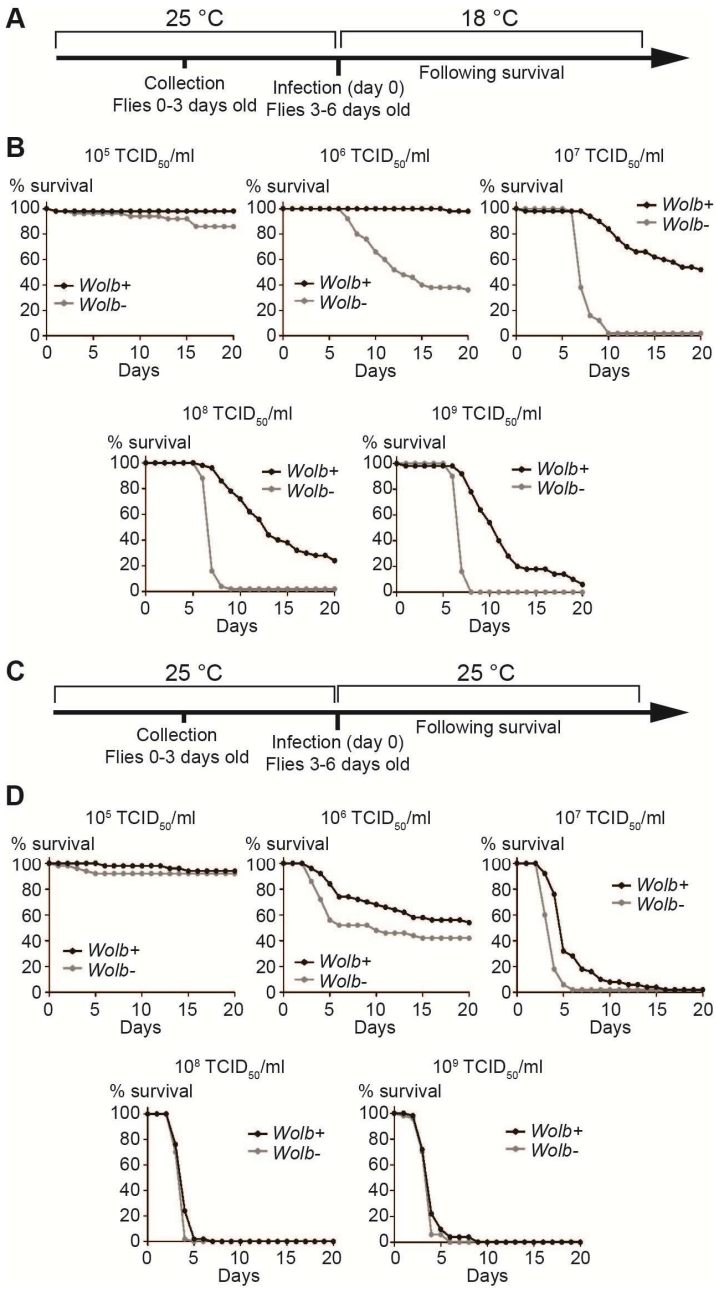

Figure. 1. Thermal regime and dose determine the strength of *Wolbachia*-conferred antiviral protection.

Continued on the next page.

Figure. 1. (continued) (A) The scheme of the standard DCV infection protocol, with the flies raised and kept at 25 °C before and 18 °C after the DCV infection. (B) *Wolbachia*-positive and *Wolbachia*-free flies, fifty per *Wolbachia* status per dose, were pricked with DCV and checked for survival every day. Flies were subjected to temperature regime illustrated in A. (C) The scheme of the experimental DCV infection protocol, with the constant temperature of 25 °C. (D) *Wolbachia*-positive and *Wolbachia*-free flies, fifty per *Wolbachia* status per dose, were pricked with DCV and checked for survival every day. Flies were subjected to temperature regime illustrated in C.

When analysing the data altogether, we also observed a significant *Wolbachia* × temperature interaction (comparison of mixed effects Cox models, X^2_1=19.5, p<0.001), indicating that the protective effect of *Wolbachia* varies with temperature, with the protection being stronger at 18 °C. Pairwise comparisons between *Wolb*+ and *Wolb*- flies at different doses under different temperature regimes showed that *Wolbachia* conferred antiviral protection is significant for all but one dose at 18 °C (after infection with 10^5 $TCID_{50}$/ml of DCV, mortality was very low both in *Wolb*- and *Wolb*+ flies, Figure 1B and Figure S1). In contrast, the protection at 25 °C was only significant at one of the doses (10^7 $TCID_{50}$/ml, Figure S1). Additionally, comparisons of protection conferred at both temperatures for each dose showed significant differences (measured by a significant interaction term between *Wolbachia* status and temperature), apart from the lowest and the middle dose. The lowest dose (10^5 $TCID_{50}$/ml) elicits little mortality even in *Wolb*- flies, thus very little protection can be detected at either temperature (Figures 1B and 1D, comparison of mixed effects Cox models, X^2_1=1.8, p=0.18). At 10^7 $TCID_{50}$/ml, the protection, measured by Cox hazard ratios, was identical at both temperatures (Figures 1B and 1D, comparison of mixed effects Cox models, X^2_1=0.8, p=0.37). This is because at 10^7 $TCID_{50}$/ml the survival curves at two temperatures for *Wolb*+ and *Wolb*- flies are shifted

proportionally. Therefore, despite the changes in the absolute risk of death at a given point in time, the average Cox hazard ratios are not different at both temperatures.

Overall, temperature after viral challenge affects the survival of the flies with and without *Wolbachia*, and protection is stronger at lower temperature and intermediate doses. The combination of high temperature and dose can completely mask the protective *Wolbachia* effect.

Upon pathogen attack the organism can defend itself either by supressing the pathogen loads (resistance to infection) or by limiting the pathogen associated negative fitness effect (tolerance to infection). *Wolbachia* was described to induce resistance to DCV in *D. melanogaster* under our standard conditions (25 °C - 18 °C and intermediate virus dose) (Teixeira *et al.*, 2008; Chrostek *et al.*, 2013, 2014). The potential of *Wolbachia* to induce tolerance is also known (Teixeira *et al.*, 2008). We speculated that manipulating temperature could change *Wolbachia*-induced response from resistance to tolerance. To distinguish between these two possible modes of protection, we checked if lethality of the flies corresponded to the viral titres at the same conditions. Viral titres were measured by quantitative real time PCR (RT-qPCR) three days post infection (dpi) (Figure 2A). As expected, *Wolbachia* had a significant effect on viral loads (linear model *Wolbachia* effect, $F_{1,80}$=164.5, p<0.001), with flies with *Wolbachia* having lower median viral loads than flies without *Wolbachia*. Overall, temperature also influences viral loads (linear model temperature effect, $F_{1,80}$=151.24, p<0.001), that were higher at 25 °C. There was also a significant *Wolbachia* × temperature interaction, (linear model *Wolbachia* × temperature interaction, $F_{1,80}$=9.3, p=0.003) with *Wolbachia* conferred protection being stronger at 18 °C and dependent on the dose of the

virus (linear models dose effect, $F_{4,80}$=36.05, p<0.001 and dose × *Wolbachia* × temperature interaction $F_{4,80}$=8.07, p<0.001).

To check if protection in terms of survival between *Wolb*+ and *Wolb*- flies correlates with differences in DCV titres we used Pearson product-moment correlation. As global median time to death for all survival curves was day 8, we used survival proportions at this day. We correlated them with median DCV loads at each dose, *Wolbachia* and temperature combination. There is a strong negative correlation (r= -0.902, t_{18}=-8.85, p <0.001) between DCV load at day 3 and probability of surviving at a later time point, which implies that *Wolbachia* at different doses and temperatures consistently provide *Drosophila* with resistance to DCV infection.

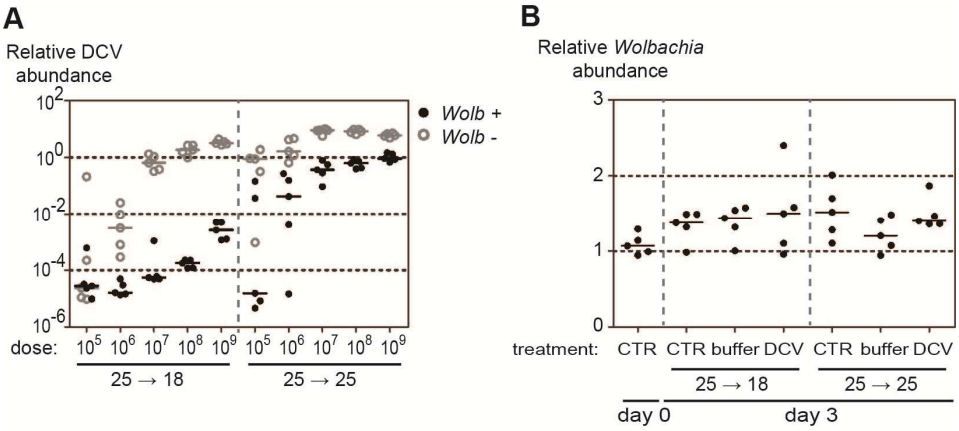

Figure 2. DCV titres and *Wolbachia* densities in DCV infected flies under different thermal regimes.

(A) DCV titres in flies infected with different doses of virus and sacrificed for RT-qPCR 3 dpi. Flies were kept at 25 °C before the infection and either at 18 °C or 25 °C after the infection, according to the schemes in Figures 1A and 1C. (B) *Wolbachia* levels measured by qPCR in flies at the day of infection (3-6 days old), or 3 days after in unchallenged (CTR), buffer- or DCV-challenged (10^7 $TCID_{50}$/ml) flies. Continued on the next page.

Figure 2. (continued) For (A) and (B) each dot is a sample, each sample consists of ten flies. Horizontal lines are medians of the replicate samples. Vertical dashed lines separate different temperature regimes.

Antiviral protection is known to be correlated with *Wolbachia* density: more *Wolbachia* translates into more antiviral protection (Osborne *et al.*, 2009, 2012; Frentiu *et al.*, 2010; Lu, Bian, *et al.*, 2012; Chrostek *et al.*, 2013, 2014; Martinez *et al.*, 2014). Thus, we asked about the effect of these two temperature regimes and the virus infection on *Wolbachia* endosymbiont. We measured *Wolbachia* densities in control flies (before the infection) and DCV infected, buffer pricked (treatment control) or unmanipulated flies (control for the temperature effect itself) after 3 days. We see that *Wolbachia* densities are constant throughout all these treatments (treatment effect, linear model, $F_{6,28}$=1.42, p=0.24, Figure 2B). This means that *Wolbachia* respond neither to the wounding nor DCV infection by increased proliferation. Importantly, in this case temperature affects *Wolbachia*-conferred protection *via* a mechanism independent of *Wolbachia* densities.

As all previous experiments contrasted constant 25 °C with the temperature shift situation (25 °C before and 18 °C after the infection) we hypothesized that the stress associated with the change of temperature triggers *Wolbachia* protection. Thus, we assessed protection under other combinations of 25 °C and 18 °C, namely constant 18 °C and 18 °C to 25 °C shift. We also included fluctuating temperature setup to approximate natural daily temperature changes (gradual increase from 18 °C to 25 °C during 12 h and decrease to 18 °C during the subsequent 12 h, both before and after infection). The results of the experiment including *Wolb*+ and *Wolb*- flies at all five thermal conditions: (i) 25 °C before and 18 °C after the infection, (ii) constant 25 °C, (iii) 18 °C before and 25 °C after the infection, (iv) constant 18 °C, and (v) fluctuations are shown in Figure

3. Analysis of the data presented in Figure 3A and 3B revealed a strong *Wolbachia* × pre-infection temperature interaction (Cox hazard ratio = -1.04±0.4, |z|=2.6, p=0.009), indicating that *Wolbachia*-conferred antiviral protection is stronger in the flies raised at 25 °C (Figure 3A) than in the flies raised at 18 °C (Figure 3B). Thus, the temperature before the infection (development from egg to adults of 0-3 days and 3 days of aging) is crucial for *Wolbachia*-conferred antiviral protection. This is confirmed by the pairwise comparisons of *Wolb*- and *Wolb*+ flies at four conditions, where the only significant protection is observed for *Drosophila* raised at 25 °C and transferred to 18°C after the infection (Cox hazard ratio=-1.72±0.42, |*z*|=4.08, p<0.001, Figure 3A) and the flies raised and kept at 25 °C after the infection (Figure 3A, Cox hazard ratio=1.10±0.39, |z|=2.85, p<0.001). Almost no *Wolbachia*-conferred protection was observed for the flies raised at 18 °C, independently of the post-infection temperature (Cox hazard ratio = -0.04±0.39 and -0.64±0.38, |*z*|<1.7, *p*>0.09 for post infection temperatures of 18 °C and 25 °C, respectively, Figure 3A), hence disproving our hypothesis about the temperature shift induction of antiviral protection.

No interaction between *Wolbachia* and post-infection temperature was detected in this experiment, contrarily to what was found in the experiment reported in Figure 1. This could be due to the pathogen dose, since at the dose chosen flies with *Wolbachia* raised at 25 °C are protected at both post-infection temperatures (Figure 3B).

Nevertheless, these data support our previous conclusion concerning temperature dependence of DCV infection. Overall, virus induced lethality in flies without *Wolbachia* is the same, but occurs ~5 days later at 18 °C than at 25 °C (Figures 3A and 3B) (temperature effect, Cox hazard ratio = -1.01±0.19, |z|=5.46, p<0.001).

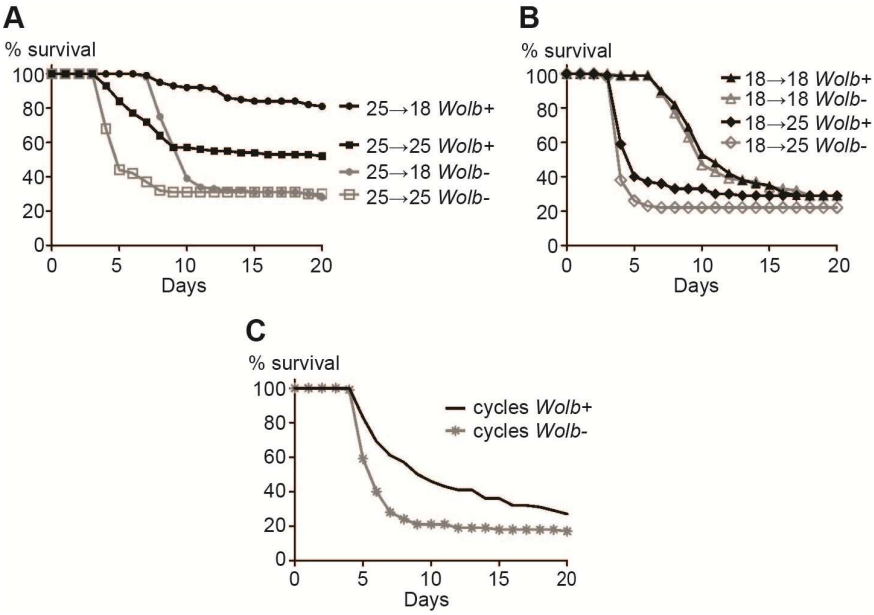

Figure 3. Pre-infection temperature is crucial for *Wolbachia*-conferred antiviral protection.

(A,B,C) *Wolbachia*-positive (black lines) and *Wolbachia*-free (grey lines) flies, fifty per *Wolbachia* status per condition, were pricked with DCV (10^8 $TCID_{50}$/ml) and checked for survival every day. Flies were kept at 25 °C (A) or 18 °C (B) before the infection and at either 18 °C or 25 °C after the infection (A,B). (C) Flies were kept at fluctuating temperature (18 °C - 25 °C - 18 °C, 24h) before and after the infection.

Fluctuating temperature regime resulted in moderate, but statistically significant, *Wolbachia*-conferred protection (*Wolbachia* effect, Cox hazard ratio = -0.67±0.27, |z|=2,45, p=0.014, Figure 3C).

To test if differences in survival were reflected in virus titres we performed RT-qPCR three dpi. We see that, due to the low statistical power, the only significant protection can be detected under our initial protocol conditions (Figure 4A) (Wilcoxon rank sum test, p=0.002). Flies with *Wolbachia* have 341× less virus than the flies without *Wolbachia.* Constant 25 °C and cycling between 25 °C and 18 °C degrees produced

8-fold and 12-fold non-significant differences (Wilcoxon rank sum test, p=0.24 and p=0.13, respectively). An even smaller, 5-fold change was observed for constant 18 °C (Wilcoxon rank sum test, p=0.065). Additionally, Kruskal-Wallis rank sum test did not detect any significant differences between DCV levels at different thermal regimes in flies without *Wolbachia* (p=0.542). This may mean that the virus replication is temperature independent in the *Wolb*- flies or that the DCV titres already reached maximum at all treatments, so no differences can be detected three dpi. In contrast, differences in DCV loads were detected in *Wolbachia*-harbouring flies (Kruskal-Wallis rank sum test, p=0.003), suggesting that, in the presence of *Wolbachia*, some temperature conditions may be more restrictive for DCV proliferation.

As neither DCV infection nor 3 days at different temperature influence *Wolbachia* densities (Figure 2B), we tested if raising the flies at different temperatures does (Figure 4B). *Wolbachia* densities measured by qPCR in whole flies confirmed that the flies raised at 25 °C have ~50% higher *Wolbachia* densities than the flies raised at 18 °C (mean difference in *Wolbachia* densities between 18 and 25 °C, t=-4.13, p=0.040). *Wolbachia* levels at constant 25 °C are not different from these at fluctuating temperature (mean difference in *Wolbachia* densities between 25 °C and fluctuating thermal regimes, t=-1.54 p=0.287). These data are consistent with survival data and the trends observed in the DCV titres measurements. Therefore, *Wolbachia* levels could be responsible for the differences in protection between flies raised at different thermal regimes.

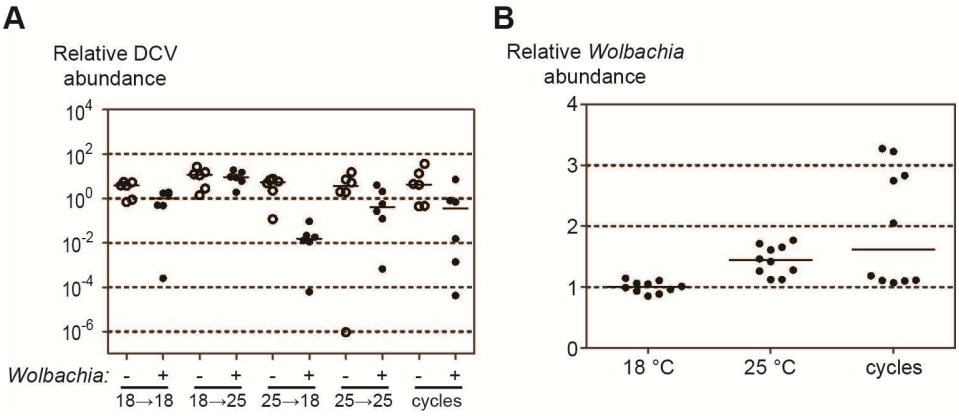

Figure 4. DCV titres and *Wolbachia* densities in flies under different thermal regimes.

(A) DCV titres in flies infected at the age of 3-6 days and sacrificed for RT-qPCR 3 dpi. Flies were kept at thermal regimes indicated, matching these form Figure 3. (B) *Wolbachia* levels measured by qPCR in 3-6 days old flies kept at indicated temperatures.

In conclusion, flies raised at 25 °C and kept at 18 °C post infection exhibit the strongest *Wolbachia*-conferred antiviral protection in terms of virus titres and survival and have more *Wolbachia* than flies raised at 18 °C, which are not protected by *Wolbachia*. Flies kept at cycling temperature exhibit significant protection in terms of survival, and *Wolbachia* densities are not different between these and 25 °C raised flies.

Finally, we asked if *Wolbachia*-conferred protection acts immediately after viral challenge or if it is only expressed later in the course of infection. We measured viral loads at early time-points post infection in *Wolb*+ and *Wolb*- flies (raised and aged at 25 °C and moved to 18 °C after the infection). On average, *Wolb*+ flies have lower DCV titres than *Wolb*- flies (*Wolbachia* effect, censored Gaussian linear model $\chi^2_1 = 9.64$, $p < 0.016$, Figure 5). Also, at 0 and 6 hours post infection we cannot detect any viral RNA in most of our samples, and at 12 hours post infection we do not detect virus only in *Wolb*+ flies. Interestingly, from 24

hours on, virus grows at the same rate in flies with and without *Wolbachia* (*Wolbachia* × time interaction, censored Gaussian linear model, χ^2_5= 5.64, *p*= 0.34).

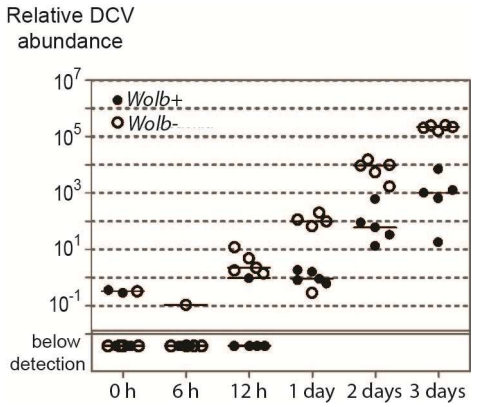

Figure 5. Time course analysis of DCV titres in flies with and without *Wolbachia*.

DCV titres in flies sacrificed for RT-qPCR at the time of DCV infection (0 h) and at different time points after the infection. Flies were kept at 25 °C before the infection and at 18 °C after the infection. Each point is a sample, each sample consisted of 10 flies, lines are medians of the replicates.

Discussion

We have shown that temperature greatly affects natural *Wolbachia*-*Drosophila* defensive symbiosis and that *Wolbachia*-conferred antiviral protection is only present at certain environmental conditions.

We identified pre-infection temperature as a factor crucial for the expression of the protection. Although *Wolbachia* densities may be responsible for this effect (Figures 3), we consider this unlikely. The density difference between flies raised at 25 °C and 18 °C is small (Figure 4B), and a 2-fold densities difference between *w*Mel-like and *w*MelCS-like genotypes reported before produced less pronounced effect (Chrostek *et al.*, 2013).

Wolbachia may also "prime" *Drosophila* for protection during development or early adulthood at 25 °C. The existence of a ready protective mechanism is further strengthened by the observation that the flies are protected very early in the course of the viral infection, and from 24 hours on virus growth is the same in the flies with and without *Wolbachia* (Figure 5). This also suggests that the difference generated in the first hours after the viral challenge can produce significant differences in survival many days later. The nature of the potential *Wolbachia*-induced priming remains to be uncovered, as classical immune priming by *Wolbachia* has already been excluded (Wong *et al.*, 2011; Rancès *et al.*, 2012; Chrostek *et al.*, 2014), along with the whole genome, microarray measured transcriptional activation (Teixeira, 2012).

Another possible explanation of the differences in antiviral protection between flies raised at different temperatures is the difference in *Wolbachia* tissue tropism. If true, this would imply that once endosymbiont colonizes certain organs, its influence on animal physiology is determined. Also, a small difference in endosymbionts densities in whole fly may reflect huge, tissue specific abundance

changes. Dependence of virus distribution on post-infection temperature may also explain higher flies mortality at higher temperature. Therefore, spatial characterisation of viral infection and endosymbiont niche could help to understand host-endosymbiont-pathogen interaction dynamics.

We demonstrated that *Wolbachia* densities in flies kept at 25 °C and flies raised at 25 °C and shifted to 18 °C are the same three days after infection. This indicates that *Wolbachia* does not respond to the presence of virus by, e.g. increased replication, and is also consistent with our priming hypothesis. However, early *Wolbachia* response in terms of proliferation, cell death or metabolic activity remains to be assessed.

As mentioned in the introduction, a recent study by Murdock *et al.* on *Wolbachia* transinfected *Anopheles stephensi* addressed the question of temperature dependence in *Wolbachia*-induced pathogen blocking (Murdock *et al.*, 2014). The effect of temperature on *Wolbachia*-conferred protection and *Plasmodium* infection dynamics was non-linear, and depended on the readout used to access parasitaemia, while *Wolbachia* densities were directly correlated with temperature (Murdock *et al.*, 2014). Consistently, we see that the strength of protection or its presence is not always correlated with *Wolbachia* densities, as is the case of the flies raised at 25 °C and placed at different temperatures after the infection (Figures 1 and 2). However, in contrast to Murdock *et al.* (2014), we have never observed enhancement of the viral infection in the flies with *Wolbachia*. This can be due to the biological differences between the two systems (artificial mosquito-*Wolbachia* and natural *Drosophila*-*Wolbachia,* parasite and virus), temperature regimes chosen or our unilateral assessment of viral titres (that does not include other possible readouts, e.g. virus infectivity).

This study shows that the fitness benefit provided by *Wolbachia* can be abolished by temperature. The literature indicates that so does the cost

of harbouring the symbiont. One of the most striking examples is pathogenic *w*MelPop *Wolbachia* variant that shortens the lifespan dramatically at 29 and 25 °C, but not at 19 °C (Reynolds *et al.*, 2003). Some of the natural *Wolbachia* endosymbionts of *Drosophila melanogaster* belonging to *w*MelCS-like group, including *w*MelCS_b used there, also exert a lifespan cost (Teixeira *et al.*, 2008). This indicates that *Wolbachia* may be more neutral, at least taking into account protection and lifespan, in cooler climates.

We also described thermal dependence of DCV infection in *Drosophila.* Overall, virus induced lethality in flies without *Wolbachia* is the same, but occurs 5 days later at 18 °C than at 25 °C. This strongly suggests that virus infectivity is not affected. The delay can be caused by virus replication machinery being slower or fly immune response and damage control being more potent at lower temperature (Linder *et al.*, 2008). In our system we cannot untangle viral replication and host's response as viruses only replicate intracellularly, meaning that they are always subjected to cellular immune responses. Also, no simple readout for the activation of main antiviral siRNA pathway in *Drosophila* exists, and only a transcriptomic study, especially small RNAseq, could answer why virus induced mortality depends on ambient temperature. Interestingly, similar temperature dependence that we observe for DCV infection was described before for *Drosophila melanogaster* infected with bacteria (Linder *et al.*, 2008). Flies raised at 25 °C and placed at 25 °C after the infection die much faster than the ones placed at 17 °C. The same study showed that pre-infection adult temperature does not influence mortality, but lower temperature during only 3 h post infection changes its outcome (Linder *et al.*, 2008). Testing all these permutations can provide further insights into dynamics of the response in our system.

As shown above, laboratory assay conditions can change results and conclusions taken from an experiment. In the case of *Wolbachia*-

conferred antiviral protection temperature turned out to be crucial. Many other factors, like genetic and environmental variation within hosts, pathogens or symbionts and age and sex of animals, remain to be tested. In our model system, comparative transcriptomic and metabolomic analysis of insects at protective and non-protective setup could be used to understand the mechanism of *Wolbachia* antiviral effect.

Including additional variables in our experimental designs will provide deeper insight into the biology of symbiotic associations. In particular, we can learn how hosts and their microbial partners are adapted to certain conditions and how environmental changes challenge these relationships. Understanding the ecology of biological systems, currently and over evolutionary timescales, may help to predict the changes associated with climate change or species reallocation in the future. Currently, assessment of temperature influence on *Wolbachia*-induced pathogen blocking in the field seems essential for all practical *Wolbachia* applications.

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Chapter 6 - General Discussion

1. Phenotypes of *Wolbachia* in *Drosophila melanogaster*

1.1. Genetic bases of *Wolbachia* phenotypes

*w*Mel variants have been shown to differ in terms of genomic markers (Riegler *et al.*, 2005) and to belong to two major clades (Richardson *et al.*, 2012). Also, there are evidences for the recent global replacement of *w*MelCS-like variants by *w*Mel-like variants in the natural *Drosophila melanogaster* populations (Riegler *et al.*, 2005; Nunes *et al.*, 2008; Richardson *et al.*, 2012). We have discovered phenotypic differences between *w*MelCS-like and *w*Mel-like variants that are the products of their divergent genomes and a likely cause of the variant frequencies shift in natural populations. *w*MelCS genotypes are more protective against viral infections, reach higher densities within the host and are more costly to the host than *w*Mel genotypes (Chrostek *et al.*, 2013). We anticipated that genomic characterisation of these *w*Mel variants could provide insight into the molecular bases of these phenotypes. Using complete endosymbionts' genomes sequences we identified 108 SNPs, a tandem duplication and seven indels differentiating between *w*Mel-group and *w*MelCS-group. These are located in many genes and could potentially explain phenotypic differences we observed. We identified polymorphisms in six ankyrin repeat-containing genes (WD0073, WD00514, WD0550, WD0636, WD0754, WD0766), which could mediate interactions with host proteins, in two Octomom genes (WD0513 and WD0514) implicated in *w*MelPop titres control, and a protein with a Piwi/Argonaute/Zwille siRNA-binding domain (WD0033), that could interact with antiviral machinery of *Drosophila* (Chrostek *et al.*, 2013). However, any of the 116 identified genetic differences can cause the *w*Mel variants phenotypes and the number of candidates is, for now, experimentally unmanageable. To make further studies feasible, phenotypic characterisation of additional *w*Mel variants would be

necessary, especially the ones representing more basal *Wolbachia* clades.

*w*MelPop *Wolbachia* strain, very closely related to *w*MelCS_b, exhibits all *w*MelCS-like phenotypes with the increased strength. *w*MelPop-conferred antiviral protection is stronger, endosymbiont densities are higher and so is the cost associated with this variant (Chrostek *et al.*, 2013). Comparison of the genomes of *w*MelPop and *w*MelCS_b allowed identification of Octomom as responsible for phenotypic difference, providing the first link between genotype and phenotype in *Wolbachia* (Chapter 3 and Chrostek *et al.*, 2013). The potential involvement of Octomom genes in generation of the phenotypes encourages speculation on the exact mechanism of their action. Faster *Wolbachia* growth could be an outcome of increase of the speed of replication (as indicated by the presence of DNA processing genes in Octomom region) or by inhibition of host control of endosymbiont growth (as the genes with eukaryotic like domains potentially enabling interaction with the host are also present in this region). However, Octomom amplification changing the chromosome structure and regulation of non-Octomom genes is also possible. Demonstrating the importance of Octomom genes requires either genetic *Wolbachia* manipulation, expression of these genes in flies or other heterologous system or exploring the naturally existing variation.

A strain exogenous for *D. melanogaster*, *w*Au, also provides protection, which is much stronger than that provided by any of the natural *w*Mel variants (Chrostek *et al.*, 2014). Counterintuitively, this protection is not associated with immune upregulation of *Drosophila* immune system (Chrostek *et al.*, 2014). Genome sequence of *w*Au is not available, but it could contribute to further elucidation of the mechanisms of *Wolbachia*-fly symbiosis. However, this approach should not be overestimated, as *w*Au-*Drosophila* association was created artificially, and identified

phenotypic effects may reflect the lack of coadaptation rather than the presence of specific genes or alleles.

1.2. Mechanism of *Wolbachia*-conferred antiviral protection

Different *Wolbachia* genotypes consistently provide *Drosophila melanogaster* with antiviral protection under our standard assay conditions. However, the degree of protection differs, with *w*MelPop and *w*Au being most protective (they were never compared directly, but both are more protective than *w*MelCS_b), *w*MelCS-like variants conferring less protection and *w*Mel-like variants being least protective.

The lack of a non-protective *Wolbachia* among the *w*Mel genotypes tested suggested that our genotypic and phenotypic analyses could only identify factors modulating the protection. However, the opposite can also be true, and mutations in the "*Wolbachia*-protective genes" may produce different strengths of protection. Direct juxtaposition of protective and the non-protective *Regiella* strains enabled many inferences about the mode of *Regiella* anti-parasitiod action in aphids (Hansen *et al.*, 2012). In particular, the non-protective bacteria miss O-antigen biosynthetic pathway, an intact Type 1 and Type 3 Secretion System and their effectors, hemin transport, and the two-component system PhoPQ. All of these could mediate endosymbionts' virulence against parasitoids, resulting in aphid protection (Hansen *et al.*, 2012). Comparative analysis of closely related protective and non-protective *Wolbachia* could also give rise to many specific hypotheses.

Despite the numerous efforts, several basic questions about *Wolbachia*-conferred antiviral protection remain unanswered. As we do not know the mechanism, we cannot answer if protection is always present and active or what governs its temperature dependence. We do not know if

Wolbachia influence virus directly, if they act through host's pathways or modify intracellular niche making it less hospitable for the virus.

One of the guiding questions that may help to understand this complex phenomenon is whether *Wolbachia*-conferred antiviral-protection is cell autonomous, more specifically, if only cells with *Wolbachia* are protected against infection or if a *Wolbachia*-harbouring cell can protect *Wolbachia*-free neighbours. Cell autonomous protection would open the door for further *in vitro* studies. If the protection is non-cell autonomous, it would be interesting to isolate the responsible signal and address the systemic spread of protection *in vivo*. Moreira and colleagues saw that dengue virus does not colocalize with *Wolbachia* in mosquito fat body cells (Moreira *et al.*, 2009), suggesting a cell autonomous effect. Conversely, we have evidence for *Drosophila* C virus replication within *Wolbachia* infected cells in *Drosophila melanogaster* cell line (data not shown). *Wolbachia* was also shown to block apoptosis in nematodes using a non-cell autonomous mechanism (Landmann *et al.*, 2011).

Another complementary route towards understanding of *Wolbachia*-induced pathogen blocking would be to determine which insect tissues are crucial for protection, where protection starts and what the dynamics of virus spread and replication in the presence and absence of *Wolbachia* are. Focusing further research on specific tissue could prevent the "phenotypes dilution" that we probably observe by assessing amounts of virus, *Wolbachia* and substances potentially involved in *Wolbachia*-virus competition in whole insects (e.g. Teixeira *et al.*, 2008; Caragata *et al.*, 2013, 2014; Chrostek *et al.*, 2013, 2014). This is interesting, especially because it seems that in *Drosophila melanogaster* *Wolbachia* interferes with initial DCV processes and replication, and from the 24 hours post infection on the rate of virus replication is the same in the flies with and without *Wolbachia* (Chapter 5). Therefore, it is likely

that *Wolbachia* blocks early virus replication in a certain tissue crucial for the outcome of the infection.

1.3. *Wolbachia* densities and antiviral protection

Overall, we observed that for all *Wolbachia* genotypes tested: natural *w*Mel variants, *w*MelPop and *w*Au, the higher the *Wolbachia* levels – the higher the antiviral protection. Of course, as we compared different endosymbiont genotypes we cannot exclude the possibility that genetic differences regulating titres are independent of these responsible for protection. Similar limitation, concerning both, host and symbiont genotypes, is also associated with the data of Osborne *et al.* (2009). However, by now, other groups correlated protection and symbiont density, using gradient antibiotic treatments of flies (Osborne *et al.*, 2012) or cells in culture (Lu *et al.*, 2012). As different antibiotic concentrations may influence other symbionts of flies and mitochondria of the eukaryotic cells (reviewed in Zug and Hammerstein, 2014) we believe that our studies on unmanipulated associations also contribute important evidence.

Additionally, the recent study by Martinez *et al.* provided endosymbiont densities and antiviral protection data for 19 *Wolbachia* strains originating from 12 different *Drosophila* species (Martinez *et al.*, 2014). Comparison of phenotypes of these *Wolbachia* strains in a single *D. simulans* genetic background revealed that approximately half of the strains protects against two viruses tested (DCV and FHV) and that the strength of protection is strongly correlated with the endosymbiont densities. Moreover, the association between densities and protection does not seem to be a consequence of phylogenetic relatedness of the *Wolbachia* strains, indicating that any strain present in high enough densities can protect *D. simulans* against viruses (Martinez *et al.*, 2014).

*w*MelPop, with more copies of Octomom region consistently resulting in higher endosymbiont densities and higher protection, also proves that symbiont densities are crucial for the expression of antiviral protection (Chapter 3).

1.4. Control of *Wolbachia* densities

Importantly, using different *Wolbachia* genotypes in the same *Drosophila* nuclear background showed that endosymbionts, at least partially, control their own proliferation, instead of leaving it under hosts' restraint (Chapter 3, Chrostek *et al.*, 2013, 2014; Martinez *et al.*, 2014).

Previous reports on *Wolbachia* control over their own densities used mainly different *Wolbachia* strains co-infecting a single host. They showed that in multiply infected insects each strain reaches its final densities independently from other strains (Mouton *et al.*, 2003; Lu *et al.*, 2012). There are also several studies showing that densities of *Wolbachia* vary between natural *Drosophila*-endosymbiont associations (Veneti *et al.*, 2003; Osborne *et al.*, 2009). In these cases, the influences of the host and symbiont genotypes are impossible to untangle.

We also identified the first endosymbiont genomic region, Octomom, responsible for control of *Wolbachia* densities (Chapter 3). Moreover, Octomom amplification in *w*MelPop demonstrates that the regulation of endosymbiont titres, reached by co-evolution, can be disrupted with a single genetic change in the symbiont. This implies that the symbiont must be under a constant selection for density control.

Endosymbionts densities can also be controlled by the host (McGraw *et al.*, 2002; Kondo *et al.*, 2005; Mouton *et al.*, 2007), which was demonstrated by *Wolbachia* transfers between hosts' genotypes and subsequent comparisons of their phenotypes. However, a link between different insect alleles and particular effects on symbionts is still missing.

1.5. Cost of harbouring defensive symbionts

Another recurrent theme in our data is the cost associated with the presence of *Wolbachia*. Natural *w*Mel variants, *w*MelPop and *w*Au seem to follow the same pattern: high *Wolbachia* titres lead to high antiviral protection, but impact lifespan negatively in the absence of the viral challenge. For *w*Au and natural protective *w*MelCS variants, the life-shortening we observed (by 11 % to 31 % relatively to non-life-shortening *Wolbachia*) (Chrostek *et al.*, 2013, 2014) would probably have a limited influence on the fitness in the wild, as most of the *Drosophila* progeny is produced earlier (Ashburner *et al.*, 2005). However, as mentioned before, the longevity cost in the lab could reflect susceptibility to other, ecologically relevant stress in the wild. The nature of this potential stress remains to be determined. Additionally, assessment of the influence of different *Wolbachia* on reproductive output of the flies is missing from our analysis. Higher egg laying early in life could potentially neutralize the longevity cost, while lower could be interpreted as an extra price to pay for high *Wolbachia* levels and antiviral protection.

The fitness cost associated with the presence of defensive symbionts has been reported before. The pea aphids harbouring anti-parasitoid *Hamiltonella defensa* are more susceptible to predation by ladybirds (Polin *et al.*, 2014), and black bean aphids with *H. defensa* have reduced lifespan and, as a consequence, lower lifetime reproduction (Vorburger and Gouskov, 2011; Vorburger *et al.*, 2013). The existence of this cost is intuitive, as intracellular bacteria use metabolites produced by host cells, probably competing with other intracellular processes. Their replication may also cause direct damage. Finally, as protective symbionts constitute part of host immune defences, their cost may also be interpreted as a price of maintenance of the protective mechanism (Schmid-Hempel, 2003). We suspect that for *w*MelPop the most probable

cause of life-shortening effect is the direct tissue damage associated with *Wolbachia* over-replication (Min and Benzer, 1997). Even though *w*Au and other *w*MelCS-like variants grow at the smaller rate than *w*MelPop, they may damage the host sufficiently to cause premature death. Finally, *w*Mel-like variants that are present at lower densities do not affect hosts' longevity.

Antiviral protection associated with high *Wolbachia* densities and the cost of harbouring defensive symbiont seem to be inextricably tied. Moreover, they would have opposite effects on fitness of insects in the wild. High virus pressure would cause the spread of protective variants, while other stresses may promote variants exerting minimal cost. We suspect that the selective pressure driving the recent global expansion of *w*Mel-like variants was not increase resistance to viruses but, probably, reduced *Wolbachia* titers (Chrostek *et al.*, 2013). Yet, only the ecological data describing prevalence and geographic distribution of *Wolbachia*, viruses, and other potential interacting partners could help to predict the fate of protective but costly *Wolbachia* in the wild. Net effect of symbiosis in nature may also include other benefits that add up to the antiviral protection. As it was discussed in the Introduction, presence/absence of these depends strictly on fly and, probably, symbiont genetic backgrounds and on experimental conditions. These considerations are especially important for practical application of protective *Wolbachia* in the prevention of spread of arboviral human diseases. Further phenotypic screens for *Wolbachia* exerting high protection at minimal cost could inform these practical approaches and provide insight into the molecular mechanism of protection.

1.6. Temperature and protective symbiosis

We identified temperature as a factor able to reduce or abolish *w*MelCS_b-conferred protection. By testing combinations of two temperatures, 25 and 18 °C, we were able to show that the pre-infection temperature determines the presence or absence of the *Wolbachia*-conferred antiviral protection (Chapter 5). Although the difference in protection between flies raised at 25 and 18 °C is profound, there is only 50 % difference in *Wolbachia* densities. We find this difference unlikely to cause the shift from a very strong to no protection. *w*Mel-like variants reach 2-fold lower *Wolbachia* densities than *w*MelCS-like variants at 25 °C, and still have a significant antiviral effect (Chrostek *et al.*, 2013).

Curiously, differences in the viral titres between flies with and without *Wolbachia* arise within 12 hours post infection, and remain constant up to 3 days after the challenge. Quick response may indicate that the resistance potential of flies with *Wolbachia* is predetermined, i.e. the system is ready to respond before viral challenge occurs. These dynamics are in support of some hypotheses explaining antiviral protection, like deployment of proteolytic ready-to-use host's pathway, e.g. apoptotic pathway (see Chapter 1, Section 2.3). It also indicates that the early virus replication is essential for the outcome of the infection.

Finally, we have found that post infection temperature influences virus infection severity, and depending on the dose, may also mask antiviral *Wolbachia* effect. Importantly, the temperature after the infection was able to influence the protection independently of the *Wolbachia* densities. Our data and these provided by Murdock *et al.* (2014) indicate that the high endosymbiont densities provide protection only at some environmental conditions.

We suspect that *Wolbachia* and virus tissue distributions could be crucial for understanding the temperature dependence of this phenomenon. It

would be interesting to find that the small or undetectable *Wolbachia* densities differences in the whole fly are indeed huge but precisely localised. Additionally, these studies could reveal which tissues are crucial for the *Wolbachia*-conferred antiviral protection.

If *Wolbachia*-conferred protection can be influenced by temperature without being dependent on *Wolbachia* density even locally, in a specific tissue, high protection with low *Wolbachia* titres may also exist. Again, studies on the exceptions from "more *Wolbachia*, more protection" rule should answer what, apart from *Wolbachia* presence, is required for the protection to occur.

Different temperatures may be a useful system to explore the biology of *Wolbachia*. Especially because temperature affects both, *Wolbachia* associated benefits (Chapter 5) and costs (Reynolds *et al.*, 2003). In particular, *w*MelPop harbouring flies do not die prematurely when housed at 18 °C (Reynolds *et al.*, 2003). Thus, it may be interesting to test how the balance between cost and benefit changes at different thermal environments for *w*MelCS_b and other *Wolbachia* genotypes.

As *Wolbachia*-conferred protection holds a promise for helping to control vector borne diseases, many host-*Wolbachia*-pathogen associations have been examined recently. Apart from many instances of pathogen-blocking, some symbioses turned out to be neutral for the pathogen, while others enhanced the infection (Hughes *et al.*, 2012; Baton *et al.*, 2013; Martinez *et al.*, 2014; Zélé *et al.*, 2014; Murdock *et al.*, 2014; Dodson *et al.*, 2014). However, all these authors, except from Murdock *et al.* (2014), have examined different genotypes only under single assay conditions. Thus, a spectrum of possible *Wolbachia* phenotypes, including *w*Mel and *w*Au effects, depending on the environment remains to be uncovered.

Again, our results highlight the need to understand environmental conditions in the wild in order to evaluate importance of antiviral protection for *Wolbachia* maintenance in nature. Especially because the recent estimates of *Wolbachia* infection prevalence in arthropods (Zug and Hammerstein, 2012) and the protective capabilities of *Wolbachia* strains (Martinez *et al.*, 2014) suggest that 20 % of arthropods may benefit from *Wolbachia*-conferred protection (Martinez *et al.*, 2014). Climatic restrictions may diminish that number or cause local adaptations of *Drosophila*-*Wolbachia* symbiosis to thermal conditions. Finally, understanding how host and symbiont will evolve under different conditions can help to predict the fate of *Wolbachia*-carrying mosquitoes in the wild.

2. *w*MelPop and its virulence

2.1. Plasticity of bacterial genomes

Although genome reduction limits adaptive capacity of intracellular bacteria (Moran and Wernegreen, 2000), *Wolbachia* genomes, due to several mobile genetic elements (Wu *et al.*, 2004; Klasson *et al.*, 2008, 2009), were shown to be plastic over evolutionary timescales (Cordaux *et al.*, 2008; Klasson *et al.*, 2009; Leclercq *et al.*, 2011). Comparisons of different *Wolbachia* strains show general lack of synteny between their genomes (Cordaux *et al.*, 2008; Klasson *et al.*, 2009; Leclercq *et al.*, 2011). Most gene-order breakpoints are flanked by mobile genetic elements and other repeated sequences, while others are located within prophage regions (Cordaux *et al.*, 2008; Klasson *et al.*, 2009; Leclercq *et al.*, 2011). Although rearrangements shaped *Wolbachia* genomes throughout evolution, they were never detected in a shorter periods of time, e.g. within one *Wolbachia* strain (Klasson *et al.*, 2009).

Knowing the potential of insertion sequences (IS) to mediate rearrangements in *Wolbachia* genomes (Cordaux *et al.*, 2008), we assume that in the case of *w*MelPop recombination across identical IS repeats (flanking Octomom region in *w*MelCS-like strain) produced virulent copy number variants (Andersson and Hughes, 2009). Therefore, *w*MelPop is the first example in which we detect *Wolbachia* genome plasticity products within the single *Wolbachia* strain.

Importantly, we are not sure if Octomom copy number variants arise every generation in the fly germline and during fly development or if all of this variability was present in the original stock. In our experiment, low copy number flies are more likely to produce the offspring with the same copy number between generations than the high copy variants. In one copy line we have never observed appearance of any other variation, and among two copy variants this happens relatively rarely. Also, with

the progress of selection even the high copy lines seemed to become more stable. This suggests that *w*MelPop variants with different Octomom copy numbers were generated once and come from the initial stock and we just isolated them in the process of selection. On the other hand, we can reverse a direction of selection, and select for low copy females starting with high copy mothers and the other way round. If all the copy number variants come from the initial pool, the whole pool has to be always transmitted to the next generation inside an oocyte. Also, the presence of undetectable low copy number variants in the flies harbouring high Octomom copy numbers is conceivable, but the production of the low copy flies with the cryptic high copy *Wolbachia* is more difficult to conceptualize. This is because the high copy number *w*MelPop variants should outcompete the low copy number *w*MelPop variants and produce high copy progeny. Therefore, the heritability of the whole variation in Octomom copy numbers seems unlikely and probably frequent generation of copy number variants occurs.

Also, the reverse selection starting with high copy mothers produced desired outcomes faster than the reverse selection starting with low copy mothers. The high copy number *w*MelPop variants containing many flanking direct repeats may recruit more proteins responsible for recombination. This suggests that high Octomom copy numbers render the region more dynamic and the variation is generated at higher frequency in high copy variants.

Recombination between insertion sequences has been shown before to produce duplications over a short timescales in other bacteria (rewieved in Andersson and Hughes, 2009). An additional copy of 165 kb genomic region in the genome of *Pseudomonas syringae,* pv. *tomato* (Pst) DC3000, a plant pathogen, provided the bacteria with an advantage in some culture conditions, but did not influence pathogenicity (Bao *et al.*, 2014). Similar structural polymorphism, mediated by direct repeats, was

also found in the genome of *Portiera,* obligate endosymbiont of whiteflies (Sloan and Moran, 2013). Sloan and Moran proposed two alternative genome structure models (Figure 1), and using Southern blot demonstrated that variable number of tandem copies is present within *Portiera* genomes (Figure 1B).

Similar experimental design as in Sloan and Moran, 2013 would help us to make a definitive statement about Octomom region organization. Next to the two hypotheses illustrated in Figure 1, namely the existence of the subcircle containing extra copies of the amplified sequence and tandem localization of the copies in the main chromosome, we consider the possibility that Octomom copies may be located in different places of bacterial chromosome.

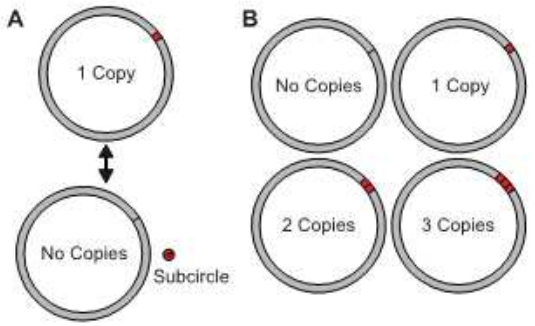

Figure. 1. Two alternative structural models of copy number variants

(A) The sequence (red) flanked by identical repeats (black lines) could interconvert between an integrated form and a separate subcircle form via repeat-mediated recombination. (B) Alternatively, the sequence could exist in a variable number of tandem copies. Sloan & Moran, The evolution of genomic instability in the obligate endosymbionts of whiteflies, Genome biology and evolution, 2013, volume 5, issue 5, 783-93, by permission of Oxford University Press.

2.2. Evolution of amplified sequence

Gene duplications and amplifications provide raw material for evolution, as new copies of sequence can undergo mutations without a loss of the primary function. As potential mutated and non-mutated copies should co-exist within individuals, sequencing of a single copy Octomom variant *w*MelPop would answer if the duplicated region was subjected to accelerated sequence evolution. Encountering original Octomom region sequence is however likely, as beneficial mutations are rare, and single copy Octomom variant with detrimental sequence changes could be counterselected within the host.

2.3. Octomom in the literature

Dynamic nature expressed as a potential to shrink and expand, association with pathogenicity, or both of these reasons led to a complete knockout of Octomom region in the process of *w*MelPop adaptation to mosquito intracellular environment (Figure 2B) (McMeniman *et al.*, 2008; Woolfit *et al.*, 2013). Testing the adapted *w*MelPop-PGYP variant in flies could provide insights into the function of the genes included in this region. However, other genetic changes between this variant and *w*MelPop (Figure 2A and 2C) may obscure an interpretation of a final result.

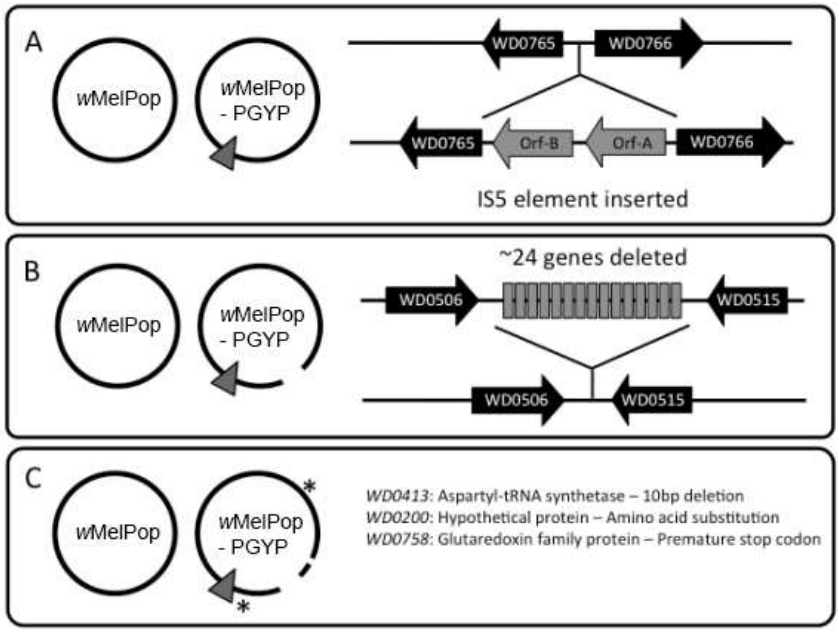

Figure 2. The genomic differences detected between *w*MelPop and *w*MelPop-PGYP.

(A) Insertion of an additional IS5 element between the orthologs of *w*Mel genes WD0765 and WD0766. (B) Deletion of Octomom region. (C) Two point mutations and one 10 nucleotides deletion. Woolfit *et al*., Genomic Evolution of the Pathogenic Wolbachia Strain, wMelPop, Genome Biology and Evolution, 2013, volume 5, issue 11, 2189-204, by permission of Oxford University Press.

Interestingly, one previous study performed phenotypic selection on *w*MelPop infected *D. melanogaster,* looking for changes in pathogenicity (Carrington *et al.*, 2009). The flies were selected for early and late reproduction, which was supposed to produce more and a less virulent *w*MelPop, respectively. In the light of our data, we expect the flies selected for late reproduction to be selected for lower Octomom copy numbers and therefore, indirectly, for the extended lifespan. However, almost no *Wolbachia* associated effects on longevity were found (Carrington *et al.*, 2009), probably due to insufficient selective pressure

and/or low initial Octomom copy number. It is also possible that the genetic background of the flies has stronger effect than the Octomom copy number. These potential host background effect should be tested, as host encoded genetic suppressors of *Wolbachia* pathogenicity were described before in the context of reproductive manipulations (Hornett *et al.*, 2006). In particular, *Wolbachia*-induced male killing was shown to be absent from some male-killer carrying butterfly populations (Hornett *et al.*, 2006). This proves that the suppressors can arise in the populations and can reach fixation as they confer a strong fitness advantage. Yet, *w*MelPop laboratory origin does not support existence of natural blocking genotypes and unstable nature of its virulence may hinder screening approaches. On the other hand, if *w*MelPop suppressors exist, it may be possible for *w*MelPop-harbouring flies to live in the wild, taking advantage of the protective symbiont without paying the price.

2.4. Potential of *w*MelPop and virulent endosymbiont variants to survive in nature

In nature, genetic conflict between virulent *Wolbachia* and a host may not lead to evolution of genetic suppressors of endosymbiont. The most probable outcome of a virulent mutation is extinction of the infected line. In the absence of sufficient fitness benefits, symbiont could also revert to non-pathogenicity (which is easily achieved by *w*MelPop by Octomom copy loss), or could be lost by *Drosophila*. Until now, we have never observed *w*MelPop imperfect transmission in our multiple Octomom copies lines. However, increasing competition between females at different environmental conditions could show which strategy is preferred and if the *w*MelPop-associated cost both early in life (reduced fecundity – Sara Esteves, unpublished data) and later (longevity) is enough for *Drosophila* to lose its endosymbiont. However, as pathogenic *w*MelPop also provides high protection to viruses, it could be selected for in the

presence of viruses. Finally, *w*MelPop does not kill flies if adults are maintained at lower temperatures (Reynolds *et al.*, 2003), while providing antiviral protection (Chapter 3). Establishment of this variant in natural populations could be possible, depending on the local thermal conditions.

2.5. Implications of Octomom region discovery

We have demonstrated that *Wolbachia* can evolve fast due to gene amplification and despite a low nucleotide substitution rate. This brings in a new mechanism of dynamic evolution in *Wolbachia,* which can be extended to other endosymbionts that have reduced genomes. Octomom instability or selectability has also important consequences for the use of *Wolbachia* to control vector-transmitted diseases.

Finally, we have shown for the first time a link between genotype and phenotype in *Wolbachia:* a set of genes involved in *Wolbachia* growth regulation. These genotype-phenotype links are very rarely established in endosymbionts due to the impossibility to grow many of them *in vitro*. The only strong associations shown before relate to aphid thermal tolerance and *Buchnera* (Dunbar *et al.*, 2007) and aphid protection to parasitoids and *Hamiltonella* (Oliver *et al.*, 2009). The involvement of Octomom genes in *Wolbachia* virulence provides a unique point of entry into understanding *Wolbachia*-host interactions at the molecular level.

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Appendix I - *Wolbachia* variants induce differential protection to viruses in *Drosophila melanogaster*: a phenotypic and phylogenomic analysis

Table S1. Statistics of linear models based on *w*Mel variants titres change over time.

The data for *w*Mel, *w*MelCS_a and *w*MelCS_b analysis are represented in Figure 4D, *w*MelPop data are represented in Figure 6D.

*w*Mel variant	linear model of titres		linear model of log of titres	
	R^2	*p*	R^2	*p*
*w*Mel	0.6863	1.589×10^{-8}	0.6639	4.23×10^{-8}
*w*MelCS_a	0.934	1.801×10^{-14}	0.8691	3.46×10^{-11}
*w*MelCS_b	0.893	4.045×10^{-15}	0.8751	3.592×10^{-14}
*w*MelPop	0.6247	9.473×10^{-5}	0.8146	3×10^{-7}

Table S2. *w*Mel variants phenotypic data for cluster analysis.

Natural logarithm of Cox hazard ratios (CHR), relative to w^{1118} *iso*, of survival to infection with DCV and FHV and long-term survival. Median of relative titres of DCV and FHV, three days after infection, and of *Wolbachia*, three and six days after eclosion.

Variant	DCV CHR	FHV CHR	Longterm CHR	DCV titres	FHV titres	*Wolbachia* titres
*w*Mel	-1.619	-0.637	-0.001	1.880	0.603	0.393
*w*Mel2_a	-1.925	-1.175	-0.008	0.787	0.557	0.380
*w*Mel2_b	-2.145	-0.554	-0.008	1.252	0.527	0.288
*w*Mel3	-1.593	-0.858	0.175	6.539	1.178	0.506
*w*MelCS_a	-2.633	-2.748	1.564	0.514	1.212	0.980
*w*MelCS_b	-2.698	-2.255	0.241	1.095	1.053	1.032
*w*MelCS2_a	-2.728	-2.203	0.075	0.535	0.655	0.912
*w*MelCS2_b	-2.649	-2.209	0.521	0.635	0.184	1.022

Table S3. Synonymous and non-coding SNPs between *w*Mel-like and *w*MelCS-like variants.

Gene predictions according to annotation of AE017196 (Wu *et al.*, 2004). (a) Indicates common ambiguous nucleotide call in the sequence of all *w*MelCS-like variants (IUPAC nucleotide code).

Position	Nucleotide *w*Mel-like	Nucleotide *w*MelCS-like	Gene name
15742	A	G	WD0016
25603	G	A	WD0024
45292	C	T	Non-coding region
89970	A	G	Non-coding region
94978	T	C	WD0103
151274	T	A	Non-coding region
183394	G	A	Non-coding region
201340	G	A	WD0221
278050	G	A	Non-coding region
287099	G	A	Non-coding region
346902	G	A	Non-coding region
372405	A	G	Non-coding region
398613	A	G	Non-coding region
416891	C	T	WD0435
440973	A	C	WD0460
449370	A	G	Non-coding region
463714	T	C	Non-coding region
537479[a]	C	Y	WD0550
537486[a]	T	Y	WD0550
537512[a]	T	Y	WD0550
571424	G	T	Non-coding region
587192	G	T	WD0609
588436	T	C	Non-coding region
654265	T	C	WD0675
738991	A	G	Non-coding region
739429[a]	A	R	WD0766
739618[a]	G	S	WD0766
811613	G	A	WD0847
811613	G	A	WD0848
812321	A	G	Non-coding region
830307	C	G	Non-coding region

Table S3. (continued)

Position	Nucleotide *w*Mel-like	Nucleotide *w*MelCS-like	Gene name
840037	A	G	Non-coding region
854922	T	K	Non-coding region
872208	G	A	Non-coding region
889384	G	A	WD0924
914712	G	C	Non-coding region
917945	A	G	Non-coding region
949888	G	A	WD0992
988727	C	T	Non-coding region
1017650	T	C	WD1055
1135851	T	C	Non-coding region
1145254	T	C	WD1199
1152452	T	C	WD1203
1165158	G	A	WD1217
1183214	C	T	WD1237
1206452	T	C	Non-coding region
1207767	G	A	WD1262
1217973	G	A	WD1277
1247609	A	G	Non-coding region

Table S4. Indels between *w*Mel-like and *w*MelCS-like variants.

a) The type of polymorphism is defined relative to the reference genome AE017196. b) This insertion matches the IS5 insertion in WD1310 described in Riegler *et al.* (2005).

Type [a]	Size	Inserted sequence	Start	End	Gene name	Protein domains
long insertion[b]	unknown	unknown	1251892	1251892	WD1310	P-loop NTPase
deletion	6	none	45287	45294	Non-coding region	-
deletion	1	none	222378	222380	Non-coding region	-
deletion	9	none	864708	864718	WD0898	no predicted domains
short insertion	5	AGAGT	156880	156881	Non-coding region	-
short insertion	1	T	279146	279147	Non-coding region	-
short insertion	1	A	432673	432674	Non-coding region	-
tandem duplication	99	-	537421	537521	WD0550	ankyrin-repeat containing protein

Table S5. Predicted genes present in the *w*Mel Octomom region. Gene predictions according to annotation of AE017196 (Wu *et al.*, 2004). Domains and predicted functions are based on NCBI CD-Search tool (Marchler-Bauer *et al.*, 2013). (a) gene is annotated as a pseudogene, however it contains a valid start site and open reading frame. (b) WD0515 in wMelCS-like variants, including wMelPop, is identical to WD0506.

Predicted gene	Size (aa)	Domain / predicted function
WD0506[a]	329	Reverse transcriptase (RTs) with group II intron origin
WD0507	135	RadC domain - DNA repair protein
WD0508	312	Helix-turn-helix XRE-family like proteins - DNA binding protein
WD0509 /MultL-2	598	MutL - DNA mismatch repair protein
WD0510[a]	146	RNase HI prokaryote like
WD0511	309	PD-(D/E)XK nuclease family transposase – putative transposase, DNA invertase (resolvase), or recombinase
WD0512	1120	-
WD0513	2843	RHS repeat-associated core domain
WD0514	469	Ankyrin repeats
WD0515[b]	329	Reverse transcriptase (RTs) with group II intron origin

Table S6. Summary of comparisons between wMel variants phenotypes.

Comparison	DCV infection	FHV infection	*Wolbachia* levels	Lifespan
wMelCS-like compared to wMel-like	- Better survival - 2-fold lower titres - Higher resistance - Higher tolerance?	- Better survival - No difference in titres - Higher tolerance	- Higher titres - Higher growth rate	- Some lines have reduced survival
wMelPop compared to wMelCS_b	- Strongly reduced titres - Higher resistance	- Strongly reduced titres - Higher resistance	- Higher titres - Much higher growth rate	- Strongly reduced survival

Table S7. Oligonucleotide primers used in real-time quantitative PCR experiments.

(a) published in (Deddouche *et al.*, 2008), (b) published in (Berry *et al.*, 2009).

Target	Forward primer sequence (5'- 3')	Reverse primer sequence (5'- 3')
DCV[a]	TCATCGGTATGCACATTGCT	CGCATAACCATGCTCTTCTG
FHV[b]	ACCTCGATGGCAGGGTTT	CTTGAACCATGGCCTTTTG
Rpl32	CCGCTTCAAGGGACAGTATC	CAATCTCCTTGCGCTTCTTG
wsp	CATTGGTGTTGGTGTTGGTG	ACCGAAATAACGAGCTCCAG
WD0505	TGTTCCTGGTGGATCATCTG	ACGCGAGCATCTTCCATAAG
WD0506/WD0515	TTTGCGTCTTCTTCCCTCTC	ATCAAGGCACACCACAAGGT
WD0507	GCATGACAGGGAAGAAGCTC	CTTTGCAGCTTCCTTTAGGC
WD0508	TCTAGCTTGCGGACAAGAAG	CTGCCTTTCCACTTTCTTCC
WD0509	CCGTATAGCAGCAGGAGAGG	AGTGGCATGCCTCATAAGTG
WD0510	CCACTTGTTGATCCATCCTG	GGCAGCCGTGGTAATGTATG
WD0511	CTTGGCTGCTATTCACGATG	CGAAGCCCTTGGTCTTAGTG
WD0512	ATGCTGCTAATTGGGACTGG	AGGCAATCGACCATACTTGC
WD0513	TTAACCGGCCAGTCTTATCG	AGCATGTCCTCTCTGCCATC
WD0514	CTGTGCCTGAGAATCAAGAGG	CCTTCAAGCGAGGAGATTTG
WD0519	TGCAAGAAGAGAAAATCAAATAAGAG	TCCCTTGTAAGCGTTCTTTC
rpoD	AAGAGGCCTTGATCTGCTTG	CCACCAAGTGCCATAAGTTG
Gmk	ACTGGCAAGGAGCATTTCAC	CGCTCTATTTCGCTTGCATC

Appendix II - Mutualism breakdown by amplification of *Wolbachia* genes

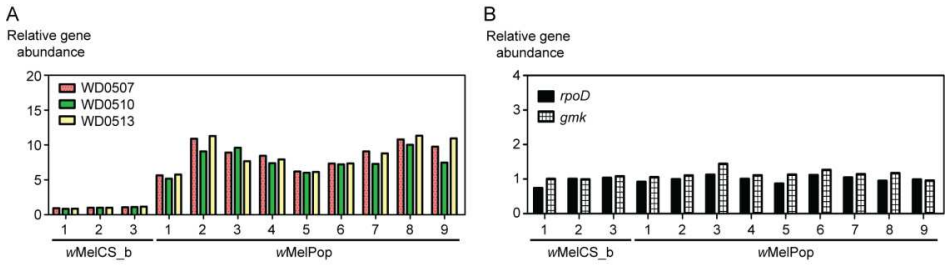

Figure S1. Different Octomom genes are amplified to the same extent in individual *w*MelPop flies.

Octomom genes copy number variability between *w*MelPop *iso* flies relative to *wsp*. qPCR was performed on DNA from single females from the *iso* line three (Figure 1A) for WD0507, WD0510 and WD0513 (a) and *rpoD* and *gmk* (b). *w*MelCS_b flies were used for copy number normalization.

>WD0514_WD0507_Octomom_Junction

TTCTTTATCTCCTCAATCTCCTTTTTTAACTCCCTCACTTTATTTTCAAGCCCGATAATGATTTCCTCTTGATTCTCAGGCACAGT
CTCCATCTCTGAGAGTGACTCGTTAGTAGAACGTTTAAAGCGTTGGCTCTTTACATACGTTGAAACTTCTACCAGCTCACCGGTGT
CTCTTAAATCTTTGAACTCATCTCTTATTTCTGTAAACCTACCATCAAGGTCTCTTTCAACAACTTCAATTTCTTTTCTTACATTG
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AAGATTATTATCAAAAGGTCTGGTAGAGACTCATGACCTTGAAACCAGCGATTTGGTCTCATAACCATAGTCTCCCCAAAAGAACC
GTGCTTGCGGATTTCCCGCACACGGCTCCACAATACAGCGTTCACACTAGTGCTCCTGTGTATAAAGAAACATATACCTTTGGTTT
TGGTAATGGATTTACTTTTAAATAGTGTATAAATTGTTCCCAATCCATACTTTTCTTTTGACTACGTCGGTTTATCCACTTAAATG
CTAACTTTGTTACCGGTCGATAGAATTGAATCAAACACCGATAATTTCCGCTAACTCCGAAGTAGCTATAGTGTCCTGTTAGTTTG
GCTTTAAGTTTCTGCCACCAATCTTTGAGACAGATACGACTTCGTACCATCTTCAACCATTCTTTGATTTCTTTAATCTTTCTGGC
TAGGCTTATTTTTGAAGTTTTCTGCTTCATCATAAGTTTACCATTACGACTTTTTCCACAATAATGTGTAAATCCTAGAAAGTTGA
AGCTAGCCGTCCTACGTTTCTCTCTTTCTGCTTGATACCATTCTTTCTTACCAAACTTTACTATTTTTGTTTTATTTTCAGCTATT
TCCAACCCAAATTTACTTAGTCTTTGTTTCAGTAATTCTAGAAATTCTTTTGCGTCTTCTTCCCTCTCGCAGCCAACTACAAAATC
GTCGCAAAACCTTATTAGCTGTAAATATCCTCTGGCTTTTGGCTTAAATTTCTTTTCAAACCATAAGTCCAGCACATAGTGTAAGT
ATATATTAGCTAAGACAGGGCTTACTATACCACCTTGTGGTGTGCCTTGATCGGTTGCTTTATAACATCCAACTTCGACTATTCCT
GCCTTTAGAAATCGTTTTATTAACCACAATAAATTTGGGTCAGCTATTCGTTCCCTTAGACAATTCATTAGCCATTTATGTTGAAC
ATTATCAAAGAACTTCTTGATATCCACTTCTACAATATAGTTAATTGGTTTGTGCATAACTGCTTTATCTAGAGCGTTTATCGCCT
GATGACAATTTCTTCCTGGTCGAAATCCATACGAGCTGTCCATAAAGTTTGCTTCATAAATATTTTCTAATATCTTCTTTAGCATT
ACCTGTACCAACTTATCTTCCGTTGATGGTATTCCGAGACCGCGCTTCTCTTTGCTCCCGGCTTTGGGTATGTATACCCTTTTCAC
TGGTAGCGGTGATATTGTTTTCTCTTCATACTATCCACTAGTGTTTTAAGTTTCTCTTCTAGATTTTCTCCATAAGCTTCCACTGT
CACACGATCTATACCACAAGCCTTGTTGCGTTTTAGTTCCTTATAACACTCTGCAAGATTCTCTTCATTAATCAAATGAACTAATG
ATGTAAATTTTACTCGCTTATCTTGCTTAGCCCTTACTGCTATCTGGTTTAGTTTTCCTTGCATACTCCTCTAATCTCTGTTGTAT
TGGTATAGCAATCCCTATACCAGTCACTATATTGCTAACCGCTTCCCCTTGTATGTGGCTTTCCCACACTCCGAGTACTATCAGTT
AGTCCGACTTCCTTTGCATCTTCCTCCAGTCCTCGCCTTTATTGACTTGTTCCAGAGTACCCTTACAGGAATACAAAGGATCTCCC
AAGTTCACGTAAGGTCCATCTCAACATATGACGCGGCCTTCGATCCCGATGAGGTGAGTAATATCTTGCCTTAGCGTTATTACCCA
TGTTGCCTTCCACCAAATGTAAAGTGTCAGCCCCCATGACGCGTGAATTACGGGACTCAATACCTTCACTTTCGTTGCACCCTATG
TTGTCCATTCCATTAGCTTTACTACGCTCGTTACCTTACGCAGCATAATGGTTTGTTCCAGGTTGCCGGCTAAGCTTTACCCGTGT
TGGATTTTCACCAACTGTTCCTTACGCACTTCTTGGCGCACACCAATACTCACCCTCAAATATTTTACTATGGTTTCCAAGTTATC
CATTACAGGTCCTTTCTTTAACTCTTCTCTTGGTACTCTCTTTGCAGCTTCCTTTAGGCA

Figure S2. Sequence of new WD0514-WD0507 junction.

The sequencing of the PCR band (Figure 1C) was performed with primers Link_seq_1-7 (Table S3).

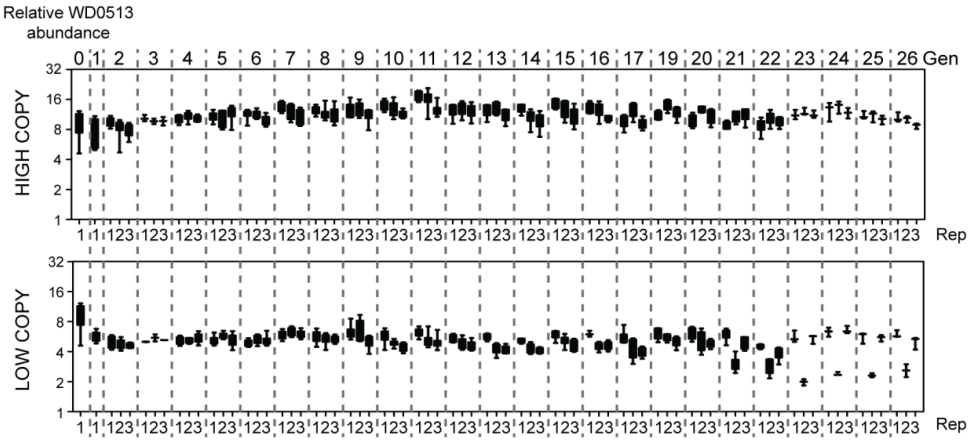

Figure S3. Selection for high and low Octomom copy number in *iso* flies.

Selection for high or low WD0513 copy number *w*MelPop in *iso* flies. The bars for generation zero correspond to the data for *iso* line three from Figure 1A. The female with the highest or lowest WD0513 copy number was always the founder of the next generation. After the first generation three females of high and low copy number gave rise to three replicate lines that were maintained separately for the consecutive generations. The boxes extend from the 25th to 75th percentiles and whiskers include all the values. Dashed lines separate the generations. Gen = generation, Rep = replicate.

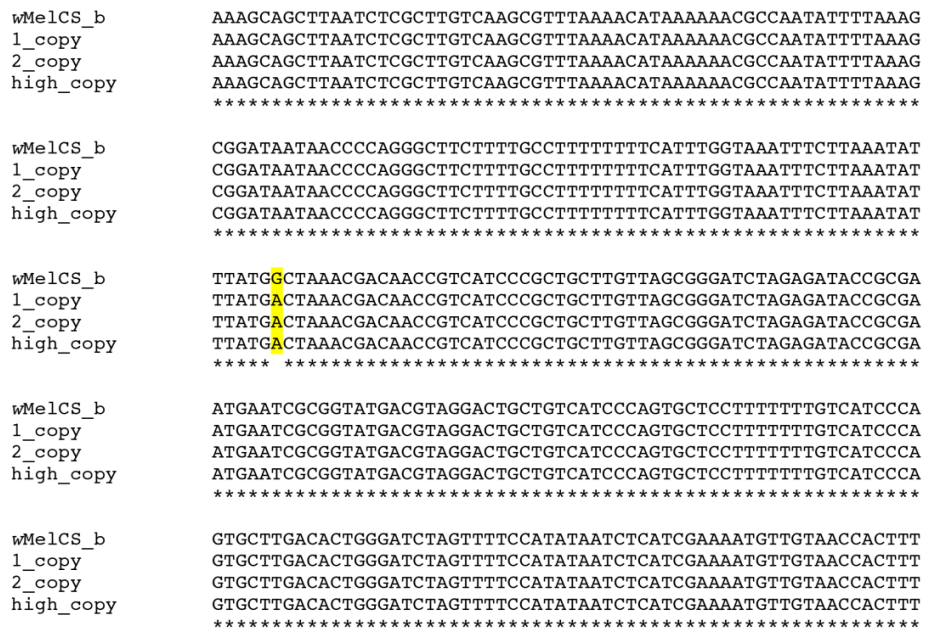

Figure S4. Alignment of the sequences containing *w*MelPop unique SNP site from *w*MelCS_b and *w*MelPop selection lines with one, two and high number of Octomom copies.

CLUSTAL O (1.2.1) multiple sequence alignment (Goujon *et al.*, 2010; Sievers *et al.*, 2011; McWilliam *et al.*, 2013) of sequences surrounding *w*MelPop unique SNP at position 943,443 in w^{1118} selection lines. Position 943,443 for *w*MelCS_b, one copy, two copies and high number of Octomom copies selection lines is highlighted in yellow.

References:

Goujon M, McWilliam H, Li W, Valentin F, Squizzato S, Paern J, *et al.* (2010). A new bioinformatics analysis tools framework at EMBL-EBI. *Nucleic Acids Res* **38**: W695–9.

Sievers F, Wilm A, Dineen D, Gibson TJ, Karplus K, Li W, *et al.* (2011). Fast, scalable generation of high-quality protein multiple sequence alignments using Clustal Omega. *Mol Syst Biol* **7**: 539.

McWilliam H, Li W, Uludag M, Squizzato S, Park YM, Buso N, *et al.* (2013). Analysis Tool Web Services from the EMBL-EBI. *Nucleic Acids Res* **41**: W597–600.

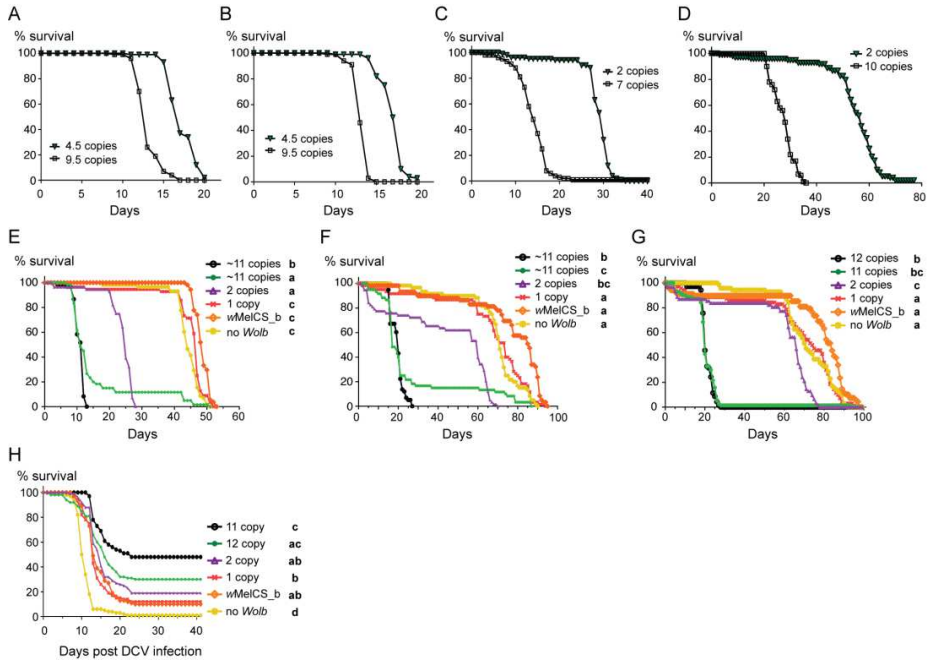

Figure S5. Octomom amplification determines *w*MelPop phenotypes.

(A,B) One hundred *iso* females from high and low selection regimes were checked for survival at 25 °C every day. Mixed effects Cox model fit, high versus low for both replicates, $p<0.001$. (C,D) One hundred w^{1118} females from high and low selection regimes were checked for survival at 25 °C (C) or 29 °C (D) every day. Mixed effects Cox model fit, high versus low at both temperatures, $p<0.001$. (E,F,G) Sixty-seventy females with different *w*MelPop Octomom copy numbers were monitored daily for their survival at 29 °C (E) or at 25 °C (F,G). Females are the progeny from crosses between *iso* and w^{1118} lines. Letters refer to groups of significantly different survival curves according to Tukey's test of all pairwise comparisons of Cox hazard ratios. The experiment at 29 °C is a replicate of the one presented in Figure 3A. Continued on the next page.

Figure S5. (continued) (H) One hundred females with different *w*MelPop Octomom copy numbers were pricked with *Drosophila* C virus (10^9 $TCID_{50}$/ml) and survival was followed daily. Females are the progeny from crosses between *iso* and w^{1118} lines. Letters refer to groups of significantly different survival curves according to Tukey's test of all pairwise comparisons of Cox hazard ratios. This is a replicate of the experiment shown in Figure 3E.

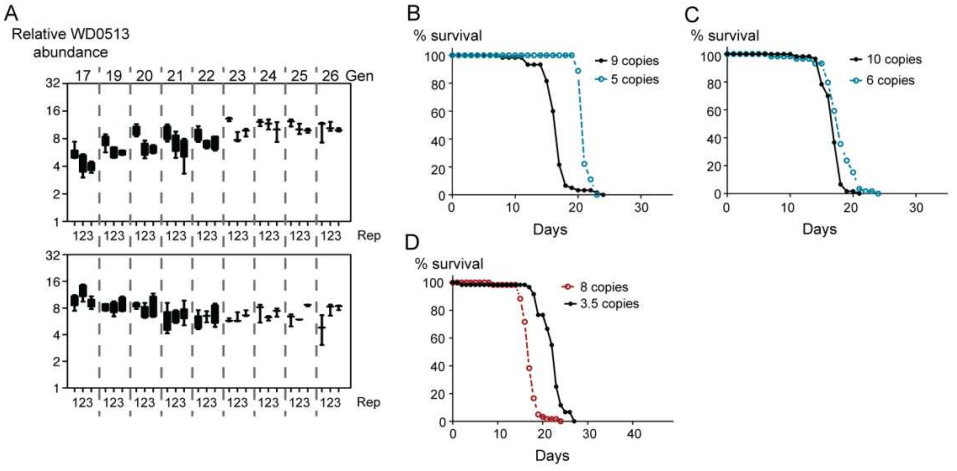

Figure S6. Phenotypic responses to reverse selection.

(A) At generation 17 of the selection for high and low WD0513 copy number *w*MelPop *iso* lines (Figure S3) the selection was reversed. This reverse selection was performed in all three replicate lines from high and low selection regimes by selecting the female with the highest WD0513 abundance from each low copy line and the lowest from each high copy line (forward selection also continued as shown in Figure S3). The boxes extend from the 25th to 75th percentiles and whiskers include all the values. Dashed lines separate the generations. Gen = generation, Rep = replicate. (B,C) Lifespan of females of reversely selected high copy lines was compared with high copy females under the forward selection at generation 22. Fifty females per line were used. (B) High copy line one (9 Octomom copies) *vs* reverse high copy line one (5 copies) (C) High copy line three (10 copies) *vs* reverse high copy line three (6 copies). Tukey's test on the mixed effects Cox model fit, high *vs* low, $p<0.001$ and $p=0.0321$, respectively. (D) Lifespan of females from forward selection low copy line three (3.5 Octomom copies) and corresponding reverse selection line (8 copies) at generation 22. Fifty females per line were used. Tukey's test on the mixed effects Cox model fit, high *vs* low, $p<0.001$.

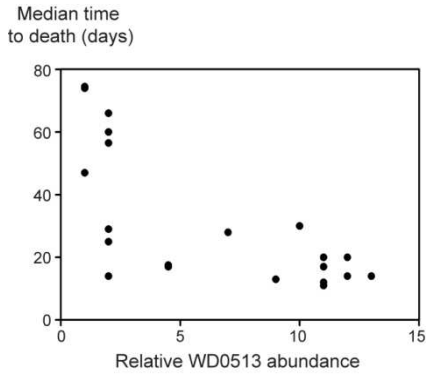

Figure S7. Negative correlation between Octomom copy numbers and host longevity.

Median time to death (days) for lifespan experiments performed (Figures 3A and 5A-G) is plotted as a function of Octomom copy number (relative WD0513 copy number). These data refer to flies with two different genetics backgrounds and two different temperatures. The two variables are negatively correlated (Spearman correlation *rho* = -0.701, $p<0.001$).

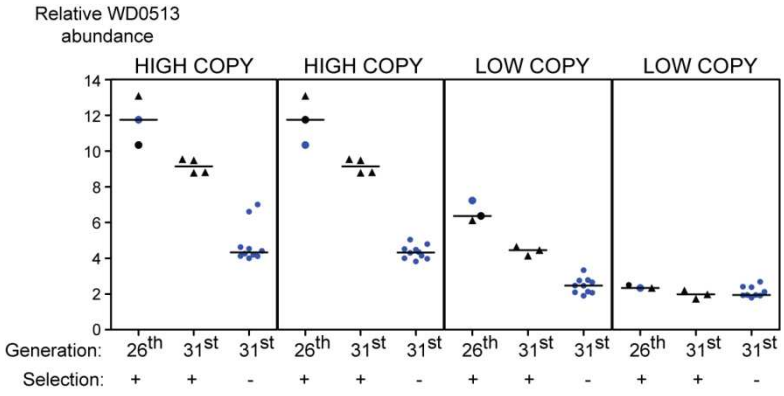

Figure S8. Release of selection pressure leads to a change in Octomom copy number.

Selection was released in *w*MelPop *iso* flies at generation 26. The progeny of single females from generation 26 was kept without any selection for five generations by passing all the flies to a new tube every 20 days. After these five generations ten females per line were scored for WD0513 copy number. Plotted are the original selection lines at generation 26, the same selected lines at generation 31 (the high copy number was selected for ten Octomom copies from generation 29 onwards) and released selection lines at generation 31. The mothers of selected lines are the triangular data points, the mothers of the released selection are the blue circular data points. Lines are medians of the points at each generation/treatment. Octomom copy number decreased in three out of four lines released from selection. The only line that did not show a decrease started with two copies of Octomom.

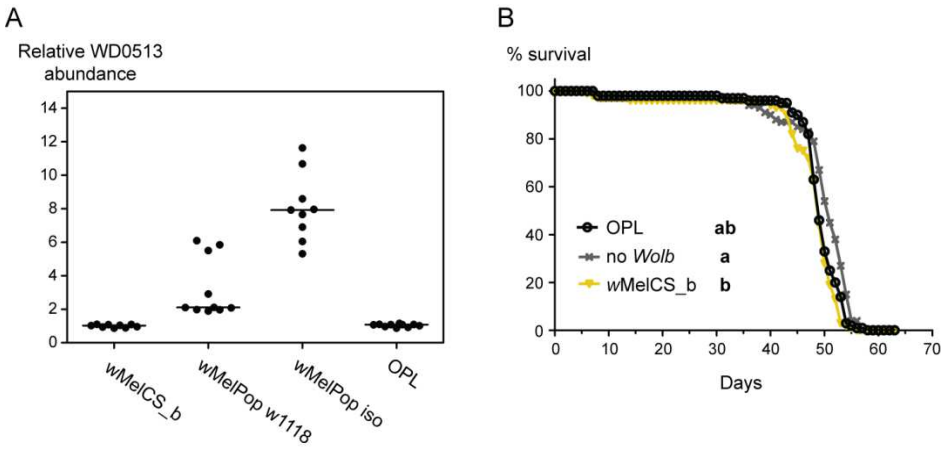

Figure S9. Lack of Octomom amplification and virulent phenotype in a different *w*MelPop stocks.

(A) Comparison of WD0513 copy number within different *w*MelPop *iso* and w^{1118} stocks kept in Teixeira lab from Figure 1A with *w*MelPop stock obtained from William Sullivan lab (*w*MelPop OPL (original Popcorn line)). DNA from single females was extracted for qPCR. *w*MelCS_b *iso* flies were used for copy number normalization, *wsp* was used as a reference gene. Lines are medians of the replicates. (B) Lifespan of females without *Wolbachia*, with *w*MelCS_b and with *w*MelPop OPL. Females are the progeny from crosses between flies of the *iso* and the *w*MelPop OPL genetic backgrounds. One hundred females were collected at eclosion, allowed to mate for 24 h, separated from males and scored daily for survival at 29 °C. Letters refer to groups of significantly different survival curves according to Tukey's test of all pairwise comparisons of Cox hazard ratios.

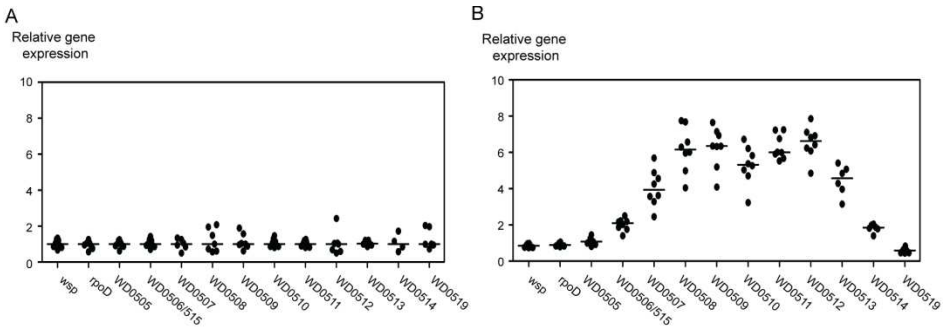

Figure S10. Octomom amplification leads to higher expression of Octomom genes.

Expression of the genes in the Octomom region (WD0507-WD0514), the flanking repeated region (WD0506/WD0515), immediately adjacent region (WD0505 and WD0519) and genes in other locations of the chromosome (*gmk* and *rpoD*) in *w*MelCS_b (A) and *w*MelPop (B). Relative expression for each gene is calculated using *gmk* as a reference gene and is relative to *w*MelCS_b samples. RNA was extracted from eight samples of ten 3-6 days old *iso* males and real time qPCR was performed on cDNA with specific primers. Lines are medians of the replicates. Cycle threshold values for the genes WD0507, WD0513 and WD0514 are high indicating low gene expression levels of these genes. These cycle threshold values fall in a non-linear section of the standard curve making the quantification inaccurate. Moreover, cycle threshold values for some reactions were below the detection limit.

Table S1. Oligonucleotide primers used for sequencing of WD0514-WD0507 junction.

Name	Primer sequence (5'- 3')
Link_seq_1	CCTTCAAGCGAGGAGATTTG
Link_seq_2	GCCTAAAGGAAGCTGCAAAG
Link_seq_3	TTGTTCCCAATCCATACTTTTC
Link_seq_4	TTGGGTCAGCTATTCGTTCC
Link_seq_5	CTACAATATAGTTAATTGG
Link_seq_6	TAACCGCTTCCCCTTGTATG
Link_seq_7	AATGGTTTGTTCCAGGTTGC

Table S2. Genetic background of females used in reciprocal crosses to generate w^{1118} × *iso* hybrids.

***Wolbachia* variant**	**Female genetic background**
no *Wolbachia*	*iso*
*w*MelCS_b	*iso*
1 copy *w*MelPop	w^{1118}
2 copies *w*MelPop	w^{1118}
>10 copies *w*MelPop	*iso* and w^{1118}

Table S3. Selection generation number origin of mothers of the flies used for phenotypic analysis.

Figure	Generation number	
	***w*^{1118}**	***Iso***
1B	5th	-
3A	10th	14th
3B	-	22nd
3C	11th	15th
3D	-	24th
3E	13th	17th
S2	4th	-
S5A	-	3rd
S5B	-	5th
S5C	3rd	-
S5D	6th	-
S5E	8th	12th
S5F	8th	12th
S5G	10th	14th
S5H	12th	16th
S6	-	22nd

Appendix III - High anti-viral protection without immune upregulation after interspecies *Wolbachia* transfer

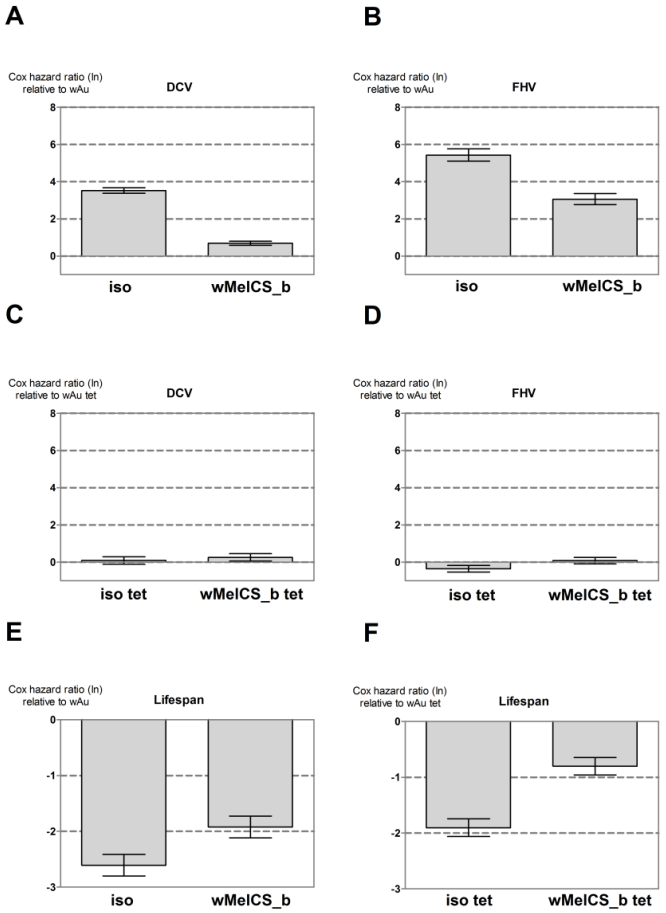

Figure S1. Statistical analysis of survival curves.

(A,B,E) Hazard ratios between either *iso Wolbachia*-free control or *w*MelCS_b carrying line and *w*Au line for: (A) DCV infection, (B) FHV infection, (E) uninfected flies. (C,D,F) Hazard ratios between either *iso* or *w*MelCS_b tetracycline-treated line and *w*Au tetracycline-treated line for: (C) DCV infection, (D) FHV infection, (F) uninfected flies. In all panels error bars represent standard errors of the estimated hazard ratios. The only non-significant differences in Cox hazard ratios are: *iso* tet vs. *w*Au tet for DCV infection (C) and both *iso* tet and *w*MelCS_b tet vs. *w*Au tet for FHV infection (D).

Appendix IV - Temperature dependence of *Wolbachia*-conferred antiviral protection

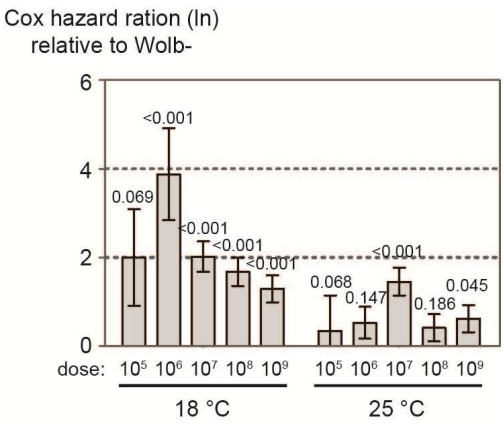

Figure S1. Statistical analysis of survival curves.

Hazard ratios between *Wolb*+ line and *Wolb*- line, raised at 25 °C and then transferred to either 25 °C or 18 °C infected with different doses of DCV.