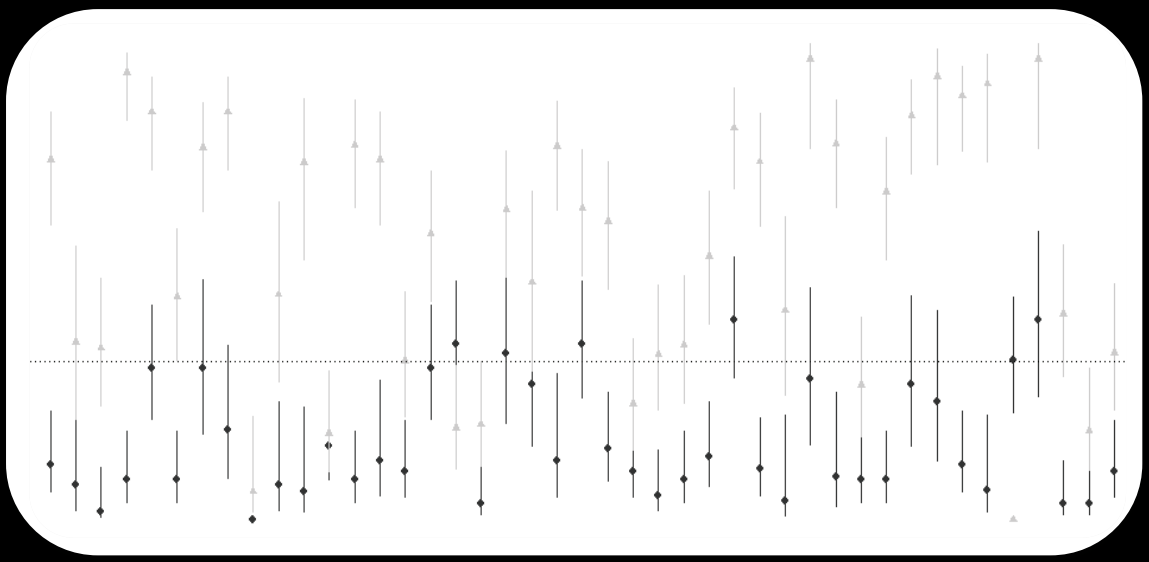
Host-Microbe Interaction and Evolution:

Infection, Symbiosis, Immunity and Adaptation

Vitor Gouveia Faria



Dissertation presented to obtain the Ph.D degree in Evolutionary Biology

Instituto de Tecnologia Química e Biológica António Xavier | Universidade Nova de Lisboa

Oeiras,
October, 2016

INSTITUTO
DE TECNOLOGIA
QUÍMICA E BIOLÓGICA
ANTÓNIO XAVIER /UNL

Knowledge Creation

Host-microbe interaction and evolution: infection, symbiosis, immunity and adaptation

Vitor Gouveia Faria

Dissertation presented to obtain the Ph.D. degree in Evolutionary Biology

Instituto de Tecnologia Química e Biológica
Universidade de Lisboa

Research work coordinated by Doctor **José Élio da Silva Sucena**

Oeiras, October 2016

The study described in this Ph.D. Thesis was performed at Instituto Gulbenkian de Ciência (IGC), Oeiras, Portugal and was financially supported by Fundação para a Ciência e a Tecnologia (FCT), Portugal (SFRH/BD/82299/2011).

Acknowledgements

- Thank you Élio. For everything. For guidance, mentoring and friendship. For being a true scientist and a full humanist. For respecting the differences, despite the differences. For liking the simple, despite the rush. If I ever gave any contribution to Science, it was only possible due to having had the opportunity, in your Lab, to be free to think and to do.
- Thank you Nelson. A big chunk of this Thesis is also yours. So many hours, so many numbers, so many plans. Together we proved that a result can be more that a sum of the parts.
- Thank you Tânia. Helping to guide you was a wonderful choice. You'll always have my support.
- Thank you Lab. For intelligence, dynamism and friendship.
- Thank you Sara and Luís. For discussion, alliance and care.
- Thank you Christian and Viola. For collaboration and for opening the doors for new tools.
- Thank you Patrícia's, Christen's and Luís's Labs. For input, companionship and joy.
- Thank you Fly Facility. For being so helpful and tireless.
- Thank you IGC. For being a sweet home and a pleasant inspiration.

- Thank you to both sides of my beloved transatlantic family. For all support, affection and unconditional love.
- Thank you adored friends. A priceless group of crazy-amazing people.
- Thank you Team Parallel – From Aroeira to Duque, from Barça to anywhere.

Thank you, my dear Inês.
Words were jaded by dreamers. Poets try to rearrange them, craving for survival. Well protected, love always remains in the coherence of actions. 44.

MANIFESTO À DISSEMELHANÇA

Formatemos todos então,
Em nome dos antigos e dos passivos.
Confundamos, vigorosamente, diferença com desigualdade.
Preguemos os não decassilábicos,
queimemos os hereges e todos os que blasfemam.
Certifiquemos que os ovos são todos iguais,
E que todas as terras produzem o mesmo.
Industrializemos as mentes, os gostos e o livre arbítrio.
Promulguemos o poder hereditário,
E ensinemos que o uniforme é a semente do porvir.
Ou então, por não ser assim tão descabido,
Aprendamos simplesmente a dizer não.
Saibamos que não compensa o boicote pessoal
em nome da mera incompreensão.
Que os loucos o sejam até já não o serem,
E que os pintores pintem o que bem entenderem.
Resistamos à inevitabilidade do indiscutível.
Libertemos todos então.

Table of contents

Table of Figures

Table Index

Preface

This dissertation comprises the data obtained during the PhD research project, from January 2012 to October 2016, developed at Instituto Gulbenkian de Ciência (IGC) under the supervision of Doctor Élio Sucena.

This Thesis is structured in 5 Chapters, preceded by a summary written in English and Portuguese, outlining the aims, results and outcomes of the project. The first and fifth Chapters correspond to the Introduction and Final Remarks of this dissertation, whereas Chapters two, three and four contain research work produced during my PhD Thesis.

This Thesis contains 7 published papers and 3 manuscripts in preparation. All papers are signed as first or co-first author. Prologues and Concluding Remarks may contain excerpts of cover letters, author summaries and abstracts.

Summary

Evolution has been shaping the genetic structure of populations across generations, using mutation and recombination, migration, drift and selection to create and/or corrode variation. The array of traits presented by individuals in a population is dependent on several factors, such as their heritability or the genetic pool available to the adaptive process. Additionally, the multitude of complex relationships within and between species creates another level of complexity that can compromise the pinpointing of the contributing factors and their relative weight to such changes. As so, understandably, disentangling the factors that influence the course of evolution in natural populations is of extreme importance but also of great difficulty.

Host-microbe interactions represent a strong and constant pressure to both partners, driven by an intense race between infection and immunity. In this context, a co-evolutionary process can emerge that may lead to a mutualistic relationship mitigating or even suspending hostilities. Considering the multifactorial nature of these interactions, host-microbe relationships are challenging but rewarding models of laboratorial research to assess a vast range of questions. A prominent model is *Drosophila* and its natural microbes: from pathogens, such as DCV (*Drosophila* C virus), to intracellular endosymbionts, such as *Wolbachia.* The complexity of this network, potentiated by the knowledge and tools of model organisms, constitutes a unique system to reveal important features of the evolution of pathogenicity, immune response and symbiosis.

The combination of different methodologies, techniques and technologies is also an efficient way to transversally approach biological questions. For example, experimental evolution studies performed in the laboratory have proven to be a valuable source of information, namely as a

proxy to the evolutionary crosstalk between different species and their influence in adaptation upon certain selective pressures. Also, with the advent of genomics, we can take our understanding of the genetic basis of each phenomenon to a new level.

This Thesis seeks therefore, by combining these different concepts and tools, to understand the evolutionary consequences of host-pathogen interactions and the relative importance of symbiotic relationships to adaptation, at the population, physiological and genetic levels.

We have established laboratory-controlled outbred populations of *Drosophila melanogaster* originally infected with the intracellular symbiont *Wolbachia* and followed their response and genetic configuration upon different pathogenic challenges and different infection routes. We could observe that populations infected with different natural pathogens, *Pseudomonas entomophila* or *Drosophila* C Virus, followed distinct evolutionary paths and immune strategies in their response against these pathogens. Moreover, we uncovered and characterized the corresponding genomic regions of differentiation and several causative genes and alleles, responsible for providing the genetic basis of the selected phenotypes. We further studied if adaptation processes against pathogens entails evolutionary costs in evolved populations. By removing the selective pressures exerted, as well as measuring fitness and other possibly relevant traits, we found that there were no maintenance costs in the ancestral environment, nor trade-offs were observed in tested traits.

We further investigated the evolutionary relationships of microbial endosymbionts and hosts by focusing on the effect of the presence of *Wolbachia* in *Drosophila* populations to the evolution of both parties. Using the fact that the initial population carried different *Wolbachia* haplotypes,

we discovered that the strains that conferred higher anti-viral protection were fixed by selection in virus-adapted populations. This fact makes host selection dependent on endosymbiont genotypes and *vice-versa*. In addition, we show that by removing *Wolbachia* from this equation in the same experimental setup as before, *Drosophila* populations returned to being highly susceptible to viral challenge, but soon recovered by engaging in a new adaptive process. This new wave of adaptation revealed the same previously reported regions of interest and the increase in frequency of highly differentiated protective alleles, differing from the initial *Wolbachia*-free population.

Finally, we explored the mechanisms of *Wolbachia* transmission in a broad evolutionary context. We engaged in a systematic review of the literature, to propose the thesis that endosymbiosis can be a mechanism for rapid and cyclic speciation, provided that vertical and horizontal transmission occurs. In this broadening of the role of endosymbionts in the evolutionary process, we challenged also the view that *Wolbachia* allocated to somatic tissues faces an evolutionary dead-end. We hypothesize that a fraction of the bacteria present in the germarium, and will be vertically transmitted, actually originates from somatic tissues, namely from malpighian tubules. Furthermore, as horizontal transmission has not yet been observed directly in this particular context, we tested cannibalism as a possible route of *Wolbachia* horizontal transmission. We could not observe this process and conclude that, if existing, it is either occurring very rarely or requiring additional factors not included in our experimental setup.

Altogether, the results here presented contribute directly and in different ways to our understanding of the evolutionary processes and consequences underlying host-pathogen and symbiotic interactions.

Resumo

A Evolução tem vindo a moldar a estrutura genética das populações ao longo de gerações, usando mutação e recombinação, migração, deriva e seleção para criar e/ou corroer a variabilidade. A gama de caracteres apresentados por indivíduos numa população depende de vários factores, tais como a heritabilidade ou o *pool* genético disponível para o processo adaptativo. Adicionalmente, a multiplicidade de complexas relações intra- e inter-espécies, cria um outro nível de complexidade que poderá comprometer a determinação dos factores envolvidos e a sua contribuição relativa para tais mudanças. Assim, compreensivelmente, destrinçar os factores que influenciam o decorrer da evolução em populações naturais é extremamente importante porém bastante difícil.

As interações patogénio-hospedeiro representam uma pressão elevada e constante para ambos os intervenientes, advinda de uma intensa batalha entre infeção e imunidade. Neste contexto, poderá emergir um processo co-evolutivo, podendo levar a uma relação mutualista, mitigando ou mesmo suspendendo as hostilidades. Tendo em conta a natureza multifactorial destas interações, as relações hospedeiro-microorganismo são desafiantes porém recompensadores elementos de investigação laboratorial para abordar um vasto leque de perguntas. Um modelo proeminente consiste de *Drosophila* e seus micróbios associados: desde patogénios como o DCV (*Drosophila* C virus), até aos endosimbiontes intracelulares, como a *Wolbachia*. A complexidade desta rede, potenciada pelo conhecimento e ferramentas existentes em organismos-modelo, revela propriedades importantes da evolução da patogenicidade, resposta imunitária e simbiose.

A combinação de diferentes metodologias, técnicas e tecnologias é também uma forma eficiente de abordar transversalmente questões

biológicas. Por exemplo, estudos de evolução experimental realizados em laboratório têm provado ser uma valiosa fonte de informação, nomeadamente como representantes do diálogo evolutivo entre diferentes espécies e da sua influência na sua adaptação quando sujeitas a pressões selectivas. Acresce que, com o advento da genómica, podemos estender a nossa compreensão da base genética de cada fenómeno para um novo nível.

Esta Tese procura, portanto, ao combinar estes diferentes conceitos e ferramentas, compreender as consequências evolutivas das interações patogénio-hospedeiro e a importância relativa das relações simbióticas para a adaptação, aos níveis populacional, fisiológico e genético.

Estabelecemos populações *outbred* de *Drosophila melanogaster* controladas em laboratório, originalmente infectadas com o simbionte intracelular *Wolbachia* e seguimos a sua resposta e configuração genética face a diferentes investidas de patogénios por diferentes vias infecciosas. Pudemos observar que populações infectadas com diferentes patogénios naturais, *Pseudomonas entomophila* ou *Drosophila* C Virus, seguiram vias evolutivas e estratégias imunitárias distintas na sua resposta contra estes patogénios. Mais ainda, deslindámos e caracterizámos as respectivas regiões genómicas de diferenciação e vários genes e alelos responsáveis pela base genética dos fenótipos selecionados. Estudámos também se os processos adaptativos contra patogénios implicam custos evolutivos para as populações evoluídas. Ao remover as pressões seletivas exercidas, bem como medindo *fitness* e outros caracteres possivelmente relevantes, descobrimos que não existem custos de manutenção no ambiente ancestral, nem foram observados *trade-offs* nos caracteres testados.

Investigámos ainda as relações evolutivas de endosimbiontes microbianos e hospedeiros, tendo como foco o efeito da presença de

Wolbachia em populações de *Drosophila* para a adaptação de ambas as partes. Usando o facto de que a população inicial continha diferentes haplótipos de *Wolbachia*, descobrimos que as estirpes que conferiram maior proteção anti-viral foram fixadas por seleção nas populações adaptadas contra vírus. Este facto faz com que a seleção do hospedeiro seja dependente do genótipo de endosimbiontes, e *vice-versa*. Adicionalmente, mostrámos que ao remover a *Wolbachia* desta equação no mesmo *setup* experimental, regressa a alta susceptibilidade à infeção viral nas populações de *Drosophila*, seguindo-se contudo uma rápida recuperação através de novo processo adaptativo. Esta nova onda de adaptação baseou-se nas mesmas regiões do genoma já reportadas, mais especificamente no aumento de frequência dos alelos mais protectores anteriormente identificados na presença de *Wolbachia*.

Finalmente, explorámos os mecanismos de transmissão de *Wolbachia*. Através de uma revisão sistemática da literatura, postulámos que a endossimbiose pode constituir um mecanismo de especiação rápida e cíclica, desde que ocorra transmissão vertical e horizontal. Neste alargamento do papel dos endossimbiontes no processo evolutivo, desafiámos também a perspectiva de que a *Wolbachia* alocada aos tecidos somáticos esteja perante um beco evolutivo. Colocamos a hipótese de que uma fração das bactérias alocadas ao germário, que será transmitida verticalmente, tenha a sua origem em tecidos somáticos, nomeadamente nos túbulos de malpighi. Adicionalmente, como nunca foi observada transmissão horizontal neste contexto em particular, testámos o canibalismo como uma possível via de transmissão horizontal de *Wolbachia*. Este processo não foi observado e concluímos que, caso exista, ocorrerá raramente ou requererá factores não contemplados no nosso desenho experimental.

Em conjunto, os resultados aqui apresentados contribuem diretamente e em diferentes formas para a nossa compreensão dos processos evolutivos e suas consequências para as interações patogénio-hospedeiro e as interações simbióticas.

1

General Introduction

An overview on host-microbe interaction and evolution

1.1. Evolutionary strategies as the basis to life perpetuation

As soon as life began on our planet, the necessity for survival strategies became a *sine qua non* requisite for lineage perpetuation. Charles Darwin, together with Alfred Wallace, structured the idea that the major driver of evolution would be Natural Selection. This idea is based on competition, where well-adapted individuals that respond against selective pressures will have higher fitness, generating more offspring. This progeny will carry the parental information and, by (re)combining with other characteristics on each subsequent generation, will go through an adaptive trajectory without a pre-defined destination. With the discovery of the rules of heredity by Gregor Mendel, also in the middle of the 19th century, and subsequently its genetic basis, the DNA code, a direct relationship between phenotype and genotype was built. It was possible to begin to understand the dynamics of populational structures, as well as the creative and destructive forces of variation, responsible for the evolution and diversity of species.

These evolutionary paths lead to the continuous emergence and extinction of lineages that, by ecological necessity, have to interact recurrently with each other. Several strategies arise from these interactions, marked by different selective pressures that drive this process over time, such as the access to nutrients or fighting for habitat. The Thesis here presented focuses mainly on one of these transversal selective pressures, which can be more or less intense, but certainly critical for the organisms on our planet: the host-microbe relationships.

Infection and immunity: the two sides of an endless arms race

Over many millions of years, the evolution of life on earth enabled the emergence of an extensive variety of organisms, which exhibit strategies of perpetuation with different degrees of complexity [1]. Uni- or multicellular organisms are able to use diverse base resources for their metabolic functions, and all organisms that survive and spread part of their genetic information need to attack and defend. Throughout the tree of life, specialization in one of these two faculties is common, as well as a balance between both.

Microorganisms, small and ancestral forms of life, inhabit almost all habitats of our planet [2–6]. This ability to explore a variety of resources is based on an enormous capacity to adapt, using strategies ranging from a fast generation time to recurrent lateral gene transfer [7–9]. Apart from the lineages that have specialized in extracting resources from abiotic substrates, many others evolved to obtain the supplies required for survival from other living organisms [10,11]. However, while some thrive without causing injury to its partners (at least temporarily on the evolutionary scale), others depend or take advantage of the energy stored by them, triggering a cost.

Infectious agents, such as pathogens and parasites, are an example of the latter group, which have built their strategy on the ability to explore, directly or indirectly, other individuals, causing disease. These organisms are able to exploit the available resources and multiply inside or on the surface of their usually larger hosts. Classic examples of these infection agents are viruses [12,13], prions [14,15] or bacteria [16], but also multicellular organisms, such arthropods [17,18], helminths [19,20] or fungi (also unicellular) [21,22]. Some of these are designated as primary pathogens, characterized by having developed a strategy to directly infect

healthy hosts, requiring these individuals for completion of their life cycle. Others, called opportunistic pathogens, take advantage of periods of debility of hosts to establish the infection [23]. With the advancement of fundamental and medical research, special attention has been paid in the attempt to understand how these invading microorganisms operate. Comprehending and recognizing the mechanisms of pathogenic infection is the first step to uncover how the host-parasite relationships evolve, and thus hope to develop strategies to tackle them.

Hosts, on their side, have developed several response strategies and defence mechanisms to riposte against pathogen attacks. Some are generic and conserved across large groups of organisms, whilst others are particular specializations resultant of host-pathogen co-evolution. The most established group of protective adaptations consist of the immune system.

Generically, as an evolutionary gain shared by a wide range of multicellular organisms, we find the innate immune system. Innate immunity relates to the set of physical, chemical and even biological barriers (created by the presence of others species) that impedes the entry of pathogens, including cellular barriers, which fight infection after a contact [24,25]. The innate immune system protects from infections and responds in a general way, but not necessarily in a non-specific manner, where cell receptors with high affinity to specific transversal proteins in numerous invaders are also present [26].

In jawed vertebrates, and analogously in agnathans, an even more complex defensive structure evolved: the adaptive immune system [27]. With the co-option of DNA-editing proteins, namely RAG (Recombination Activating-Gene) and AID (Activation-Induced cytidine Deaminase) to immune functions, this group developed an outstanding evolutionary novelty [28–30]. Throughout the development of the organism, but also

across life, these animals originate specialized hematopoietic cells, the lymphocytes, which display a virtually endless variety of rearranged defensive receptors that may recognize the invading proteins (non-self). Protein fragments are exposed in cell surface by the Major Histocompatibility Complex (MHC), a process called antigen presentation, allowing the activation of T lymphocytes. From that, a refined immune regulation by T lymphocytes and a specialized production of reactive proteins (antibodies/immunoglobulins) by B lymphocytes, closely articulated with the innate defensive mechanisms, create an effective and efficient protective network (for review see [25]). The diversity of receptors necessary to attack a massive variety of invasive agents is only possible through the rearrangement of gene segments and high mutation rate of the genes involved as source material for the process [31]. In this way, progressively in the phylogenetic tree, this group of animals was capable of maximizing the diversity of receptors without exponentially increasing the size of the genome, which is itself a known selective pressure on organisms [26].

In the process of creation of this undifferentiated army for specialized attacks, the lymphocytes are tested with self-proteins and the autoreactive clones are eliminated before entry into circulation. The released lymphocytes that are stimulated by non-self proteins will multiply and trigger an extremely effective systemic response. Moreover, this response mechanism acts not only when facing a particular infection, but also allows a capacity of a large-scale response in future infections from the same pathogen (or any other with the same epitope recognized by this receptor) [25]. This characteristic of memory of infection in adaptive immune system is the basis for effective immunization programs developed by humans, namely vaccination against several pathogens. Such adaptation is a remarkable evolutionary strategy that although configured as very

efficient, has been associated to a significant expenditure of energy. This is because the majority of the produced lymphocytes are useless, being automatically destroyed when detected as auto-reactive, or never being activated by a compatible ligand to its receptor.

Moreover, as important as the effectiveness of the adaptive immune system against infections, is the quality of the regulation of autoreactive defensive cells elimination. Several studies in mice, and also clinical cases in humans, have shown the adverse effects of errors in this process [32,33]. This observation suggests that acquired immunity must be under an extreme selective pressure, either positive or negative, according to the success (immunity) or failure (auto-immunity) of the process. Therefore, it is essential that organisms balance the direct and indirect costs of increasing the immune response versus spending resources on reproduction, according to the selective pressure imposed by each feature.

Nevertheless, the possibility of self-damage is not exclusive to the adaptive immune system. The innate immune system is also conditioned by the collateral effects of defensive activity, triggered by, for example, the deregulation of pH, reactive oxygen species (ROS) levels or cell damage by phagocytosis [34–38].

Another interesting defensive line of many organisms is based on the evolution of behaviour. Although not usually considered a generalized mechanism, but more as specific adaptations resulting from co-evolution, behavioural barriers are extremely successful in some cases [39,40]. They consist in the avoidance of pathogens (or even toxic substances produced by other organisms), identified by the potential hosts with sensory characteristics, such as distinctive flavours or smells.

Still another type of defence, belonging to a field that is increasingly growing in knowledge and relevance, is symbiotic immunity, where the

presence of some microorganisms, such as the gut microbiota, can protect the host from the potential entry of harmful microbes [41,42]. Intracellular endosymbionts belong to yet another level of symbiotic immunity, which directly or indirectly (as we will see later), can increase host's fitness helping to prevent or fight pathogenic infections.

Thus, with so many defensive barriers, it is necessary for microorganisms to adapt quickly, avoiding or neutralizing the evolved arms of the potential new hosts. However, a very fast and violent adaptation by specialized pathogens can be a dead-end, disappearing by increase of virulence to the point of decimating their hosts. Therefore, this battle has a very large number of constrains for both sides, creating a necessary balance between different strategies.

1.2. The paths for host-microbe evolution

The constant battle where pathogenic virulence and host defence are committed can be sustained for a long time, triggered by the continuous adaptation of both sides. In 1871, Lewis Carroll described in one of his famous books how a enigmatic Red Queen explained to Alice that, in her country, she had to run as fast she could just to stay in the same place, and, to go further, it was necessary run twice as fast. This puzzling vision, also entitled Red Queen Paradigm, was later applied to some evolutionary processes, where host-pathogen interaction is a perfect example [26]. The idea that it is necessary keep adapting just to maintain the balance of forces cannot be more appropriate between infection and immunity. In order that one part gains some advantage, it is essential to create new strategies even faster, where the respective evolutionary consequences, beneficial or costly, will be proportional to the magnitude of the change.

As a result of the intrinsic constraints on this process, the frontier between parasitism and mutualism can be a thin line in the coevolution of host-microbe systems, where the outcomes are not always strict [43,44]. Symbionts can be mutualistic or pathogenic according to the environmental conditions, such as temperature fluctuations or nutritional status, stimulating and further complicating the evolution of these interactions [45,46] . Therefore, in this shared road of action-reaction between both players, fitness is constantly brought into question, proportionally to the degree of interdependence. According to that, the level of host-microbe specialization may be decisive for the increase of hostility or, on the other side, for a mutualistic path, a necessary commitment for the good of both [47].

To better understand the possible outcomes of these relationships it is important to realize that all host-microbe pairs establish a type of physical interaction, and the nature of this contact will condition the future of the relationship [48]. From parasitism to mutualism, symbioses can be categorized by these types of physical interface between players, namely ecto or endosymbiosis. Ectosymbiosis includes interactions between organisms outside the host's cells, more specifically on the organism's surface. The perpetuation of this type of relationship is based on horizontal transmission, creating *de novo* infections across individuals and generations [49].

In endosymbiosis, the central feature is the presence of a microorganism within the host, intra or extracellularly. This type of relationship is widespread across a vast range of phylogenetic groups [50]. Actually, some basilar and diagnostic characteristics of the kingdoms of life on Earth are defined by the occurrence of intracellular endosymbiosis in the past [51,52]. The prevailing theory clearly points that the origin of

mitochondria or plasts are resultant of successful intracellular symbiosis, a process called symbiogenesis [53–55]. These examples seem to show that a putative infection can originate massive adaptive advantages over time, constructed by the intense coevolutionary process. Thus, as attested by the history of life, this path to mutualism can generate great rearrangements of evolutionary novelties, such as metabolic functions.

In invertebrates, a wide range of endosymbionts has been described in the last decades [56], revealing several symbiogenic processes in distinct steps of coevolution with hosts [57]. For example, in insects, although diverse and presenting different strategies, the identified heritable symbionts can no longer be perpetuated, or even replicate, without the host. On the host's side, some also cannot develop or reproduce without the endosymbiont, making these microorganisms classified as obligatory (or primary) [58]. Obligatory endosymbionts result from a prolonged coevolution with the host, reducing their genome and usually concentrating in specialized organs, the bacteriomes, which can vary in cellular origin according to the host group. One central model of the host/obligatory endosymbiont relationship is aphids and their endosymbiont *Buchnera aphidicola* (a generic name for the related obligatory endosymbiont of all aphids), which shows the typical characteristics of these relationships mentioned above [59–61].

On the other hand, those endosymbionts that are not vital to the host are designated as facultative or secondary. Contrarily to the obligatory symbionts, facultative symbionts retain their ability to invade new tissues and hosts (with different levels of effectiveness), showing an irregular presence among host tissues and populations [58].

For the endosymbionts that have the capacity of perpetuation by horizontal transmission, the escalation of virulence will increase fitness

since they will always have available another host to infect. Thus, the limiting factor is not the survival of the current individual host but, instead, the viability of the host population as a whole (constituted by the set of infectable individuals, from one or more species). When endosymbionts start to lose the capacity to invade new hosts, progressively adapting to a vertical transmission strategy (maternal and/or paternal) as the mechanism of heritability, the fitness of the current host becomes a key feature. Thus, in this case of intensification of dependency, the fitness of their host and respective progeny is increasingly important, being then directly interconnected. Simultaneously, the door is open to the reduction in virulence and construction of a mutualistic relationship, triggered by the elaboration of new strategies that will increase the fitness of both sides [62,63].

Coupled with the process of the suspension of hostilities and a joint perpetuation, evolution can now use the genetic redundancy between these new allies as raw material to further accelerate the evolution of this new lineage. Redundancy relieves the selective pressure on copies of genes that encode proteins performing similar functions, allowing the sub or neofuncionalization of genes (or alleles) on one side, or even leading to their disappearance [64–66]. Over time, this path consolidates the irreversibility of the process and turns what used to be two entities into one. The above-mentioned examples, such as alpha-proteobacteria and cyanobacteria, which respectively originated the mitochondria and chloroplast, are great examples of current results of ancient symbiogenic processes.

It is thus clear that the invasion/internalization process, which initially resulted from a pathogenic infection, can evolve into a beneficial system for this new combined entity, in which one fails to survive without the other. However, throughout this process, it is also possible to loose the

endosymbiont: firstly, through elimination by selection while it is not obligatory to the host, or, after this step, by replacement with of a new endosymbiont that satisfies the necessary vital functions and adds extra adaptive advantages [61,67–69].

In these relationships, where the fitness of host arthropods and endosymbionts are now interconnected by vertical transmission, a possible mechanism that could effectively spread the proliferation of this new entity is the modification of the reproductive rules of the host species. These mechanisms are triggered by some endosymbionts to increase their own transmission, using for this the increase of the progeny of the host. In insects, several species of facultative endosymbionts can modify the host reproduction, such as bacteria of Genus *Cardinium* [70–72], *Arsenophonus* [73,74] or *Spiroplasma* [75–77]. However, and now approached further, *Wolbachia* is the most paradigmatic and well-studied case, triggering different manipulations in a vast spectrum of hosts.

Although a large range of immune responses are found along the tree of life, this Thesis focuses in the immune response of invertebrate animals, and in particular *Drosophila*, which as described further on, presents itself as the central model for the studies in this Thesis.

1.3. *Drosophila* as a transversal host-model

In this long road of scientific discoveries, some organisms have become central in biological research [78]. Among them, with special emphasis on the study of invertebrates, but also with a huge impact on the understanding of Vertebrates, *Drosophila* flies occupy a central place [79]. *Drosophila* is a Genus belonging to Family Drosophilidae (Order: Diptera; Class: Insecta; Filo: Arthropoda), generically designated as “vinegar flies” or “fruit flies”. This Genus includes more than 1,500 species, with large

differences in appearance, behavior and reproductive habitats [80–82]. In particular, one species, *Drosophila melanogaster*, has been massively used as an *in vivo* model organism in basic and applied research across several areas [83]. The popularization of *Drosophila* started with the morphological and genetic experiments by the geneticist Thomas Hunt Morgan, whose lab contributed fundamentally to the establishment of a chromosomal theory of inheritance [84]. To achieve that, and still today in all fields where these flies are used, several characteristics of *Drosophila* were key. A short generation time (from 2 to 4 weeks), the easy and not expensive maintenance and the possibility to keep a high effective population size, make this organism an accessible genetic analysis tool. Posteriorly, a vast range of public genomic resources and genetic tools allowed an orchestrated manipulation of physiology, genomics and proteomics at the global scale. Great examples of these tools are the GAL4/UAS system (upstream activation sequence) [85,86] or the RNA interference constructs [87,88]. But also the easy access to transgenics banks or genomic libraries and platforms, with thousands of sequencing and annotated genomes, creating a very efficient network to approach several biological questions [89–92]. On top of that, a large community of fly users, sharing tools and knowledge but also using different developmental stages and tissues, exponentially increased the speed at which discoveries under complementary fields are made.

Moreover, the tools generated in this model, associated with diverse natural interacting microorganisms, also make *Drosophila* an excellent host for studies of the symbiotic relationship [93,94]. As a consequence, *D. melanogaster* was also established as an effective test-model for human pathogens, by taking advantage of the conservation of some immune mediators and pathways to better understand the specificities of microbes, including of our species [95–97]. As a result of this context, much has been

learned about the immunity and immune response mechanisms against infections, but also about the strategies to suspend or relieve immunological hostilities in order to live with mutualistic microorganisms.

Half a century of *Drosophila* immunity

As stated before, *Drosophila* is a global model to several research areas in Biology. Moreover, the evolutionary conservation of pathways and transcriptional regulators transform *Drosophila* in a very important platform of experimentation across species [98,99]. The attention on *Drosophila* immunity starts in Hans Boman's Lab, that in 1972 recognized for the first time an inducible humoral immune response [100], opening the road to the finding and understanding of antimicrobial peptides (AMPs) production and regulation. Almost half a century since this game-changing event, many lessons have been learned about the multifaceted immune response of *Drosophila*, serving as paradigm to insect immunity (for review see [99,101]).

Two main features define the immune response's progression: the route of infection and the type of invading microorganism.

In the natural environment, ingestion appears to be the most common port of entry of microbes into the host's body. For microorganisms that need to access the digestive system to complete their life cycle, this is a quick way to ensure horizontal transmission in a cyclic colonization manner [102]. Moreover, this may be an eased path because of the constant energy availability on this milieu. However, the digestive system has several tools to combat an infection or colonization, selecting microbes equipped to avoid these defences [103]. Across this road, the microbes will have to fight against a particularly aggressive environment, including low pH and

digestive enzymes, as well as local production of reactive oxygen species (ROS) and AMP (37). After bacterial ingestion, the epithelia of the midgut increases the production of ROS by dDuox enzyme activity [104]. Then, bacteria that survive these initial challenges in the gut can have their own peptidoglycan fragments recognized by peptidoglycan recognition proteins (PGRPs), responsible to trigger AMP production by the epithelium [105]. This immune response is conducted by the Imd (Immune deficiency) pathway and is mediated by the nuclear translocation of the (NF)-κB transcription factor Relish [106,107].

Some pathogens have evolved not only to survive these local responses but also to cross the protective barriers of the gut and become lodged inside the organism, systemically, intracellularly, or even inside other organs [108]. In other cases, direct systemic invasions from wounds in the cuticle, or through parasitoids vectors, may also occur [108]. When this happens, AMP production is triggered in the fat body, and systemically released to fight infection [109]. In parallel, plasmatocytes start their phagocytic activity, also having their independent internal AMP production [110]. Moreover, other hematopoietic lineages can perform other immune functions, namely lamellocytes, which perform encapsulation and crystal cells that undergo melanization [111].

AMPs are thus one major tool in the fight against pathogens. Unlike the local immune response of epithelial tissues, the systemic response is not only controlled by the Imd pathway, but also by the Toll-pathway [112]. While Imd is mostly activated by Gram-negative bacteria, the Toll pathway is deployed in response to Gram-positive bacteria and fungi, leading to the production of AMPs in the fat body [113]. The Toll pathway, similarly to Imd, is also activated by the recognition of bacterial peptidoglycan by PGRPs. However, while Imd pathway receptors PGRP-LC and PGRP-LE are activated by the recognition of diaminopimelic acid peptidoglycans (from

Gram-negative and some Gram-positives), Toll pathway receptors PGRP-SA and GNBP1 are activated by lysine-type peptidoglycans (Gram-positive bacteria) and the receptor GNBP3 by β-glucans (yeast). This binding to Toll pathway receptors, as well as other danger signals coming from the body, lead to cleavage of the Toll ligand Spätzle, activating the transcription factor DIF, that will trigger the production of some AMPs, for example Drosomycin (for review see [101]).

Therefore, independently of the route of infection or species of microbe, different defensive layers will be ready to act. However, some pathogens can overcome these defensive barriers and proceed to successful infection, such as the bacterial species *Erwinia carotovora*, *Serratia marcescens* and *Pseudomonas entomophila*. The latter is studied as an oral and systemic infection model – and also a central element in many works presented in this Thesis. *P. entomophila* infects both adults and larvae, leading to death in a few hours by destruction of the midgut [114]. *Drosophila* attempts to fight the infection with AMP production, especially with local diptericin [115]. However, *P. entomophila* can evade *Drosophila*'s immune response. One of the key weapons to achieve escape from the gut defense system is in the production of the zinc metalloprotease AprA [115], although a wide range of other putative virulence factors are produced during infection [116].

Another successful case of systemic infection in *Drosophila* is viral pathogens. Several virus have been identified in flies [117]. Sigma Virus (DmelSV; Rhabdoviridae), Nora virus, (Picornavirales) [118] and *Drosophila* C Virus (DCV) are some of the most studied. The latter, also central to the work further presented herein, can cause a potent infection in *Drosophila*, both systemically and orally.

Drosophila, as well as the majority of insects, predominantly

responds against viral infection through RNA pathways. Despite other two related RNAi pathways having been described, namely micro-RNA and PIWI-RNA, the small interfering RNA (siRNA) pathway is the most relevant to this response (for review see [119–121]). This pathway is dependent on Dicer-2 protein, that produces small interfering RNA that marks the viral RNA to degradation, consecutively blocking replication [122–124]. Additionally, Imd and Toll pathways have also an important role against viral infection [125,126], as well as the Jak-STAT pathway [127], although the mechanism of regulation is not fully understood.

DCV expresses a suppressor of RNAi, the protein 1A that prevents the Dicer-2 activity [128]. Interestingly, the 1A protein of the closely related virus, cricket paralysis virus (CrPV), has a different target (the endonuclease Ago2), making a much faster and lethal infection than DCV [128]. This evidence shows how close species of virus can rapidly evolve different strategies of infection by changing the targets of action.

Despite several successful strategies developed by pathogens, the enormous success of artropods, in particular insects, shows that the immune response mechanisms are truly effective, making them prosperous in the midst of their natural invaders. However, while most of the invading microorganisms need a cyclic colonization of their hosts, heritable endosymbionts are permanently inside, only depending on the host's reproduction to ensure their perpetuation [58, 129].

As discussed above, the presence of endosymbionts is widespread in insects, and its particularities drive the evolution to a different path. These relations reveal the outcomes that enable those processes, such as the increase of tolerance or avoidance of immune response activation. In *Drosophila*, two Genera of intracellular endosymbionts can be found: *Spiroplasma* and *Wolbachia* [75,130]. It is possible to find both in the same

individual, where the abundance and location of one is related to the presence of the other [131].

Spiroplasma is a helical and motile bacterium, related to Gram-positive bacteria, which can be located both intra or extracellularly [132]. *Spiroplasma* is invisible to the immune response of *Drosophila*, as it does not induce the expression of antimicrobial genes [133,134]. This is likely due to the absence of wall structure components. Even more remarkable – and central for the Thesis here presented – *Wolbachia* is an even bigger case of endobacterial success, being the comprehension of its strategies a central piece for the understanding of virulence equilibrium and endosymbiosis evolution.

1.4. *Wolbachia pipientis*: a widespread and multifaceted endobacterium

Among the several endosymbiotic relationships observed in invertebrates, *Wolbachia pipientes* certainly stands out, not only by the frequency of infection but also by the number of phenotypic changes that it can cause to its hosts. *Wolbachia* was first identified in 1924 by Hertig & Wolbach [135] in reproductive tissues of the mosquito *Culex pipientis* [136]. *Wolbachia* belongs to the Rickettsiales Order, a genetically diverse group of gram-negative alpha-Proteobacteria that mainly has the characteristic of cellular endosymbiosis and a remarkable reduction of genome size as a consequence of the co-evolutionary processes [137]. Although it can be obligatory for some hosts, members of this Order are usually pathogenic, triggering a variety of diseases in their hosts [138,139].

Several characteristics revealed by *Wolbachia*, and other "Rickettsia-like" bacteria, are indicative of a co-evolution that is moving

towards an obligatory relationship to both parts, or even culminate in organelle evolution, such as the case of mitochondria [140,141]. Some characteristics are shared by these bacteria strains, such as: their strong specialization in maternal transmission; their localization at different cells of various host tissues; being surrounded by lipid membranes; and having a considerable loss of cell wall structures, having an important influence in the host's metabolism or even transferring their genes to the nuclear genome of the host [138].

Wolbachia shows a widespread incidence, being present in approximately 40% of terrestrial arthropods [142], including spiders, mites, terrestrial crustaceans and in every insect order [143]. In addition, *Wolbachia* can also be found in Nematodes, with which it maintains a mutualistic relationship [144]. Even though Nematodes can survive without *Wolbachia*, the removal of these bacteria promotes a strong decrease of hosts' fitness, directly affecting the viability of embryos, female fertility or even the survival of adults [145].

For historical and technical reasons, *Wolbachia* is considered a single species, although it shows extensive genetic variation [146]. Several supergroups, catalogued from A to H, organize the existent diversity of *Wolbachia*, where supergroups A e B concentrate the majority of *Wolbachia* strains of Arthropods [147]. In *Drosophila melanogaster*, only one *Wolbachia* strain was found, *w*Mel, although with several variants [148]. This variety is characterized by having a high number of repetitions of mobile elements and several duplications of short open reading frames (ORFs) in its genome [149]. Posteriorly, using different genetic markers, *w*Mel haplotypes were catalogued and organized in several Clades [150].

Another interesting feature of *Wolbachia* strains of Arthropods is the presence of WO prophages [149,151,152]. 90% of *Wolbachia* strains

tested, including *w*Mel, contain the WO-B prophage variant [153,154]. On the other hand, *Wolbachia* strains of Nematodes (mutualistic) do not have phages [155]. It is still unknown which are the consequences of this phage in phenotypes induced by *Wolbachia*, although some efforts to study their correlation have already been made [151,156–158]. Recently, new insights into the horizontal transmission capacity and lateral gene transfer of WO have been revealed [159,160].

In Arthropods, where *Wolbachia* is a facultative endosymbiont perpetuated predominantly through maternal transmission, these bacteria use several manipulations of the host's reproduction to enhance infected-female fitness and consequently their own transmission [161]. The main modifications reported are feminization of genetic males [162–164], killing of males during embryogenesis [165–168], parthenogenesis of females [169–172] or cytoplasmatic incompatibility (CI) between individuals with different infection status [173–176]. The outcomes can be even more complex and multifactorial, with combination and interaction of the different phenotypes. CI is the more usual and studied manipulation, where *Wolbachia*-uninfected females have less progeny when crossed with infected males [177]. CI was first described in mosquitoes, in 1971 [178], and although the mechanism is not yet fully understood, it is known that a delay in the synchronization for male nuclear envelope breakdown is responsible for the phenotype and, consequently, for the fitness differences [179]. Thus, the absence or an incorrect genetic contribution of males will lead to different consequences according to the sexual genetic structure of the host species – haploid, diploid or haplodiploid.

Although directly influencing spermatogenesis, as seen in the reproductive manipulations, *Wolbachia* is not transmitted by sperm due to the small amount of cytoplasm present in these cells [180]. Thus, a high

level of *Wolbachia* specialization evolved in oogenesis, developing an efficient perpetuation by female contribution. *Wolbachia* uses the host's microtubules and proteins, such as dynein and actin, to migrate efficiently during oogenesis to the zone that will originate the polar cells and consecutively ensure their presence in the germinal tissue cells [181–184]. Moreover, by performing a successful cellular distribution into the embryo, for example by concentrating in the proximity of mitotic spindles, *Wolbachia* also ensures a very high rate of infection in several tissues of the progeny [185], especially in reproductive cells [186–188].

Nevertheless, despite the high specialization and efficiency of maternal vertical transmission of *Wolbachia*, sporadic horizontal transmission is also detected in some hosts species, namely in Arthropods. The first evidence is the lack of phylogenetic concordance between *Wolbachia* and host lineages, where sometimes similar strains of *Wolbachia* are found in distant hosts [189,190]. Furthermore, in a vast range of experiments using different species, it was possible to create a stable new infection, transferring cytoplasm of infected embryos to uninfected ones [191–194].

The combination these two mechanisms of transmission makes it understandable that *Wolbachi*a's presence in nature is so diverse and successful. Importantly, although *Wolbachia* is present in so many species, its incidence of infection intra and inter populations is highly variable [195–197]. Therefore, *Wolbachia*'s presence or absence in populations, as well as its frequency fluctuations are probably related to the balance between the benefits and costs that it can trigger in each moment, either in a sporadic or cyclic fashion. With this in mind, new questions have emerged to try to understand in a more complete manner the influence of these endosymbionts in host populations and new factors that could explain the dispersal and populational structure of *Wolbachia*.

Antiviral protection mediated by *Wolbachia*

In 2008, two studies unveiled new consequences for the presence of *Wolbachia* in *Drosophila*, reporting that this endosymbiont can confer protection to the host against viral infection [198,199]. Teixeira and colleagues went one step further, confirming categorically that this protective phenotype was induced by *Wolbachia*, as well as characterizing that this phenomenon was not only verified with DCV but also with other RNA viruses [198]. Thus began a new phase in the investigation of symbiotic viral immunity in insects. Since then, several studies have revealed the *Wolbachia*-mediated protection against virus in different species and circumstances [200–207].

This host-protective characteristic, associated with the capacity of reproductive manipulation, namely CI, led to the development of disease control projects through *Wolbachia* infection of vectors agents [208–211]. Moreover, it was also found that, in mosquitos, *Wolbachia* also protects against filarial nematodes [212], *Plasmodium* parasites [201,213,214] and pathogenic bacteria [215].

Subsequently, it was shown that different clades of *w*Mel promote different protection against systemic viral infection [216]. This difference seems to be directly related to the amount of *Wolbachia* into the host, where a larger load of *Wolbachia* corresponds to a lower viral titer and, consequenly, higher protection [217]. A good example of this relation between the amount of *Wolbachia* and the increase of protection is visible in the most extreme variant, *w*MelPop, which presents a massive bacterial growth in numerous host tissues [218,219]. However, these high bacterial loads have a cost in the host's lifespan in absence of viral infection, in both *Drosophila* and transinfected mosquitos, namely the dengue vector *Aedes aegypti* [220–222]. It was shown that virulence is triggered by a specific region in the *Wolbachia* genome, called Octomon, where the fluctuation in

copy number is responsible for driving the bacterial phenotypes and, consequently, the associated costs [223]. Due to these characteristics and particularities, *w*MelPop is extensively used for transinfections and tested in many of the above-mentioned studies of *Wolbachia* protection against pathogens and parasites. However, although this inversely proportional relationship between *Wolbachia* load and virus titer is well established, the full mechanism behind this protection is not well known.

One hypothesis for the conferred protection may be through oxidative stress, where it has been shown in *Drosophila* that there is a correlation between the increase of hydrogen peroxide and a decrease in susceptibility to viral infection [224]. On the other hand, different evidence exclude other hypotheses, at least under specific conditions. First, several reports demonstrated that *Wolbachia* does not activate or up-regulate the immune response of naturally-infected hosts, that is, where it is vertically transmitted [217,225–229]. However, in trans-infection experiments (forced horizontal transmission) where mosquitoes received *Wolbachia* from *Drosophila*, activation of the immune response could be observed [212,230,231] but no activation was seen in *D. melanogaster* after receiving a non-native strain, *w*MelAu from *D. simulans* [225]. Thus, the *Wolbachia* relation with the host immunity appears not to be transversal in all associations or an intrinsic feature of bacteria, but probably a specificity of each co-evolutionary process.

In another perspective, in a recent report it was demonstrated that diet had an effect in viral titers in *Drosophila* [232]. Linking this fact with the results that support that nutrition may also have a role in *Drosophila*-*Wolbachia* relationship [233], a new hypothesis can emerge to try to explain the physiological basis of protection. Indeed, an interesting case of the bedbug *Cimex lectularius*, where *Wolbachia* evolved to an obligate

nutritional mutualist, demonstrated how nutrition can drive the evolution of the *Wolbachia*-host relationship [234].

It is still to be determined whether the viral protection conferred by *Wolbachia* is a trigger by direct competition against the virus or an indirect response by the interaction with the host. It is therefore crucial look carefully at the immune mechanisms of hosts to better understand the possible links of responses against infections, as well as the outcomes of the symbiotic relationships.

1.5. How to contribute to a better understanding of host-microbe interactions?

Scientific knowledge has grown exponentially in the last decades. An enormous amount of data has been generated by a vast research community, where classical and new biological models contribute to a progressive understanding of life on Earth, from phylogenetic patterns to what make a species unique. Here, it is my fair expectation use the vast range of available tools to help to better comprehend the system host-endosymbiont-pathogen and unveil the particularities of the evolutionary paths of this complex interactions. This is the main goal for the Thesis here presented.

References

1. **Mechanisms of life history evolution: the genetics and physiology of life history traits and trade-offs**. Edited by Flatt T, Heyland A. Oxford: Oxford University Press; 2011.

2. Pace NR: **A molecular view of microbial diversity and the biosphere.** *Science* 1997, **276**:734–740.

3. Kent AD, Triplett EW: **Microbial communities and their interactions in soil and rhizosphere ecosystems.** *Annual Review of Microbiology* 2002, **56**:211–236.

4. Sogin ML, Morrison HG, Huber JA, Welch DM, Huse SM, Neal PR, Arrieta JM, Herndl GJ: **Microbial diversity in the deep sea and the underexplored "rare biosphere"**. *Proceedings of the National Academy of Sciences of the United States of America* 2006, **103**:12115–12120.

5. Lynch MDJ, Neufeld JD: **Ecology and exploration of the rare biosphere.** *Nature Reviews Microbiology* 2015, **13**:217–229.

6. Cordero OX, Polz MF: **Explaining microbial genomic diversity in light of evolutionary ecology**. *Nature Reviews Microbiology* 2014, **12**:263–273.

7. Garcia-Vallvé S, Romeu A, Palau J: **Horizontal gene transfer in bacterial and archaeal complete genomes.** *Genome Research* 2000, **10**:1719–1725.

8. Dagan T, Artzy-Randrup Y, Martin W: **Modular networks and cumulative impact of lateral transfer in prokaryote genome evolution.** *Proceedings of the National Academy of Sciences of the United States of America* 2008, **105**:10039–10044.

9. Polz MF, Alm EJ, Hanage WP: **Horizontal gene transfer and the evolution of bacterial and archaeal population structure**. *Trends in Genetics* 2013, **29**:170–175.

10. Woese CR: **Bacterial evolution**. *Microbiology* 1987, **51**:221–271.

11. DeLong EF, Pace NR: **Environmental diversity of bacteria and archaea.** *Systematic Biology* 2001, **50**:470–478.

12. Marsh M, Helenius A: **Virus entry: open sesame**. *Cell* 2006, **124**:729–740.

13. Feschotte C, Gilbert C: **Endogenous viruses: insights into viral evolution and impact on host biology**. *Nature Reviews Genetics* 2012, **13**:283–296.

14. Mabbott NA, MacPherson GG: **Prions and their lethal journey to the brain**. *Nature Reviews Microbiology* 2006, **4**:201–211.

15. Halfmann R, Lindquist S: **Epigenetics in the extreme: prions and the inheritance of environmentally acquired traits**. *Science* 2010, **330**:2010.

16. Escoll P, Mondino S, Rolando M, Buchrieser C: **Targeting of host organelles by pathogenic bacteria: a sophisticated subversion strategy**. *Nature Reviews Microbiology* 2015, **14**:5–19.

17. Benoit JB, Denlinger DL: **Meeting the challenges of on-host and off-host water balance in blood-feeding arthropods**. *Journal of Insect Physiology* 2010, **56**:1366–1376.

18. Petney TN, Skuballa J, Muders S, Pfäffle M: **Arthropods as vectors of emerging diseases**. *Arthropods As Vectors of Emerging Diseases* 2012, **3**:151–166.

19. Maizels RM, Pearce EJ, Artis D, Yazdanbakhsh M, Wynn TA: **Regulation of pathogenesis and immunity in helminth infections.** *The Journal of Experimental Medicine* 2009, **206**:2059–2066.

20. McSorley HJ, Maizels RM: **Helminth infections and host immune regulation**. *Clinical Microbiology Reviews* 2012, **25**:585–608.

21. Casadevall A: **Determinants of virulence in the pathogenic fungi**. *Fungal Biology Reviews* 2007, **21**:130–132.

22. Barelli L, Moonjely S, Behie SW, Bidochka MJ: **Fungi with multifunctional lifestyles: endophytic insect pathogenic fungi**. *Plant Molecular Biology* 2016, **90**:657–664.

23. Peterson JW: **Bacterial pathogenesis**. In *Medical Microbiology*. Edited by Baron S. University of Texas Medical Branch at Galveston; 1996.

24. Parkin J, Cohen B: **An overview of the immune system**. *Lancet* 2001, **357**:1777–1789.

25. Janeway CA, Travers P, Walport M, Shlomchik M: **Immunobiology: the immune system in health and disease**; 2001.

26. Jack RS: **Evolution of immunity and pathogens**. In *Pathogen-host interactions: antigenic variation v. somatic adaptations*. Edited by Hsu E, Du Pasquier L. Springer 2015:1–20.

27. Boehm T, Swann JB: **Origin and evolution of adaptive immunity.** *Annual Review of Animal Biosciences* 2014, **2**:259–283.

28. Schatz DG, Oettinger MA, Baltimore D: **The V(D)J recombination activating gene, RAG-1**. *Cell* 1989, **59**:1035–1048.

29. Oettinger MA, Schatz DG, Gorka C, Baltimore D: **RAG-1 and RAG-2, adjacent genes that synergistically activate V(D)J recombination.** *Science* 1990, **248**:1517–1523.

30. Muramatsu M, Sankaranand VS, Anant S, Sugai M, Kinoshita K, Davidson NO, Honjo T: **Specific expression of activation-induced cytidine deaminase (AID), a novel member of the rna-editing deaminase family in germinal center b cells**. *Journal of Biological Chemistry* 1999, **274**:18470–18476.

31. Hozumi N, Tonegawa S: **Evidence for somatic rearrangement of immunoglobulin genes coding for variable and constant regions.** *Proceedings of the National Academy of Sciences of the United States of America* 1976, **73**:3628–3632.

32. Roth DB: **Restraining the V(D)J recombinase.** *Nature Reviews Immunology* 2003, **3**:656–666.

33. Wang JH: **The role of activation-induced deaminase in antibody diversification and genomic instability**. *Immunologic Research* 2013, **55**:287–297.

34. Read AF, Allen JJE: **The economics of immunity**. *Science* 2000, **290**:1104–1105.

35. Moret Y: **Explaining variable costs of the immune response: selection for specific versus non-specific immunity and facultative life history change**. *Oikos* 2003, **102**:213–216.

36. Nappi AJ, Christensen BM: **Melanogenesis and associated cytotoxic reactions: applications to insect innate immunity**. *Insect Biochemistry and Molecular Biology* 2005, **35**:443–459.

37. Sorci G, Faivre B: **Inflammation and oxidative stress in vertebrate host-parasite systems.** *Philosophical Transactions of the Royal Society of London. Series B, Biological sciences* 2009, **364**:71–83.

38. Chatzinikolaou G, Karakasilioti I, Garinis GA: **DNA damage and innate immunity: links and trade-offs**. *Trends in Immunology* 2014, **35**:429–435.

39. Sadd BM, Schmid-Hempel P: **Principles of ecological immunology**. *Evolutionary Applications* 2009, **2**:113–121.

40. Schulenburg H, Kurtz J, Moret Y, Siva-Jothy MT: **Introduction. Ecological immunology.** *Philosophical transactions of the Royal Society of London. Series B, Biological Sciences* 2009, **364**:3–14.

41. Dillon R, Dillon V: **The gut bacteria of insects: nonpathogenic interactions**. *Annual Reviews in Entomology* 2004, **49**:71–92.

42. Caballero S, Pamer EG: **Microbiota-mediated inflammation and antimicrobial defense in the intestine**. *Annual Reviews in Immunol*ogy 2015, **33**:227–256.

43. Hentschel U, Steinert M, Hacker J: **Common molecular mechanisms of symbiosis and pathogenesis**. *Trends in Microbiology* 2000, **8**:226–231.

44. Dybdahl MF, Storfer A: **Parasite local adaptation: red queen versus suicide king**. *Trends in Ecology and Evolution 2003*, **18**:523–530.

45. Cheney KL, Côté IM: **Mutualism or parasitism? the variable outcome of cleaning symbioses**. *Biology Letters* 2005, **1**:162–165.

46. Newton AC, Fitt BDL, Atkins SD, Walters DR, Daniell TJ: **Pathogenesis, parasitism and mutualism in the trophic space of microbe-plant interactions**. *Trends in Microbiology* 2010, **18**:365–373.

47. Boots M, Mealor M: **Local interactions select for lower pathogen infectivity**. *Science* 2007, **315**:1284–1286.

48. Moran NA: **Symbiosis**. *Current Biology* 2006, **16**:866-871.

49. Bermudes D: **Symbiosis: an introduction to biological associations**. Edited by Paracer S, Ahmadjian V . Oxford: Oxford University Press; 2000.

50. Lane CE, Archibald JM: **The eukaryotic tree of life: endosymbiosis takes its TOL**. *Trends in Ecology and Evolution* 2008, **23**:268–275.

51. Sapp J: **Evolution by association: a history of symbiosis**. New York: Oxford University Press; 1994.

52. Archibald JM: **Endosymbiosis and eukaryotic cell evolution**. *Current Biology* 2015, **25**:911–921.

53. Margulis L: **Symbiotic planet : a new look at evolution**. Science Masters*;* 1998.

54. Sapp J, Carrapiço F, Zolotonosov M: **Symbiogenesis: the hidden face of constantin merezhkowsky.** *History and Philosophy of the Life Sciences* 2002, **24**:413–440.

55. Cavalier-Smith T: **Symbiogenesis: mechanisms, evolutionary consequenses, and systematic implications**. *Annual Review of Ecology, Evolution, and Systematics* 2013, **44**:145–172.

56. Weinert LA, Araujo-Jnr E V., Ahmed MZ, Welch JJ: **The incidence of bacterial endosymbionts in terrestrial arthropods.** *Proceedings of the Royal Society B* 2015, **282**:20150249.

57. Perotti MA, Clarke HK, Turner BD, Braig HR: ***Rickettsia* as obligate and mycetomic bacteria.** *The FASEB Journal* 2006, **20**:2372–2374.

58. Moran NA, McCutcheon JP, Nakabachi A: **Genomics and evolution of heritable bacterial symbionts.** *Annual Review of Genetics* 2008, **42**:165–190.

59. van Ham RCHJ, Kamerbeek J, Palacios C, Rausell C, Abascal F, Bastolla U, Fernández JM, Jiménez L, Postigo M, Silva FJ, Tamames J, Viguera E, Latorre A, Valencia A, Moran F, Moya A: **Reductive genome evolution in *Buchnera* aphidicola.** *Proceedings of the National Academy of Sciences of the United States of America* 2003, **100**:581–586.

60. Nikoh N, McCutcheon JP, Kudo T, Miyagishima SY, Moran NA, Nakabachi A: **Bacterial genes in the aphid genome: absence of functional gene transfer from buchnera to its host**. *PLoS Genetics* 2010, **6**:1000827.

61. Bennett GM, Moran NA: **Heritable symbiosis: the advantages and perils of an evolutionary rabbit hole**. *Proceedings of the National Academy of Sciences of the United States of America* 2015, **112**:1–8.

62. Little TJ, Shuker DM, Colegrave N, Day T, Graham AL: **The coevolution of virulence: tolerance in perspective**. *PLoS Pathogens* 2010, **6**:1001006.

63. Pagán I, Montes N, Milgroom MG, García-Arenal F: **Vertical transmission selects for reduced virulence in a plant virus and for increased resistance in the host**. *PLoS Pathogens* 2014, **10**:e1004293.

64. Wernegreen JJ: **Genome evolution in bacterial endosymbionts of insects.** *Nature Reviews Genetics* 2002, **3**:850–861.

65. McCutcheon JP, Moran NA: **Parallel genomic evolution and metabolic interdependence in an ancient symbiosis**. *Proceedings of the National Academy of Sciences of the United States of America* 2007, **104**:19392–19397.

66. Mendonça AG, Alves RJ, Pereira-Leal JB: **Loss of genetic redundancy in reductive genome evolution**. *PLoS Computational Biology* 2011, **7**:1001082.

67. Toenshoff ER, Gruber D, Horn M: **Co-evolution and symbiont replacement shaped the symbiosis between adelgids (Hemiptera: Adelgidae) and their bacterial symbionts**. *Environmental Microbiology* 2012, **14**:1284–1295.

68. Koga R, Moran NA: **Swapping symbionts in spittlebugs: evolutionary replacement of a reduced genome symbiont.** *The ISME journal* 2014, **8**:1237–1246.

69. Hall AAG, Morrow JL, Fromont C, Steinbauer MJ, Taylor GS, Johnson SN, Cook JM, Riegler M: **Codivergence of the primary bacterial endosymbiont of psyllids versus host switches and replacement of their secondary bacterial endosymbionts**. *Environmental Microbiology* 2016, **18**:2591–2603.

70. Zchori-Fein E, Perlman SJ: **Distribution of the bacterial symbiont *Cardinium* in arthropods**. *Molecular Ecology* 2004, **13**:2009–2016.

71. Gotoh T, Noda H, Ito S: ***Cardinium* symbionts cause cytoplasmic incompatibility in spider mites.** *Heredity* 2007, **98**:13–20.

72. Penz T, Schmitz-Esser S, Kelly SE, Cass BN, Müller A, Woyke T, Malfatti SA, Hunter MS, Horn M: **Comparative genomics suggests an independent origin of cytoplasmic incompatibility in *Cardinium hertigii***. *PLoS Genetics* 2012, **8**:1003012.

73. Taylor GP, Coghlin PC, Floate KD, Perlman SJ: **The host range of the male-killing symbiont *Arsenophonus nasoniae* in filth fly parasitioids**. *Journal of Invertebrate Pathology* 2011, **106**:371–379.

74. Jousselin E, Cœur D'Acier A, Vanlerberghe-Masutti F, Duron O: **Evolution and diversity of *Arsenophonus* endosymbionts in aphids**. *Molecular Ecology* 2013, **22**:260–70.

75. Montenegro H, Solferini VN, Klaczko LB, Hurst GDD: **Male-killing *Spiroplasma* naturally infecting *Drosophila melanogaster***. *Insect Molecular Biology* 2005, **14**:281–287.

76. Anbutsu H, Fukatsu T: ***Spiroplasma* as a model insect endosymbiont**. *Environmental Microbiology Reports* 2011, **3**:144–153.

77. Harumoto T, Anbutsu H, Fukatsu T: **Male-killing *Spiroplasma* induces sex-specific cell death via host apoptotic pathway**. *PLoS Pathogens* 2014, **10**:1003956.

78. Hedges SB: **The origin and evolution of model organisms**. *Nature Reviews Genetics*, **3**:838–849.

79. Schneider D: **Using *Drosophila* as a model insect.** *Nature Reviews Genetics* 2000, **1**:218–226.

80. Lachaise D, Cariou M-L, David JR, Lemeunier F, Tsacas L, Ashburner M: **Historical biogeography of the *Drosophila melanogaster* species subgroup**. *Evolutionary Biology* 1988, **22**:159–225.

81. Ashburner M: ***Drosophila*: a laboratory handbook**. Plainview NY: Cold Spring Harbor Laboratory Press; 1989.

82. Markow TA, O'Grady P: ***Drosophila*: a guide to species identification and use**. Elsevier; 2006.

83. Markow TA: **The secret lives of *Drosophila* flies**. *eLife* 2015, **4**:e06793.

84. Morgan TH, Sturtevant a. H, Muller HJ, Bridges CB: **The mechanism of mendelian heredity**. *New York: Johnson Reprint 1972.* 1915:471–535.

85. Fischer JA, Giniger E, Maniatis T, Ptashne M: **GAL4 activates transcription in *Drosophila*.** *Nature* 1988, **332**:853–856.

86. Brand AH, Perrimon N: **Targeted gene expression as a means of altering cell fates and generating dominant phenotypes**. *Development* 1993, **118**:401–415.

87. Hammond SM, Bernstein E, Beach D, Hannon GJ: **An RNA-directed nuclease mediates post-transcriptional gene silencing in *Drosophila* cells**. *Nature* 2000, **404**:293–296.

88. Dykxhoorn D, Novina C, Sharp P: **Killing the messenger: short RNAs that silence gene expression.** *Nature Reviews Molecular Cell Biology* 2003, **4**:457–467.

89. Adams MD, Celniker SE, Holt RA, Evans CA, Gocayne JD, Amanatides PG, et al.: **The genome sequence of *Drosophila melanogaster*.** *Science* 2000, **287**:2185–2195.

90. Drysdale R, FlyBase Consortium: **Flybase : a database for the *Drosophila* research community**. *Methods in Molecular Biology* 2008, **420**:45–59.

91. Mackay TFC, Richards S, Stone EA, Barbadilla A, Ayroles JF, Zhu D, Casillas S, Han Y, Magwire MM, Cridland JM, Richardson MF, Anholt RRH, Barrón M, Bess C, Blankenburg KP, Carbone MA, Castellano D, Chaboub L, Duncan L, Harris Z, Javaid M, Jayaseelan JC, Jhangiani SN, Jordan KW, Lara F, Lawrence F, Lee SL, Librado P, Linheiro RS, Lyman RF, et al.: **The *Drosophila melanogaster* Genetic Reference Panel**. *Nature* 2012, **482**:173–178.

92. Mohr SE, Hu Y, Kim K, Housden BE, Perrimon N: **Resources for functional genomics studies in *Drosophila melanogaster***. *Genetics* 2014, **197**:1–18.

93. Vodovar N, Acosta C, Lemaitre B, Boccard F: ***Drosophila*: a polyvalent model to decipher host-pathogen interactions**. *Trends in Microbiology* 2004, **12**:235–242.

94. Tzou P, De Gregorio E, Lemaitre B: **How *Drosophila* combats microbial infection: a model to study innate immunity and host-pathogen interactions**. *Current Opinion in Microbiology* 2002, **5**:102–110.

95. Apidianakis Y, Rahme LG: ***Drosophila melanogaster* as a model for human intestinal infection and pathology.** *Disease Models & Mechanisms* 2011, **4**:21–30.

96. Panayidou S, Ioannidou E, Apidianakis Y: **Human pathogenic bacteria, fungi, and viruses in *Drosophila*: disease modeling, lessons, and shortcomings.** *Virulence* 2014, **5**:253–269.

97. Hughes TT, Allen AL, Bardin JE, Christian MN, Daimon K, Dozier KD, Hansen CL, Holcomb LM, Ahlander J: ***Drosophila* as a genetic model for studying pathogenic human viruses**. *Virology* 2012, **423**:1–5.

98. Hultmark D: ***Drosophila* immunity: paths and patterns**. *Current Opinion in Immunology* 2003, **15**:12–19.

99. Lemaitre B, Hoffmann J: **The host defense of *Drosophila melanogaster***. *Annual Review of Immunology* 2007, **25**:697–743.

100. Boman HG, Nilsson I, Rasmuson B: **Inducible antibacterial defence system in *Drosophila***. *Nature* 1972, **237**:232–235.

101. Buchon N, Silverman N, Cherry S: **Immunity in *Drosophila melanogaster* - from microbial recognition to whole-organism physiology.** *Nature Reviews Immunology* 2014, **14**:796–810.

102. Lestradet M, Lee KZ, Ferrandon D: ***Drosophila* as a model for intestinal infections**. *Methods in Molecular Biology* 2014, **1197**:11–40.

103. Buchon N, Broderick NA, Lemaitre B: **Gut homeostasis in a microbial world: insights from *Drosophila melanogaster***. *Nature Reviews Microbiology* 2013, **11**:615–626.

104. Ha EM, Oh CT, Bae YS, Lee WJ: **A direct role for dual oxidase in *Drosophila* gut immunity**. *Science* 2005, **310**:847–850.

105. Zaidman-Rémy A, Hervé M, Poidevin M, Pili-Floury S, Kim MS, Blanot D, Oh BH, Ueda R, Mengin-Lecreulx D, Lemaitre B: **The *Drosophila* amidase PGRP-LB modulates the immune response to bacterial infection**. *Immunity* 2006, **24**:463–473.

106. Tzou P, Ohresser S, Ferrandon D, Capovilla M, Reichhart JM, Lemaitre B, Hoffmann JA, Imler JL: **Tissue-specific inducible expression of antimicrobial peptide genes in *Drosophila* surface epithelia.** *Immunity* 2000, **13**:737–748.

107. Tingvall TÖ, Roos E, Engström Y: **The imd gene is required for local cecropin expression in *Drosophila* barrier epithelia**. *EMBO Reports* 2001, **2**:239–243.

108. Vallet-Gely I, Lemaitre B, Boccard F: **Bacterial strategies to overcome insect defences.** *Nature Reviews Microbiology* 2008, **6**:302–313.

109. Ferrandon D, Imler JL, Hetru C, Hoffmann JA: **The *Drosophila* systemic immune response: sensing and signalling during bacterial and fungal infections.** *Nature Reviews Immunology* 2007, **7**:862–874.

110. Charroux B, Royet J: **Elimination of plasmatocytes by targeted apoptosis reveals their role in multiple aspects of the *Drosophila* immune response.** *Proceedings of the National Academy of Sciences of the United States of America* 2009, **106**:9797–9802.

111. Meister M: **Blood cells of *Drosophila*: cell lineages and role in host defence**. *Current Opinion in Immunology* 2004, **16**:10–15.

112. Lemaitre B, Reichhart JM, Hoffmann JA: ***Drosophila*** **host defense: differential induction of antimicrobial peptide genes after infection by various classes of microorganisms**. *Immunology* 1997, **94**:14614–14619.

113. Lemaitre B, Nicolas E, Michaut L, Reichhart JM, Hoffmann JA: **The dorsoventral regulatory gene cassette spätzle/Toll/cactus controls the potent antifungal response in *Drosophila* adults.** *Cell* 1996, **86:**973–983.

114. Vodovar N, Vinals M, Liehl P, Basset A, Degrouard J, Spellman P, Boccard F, Lemaitre B**: *Drosophila* host defense after oral infection by an entomopathogenic *Pseudomonas* species.** *Proceedings of the National Academy of Sciences of the United States of America* 2005**, 102:**11414–11419.

115. Liehl P, Blight M, Vodovar N, Boccard F, Lemaitre B: **Prevalence of local immune response against oral infection in a *Drosophila*/*Pseudomonas* infection model**. *PLoS Pathogens* 2006, **2**:e56.

116. Vodovar N, Vallenet D, Cruveiller S, Rouy Z, Barbe V, Acosta C, Cattolico L, Jubin C, Lajus A, Segurens B, Vacherie B, Wincker P, Weissenbach J, Lemaitre B, Médigue C, Boccard F: **Complete genome sequence of the entomopathogenic and metabolically versatile soil bacterium *Pseudomonas entomophila*.** *Nature Biotechnology* 2006, **24**:673–679.

117. Webster CL, Waldron FM, Robertson S, Crowson D, Ferrari G, Quintana JF, Brouqui JM, Bayne EH, Longdon B, Buck AH, Lazzaro BP, Akorli J, Haddrill PR, Obbard DJ: **The discovery, distribution, and evolution of viruses associated with *Drosophila melanogaster***. *PLoS Biology* 2015, **13**:e1002210.

118. Habayeb MS, Ekengren SK, Hultmark D: **Nora virus, a persistent vitus in *Drosophila*, defines a new picorna-like virus family**. *Journal of General Virology* 2006, **87**:3045–3051.

119. Kim VN, Han J, Siomi MC: **Biogenesis of small RNAs in animals.** *Nature Reviews Molecular Cell Biology* 2009, **10**:126–139.

120. Ding SW: **RNA-based antiviral immunity.** *Nature Reviews Immunology* 2010, **10**:632–644.

121. Kingsolver MB, Huang Z, Hardy RW: **Insect antiviral innate immunity: pathways, effectors, and connections**. *Journal of Molecular Biology* 2013, **425**:4921–4936.

122. Galiana-Arnoux D, Dostert C, Schneemann A, Hoffmann JA, Imler JL: **Essential function *in vivo* for Dicer-2 in host defense against RNA viruses in *Drosophil*a.** *Nature Immunology* 2006, **7**:590–597.

123. Van Rij RP, Saleh MC, Berry B, Foo C, Houk A, Antoniewski C, Andino R: **The RNA silencing endonuclease Argonaute 2 mediates specific antiviral immunity in *Drosophila melanogaster***. *Genes and Development*. 2006 **20**:2985–2995.

124. Zambon RA, Vakharia VN, Wu LP: **RNAi is an antiviral immune response against a dsRNA virus in *Drosophila melanogaster***. *Cellular Microbiology* 2006, **8**:880–889.

125. Zambon RA, Nandakumar M, Vakharia VN, Wu LP: **The Toll pathway is important for an antiviral response in *Drosophila*.** *Proceedings of the National Academy of Sciences of the United States of America* 2005, **102**:7257–7262.

126. Costa A, Jan E, Sarnow P, Schneider D: **The Imd pathway is involved in antiviral immune responses in *Drosophila***. *PLoS ONE* 2009, **15**:e7436.

127. Dostert C, Jouanguy E, Irving P, Troxler L, Galiana-Arnoux D, Hetru C, Hoffmann JA, Imler JL: **The Jak-STAT signaling pathway is required but not sufficient for the antiviral response of *Drosophila*.** *Nature Immunology* 2005, **6**:946–953.

128. Nayak A, Berry B, Tassetto M, Kunitomi M, Acevedo A, Deng C, Krutchinsky A, Gross J, Antoniewski C, Andino R: **Cricket paralysis virus antagonizes Argonaute 2 to modulate antiviral defense in *Drosophila*.** *Nature Structural & Molecular Biology* 2010, **17**:547–554.

129. Werren JH, O'Neill SL: **The evolution of heritable symbionts**. In *Influential passengers: inherited microorganisms and arthropod reproduction*. Edited by O'Neill SL, Werren JH. Oxford: Oxford University Press; 1997:1–41.

130. Duron O, Bouchon D, Boutin SSS, Bellamy L, Zhou L, Engelstadter J, Hurst GD: **The diversity of reproductive parasites among arthropods: *Wolbachia* do not walk alone**. *BMC Biology* 2008, **6**:27.

131. Goto S, Anbutsu H, Fukatsu T: **Asymmetrical interactions between *Wolbachia* and *Spiroplasma* endosymbionts coexisting in the same insect host**. *Applied and Environmental Microbiology* 2006, **72**:4805–4810.

132. Regassa LB, Gasparich GE: **Spiroplasmas: evolutionary relationships and biodiversity.** *Frontiers in Bioscience* 2006, **11**:2983–3002.

133. Hurst GD, Anbutsu H, Kutsukake M, Fukatsu T: **Hidden from the host: *Spiroplasma* bacteria infecting *Drosophila* do not cause an immune response, but are suppressed by ectopic immune activation**. *Insect Molecular Biology* 2003, **12**:93–97.

134. Herren JK, Lemaitre B: ***Spiroplasma* and host immunity: activation of humoral immune responses increases endosymbiont load and susceptibility to certain Gram-negative bacterial pathogens in *Drosophila melanogaster***. *Cellular Microbiology* 2011, **13**:1385–1396.

135. Hertig M, Wolbach SB: **Studies on *Rickettsia*-like micro-organisms in insects**. *The Journal of Medical Research* 1924, **44**:329–374.

136. Hertig M: **The *Rickettsia*, *Wolbachia pipientis* and associated inclusions of the mosquito *Culex pipiens***. *Parasitology* 1936, **28**:453–486.

137. Darby AC, Cho NH, Fuxelius HH, Westberg J, Andersson SGE: **Intracellular pathogens go extreme: genome evolution in the *Rickettsiales***. *Trends in Genetics* 2007, **23**:511–20.

138. Perlman SJ, Hunter MS, Zchori-Fein E: **The emerging diversity of *Rickettsia***. *Proceedings of the Royal Society B* 2006, **273**:2097–2106.

139. Weinert LA, Werren JH, Aebi A, Stone GN, Jiggins FM: **Evolution and diversity of *Rickettsia* bacteria.** *BMC Biology* 2009, **7**:6.

140. Fitzpatrick DA, Creevey CJ, McInerney JO: **Genome phylogenies indicate a meaningful alpha-proteobacterial phylogeny and support a grouping of the mitochondria with the *Rickettsiales***. *Molecular Biology and Evolution* 2006, **23**:74–85.

141. Wang Z, Wu M: **An integrated phylogenomic approach toward pinpointing the origin of mitochondria.** *Scientific Reports* 2015, **5**:7949.

142. Zug R, Hammerstein P: **Still a host of hosts for *Wolbachia*: analysis of recent data suggests that 40% of terrestrial arthropod species are infected**. *PLoS ONE* 2012, **7**:e38544.

143. Hilgenboecker K, Hammerstein P, Schlattmann P, Telschow A, Werren JH: **How many species are infected with *Wolbachia*? – a statistical analysis of current data**. *FEMS Microbiology Letters* 2008, **281**:215–220.

144. Taylor MJ, Bandi C, Hoerauf A: ***Wolbachia* bacterial endosymbionts of filarial nematodes**. *Advances in Parasitology* 2005, **60**:245–284.

145. Bandi C, Trees AJ, Brattig NW: ***Wolbachia* in filarial nematodes: evolutionary aspects and implications for the pathogenesis and treatment of filarial diseases**. *Veterinary Parasitology* 2001, **98**:215–238.

146. Baldo L, Lo N, Werren JH: **Mosaic nature of the *Wolbachia* surface protein**. *Journal of Bacteriology* 2005, **187**:5406–5418.

147. Werren JH, Zhang W, Guo LR: **Evolution and phylogeny of *Wolbachia*: reproductive parasites of arthropods**. *Proceedings of the Royal Society B* 1995, **261**:55–71.

148. Zhou W, Rousset F, O'Neil S: **Phylogeny and PCR-based classification of *Wolbachia* strains using *wsp* gene sequences**. *Proceedings Biological Sciences / The Royal Society* 1998, **265**:509–515.

149. Wu M, Sun L V., Vamathevan J, Riegler M, Deboy R, Brownlie JC, McGraw EA, Martin W, Esser C, Ahmadinejad N, et al.: **Phylogenomics of the reproductive parasite *Wolbachia pipientis* wmel: a streamlined genome overrun by mobile genetic elements**. *PLoS Biology* 2004, **2**:e69.

150. Riegler M, Sidhu M, Miller WJ, O'Neill SL: **Evidence for a global *Wolbachia* replacement in *Drosophila melanogaster*.** *Current Biology* 2005, **15**:1428–1433.

151. Bordenstein SR, Marshall ML, Fry AJ, Kim U, Wernegreen JJ: **The tripartite associations between bacteriophage, *Wolbachia*, and arthropods**. *PLoS Pathogens* 2006, **2**:384–393.

152. Masui S, Kamoda S, Sasaki T, Ishikawa H: **Distribution and evolution of bacteriophage WO in *Wolbachia*, the endosymbiont causing sexual alterations in arthropods.** *Journal of Molecular Evolution* 2000, **51**:491–497.

153. Bordenstein SR, Wernegreen JJ: **Bacteriophage flux in endosymbionts (*Wolbachia*): infection frequency, lateral transfer, and**

recombination rates. *Molecular Biology and Evolution* 2004, **21**:1981–1991.

154. Chauvatcharin N, Ahantarig A, Baimai V, Kittayapong P: **Bacteriophage WO-B and *Wolbachia* in natural mosquito hosts: infection incidence, transmission mode and relative density**. *Molecular Ecology* 2006, **15**:2451–2461.

155. Foster J, Ganatra M, Kamal I, Ware J, Makarova K, Ivanova N, Bhattacharyya A, Kapatral V, Kumar S, Posfai J, Vincze T, Ingram J, et al.: **The *Wolbachia* genome of *Brugia malayi*: endosymbiont evolution within a human pathogenic nematode**. *PLoS Biology* 2005, **3**:599–614.

156. Fujii Y, Kubo T, Ishikawa H, Sasaki T: **Isolation and characterization of the bacteriophage WO from *Wolbachia*, an arthropod endosymbiont**. *Biochemical and Biophysical Research Communications* 2004, **317**:1183–1188.

157. Williams EH, Fields S, Saul GB: **Transfer of incompatibility factors between stocks of *Nasonia* (= *Mormoniella*) *vitripennis*.** *Journal of invertebrate pathology* 1993, **61**:206–210.

158. Pichon S, Bouchon D, Liu C, Chen L, Garrett R a, Grève P: **The expression of one ankyrin *pk2* allele of the WO prophage is correlated with the *Wolbachia* feminizing effect in isopods.** *BMC Microbiology* 2012, **12**:55.

159. Wang GH, Jia LY, Xiao JH, Huang DW: **Discovery of a new *Wolbachia* supergroup in cave spider species and the lateral transfer of phage WO among distant hosts**. *Infection, Genetics and Evolution* 2016, **41**:1–7.

160. Wang N, Jia S, Xu H, Liu Y, Huang D: **Multiple horizontal transfers of bacteriophage WO and host *Wolbachia* in fig wasps in a closed community**. *Frontiers in Microbiology* 2016; **7**:136.

161. Stouthamer R, Breeuwer JA, Hurst GD: ***Wolbachia pipientis*: microbial manipulator of arthropod reproduction.** *Annual Review of Microbiology* 1999, **53**:71–102.

162. Hiroki M, Kato Y, Kamito T, Miura K: **Feminization of genetic males by a symbiotic bacterium in a butterfly, *Eurema hecabe* (Lepidoptera: Pieridae)**. *Naturwissenschaften* 2002, **89**:167–170.

163. Negri I, Pellecchia M, Mazzoglio PJ, Patetta A, Alma A: **Feminizing *Wolbachia* in *Zyginidia pullula* (Insecta, Hemiptera), a leafhopper**

with an XX/X0 sex-determination system. *Proceedings Biological Sciences / The Royal Society* 2006, **273**:2409–2416.

164. Kageyama D, Nishimura G, Hoshizaki S, Ishikawa Y: **Feminizing *Wolbachia* in an insect, *Ostrinia furnacalis* (Lepidoptera: Crambidae).** *Heredity* 2002, **88**:444–449.

165. Jiggins FM, Hurst GD, Schulenburg JH, Majerus ME: **Two male-killing *Wolbachia* strains coexist within a population of the butterfly *Acraea encedon*.** *Heredity* 2001, **86**:161–166.

166. Fialho RF, Stevens L: **Male killing *Wolbachia* in a flour beetle**. *Proceedings of the Royal Society B* 2000, **267**:1469–1473.

167. Kageyama D, Traut W: **Opposite sex-specific effects of *Wolbachia* and interference with the sex determination of its host *Ostrinia scapulalis*.** *Proceedings Biological Sciences / The Royal Society* 2004, **271**:251–258.

168. Sheeley SL, McAllister BF: **Mobile male-killer: similar *Wolbachia* strains kill males of divergent *Drosophila* hosts**. *Heredity* 2009, **102**:286–292.

169. Stouthamer R, Luck RF, Hamilton WD: **Antibiotics cause parthenogenetic *Trichogramma* (Hymenoptera/ Trichogrammatidae) to revert to sex.** *Proceedings of the National Academy of Sciences of the United States of America* 1990, **87**:2424–2427.

170. Weeks AR, Breeuwer JA: ***Wolbachia*-induced parthenogenesis in a genus of phytophagous mites.** *Proceedings Biological Sciences / The Royal Society* 2001, **268**:2245–2251.

171. Arakaki N, Miyoshi T, Noda H: ***Wolbachia*-mediated parthenogenesis in the predatory thrips *Franklinothrips vespiformis* (Thysanoptera: Insecta).** *Proceedings of the Royal Society B* 2001, **268**:1011–1016.

172. Kremer N, Charif D, Henri H, Bataille M, Prevost G, Kraaijeveld K, Vavre F: **A new case of *Wolbachia* dependence in the genus *Asobara*: evidence for parthenogenesis induction in *Asobara japonica***. *Heredity* 2009, **103**:248–256.

173. Hoffmann AA: **Unidirectional incompatibility between populations of *Drosophila simulans***. *Evolution* 1986, **40**:692–701.

174. Hoffmann AA, Clancy DJ, Merton E: **Cytoplasmic incompatibility in australian populations of *Drosophila melanogaster***. *Genetics* 1994, **136**:993–999.

175. Giordano R, O'Neill SL, Robertson HM: ***Wolbachia* infections and the expression of cytoplasmic incompatibility in *Drosophila sechellia* and *D. mauritiana***. *Genetics* 1995, **140**:1307–1317.

176. Werren JH: **Biology of *Wolbachia***. *Annual Review of Entomology* 1997, **42**:587–609.

177. Werren JH, Baldo L, Clark ME: ***Wolbachia***: **master manipulators of invertebrate biology**. *Nature Reviews Microbiology* 2008, **6**:741–751.

178. Yen JH, Barr AR: **New hypothesis of the cause of cytoplasmic incompatibility in *Culex pipiens* L.** *Nature* 1971, **232**:657–658.

179. Tram U, Sullivan W: **Role of delayed nuclear envelope breakdown and mitosis in *Wolbachia*-induced cytoplasmic incompatibility**. *Science* 2002, **296**:1124–1126.

180. Clark ME, Veneti Z, Bourtzis K, Karr TL: **The distribution and proliferation of the intracellular bacteria *Wolbachia* during spermatogenesis in *Drosophila***. *Mechanisms of Development* 2002, **111**:3–15.

181. Ferree PM, Frydman HM, Li JM, Cao J, Wieschaus E, Sullivan W: ***Wolbachia* utilizes host microtubules and dynein for anterior localization in the *Drosophila* oocyte**. *PLoS Pathogens* 2005, **1**:e14.

182. Serbus LR, Sullivan W: **A cellular basis for *Wolbachia* recruitment to the host germline**. *PLoS Pathogens* 2007, **3**:e190.

183. Newton ILG, Savytskyy O, Sheehan KB: ***Wolbachia* utilize host actin for efficient maternal transmission in *Drosophila melanogaster***. *PLoS Pathogens* 2015, **11**:e1004798.

184. Serbus LR, Casper-Lindley C, Landmann F, Sullivan W: **The genetics and cell biology of *Wolbachia*-host interactions.** *Annual Review of Genetics* 2008, **42**:683–707.

185. Dobson SL, Bourtzis K, Braig HR, Jones BF, Zhou W, Rousset F, O'Neill SL: ***Wolbachia* infections are distributed throughout insect somatic and germ line tissues**. *Insect Biochemistry and Molecular Biology* 1999, **29**:153–160.

186. Callaini G, Riparbelli MG, Dallai R: **The distribution of cytoplasmic bacteria in the early *Drosophila* embryo is mediated by astral microtubules.** *Journal of Cell Science* 1994, **107**:673–682.

187. Kose H, Karr TL: **Organization of *Wolbachia pipientis* in the *Drosophila* fertilized egg and embryo revealed by an anti-*Wolbachia* monoclonal antibody**. *Mechanisms of Development* 1995, **51**:275–288.

188. Lassy CW, Karr TL: **Cytological analysis of fertilization and early embryonic development in incompatible crosses of *Drosophila simulans***. *Mechanisms of Development* 1996, **57**:47–58.

189. Vavre F, Fleury F, Lepetit D, Fouillet P, Bouletreau M: **Phylogenetic evidence for horizontal transmission of *Wolbachia* in host-parasitoid associations**. *Molecular Biology and Evolution* 1999, **16**:1711–1723.

190. Jamnongluk W, Kittayapong P, Baimai V, O'Neill SL: ***Wolbachia* infections of tephritid fruit flies: molecular evidence for five distinct strains in a single host species**. *Current Microbiology* 2002, **45**:255–260.

191. Boyle L, O'Neill SL, Robertson HM, Karr TL: **Interspecific and intraspecific horizontal transfer of *Wolbachia* in *Drosophila***. *Science* 1993, **260**:1796–1799.

192. Braig HR, Guzman H, Tesh RB, O'Neill SL: **Replacement of the natural *Wolbachia* symbiont of *Drosophila simulans* with a mosquito counterpart**. *Nature* 1994, **367**:453–455.

193. hang NW, Wade MJ: **The transfer of *Wolbachia pipientis* and reproductive incompatibility between infected and uninfected strains of the flour beetle, *Tribolium confusum*, by microinjection**. *Canadian Journal of Microbiology* 1994, **40**:978–981.

194. Grenier S, Pintureau B, Heddi A, Lassabliere F, Jager C, Louis C, et al.: **Successful horizontal transfer of *Wolbachia* symbionts between *Trichogramma* wasps**. *Proceedings of the Royal Society B* 1998, **265**:1441–1445.

195. Weeks AR, Turelli M, Harcombe WR, Reynolds KT, Hoffmann AA: **From parasite to mutualist: rapid evolution of *Wolbachia* in natural populations of *Drosophila***. *PLoS Biology* 2007, **5**:e114.

196. Kriesner P, Hoffmann AA, Lee SF, Turelli M, Weeks AR: **Rapid sequential spread of two *Wolbachia* variants in *Drosophila simulans***. *PLoS Pathogens* 2013, **9**:e1003607.

197. Hu L, Huang M, Tang M, Yu J, Zheng B: ***Wolbachia* spread dynamics in stochastic environments**. *Theoretical Population Biology* 2015, **106**:32-44.

198. Teixeira L, Ferreira A, Ashburner M: **The bacterial symbiont *Wolbachia* induces resistance to RNA viral infections in *Drosophila melanogaster***. *PLoS Biology* 2008, **6**:e2.

199. Hedges LM, Brownlie JC, O'Neill SL, Johnson KN: ***Wolbachia* and virus protection in insects**. *Science* 2008, **322**:702.

200. Osborne SE, Leong YS, O'Neill SL, Johnson KN: **Variation in antiviral protection mediated by different *Wolbachia* strains in *Drosophila simulans***. *PLoS Pathogens* 2009, **5**:e1000656.

201. Moreira LA, Iturbe-Ormaetxe I, Jeffery JA, Lu G, Pyke AT, Hedges LM, Rocha BC, Hall-Mendelin S, Day A, Riegler M, Hugo LE, Johnson KN, Kay BH, McGraw EA, van den Hurk AF, Ryan PA, O'Neill SL: **A *Wolbachia* symbiont in *Aedes aegypti* limits infection with dengue, chikungunya, and plasmodium.** *Cell* 2009, **139**:1268–1278.

202. Frentiu FD, Robinson J, Young PR, McGraw EA, O'Neill SL: ***Wolbachia*-mediated resistance to dengue virus infection and death at the cellular level**. *PLoS ONE*. 2010, **5**:e13398.

203. Glaser RL, Meola MA: **The native *Wolbachia* endosymbionts of *Drosophila melanogaster* and *Culex quinquefasciatus* increase host resistance to West Nile virus infection**. *PLoS ONE* 2010, **5**:e11977.

204. Unckless RL, Jaenike J: **Maintenance of a male-killing *Wolbachia* in *Drosophila innubila* by male-killing dependent and male-killing independent mechanisms**. *Evolution* 2012, **66**:678–689.

205. Blagrove MSC, Arias-Goeta C, Failloux A-B, Sinkins SP: ***Wolbachia* strain *w*Mel induces cytoplasmic incompatibility and blocks dengue transmission in *Aedes albopictus*.** *Proceedings of the National Academy of Sciences of the United States of America* 2012, **109**:255–260.

206. Stevanovic AL, Arnold PA, Johnson KN: ***Wolbachia*-mediated antiviral protection in *Drosophila* larvae and adults following oral infection**. *Applied and Environmental Microbiology* 2015, **81**:8215–8223.

207. Bian G, Xu Y, Lu P, Xie Y, Xi Z: **The endosymbiotic bacterium *Wolbachia* induces resistance to dengue virus in *Aedes aegypti*.** *PLoS Pathogens* 2010, **6**:e1000833.

208. Bourtzis K: ***Wolbachia*-based technologies for insect pest population control.** *Advances in Experimental Medicine and Biology* 2008, **627**:104–113.

209. Walker T, Johnson PH, Moreira LA, Iturbe-Ormaetxe I, Frentiu FD, McMeniman CJ, Leong YS, Dong Y, Axford J, Kriesner P, Lloyd AL, Ritchie SA, O'Neill SL, Hoffmann AA: **The *w*Mel *Wolbachia* strain blocks dengue and invades caged aedes *Aegypti* populations.** *Nature* 2011, **476**:450–453.

210. Hoffmann AA, Montgomery BL, Popovici J, Iturbe-Ormaetxe I, Johnson PH, Muzzi F, Greenfield M, Durkan M, Leong YS, Dong Y, Cook H, Axford J, Callahan AG, Kenny N, Omodei C, McGraw EA, Ryan PA, Ritchie SA, Turelli M, O'Neill SL: **Successful establishment of *Wolbachia* in *Aedes* populations to suppress dengue transmission**. *Nature* 2011, **476**:454–457.

211. Bian G, Joshi D, Dong Y, Lu P, Zhou G, Pan X, Xu Y, Dimopoulos G, Xi Z: ***Wolbachia* invades *Anopheles stephensi* populations and induces refractoriness to *Plasmodium* infection.** *Science* 2013, **340**:748–751.

212. Kambris Z, Cook PE, Phuc HK, Sinkins SP: **Immune activation by life-shortening *Wolbachia* and reduced filarial competence in mosquitoes.** *Science* 2009, **326**:134–136.

213. Hughes GL, Koga R, Xue P, Fukatsu T, Rasgon JL: ***Wolbachia* infections are virulent and inhibit the human malaria parasite *Plasmodium falciparum* in *Anopheles gambiae*.** *PLoS Pathogens* 2011, **7**:e1002043.

214. Zélé F, Nicot A, Duron O, Rivero A: **Infection with *Wolbachia* protects mosquitoes against *Plasmodium*-induced mortality in a natural system**. *Journal of Evolutionary Biology* 2012, **25**:1243–1252.

215. Ye YH, Woolfit M, Rancès E, O'Neill SL, McGraw EA: ***Wolbachia*-associated bacterial protection in the mosquito *Aedes aegypti***. *PLoS Neglected Tropical Diseases* 2013, **7**:e2362.

216. Chrostek E, Marialva MS, Esteves SS, Weinert LA, Martinez J, Jiggins FM, Teixeira L: ***Wolbachia* variants induce differential protection to viruses in *Drosophila melanogaster*: a phenotypic and phylogenomic analysis**. *PLoS Genetics*. 2013, **9**:e1003896.

217. Martinez J, Longdon B, Bauer S, Chan Y-S, Miller WJ, Bourtzis K, Teixeira L, Jiggins FM: **Symbionts commonly provide broad spectrum resistance**

to viruses in insects: a comparative analysis of *Wolbachia* strains. *PLoS Pathogens* 2014, **10**:e1004369.

218. Min KT, Benzer S: ***Wolbachia*, normally a symbiont of *Drosophila*, can be virulent, causing degeneration and early death.** *Proceedings of the National Academy of Sciences of the United States of America* 1997, **94**:10792–10796.

219. Reynolds KT, Thomson LJ, Hoffmann AA: **The effects of host age, host nuclear background and temperature on phenotypic effects of the virulent *Wolbachia* strain *popcorn* in *Drosophila melanogaster***. *Genetics* 2003, **164**:1027–1034.

220. Sun LV, Riegler M, O'Neill SL: **Development of a physical and genetic map of the virulent *Wolbachia* strain *w*MelPop**. *Journal of Bacteriology* 2003, **185**:7077–7084.

221. McMeniman CJ, Lane R V., Cass BN, Fong AWC, Sidhu M, Wang YF, O'Neill SL: **Stable introduction of a life-shortening *Wolbachia* infection into the mosquito *Aedes aegypti***. *Science*.2009, **323**:141–144.

222. McMeniman CJ, O'Neill SL: **A virulent *Wolbachia* infection decreases the viability of the dengue vector *Aedes aegypti* during periods of embryonic quiescence**. *PLoS Neglected Tropical Diseases* 2010, **4**:e748.

223. Chrostek E, Teixeira L: **Mutualism breakdown by amplification of *Wolbachia* genes**. *PLoS Biology* 2015, **13**:e1002065.

224. Wong ZS, Brownlie JC, Johnson KN: **Oxidative stress correlates with *Wolbachia*-mediated antiviral protection in *Wolbachia-Drosophila* associations**. *Applied and Environmental Microbiology* 2015, **81**:3001–3005.

225. Chrostek E, Marialva MSP, Yamada R, O'Neill SL, Teixeira L: **High anti-viral protection without immune upregulation after interspecies *Wolbachia* transfer**. *PLoS ONE* 2014, **9**:e99025.

226. Bourtzis K, Pettigrew MM, O'Neill SL: ***Wolbachia* neither induces nor suppresses transcripts encoding antimicrobial peptides**. *Insect Molecular Biology* 2000, **9**:635–639.

227. Rottschaefer SM, Lazzaro BP: **No effect of *Wolbachia* on resistance to intracellular infection by pathogenic bacteria in *Drosophila melanogaster***. *PLoS ONE* 2012, **7**:e40500.

228. Wong ZS, Hedges LM, Brownlie JC, Johnson KN: ***Wolbachia*-mediated antibacterial protection and immune gene regulation in *Drosophila***. *PLoS ONE* 2011, **6**:e25430.

229. Rancès E, Johnson TK, Popovici J, Iturbe-Ormaetxe I, Zakir T, Warr CG, O'Neill SL: **The Toll and Imd pathways are not required for *Wolbachia*-mediated dengue virus interference.** *Journal of Virology* 2013, **87**:11945–11949.

230. Kambris Z, Blagborough AM, Pinto SB, Blagrove MSC, Godfray HCJ, Sinden RE, Sinkins SP: ***Wolbachia* stimulates immune gene expression and inhibits *Plasmodium* development in *Anopheles gambiae***. *PLoS Pathogens* 2010, **6**:e1001143.

231. Pan X, Zhou G, Wu J, Bian G, Lu P, Raikhel AS, Xi Z: ***Wolbachia* induces reactive oxygen species (ROS)-dependent activation of the Toll pathway to control dengue virus in the mosquito *Aedes aegypti***. *Proceedings of the National Academy of Sciences of the United States of America* 2012, **109**:23–31.

232. Serbus LR, White PM, Silva JP, Rabe A, Teixeira L, Albertson R, Sullivan W: **The impact of host diet on *Wolbachia* titer in *Drosophila***. *PLoS Pathogens* 2015, **11**:1–25.

233. Ponton F, Wilson K, Holmes A, Raubenheimer D, Robinson KL, Simpson SJ: **Macronutrients mediate the functional relationship between *Drosophila* and *Wolbachia***. *Proceedings of the Royal Society B* 2015, **282**:20142029.

234. Hosokawa T, Koga R, Kikuchi Y, Meng XY, Fukatsu T: ***Wolbachia* as a bacteriocyte-associated nutritional mutualist**. *Proceedings of the National Academy of Sciences of the United States of America* 2010, **107**:769–774.

2

Wolbachia-positive host adaptation against pathogens

2.1. Prologue

To study and the evolution of species in a controlled and accurate way is undoubtedly a challenge. The complexity of factors acting simultaneously on individuals creates a worrying probability of blurring the conclusions that can be drawn. To minimize most of this risk, and to facilitate the control of experimental conditions, it has become essential to bring populations from nature to the laboratory, keeping these individuals in parental lineages (isolines) or recreating a new population with a collection of individuals (outbred populations).

As soon as such populations leave nature, they no longer undergo certain selective pressures but are submitted instead to other intrinsic to the laboratory environment. Some research groups are precisely dedicated to explore the underpinnings of laboratorial adaptation. In other cases, as ours, the purpose is to test other and new selective pressures in a controlled manner. For this, one must first wait for the outbred populations to adapt to the lab and stabilization of its genetic pool, in order to avoid simultaneous selective pressures that would lead to the confounding effects and confusing findings.

It is always necessary to keep in mind that adaptation is largely influenced by the genetic diversity that was included in the founding samples of populations. Thus, it is possible, and even likely in some cases, that populations collected in different locations may have different adaptive responses. Moreover, when populations originate from the same location but the effective population size is not representative of the total population, the response to the tested feature may vary.

Another important characteristic that should be considered is the presence of symbionts in these nature-collected individuals. In the case of *Drosophila melanogaster*, the intracellular endosymbionts *Wolbachia* and

Spiroplasma are pervasive between and within populations. However, other endosymbionts must also be taken into account, namely the gut microbiome. It is likely that the microbiota undergoes major changes after the population's arrival to the lab, not only due to the temperature of maintenance but mostly due to the switching of food source (different, standardized and sometimes with antibiotics and antifungals). Be it as it may, it is a factor which is no longer neglected by researchers, with proven influence on numerous traits, with emphasis on health and response capacity of individuals to diverse biotic and abiotic challenges.

As so, the goal in this Chapter is, first, to show the potential of these biological tools that can be created by bringing new specimens from nature to the lab, contributing to maximize the exploration of these resources, as well as trying to standardize the protocol to the *Drosophila* community. This work will facilitate the process of establishing these resources in other labs, simultaneously allowing better comparisons and parallelisms between studies using different populations in several parts of the world.

We used these tools with special emphasis on outbred populations, to approach the study of the evolution of the immune response. Using different methodologies and techniques, we addressed different levels of the dynamics and mechanisms (cellular, physiological, genetic and genomic) underlying host adaptation against different pathogens infecting through different routes. Moreover, we also want to understand which are the costs involved in these processes, thus revealing the possible trade-offs coupled to these evolutionary processes.

This Chapter thus intends to search for a large spectrum of immunological and evolutionary questions, which we are convinced will represent significant contributions to approaching and understanding host-parasite interactions.

2.2. From nature to the lab: establishing *Drosophila* resources for evolutionary genetics

Vítor G. Faria[1#], Élio Sucena[1, 2#]

1 - Instituto Gulbenkian de Ciência, Apartado 14, 2781-901 Oeiras, Portugal

2 - Departamento de Biologia Animal, Faculdade de Ciências, Universidade de Lisboa, edifício C2, Campo Grande, 1749-016 Lisboa, Portugal

- Email : vfaria@igc.gulbenkian.pt; esucena@igc.gulbenkian.pt

keywords: experimental evolution, outbred populations, cryptic species, isofemale lines, wild-specimen collection, multiplex PCR, introgression

Article in preparation for publication

Author Contributions: Conceived and designed the experiments: V.G.F., É.S. Performed the experiments: V.G.F., É.S. Analyzed the data: V.G.F., É.S. Contributed reagents/ materials/analysis tools: É.S. Wrote the paper: V.G.F., É.S.

The authors declare no conflict of interest.

Funding**:** Fundação para a Ciência e a Tecnologia (FCT, Portugal) supports V.G.F (SFRH/BD/ 82299/2011). This work was supported by Instituto Gulbenkian de Ciência/Fundação Calouste Gulbenkian. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Abstract

In recent years many tools have been developed in *Drosophila* to capture with the greatest possible accuracy the variation found in nature. Efforts such as the Drosophila Genetics Reference Panel (DGRP) or the Drosophila Synthetic Population Resource Panel (DSRP) allied to the advances in whole-genome sequencing and analysis have propelled to unprecedented level our capacity to dissect the genotype-phenotype map. However, several practical problems arise upstream of these analyses starting with the collection and identification of wild specimens. These problems are dealt with in different ways by each researcher generating solutions not necessarily compatible across laboratories. Here, we provide a systematic coverage of every phase of this process based on our experience, and suggest procedures to maximize the generated resources potentiating future applications across laboratories. We provide a detailed pipeline to guide researchers from collection in the wild to the development of a large array of molecular and genetic resources. We designed a multiplex PCR that distinguishes the two sister species *D. melanogaster* and *D. simulans* and is diagnostic of the presence/absence of *Wolbachia* bacteria infection. These procedures may extended to other cryptic species pairs and endosymbionts. We developed a standardized protocol to create, replicate and maintain isofemale lines and outbred populations. Finally, we explore the potential of outbred populations across several applications from experimental evolution, to genetic introgression of transgenic constructs or mutant alleles, and genomic analyses. We have generated a systematic coverage of all steps taken between collection of wild *Drosophila* and the laboratory usage of its derived analytical tools. With this we wish to contribute to the success in developing *Drosophila* resources for evolutionary genetics studies and facilitate exchanges across laboratories based on a common set of procedures.

Introduction

Maintaining the original populations variation after bring populations from nature to the lab is certainly a challenge and can be achieved through two different methods, using parental lineages (isolines) or recreating a new outbred population, the central element to experimental evolution studies.

Experimental evolution can establish direct causation between selection in a given environment and the genetic and phenotypic changes observed in a population. This powerful approach departs from the comparative method in three fundamental aspects: (i) knowledge of the ancestral state, (ii) knowledge of the adaptive trajectories in real-time, (iii) high degree of replication under controlled selection and control regimes [1–4].

At a different plane, recent years have witnessed the rise of genomic studies, which have provided significant insights into the genetic basis of adaptation for a variety of complex traits. Examples cross all biological organization levels and include studies on transposable element population dynamics [5], developmental time [6], immune response [7,8], hypoxia tolerance [9], body size [10], adaptation to high/low temperatures [11,12], courtship behaviour [13] and life span [14,15].

The combination of genomics with experimental evolution can provide a nearly unbiased estimate of the genetic changes that underlie the adaptation of populations to a given selective pressure, a central issue in evolutionary biology [3]. The success of this methodology lies in the choice of the model species (with solid genomic tools, such as *Drosophila*) and the availability of outbred populations (with high levels of genetic variability) in which adaptation relies mostly on standing genetic variation (SGV) [16,17]. The potential of this methodology has been confirmed in a number of recent

studies [7,8,12,15,18–21], and expectedly this approach will gain more and more followers in the coming years [22,23].

Other important, and complementary approaches, underscore the importance of describing and understanding the nature of standing genetic variation in natural and laboratory populations. For example, the increased use of isofemale lines propelled by the DGRP-Drosophila Genome Reference Panel [24], has inaugurated an era of unprecedented success in *Drosophila* GWAS studies [25–30]. In parallel, the development of recombinant inbred lines that constitute the Drosophila Synthetic Population Resource Panel (DSRP) have provided another extraordinary resource for the dissection of the genetic basis of complex traits [31-34]. Before this, isofemale lines had been at the core of fecund research programs aiming at describing and comparing genomic variation between *D. melanogaster* and its sister species [35], and comprehending their genotype-phenotype map [36–41]. Finally, individually wild-collected flies have provided valuable information in describing and quantifying natural variants [42,43], characterizing ecological and evolutionary dynamics of natural populations [44–46], estimating the spread dynamics of endosymbionts in natural populations [47], validating laboratory results [48] and testing high throughput re-sequencing techniques [40].

Though these different approaches differ in the nature and level at which they ask their questions, they share a common founding feature: the collection of material from nature and its subsequent establishment as a laboratory resource. In the case of *Drosophila melanogaster*, one seemingly trivial yet important question that must be resolved consists of its co-existence with the cryptic species *D. simulans.* Here, we present a protocol to streamline the collection, identification and establishment of *D. melanogaster* in the laboratory. We provide a high-throughput method, that not only identifies the species but also the individual's status of *Wolbachia*

infection, and that can be easily extended to other species. We provide a pipeline to maximize the resources generated, namely the establishment of outbred populations, isofemale lines, and DNA/RNA banks for genomic studies.

Results

Collection

Generic methods to collect *Drosophila* species have been described in Markow and O´Grady [49]. For the specific collection of *Drosophila melanogaster*, our own experience favours the choice of a vineyard as the collection site, given the advantages that collecting from large populations provide (particularly during harvest, which corresponds roughly to the period from August to October in the northern hemisphere) (Figure 2.2.1). Using a hand vacuum cleaner adapted to this purpose (see M&M), in 2007 and 2013, we collected in a single afternoon around 5000 females from the

Figure 2.2.1 – Vineyards as a large-scale collection site for wild *Drosophila* specimens. (**A**) Winery dump site in Southern Portugal (**B**) portable vacuum cleaner coupled to a acrylic tube with a soft net (**C**) collecting a large number of individuals (**D**) flies are transferred directly to bottles containing standard fly food.

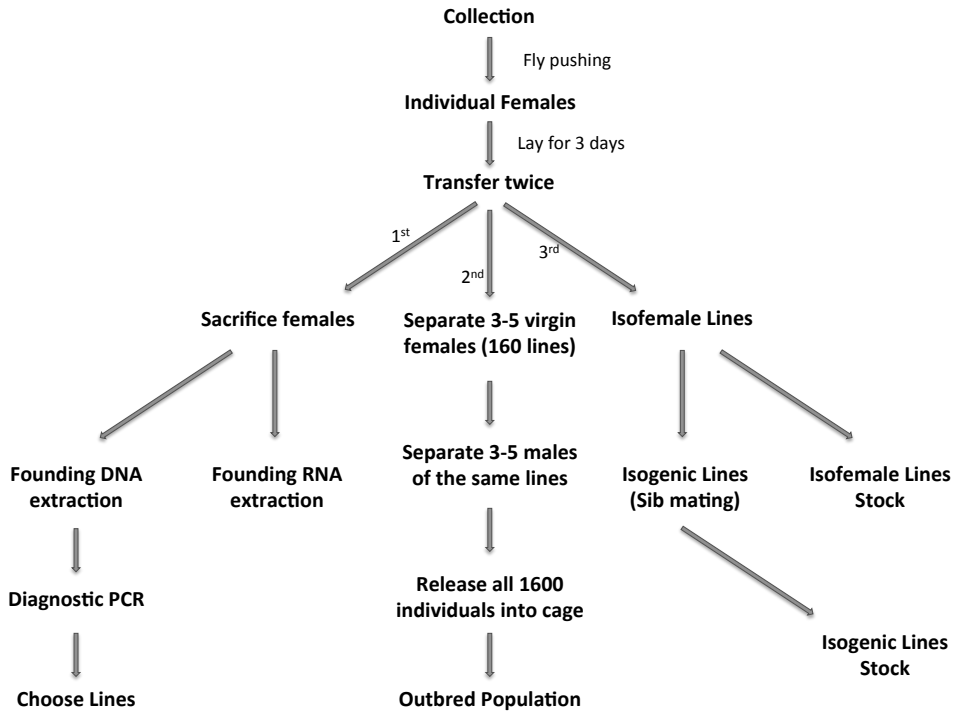


Figure 2.2.2 – Pipeline of how to establish *Drosophila* laboratory resources upon collection from nature.

José Maria da Fonseca winery in Southern Portugal (Azeitão, Portugal GPS: 38º 31' 04.91" N9º 00' 56.24").

Back to the lab, we followed a sequential approach in order to maximize the collected resources. These steps are schematically presented in Figure 2.2.2. First, single females were separated in vials to lay eggs and ensure the next generation. Then, the progenitor females were sacrificed and used as starting material for to individual nucleic acids extraction (both DNA and RNA) in 96-well plates.

Screening isofemale lines

Guided by Alberto Civetta [50], we have scanned the genomes of *D. melanogaster* and *D. simulans* for large indels that would be diagnostic of

species through a simple PCR followed by electrophoresis. We chose Slif (CG11128) for which we designed a primer pair (see M&M) that amplifies fragments of 939bp in *D. melanogaster* and 1058bp in *D. simulans*. To detect *Wolbachia*, we used primers designed for *wsp* (*Wolbachia* surface protein) by Zhou and co-workers [51] (see M&M), which produce two differently sized fragments of 610bp and 590bp, for *D. melanogaster* and *D. simulans*, respectively. The *Wolbachia wsp* gene amplification product serves therefore two purposes: the characterization of the individual's infection status and a second (indirect) confirmation of the *Drosophila* species itself, conveyed by the different amplicon sizes generated by respective *Wolbachia* strains.

Figure 2.2.3 shows how this combination of primers reveals simultaneously, in a multiplex PCR (see M&M), the status of *Wolbachia* infection and which of the cryptic species it is. The PCR program used permits the simultaneous amplification of both fragments without any primer incompatibility or confounding effects from nonspecific bands. We have performed this method at high throughput rate using 96-well plates.

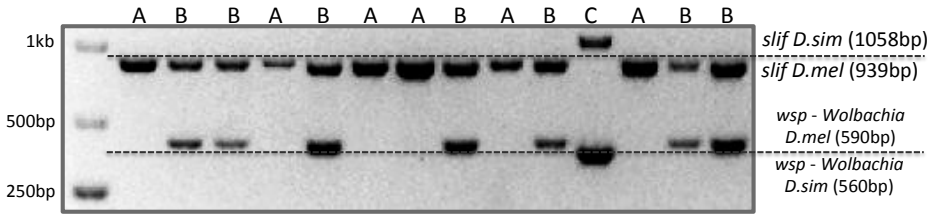


Figure 2.2.3 – Rapid *Drosophila* species identification and infection status by PCR multiplex. Each lane contains the PCR amplification products using *Wolbachia* specific primers and primers for the gene *slif*. In both cases, amplification products have distinctive sizes that allow identification of the species and determination of *Wolbachia* infection status. Letters A, B or C refer to *D. melanogaster*/ *Wolbachia*-negative, *D. melanogaster*/ *Wolbachia*-positive and *D. simulans*/ *Wolbachia*-positive samples, respectively.

To validate the species diagnosis, we performed in parallel a blind test running our method and performing the classical male genitalia classification based on morphological analysis. For this we took 50 males from one collection and mounted their genitalia (Figure 2.2.4) as described in Ashburner [52] and used the carcass to prepare genomic DNA for each individual. We found a 100% match (50 in 50) fit between the two classification methods, leading to the conclusion that the method we developed is at least as good as the more time-consuming (and more subjective) classical method.

Figure 2.2.4 – Species confirmation by male genitalia. As described in [52] we show the distinctive male genital arch (arrows) of *D. simulans* (right) and *D. melanogaster* (left).

As illustration of the proportions found in our specific case, in the 2013 collection we genotyped 576 (96x6) fertile females (progenitors of isofemale lines), being 341 (*D.mel*/*Wol*+), 189 (*D.mel*/*Wol*-), 36 (*D.sim*/*Wol*+), 1 (*D.sim*/*Wol*+) and 9 failed extractions/amplifications.

Isofemale lines and outbred population establishment

From the collection described above, we established an outbred population from 160 fertilized *Wolbachia*-positive females. We used 3-5 virgin females and 3-5 males from the F1 of each previously screened line.

In parallel, we started 160 isofemale lines, kept in similar maintenance conditions but under an inbreeding regime, in vials with uncontrolled census and overlapping generations.

Experimental evolution

After the establishment of laboratory-controlled outbred populations, namely of *Drosophila melanogaster* infected with *Wolbachia,* we waited several generations for adaptation to the lab and consecutive stabilization of genetic pool. After that, we sequentially expanded the population in order to generate several identical replicas (see M&M). In our case, we used those populations to follow their response and genetic configuration upon different pathogenic challenges and different infection routes [7,8]. Additionally, we also approached the eventual costs of adaptation [53] and the influence of the increase of immunocompetence on *Wolbachia* population [54].

In each generation of experimental evolution, 200-400 flies (males and females) were frozen and posteriorly used for different analyses, such as PoolSeq and genotyping [8,54].

Introgression

After the foundation of outbred populations, the introgression of specific alleles in the populations can be performed. In the case of visible markers this procedure is rather simple, though laborious, as exemplified by the scheme below (M&M) used to introgress the white mutant allele (w^{1118}) into the outbred population. Using over 100 single female crosses per generation, we recombined the white mutant allele into "wild" chromossomes 6 times. Also, all other chromosomes were outbred as they were replaced in full (using balancer chromosomes) from the initial generations of the crossing scheme (see M&M). With this number of

recombination rounds the estimated proportion of the white allele-carrying X chromossome in the final population is theoretically inferior to 2% (2^6 = 1/64). Except for the fragment of the X in linkage with the white locus, every other fragment from the w^{1118} stock was randomized and should have virtually no impact on the phenotypes observed at the population level. Effectively, we have generated an outbred population genetically indistinguishable from the wild-derived initial outbred population except for the fact it carries the w^{1118} allele.

Additionally, we have started a systematic introgression of transgenic lines into the outbred background.

Discussion

We have attempted to propose a systematic and normalized set of procedures when establishing *Drosophila* tools upon collection of flies from nature. This approach can be extended to any cryptic species pair, including both intra and extracellular symbionts. In our case, a collection in a vineyard in Portugal (Figure 2.2.1) potentially allows the establishment of a total of 8 different populations: *D. melanogaster* and *D. simulans*, with *Wolbachia* and/or *Spiroplasma* or none. Interestingly, we also observed in our collections the sporadic presence of parasitoid wasps, in particular species belonging to the genus *Leptopilina*. Although it is possible to start some laboratorial isolines with captured wasps, the low frequency indicates that the open-air method (or even the location) used to flies is not the most suitable to catch high number of wasps to, for example, initiate a outbred population of wasps. Other relevant studies could be performed with these flies concerning the gut microbiome of different capture regions and seasons, which probably reflects the difference in locations and diets.

The pipeline here presented (Figure 2.2.2) can be applied to any collection regardless of the fly species and initial object of study as it

preserves to the fullest the potential of samples for future analysis. Inbred lines and the outbred population have, in principle, retained to a great extent the same qualitative variation of the sampled population. Yet, these different methods of maintaining specimens impact differently this genetic variation, namely in what regards the frequencies of deleterious recessive alleles and epistatic complexes [55,56]. This has been eloquently demonstrated by Huang and co-workers that found distinct genetic bases for the same traits analysed by GWAS on the DGRP panel or on a population reconstituted from the same DGRP panel lines [28]. However a recent study shows that no significant allele differences are found between an ancestral population and a reconstituted counterpart generated by isofemale lines derived from the same original population [57]. Be it as it may, isofemale lines or isogenic lines, on one hand, and outbred populations, on another, are best suited for different purposes and questions. However, these tools may be regarded also as complementary and yield distinct but equally informative results such as resistance against oral infection with *Pseudomonas entomophila* where GWAS analysis using the DGRP panel [58] and Pool-seq on experimentally-evolved populations (our unpublished results), showed qualitatively different genetic bases. Moreover, these approaches may reveal comparable outcomes and corroborate one another as illustrated by resistance against DCV infection, for example [8,27].

The sequential 96 well-plate protocols of DNA extraction, multiplex PCR and agarose gel electrophoresis allows a quick analysis of a large number of specimens. Moreover, the method is also very reliable to distinguish between *D. melanogaster* and *D. simulans*, as evidenced by the comparison with the male genitalia method (Figure 2.2.4). However, while the preparation of the genitalia is a time-consuming procedure that may need an experienced manipulator to dissect, assemble and efficiently distinguish both species, the PCR here described permits high-throughput

easily. This method allows the testing of large amounts of individuals, necessary for the foundation of outbred populations. In addition, after this first nucleic acid extraction, the resulting material may be used for further genetic tests. In addition, in this setup, other primers may be included or changed to quickly diagnose different species and/or strains.

Prior to starting selection experiments, it is essential to adapt the outbred population to the lab, in itself a novel environment to which the population is exposed [59,60]. Major changes may occur in the populational structure during this period of adaptation to laboratorial conditions, both in flies and associated microbiome. After this step, performing pilot tests is essential to confirm that replicate populations respond similarly against the chosen selective pressure. Another complementary approach to caracterize and validate the populations before the beginning of experimental evolution is the estimation of heterozygosity and initial effective population size. This information provides a clear idea of the potential of this tool in future studies.

Populational replicates are therefore ready to be submitted to against a vast range of selective pressures. In our case, as already mentioned, we evolved those populations in the presence or absence of pathogens, namely *Pseudomonas entomophila* and *Drosophila* C Virus (DCV). Throughout experimental evolution, we have frozen adult flies in each generation, creating a bank to explore genetic questions about the adaptive processes.

The posterior introgression of genetic markers could also create very useful tools to evolutionary and genetic questions. The outbred w^{1118} population can be compared and/or used as control as it is easily distinguishable from the outbred population, though virtually identical from a genetic perspective. Behavioural and competition experiments are also important applications of this tool. However, caution is advised in this case,

as the *white* mutation is far from being a fully innocuous marker. Indeed, *white* codes for an ABC transporter subunit [61] described to play a role in a number of homeostatic functions, namely in the nervous system and may impinge functionally on a number of traits [62,63].

Finally, this population can be used to introgress transgenic constructs into the outbred background. Following the same crossing scheme (see M&M) it is straightforward to introgress into the outbred background mini-white containing transgenics, namely of the vast available collection of UAS and GAL4 lines. In this case, upon recombination in heterozygous females, non-white males can be selected to cross against outbred white females. This may prove to be and interesting tool to test the effects of such transgenes in a properly controlled genetic background.

Here, we have attempted to provided the community with a comprehensive guide for the establishment and development of the necessary laboratory resources stemming from the initial collection of wild *Drosophila* specimens. With this we hope also to contribute to the standardization of procedures permitting an easier exchange of resources across researchers engaging in the study of natural variation in laboratory conditions.

Material and Methods

Collection

Using a portable vacuum cleaner coupled to a simple acrylic tube custom made with a soft net on one end (Figure 2.2.1B), we collected large numbers of flies from a vineyard dump site (Figure 2.2.1C). After collection, flies were transferred directly to bottles containing standard cornmeal-agar medium (Figure 2.2.1D). Around 1000 females were separated and individually distributed to vials with standard food. These females were transferred twice to new vials, laying eggs during 3 days in each vial.

Nucleic acid extraction

To perform nucleic acid extraction, after the second turn of egglaying, each vial with fertile progeny (checking the presence of F1 larvae in the food) was numbered and the respective female was anesthetized with CO_2. A 96-well plate was previously prepared for DNA extraction, cooled over dry ice to facilitate the placing of the anesthetized females in the respective wells.

Nucleic acid extraction was performed according to (http://www.drosdel.org.uk/molecular_methods.php) [64] with minor modifications. Briefly, each biological sample was homogenized with metallic beads and detergent for cell lysis. After removing the cellular waste by centrifugation, the supernatant was transferred to a new 96-well plate and nucleic acids were separated and precipitated with KCl and isopropanol. Samples were further washed in EtOH 70% and afterwards ressuspended in milliQ H_2O. All steps were alternated with centrifugation steps. DNA or RNA were isolated after incubation with either RNase or Dnase, respectively.

Diagnostic PCR

After DNA extraction, a multiplex PCR reaction was performed in 96-well plates using 1µL of diluted DNA, GoTaq DNA polymerase (Promega) in a 20µL total reaction volume per well, using the primers Slif (Fwd – 5´-GTTAGCGCCTATTAGCACAT-3'; Rev – 5´-CGGGACAACTCAGTCTGTAA-3') to distinguish between *D. melanogaster* and *D. simulans* and *wsp* (81F- 5´-TGGTCCAATAAGTGATGAAGAAAC-3'; 691R- 5´-AAAAATTAAACGCTACTCCA -3'), to diagnose for the presence or absence of *Wolbachia*. The PCR program was used as follows: 95 ºC for 10 min; 30 cycles at 95 ºC for 30s (denaturation), 60 ºC for 1 min (annealing), 72 ºC for 1 min (elongation)

and a final extension step at 72 ºC for 10 min. PCR amplification products were visualized after electrophoresis in agarose gel (1.5% in TAE supplied with 0.5% RedSafe).

After analysis, the F1 vials of each tested-isofemale line could be separated into four groups, *D. simulans* or *D. melanogaster*, and *Wolbachia* positive or negative.

Outbred populations foundation

From 160 isofemale lines of each group previously diagnosed, 10 F1-flies, 3-5 virgin females and 3-5 males were separated. Groups of males and virgin females were placed simultaneously in populational acrylic boxes (50x30x25cm), thus minimizing sib-mating. Around 1500 individuals founded each populations.

Populations were kept on a three-week non-overlapping generations. Treatments were always performed 3-5 days after eclosion and reproduction occurred 5-7 days after treatment. Reproduction was performed in 10 plastic cups (5 per day) with standard food. Egg density was limited to 400 per cup, a density determined experimentally to enable optimal larval development and population effective numbers. Flies were maintained under constant temperature (25 ^{o}C), humidity (60-70%) and light-darkness cycle (12:12 hours), and fed with standard cornmeal-agar medium.

Each population was kept in laboratory cages with high census (between 1500 and 2000 individuals). Census above 2000 flies will lead to excess moisture inside the boxes that compromises egglaying, mobility and viability of flies and promotes bacterial and fungal proliferation.

Starting Experimental Evolution

Before the initiation of experimental evolution experiments, populations were maintained under the laboratory previously described conditions for a minimum of 15 generations and then serially expanded for two generations to allow the establishment of the new replicate populations. In our case, all lines of all treatments were derived from the same base population (from 1 to 6 in first generations and from 6 to 36 in the second). The egglaying for the foundation should be randomly distributed across the replicates to avoid any selection for fertility. In each generation, pools of with 200-300 flies of each replicate were frozen in liquid nitrogen and kept at -80 ºC.

Isofemale lines

Isofemale lines were established using the vials of each original line,. The successive backcrosses in consecutive generations led to a considerable increase in inbreeding of each isofemale line. These lines may have kept genetic information of the founding populations, which is expected to eventually be lost in the future process of adaptation.

Starting from the isofemale lines, one can also establish isogenic lines. To do so, each isofemale line can be taken through 20 generations of full-sib mating as done by MacKay and co-workers [24]. This procedure should purge deleterious alleles and provide, at a reasonable frequency, fertile and viable genetically-homogeneous lines.

Introgression

We introgressed the *white* mutant allele (w^{1118}) into the outbred population. Using 80 to 100 single female crosses in the first two generations, we replaced all second and third chromosomes from the w^{1118} stock by "wild" chromosomes of the outbred population. In each odd generation (F1, F3, F5, F7, F9, F11) recombination in females reduces the

contribution of the w^{1118} stock. From these crosses, 2 white-eyed males were used to establish at least 100 single female crosses with virgins from the outbred stock (all even generations F2, F4, F6, F8, and F10). In the F12 generation, 3 to 5 virgin females and 3 to 5 males from 140 F11 single-female crosses were released into a population cage to establish the white introgressed population.

Crossing scheme:

(P) Outbred females (O;O;O) x w; If/CyO; MKRS/TM6b males

(F1) O/w; O/CyO; O/TM6b x Outbred males (O; O; O)

(F2) Outbred females (O;O;O) x w; O; O

(F3) O/w; O; O x Outbred males (O; O; O)

F2 and F3 crosses were repeated 4 more rounds

(F12) O/w; O; O x O/w; O; O

Number of individuals used in each single-female cross throughout the introgression procedure.

	Females	Males
F1	80	160
F2	100	60
F3	100	200
F4	120	120
F5	120	200
F6	120	100
F7	110	220
F8	120	100
F9	100	200
F10	140	280
F11	140	280
F12	(3-5) *140	(3-5) *140

Acknowledgements

We thank the Sucena, Beldade and Mirth Labs for discussion. We are indebted to Alberto Civetta for sharing unpublished data and for advice on the choice of the species-diagnostic polymorphisms. We thank Ivo Chelo, Henrique Teotónio, Nelson Martins and Tânia Paulo for their help with the genomic analyses.

References

1. Rose MR, Nusbaum TJ, Chippindale AK: **Laboratory evolution: the experimental wonderland and the Cheshire cat syndrome**. In *Adaptation*. Edited by Rose MR, Lauder GV. San Diego, CA: Academic Press; 1996:221–241.

2. Gibbs AG, Chippindale AK, Rose MR: **Physiological mechanisms of evolved desiccation resistance in *Drosophila melanogaster***. *Journal of Experimental Biology* 1997, **200**:1821–1832.

3. Kawecki TJ, Lenski RE, Ebert D, Hollis B, Olivieri I, Whitlock MC: **Experimental evolution.** *Trends in Ecology and Evolution* 2012, **27**:547–560.

4. Magalhães S, Matos M: **Strengths and weaknesses of experimental evolution**. *Trends in Ecology and Evolution* 2012, **27**:649–650.

5. Barrón MG, Fiston-Lavier AS, Petrov DA, González J: **Population genomics of transposable elements in *Drosophila***. *Annual Review of Genetics* 2014, **48**:561–581.

6. Burke MK, Dunham JP, Shahrestani P, Thornton KR, Rose MR, Long AD: **Genome-wide analysis of a long-term evolution experiment with *Drosophila*.** *Nature* 2010, **467**:587–590.

7. Martins NE, Faria VG, Teixeira L, Magalhães S, Sucena É: **Host adaptation is contingent upon the infection route taken by pathogens**. *PLoS Pathogens* 2013, **9**:e1003601.

8. Martins NE, Faria VG, Nolte V, Schlötterer C, Teixeira L, Sucena É, Magalhães S: **Host adaptation to viruses relies on few genes with different cross-resistance properties**. *Proceedings of the National Academy of Sciences of the United States of America* 2014, **111**:5938–5943.

9. Zhou D, Udpa N, Gersten M, Visk DW, Bashir A, Xue J, Frazer K a, Posakony JW, Subramaniam S, Bafna V, Haddad GG: **Experimental selection of hypoxia-tolerant *Drosophila melanogaster*.** *Proceedings of the National Academy of Sciences of the United States of America* 2011, **108**:2349–2354.

10. Turner TL, Stewart AD, Fields AT, Rice WR, Tarone AM: **Population-based resequencing of experimentally evolved populations reveals**

the genetic basis of body size variation in *Drosophila melanogaster*. *PLoS Genetics* 2011, **7**:e1001336.

11. Orozco-terWengel P, Kapun M, Nolte V, Kofler R, Flatt T, Schlötterer C: **Adaptation of *Drosophila* to a novel laboratory environment reveals temporally heterogeneous trajectories of selected alleles.** *Molecular Ecology* 2012, **21**:4931–4941.

12. Tobler R, Franssen SU, Kofler R, Orozco-Terwengel P, Nolte V, Hermisson J, Schlötterer C: **Massive habitat-specific genomic response in *D. melanogaster* populations during experimental evolution in hot and cold environments**. *Molecular Biology and Evolution* 2014, **31**:364–375.

13. Turner TL, Miller PM, Cochrane VA: **Combining genome-wide methods to investigate the genetic complexity of courtship song variation in *Drosophila melanogaster*.** *Molecular Biology and Evolution* 2013, **30**:2113–2120.

14. Remolina SC, Chang PL, Leips J, Nuzhdin S V, Hughes KA: **Genomic basis of aging and life-history evolution in *Drosophila melanogaster*.** *Evolution* 2012, **66**:3390–3403.

15. Durham MF, Magwire MM, Stone EA, Leips J: **Genome-wide analysis in *Drosophila* reveals age-specific effects of SNPs on fitness traits**. *Nature Communications* 2014, **5**:4338.

16. Barrett RDH, Schluter D: **Adaptation from standing genetic variation**. *Trends in Ecology and Evolution* 2008, **23**:38–44.

17. Teotónio H, Chelo IM, Bradić M, Rose MR, Long AD: **Experimental evolution reveals natural selection on standing genetic variation**. *Nature Genetics* 2009, **41**:251–257.

18. Turner TL, Miller PM: **Investigating natural variation in *Drosophila* courtship song by the evolve and resequence approach**. *Genetics* 2012, **191**:633–642.

19. Abbott JK, Innocenti P, Chippindale AK, Morrow EH: **Epigenetics and sex-specific fitness: an experimental test using male-limited evolution in *Drosophila melanogaster***. *PLoS ONE* 2013, **8**:e70493.

20. Klepsatel P, Gáliková M, De Maio N, Huber CD, Schlötterer C, Flatt T: **Variation in thermal performance and reaction norms among populations of *Drosophila melanogaster*: thermal performance in *Drosophila***. *Evolution* 2013, **67**:3573–3587.

21. Klepsatel P, Gáliková M, De Maio N, Ricci S, Schlötterer C, Flatt T: **Reproductive and post-reproductive life history of wild-caught *Drosophila melanogaster* under laboratory conditions**. *Journal of Evolutionary Biology* 2013, **26**:1508–1520.

22. Kawecki TJ, Lenski RE, Ebert D, Hollis B, Olivieri I, Whitlock MC: **Experimental evolution**. *Trends in Ecology and Evolution* 2012, **27**:547–60.

23. Kofler R, Schlötterer C: **A guide for the design of evolve and resequencing studies**. *Molecular Biology and Evolution* 2014, **31**:474–483.

24. Mackay TFC, Richards S, Stone EA, Barbadilla A, Ayroles JF, Zhu D, Casillas S, Han Y, Magwire MM, Cridland JM, Richardson MF, Anholt RRH, Barrón M, Bess C, Blankenburg KP, Carbone MA, Castellano D, Chaboub L, Duncan L, Harris Z, Javaid M, Jayaseelan JC, Jhangiani SN, Jordan KW, Lara F, Lawrence F, Lee SL, Librado P, Linheiro RS, Lyman RF, et al.: **The *Drosophila melanogaster* Genetic Reference Panel**. *Nature* 2012, **482**:173–178.

25. Weber AL, Khan GF, Magwire MM, Tabor CL, Mackay TFC, Anholt RRH: **Genome-wide association analysis of oxidative stress resistance in *Drosophila melanogaster***. *PLoS ONE* 2012, **7**:e34745.

26. Jordan KW, Craver KL, Magwire MM, Cubilla CE, Mackay TFC, Anholt RRH: **Genome-wide association for sensitivity to chronic oxidative stress in *Drosophila melanogaster***. *PLoS ONE* 2012, **7**:e38722.

27. Magwire MM, Fabian DK, Schweyen H, Cao C, Longdon B, Bayer F, Jiggins FM: **Genome-Wide Association Studies Reveal a Simple Genetic Basis of Resistance to Naturally Coevolving Viruses in *Drosophila melanogaster***. *PLoS Genetics* 2012, **8**:e1003057.

28. Huang W, Richards S, Carbone MA, Zhu D, Anholt RRH, Ayroles JF, Duncan L, Jordan KW, Lawrence F, Magwire MM, Warner CB, Blankenburg K, Han Y, Javaid M, Jayaseelan J, Jhangiani SN, Muzny D, Ongeri F, Perales L, Wu Y-Q, Zhang Y, Zou X, Stone EA, Gibbs RA, Mackay TFC: **Epistasis dominates the genetic architecture of *Drosophila* quantitative traits**. *Proceedings of the National Academy of Sciences of the United States of America* 2012, **109**:15553–15559.

29. Swarup S, Huang W, Mackay TFC, Anholt RRH: **Analysis of natural variation reveals neurogenetic networks for *Drosophila* olfactory**

behavior. *Proceedings of the National Academy of Sciences of the United States of America* 2013, **110**:1017–1022.

30. Kislukhin G, King EG, Walters KN, Macdonald SJ, Long AD: **The genetic architecture of methotrexate toxicity is similar in *Drosophila melanogaster* and humans**. *G3* 2013, **3**:1301–1310.

31. King EG, Macdonald SJ, Long AD: **Properties and Power of the *Drosophila* Synthetic Population Resource for the Routine Dissection of Complex Traits**. *Genetics* 2012, **191**:935–949.

32. Burke MK, King EG, Shahrestani P, Rose MR, Long AD: **Genome-Wide Association Study of Extreme Longevity in *Drosophila melanogaster***. *Genome Biology and Evolution* 2014, **6**:1–11.

33. Marriage TN, King EG, Long AD, Macdonald SJ: **Fine-mapping nicotine resistance loci in *Drosophila* using a multiparent advanced generation inter-cross population**. *Genetics* 2014, **198**:45–57.

34. Cogni R, Cao C, Day JP, Bridson C, Jiggins FM: **The genetic architecture of resistance to virus infection in *Drosophila***. *Molecular Ecology* 2016, doi: 10.1111/mec.13769 [Epub ahead of print].

35. Begun DJ, Holloway AK, Stevens K, Hillier LW, Poh Y-P, Hahn MW, Nista PM, Jones CD, Kern AD, Dewey CN, Pachter L, Myers E, Langley CH: **Population genomics: whole-genome analysis of polymorphism and divergence in *Drosophila simulans***. *PLoS Biology* 2007, **5**:e310.

36. Hoffmann AA, Turelli M, Harshman LG: **Factors affecting the distribution of cytoplasmic incompatibility in *Drosophila simulans***. *Genetics* 1990, **126**:933–948.

37. Turelli M, Hoffmann AA: **Cytoplasmic incompatibility in *Drosophila simulans*: dynamics and parameter estimates from natural populations**. *Genetics* 1995, **140**:1319–1338.

38. Lazzaro BP, Sackton TB, Clark AG: **Genetic variation in *Drosophila melanogaster* resistance to infection: a comparison across bacteria**. *Genetics* 2006, **174**:1539–54.

39. Scott D, Shields A, Straker M, Dalrymple H, Dhillon PK, Harbinder S: **Variation in the male pheromones and mating success of wild caught *Drosophila melanogaster***. *PLoS ONE* 2011, **6**:e23645.

40. Zhu Y, Bergland AO, González J, Petrov DA: **Empirical validation of pooled whole genome population re-sequencing in *Drosophila melanogaster***. *PLoS ONE* 2012, **7**:e41901.

41. Ventura IM, Costa T, Klaczko LB: **Low temperature reveals genetic variability against male-killing *Spiroplasma* in *Drosophila melanogaster* natural populations**. *Microbial Ecology* 2013, **67**:229–235.

42. Nunes MDS, Neumeier H, Schlötterer C: **Contrasting patterns of natural variation in global *Drosophila melanogaster* populations**. *Molecular Ecology* 2008, **17**:4470–4479.

43. Clark ME, Veneti Z, Bourtzis K, Karr TL: **The distribution and proliferation of the intracellular bacteria *Wolbachia* during spermatogenesis in *Drosophila***. *Mechanisms of Development* 2002, **111**:3–15.

44. Fabian DK, Kapun M, Nolte V, Kofler R, Schmidt PS, Schlötterer C, Flatt T: **Genome-wide patterns of latitudinal differentiation among populations of *Drosophila melanogaster* from North America**. *Molecular Ecology* 2012, **21**:4748–4769.

45. Fabian DK, Lack JB, Mathur V, Schlötterer C, Schmidt PS, Pool JE, Flatt T: **Spatially varying selection shapes life history clines among populations of *Drosophila melanogaster* from sub-Saharan Africa**. *Journal of Evolutionary Biology* 2015, **28**:826–840.

46. Bergland AO, Behrman EL, O'Brien KR, Schmidt PS, Petrov DA: **Genomic evidence of rapid and stable adaptive oscillations over seasonal time scales in *Drosophila***. *PLoS Genetics* 2014, **10**:e1004775.

47. Kriesner P, Hoffmann AA, Lee SF, Turelli M, Weeks AR: **Rapid Sequential Spread of Two *Wolbachia* Variants in *Drosophila simulans***. *PLoS Pathogens* 2013, **9**:e1003607.

48. Macdonald SJ: **A Potential Regulatory Polymorphism Upstream of hairy Is Not Associated With Bristle Number Variation in Wild-Caught *Drosophila***. *Genetics* 2004, **167**:2127–2131.

49. Markow TA: ***Drosophila*: A Guide to Species Identification and Use**. Amsterdam, Boston: Elsevier/AP; 2006.

50. Minuk L, Civetta A: ***Drosophila*** **genomes and the development of affordable molecular markers for species genotyping**. *Genome* 2011, **54**:341–347.

51. Zhou W, Rousset F, O'Neil S: **Phylogeny and PCR-based classification of *Wolbachia* strains using *wsp* gene sequences**. *Proceedings Biological Sciences / The Royal Society* 1998, **265**:509–515.

52. Ashburner M, Golic K, Hawley RS: *Drosophila*: **A Laboratory Handbook**. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press; 2011.

53. Faria VG, Martins NE, Paulo T, Teixeira L, Sucena É, Magalhães S: **Evolution of *Drosophila* resistance against different pathogens and infection routes entails no detectable maintenance costs**. *Evolution* 2015, **69**:2799–2809.

54. Faria VG, Martins NE, Magalhães S, Paulo TF, Nolte V, Schlötterer C, Sucena É, Teixeira L: ***Drosophila*** **adaptation to viral infection through defensive symbiont evolution**. *PLoS Genetics* 2016, **12**:e1006297.

55. Rose MR: **Genetic covariation in *Drosophila* life-history: untangling the data**. *American Naturalist* 1984,**123**:565–569.

56. Mackay TFC: **Epistasis and quantitative traits: using model organisms to study gene–gene interactions**. *Nature Reviews Genetics* 2013, **15**:22–33.

57. Nouhaud P, Tobler R, Nolte V, Schlötterer C: **Ancestral population reconstitution from isofemale lines as a tool for experimental evolution**. *Ecology and Evolution* 2016, doi: 10.1002/ece3.2402 [Epub ahead of print].

58. Bou Sleiman MS, Osman D, Massouras A, Hoffmann AA, Lemaitre B, Deplancke B: **Genetic, molecular and physiological basis of variation in *Drosophila* gut immunocompetence**. *Nature Communications* 2015, **6**:7829.

59. Santos M, Fragata I, Santos J, Simoes P, Marques A, Lima M, Matos M: **Playing Darwin. Part B. 20 years of domestication in *Drosophila* subobscura**. *Theory Bioscience* 2010, **129**:97–102.

60. Simões P, Rose MR, Duarte A, Gonçalves R, Matos M: **Evolutionary domestication in *Drosophila* subobscura**. *Journal of Evolutionary Biology* 2007, **20**:758–766.

61. Sullivan DT, Sullivan MC: **Transport defects as the physiological basis for eye color mutants of *Drosophila melanogaster***. *Biochemical Genetics* 1975, **13**:603–613.

62. Sitaraman D, Zars M, LaFerriere H, Chen Y-C, Sable-Smith A, Kitamoto T, Rottinghaus GE, Zars T: **Serotonin is necessary for place memory in *Drosophila***. *Proceedings of the National Academy of Sciences of the United States of America* 2008, **105**:5579–5584.

63. Diegelmann S: **Genetic dissociation of acquisition and memory strength in the heat-box spatial learning paradigm in *Drosophila***. *Learning & Memory* 2006, **13**:72–83.

64. Ryder E, Blows F, Ashburner M, Bautista-Llacer R, Coulson D, Drummond J, Webster J, Gubb D, Gunton N, Johnson G, O'Kane CJ, Huen D, Sharma P, Asztalos Z, Baisch H, Schulze J, Kube M, Kittlaus K, Reuter G, Maroy P, Szidonya J, Rasmuson-Lestander A, Ekström K, Dickson B, Hugentobler C, Stocker H, Hafen E, Lepesant JA, Pflugfelder G, Heisenberg M, Mechler B, Serras F, Corominas M, Schneuwly S, Preat T, Roote J, Russell S. **The DrosDel collection: a set of P-element insertions for generating custom chromosomal aberrations in *Drosophila melanogaster***. *Genetics*. 2004, **167**:797–813.

2.3. Host adaptation is contingent upon the infection route taken by pathogens

Nelson E. Martins[1*], **Vitor G. Faria[1*]**, LuisTeixeira[1], Sara Magalhães[2,¶#], Élio Sucena[1,3¶#]

1 - Instituto Gulbenkian de Ciência, Apartado 14 , 2781-901 Oeiras, Portugal

2 - Centro de Biologia Ambiental, Faculdade de Ciências, Universidade de Lisboa, edifício C2, Campo Grande, 1749-016 Lisboa, Portugal

3 - Departamento de Biologia Animal, Faculdade de Ciências, Universidade de Lisboa, edifício C2, Campo Grande, 1749-016 Lisboa, Portugal

*** - These authors contributed equally to this work**

¶ - Joint last authors

- Email: snmagalhaes@fc.ul.pt, esucena@igc.gulbenkian.pt

Keywords: *Drosophila*, immunity, infection route, *Pseudomonas*, systemic infection, oral infection, experimental evolution

This article was published in the journal **PLoS Pathogens** (vol. 9, issue. 9, e1003601). Peer-Reviewed. September 2013.
DOI - 10.1371/journal.ppat.1003601.
PubMed ID - 24086131.

Author Contributions: Conceived and designed the experiments: É.S., S.M., L.T. Performed the experiments: N.E.M., V.G.F. Analyzed the data: N.E.M. Contributed reagents/ materials/ analysis tools: É.S. Wrote the paper: N.E.M., V.G.F., L.T., S.M., É.S.
The authors declare no conflict of interest.

Funding**:** Fundação para a Ciência e a Tecnologia (FCT, Portugal) supports N.E.M (#SFRH/BPD/62964/2009) and V.G.F (#SFRH/BD/82299/2011). This work was supported by Instituto Gulbenkian de Ciência/Fundação Calouste Gulbenkian and FCT (#POCTI/BIA -BDE/60950/2004) to É.S. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Abstract

Evolution of pathogen virulence is affected by the route of infection. Also, alternate infection routes trigger different physiological response on hosts, impinging on host adaptation and on its interaction with pathogens. Yet, how route of infection may shape adaptation to pathogens has not received much attention at the experimental level. We addressed this question through the experimental evolution of an outbred *Drosophila melanogaster* population infected by two different routes (oral and systemic) with *Pseudomonas entomophila*. The two selection regimes led to markedly different evolutionary trajectories. Adaptation to infection through one route did not protect from infection through the alternate route, indicating distinct genetic bases. Finally, relatively to the control population, evolved flies were not more resistant to bacteria other than *Pseudomonas* and showed higher susceptibility to viral infections. These specificities and trade-offs may contribute to the maintenance of genetic variation for resistance in natural populations. Our data shows that the infection route affects host adaptation and thus, must be considered in studies of host-pathogen interaction.

Introduction

The transmission route taken by pathogens to infect their hosts has a profound impact on the evolution of host-pathogen interactions. A body of theory [1–3] and several experiments [4–7] have addressed the effect of vertical or horizontal transmission on the evolution of pathogen virulence. Moreover, virulence in vector-borne or directly transmitted pathogens is expected to be differentially-affected by several factors, such as the timing of infection or inoculum size [8–10]. Recently, a meta-analysis has also shown that systemically-infecting pathogens are more virulent than those that infect via ingestion [11]. However rich this body of literature may be, it concerns the effect of transmission routes on the evolution of pathogens, not hosts (even though this implies measuring host traits, as pathogen virulence is defined as the harm imposed on hosts) [12, 13]. Pathogens that infect hosts via different routes (e.g., orally *vs* systemically) also trigger different physiological responses in hosts. This in turn may affect the evolution of host responses to pathogens, which will affect the outcome of the host-pathogen interaction. Therefore, addressing the evolutionary consequences of transmission route for host-parasite interactions calls for a characterization of its effects in the evolution of both pathogen and host.

It has been suggested that the immune response follows a hierarchical structure, starting with behavioural avoidance, through physical barriers and culminating in a humoral/cellular response [14–16]. Different infection routes will impact this cascade of events at different levels. Thus, the route taken by the pathogen will be crucial in defining the evolutionary consequences of infection to the individual and population. Yet, the distribution of variants across different levels in this cascade of events is unknown: which level is more likely to evolve in a population exposed to a particular immune challenge? If host adaptation occurs through changes in a shared downstream portion of the cascade such as the

humoral effectors, then adapted populations are expected to show a positive correlated response to challenges acting on any part of the cascade. Conversely, if there is at least partial independence in the defence pathways activated by each infection route, then adaptation to pathogens infecting through different routes should be uncorrelated. Thus, testing host evolutionary responses to infection through different routes is crucial to ecological immunology as it will, (a) establish whether responses are general or specific for distinct routes of pathogen access and, (b) provide insight into which part of the defense cascade may be modified by evolution.

In recent years much attention has been given to the mechanistic distinction between resistance (capacity to limit pathogen loads) and tolerance (capacity to survive damage caused by a given pathogen load) [17–19]. Yet, although a few recent studies have determined if resistance or tolerance mechanisms are involved in insect host responses to pathogens [20–22], whether and how different transmission routes affect the evolution of these mechanisms is still unknown. Indeed, no study has yet addressed the consequences of different infection routes of horizontally-transmitted pathogens for the evolution of host responses.

Routes of infection observed in nature are paralleled by the infection protocols used in the *Drosophila melanogaster* laboratory model of insect immunity [23–25]. Traditionally, the study of *Drosophila* immunity is done with systemic infections [26–29], but more recently, several studies have addressed the immune response to ingested bacteria [30–34], as the ecological relevance of this route of infection is most likely higher (for a review see [35]). These studies have shown that several responses are specific to the infection route, even if some overlap can be observed [30, 33, 36]. Indeed, to infect hosts, ingested pathogens need to avoid evacuation, resist oxidative burst and/or breach the epithelial gut barrier [32, 37–39].

For example, Kuraishi and co-workers [40] have found that loss of Drosocrystallin, a protein involved in the formation of the peritrophic matrix, leads to increased mortality after ingestion *of P. entomophila* and *S. marcescens*, but does not seem to play a role in systemic infections. Conversely, systemic infections bypass those defence levels [25] leading, in most cases, to virulence at much lower doses [31] and inducing melanisation responses that are not observed in oral infections [41]. However, besides the local specific response, oral infection may induce, a systemic response [31, 34, 38], although not always [30].

Because it is a model system for both invertebrate immunity [23, 42] and experimental evolution [43], *Drosophila melanogaster* stands out as the ideal organism to address the evolutionary consequences for hosts of different infection routes. In particular, recent years have witnessed the use of experimental evolution in *Drosophila* to unravel the evolution of host responses to pathogens [44–48]. However, all these studies concern host evolution to one specific immune challenge, and hence they do not address how different infection routes affect the host response. In the work here presented, we bridge this gap using experimental evolution on an outbred population of *D. melanogaster* responding to two routes of infection of the bacteria *Pseudomonas entomophila*. In brief, we will, (a) compare the rate of adaptation to each challenge, (b) test whether pathogen loads after infection changes with the evolutionary history of populations, (c) address whether adaptation is specific to each infection route and (d) test the generality of the response towards other pathogens.

Results

1. Adaptation to *P. entomophila* oral and systemic infections

In Figure 2.3.1, we present the survival along of the selected and control populations across 24 and 34 generations of experimental

evolution, upon exposure to the natural pathogen *P. entomophila*, by oral (Figure 2.3.1A) and systemic infection (Figure 2.3.1B).

Both the selection regime and selection regime by generation effects were significant ($P < 0.0001$), either in the BactOral scenario ($\chi^2_1 = 35.452$ and $\chi^2_{17} = 60.522$ for the selection regime and selection regime by generation effects, respectively) and the BactSys scenario ($\chi^2_1 = 16.336$ and $\chi^2_{25} = 265.756$, respectively).

Upon oral infection, the mean number of live individuals at day 10 after infection rose from the control 33% to a stable 90% after approximately 5 generations (Figure 2.3.1A). This rise is quite spectacular in that in only 3 generations the number of alive orally-infected flies had doubled (Figure 2.3.1A). Concomitantly, pairwise comparisons at each

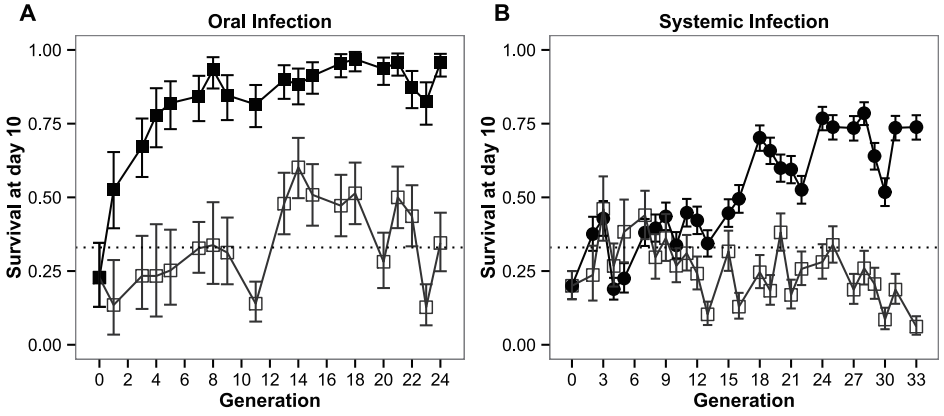


Figure 2.3.1 – Response to selection. Experimental evolution trajectories of populations evolving with a *Pseudomonas entomophila* oral (**A**) or systemic (**B**) infection and their respective control populations. Shown is the survival of flies from each selection regime when infected with *P. entomophila* either by (**A**) ingestion (orally) or, (**B**) pricking (systemic). Closed symbols: populations evolving in presence of the pathogen; open symbols: control lines. Vertical bars correspond to standard error across means of replicate lines; the straight dotted line corresponds to the original mortality rate imposed on the populations (66%).

generation reveal significant differences among selection regimes for this treatment starting at generation 3 ($|z| > 3.072$; $P < 0.05$ for all comparisons beyond that generation). In contrast, selection via systemic infection with the same bacterium, only led to significant differences at generation 13 ($|z| > 4.160$; $P < 0.001$). This difference was consistently significant after generation 16 ($|z| > 3.887$; $P < 0.01$), except for generation 20 ($z = 3.065$; $P = 0.05$), The lines selected in presence of the pathogen never exceeded 80% survival (Figure 2.3.1B).

2. Pathogen loads of control and selected flies

Next, we asked whether the increased levels of survival observed after 24 generations of selection corresponded to differences in pathogen loads after infection. For both modes of infection and for the early time point corresponding to the onset of mortality (left bars on Figure 2.3.2A and 2.3.2.B), the profile was the same, displaying a significantly higher number of bacteria in controls relatively to the evolved populations ($|z| = 3.287$ and 3.430, for oral and systemic infections, respectively, $P < 0.01$ for both comparisons). At the later time point, after which no more death is observed between populations (right bars on Figure 2.3.2A and 2.3.2B), there were no statistical differences between bacteria titers in the two time points for each of the infection routes ($|z| > 0.175$ for oral and systemic infections, respectively; $P = 0.998$ for both comparisons). The absolute number of bacteria was significantly reduced between the first and second time points in all treatments and selection regimes ($|z| > 4.883$, $P < 0.001$ for all pairwise comparisons) (Figure 2.3.2A and 2.3.2B). Under oral challenge, infection-free samples raised from 6/48 to 33/48 in control populations, and from 11/48 to 35/48 in selected populations. As for systemic infection, samples without bacterial counts increased from 0/48 to 11/22 in control populations, and 0/48 to 22/48 for selected populations.

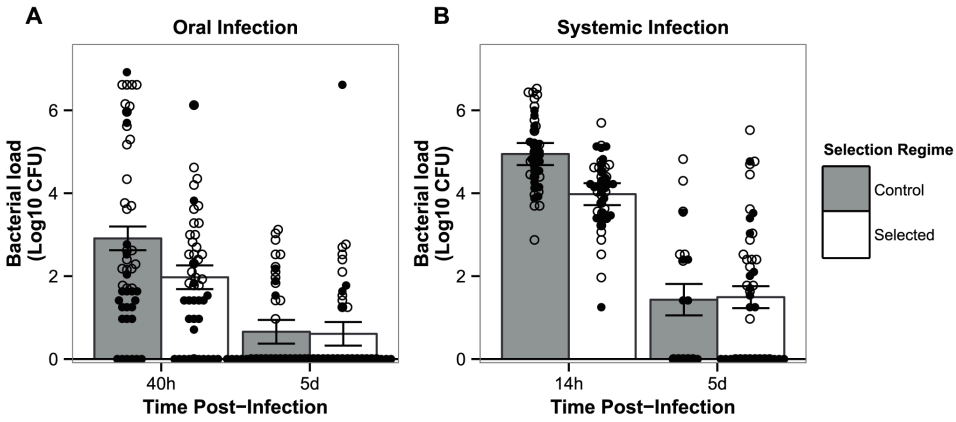


Figure 2.3.2 – Flies have evolved resistance against *P. entomophila* infection. Bacterial loads in flies from both control populations (grey bars) and populations evolving in presence of a pathogen (white bars) when exposed to oral (**A**) or systemic (**B**) infection. Males (full diamonds) and females (empty diamonds) are represented separately. Vertical bars correspond to the standard error of the mean pathogen load of each selection regime at each time point. (N = 48, except for panel B) systemic infection on control lines after 5 days where N = 22).

---

3. Correlated responses to selection of alternative routes of infection

We wondered how much of the adaptation to one route of infection would protect individuals infected through a different route. To address this, individuals of both sexes from control and selected populations were infected by pathogens via each of the two alternative routes of infection at two different time points (generations 14-15 and 24-25) (Figure 2.3.3).

For both the oral and systemic infection treatments, there was a significant overall interaction effect between sex, selection regime and generation (χ^2_6 = 67.795 and χ^2_6 = 15.420, $P < 0.0001$ and $P < 0.05$ for oral and systemic infections, respectively). We therefore compared the hazard ratios between the selection regime and their respective controls, independently for the two time points and averaging the effect of sex.

Concurrently with the survival data obtained for generations 14-15 and 24-25 in Figures 2.3.1A and 2.3.1B, evolved populations tested in the conditions in which they evolved (hereafter homologous environment) had a significantly higher survival relative to their controls. This is shown by the significant departure from zero of their hazard ratios (Figure 2.3.2: oral infection: $|z| > 8.003$, $P < 0.001$ in both generations; systemic infection: $|z| > 6.229$; $P < 0.0001$ in both generations). In contrast, exposing the adapted populations to the challenge they have not evolved in (hereafter heterologous environment), revealed no difference between control and selected lines for the BactOral selection regime ($|z| < 1.292$, $P > 0.784$ in both generations). For the BactSys selection regime, a significant difference was found in generations 14-15 (in which Bactsys < Control), but not in the later generations ($|z| = 3.062$, $P < 0.01$, and $|z| = 0.656$, $P = 0.939$,

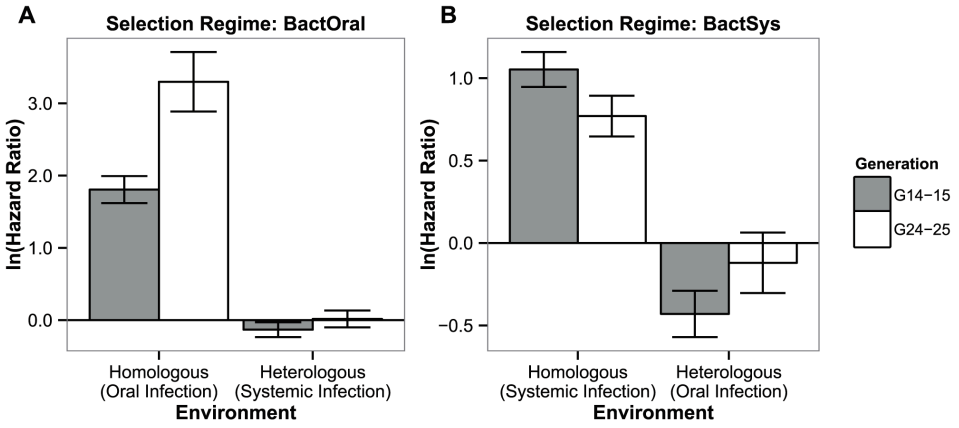


Figure 2.3.3 – Test of adaptation and its correlated response. Hazard ratios of lines evolving in presence of a pathogen relative to controls at generations 14-15 (grey bars) and 24-25 (white bars) of adaptation, when exposed to the challenge they have evolved with or to the other infection route. (**A**) Oral infection selection regime (BactOral) and (**B**) systemic infection evolved flies (BactSys). All populations spent one generation in a common environment before being tested. Vertical bars correspond to the standard error of the estimated ratio between the two selection regimes.

respectively). Therefore, adaptation to *P. entomophila* through one infection route infection did not affect susceptibility to the same pathogen infecting from a different route.

4. Correlated responses to other pathogens

Subsequently, we tested whether specificity of the evolved response could extend to other pathogens when infected via the same route (Figure 2.3.4).

Hazard ratios between the BactSys and ContSys populations after infection with the closely related species (same genus) *P. putida* were equivalent to those obtained with the original challenge, *P. entomophila* (|z| = 6.001 and 8.790, for *P. entomophila* and *P. putida*, respectively, $P < 0.001$ in both comparisons). In contrast, challenges with other known *Drosophila* pathogens such as *Serratia marcescens* and *Erwinia carotovora*, also Gram-negative Gammaproteobacteria, or *Enteroccocus faecalis*, a Gram-positive bacterium, caused equal degrees of mortality between evolved populations and their controls (|z| = 0.670, $P = 0.503$; |z| = 0.031, $P = 0.976$ and |z| = 1.374, $P = 0.170$ for *S. marcescens*, *E. carotovora and E. faecalis*, respectively). We therefore conclude that the response obtained is specific, at least, to the *Pseudomonas* genus level but not for all Gammaproteobacteria. Finally, fly populations evolving with *P. entomophila* infection were more susceptible than control populations to infections with Drosophila C Virus (DCV) and Flock House Virus (FHV) (|z| = 4.043 and 2.855, $P < 0.001$ and $P < 0.05$ for DCV and FHV infections respectively).

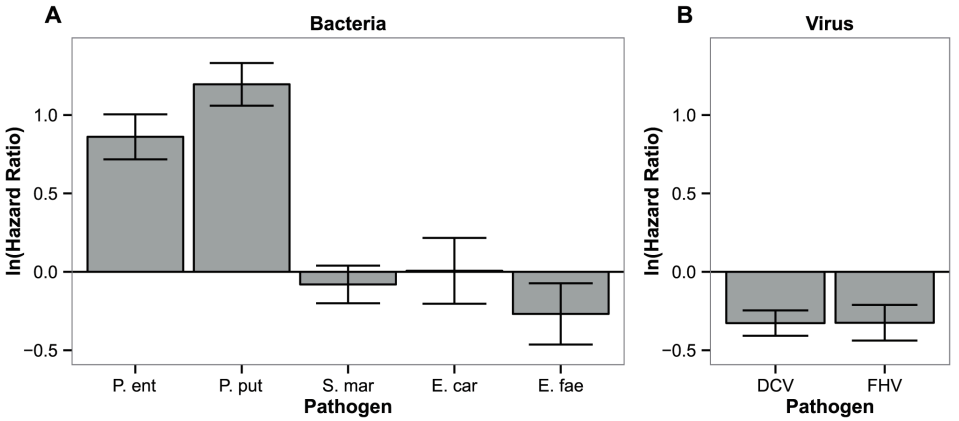


Figure 2.3.4 – Specificity of the response. Differences in hazard ratios between control lines (ContSys) and evolved lines with *Pseudomonas entomophila* systemic infection (BactSys), when exposed to (**A**) bacterial pathogens, P.e (*P. entomophila*), P. put (*Pseudomonas putida*), S.mar (*Serratia marcescens*), E.fae (*Enterococcus faecalis*); and (**B**) viral pathogens, DCV (*Drosophila* C Virus), FHV (Flock House Virus). Vertical bars correspond to the standard error of the estimated ratio between the selection regime and controls.

Discussion

Here, we report the first study addressing the impact of different infection routes taken by horizontally-

pathogens on the evolutionary trajectories and outcomes of their hosts. Our main conclusions are:

i) both exposure to systemic or oral infection results in the evolution of resistance in hosts, albeit at a different pace;

ii) adaptation is route-specific: hosts that adapt to pathogens from one infection route do not become less susceptible to the same pathogen infecting through a different route;

iii) the populations that evolved under systemic challenge by *P. entomophila* do not exhibit a generalized response outside the

Pseudomonas genus; rather, resistance to this bacteria trades off with survival to infection with viruses.

Different genetic bases for adaptation to distinct infection routes

Despite using the same pathogen in both infection protocols, we observed a lack of cross-resistance after a heterologous challenge with the same pathogen. Indeed, fly populations adapted to an oral infection by *P. entomophila* are equally susceptible to a systemic infection by the same bacterium species as populations evolved without the pathogen. The same holds true for populations evolved under a systemic infection challenged with an oral infection. This indicates that the response to each challenge has a different genetic basis.

Several genes and pathways are known to specifically participate in each infection route [23, 25, 33, 40] and our results are compatible with these findings. Yet, both humoral and epithelial responses may lead to the activation of anti-microbial peptides (AMPs) [25, 36, 49]. Moreover, the same pathways may be activated and required for survival in both infection routes. For instance, the Imd pathway has a role in protection against both orally and systemic infection with *P. entomophila* [38, 50]. Therefore, some of these effector elements could constitute a common target for selection and a general basis for adaptation to the pathogens, irrespective of infection route [51]. This is probably not the case, otherwise we would observe a positive correlation among responses.

A rapid response

A few studies have previously shown that evolution of the response to different pathogens in *Drosophila* occurs at a rapid [44, 46]. Our results confirm this rapid evolution but they also show that the rate of adaptation is contingent upon the infection route taken by this pathogen. Specifically, the increase in survival to oral infection in our fly population occurs within

fewer generations than the response to systemic infection, and it reaches a higher plateau. Because this is the first study that compares adaptation to different infection routes, whether these differences in dynamics are a general feature remains to be established. It would be interesting in the future to compare other pathogens that can infect through these different routes.

The observed differences in the evolutionary dynamics of populations exposed to each challenge may be due to the different genetic bases underlying each adaptation process. However, other factors may account for different dynamics. For example, systemic infection may be associated with more environmental variance (Ve) than oral infection. These differences in Ve would lead to the observed differences in dynamics even in the absence of different genetic bases for the traits underlying adaptation to each challenge. Quantitative genetic designs allowing measures of environmental and additive genetic variance for these traits are needed to distinguish between such alternatives.

Evolution of resistance

Interestingly, in our experiments the only aspect in which the adaptive responses to oral or systemic infections are parallel, regards the evolution of resistance (Figure 2.3.4A and 2.3.4B). Indeed, we find a significant difference between the bacterial counts of control and evolved lines at the onset of mortality for each selection regime. At a later time point (120h), control and evolved flies have the same bacterial load. However, at this point, we are only measuring bacterial loads in flies that survive infection, hence this information is irrelevant to the clarification of the mechanism involved in the adaptation process. Our results thus reiterate the need to follow the infection dynamics to discriminate between resistance and tolerance. Yet, with our data, we cannot exclude a role for

tolerance, as the infected flies from evolved and control populations that survive may have different abilities to cope with the infection (e.g., in terms of fecundity or subsequent mortality). Given that theory predicts different evolutionary outcomes depending on whether host responses involve tolerance or resistance [52], it is important to establish experimentally which of these mechanisms is acting in an evolving population.

The similarity observed among responses to each challenge does not imply an equivalence of mechanisms. The clearance of bacteria in fed versus pricked flies is likely bound to rely upon very different processes [33]. Bacterial loads are much lower in orally infected flies (two orders of magnitude) than in systemic infections (compare panels a and b of Figure 2.3.4), despite the fact that in the oral infection treatment the bacteria density administrated was four orders of magnitude higher than in systemic infections, indicating that elimination mechanisms are much more effective in this route of infection. This is consistent with published work showing that oral infection provokes strong epithelial responses namely by the modulation of physical barriers blocking pathogen access to the body cavity and of gut epithelium renewal, and there is limited crossing of the bacteria to the body cavity [33, 40, 41, 53]. In contrast, in a systemic infection the pathogen is inside the body cavity. Thus, any reduction in pathogen loads in the populations adapted to systemic infection must rely on active methods of identifying and eliminating bacterial invaders, namely through the canonical action of AMPs and plasmatocytes [23, 25, 42].

Pathogen specificities

The evolved populations only respond to infections with the bacterium used for selection, *P. entomophila*, and to its close relative *P. putida*. Other bacteria cause the same levels of lethality as in controls. This genus-specific response is somewhat surprising in that systemic infection

with different bacteria can induce a wide-spectrum of AMPs and other immune responsive genes with large overlaps, yet closely related pathogens induce considerably divergent responses [54–56]. Other studies using inbred lines have also established a lack of correlation between bacterial loads of different bacteria [57]. Finally, this specific adaptation to the *Pseudomonas* genus comes at a cost in survival to viral infections (Figure 2.3.3). Other studies provide contradictory evidence regarding the existence of trade-offs between susceptibility to different pathogens [54, 58–60]. This study, however, strongly points to the occurrence of a trade-off, where adapting to one pathogen increases susceptibility to others. This trade-off may underlie the maintenance of variation for resistance to *Pseudomonas* in the population.

Implications for the evolution of host-pathogen interactions

Several studies have shown that infection routes affect the evolution of virulence in pathogens [4–7, 11]. Here, we show that host adaptation to pathogens is also contingent upon those infection routes. Therefore, host responses may confound the conclusions drawn from studies on the evolution of virulence in pathogens in natural populations. For example, most pathogens that infect invertebrate hosts systemically are transmitted by vectors [14]. Several factors are expected to differentially affect virulence in vector-borne or directly-transmitted pathogens [8–10]. However, here we show that hosts adapt slower to a systemic than to an oral infection. This may confound the conclusions drawn from the observation of virulence patterns in natural populations. Hence, instead of merely observing patterns, studies on the effect of transmission modes in the evolution of host-pathogen interactions should follow the processes of adaptation in hosts and pathogens separately, to pinpoint the real cause underlying the observed patterns. In this sense, experimental evolution is a

powerful, yet underexploited tool to unravel the selection pressures underlying host-pathogen interactions. Our findings reinforce the necessity of including the mechanism of pathogen access into the set of criteria used to categorize and study host-pathogen interactions in ecological immunity, physiology and evolution [14–16].

Materials and Methods

Foundation and maintenance of *Drosophila melanogaster* populations.

An outbred population of *Drosophila melanogaster* was established in the laboratory in 2007, from 160 *Wolbachia*-infected fertilized females, collected in Azeitão, Portugal. Variability in this base population was assessed using multiple methods, based on 103 SNPs located in the left arm of the 3rd chromosome (supplementary methods). It contains high and relatively constant levels of polymorphism (SI, Figure S1). The population was kept in the laboratory cages for over 50 non-overlapping generations (generation time: three weeks) with high census (>1500 individuals). Flies were maintained under constant temperature (25 ºC), humidity (60-70%) and light-darkness cycle (12:12), and fed with standard cornmeal-agar medium. Prior to the initiation of experimental evolution, the initial population was serially expanded for 2 generations to allow the establishment of 16 new populations used in this work (see below).

Pathogen cultures

Experimental evolution of *D. melanogaster* populations was performed using *Pseudomonas entomophila*. In addition, we used other pathogens in some assays, namely, *Pseudomonas putida, Serratia marcescens, Erwinia carotovora, Enterococcus faecalis*, DCV (Drosophila C Virus) and FHV (Flock House Virus). For each round of infections, bacterial pathogens were grown in LB inoculated with a single bacterial colony, taken

from solid medium cultures grown from glycerol stocks kept at -80 ºC and streaked in fresh (<1 week) Petri dishes. Excluding *P. entomophila*, grown at 30 ºC, all bacteria were prepared from an overnight culture grown exponentially at 37 ºC, centrifuged and adjusted to the desired OD (see below). The *P. entomophila* strain used for experimental evolution was a generous gift from Bruno Lemaitre. It is resistant to rifampicin, which was used as a marker trait. The remainder bacterial pathogens were generous gifts from Karina Xavier (*P. putida*), Dominique Ferrandon (*S. marcescens*) and Thomas Rival (*E. carotovora* and *E. faecalis*). Viruses were produced as described elsewhere [61] and aliquots were kept at -80 ºC and thawed prior to infection.

Experimental evolution

Lines of all treatments were derived from the same base population (four lines per treatment). Four selection regimes were created, to which the following treatments were applied: systemic infection, in which flies were pricked in the thoracic region [32] with *P. entomophila* (OD600 = 0.01) (BactSys regime); a control for injection, following the same procedure except that the needle was dipped in sterile LB as a control (ContSys regime); oral infection, in which the food plates were covered for 24 hours with filter papers soaked with a *P. entomophila* culture (OD600 = 100) diluted 1:1 with sterile 5% sucrose solution (BactOral regime) (adapted from [41]); and control lines, where flies were kept in standard food (Control regime). The dose of *P. entomophila* for both bacterial treatments was determined at the start of the selection experiment to cause an average mortality of 66% in the base population, which corresponds to an OD of 0.01 for the systemic and of 50 for the oral infection treatments, respectively (SI, Figure S2).

These treatments were administrated at each generation to 310 males and 310 females (4-6 days old since eclosion). The subsequent generation was founded by all survivors at days 5 and 6 after treatment. The density of eggs was limited to 400 eggs in each cup, a density determined experimentally to enable optimal larval development. Each generation cycle lasted 3 weeks. Absence of transmission of the pathogen to the progeny was tested by plating whole pupae homogenates in LB agar plates supplemented with 100µg/ml rifampicin. No evidence of transmission of the pathogen to the next generation was found for either infection route, as plating of the progeny of infected flies (pupae) resulted in no *P. entomophila* colony. Altogether, populations evolved in their specific treatments for 24 generations in the case of the BactOral regime and 34 generations in the case of the BactSys regime.

At each generation, a sample of individuals from each selection regime was used to monitor survival across generations. To this aim, individuals from each replicate population of the BactSys and the ContSys selection regimes were exposed to systemic infection with *P. entomophila*, whereas individuals from the BactOral and ContOral selection regime were exposed to oral infection with the same bacteria species, and their mortality was monitored in vials for at least 10 days. For systemic infections, 100 individuals were placed in vials of 10 individuals. For the oral infection treatments, 120 individuals were placed for 24 hours in groups of 20 in vials where the food was covered with a filter paper disk soaked in bacteria solution, and subsequently transferred to standard food vials. A mixed sample of 200 individuals of the four populations of the Control selection regimes (ContSys and ContOral) were used as controls in these experiments. To further confirm that persistent infection was not affecting the results, e.g., due to immune priming, at generation 20, these tests were also performed using individuals whose eggs were previously

decontaminated in 50% bleach for 2 minutes. Evolved populations showed the same proportion of individuals surviving after infection with/without bleaching.

Pathogen loads in controlled and selected populations

P. entomophila quantifications were performed in two assays at generations 23 to 25, as described in Nehme et al (2007) [30] with minor modifications. For these assays, 150 to 250 flies (males and females) from each control and selected population were infected as in the survival assays. Flies were collected at 14 and 120 hours after systemic infection for BactSys and ContSys regimes, and at 40 and 120 hours after oral infection, for the BactOral and Control regimes. These time points were selected as the ones that correspond to the point before the onset of mortality in both modes of infection, and to the first day of egg-laying, after which no further mortality occurs (Figure S2). Six replicates of pools of 3 infected flies were homogenized in 50µL of sterile 1mM MgCl medium and serially diluted. Homogenates (4µL) were plated in triplicate on LB agar plates, supplemented with 100µg/mL Rifampicin and incubated overnight. The next day, we counted the number of colony-forming units (CFUs) on those plates. To avoid possible artifacts due to different maternal effects, flies used in these tests were the progeny of unselected flies that spent one generation in a common environment.

Adaptation and its consequences in heterologous environments

To test how host adaptation to pathogens from one infection route affected the host response to pathogens from a different route, 100 individuals (males and females) from each of the replicate populations of the BactSys and BactOral selection regimes, and the matching controls were exposed to the environment they evolved in as well as to that of the

heterologous selection regime (orthogonal assay), following the same protocol of the survival assays, at generations 15 and 25. To avoid possible artifacts due to different maternal effects, flies used in these tests were the progeny of flies that spent one generation without being exposed to pathogens, thus all in the standard environment of the base population.

Testing the generality of the response

To test how adaptation to a specific pathogen affected host responses to other pathogens, 100 individuals (males and females) from each replicate population of the BactSys and ContSys selection regimes were systemically infected with the following pathogens: *Pseudomonas putida* (OD_{600} = 10); *Serratia marcescens* (OD_{600} = 0.01); *Erwinia carotovora* (OD_{600} = 150); *Enterococcus faecalis* (OD_{600} = 3); DCV ($TCID_{50}$ = $2x10^7$); FHV ($TCID_{50}$ = $5x10^6$). These tests were performed between generations 27 and 30, and were repeated at least twice for each pathogen. The protocol followed was the same as that used for the cross-testing experiments. We could not perform this experiment with oral infections because we were unable to find another pathogen that caused mortality in our population via this infection route.

Statistics

All statistical analyses were done using R (v 2.15). To compare survival across generations in flies evolving with or without pathogens, the proportion of individuals surviving at day 10 after infection in each vial was first estimated using the Kaplan-Meier method. Individuals alive at the end of the experiment, stuck in the food or escaped from vials during the period of observation were counted as censored observations. Afterwards, the square root of the proportion of surviving individuals was arcsin transformed and analyzed using a general linear mixed model, with sex,

generation and selection regime as fixed factors and replicate population as a random factor. To test for the effect of the selection regime, a model with sex and generation as fixed factors was compared with a model with sex, generation and selection line as fixed factors. To test the different effects of the selection line across generations a model with interaction between selection line and generation was compared with the model without this interaction. To compare the proportion of individuals surviving at each generation, each selection regime was contrasted with its control at a given generation and corrected for multiple comparisons using the Bonferroni correction.

To compare survival between the control and selected population in the homologous and in heterologous selection environment, and after infection with different pathogens, we used a Cox's proportional hazards mixed effect model. The model included sex, selection regime and generation as fixed factors and test vials nested into population as random factor, thus accounting for variation in survival rates between populations within each selection line and between vials [62].

To compare pathogen loads, a linear mixed model on the natural logarithm of bacterial counts was employed, with selection regime, time after infection and sex as fixed factors and population as random factor. Interactions among all fixed factors were included in the full model, and sequentially removed if non-significant ($P > 0.05$). These tests were done using the R libraries *lme4* (v0.999999, generalized and linear mixed models), *coxme* (v2.2, mixed effects Cox proportional hazards model) and *glht* (v1.2, multiple comparisons).

Acknowledgements

The authors wish to thank Margarida Matos for critical reading of the manuscript and suggestions, the laboratories of Élio Sucena, Patrícia Beldade and Christen Mirth for inspiring discussions on the project, Henrique Teotónio and Ivo Chelo for help with SNP analysis and Bruno Lemaitre, Karina Xavier, Dominique Ferrandon and Thomas Rival for the bacterial strains used for the infections.

References

1. Anderson RM, May RM: **Coevolution of hosts and parasites**. *Parasitology* 1982, **85**:411–426.

2. Ewald PW: **The evolution of virulence: a unifying link between parasitology and ecology**. *Journal of Parasitololy* 1995, **81**:659–669.

3. Lipstich M, Siller S, Norwak MA: **The evolution of virulence in pathogens with vertical and horizontal transmission**. *Evolution* 1996, **50**:1729–1741.

4. Ebert D, Mangin KL: **The influence of host demography on the evolution of virulence of a microsporidian gut parasite**. *Evolution* 1997, **51**:1828–1837.

5. Messenger SL, Molineux IJ, Bull JJ: **Virulence evolution in a virus obeys a trade-off**. *Proceedings Biological Sciences / The Royal Society* 1999, **266**:397–404.

6. Stewart AD, Logsdon JMJ, Kelley SE: **An empirical study of the evolution of virulence under both horizontal and vertical transmission**. *Evolution* 2005, **59**:730–739.

7. Magalon H, Nidelet T, Martin G, Kaltz O: **Host growth conditions influence experimental evolution of life history and virulence of a parasite with vertical and horizontal transmission**. *Evolution* 2010, **64**:2126–2138.

8. Ewald PW: **Host–parasite relations, vectors, and the evolution of disease severity**. *Annual Review of Ecology, Evolution and Systematics* 1983, **14**:465–485.

9. Day T: **Parasite transmission modes and the evolution of virulence**. *Evolution* 2001, **55**:2389–2400.

10. Day T: **The evolution of virulence in vector-borne and directly transmitted parasites**. *Theoretical Population Biology* 2002, **62**:199–213.

11. Leggett HC, Cornwallis CK, West SA: **Mechanisms of pathogenesis, infective dose and virulence in human parasites**. *PLoS Pathogens* 2012, **8**:e1002512.

12. Vizoso DB, Ebert D: **Phenotypic plasticity of host-parasite interactions in response to the route of infection**. *Journal of Evolutionary Biology* 2005, **18**:911–921.

13. Ben-Ami F, Rigaud T, Ebert D: **The expression of virulence during double infections by different parasites with conflicting host exploitation and transmission strategies**. *Journal of Evolutionary Biology* 2011, **24**:1307–1316.

14. Schmid-Hempel P: **Evolutionary Parasitology: The integrated study of infections, Immunology, Ecology, and Genetics**. Oxford: Oxford Biology; 2011.

15. Schmid-Hempel P, Ebert D: **On the evolutionary ecology of specific immune defence**. *Trends in Ecology and Evolution* 2003, **18**:27–32.

16. Boughton RK, Joop G, Armitage SAO: **Outdoor Immunology: methodological considerations for ecologists**. *Functional Ecology* 2011, **25**:81–100.

17. Raberg L, Sim D, Read AF: **Disentangling genetic variation for resistance and tolerance to infectious diseases in animals**. *Science* 2007, **318**:812–814.

18. Schneider DS, Ayres JS: **Two ways to survive infection: what resistance and tolerance can teach us about treating infectious diseases**. *Nature Reviews Immunology* 2008, **8**:889–895.

19. Medzhitov R, Schneider DS, Soares MP: **Disease tolerance as a defense strategy.** *Science* 2012, **335**:936–941.

20. Lhocine N, Ribeiro PS, Buchon N, Wepf A, Wilson R, Tenev T, Lemaitre B, Gstaiger M, Meier P, Leulier F: **PIMS modulates immune tolerance by negatively regulating *Drosophila* innate immune signaling**. *Cell Host and Microbe* 2008, **4**:147–158.

21. Ayres JS, Schneider DS: **A signaling protease required for melanization in *Drosophila* affects resistance and tolerance of infections**. *PLoS Biology* 2008, **6**:2764–2773.

22. Neyen C, Poidevin M, Roussel A, Lemaitre B: **Tissue- and ligand-specific sensing of gram-negative infection in *Drosophila* by PGRP-LC isoforms and PGRP-LE**. *Journal of Immunology* 2012, **189**:1886–1897.

23. Lemaitre B, Hoffmann J: **The host defense of *Drosophila melanogaster***. *Annual Review of Immunology* 2007, **25**:697–743.

24. Shirasu-Hiza MM, Dionne MS, Pham LN, Ayres JS, Schneider DS: **Interactions between circadian rhythm and immunity in *Drosophila melanogaster***. *Current Biology* 2007, **17**:353–355.

25. Kounatidis I, Ligoxygakis P: ***Drosophila* as a model system to unravel the layers of innate immunity to infection**. *Open Biology* 2012, **2**:120075.

26. Boman HG, Nilsson I, Rasmuson B: **Inducible antibacterial defence system in *Drosophila***. *Nature* 1972, **237**:232–235.

27. Lemaitre B, Nicolas E, Michaut L, Reichhart JM, Hoffmann JA: **The dorsoventral regulatory gene cassette spatzle/Toll/cactus controls the potent antifungal response in *Drosophila* adults**. *Cell* 1996, **86**:973–983.

28. Irving P, Troxler L, Heuer TS, Belvin M, Kopczynski C, Reichhart J, Hoffmann JA, Hetru C: **A genome-wide analysis of immune responses in *Drosophila***. *Proceedings of the National Academy of Sciences of the United States of America* 2001, **98**:15119–15124.

29. De Gregorio E, Spellman PT, Rubin GM, Lemaitre B: **Genome-wide analysis of the *Drosophila* immune response by using oligonucleotide microarrays**. *Proceedings of the National Academy of Sciences of the United States of America* 2001, **98**:12590–12595.

30. Nehme NT, Liegeois S, Kele B, Giammarinaro P, Pradel E, Hoffmann JA, Ewbank JJ, Ferrandon D: **A model of bacterial intestinal infections in *Drosophila melanogaster***. *PLoS Pathogens* 2007, **3**:e173.

31. Basset A, Khush RS, Braun A, Gardan L, Boccard F, Hoffmann JA, Lemaitre B: **The phytopathogenic bacteria *Erwinia carotovora* infects *Drosophila* and activates an immune response**. *Proceedings of the National Academy of Sciences of the United States of America* 2000, **97**:3376–3381.

32. Vodovar N, Vinals M, Liehl P, Basset A, Degrouard J, Spellman P, Boccard F, Lemaitre B: ***Drosophila* host defense after oral infection by an entomopathogenic *Pseudomonas* species**. *Proceedings of the National Academy of Sciences of the United States of America* 2005, **102**:11414–11419.

33. Buchon N, Broderick NA, Poidevin M, Pradervand S, Lemaitre B: ***Drosophila*** **intestinal response to bacterial infection: activation of host defense and stem cell proliferation**. *Cell Host and Microbe* 2009, **5**:200–211.

34. Limmer S, Haller S, Drenkard E, Lee J, Yu S, Kocks C, Ausubel FM, Ferrandon D: ***Pseudomonas aeruginosa*** **RhlR is required to neutralize the cellular immune response in a** ***Drosophila melanogaster*** **oral infection model**. *Proceedings of the National Academy of Sciences of the United States of America* 2011, **108**:17378–17383.

35. Vallet-Gely I, Lemaitre B, Boccard F: **Bacterial strategies to overcome insect defences**. *Nature Reviews Microbiology* 2008, **6**:302–313.

36. Teixeira L: **Whole-genome expression profile analysis of** ***Drosophila melanogaster*** **immune responses**. *Briefings in Functional Genomics* 2012, **11**:375–386.

37. Ha EM, Oh CT, Bae YS, Lee WJ: **A direct role for dual oxidase in** ***Drosophila*** **gut immunity**. *Science* 2005, **310**:847–850.

38. Liehl P, Blight M, Vodovar N, Boccard F, Lemaitre B: **Prevalence of local immune response against oral infection in a** ***Drosophila*****/*****Pseudomonas*** **infection model**. *PLoS Pathogens* 2006, **2**:e56.

39. Ryu JH, Ha EM, Oh CT, Seol JH, Brey PT, Jin I, Lee DG, Kim J, Lee D, Lee WJ: **An essential complementary role of NF-kappaB pathway to microbicidal oxidants in** ***Drosophila*** **gut immunity**. *EMBO Journal* 2006, **25**:3693–3701.

40. Kuraishi T, Binggeli O, Opota O, Buchon N, Lemaitre B: **Genetic evidence for a protective role of the peritrophic matrix against intestinal bacterial infection in** ***Drosophila melanogaster***. *Proceedings of the National Academy of Sciences of the United States of America* 2011, **108**:15966–15971.

41. Buchon N, Broderick NA, Chakrabarti S, Lemaitre B: **Invasive and indigenous microbiota impact intestinal stem cell activity through multiple pathways in** ***Drosophila***. *Genes and Development* 2009, **23**:2333–2344.

42. Dionne MS, Schneider DS: **Models of infectious diseases in the fruit fly** ***Drosophila melanogaster*****.** *Disease Models and Mechanisms* 2008, **1**:43–49.

43. Kawecki TJ, Lenski RE, Ebert D, Hollis B, Olivieri I, Whitlock MC: **Experimental evolution**. *Trends in Ecology and Evolution* 2012, **27**:547–560.

44. Kraaijeveld AR, Godfray HC: **Trade-off between parasitoid resistance and larval competitive ability in *Drosophila melanogaster***. *Nature* 1997, **389**:278–280.

45. Kraaijeveld AR, Godfray HC: **Selection for resistance to a fungal pathogen in *Drosophila melanogaster***. *Heredity* 2008, **100**: 400–406.

46. Ye YH, Chenoweth SF, McGraw EA: **Effective but costly, evolved mechanisms of defense against a virulent opportunistic pathogen in *Drosophila melanogaster***. *PLoS Pathogens* 2009, **5**:e1000385.

47. Wolfle S, Trienens M, Rohlfs M: **Experimental evolution of resistance against a competing fungus in *Drosophila melanogaster***. *Oecologia* 2009, **161**:781–790.

48. Wertheim B, Kraaijeveld AR, Hopkins MG, Walther Boer M, Godfray HC: **Functional genomics of the evolution of increased resistance to parasitism in *Drosophila***. *Molecular Ecology* 2011, **20**:932–949.

49. Davis MM, Engstrom Y: **Immune response in the barrier epithelia: lessons from the fruit fly *Drosophila melanogaster***. *Journal of Innate Immunity* 2012, **4**:273–283.

50. Charroux B, Royet J: **Elimination of plasmatocytes by targeted apoptosis reveals their role in multiple aspects of the *Drosophila* immune response**. *Proceedings of the National Academy of Sciences of the United States of America* 2009, **106**:9797–9802.

51. Lazzaro BP, Clark AG: **Molecular population genetics of inducible antibacterial peptide genes in *Drosophila melanogaster***. *Molecular Biology and Evolution* 2003, **20**:914–923.

52. Roy BA, Kirchner JW: **Evolutionary dynamics of pathogen resistance and tolerance**. *Evolution* 2000, **54**:51–63.

53. Chakrabarti S, Liehl P, Buchon N, Lemaitre B: **Infection-induced host translational blockage inhibits immune responses and epithelial renewal in the *Drosophila* gut**. *Cell Host and Microbe* 2012, **12**:60–70.

54. Chambers MC, Lightfield KL, Schneider DS: **How the fly balances its ability to combat different pathogens.** *PLoS Pathogens* 2012, **8**:e1002970.

55. Apidianakis Y, Mindrinos MN, Xiao W, Lau GW, Baldini RL, Davis RW, Rahme LG: **Profiling early infection responses: *Pseudomonas aeruginosa* eludes host defenses by suppressing antimicrobial peptide gene expression**. *Proceedings of the National Academy of Sciences of the United States of America* 2005, **102**:2573–2578.

56. Schlenke TA, Morales J, Govind S, Clark AG: **Contrasting infection strategies in generalist and specialist wasp parasitoids of *Drosophila melanogaster***. *PLoS Pathogens* 2007, **3**:e158.

57. Hill-Burns EM, Clark AG: **X-linked variation in immune response in *Drosophila melanogaster***. *Genetics* 2009, **183**:1477–1491.

58. Zbinden M, Haag CR, Ebert D: **Experimental evolution of field populations of *Daphnia magna* in response to parasite treatment**. *Journal of Evolutionary Biology* 2008, **21**:1068–1078.

59. Kraaijeveld AR, Layen SJ, Futerman PH, Godfray HC: **Lack of phenotypic and evolutionary cross-resistance against parasitoids and pathogens in *Drosophila melanogaster***. *PLoS ONE* 2012, **7**:e53002.

60. Hodges TK, Laskowski KL, Squadrito GL, De Luca M, Leips J: **Defense traits of larval *Drosophila melanogaster* exhibit genetically based trade-offs against different species of parasitoids**. *Evolution* 2013, **67**:749–760.

61. Teixeira L, Ferreira A, Ashburner M: **The bacterial symbiont *Wolbachia* induces resistance to RNA viral infections in *Drosophila melanogaster***. *PLoS Biology* 2008, **6**:e2.

62. Therneau TM, Grambsch PM: ***Modeling survival data : extending the Cox model***. New York: Springer; 2000.

2.4. Contrasting *Drosophila* adaptation against systemic and oral bacterial infections

Vítor G. Faria[1], Nelson E. Martins[1], Tânia F. Paulo[1], Viola Nolte[2], Christian Schlötterer[2], Luis Teixeira[1], Sara Magalhães[3,4], Élio Sucena[1,3#]

1 - Instituto Gulbenkian de Ciência, Apartado 14 , 2781-901 Oeiras, Portugal

2 - Institut für Populationsgenetik, Vetmeduni Vienna, Austria

3 - Departamento de Biologia Animal, Faculdade de Ciências, Universidade de Lisboa, edifício C2, Campo Grande, 1749-016 Lisboa, Portugal

4 - cE3c: Centre for Ecology, Evolution and Environmental Changes, Faculdade de Ciências, Universidade de Lisboa, edifício C2, Campo Grande, 1749-016 Lisboa, Portugal

- Email: esucena@igc.gulbenkian.pt

Keywords: *Drosophila* immunity, *Pseudomonas*, systemic infection, oral infection, experimental evolution, evolve and resequence

Article in preparation for publication

Author contributions: V.G.F., N.E.M., L.T., S.M., and É.S. designed research; V.G.F., N.E.M., T.P and V.N. performed research; C.S. and É.S. contributed new reagents/analytic tools; V.G.F., N.E.M., T.P. analyzed data; V.G.F., and É.S wrote the paper.
The authors declare no conflict of interest.

Funding: Fundação para a Ciência e a Tecnologia (FCT, Portugal) supports V.G.F (#SFRH/BD/82299/2011) and N.E.M (#SFRH/BPD/62964/2009). C.S. is funded by AustrianScience Fund (FWF) (P22725 and P19467) and European Research Council (ArchAdapt). This work was supported by Instituto Gulbenkian de Ciência/Fundação Calouste Gulbenkian, FCT (PTDC/SAU -IMU/120673/2010), and Vetmeduni funding. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Abstract

Pathogens are a constant selective pressure to hosts, creating a continuous arms race between both parties. In *Drosophila*, a vast range of immune challenges is triggered by several natural pathogens, such as fungi, virus or bacteria. An important example is the bacterium *Pseudomonas entomophila*, a natural pathogen shown to cause high mortality rates through both systemic and oral infection. Using this pathogen/host model, we previously adapted outbred *D. melanogaster* populations to infection through either route. We found remarkable differences in the adaptive dynamics of *Drosophila* populations dependent on both the pathogen and the infection route. Here, using Pool-Seq technology, we assessed the genetic bases of these adaptive processes. Populations that evolved against systemic infection showed an expected pattern of genomic modification, with several differentiated peaks across chromosomes. Contrastingly, populations adapted to oral infection did not present the expected genomic profile given their rapid adaptation, with the analysis displaying multiple peaks with weak differentiation. Focusing in the systemic populations, we tested several candidate loci using RNAi and uncovered multiple genes involved in the process. Finally, we performed a genome-wide association study (GWAS) challenging DGRP lines with the same initial pressure used in systemic experimental evolution. We found that, unlike other comparisons between both methods with different pathogens, namely DCV, the set of high-differentiated candidate SNPs revealed were totally different, with only one gene in common, *Cpr50Ca*. These results pinpointed new players in the evolution of host immunity, reinforcing the central role of initial genetic pool and recombination processes (and other population effects) on the evolution of multifactorial immune responses.

Introduction

Pathogens continuously impose a strong selective pressure on their host(s). Using different routes of infection, oral, systemic or even maternal, pathogenic agents challenge organisms in different forms [1]. Hosts fight these infections with several mechanisms, such as behavioral avoidance, physical barriers and cellular and humoral defense. Thus, to resist and/or tolerate infection, hosts respond to specific infectious challenges with different physiological processes using several genetic pathways [2], remarkably similar across species [3]. These diverse strategies underlie host adaptation but also the evolution of pathogen virulence itself [4,5].

In *Drosophila*, immune responses are well-characterized, involving production of reactive oxygen species (ROS) [6], release of antimicrobial peptides (AMP) [7,8], small RNAs [9,10], phagocytosis or melanization (for revision see [11]). Upon this, another layer of complexity appears with symbiotic immunity, as is the case of the endobacterium *Wolbachia* protecting *Drosophila* against viral infection [12,13] (Chapter 3.2) or *Spiroplasma* against parasitic wasps [14].

Numerous studies have granted *D. melanogaster* a central role in the study of innate immunity [15-16], uncovering how this species responds against a vast range of immune challenges [17–20]. For this, several natural pathogens have been described and used, such as *Pseudomonas entomophila* [21]. This pathogenic bacterium may be used to infect orally and systemically, promoting high mortality in *Drosophila* larvae and adults, in spite of the strong immune response triggered [22–24].

In addition, *D. melanogaster* is also a central model for a large spectrum of evolutionary questions. Several studies using experimental evolution have been revealing how populations adapt against diverse selective pressures, including pathogens [19,25,26]. This method associated with a number of available tools and technologies, for example Next

Generation Sequencing (NGS) [27], permits deep dissection of the mechanisms underlying the adaptive process, as well as the potential associated costs.

In a previous study, we adapted outbred populations of *Drosophila* against *Pseudomonas entomophila* [28]. We applied a selective pressure every generation through two different routes of infection, systemic and oral. Here, we revisited these populations and used genome-wide sequencing of DNA pools (Pool-Seq) to assess the new genetic structure of evolved populations, and performed RNAi assays to test the candidates gene responsible for this evolutionary response.

We found that, while populations adapted to systemic infection showed several genomic regions of high differentiation, populations adapted to an oral route of infection presented a comparatively weak genetic differentiation signature. We further validated functionally several of the candidate genes identified. Finally, we compared the SNPs of causative candidates found with our approach to the gene set identified though GWAS. This comparison yielded surprisingly low overlap in the candidate SNPs for immune response against *P. entomophila* infection.

Results

Genetic basis of host adaptation to *P. entomophila* infections

We have performed genome-wide sequencing of DNA pools (Pool-Seq) of outbred *D. melanogaster* populations previously adapted to *P. entomophila* through 2 distinct routes of infection, systemic and oral, with different adaptation dynamics [28].

In Figure 2.4.1, we observe the changes in allele frequencies in evolved treatments, systemic (A) and oral (B). Using a chromosome-wide cutoff (see M&M), we observed several regions of high differentiation in

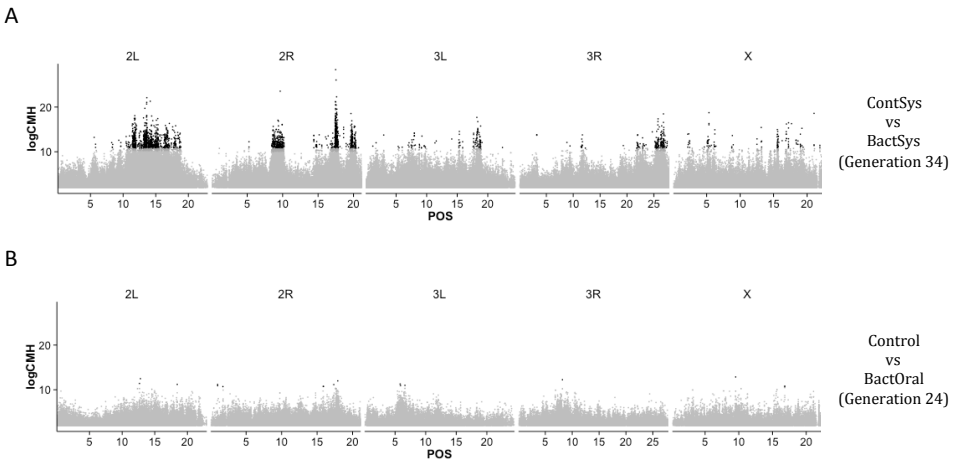


Figure 2.4.1 – Differentiation between selection regimes. –log10 values of the CMH statistic for every polymorphic SNP, across the five major chromosomal arms through pairwise comparison of allele frequencies between ContSys and BactSys (**A**) populations at generation 34 and between Control and BactOral (**B**) populations at generation 24.

each chromosome. BactSys (A) showed a large quantity of differentiated SNPs when compared with the respective control, ContSys. In all chromosomes, well-defined peaks accompanied the adaptive process. In contrast, BactOral (B) presented a small number of differentiated SNPs, without weakly-defined peaks.

Role of the microbiome in the evolved response

We wondered if the microbiome could be responsible for (part of) the evolved higher survival against bacterial infection mainly in BactOral populations where no clearly correlated SNPs could be found. To address this, we performed an antibiotic treatment with tetracycline in populations evolved with bacteria, as well as in their respective controls. With this procedure we eliminate the gut microbiome as well as *Wolbachia.* After the

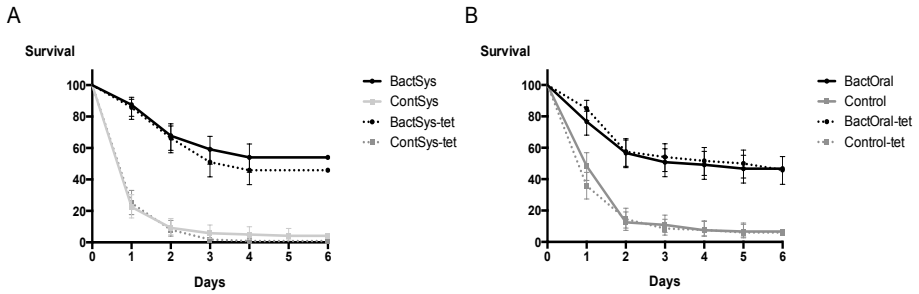


Figure 2.4.2 – No effect of microbiome in evolved response. Survival after *P. entomophila* infection of control and bacterial selected lines, (**A**) systemically and (**B**) orally, with or without antibiotic treatment.

antibiotic treatment, these populations were allowed to recover and their gut microbiome homogenized to allow proper comparisons across regimes.

As shown in Figure 2.4.2, no significant differences were found between treated and non-treated populations after bacterial challenge, both in Bactsys (p = 0.26) and in BactOral (p = 0.81). In both selection regimes, survival profiles between treated and non-treated conditions are constant between evolved and control populations.

Functional Validation of Candidate Genes

To identify putative causative genes involved in the evolved systemic response against *P. entomophila*, we used RNA interference (RNAi). We tested 13 genes associated to the highest differentiated SNPs in the different peaks throughout the genome (Figure 2.4.3A).

Knockdown of these genes (Figure 2.4.3B) revealed that 3 genes have a significant effect in the response in both sexes: *kuz* (1st of peak 2, chr.2L), *Cpr50Ca* (1st of peak 1, chr.2R) and *elF2-alpha* (2nd of peak 3, chr.3R). Moreover, other genes were also significant but exclusive to male response: *sano* (1st of peak 2, chr.2R) and *pHCI* (1st of peak 2, chr.3L).

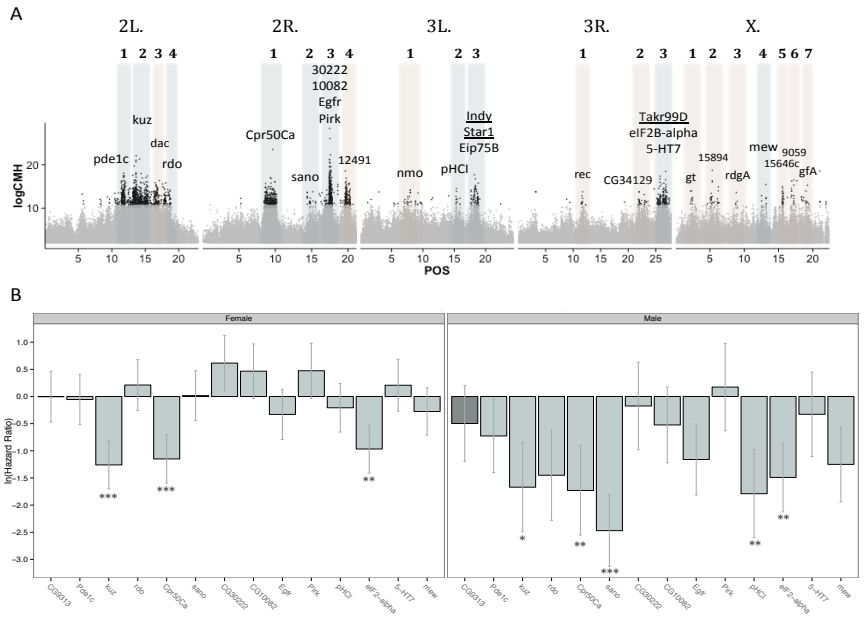


Figure 2.4.3 – RNAi knockdown of candidate genes in BactSys populations. (**A**) Schematic representation of high-differentiated peaks and genes, tested (gray blocks) and not tested (orange blocks and underline genes). (**B**) Natural logarithm of hazard ratios between survival of flies with knocked-down candidate genes and their controls (KK background construct and CG9313) upon infection with *P. putida* using as genetic background KK. Vertical bars correspond to the 95% confidence intervals of the estimated hazard ratios. **P* < 0.05; ***P* < 0.01; ****P* < 0.001.

Genome Wide Association Study (GWAS) for *P. entomophila* infection

To complement and later compare this approach for the identification of genes involved in the response to *P. entomophila* infection, we undertook a GWAS using *Drosophila* Genetics Reference Panel (DGRP) flies. We challenged 139 DGRP lines with *P. entomophila* (with the same bacterial load used in the experimental evolution protocol, OD600 = 0.01) and measured survival rates until day 6. In Figure 2.4.4A, we show the survival variance among tested lines at day 3. We performed a further

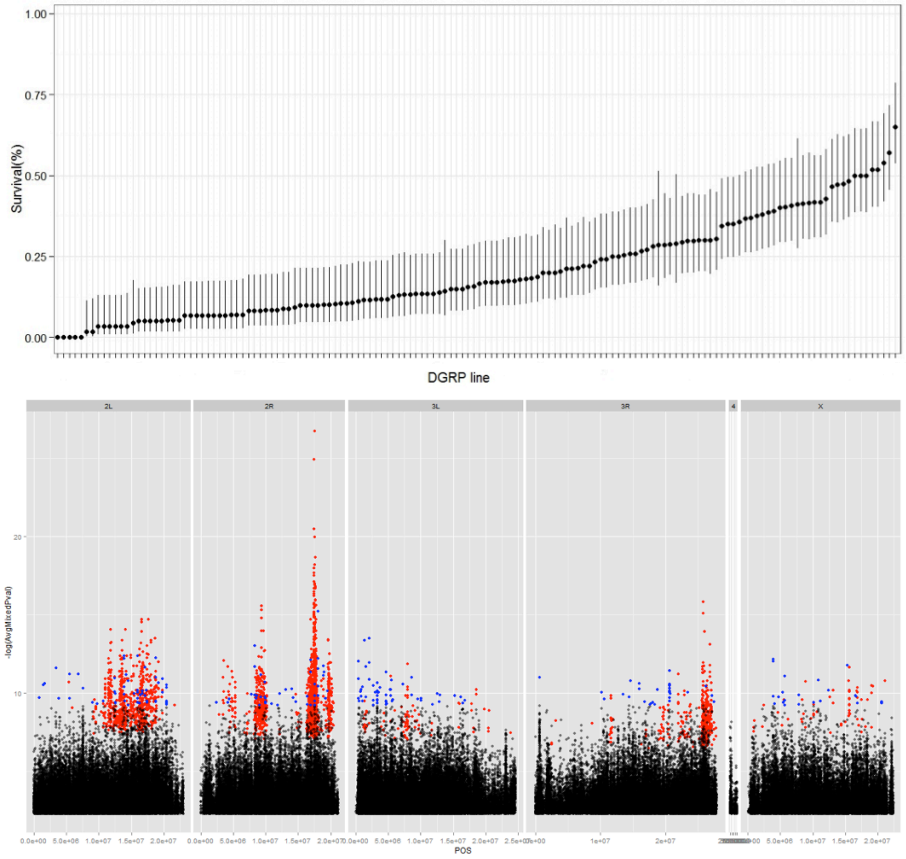


Figure 2.4.4 – GWAS with *P. entomophila* infection and comparison against Pool-Seq. (A) Representation of survival variance of 139 DGRP lines against *P. entomophila*, 3 days after systemic infection **(B)** Overlapping SNPs differentiation in Pool-Seq of BactSys (red dots) and GWAS with the 139 DGRP lines (blue dots) systemically infected with *P. entomophila.*

GWAS and compared the identified SNPs with Pool-Seq results of BactSys populations (Figure 2.4.4B). We verified no match between the highest SNPs revealed by each method. Regarding the associated genes, the only candidate shared by Pool-Seq and GWAS lines is *Cpr50Ca*, located on chromosome 2L.

Discussion

In this study, we found that resistance of *Drosophila* against *P. entomophila* via two different routes of infection, systemic and oral, has a different genetic basis and both phenotypes are fully independent of microbiome influence. Looking at BactSys candidates uncovered by Pool-Seq, we revealed several genes that contributed, alone or perhaps epistatically, to the evolved phenotype. Moreover, with one exception, the revealed high-differentiated candidates were not the same as generated by GWAS, a method also tested here under the same bacterial challenge conditions.

First, using the previously evolved *D. melanogaster* populations against *P. entomophila* by experimental evolution [28], we identified genetic changes associated to the adaptive processes. In BactSys (Figure 2.4.1A), we found several regions of differentiation, distributed by several peaks in all chromosomes. This evolved genomic profile was compatible with the slow and gradual adaptive dynamics of BactSys (survival increased from 33% to 80% of survival, between generations 13 and 34), where several partially protective alleles of different genes increased gradually their frequency to contribute to the evolved multifactorial protective phenotype. Moreover, the adaptive dynamics could reflect the necessity of recombination, grouping together these alleles or even an epistatic relationship among some of them. However, Pool-seq technology, based in short sequence reads, precludes the determination of haplotypes or linkage, thus limiting our capacity to detect epistasis [29].

On the other hand, the genome profile of BactOral (Figure 2.4.1B) had an unpredictable outcome in light of the dynamics observed for the evolved phenotype. Such small peaks of differentiation cannot directly reflect the fast adaptation performed by the oral selected populations. It

was expected that a strong genetic signature would underlie the increase of resistance from 33% to almost 90% in only four generations of selection. One hypothesis is that the causative genetic effect could be masked by the computational analysis method itself, its cutoffs or criteria. Specifically, the method only considers bi-allelic SNPs and does not account for other types of genetic variants that could explain the fast adaptive dynamics, namely multi-allelic SNPs, indels, and chromosome rearrangements. Otherwise, it would be expected that the haplotypes associated to the relevant characteristic had increased indirectly in frequency, remaining therefore a well-defined and detectable genetic mark. Finally, and although unusual, we can also consider an epigenetic mechanism as the basis of this process.

Another strong hypothesis to explain this paradox would be a role of the microbiome. A change in the frequency of bacterial strains/haplotypes, or even in the species itself, could explain the selected phenotype in evolved populations. However, antibiotic treatment with tetracycline (removing gut microbiota but also *Wolbachia*) revealed that evolved and non-evolved populations have no differences in response when compared with their respective controls (Figure 2.4.2). These results were observed not only for oral but also for systemic infection, excluding in both cases a role of symbiotic bacteria.

Additionally, we can also exclude an eventual persistent infection by maternal transmission that could have up-regulated the immune system of the progeny before selective pressure. We previously tested this immune priming hypothesis, at generation 20, challenging individuals whose eggs were previously decontaminated in bleach. Again, we have discarded this possibility since the evolved populations showed the same proportion of individuals surviving after infection in both situations [28] (Chapter 2.3).

In face of the BactOral results, the next step will be to test the immune function of genes that revealed differentiated SNPs, even if the

differentiation peaks are modest. In parallel, new genetic experiments and new computational analyses will be required to try to understand the mechanism at the basis of this adaptation. It is important to highlight that the response dynamics was very consistent among the 4 populational replicates under selection, reinforcing therefore the idea that the evolved response was driven by a major and fast selectable element, although yet unrevealed.

Back to the results of the BactSys populations, we next approached and tested the candidate genes associated to SNPs uncovered by Pool-Seq. We identified and divided the differentiated regions in small well-defined peaks across chromosomes, considering each one as putatively associated physically to the causative genes that drove the selection (Figure 2.4.3A). Importantly, other Evolve&Resequence studies have confirmed the validity of this assumption [19].

To perform these tests, and taking advantage of BactSys being equally resistant to *P. entomophila* and *P. putida* infections, we used the later to increase the definition in the measurement of survival dynamics. As virulence of *P. putida* is more spread in time than that of *P. entomophila* (used already in the lower dose recommended for an effective infection), we were able to extend the time-window of mortality and gain more detail in the analyses.

Using the criteria and conditions mentioned above, we challenged RNAi lines of 13 genes associated to the high-differentiated SNPs (Figure 2.4.3B). We found a range of responses among tested candidates. While the results of some genes are not significant, some genes show categorical roles in anti-bacterial response. Consequently, we can hypothesize that these loci contain natural variation for protective ability, which has been the target of selection throughout our experimental evolution. Three genes stood out in this experiment, *kuz*, *Cpr50Ca* and *elF2-alpha*, with a detectable role for

both sexes. *kuz* is a component of the membrane with metalloendopeptidase activity and, among other functions, a previously reported role in *Drosophila* phagocytosis of *C. albicans* and *E. coli* [30]. *Cpr50Ca* is a predicted structural component of the cuticle [31] and elF2-alpha is reported as responsible for translation initiation activity [32], neither implicated so far in immune response.

Absence of the gene products of *sano* and *pHCI* showed significant consequences in male survival against infection. These disparities between sexes in the relevance of selected genes may explain the differences of adaptive dynamics seen between males and females throughout experimental evolution. Nevertheless, other candidates also need to be tested for a more comprehensive understanding of the complexity involved in this adaptive process. For example, none of the four genes tested in peak 2 of chromosome 2R (Figure 3A) showed differences when down-regulated with RNAi. Thus, we cannot explain therefore this peak, the most prominent found in the BactSys regime. In parallel, another step will be the testing of other RNAi collections (GD) to further validate (or not) those candidate genes for which results are yet inconclusive.

In a previous work, we also used the Evolve&Resequence methodology to assess the genetic basis for *Drosophila* adaptation against systemic DCV infection [19]. We found that the gene *pastrel* was likely to be the major causative agent in evolved protection against DCV in our selected populations. The same gene was also uncovered as the leading player through a genome-wide association study (GWAS) [33]. Thus, through two different methods, using outbred populations or inbred lines, the main genetic basis for immune response against DCV was common. However, the Evolve&Resequence method also revealed 2 additional genes involved in evolved response in our populations, *CG8492* and *Ubc-E2H*, uncovering

other protective alleles recruited to respond against a long term selective pressure.

To accomplish the same comparison and compare the two methods qualitatively, we performed a GWAS with systemic *P. entomophila* infection. The results showed that, in contrast to DCV infection, the two approaches did not uncover shared players, with one exception (Figure 2.4.4). The only gene related with a highest differentiated SNP in the evolved populations also uncovered with the GWAS method was *Cpr50Ca*, although not with the same relative importance.

Moreover, a recent report has performed a GWAS with oral infection of *P. entomophila* [34]. Using 140 DGRP lines, Sleiman and colleagues revealed several SNPs associated with the immune response against this challenge. When compared with our results of BactOral Pool-Seq, once again, we did not find almost any direct parallel in candidate genes.

These differences may be intrinsic to the methods themselves. First, perhaps due to differences in the initial genetic pool between our outbred populations and the set of inbred lines tested in GWAS. Thus, for a more refined comparison between methods, a comparative analysis will be necessary on the presence/absence of the revealed SNPs in one method and the genetic pool of the other. The second major interpretation to this topic is that the genetic basis for a protective phenotype is not necessarily adaptive, and *vice-versa*, which will always be a barrier between the two methods. Evolve&Resequence cannot determine the group of protective but not adaptive alleles, for example by pleiotropy or high cost. In turn, GWAS cannot assess from the tested phenotypes the group of non-protective alleles that could be adaptive in a more realistic ecological and populational context, for example trigger by epistatic interactions.

Another important issue will be to test if, in causative genes, only the selected alleles are protective, or if, on the other hand, all alleles have

different degrees of protection. For example, in the case of *pastrel*, the major gene responsible for *Drosophila*'s response against DCV, although it is a considerably highly protective allele, other alleles also confer great protection when compared with the absence of the gene. This may explain part of the refinement of the adaptive process after a first phase of selection of large effects. This is also a determining factor in the above-mentioned differences obtained between Evolve&Resequence and GWAS methods. A detailed analysis among different protective alleles is necessary and may be achieved in future studies through allele replacement using CrispR technology, for example.

Our findings reveal the large difference in the genetic basis of distinct adaptive dynamics, even caused by the same pathogen. Certainly a word of caution is in order regarding the evolutionary implications that can and cannot be drawn from each of the methods. Further studies are necessary to realize the full implications of these differences, as well as to understand the other elements relevant to these responses, especially in the case of populations evolved orally. Moreover, our comparisons between methods seem to show that, under selection, recombination of multifactorial characteristics quickly creates new complex genetic combinations, and even possible epistasis, that strongly exceeds the immediate response capacity of a not under-selection population.

Materials and methods

Foundation, maintenance, and selection of populations

We used outbred populations of *D. melanogaster* founded, expanded, maintained and selected as described in Martins et al. (2013) [28]. Briefly, the ancestral outbred population of *Drosophila melanogaster* was established in the laboratory in 2007, from 160 *Wolbachia*-infected fertilized females, collected in Azeitão, Portugal. The population was kept in

laboratory cages for over 50 non-overlapping generations (under constant maintenance conditions) and then serially expanded to allow the establishment of new populations used in experimental evolution against pathogens. Four lines were derived per each four treatments, BactSys and BactOral, and respective controls, ContSys and Control.

Whole-Genome Sequencing of Populations (Pool-seq)

Pool-seq, Read Quality Control and Mapping, SNP Calling and Identification of Candidate SNPs were performed as described in Martins at al. (2014) [19]. Four groups of populations were sequenced and analyzed: four replicates of the base population ("ancestral") and four replicates of the BactSys and ContSys selection regimes at generation 34 and four replicates of the BactOral and Control selection regimes at generation 24.

Bacteria Stocks and Cultures

All tests were performed using *Pseudomonas entomophila*. For each round of infections, bacteria were grown in LB inoculated with a single bacterial colony, taken from solid medium cultures grown from glycerol stocks kept at -80 ºC and streaked in fresh (<1 week) Petri dishes. *P. entomophila* was prepared from an overnight culture grown exponentially at 30 ºC, centrifuged and adjusted to OD_{600} = 0.01. It is resistant to rifampicin, which was used as a marker trait. The *P. entomophila* strain used for all tests was a generous gift from Bruno Lemaitre. RNAi tests were performed using *Pseudomonas putida*. Excluding growing at 37 ºC and have a final OD_{600} = 10, *P. putida* were prepared with the same protocol as *P. entomophila*. The *P. putida* was a generous gift from Karina Xavier.

Antibiotic treatment

Wolbachia-free populations were generated at generation 24, by

raising the progeny of the 4 *Wolbachia*-infected populations for two generations on food with tetracycline (0.05 mg/mL). Flies *Wolbachia*-free were tested two generations after tetracycline treatment. 100 individuals (males and females) from each replicate population of *Wolbachia*-infected selection regimes (BactSys, ContSys, BactOral and Control) and their *Wolbachia*-free counterparts were infected with *P. entomophila* (through respective route) and their survival was followed for 6 days.

RNAi

We performed *in vivo* RNAi knockdown assays for a set of candidate genes in 2L (*pd1c*, *kuz*, *rdo*), 2R (*Cpr50Ca*, *sano*, *CG30222*, *CG10082*, *Egfr*, *pirk*), 3L (*pHCl*, *Eip75B*), 3R (*elF2B-alpha*, *5-HT7*) and X (*mew*) chromosomes, selected according to whether they had significantly differentiated SNPs in each identified peak (Figure 2.4.3A). We took advantage of one of the two large RNAi collections (KK) of the Vienna Drosophila RNAi Center, and used the Gal80ts/Tub-Gal4 inducible system as a rescue from developmental lethality.

The inducible driver constructs were previously introgressed into a common isogenic genetic background. Gal80 repression was released by transferring the adults to 29 °C 3 days before infection. *P. putida* (OD_{600} = 10) were used in these experiments, to account for increased mortality of *P. entomophila* (OD_{600} = 0.01) in this genetic background and at higher temperatures. As negative controls, males from the two genetic backgrounds without insertions (w1118 and y,w1118;P{attP,y+,w3′}) and males expressing RNAi against CG9313 (P{KK103600}VIE-260B) were crossed with the driver lines. All lines were tested at least twice. For each test, we infected 30–50 individuals of each sex.

GWAS

We systemically infected with *P. entomophila* 139 lines from the DGRP (*Drosophila* General Reference Panel). From each line, 30 females and 30 males were tested, in vials of 10 individuals. Infection protocol was the same of experimental evolution, where flies were pricked in the thoracic region with *P. entomophila* (OD_{600} = 0.01). Maintenance conditions were also the same. All vials were kept in the same maintenance conditions of experimental evolution populations and survival was followed during 6 days.

Statistical Analysis

All statistical analyses were performed using R (v 3.1.0)

Identification of candidate SNPs

The Cochran–Mantel–Haenszel (CMH) tests were performed on a SNP-wise basis for the comparisons across groups of populations. The 99.99th percentile of the P value of this statistic, both at chromosome- and genome-wide levels, was used as an empirical false discovery rate for calling a significant SNP. SNPs were mapped to the genes present in FlyBase *D. melanogaster* genome r5.38, using SNPeff (Version 3.3h). Selection coefficients on the candidate polymorphisms were estimated assuming additivity, following:

$$\ln(pt=qt) = \ln(p0=q0) + (s=2) \times t$$

where, p and q are the major and minor allele frequencies at generation t, and s is the estimated selection coefficient.

Survival of *Wolbachia*-free populations

To compare survival between *Wolbachia*-positive and negative populations after infection with *P. entomophila* infections, we used the Kaplan-Meier method (Log-rank (Mantel-Cox) test).

RNAi

To compare survival of RNAi lines against their respective genetic background controls after infection with *P. entomophila*, we used a Cox's proportional hazards mixed effect model, with line as fixed factor and the replicate vial nested within repetition as the random factor.

GWAS

The survival function of each line, after the 6 days, was estimated using the Kaplan-Meier method. The ANOVA statistical model was then applied to compare differences between groups, between each line and between sexes. After the calculation of the survival rate for each line, that survival data from the 3rd day of observations, with the survival of males and females separated, was submitted to the DGRP website to perform a genome wide association study (GWAS) regarding this phenotype, in order to identify single nucleotide polymorphisms (SNPs) that were associated to this susceptibility

Acknowledgements

We would like to thank Instituto Gulbenkian de Ciência for support for TFP. We are also grateful to the group of master students of FCUL (Ana Morais, Ana Eugénio, Joaquim Santos e Sónia cunha) for help in GWAS.

References

1. Vallet-Gely I, Lemaitre B, Boccard F: **Bacterial strategies to overcome insect defences**. *Nature Reviews Microbiology* 2008, **6**:302–313.

2. Lemaitre B, Hoffmann J: **The host defense of *Drosophila melanogaster***. *Annual Review of Immunology* 2007, **25**:697–743.

3. Kimbrell D, Beutler B: **The evolution and genetics of innate immunity.** *Nature Reviews Genetics* 2001, **2**:256–267.

4. Lipstich M, Siller S, Norwak MA: **The evolution of virulence in pathogens with vertical and horizontal transmission**. *Evolution* 1996, **50**:1729–1741.

5. Stewart AD, Logsdon JM, Kelley SE: **An empirical study of the evolution of virulence under both horizontal and vertical transmission.** *Evolution* 2005, **59**:730–739.

6. Buchon N, Broderick NA, Lemaitre B: **Gut homeostasis in a microbial world: insights from *Drosophila melanogaster***. *Nature Reviews Microbiology* 2013, **11**:615–626.

7. Boman HG, Nilsson I, Rasmuson B: **Inducible antibacterial defence system in *Drosophila***. *Nature* 1972; **237**:232–235.

8. Lemaitre B, Reichhart JM, Hoffmann JA: ***Drosophila* host defense: differential induction of antimicrobial peptide genes after infection by various classes of microorganisms**. *Immunology* 1997, **94**:14614–14619.

9. Ding SW, Voinnet O: **Antiviral immunity directed by small rnas**. *Cell* 2007, **130**:413–426.

10. Wang X-H, Aliyari R, Li W-X, Li H-W, Kim K, Carthew R, Atkinson P, Ding SW: **Rna interference directs innate immunity against viruses in adult *Drosophila*.** *Science* 2006, **312**:452–454.

11. Buchon N, Silverman N, Cherry S: **Immunity in *Drosophila melanogaster* - from microbial recognition to whole-organism physiology.** *Nature Reviews Immunology* 2014, **14**:796–810.

12. Teixeira L, Ferreira A, Ashburner M: **The bacterial symbiont *Wolbachia* induces resistance to RNA viral infections in *Drosophila melanogaster***. *PLoS Biology* 2008, **6**:e2.

13. Osborne SE, Leong YS, O'Neill SL, Johnson KN: **Variation in antiviral**

protection mediated by different *Wolbachia* strains in *Drosophila simulans*. *PLoS Pathogens* 2009, **5:**e1000656.

14. Xie J, Vilchez I, Mateos M: ***Spiroplasma* bacteria enhance survival of *Drosophila hydei* attacked by the parasitic wasp *Leptopilina heterotoma***. *PLoS ONE* 2010, **5**:e12149.

15. Kounatidis I, Ligoxygakis P: ***Drosophila* as a model system to unravel the layers of innate immunity to infection**. *Open Biology* 2012, **2**:120075.

16. Lestradet M, Lee KZ, Ferrandon D: ***Drosophila* as a model for intestinal infections**. *Methods in Molecular Biology* 2014, **1197**:11–40.

17. Buchon N, Broderick NA, Poidevin M, Pradervand S, Lemaitre B: ***Drosophila* intestinal response to bacterial infection: activation of host defense and stem cell proliferation**. *Cell Host and Microbe* 2009, **5**:200–211.

18. Teixeira L: **Whole-genome expression profile analysis of *Drosophila melanogaster* immune responses**. *Briefings in Functional Genomics* 2012, **11**:375–386.

19. Martins NE, Faria VG, Nolte V, Schlötterer C, Teixeira L, Sucena É, Magalhães S: **Host adaptation to viruses relies on few genes with different cross-resistance properties.** *Proceedings of the National Academy of Sciences of the United States of America* 2014, **111**:5938–5943.

20. Unckless RL, Rottschaefer SM, Lazzaro BP: **The complex contributions of genetics and nutrition to immunity in *Drosophila melanogaster***. *PLoS Genetics* 2015, **11**:e1005030.

21. Vodovar N, Vallenet D, Cruveiller S, Rouy Z, Barbe V, Acosta C, Cattolico L, Jubin C, Lajus A, Segurens B, Vacherie B, Wincker P, Weissenbach J, Lemaitre B, Médigue C, Boccard F: **Complete genome sequence of the entomopathogenic and metabolically versatile soil bacterium *Pseudomonas entomophila*.** *Nature Biotechnology* 2006, **24**:673–679.

22. Vodovar N, Vinals M, Liehl P, Basset A, Degrouard J, Spellman P, Boccard F, Lemaitre B**: *Drosophila* host defense after oral infection by an entomopathogenic *Pseudomonas* species.** *Proceedings of the National Academy of Sciences of the United States of America* 2005, **102**:11414–11419.

23. Liehl P, Blight M, Vodovar N, Boccard F, Lemaitre B: **Prevalence of local**

immune response against oral infection in a *Drosophila*/ *Pseudomonas* infection model. *PLoS Pathogens* 2006, **2**:e56.

24. Kuraishi T, Binggeli O, Opota O, Buchon N, Lemaitre B: **Genetic evidence for a protective role of the peritrophic matrix against intestinal bacterial infection in *Drosophila melanogaster***. *Proceedings of the National Academy of Sciences of the United States of America* 2011, **108**:15966–15971.

25. Vijendravarma RK, Kraaijeveld AR, Godfray HCJ: **Experimental evolution shows *Drosophila melanogaster* resistance to a microsporidian pathogen has fitness costs**. *Evolution* 2009, **63**:104–114.

26. Ye YH, Chenoweth SF, McGraw EA: **Effective but costly, evolved mechanisms of defense against a virulent opportunistic pathogen in *Drosophila melanogaster***. *PLoS Pathogens* 2009, **5**:e1000385.

27. Schlötterer C, Kofler R, Versace E, Tobler R, Franssen SU: **Combining experimental evolution with next-generation sequencing: a powerful tool to study adaptation from standing genetic variation.** *Heredity* 2015, **114**:431–440.

28. Martins NE, Faria VG, Teixeira L, Magalhães S, Sucena É: **Host adaptation is contingent upon the infection route taken by pathogens**. *PLoS Pathogens*, 2013, **9:**e100.

29. Schlötterer C, Tobler R, Kofler R, Nolte V: **Sequencing pools of individuals — mining genome-wide polymorphism data without big funding**. *Nature Reviews Genetics* 2014, **15**:749–763.

30. Stroschein-Stevenson SL, Foley E, O'Farrell PH, Johnson AD: **Identification of *Drosophila* gene products required for phagocytosis of *Candida albicans***. *PLoS Biology* 2006, **4**:e4.

31. Karouzou M V., Spyropoulos Y, Iconomidou VA, Cornman RS, Hamodrakas SJ, Willis JH: ***Drosophila* cuticular proteins with the r&r consensus: annotation and classification with a new tool for discriminating rr-1 and rr-2 sequences**. *Insect Biochemistry and Molecular Biology* 2007, **37**:754–760.

32. Williams DD, Pavitt GD, Proud CG: **Characterization of the initiation factor eif2b and its regulation in *Drosophila melanogaster***. *Journal of Biological Chemistry* 2001, **276**:3733–3742.

33. Magwire MM, Fabian DK, Schweyen H, Cao C, Longdon B, Bayer F, Jiggins

FM: **Genome-wide association studies reveal a simple genetic basis of resistance to naturally coevolving viruses in *Drosophila melanogaster*.** *PLoS Genetics* 2012, **8**:e1003057.

34. Bou Sleiman MS, Osman D, Massouras A, Hoffmann AA, Lemaitre B, Deplancke B: **Genetic, molecular and physiological basis of variation in *Drosophila* gut immunocompetence**. *Nature Communications* 2015, **6**:7829.

2.5. Host adaptation to viruses relies on few genes with different cross-resistance properties

Nelson E. Martins[1*], **Vítor G. Faria[1*]**, Viola Nolte[2], Christian Schlötterer[2], Luis Teixeira[1], Élio Sucena[1,3¶#], Sara Magalhães[3,4¶#]

1 - Instituto Gulbenkian de Ciência, Apartado 14 , 2781-901 Oeiras, Portugal

2 - Institut für Populationsgenetik, Vetmeduni Vienna, Austria

3 - Universidade de Lisboa, Faculdade de Ciências, Departamento de Biologia Animal, edifício C2, Campo Grande, 1749-016 Lisboa, Portugal

4 - cE3c: Centre for Ecology, Evolution and Environmental Changes, Faculdade de Ciências, Universidade de Lisboa, edifício C2, Campo Grande, 1749-016 Lisboa, Portugal

*** - These authors contributed equally to this work**

¶ - Joint last authors

- Email: esucena@igc.gulbenkian.pt, snmagalhaes@fc.ul.pt

Keywords: Host-parasite interactions, experimental evolution, *Drosophila* immunity, evolve and resequence

This article was published in the journal **Proceedings of the National Academy of Science** (vol. 111 no. 16, 5938-5943). April 2014.
DOI - 10.1073/pnas.1400378111.
PubMed ID - 24711428.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1400378111/-/DCSupplemental.

Author contributions: N.E.M., V.G.F., L.T., É.S., and S.M. designed research; N.E.M., V.G.F., and V.N. performed research; C.S. and É.S. contributed new reagents/analytic tools; N.E.M., V.G.F., C.S., L.T., É.S., and S.M. analyzed data; and N.E.M., É.S., and S.M. wrote the paper.
The authors declare no conflict of interest.

Funding: Fundação para a Ciência e a Tecnologia (FCT, Portugal) supports N.E.M (#SFRH/BPD/62964/2009) and V.G.F (#SFRH/BD/82299/2011). C.S. is funded by AustrianScience Fund (FWF) (P22725 and P19467) and European Research Council (ArchAdapt). This work was supported by Instituto Gulbenkian de Ciência/Fundação Calouste Gulbenkian, FCT (PTDC/SAU -IMU/120673/2010), and Vetmeduni funding. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Data deposition: The sequences reported in this paper have been deposited in the Sequence Read Archive of the European Nucleotide Archive, www.ebi.ac.uk/ena/about/ search_and_browse (accession nos. ERS409776 –ERS409787).

Abstract

Host adaptation to one parasite may affect its response to others. However, the genetics of these direct and correlated responses remains poorly studied. The overlap between these responses is instrumental for the understanding of host evolution in multiparasite environments. We determined the genetic and phenotypic changes underlying adaptation of *Drosophila melanogaster* to *Drosophila* C virus (DCV). Within 20 generations, flies selected with DCV showed increased survival after DCV infection, but also after cricket paralysis virus (CrPV) and flock house virus (FHV) infection. Whole-genome sequencing identified two regions of significant differentiation among treatments, from which candidate genes were functionally tested with RNAi. Three genes were validated—*pastrel*, a known DCV-response gene, and two other loci, *Ubc-E2H* and *CG8492*. Knockdown of *Ubc-E2H* and *pastrel* also led to increased sensitivity to CrPV, whereas knockdown of *CG8492* increased susceptibility to FHV infection. Therefore, *Drosophila* adaptation to DCV relies on few major genes, each with different cross-resistance properties, conferring host resistance to several parasites.

Introduction

Parasites impose a strong fitness cost on their hosts as they develop and reproduce at the expenses of host resources. Therefore, it is expected that host strategies will be selected to cope with parasite burden. There is ample variety of such strategies, from behavioural to intracellular responses [1]. Because the range of possibilities is very broad, it is difficult to predict which strategy, if any, will evolve in host populations upon parasite attack. Moreover, in natural populations, hosts are exposed simultaneously to several parasite species and many other selection pressures. If these selection pressures do not vary independently of each other, a clear establishment of causality between changes in host traits and the selection pressure posed by a given parasite species may be hampered.

Experimental evolution enables the establishment of a direct link between the selection imposed by a given environment and the genetic and phenotypic changes observed in a population. The explanatory power of this methodology relies on three major characteristics: (i) knowledge of the ancestral state, (ii) control of the selection forces driving different sets of replicated populations and (iii) the ability to follow the dynamics of a process, instead of measuring only its end-product [2]. In addition, this methodology allows addressing the consequences of the adaptation process for the performance in other environments [3–5].

Experimental evolution coupled with whole-genome approaches can provide a nearly unbiased view of the actual targets of selection, a long-standing aim of evolutionary biology [2]. To this day few examples exist in which these combined methodologies have been used in multicellular sexual organisms, in which most adaptation comes from standing genetic variation (SGV) instead of novel mutations [6–10]. However, despite the centrality of host-parasite interactions in evolutionary biology, and several experimental evolution studies in host-parasite systems [11–16], no study

of host-parasite interactions has combined experimental evolution with genomics.

Another important aspect of experimental evolution is that it allows measuring the consequences of evolving in one environment for the performance in other environments [3]. Indeed, adaptation to one environment may entail a fitness decrease in other environments, possibly hampering future evolution in such settings [17,18]. Despite being common, these costs are not universal [4] even within experiments [17]. Moreover, adapting to one environment may even lead to increased performance in other environments [e.g., 5, 19]. In host-parasite interactions, the question is particularly important because of the epidemiological consequences of infecting or resisting multiple hosts or parasites, respectively.

Despite ample knowledge of the genes triggered by parasite attacks against *Drosophila*, only a few key studies have analysed how an outbred fly population may adapt to a given parasite [11–13, 15]. Yet, the genetic basis and the consequences of such adaptation for host susceptibility to other parasites have not been determined.

It has been shown that natural *D. melanogaster* populations contain standing genetic variation for resistance against natural viruses. Whereas some studies show that most of this variation can be attributed to a limited number of genes with major effect [20–23], others indicate that a significant fraction of the genetic variation for resistance is polygenic [24,25]. Interestingly, the alleles that contribute to the variation in resistance to a given virus are in genes unrelated to the canonical insect anti-viral defence pathways [26]. Moreover, this variation may be rather specific in mediating responses to distinct natural pathogens [21].

Here, we addressed the genetics of host adaptation to parasites and the effects in cross-resistance in a *D. melanogaster*-virus system. To this aim, we performed experimental evolution of an outbred *D. melanogaster*

population exposed to a natural viral parasite (*Drosophila* C virus - DCV), analysed the basis for the response using a genome-wide approach, and functionally tested candidate genes for their role in the response against DCV and other parasites.

Results

Adaptation to DCV Infection

We have performed experimental evolution of an outbred *D. melanogaster* population exposed to recurrent systemic DCV infection (VirSys). DCV infection was imposed at every generation using the same (not coevolved) ancestral virus strain. In parallel, two control conditions were established, where individuals were subjected to the same procedure as the virus-selected population but pricked with a buffer solution only (ContSys) or not pricked at all (Control). The experiment was performed with four replicates for each condition.

When exposed to DCV, VirSys populations showed higher survival than individuals from control lines [Figure 2.5.1A; general linear mixed model (GLMM), $\chi^2_1 = 154.98$, $P < 0.0001$]. Changes in survival in the VirSys selection regime were consistent among replicate populations (Figure S1A). The difference in survival was absent in the early generations and increased with time, leading to a significant interaction between generation and selection regime (Figure 2.5.1A, Dataset S1, and Figure S1A; GLMM, $\chi^2_{30} = 163.54$, $P < 0.0001$). When tested independently in the two sexes, both effects of selection regime (GLMM, $\chi^2_1 = 20.489$ and 24.288, $P < 0.0001$ for males and females, respectively) and interaction with generation (GLMM, $\chi^2_{30} = 236.95$ and $\chi^2_{26} = 145.89$, $P < 0.0001$ for males and females, respectively) were significant. Given that we were comparing control with VirSys individuals, and that ContSys populations were used in all subsequent tests, survival rates of ContSys and control populations were

directly compared at generations 15 and 25. No significant differences were observed between the two sets of control lines (Table S1).

VirSys lines showed a strong reduction of virus numbers compared with ContSys lines (Figure 2.5.1B; ANOVA, $F_{1,6} = 39.55$, $P = 0.0008$) indicating that selection has relied (at least partially) on the evolution of resistance.

Next, we tested the contribution of *Wolbachia* to the evolution of resistance in our populations as this endosymbiont has been shown to protect *Drosophila* against viral infections [27]. To this end, we removed *Wolbachia* from replicates of VirSys and ContSys populations, after 25 generations of selection and measured survival upon DCV infection (Figure 2.5.1C). A significant interaction was found between sex and both

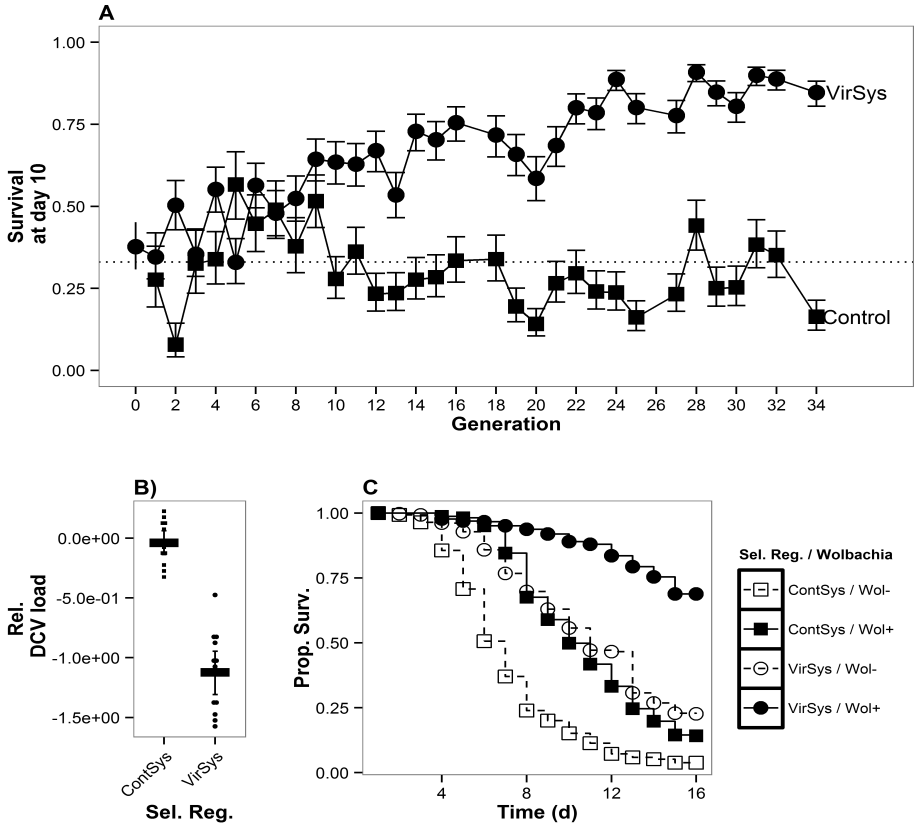


Figure 2.5.1 – Evolution of increased resistance to DCV. (**A**) Experimental evolution trajectories of control (Control) and virus-exposed (VirSys) populations over 34 generations of experimental evolution. Circles represent populations exposed to the virus. Squares represent control lines. Vertical bars correspond to the SEM survival among the four selected populations (VirSys) and of the pool of control individuals. The straight dotted line corresponds to the original mortality rate imposed on the populations (66%). (**B**) Relative DCV loads (DCV/*RpL32* copies) in females, 5 d postinfection, of ContSys and VirSys populations. Points represent individual measurements; horizontal lines the mean and 95% confidence intervals). (**C**) Survival after DCV infection of control and virus selected lines, with or without *Wolbachia* (solid lines/filled symbols, Wol+ or dotted lines/open symbols, Wol–, respectively).

Wolbachia and selection regime (Cox model, χ^2_1 = 56.705 and 17.150, respectively and $P < 0.0001$ in both comparisons). Therefore, we tested the effects of *Wolbachia* and selection regime independently for both sexes (Figure S1B). In both cases there was a significant *Wolbachia* and selection-regime effect (Cox model, χ^2_1 = 29.110 and 34.94, for *Wolbachia* and selection-regime effect in males; χ^2_1 = 24.865 and 22.824 for *Wolbachia* and selection-regime effects in females, respectively; $P < 0.0001$ in all comparisons). Therefore, the protective role of *Wolbachia* against viral infections [27] is confirmed in this study on both experimental and control lines. However, no significant effect of the interaction *Wolbachia* X selection regime was found for either sex (Cox model, $\chi^2_1 = 0.255$, $P = 0.613$ and $\chi^2_1 = 1.007$, $P = 0.316$ for males and females, respectively). This indicates a significant contribution of the host genome to the evolution of resistance, which is statistically independent of the effect of *Wolbachia* infection status.

Cross-Resistance to Other Parasites

As shown in Figure 2.5.2, VirSys populations also had on average higher survival, relative to ContSys, after infection with the parasites cricket paralysis virus (CrPV) or flock house virus (FHV) (Cox model, |z| = 19.857, 11.329, and 5.226 for infection with DCV, CrPV, and FHV, respectively; $P < 0.0001$ for all comparisons). There was a significant interaction effect with the generation at which the test was conducted for the different parasites (Cox model, $\chi^2_3 = 31.276$, $P < 0.001$ for DCV; $\chi^2_1 = 4.192$, $P < 0.05$ for CrPV; and $\chi^2_2 = 6.819$, $P < 0.05$ for FHV). However, the difference between the VirSys and ContSys regimes was significant in all separate tests performed at different generations and for the different viruses (Cox model, |z| = 14.480, 10.790, 13.454, and 7.337 for DCV infections performed at generations 15, 20, 25, and 30; |z| = 1.122 and 1.438 for CrPV infections at

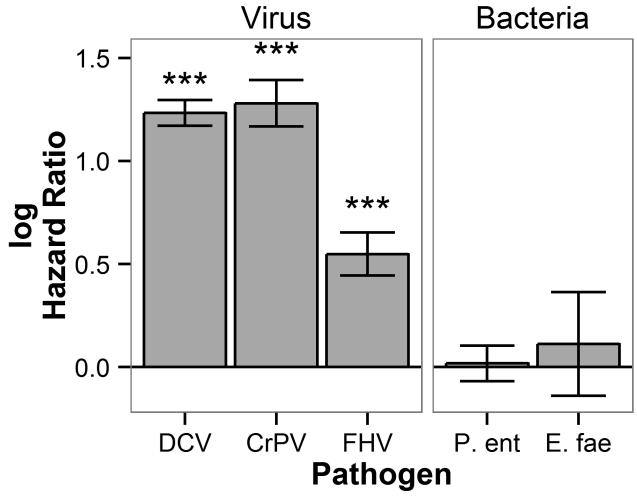


Figure 2.5.2 – Specificity of the evolved response. Hazard ratios between ContSys and VirSys populations, when exposed to DCV, CrPV, FHV, *P. entomophila* (P.ent), and *E. faecalis* (E.fae). Shown are the average hazard ratios of at least two independent experiments, done at different generations. Vertical bars correspond to the 95% confidence intervals of the estimated hazard ratios. ***$P < 0.001$.

generations 15 and 30; and |z| = 0.514, 0.327, and 0.804 for FHV infections at generations 15, 20, and 30. $P < 0.001$ in all comparisons, except for the FHV infection at generation 20, where $P < 0.05$). However, the hazard ratios between ContSys and VirSys exposed to FHV infection are significantly lower than those observed upon exposure to DCV (used for selection) or against CrPV, a very close DCV relative (Figure 2.5.2).

No significant difference in survival among selection regimes was found when flies were infected with the bacteria *Pseudomonas entomophila* and *Enterococcus faecalis* (Cox model, |z| < 0.446, $P > 0.66$ for all comparisons after infection with *P. entomophila* at generations 15 and 25 or with *E. faecalis* at generations 34 and 35). We therefore conclude that evolution of resistance to DCV leads to partial protection against other positive strand RNA viruses, but not against bacterial pathogens.

Genetic Basis of Host Adaptation

To identify the changes in allele frequencies underlying the observed increased resistance of *Drosophila* populations evolving in presence of DCV, we performed genome-wide sequencing of DNA pools (Pool-Seq) of all populations (Figure 2.5.3) [28]. Patterns of overall genetic diversity are presented in Figure S2.

Using a chromosome-wide cutoff, we observed consistent significant changes in allele frequencies of 853 SNPs over a region that spans ~4 Mb on the left arm of the third chromosome (*3L*) (most 5′ SNP, 3L:5127093 and most 3′ SNP, 3L:9149494) and 5 SNPs on the *X* chromosome across a 300-kb region (X:7638809–7984449). This result did not change qualitatively using a genome-wide cutoff, but the region of significance was reduced to positions 3L:5221901–8901948 (i.e., 384 SNPs), and to 2 SNPs on the *X* chromosome. The most significantly differentiated SNP in the *3L* region

corresponds to position 3L:7350895 and maps to the gene *pastrel* (*pst*). The two significantly differentiated SNPs on the *X* chromosome (X:7984325 and X:7984449) are located in the introns of the gene *Ubc-E2H*. Initial and final frequencies of the most significantly differentiated SNPs were 0.167 and 0.7 for 3L:7350895 (*pst*) and 0.267 and 0.6 for X:7984325 (*Ubc-E2H*), respectively. Considering these changes in frequency, and assuming additive effects only, the estimated selection coefficients are 0.24 and 0.14 for the SNP in *pst* and *Ubc-E2H*, respectively. Changes in other significantly

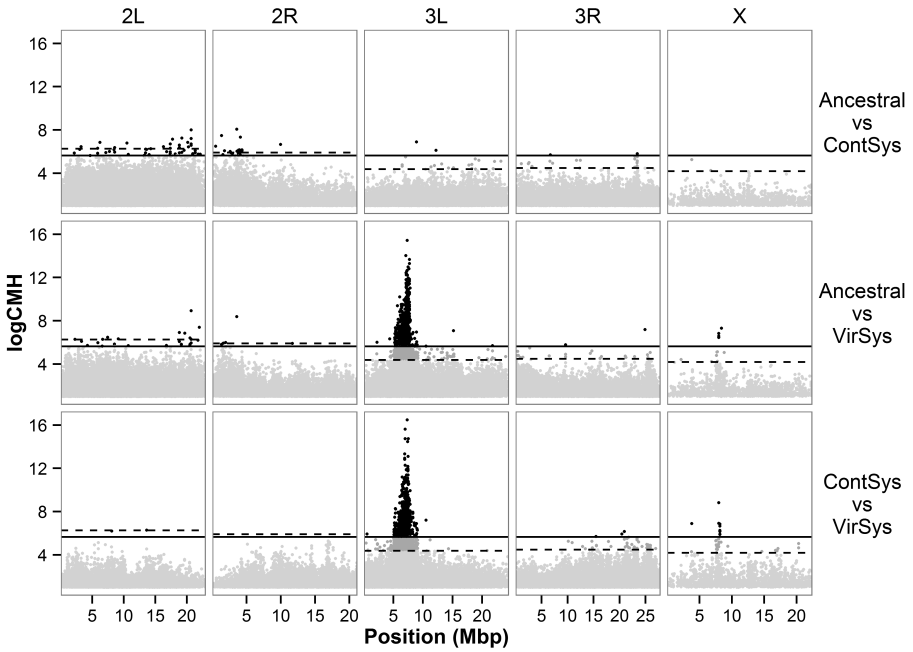


Figure 2.5.3 – Differentiation between selection regimes. –log10 values of the CMH statistic for every polymorphic SNP, across the five major chromosomal arms through pairwise comparison of allele frequencies between ancestral and ContSys populations at generation 20 (*Top*), ancestral and VirSys populations at generation 20 (*Middle*), and between ContSys and VirSys at generation 20 (*Bottom*). The solid and dotted lines represent the 99.99% quantile of the *P* values in the ancestral vs. ContSys comparison at genome-wide and chromosome-wide levels, respectively.

differentiated SNPs are described in Dataset S2.

Functional Validation of the Candidate Genes

We then used RNAi to functionally validate the two genes associated to the most significant SNPs identified in the genome-wide analysis. We further tested 12 genes in the *3L* region, which contained nonsynonymous mutations (Figure 2.5.4).

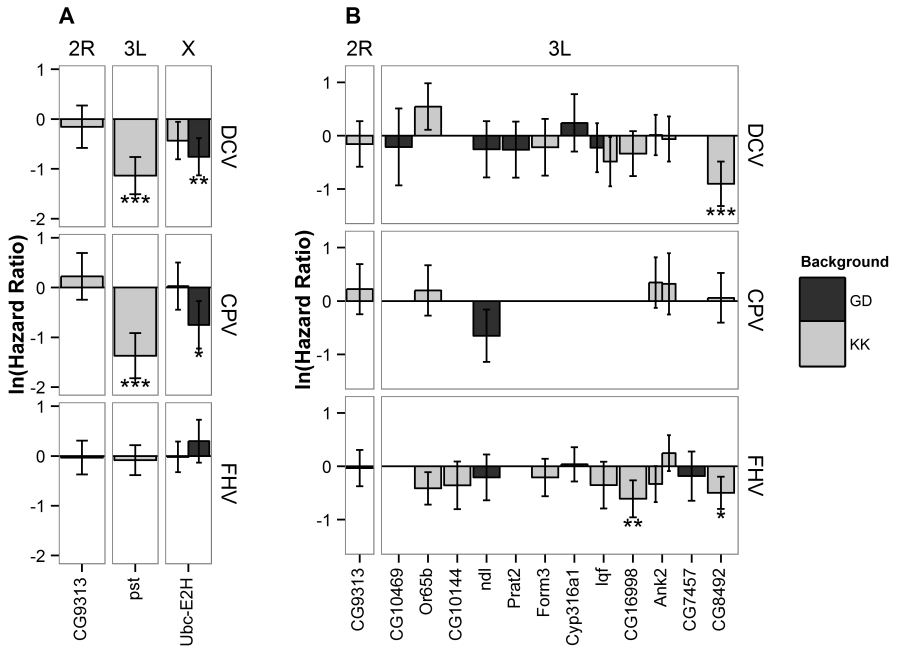


Figure 2.5.4 – RNAi knockdown of candidate genes. Natural logarithm of hazard ratios between survival of flies with knocked-down candidate genes and their controls upon infection with DCV (*Top*), CrPV (*Middle*), and FHV (*Bottom*), using as genetic background KK (gray bars), GD (black bars) or both, whenever a construct was available in both backgrounds. (**A**) RNAi interference against the candidate genes identified by the peaks in Figure 2.5.3, *pst* and *Ubc-E2H*. (**B**) Tests to other genes in the large *3L* peak. Vertical bars correspond to the 95% confidence intervals of the estimated hazard ratios. **P* < 0.05; ***P* < 0.01; ****P* < 0.001.

Knockdown of *pastrel* and *Ubc-E2H* (with stock w1118; P{GD9765}v33510; see Table S2 for details) led to reduced survival of flies when exposed to DCV or to CrPV infection (Figure 2.5.4A, *Ubc-E2H*: |z| = 3.98 and 3.09, $P < 0.01$ and $P < 0.05$, after DCV and CrPV infection, respectively; and Figure 2.5.4B, *pst* |z| = 5.94 and 5.93, $P < 0.001$ after DCV and CrPV infection), but not when exposed to FHV infection (*Ubc-E2H*: |z| = 1.35, $P > 0.9$ and *pst*: |z| = 0.08 for knockdown of both genes). Using another RNAi line targeting *Ubc-E2H* (with stock P{KK108626}VIE-260B; see Table S2 for details) did not show differences in survival against any of the viruses (|z| = 2.25, 0.11, and 0.12 for DCV, CrPV, and FHV respectively, $P > 0.3$) (Figure 2.5.4A). We attribute this survival difference using two different RNAi lines to a lower knockdown efficiency of this construct, as revealed by semiquantitative gene expression analysis (Figure S3). No differences in susceptibility to viruses were observed when comparing the negative control with the respective genetic background (|z| = 0.71, 0.93, and 0.19 for DCV, CrPV and FHV respectively; $P > 0.97$).

RNAi knockdown of another 12 genes within the *3L* region revealed only one other case, gene *CG8492* (stock P{KK100300}VIE-260B), with reduced survival upon exposure to DCV and to FHV (Figure 2.5.4B, |z| = 4.23 and 3.23, $P < 0.001$ and $P < 0.05$ for DCV and FHV, respectively), but not to CrPV (|z| = 0.24, $P = 1$). All *P* values were Bonferroni corrected for the number of performed comparisons.

Discussion

In this study, we found that resistance to DCV evolved rapidly in experimental *Drosophila* populations. Cross-resistance was detected for infection with other viruses (CrPV and FHV) but not with bacteria. Using whole-genome sequencing, we identified two regions in which genetic changes occurred in populations evolving under DCV challenge, one in the

3L chromosome arm and a smaller region on the *X* chromosome. Through RNAi assays against candidate genes in these regions, we confirmed the role of *pst*, a gene with variants previously associated with a differential response to DCV infection in *Drosophila* [21], as well as two loci that had not been linked previously to antiviral responses: *Ubc-E2H* on the *X* chromosome and *CG8492* on the *3L* chromosome arm. Knockdown of *pst* and *Ubc-E2H* led to increased sensitivity to CrPV but not to FHV, whereas the opposite pattern was found in *CG8492*. Hence, flies that have adapted to resist DCV are also better at surviving infection with other viruses, but these correlated responses rely on different sets of genes.

Genetic Basis of Resistance

Using a combination of genomics with experimental evolution, we identified the genetic changes underlying the evolution of a host population (*D. melanogaster*) adapting to a natural parasite (DCV). We find two regions of differentiation between the populations evolving in presence of a virus and control populations. These changes were parallel across four replicates (Figure S2 and Dataset S2) and correlate with the observed parallel changes in survival (Figure S1A). This indicates that selection, rather than drift, shaped this adaptive response. In one region, the peak of differentiation matched *pst*, a gene previously shown to be involved in the *Drosophila* response to DCV through an association study [21]. The high number of differentiated SNPs around this locus, extending to a region of ~4 Mb, and the observed pattern of local decrease of heterozygosity suggest the occurrence of an incomplete soft sweep around *pst* [29].

However, the influence of other genes in the region cannot be excluded, as shown by the increased susceptibility of flies expressing RNAi against *CG8492*, a gene located near the centromeric end of the peak. The determination of the haplotype structure in this region, as well as the effect

in virus resistance of the variants of *CG8492* and their possible interactions with *pst*, deserve further examination.

This result is particularly interesting in that it departs from the inconsistency observed when comparing genome-wide association studies (GWASs) using inbred lines vs. outbred populations [30]. Thus far, only a weak but significant correlation has been found between SNPs associated with polygenic traits by GWAS and evolve-and-resequence (E&R) approaches [31]. Here, we confirm *pst*, a gene found through a GWAS approach [21], as a central player in the adaptation of an outbred population of *Drosophila* to DCV infection.

Furthermore, using RNAi we confirmed the role of *pst*, and unraveled an effect of *Ubc-E2H* and *CG8492* in antiviral defense. These results confirm the power of the E&R approach in the identification of targets of selection [32]. This methodology has been used to identify changes in allele frequencies following selection in complex traits such as developmental time [7], body size [8], hypoxia tolerance [6], increased life span [33], adaptation to high/low temperatures [9, 34], and courtship behavior [10, 31]. These studies have identified a polygenic basis for the studied traits, hampering the identification of candidate genes and a subsequent functional analysis. One exception is the study of Zhou et al. [6], in which most of the differentiated genes belonged to the Notch signaling pathway, thus permitting a functional validation of this pathway in hypoxia tolerance evolution. However, the relatively high number of genes involved in these responses do not permit the assessment of the role played by each gene and how the phenotypic effect may be partitioned. In our case, the few genes underlying the evolution of resistance to DCV seem to work in an (partially) additive fashion, as each gene tested independently confers resistance. However, further studies are needed to establish the relative role of additivity and genetic interactions in this response.

Cross-Resistance

We found a strong positively correlated response with CrPV, but only a moderate response to FHV, and no response to bacteria. Hence, the correlated response is positive and diminishes with decreasing similarity to DCV. Both these findings match recent theoretical predictions for one-sided host evolution [14]. However, other studies on host evolution have found tradeoffs [16, 35] or no significant correlated response [36, 37] among resistance to different parasites, hence the generality of our finding remains to be shown.

We analyzed the correlated responses of the genes involved in DCV resistance when flies were infected with other viruses. To our knowledge, this constitutes the first direct test of the genetic basis of correlated responses to selection driven by SGV. Analysis of the effects of de novo mutations that arise in *Escherichia coli* populations adapting to a glucose-limited environment when placed in other environments, had also shown that the set of mutations conferring fitness increases varies between environments [38]. Similarly to that study, we find that distinct genes for which allelic frequencies have changed in response to DCV infection affect correlated responses differently. Indeed, knockdown of *pst* does not affect susceptibility to FHV, confirming earlier results [21]; but knockdown of either *pst* or *Ubc-E2H* affects cross-resistance to CrPV. In contrast, knockdown of *CG8492* does not affect the response to CrPV but leads to higher susceptibility to FHV. Therefore, in our populations, the evolution of a generalized response to viral parasites is specifically partitioned into different loci.

Until now, the genetic analysis of correlated responses has relied on measuring the genetic correlation among traits in different environments using quantitative genetics designs [3]. This methodology has also been used in the study of host–parasite interactions [39, 40]. However, it has

been shown that genetic correlations are poor predictors of the evolution of correlated responses to selection, mainly because the latter hinges on the genetic architecture of traits under each environment [41]. In our study, we do not measure the whole genetic architecture of the traits under selection, primarily because we miss genes involved in resistance that are fixed and those with changes occurring below our threshold value. Still, we detect those genes in which allele frequencies change across generations, and hence contribute to the evolutionary response. By describing that these genes have different cross-resistance properties against different parasites, we show that the genetics of correlated responses may be complex, even in cases where the genetic basis of adaptation is relatively simple.

Our findings raise an important issue: Which forces maintain the SGV upon which is based host adaptation to viral infection? We have not found costs in susceptibility to other parasites associated to the evolution of resistance to DCV. Hence, our results do not support the maintenance of diversity via antagonistic pleiotropy [3]. This does not rule out that tradeoffs with susceptibility to other parasites exist, which we have not included in our tests. Still, for the parasites tested, we show evolution of positively correlated responses, which depend on different genetic architectures in a parasite-specific manner. This raises the possibility that, even in cases where a generalized response evolves, specificities at the genetic level may lead to different genetic responses in environments with qualitatively different parasite challenges. This extends the possibility of maintaining genetic diversity across host populations [42], even when phenotypic responses suggest a generalized response to several parasites. A formal test of this hypothesis will require evolving and resequencing outbred populations in environments with different combinations of viruses.

It is generally believed that the occurrence of specific host genotype x parasite genotype interactions (Gh × Gp) relies on simple genetic bases [43–45]. Here, we show that although the genetic basis of host adaptation to a parasite is simple, a generalist response has evolved. Therefore, a simple genetic basis is a necessary but not sufficient condition for the evolution of specific interactions. However, it should be noted that our findings concern the outcome of an evolutionary process in which no coevolution has occurred. Therefore, more studies identifying the genetic basis of coevolution are required [44, 46]. In particular, it will be highly informative to compare the genetic architecture of cross-correlations in coevolved systems with that of the present study.

Materials and Methods

Fly Populations

We used an outbred population of *D. melanogaster* founded and maintained as described in Martins et al. [15] and kept it at a high effective populations size (*SI Materials and Methods*). Before the initiation of experimental evolution, this population was serially expanded for two generations to allow the establishment of 36 new populations of which 12 were used in this work. Unless otherwise noted, flies were maintained under constant temperature (25 °C), humidity (60–70%), and light–darkness cycle (12:12); and fed with a standard cornmeal–agar medium. The populations were fully infected with *Wolbachia* at the onset of the experiment, and this infection status of the populations was monitored throughout the experiment.

Parasite Stocks and Cultures

DCV, CrPV (a gift from Peter Christian, National Institute for Biological Standards and Control, Potters Bar, United Kingdom), and FHV,

were grown and titrated as described before [27]. Virus aliquots were kept at −80 °C and thawed before infection. *P. entomophila* and *E. faecalis* were generous gifts from B. Lemaitre (École Polytechnique Fédérale de Lausanne, Lausanne, Switzerland) and T. Rival (Aix-Marseille Université, Centre National de la Recherche Scientifique, Institut de Biologie du Développement de Marseille-Luminy, Marseille, France), respectively. Bacteria stocks were kept in glycerol at −80 °C. Before use, they were streaked in fresh Petri dishes, then a single colony was picked and let to grow in LB at 30 °C (*P. entomophila*) or 37 °C (*E. faecalis*). The culture was then centrifuged and adjusted to the desired OD.

Experimental Evolution

Starting from the base population, we derived 12 lines evolving under 3 different regimes (4 replicates per treatment). In the VirSys treatment, adult flies were pricked in the thoracic region with DCV [2 × 107 tissue culture ID50 (TCID50)] at each generation. A second treatment consisted of a control for pricking, in which the needle was dipped in sterile medium (ContSys). Finally, a second group of control lines consisted of flies kept in standard food without being pricked (control). No differences between ContSys and control lines were found for any test made with both sets of lines. The dose of DCV that was used caused an average mortality of 66% in the initial population 10 d after infection (Figure S4).

These treatments were administrated to 310 males and 310 females (4–6 d after eclosion). Selection lines were kept in large population cages and surviving individuals mated randomly; reproduction took place at days 5–7 after infection by providing fresh oviposition substrate. The number of individuals in the control populations was always reduced to the initial number of infected individuals (i.e., 600).

Egg density was limited to 400 per cup, a density determined

experimentally to enable optimal larval development. Each generation cycle lasted 3 weeks. Before the beginning of the experiment, absence of vertical transmission of the parasite to the progeny was verified (Figure S5).
To monitor survival across generations, we infected at each generation additional sample males and female flies from each of the VirSys lines and control lines and monitored their survival in vials for at least 10 days (Dataset S1).

Parasite Loads

Virus quantifications were performed as described in Teixeira et al. [27] with minor modifications. For each assay, 75–125 females from each population of ContSys and VirSys at generation 33 were infected as in the survival assays. Surviving flies were collected on day 5 after infection, pooled in 5 replicates of 10 individuals per population, and snap-frozen in liquid N2. RNA was extracted using TRIZOL. To avoid possible artifacts due to different maternal effects, flies used in these tests were the progeny of flies that spent one generation in a common environment without the virus.

Wolbachia

Wolbachia-free replicates of the ContSys and VirSys populations were derived at generation 25, by raising the progeny for two generations on food with tetracycline (0.05 mg/mL). Two generations after tetracycline treatment, 100 individuals (males and females) from each replicate population of the VirSys and ContSys selection regimes and their *Wolbachia*-free counterparts, were systemically infected with DCV and their survival was followed for 16 days.

Cross-Resistance with Other Parasites

To test how adaptation to a specific parasite affected host responses

to other parasites, 100 individuals (males and females) from each replicate population of the VirSys and ContSys selection regimes, which had spent one generation in a common environment, were systemically infected with the following parasites: CrPV (undetermined TCID50), FHV (TCID50 = 5 × 106), *P. entomophila* (OD600 = 0.01), and *E. faecalis* (OD600 = 3). These tests were performed at generations 15, 20, 25, and 30 (DCV); 15, 20, and 30 (FHV); 15 and 25 (*P. entomophila*); 15 and 35 (CrPV); and at 34 and 35 (*E. faecalis*).

Whole-Genome Sequencing

Genomic DNA preparation and sequencing were done as in Orozco-terWengel et al. [9]. Briefly, a pool of 200 individuals of each selection line was homogenized with an Ultraturrax T10 (IKA-Werke), and DNA was extracted from the homogenate using a high-salt extraction protocol. Genomic DNA was sheared using a Covaris S2 device (Covaris, Inc.) and paired-end libraries were prepared using the TruSeq v2 DNA Sample Prep Kit (Illumina). Libraries were size-selected for a mean insert size of 300 bp on agarose gels and amplified with 10 PCR cycles, and 2× 100-bp paired-end reads were sequenced on a HiSeq 2000 (Illumina). Three groups of populations were sequenced: four replicates of the base population ("ancestral") and four replicates of the ContSys and VirSys selection regimes at generation 20.

Read Quality Control and Mapping

Reads were mapped following the previously described pipeline for Pool-Seq analysis. Briefly, 100-bp paired-end reads were filtered for a minimum average base quality score of 18 and trimmed using PoPoolation [28]. Reads with a minimum length ≥50 bp were then mapped against a reference containing the FlyBase *D. melanogaster* genome r5.38

(http://flybase.org). For details on filtering parameters and coverage, see *SI Materials and Methods*.

SNP Calling

Only SNPs that met the following quality criteria were considered: (*i*) occurrence in at least 2 replicate populations, (*ii*) the minor allele was covered by at least 10 reads across all populations analyzed, and (*iii*) the maximum coverage did not exceed 500.

Genetic Diversity

To characterize genome-wide patterns of genetic diversity, we estimated per-site heterozygosity (π), following the PoPoolation analysis pipeline [28]. We only considered polymorphic sites for which the minor allele was supported by at least two reads after standardizing the coverage to 30 reads per site, and used unbiased estimators for pooled data that correct for pool size and coverage [28, 47]. For graphical representation, we calculated average values in sliding 500-kb windows, with a step size of 100 kb across the entire genome (Figure S1A).

Identification of Candidate SNPs

We used the Cochran–Mantel–Haenszel (CMH) test, as implemented in PoPoolation2 [48] to identify SNPs with changes in allele frequencies between the different regimes that were consistent among replicates as described in Orozco-terWengel et al. [9] (*SI Materials and Methods*).

RNAi

We performed in vivo RNAi knockdown assays for the candidate genes in the *3L* and *X* (*pst* and *Ubc-E2H*) and for a set of genes in the *3L* peak of differentiation, selected according to whether (*i*) they had significantly

differentiated nonsynonymous SNPs or (*ii*) gene ontology or previous functional assays suggested a role in antiviral immunity. We took advantage of the two large RNAi collections of the Vienna *Drosophila* RNAi Center [49], and used the Gal80ts/Tub-Gal4 inducible system as a rescue from developmental lethality. The tested constructs are shown in Table S2. More details are available in *SI Materials and Methods*.

Statistical Analysis

All statistical analyses were done using R (Version 2.15; www.r-project.org). Full details are provided in *SI Materials and Methods*.

Acknowledgments

The authors thank Dieter Ebert and Britt Koskella for critical comments that significantly improved the manuscript; the laboratories of Patrícia Beldade, Christen Mirth, and É.S.; and Margarida Matos for inspiring discussions on the project.

References

1. Schmid-Hempel P: **Evolutionary Parasitology: the integrated study of infections, Immunology, Ecology, and Genetics**. Oxford: Oxford Biology; 2011.

2. Kawecki TJ, Lenski RE, Ebert D, Hollis B, Olivieri I, Whitlock MC: **Experimental evolution.** *Trends in Ecology and Evolution* 2012, **27**:547–560.

3. Kassen R: **The experimental evolution of specialists, generalists, and the maintenance of diversity**. *Journal of Evolutionary Biology* 2002, **15**:173–190.

4. Lopes PC, Sucena E, Santos ME, Magalhães S: **Rapid experimental evolution of pesticide resistance in *C. elegans* entails no costs and affects the mating system.** *PloS ONE* 2008, **3**:e3741.

5. Magalhães S, Blanchet E, Egas M, Olivieri I: **Are adaptation costs necessary to build up a local adaptation pattern?** *BMC Evolutionary Biology* 2009, **9**:182.

6. Zhou D, Udpa N, Gersten M, Visk DW, Bashir A, Xue J, Frazer K a, Posakony JW, Subramaniam S, Bafna V, Haddad GG: **Experimental selection of hypoxia-tolerant *Drosophila melanogaster*.** *Proceedings of the National Academy of Sciences of the United States of America* 2011, **108**:2349–2354.

7. Burke MK, Dunham JP, Shahrestani P, Thornton KR, Rose MR, Long AD: **Genome-wide analysis of a long-term evolution experiment with *Drosophila*.** *Nature* 2010, **467**:587–590.

8. Turner TL, Stewart AD, Fields AT, Rice WR, Tarone AM: **Population-based resequencing of experimentally evolved populations reveals the genetic basis of body size variation in *Drosophila melanogaster*.** *PLoS Genetics* 2011, **7**:e1001336.

9. Orozco-terWengel P, Kapun M, Nolte V, Kofler R, Flatt T, Schlötterer C: **Adaptation of *Drosophila* to a novel laboratory environment reveals temporally heterogeneous trajectories of selected alleles.** *Molecular Ecology* 2012, **21**:4931–4941.

10. Turner TL, Miller PM: **Investigating natural variation in *Drosophila* courtship song by the evolve and resequence approach.** *Genetics* 2012, **191**:633–642.

11. Ye YH, Chenoweth SF, McGraw EA: **Effective but costly, evolved mechanisms of defense against a virulent opportunistic pathogen in *Drosophila melanogaster*.** *PLoS Pathogens* 2009, **5**:e1000385.

12. Kraaijeveld AR, Godfray HC: **Trade-off between parasitoid resistance and larval competitive ability in *Drosophila melanogaster*.** *Nature* 1997, **389**:278–280.

13. Vijendravarma RK, Kraaijeveld AR, Godfray HCJ: **Experimental evolution shows *Drosophila melanogaster* resistance to a microsporidian pathogen has fitness costs.** *Evolution* 2009, **63**:104–114.

14. Antonovics J, Boots M, Ebert D, Koskella B, Poss M, Sadd BM: **The origin of specificity by means of natural selection: evolved and nonhost resistance in host-pathogen interactions.** *Evolution* 2013, **67**:1–9.

15. Martins NE, Faria VG, Teixeira L, Magalhães S, Sucena É: **Host adaptation is contingent upon the infection route taken by pathogens.** *PLoS Pathogens* 2013, **9**:e1003601.

16. Zbinden M, Haag CR, Ebert D: **Experimental evolution of field populations of *Daphnia magna* in response to parasite treatment.** *Journal of Evolutionary Biology* 2008, **21**:1068–1078.

17. Bennett AF, Lenski RE: **An experimental test of evolutionary trade-offs during temperature adaptation.** *Proceedings of the National Academy of Sciences of the United States of America* 2007, **104**:8649–8654.

18. Kubinak JL, Ruff JS, Hyzer CW, Slev PR, Potts WK: **Experimental viral evolution to specific host MHC genotypes reveals fitness and virulence trade-offs in alternative MHC types.** *Proceedings of the National Academy of Sciences of the United States of America* 2012, **109**:3422–3427.

19. Vogwill T, Lagator M, Colegrave N, Neve P: **The experimental evolution of herbicide resistance in *Chlamydomonas reinhardtii* results in a positive correlation between fitness in the presence and absence of herbicides.** *Journal of Evolutionary Biology* 2012, **25**:1955–1964.

20. Magwire MM, Bayer F, Webster CL, Cao C, Jiggins FM: **Successive increases in the resistance of *Drosophila* to viral infection through a transposon insertion followed by a duplication.** *PLoS Genetics* 2011, **7**:e1002337.

1. Magwire MM, Fabian DK, Schweyen H, Cao C, Longdon B, Bayer F, Jiggins FM: **Genome-wide association studies reveal a simple genetic basis of resistance to naturally coevolving viruses in *Drosophila melanogaster*.** *PLoS Genetics* 2012, **8**:e1003057.

21. Bangham J, Knott SA, Kim K-W, Young RS, Jiggins FM: **Genetic variation affecting host-parasite interactions: major-effect quantitative trait loci affect the transmission of sigma virus in *Drosophila melanogaster*.** *Molecular Ecology* 2008, **17**:3800–3807.

22. Contamine D, Petitjean AM, Ashburner M: **Genetic resistance to viral infection: the molecular cloning of a *Drosophila* gene that restricts infection by the rhabdovirus sigma.** *Genetics* 1989, **123**:525–533.

23. Bangham J, Kim K-W, Webster CL, Jiggins FM: **Genetic variation affecting host-parasite interactions: different genes affect different aspects of sigma virus replication and transmission in *Drosophila melanogaster*.** *Genetics* 2008, **178**:2191–2199.

24. Carpenter J a, Hadfield JD, Bangham J, Jiggins FM: **Specific interactions between host and parasite genotypes do not act as a constraint on the evolution of antiviral resistance in *Drosophila*.** *Evolution* 2012, **66**:1114–1125.

25. Xu J, Cherry S: **Viruses and antiviral immunity in *Drosophila*.** *Developmental and Comparative Immunology* 2014, **42**:67–84.

26. Teixeira L, Ferreira Á, Ashburner M: **The bacterial symbiont *Wolbachia* induces resistance to RNA viral infections in *Drosophila melanogaster*.** *PLoS Biology* 2008, **6**:e2.

27. Kofler R, Orozco-terWengel P, De Maio N, Pandey RV, Nolte V, Futschik A, Kosiol C, Schlötterer C: **PoPoolation: a toolbox for population genetic analysis of next generation sequencing data from pooled individuals.** *PloS ONE* 2011, **6**:e15925.

28. Burke MK: **How does adaptation sweep through the genome? Insights from long-term selection experiments**. *Proceedings Biological Sciences / The Royal Society* 2012, **279**:5029–5038.

29. Huang W, Richards S, Carbone MA, Zhu D, Anholt RRH, Ayroles JF, Duncan L, Jordan KW, Lawrence F, Magwire MM, Warner CB, Blankenburg K, Han Y, Javaid M, Jayaseelan J, Jhangiani SN, Muzny D, Ongeri F, Perales L, Wu Y-Q, Zhang Y, Zou X, Stone EA, Gibbs RA, Mackay TFC: **Epistasis dominates the genetic architecture of *Drosophila* quantitative traits.** *Proceedings*

of the National Academy of Sciences of the United States of America 2012, **109**:15553–15559.

30. Turner TL, Miller PM, Cochrane VA: **Combining genome-wide methods to investigate the genetic complexity of courtship song variation in *Drosophila melanogaster***. *Molecular Biology and Evolution* 2013, **30**:2113–2120.

31. Kofler R, Schlötterer C: **A guide for the design of evolve and resequencing studies.** *Molecular Biology and Evolution* 2013, **31**:474–483.

32. Remolina SC, Chang PL, Leips J, Nuzhdin S V, Hughes KA: **Genomic basis of aging and life-history evolution in *Drosophila melanogaster*.** *Evolution* 2012, **66**:3390–3403.

33. Tobler R, Franssen SU, Kofler R, Orozco-Terwengl P, Nolte V, Hermisson J, Schlötterer C: **Massive habitat-specific genomic response in *D. melanogaster* populations during experimental evolution in hot and cold environments.** *Molecular Biology and Evolution* 2013, **31**:364–375.

34. Gwynn DM, Callaghan A, Gorham J, Walters KF a, Fellowes MDE: **Resistance is costly: trade-offs between immunity, fecundity and survival in the pea aphid.** *Proceedings Biological Sciences / The Royal Society* 2005, **272**:1803–1808.

35. Lazzaro BP, Sackton TB, Clark AG: **Genetic variation in *Drosophila melanogaster* resistance to infection: a comparison across bacteria.** *Genetics* 2006, **174**:1539–1554.

36. Kraaijeveld AR, Layen SJ, Futerman PH, Godfray HCJ: **Lack of phenotypic and evolutionary cross-resistance against parasitoids and pathogens in *Drosophila melanogaster*.** *PLoS ONE* 2012, **7**:e53002.

37. Ostrowski EA, Rozen DE, Lenski RE: **Pleiotropic effects of beneficial mutations in *Escherichia coli*.** *Evolution* 2005, **59**:2343–2352.

38. Luong LT, Polak M: **Costs of resistance in the *Drosophila*-macrocheles system: a negative genetic correlation between ectoparasite resistance and reproduction.** *Evolution* 2007, **61**:1391–1402.

39. Magalhães S, Fayard J, Janssen A, Carbonell D, Olivieri I: **Adaptation in a spider mite population after long-term evolution on a single host plant.** *Journal of Evolutionary Biology* 2007, **20**:2016–2027.

40. Czesak ME, Fox CW, Wolf JB: **Experimental evolution of phenotypic plasticity: how predictive are cross-environment genetic correlations?** *The American Naturalist* 2006, **168**:323–335.

41. Thompson JN: **The geographic mosaic of coevolution**. Chicago: University Of Chicago Press; 2005.

42. Luijckx P, Ben-Ami F, Mouton L, Du Pasquier L, Ebert D: **Cloning of the unculturable parasite *Pasteuria ramosa* and its *Daphnia* host reveals extreme genotype-genotype interactions.** *Ecology Letters* 2011, **14**:125–131.

43. Luijckx P, Fienberg H, Duneau D, Ebert D: **A matching-allele model explains host resistance to parasites.** *Current Biology* 2013, **23**:1085–1088.

44. Clarke B: **The ecological genetics of host-parasite relationships**. In *Genetic aspects of host-parasite relationships*. Edited by Taylor AER, Muller R. Oxford: Blackwell; 1976:87–103.

45. Thrall PH, Laine A-L, Ravensdale M, Nemri A, Dodds PN, Barrett LG, Burdon JJ: **Rapid genetic change underpins antagonistic coevolution in a natural host-pathogen metapopulation.** *Ecology Letters* 2012, **15**:425–435.

46. Futschik A, Schlötterer C: **The next generation of molecular markers from massively parallel sequencing of pooled DNA samples.** *Genetics* 2010, **186**:207–218.

47. Kofler R, Pandey RV, Schlötterer C: **PoPoolation2: identifying differentiation between populations using sequencing of pooled DNA samples (Pool-Seq).** *Bioinformatics* 2011, **27**:3435–3436.

48. Dietzl G, Chen D, Schnorrer F, Su K-C, Barinova Y, Fellner M, Gasser B, Kinsey K, Oppel S, Scheiblauer S, Couto A, Marra V, Keleman K, Dickson BJ: **A genome-wide transgenic RNAi library for conditional gene inactivation in *Drosophila*.** *Nature* 2007, **448**:151–156.

2.6. Evolution of *Drosophila* resistance against different pathogens and infection routes entails no maintenance costs

Vítor G. Faria[1*], Nelson E. Martins[1*], Tânia F. Paulo[1], Luís Teixeira[1], Élio Sucena[1,2¶#], Sara Magalhães[3¶#]

1 - Instituto Gulbenkian de Ciência, Apartado 14, 2781-901 Oeiras, Portugal

2 - Departamento de Biologia Animal, Faculdade de Ciências, Universidade de Lisboa, edifício C2, Campo Grande, 1749-016 Lisboa, Portugal

3 - cE3c: Centre for Ecology, Evolution and Environmental Changes, Faculdade de Ciências, Universidade de Lisboa, edifício C2, Campo Grande, 1749-016 Lisboa, Portugal

*** - These authors contributed equally to this work**

¶ - Joint last authors

- Email: esucena@igc.gulbenkian.pt, snmagalhaes@fc.ul.pt

Keywords: Trade-offs, Costs, *Drosophila*, immunity, host-parasite, experimental evolution

This article was published in the journal **Evolution** (vol. 69, Issue 11, 2799-2809). November 2015.
DOI - 10.1111/evo.12782.
PubMed ID - 26496003.

This article is available online through the Evolution webpage (http://onlinelibrary.wiley.com/doi/10.1111/evo.12782/abstract).

This article contains supporting information online at http://onlinelibrary.wiley.com/doi/10.1111/evo.12782/suppinfo.

Author contributions: V.G.F., N.E.M., L.T., É.S., and S.M. designed research; N.E.M., V.G.F., and T.P. performed research; É.S. contributed new reagents/analytic tools; N.E.M., V.G.F., C.S., L.T., É.S., and S.M. analyzed data; and V.G.F, N.E.M., É.S., and S.M. wrote the paper.
The authors declare no conflict of interest.

Funding: Fundação para a Ciência e a Tecnologia (FCT, Portugal) supports V.G.F (#SFRH/BD/82299/2011) and N.E.M (#SFRH/BPD/62964/2009). This work was supported by Instituto Gulbenkian de Ciência/Fundação Calouste Gulbenkian. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Data archiving: The doi for our data is DRYAD: doi:10.5061/dryad.2533p.

Abstract

Pathogens exert a strong selective pressure on hosts, entailing host adaptation to infection. This adaptation often affects negatively other fitness-related traits. Such trade-offs may underlie the maintenance of genetic diversity for pathogen resistance. Trade-offs can be tested with experimental evolution of host populations adapting to parasites, using two approaches: (a) measuring changes in immunocompetence in relaxed-selection lines and (b) comparing life-history traits of evolved and control lines in pathogen-free environments. Here, we used both approaches to examine trade-offs in *D. melanogaster* populations evolving for over 30 generations under infection with *Drosophila* C Virus or the bacterium *Pseudomonas entomophila*, the latter through different routes. We find that resistance is maintained after up to 30 generations of relaxed selection. Moreover, no differences in several classical life-history traits between control and evolved populations were found in pathogen-free environments, even under stresses such as desiccation, nutrient limitation and high densities. Hence, we did not detect any maintenance costs associated with evolved resistance to pathogens. We hypothesize that extremely high selection pressures commonly used lead to the disproportionate expression of costs relative to their actual occurrence in natural systems. Still, the maintenance of genetic variation for pathogen resistance calls for an explanation.

Introduction

Several studies have shown that resistance to pathogens evolves rapidly in host populations [1–5]. This indicates that standing genetic variation (SGV) for host resistance to parasites is maintained in most systems. However, parasites are ubiquitous and they pose a strong fitness cost upon hosts. Hence, high resistance should be fixed in host populations. In other words, the seemingly paradoxical occurrence of SGV for traits involved in fighting pathogenic infections calls for an explanation. Such maintenance is often attributed to the occurrence of a trade-off between resistance to pathogens and other fitness-related traits (for a review see [6]).

Experimental evolution allows for robust tests of the occurrence of evolutionary-relevant genetic trade-offs. Indeed, with this methodology, the ancestral state is known, hence comparisons between control and evolved lines allows identifying traits modified by a specific selection pressure as well as correlated responses to selection. Moreover, the method avoids spurious correlations due to individuals (or their parents) having been in different conditions, or subject to different recent evolutionary histories [7, 8].

Trade-offs between immunity and fitness-related traits in experimentally-evolving lines are tested using two main approaches. The first consists in creating lines of relaxed selection [9–12]. These lines derive from populations evolving in the presence of the pathogen and are then placed for several generations in pathogen-free conditions. The occurrence of a trade-off is inferred if individuals from these lines show a lower performance when exposed to pathogens, as compared to the pathogen-resistant ancestral population they were derived from. In short, a costly defense is expected to be rapidly lost in the absence of the pathogen it targets. This logic is appealing but may not be universal. Indeed, reverting

to the ancestral state may be prevented by the loss of genetic variation allowing for such a reversion, although this possibility is seldom tested (but see [13]. Alternatively, resistance may be costly but evolution in a pathogen-free environment selects for mutations that compensate such cost. This is widely shown in antibiotic-resistant bacteria (reviewed in [14]) but has never been tested in multicellular sexual species, possibly because it relies upon the appearance of novel mutations, which require large populations and a high number of generations.

Another possible approach to test such costs is by measuring the performance of individuals from lines selected for pathogen resistance when placed in a pathogen-free environment [1–4, 12, 15–19]. Under such an approach, several life-history traits, thought to correlate with fitness, can be measured. Moreover, these tests can be done in several environments.

Irrespective of the method used, all studies addressing the consequences of the evolution of pathogen resistance have found a cost for this trait, with two exceptions. First, using both methods described above, adaptation of the cabbage looper to a virus was found to be free of cost [20]. Second, Meyer and colleagues [10] found no cost in *E. coli* resistance to phage T6 (but a cost in resistance to other phages). Therefore, such costs seem to be the rule, with few exceptions. This ubiquity of costs to immunity lends support to the hypothesis that such costs underlie the maintenance of SGV for host resistance [21].

Experimental evolution using *Drosophila* as a model host has repeatedly shown that the evolution of resistance to pathogens is costly [2, 11, 16, 18, 22]. In our previous work, we have performed experimental evolution of an outbred population of *Drosophila melanogaster* adapting to infection with different pathogens, *Drosophila* C virus (DCV) or the gram-negative bacterium *P. entomophila*, the latter being administrated via either an oral or a systemic route [5, 23].

We found that these populations increased resistance against these challenges within few generations, thereby demonstrating the presence of ample SGV for this trait. Here, we took advantage of this resource to test whether *Drosophila* resistance to such immune challenges entailed a cost. We did this using the two approaches mentioned above: 1) we created relaxed-selection lines, i.e., lines in which selection for pathogen resistance was relaxed, and tested for its maintenance over several generations; and 2) we compared the values of several life-history traits in control and evolved lines in several pathogen-free environments, including the ancestral environment.

Results

Maintenance of resistance under relaxed selection

For all pathogen challenges, significant differences in survival were found among Control, Selection, and Relaxed-Selection lines (Figure 2.6.1B and Table S1). This effect was mainly caused by the difference between Control and either Selection or Relaxed-Selection lines (Figure 2.6.1B). To get a more detailed description of mortality dynamics upon infection of the different selection lines, we also measured survival over 10 days after infection in flies from the last generation of selection (Figure 2.6.1C and Table S5).

Differences between both Selection and Relaxed-Selection lines to Controls were always significant in the BactSys, BactOral and VirSys lines (Figure 2.6.1B and 2.6.1C), either globally ($|z| > 23.5$, $P < 0.001$, $|z| > 29.3$, $P < 0.001$ and $|z| > 37.2$, $P < 0.001$, respectively), at each generation ($|z| > 7.31$, $P < 0.001$, $|z| > 5.7$, $P < 0.001$ and $|z| > 9.46$, $P < 0.001$, respectively, for all comparisons), or when comparing mortality dynamics in the last generation of selection ($|z| > 5.58$, $P < 0.001$, $|z| > 10.06$, $P < 0.001$ and $|z| > 6.30$, $P < 0.001$, respectively, for all comparisons). Excluding in the third

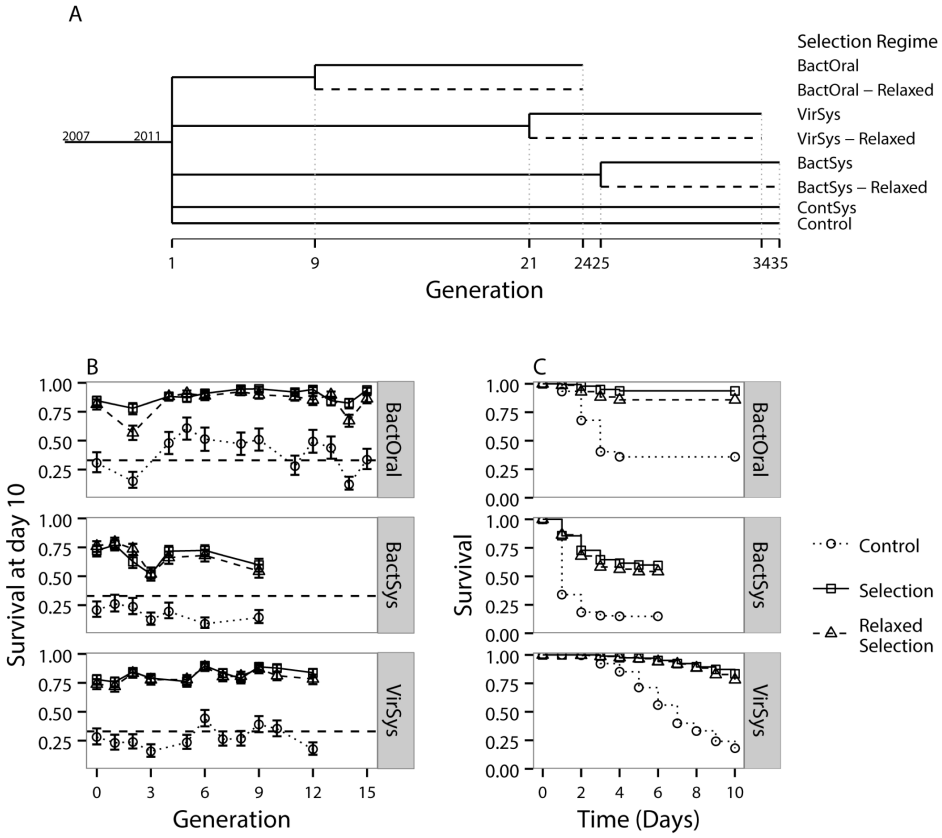


Figure 2.6.1 – Increased immunocompetence is maintained in relaxed-selection populations. (**A**) Diagram representing the different selection regimes used in this study. Lines represented by solid branches were challenged with a pathogen at every generation (Selection) or kept unchallenged (Control). From each Selection line, a line was derived and maintained in the ancestral environment (dashed lines, Relaxed-Selection). (**B**) Mean survival (± 95% CI) 10 days post-infection of individuals from Control (circles), Selection (squares) and Relaxed-Selection (triangles) lines, across 10 to 15 generations (see Materials & Methods). (**C**) Dynamics of survival after infection at the last generation of relaxed selection. Control lines die much faster than either of its counterparts, Selection or Relaxed-Selection lines, which display comparable profiles.

generation of relaxed selection, where the Relaxed-Selection lines showed significantly lower mortality the Selection lines (|z| = -2.87, P = 0.029), we

did not observe significant differences between these lines at different generations ($|z| < 1.38$, $P > 0.999$, for all comparisons), nor in the mortality dynamics in the last generation of Selection ($|z| = 0.83$, $P = 0.405$). In the VirSys *vs.* VirSys-Relaxed comparisons, no differences were found when comparing survival at each generation ($|z| < 2.49$, $P > 0.4$, for all comparisons), nor when comparing the mortality dynamics in the last generation ($|z| = 0.38$, p $P = 0.704$; Tables S2 and S6). We also did not find a significant difference in the linear slope of survival across generations between the different selection regimes (GLMM, Generation X Selection Regime effect, $\chi^2_2 < 3.79$, $P > 0.150$), despite a significant Generation effect (Generation effect, $\chi^2_1 > 18.67$, $P < 0.001$), indicating no differences between the regimes in the overall trend in survival across generations (Tables S3 and S4).

In contrast, there was a significant difference, between the BactOral lines and their matched-Relaxed Selection lines ($|z| = 5.8$, $P < 0.001$), in 4 generations across the experiment, including in the last generation of selection ($|z| = 3.63$, $P < 0.001$) (Table S2 and S4). This difference cannot be attributed to either an increased relative mortality in the Relaxed-Selection lines (comparison between Control and Relaxed-Selection lines remained constant across generations, $|z| < 1.74$, $P > 0.9$) or a decrease relative mortality in the Selection lines (comparison between Control and Selection lines remained constant, $|z| < 2.76$, $P > 0.53$).

To explore the reason for this difference, we tested changes in absolute survival across generations, separately for the Selection, Control and Relaxed Selection Lines. In this analysis, whereas in the Selection lines survival increased significantly ($|z| = 3.74$, $P < 0.001$), this trait did not change significantly in Relaxed Selection and Control lines over 11 generations ($|z| = 1.44$, $P = 0.450$ and $|z| = 1.29$, $P = 0.595$, respectively). In agreement with this finding, we also did not find a significant difference in

the linear slope of survival across generations among selection regimes (GLMM, Generation X Selection Regime effect, $\chi^2_2 = 2.91$, $P = 0.233$), again indicating no differences among regimes in changes in survival across generations (Tables S3 and S4). Therefore, we attribute the small but significant differences between Selection and Relaxed-Selection lines (less than 7% in the last generation of selection) to a marginal increase in survival in the former (approximately 9%), where selection was continued, while there was no increase (or decrease) in mortality in the latter.

At generations 60 and 70, at the moment we tested for larval competitive ability, relaxed-selection lines were still significantly more immunocompetent than control lines (lme, BactOral vs Control: z = 3.04 P = 0.0002, BactSys vs ContSys z = 8.28 $P < 0.0001$, VirSys vs ContSys z = 9.48 $P < 0.0001$).

Costs of resistance in parasite-free environments

We also tested for the occurrence of trade-offs by comparing several life-history traits between Selection and Control lines. We started by measuring the reproductive output (Figure 2.6.2A) and developmental time at generation 23 and 24 (Figure 2.6.2B) in these lines in the absence of infection. We found no effect of Selection Regime in the reproductive output ($\chi 2_4 = 0.640$, $P > 0.959$).

For developmental time (Figure 2.6.2B), and despite a statistically significant Selection Regime and Relaxed-Selection Regime by egg density interaction ($\chi^2_4 = 12.20$, $P = 0.016$, Table S7), no difference between any Selection line and their matched Controls was detected ($|t_{22}| < 2.21$, $P > 0.114$, Table S8).

Next we measured desiccation resistance and starvation resistance in Control vs Selection lines. These stressors that have putative ecological importance for *Drosophila* [25]. For both traits we failed to detect

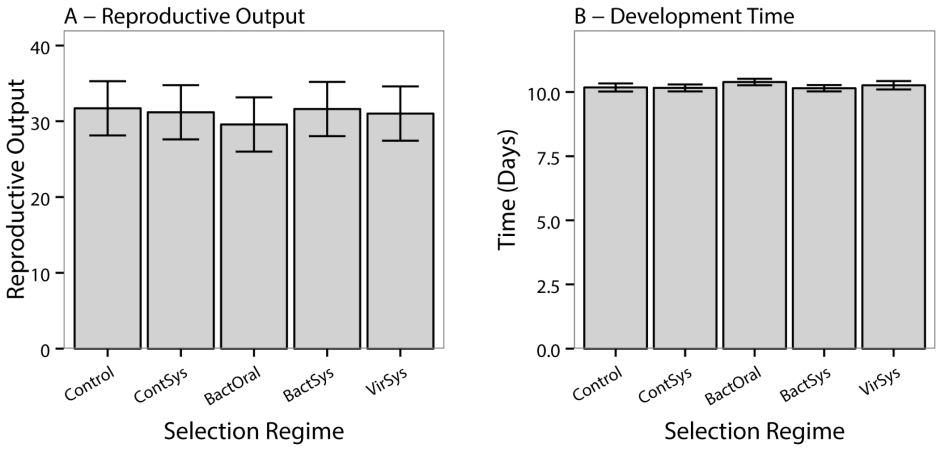


Figure 2.6.2 – Reproductive output and developmental time of individuals from Control and Selection lines in the absence of pathogens. (**A**) Mean (±95% CI) reproductive output 5-7 days after females reached adulthood, (**B**) Mean egg-to-adults developmental time from egg to adult.

statistically significant differences between selection regimes (Table S9, Selection regime effect, $\chi 2_4 < 5.21$, $P > 0.266$; $\chi 2_4 < 9.3$, $P > 0.053$ for both starvation and desiccation assays, considering either the mean time to death or the full mortality dynamics, respectively). This indicates an absence of a correlated response between adaptation to infection and both stress-related traits (Figure 2.6.3).

Moreover, because it has been often argued that costs are more easily revealed in nutrient limited environments [26], we measured egg-to-adult viability and developmental time under these conditions (Figure 2.6.4 A and B). Since these tests were done in lines that derived from the Selection lines in the end of the selection experiment, but maintained in control conditions (without selection) for > 30 generations, these lines represent a second set of Relaxed-Selection lines.

Although we detected increased mortality and developmental time in individuals raised on nutritionally-limited food relative to those raised on

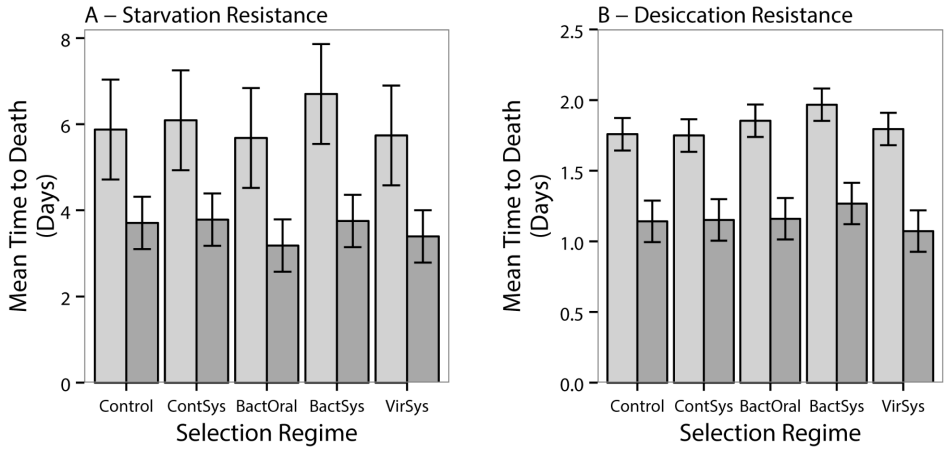


Figure 2.6.3 – Starvation and desiccation resistance of individuals from Control and Selection lines. Mean time to death (±95% CI) after (**A**) starvation or (**B**) desiccation of males (dark grey bars) and females (light grey bars).

---

standard food (Food type effect $\chi 2_2 > 141.3$, $P < 0.001$, for both traits), no differences were detected in either viability or development time among Selection regimes (Selection regime effect, $\chi 2_4 < 7.4$, $P > 0.11$, in both traits; Table S10). Since we observed a significant Regime by Food interaction in the viability assay ($\chi 2_4 < 12.99$, $P < 0.05$, Table S10), we tested for differences between Selection and their matched Control lines independently in the different food types. The absence of differences in viability among selection regimes was confirmed in both food types ($|z| < 1.94$. and $|z| < 2.14$ for comparisons in standard and nutritionally-limited food, respectively, $P > 0.194$, Table S11). Concerning differences in weight following larval development at high or low densities, the final model retained sex, density, selection regime, the interaction between sex and each of the other factors, and the triple interaction. Overall, adults were smaller at the highest density relative to the lowest, indicating an effect of competition on this trait (glm, effect of density: $F_{1,165} = 74.99$, $P < 0.0001$,

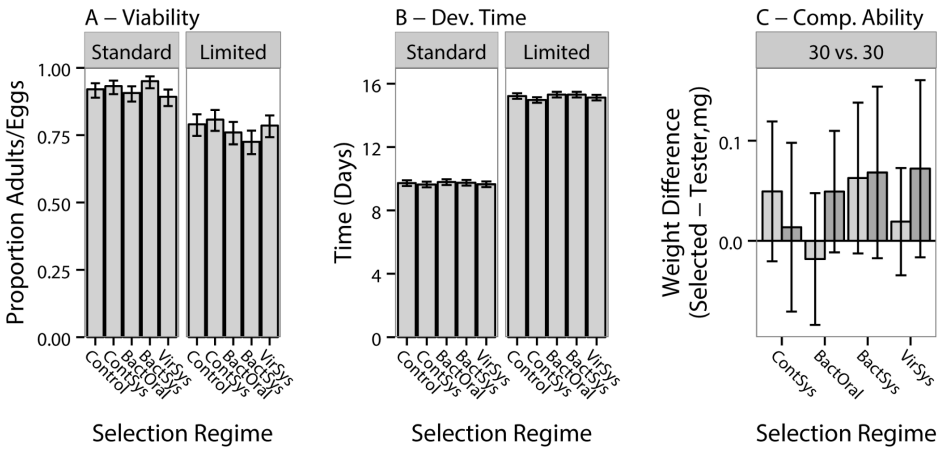


Figure 2.6.4 – Survival and developmental time of individuals from Control and Selection lines in nutrient-limiting conditions. Mean (±95% CI) (**A**) egg-to-adult viability and (**B**) development time of individuals developing in standard (left subpanel) and nutrient-limited (right subpanel) medium. (**C**) Mean (±95% CI) weight difference between individuals from the experimental lines and Tester mutants (outbred [w1118]), at high larval competition conditions (30:30 larvae in 0.5mL of food); light grey bars: females; dark grey bars: males.

Figure 2.6.4C). We then compared the weight of flies from each selection regime to that of tester flies from the same assay, at the highest density. No differences were found between tester flies and flies from ContSys, BactOral or VirSys regimes ($F_{1,24}$ = 1.996, P = 0.158; $F_{1,52}$ = 0.938 P = 0.333; $F_{1,38}$ = 2.311 P = 0.128, for ContSys, BactOral and Virsys, respectively, Figure 2.6.4C). In contrast, flies from the BactSys selection regime were on average bigger than tester flies ($F_{1,41}$ = 5. 916, P = 0.015, Figure 2.6.4C). Although the interaction between sex and selection regime was never significant ($F > 1.562$, $P > 0.211$), the factor sex was always significant ($F < 8.22$, $P < 0.004$), as males were on average lighter than females.

Discussion

In this study, we used a large-scale experimental evolution study addressing host adaptation to pathogen infection to test for the occurrence of trade-offs between immunity and other traits. We used two complementary methodologies (relaxation of selection and direct measurements of costs in selected lines), and tested 12 Selection lines, distributed over 3 different selection regimes, encompassing two distinct parasites (viruses agnd bacteria) and two infection routes (oral or systemic). Taken together our observations support the absence of maintenance costs in *Drosophila* populations evolved for higher immunocompetence against pathogens.

Using lines subject to relaxed selection allows testing the response as a whole. That is, had we observed a decrease in immunocompetence in individuals stemming from those lines, we would have concluded that a trade-off with some fitness-related trait existed. Nonetheless, we would not attribute this trade-off to a particular trait. The fact that none of the lines in this study has lost its immunocompetence suggests that these trade-offs with fitness-traits are absent in ancestral environment conditions. Still, this pattern could have also been explained by a loss of genetic variation in the selection lines, such that relaxed-selection lines would be stuck in a maladaptive peak [13]. However, two lines of evidence suggest that this is not the case: first, whole genome sequencing revealed that genetic variation in a subset of these lines was the same in Control and Selection lines, and that even loci under selection did not reach fixation [23]. Second, the performance of relaxed-selection lines in the ancestral, pathogen-free environment, showed no difference to Control for the fitness traits measured. Together, these results indicate that adaptation of our populations to pathogen infection entails no maintenance costs in conditions pertaining to the ancestral environment.

To further understand how our evolved populations respond in different pathogen-free environments, we performed direct tests for the occurrence of trade-offs between immunity and several life-history traits. The problem with this approach is that we may miss the trait in which the cost is expressed. However, we tested a comprehensive set of classical life-history traits, namely reproductive output, developmental time, starvation resistance, desiccation resistance and larval competitive ability, to maximize the possibility of detecting trade-offs. Moreover, we measured these traits in both males and females, thereby discarding the possibility of sexual antagonism for such costs [27]. This further reinforces the notion that, in the pathogen-free environment, evolution for increased survival upon infection by *P. entomophila* or DCV, has no observable costs.

Given that the large majority of studies using experimental evolution detected trade-offs between immunity and life-history traits (reviewed in [12], the absence of such a trade-off calls for an explanation. First, although we can state that maintenance costs were not present and that we did not find trade-offs related to the tested traits, some costs in other traits or environments might exist. Indeed, we did find a (relatively minor) cost of BactSys lines in presence of viruses: they performed worse than control lines [5]. The reverse, however, was not found: no costs were detected of VirSys lines in presence of other pathogens when testing the performance of these lines in presence of other pathogens [23]. Moreover, apart from survival [5, 23] and reproduction after infection (Figure S1), we did not test for the occurrence of deployment costs, or of costs in many other environments. Second, a cost may have occurred at a transient state then be compensated for during evolution. Although we know much about compensatory evolution in bacteria, we know little about its occurrence and dynamics in sexual organisms, with some remarkable exceptions in extensively-studied systems (e.g., [28]. However, compensatory evolution is

not likely in the system used here because the performance of relaxed-selection lines does not decrease and recovers across generations: it is always similar to that of evolved lines. This suggests that no transient cost was compensated for.

We hypothesize that the probability of finding a cost hinges on the selection pressure posed on the populations: a high selection pressure may sweep away most of the genetic variation that would allow for adaptation to the challenge posed, leaving only the most effective but most costly alleles. Indeed, the selection protocol we used was such that 33% of the population survived in the first generations (this percentage then increased due to adaptation). In the other studies of adaptation to pathogens, the selection pressure, when reported, was much higher, ranging from 90-95% mortality [2, 11, 22]. In contrast, in the single study that has also reported no cost in multicellular organisms, the selection procedure was such that 20-30% of the hosts (a cabbage looper) survived [29]. This reasoning may also explain why some studies failed to find a trade-off with immunity when selecting for other life-history traits [30–32]. In particular, the results reported in Sanders et al. (2005) are surprising, as the relaxed-selection process (i.e., selection for immunity and measuring consequences in life-history traits) did reveal a trade-off. The traits selected in these experiments (larval competitive ability, learning and reproductive investment, respectively) have a looser link to survival than resistance to pathogens. Hence, it may well be that the selection pressure that populations were exposed to in these studies was lower than that of studies selecting for increased immunocompetence, and this may account for the absence of a trade-off. Clearly, this hypothesis calls for a direct test. For example, one could set up selection lines evolving in presence of the same parasite but at different doses, and test whether trade-offs appeared in the treatments with higher selection pressures only. In any case, the lack of symmetry in the trade-off

between immunity and other life-history traits suggests that the trade-off is not a universal genetic characteristic of the organisms under study, but a conditional property, which may hinge upon the selection pressure posed.

Unfortunately, it is not possible to validate this hypothesis with studies that have used other approaches to test the occurrence of a cost of immunity. A cost was found in circa 50% of such studies (reviewed in [33]. However, either the evolutionary trajectories leading to host resistance are unknown or resistant clones have been generated via artificial selection, which may lead to spurious correlations among traits [34]. Hence, these data cannot be used to test whether the strength of selection underlies the probability of finding a cost (see also the discussion in [33] for other potential confounding factors in that data set).

Our hypothesis, however, is congruent with data concerning pesticide resistance. Indeed, in one of the best-documented examples of allele replacement in the wild, Labbé et al. [35] have shown that pesticide resistance in the mosquito *Culex pipiens* in Southern France first evolved via a highly-resistant but highly-costly allele. When mosquito populations were established in the treated area (hence selection for increased pesticide resistance was weaker), this allele was replaced by one conferring a lower cost. Similarly, Lopes et al. [36] found no cost for resistance to levamisole in experimentally-evolving *C. elegans* lines in which a dose killing initially 25% of individuals was used. This contrasts with most studies of natural populations, in which a cost for pesticide resistance was found [37].

Given the low prevalence of costs in this system, the question remains: what maintains genetic diversity for resistance to pathogens in our system? One possibility is that alleles conferring resistance have a large effect, such that susceptibilities differ widely in the population. This has been shown to allow for the maintenance of polymorphisms for resistance even when the cost is negligible [21]. In line with this, we have found that

the majority of the selection response for increased resistance to DCV could be attributed to alleles of 3 genes in our populations, all of which with a considerable effect upon host survival [23]. Moreover, we have shown that adaptation to all immune challenges occurred via resistance, rather than tolerance. Models predict that the maintenance of genetic variation for resistance is more likely than for tolerance mechanisms, although a cost is still necessary [38]. Another possibility is that the maintenance of genetic diversity in host populations in the field is due to coevolutionary dynamics. In that case, diversity for pathogen resistance may be maintained for a wider range of parameters than contemplated in models that consider host evolution alone [39, 40]. Coevolution in natural populations of *Drosophila* could have maintained the standing genetic variation present in our populations at the onset of experimental evolution.

Overall, this study suggests that the occurrence of maintenance costs for immunity traits is not a universal feature of organisms, raising questions as to (a) under which conditions such costs evolve and (b) what maintains genetic diversity for costless immunity traits.

Materials & Methods

Pathogen stocks and cultures

P. entomophila (a generous gift of B. Lemaitre) was grown in LB inoculated with a single bacterial colony, taken from glycerol stocks kept at -80 ºC and streaked in fresh Petri dishes. Bacteria were prepared from an overnight culture grown at 30 ºC, centrifuged and adjusted to the desired OD using fresh LB. Virus aliquots were grown and titrated as described elsewhere [24], kept at -80 ºC and thawed prior to infection.

Experimental evolution lines

From a highly outbred population of *D. melanogaster* [5], we derived

20 lines corresponding to 3 distinct immune challenges and 2 matched controls with 4 replicate lines each: a) oral infection with *P. entomophila* (BactOral), b) systemic infection by pricking flies with *P. entomophila* (BactSys), c) systemic infection by pricking flies with DCV (VirSys), d) one control under standard conditions (Control), and e) blank injected controls (ControlSys). At each generation, 600 flies were exposed to each challenge, and the survivors used to form the next generation. We selected an initial concentration of pathogens that killed approximately 66% of the fly population. At each generation, survival to infection was monitored by following the survival of 100-120 adults challenged with the same pathogen they were exposed to during selection every day until at least the 10th day post-infection. Flies were maintained under constant temperature (25 ºC), humidity (60–70%) and light-darkness cycle (12:12), and fed with standard cornmeal-agar medium. Detailed protocols for the selection experiment can be found in our previously published work ([5, 23]. We hereafter refer to lines continuously exposed to the parasites as 'Selection lines', to distinguish them from 'Relaxed-Selection lines', see below.

Relaxed-selection lines (and test to their immunocompetence)

We first established that a plateau of resistance was reached in each selection regime. This was estimated to occur whenever no difference in the response to pathogen infection was found in five consecutive generations, which took place at different periods for each selection regime. BactOral reached this plateau from generation 9 onwards, VirsSys from generation 21 onwards and BactSys from generation 25 onwards [5, 23]. We then derived Relaxed-Selection lines, one per each Selection line (i.e., 4 per Selection Regime, cf. Figure 2.6.1A). To do this, 600 indivivudals of each population of a given Selection Regime were placed in new population cages. Reproduction took place at the same days as the matching Selection

lines, and in the subsequent generations, the Relaxed-Selection population sizes (600 individuals) mirrored those of the Control lines. Survival of Relaxed-Selection following exposure to the parasites/route of infection matching to the corresponding Selection lines was monitored daily until at least the 10th day post-infection at each generation, in parallel with the Selection and Control lines.

Fitness costs in parasite-free environments

Fitness-related traits in parasite-free environments were compared between individuals from Selection and Control lines. To avoid possible artefacts due to maternal effects, flies used in these tests were the progeny of flies that spent at least one generation in a common environment without pathogens, *i.e*, in the standard environment of the base population. These assays were performed at generations 23 or 24 for reproductive output, development time and resistance to desiccation and starvation. Nutritional restriction and competition assays were done more than 30 generations after the end of the selection experiment (between generations 64 and 75 for all lines), hence evolved lines had been under a Relaxed-Selection regime for 30 generations. Therefore, a test for the maintenance of immunocompetence was performed on those lines at that moment, to ensure that differences between control and evolved lines were still present. This test was done as described in the last section.

Reproductive output

Reproductive output assays were designed to mimic the procedure followed during experimental evolution. Fifteen male-female pairs from each Selection and Control lines were transferred to fresh food vials 8-10 days post-eclosion and let to lay eggs for 48h. Reproductive output was

assayed as the number of adults emerging from pupae 12 days after oviposition.

Development time

To determine the mean fly development time, 10 replicate groups of 5 uninfected females (10-11 days old) were let to lay eggs for 1 hour in standard food vials. Egg never exceeded 52 per vial (mean density 17). The assay conditions mimic the experimental evolution procedure. The number of emerging adults was counted every 3 hours after the 9th day post-oviposition.

Resistance to starvation and desiccation

For the desiccation assay, 100 individuals (males and females) from each population were placed in groups of 10 in empty vials, and mortality was scored every 3 hours. For the starvation assay, 100 individuals (males and females) from each population were placed in groups of 10 in empty vials, with water supplied *ad libitum* by moisturizing the vial plugs.

Nutritional restriction

For each assay, 200 eggs from each population were placed in 10 groups of 20 eggs, both in standard food vials and nutritionally-restricted food (standard food diluted 1:8 with water maintaining the agar concentration). Viability in both conditions was estimated as the number of adults emerging from pupae. To determine the mean fly development time, the number of emerging adults was counted every 12 hours after the 9th and 14th day post-oviposition for standard and restricted food, respectively.

Larval competitive ability

Finally, we tested whether populations that had evolved increased immunocompetence against each pathogen had lower larval competitive ability compared to control lines. To this aim, we competed first instar larvae of the evolved populations (and their controls) against the same outbred control population carrying an introgressed white mutation. Pharates were weighted and classified as males or females, red eyes or white eyes.

Statistical analyses

Relaxed selection

To compare survival across generations in the different Selection and Relaxed-Selection lines, the proportion of individuals surviving at day 10 after infection in each vial was first estimated using the Kaplan-Meier method. Subsequently, a generalized linear mixed model (GLMM) was fitted to the data, assuming a binomial distribution and an underlying logit link function. The proportion of survivors, weighted by the number of individuals in each vial as dependent variable was fitted in a model with sex, generation and regime (Control, Selection or Relaxed-Selection) as fixed factors. Line nested within Selection Regime and sex at each generation was considered a random factor.

Subsequently, we tested for differences in survival between lines, both overall and across generations. When differences in survival between Selected and Relaxed selection lines were found, we then tested for changes in the mean difference between Control and Selection or Relaxed-Selection lines, between the first and subsequent generations after the derivation of the Relaxed-Selection lines. In addition, we also tested if there was a linear trend for change (increase or decrease) across generations in the mean survival of the different lines, by considering Generation an ordered factor.

Moreover, we tested for differences in the slope of the mean survival across generations, by fitting a logistic regression mixed model with generation as a continuous variable, assuming a binomial distribution and an underlying logit link function. The proportion of survivors, weighted by the number of individuals in each vial as dependent variable was fitted to a model with sex and regime (Control, Selection or Relaxed-Selection) as fixed factors and generation of relaxed selection as a continuous covariate.

To compare survival among Control, Selection, and Relaxed-Selection lines in the last generation of selection, we used a Cox's proportional hazards mixed effect model for each treatment, with survival time of individual flies as the dependent variable, Selection Regime and sex as fixed factors and replicate vial nested within line as a random factor.

In the tests for maintenance of immunocompetence, done at generations 60-75 we used a GLMM identical to that used for the relaxed selection analysis, comparing survival after infection between Control and Relaxed Selection lines.

Life-history traits in parasite-free environments

To compare reproductive output in the Control and Selection lines in the absence of infection, we used a linear mixed model (LMM), with the number of hatching eggs within 48h by a single female as dependent variable, Selection Regime and Generation as fixed factors and Replicate vial nested within line and generation as a random factor.

To compare development time among lines, we fitted a LMM with days to eclosion of individual flies as dependent variable, Selection Regime as fixed factor and replicate vial nested within line as a random factor.

To compare survival under starvation and desiccation conditions, we used a Cox's proportional hazards mixed effect model for each treatment (starvation or desiccation), with survival time of individual flies as the

dependent variable, Selection Regime and sex as fixed factors and replicate vial nested within line as a random variable. We also compared differences in the mean time to death (TTD) between selection regimes. For this, TTD was calculated for each vial, using the Kaplan-Meier method, and was fitted as a dependent variable in a GLMM with sex and Selection Regime as fixed factors and line nested within each Selection Regime and sex as random factor.

To compare viability in nutrient limiting conditions, we used a GLMM with the number of eclosing vs non-eclosing individuals as a binomial variable, Selection Regime and food type (Regular vs. Nutrient limited) and their interaction as fixed factors, and test vials nested into line as random factors, with an underlying logit link function. Development time was compared as above, including food type as an additional fixed factor and removing egg density as covariate. Least-square estimates of viability and development time were then compared between Selection Regimes, independently for each food type.

To test for differences in larval competitive ability, the variable weight was log-transformed to comply with normality. To confirm that a higher density implied a cost in larval weight, we compared the weight in each density using a generalised mixed model with competition level (either 15 or 30 flies from each line), selection regime and sex, and their interactions, as fixed factors and replicate as random factor. Following a significant effect of the density (cf. results) we then performed the analysis at the highest density, to address potential costs in flies derived from the selection lines. To this aim, we compared the weight of individuals from each selection regime to that of tester individuals from the same assay using a glm with selection regime (either BactSys, BactOral; ContSys, VyrSys or Tester populations), sex and their interaction as factors.

All statistical analyses were done in R (version 3.1.2). Linear mixed models were fitted using the *lmer* function and generalized linear mixed models with the *glmer* function, both in the "lme4" package in R. The effects of the fixed factors and of the hierarchical interaction terms were compared using Type II Wald $\chi 2$ tests (*Anova* function in the "car" package). Contrasts of least-square means estimates and of regression coefficients were done on the most parsimonious model, i.e. in models including only significant ($P < 0.05$) factors and interactions, using the *lsmeans* and *lstrends* function in the "lsmeans" package. Survival data was compared using the *coxme* function in "coxme" package. Hierarchically nested models were compared using likelihood ratio tests. The sex-averaged hazard ratios were then compared, using the *glht* function in the "multcomp" package in R. The reported *p*-values for tests involving multiple comparisons were adjusted using a sequential Bonferroni correction.

Acknowledgements

The authors wish to thank Margarida Matos and Alison Duncan for critical reading of the manuscript and for suggestions, Julien Marcetteau for help in the nutritional restriction assays, and the laboratories of Élio Sucena, Patrícia Beldade and Christen Mirth for inspiring discussions on the project.

References

1. Boots M, Began M: **Trade-offs with resistance to a granulosis virus in the Indian meal moth, examined by a laboratory evolution experiment**. *Functional Ecology* 1993, **7**:528–534.

2. Kraaijeveld AR, Godfray HC: **Trade-off between parasitoid resistance and larval competitive ability in *Drosophila melanogaster***. *Nature* 1997, **389**:278–280.

3. Lohse K, Gutierrez A, Kaltz O: **Experimental evolution of resistance in *Paramecium caudatum* against the bacterial parasite *Holospora undulata***. *Evolution* 2006, **60**:1177–1186.

4. Zbinden M, Haag CR, Ebert D: **Experimental evolution of field populations of *Daphnia magna* in response to parasite treatment**. *Journal of Evolutionary Biology* 2008, **21**:1068–1078.

5. Martins NE, Faria VG, Teixeira L, Magalhães S, Sucena É: **Host adaptation is contingent upon the infection route taken by pathogens**. *PLoS Pathogens* 2013, **9**:e100.

6. McKean KA, Lazzaro BP: **The costs of immunity and the evolution of immunological defense mechanisms**. In *Molecular mechanisms of life history evolution*. Edited by Heyland A, Flatt T. Oxford: Oxford University Press; 2011:299–310.

7. Kawecki TJ, Lenski RE, Ebert D, Hollis B, Olivieri I, Whitlock MC: **Experimental evolution**. *Trends in Ecology and Evolution* 2012, **27**:547–560.

8. Magalhães S, Matos M: **Strengths and weaknesses of experimental evolution**. *Trends in Ecology and Evolution* 2012, **27**:649–650.

9. Lenski RE: **Experimental studies of pleiotropy and epistasis in *Escherichia coli*. I. variation in competitive fitness among mutants resistant to virus T4**. *Evolution* 1988, **42**:425–432.

10. Meyer JR, Agrawal AA, Quick RT, Dobias DT, Schneider D, Lenski RE: **Parallel changes in host resistance to viral infection during 45,000 generations of relaxed selection**. *Evolution* 2010, **64**:3024–3034.

11. Ye YH, Chenoweth SF, McGraw EA: **Effective but costly, evolved mechanisms of defense against a virulent opportunistic pathogen in *Drosophila melanogaster***. *PLoS Pathogens* 2009, **5:**e1000184.

12. Duncan AB, Fellous S, Kaltz O: **Reverse evolution: selection against costly resistance in disease-free microcosm populations of *Paramecium caudatum***. *Evolution* 2011, **65**:3462–3474.

13. Teotónio H, Rose MR: **Variation in the reversibility of evolution.** *Nature* 2000, **408**:463–466.

14. MacLean RC, Hall AR, Perron GG, Buckling A: **The population genetics of antibiotic resistance: integrating molecular mechanisms and treatment contexts.** *Nature Reviews Genetics* 2010, **11**:405–414.

15. Schwarzenbach GA, Ward PI: **Responses to selection on phenoloxidase activity in yellow dung flies.** *Evolution* 2006, **60**:1612–1621.

16. Luong LT, Polak M: **Costs of resistance in the *Drosophila-Macrocheles* system: a negative genetic correlation between ectoparasite resistance and reproduction**. *Evolution* 2007, **61**:1391–1402.

17. Cotter SC, Myatt JP, Benskin CMH, Wilson K: **Selection for cuticular melanism reveals immune function and life-history trade-offs in *Spodoptera littoralis***. *Journal of Evolutionary Biology* 2008, **21**:1744–1754.

18. Vijendravarma RK, Kraaijeveld AR, Godfray HCJ: **Experimental evolution shows *Drosophila melanogaster* resistance to a microsporidian pathogen has fitness costs**. *Evolution* 2009, **63**:104–114.

19. Koskella B, Lin DM, Buckling A, Thompson JN: **The costs of evolving resistance in heterogeneous parasite environments**. *Proceedings of the Royal Society B* 2012, **279**:1896–1903.

20. Milks ML, Myers JH, Leptich MK: **Costs and stability of cabbage looper resistance to a nucleopolyhedrovirus**. *Evolutionary Ecology* 2002, **16**:369–385.

21. Antonovics J, Thrall PH: **The cost of resistance and the maintenance of genetic polymorphism in host-pathogen systems**. *Proceedings of the Royal Society B* 1994, **257**:105–110.

22. Fellowes MD, Kraaijeveld AR, Godfray HC: **Trade-off associated with selection for increased ability to resist parasitoid attack in *Drosophila melanogaster*.** *Proceedings of the Royal Society B* 1998, **265**:1553–1558.

23. Martins NE, Faria VG, Nolte V, Schlötterer C, Teixeira L, Sucena É, Magalhães S: **Host adaptation to viruses relies on few genes with**

different cross-resistance properties. *Proceedings of the National Academy of Sciences of the United States of America* 2014, **111**:5938–5943.

24. Teixeira L, Ferreira Á, Ashburner M: **The bacterial symbiont *Wolbachia* induces resistance to RNA viral infections in *Drosophila melanogaster***. *PLoS Biology* 2008, **6:**e2.

25. David JR, Allemand R, Van Herrewege J, Cohet Y: **Ecophysiology: abiotic factors**. In *The genetics and biology of Drosophila*. Edited by Ashburner M, Carson HL, Thompson JN. London: Academic Press; 1983:105–170.

26. McKean KA, Yourth CP, Lazzaro BP, Clark AG: **The evolutionary costs of immunological maintenance and deployment.** *BMC Evolutionary Biology* 2008, **8**:76.

27. Vincent CM, Sharp NP: **Sexual antagonism for resistance and tolerance to infection in *Drosophila melanogaster*.** *Proceedings of the Royal Society B* 2014, **281**:20140987.

28. Labbé P, Berticat C, Berthomieu A, Unal S, Bernard C, Weill M, Lenormand T: **Forty years of erratic insecticide resistance evolution in the mosquito *Culex pipiens***. *PLoS Genetics* 2007, **3**:2190–2199.

29. Milks ML, Myers JH: **The development of larval resistance to a nucleopolyhedrovirus is not accompanied by an increased virulence in the virus**. *Evolutionary Ecology* 2000, **14**:645–664.

30. Sanders AE, Scarborough C, Layen SJ, Kraaijeveld AR, Godfray HCJ: **Evolutionary change in parasitoid resistance under crowded conditions in *Drosophila melanogaster*.** *Evolution* 2005, **59**:1292–1299.

31. Kolss M, Kraaijeveld AR, Mery F, Kawecki TJ: **No trade-off between learning ability and parasitoid resistance in *Drosophila melanogaster***. *Journal of Evolutionary Biology* 2006, **19**:1359–1363.

32. Hangartner S, Sbilordo SH, Michalczyk Ł, Gage MJG, Martin OY: **Are there genetic trade-offs between immune and reproductive investments in *Tribolium castaneum*?** *Infection, Genetics and Evolution* 2013, **19**:45–50.

33. Labbé P, Vale PF, Little TJ: **Successfully resisting a pathogen is rarely costly in *Daphnia magna*.** *BMC Evolutionary Biology* 2010, **10**:355.

34. Rose MR: **Genetic covariation in *Drosophila* life history: untangling the data**. *The American Naturalist* 1984, **123**:565–569.

35. Labbé P, Sidos N, Raymond M, Lenormand T: **Resistance gene replacement in the mosquito *Culex pipiens*: Fitness estimation from long-term cline series**. *Genetics* 2009, **182**:303–312.

36. Lopes PC, Sucena É, Santos ME, Magalhães S: **Rapid experimental evolution of pesticide resistance in *C. elegans* entails no costs and affects the mating system**. *PLoS ONE* 2008, **3:**e374.

37. Coustau C, Chevillon C, Ffrench-Constant R: **Resistance to xenobiotics and parasites: can we count the cost?** *Trends in Ecology and Evolution* 2000, **15**:378–383.

38. Roy BA, Kirchner JW: **Evolutionary dynamics of pathogen resistance and tolerance** . *Evolution* 2000, **54**:51–63.

39. Best A, White A, Kisdi E, Antonovics J, Brockhurst MA, Boots M: **The evolution of host-parasite range.** *The American Naturalist* 2010, **176**:63–71.

40. Sasaki A: **Host-parasite coevolution in a multilocus gene-for-gene system.** *Proceedings of the Royal Society B* 2000, **267**:2183–2188.

2.7. Concluding remarks

In this Chapter, we challenged *Drosophila melanogaster* populations with pathogenic infections in order to determine the phenotypic and genetic changes underlying the adaptation processes. We started by founding and replicating outbred populations of *D. melanogaster*, an established model organism in immunity, experimental evolution and genomic studies. We created and standardized a protocol, trying to maximize the biological resources and contribute to a higher parallelism between different foundations and, consequently, diverse studies. Then, we evolved those populations in the presence or absence of their natural pathogens, *Pseudomonas entomophila* and *Drosophila* C Virus (DCV).

First, we studied host evolutionary consequences of two ecologically-relevant infection routes taken by a horizontally-transmitted pathogen, using experimental evolution of *D. melanogaster* infected with *P. entomophila*. We showed that host evolution, in response to pathogen infection, is not only specific to the pathogen that hosts evolve with but also to the specific route taken by these pathogens to infect hosts, thereby unraveling a novel dimension in the specificity of host responses to pathogens.

Then, we took one step further; by combining experimental evolution and genomics, we tackled the genetic basis of host adaptation to *Pseudomonas.* Using whole-genome sequencing, we identified several regions of differentiation and tested several candidates using RNAi.

Simultaneously, we also showed the dynamics of adaptation and unraveled the genetic basis of host adaptation to another parasite, DCV. In this case, we identified two regions of differentiation, and further confirmed the role of three genes within these regions using RNAi. While one of these genes had been previously associated with DCV resistance, the anti-viral

role of the other two had not been described before. Additionally, another novel aspect of this work is that we tested how the genes responsible for conferring resistance to DCV affect the performance of flies exposed to other viruses. Here we show that cross-resistance towards two different viruses (CrPV and FHV) has evolved, but each response is elicited by a different subset of the differentiated genes. Therefore, the generalist strategy relies on genes with specific cross-resistance properties.

How evolution in a particular environment affects the individuals' performance in other environments is a long-standing issue in Ecology and Evolution. So far, no study had addressed which genomic changes are responsible for the correlated responses observed. To approach this, we also used the evolved populations to test whether adaptation for resistance to parasites in *Drosophila melanogaster* leads to trade-offs with other traits. We tested 12 Selection lines, distributed over the 3 different selection regimes previously mentioned (two distinct parasites, virus and bacteria via two infection routes, oral or systemic). We used two complementary methodologies: relaxation of selection and direct measurements of costs in selected lines, including fecundity and developmental time in a standard and nutritionally-restricted environment, starvation and desiccation resistance, and larval competitive ability. This thorough analysis comprehensively demonstrated the absence of maintenance costs in the ancestral environment and also the absence of trade-offs for the tested traits. We believe these results challenge the general belief of the ubiquity of trade-offs between immunity and other fitness-related traits.

This Chapter contains therefore several main findings, where we transversally dissect several aspects of immune response and adaptation against pathogens, contributing to a more comprehensive understanding of the physiology, ecology and evolution of host-pathogen interactions.

3

Host-endosymbiont evolution: selection and adaptation

3.1. Prologue

As described in Chapter 1, it is becoming increasingly clear that microbial symbionts of multicellular organisms play a crucial role in their biology. These microbiota can influence host development, physiology and behavior. Current research focuses primarily on the identity of the microbial partners associated with different hosts and which host traits they affect. Since many microbial symbionts are co-evolving with their hosts there is a recent debate regarding the level at which selection is acting on. Part of the discussion relates to the extent selection on host may shape the presence or absence of particular symbiotic microbes. However, two key elements have to be considered and lack direct testing: i) what are the selective forces shaping the symbiont microbial genetic diversity between (and within) individuals? ii) What is the importance of symbiont genetic diversity and evolution for the adaptive process of host populations?

In recent years, the consequences of some symbiotic relationships in increasing the ability of the host to resist against pathogens became evident. More importantly for the context of this Thesis, it has been previously demonstrated that the endosymbiont *Wolbachia* strongly protects *Drosophila melanogaster* against viruses and that *Wolbachia* genetic diversity impacts on the host resistance to viruses.

As referred in Subchapter 2.5, we have performed experimental evolution on a *Drosophila* population (100% *Wolbachia*-infected) by exposing them to viral infection and determined the dynamics and the host genetic basis for adaptation. This set-up provides an ideal stage to enquire on the putative roles played and consequences suffered by the endosymbiont. In this Chapter, we have looked as primary objective for the consequences on the *Wolbachia* population of *Drosophila* adaptation against viral infection. On the other hand, it was also our aim to answer how an

eventual phenomenon of *Wolbachia* selection can influence the previously described adaptation process of *Drosophila* hosts.

Considering that outbred populations used in this work are entirely *Wolbachia*-positive, later in this Chapter we focused on two other important questions: i) which are the consequences to the hosts' adaptation after the eventual disappearance of a protective-simbiont in the population? And ii) how does the genetic basis responding in this context compare to the one identified previosly, in the presence of *Wolbachia*?

To approach these questions, we removed *Wolbachia* from the viral-adapted population and the respective control, and maintained the new sister populations under the same selective pressure. With this methodology, we answered whether a previously adapted population has the ability to increase its protection against virus from the point where *Wolbachia* infection is no longer present and also pinpointed which genes and protective alleles are responsible for this (eventual) second round of adaptation.

In this Chapter, we intended to therefore address, in two different approaches, the real impact of this symbiotic relationship for both parties. The results here obtained allow a new understanding about the actual consequences to both partners and the role of their inter-dependence to adaptation process against pathogens.

3.2. *Drosophila* adaptation to viral infection through defensive symbiont evolution

Vitor G. Faria[1]*, Nelson E. Martins[1]*, Sara Magalhães[2], Tânia F. Paulo[1], Viola Nolte[3], Christian Schlötterer[3], Élio Sucena[1, 4¶#], Luis Teixeira[1¶#]

1 - Instituto Gulbenkian de Ciências, Apartado 14, 2780-901 Oeiras, Portugal.

2- cE3c: Centre for Ecology, Evolution and Environmental Changes, Faculdade de Ciências, Universidade de Lisboa, Campo Grande, 1749-016 Lisboa, Portugal.

3 - Institut für Populationsgenetik, Vetmeduni Vienna, 1210 Wien, Austria.

4 - Universidade de Lisboa, Faculdade de Ciências, Departamento de Biologia Animal, edifício C2, Campo Grande, 1749-016 Lisboa, Portugal.

*** - These authors contributed equally to this work**

¶ - Joint last authors

- Email: esucena@igc.gulbenkian.pt, lteixeira@igc.gulbenkian.pt

Keywords: Selection, defensive symbiosis, host-pathogen interactions, *Wolbachia*, virus

This article was published in the journal **PLoS Genetics** (vol. 12, issue. 9, e1006297). Peer-Reviewed. September 2016.
DOI – 10.1371/journal.pgen.1006297.
PubMed ID - 27684942.

This article contains supporting information online.

Author contributions: V.G.F., N.E.M., S.M, É.S., and L.T. designed research; V.G.F., N.E.M., T.F.P, and V.N. performed research; C.S., É.S. and L.T. contributed new reagents/analytic tools; V.G.F., N.E.M., É.S., and L.T. analyzed data; and V.G.F., N.E.M., É.S., and L.T. wrote the paper.
The authors declare no conflict of interest.

Funding: Fundação para a Ciência e a Tecnologia (FCT, Portugal) supports V.G.F (#SFRH/BD/82299/2011) and N.E.M (#SFRH/BPD/62964/2009). C.S. is funded by AustrianScience Fund (FWF) (P22725 and P19467) and European Research Council (ArchAdapt). This work was supported by Instituto Gulbenkian de Ciência/Fundação Calouste Gulbenkia, FCT (PTDC/SAU-IMU/120673/2010), and Vetmeduni funding. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Data archiving: files are available via the European Nucleotide Archive (http://www.ebi.ac.uk/ena/about/search_and_browse), as project PRJEB8815, with reads accession nos. ERS684186- ERS684197 and ERS764859 – ERS764870, respectively.

Abstract

Microbial symbionts can modulate host interactions with biotic and abiotic factors. Such interactions may affect the evolutionary trajectories of both host and symbiont. *Wolbachia* protects *Drosophila melanogaster* against several viral infections and the strength of the protection differs between variants of this endosymbiont. Since *Wolbachia* is maternally transmitted its fitness depends on the fitness of its host. Therefore, *Wolbachia* populations may be under selection when *Drosophila* is subject to viral infection. Here we show that in *D. melanogaster* populations selected for increased survival upon infection with *Drosophila* C virus there is a strong selection coefficient for specific *Wolbachia* variants, leading to their fixation. Flies carrying these selected *Wolbachia* variants have higher survival and fertility upon viral infection when compared to flies with the other variants. These findings demonstrate how the interaction of a host with pathogens shapes the genetic composition of symbiont populations. Furthermore, host adaptation can result from the evolution of its symbionts, with host and symbiont functioning as a single evolutionary unit.

Introduction

Animals and plants live in close association with numerous symbiotic bacteria that often cause strong phenotypic changes in their hosts [1]. For example, defensive symbionts can increase host resistance to pathogens and parasitoids [2-8]. In insects, several defensive symbionts are maternally transmitted [3-7], such that the fitness of the symbiotic bacteria is dependent on that of their female hosts. Therefore, one can expect that selection on host phenotypes, including resistance to (other) parasites, impacts the evolution of the bacterial symbiont population.

Host parasite burden can impact symbiont populations. For example, experimental evolution of the pea aphid *Acyrthosiphon pisum* or of *Drosophila hydei* in the presence of parasitoid wasps, caused an increase in the frequency of individuals carrying the protective symbionts *Hamiltonella defensa* and *Spiroplasma*, respectively [9-10]. Also, the recent spread of a *Spiroplasma* symbiont in natural populations of *D. neotestacea* in North America has been associated with the arrival of a parasitic nematode to this continent [7]. In agreement with this, the frequency of *Spiroplasma* in a *D. neotestacea* population increases in the presence of the parasitic nematode during experimental evolution [11]. These studies show changes in the prevalence of endosymbiont infection in host populations, but did not address selection at the levels of the genetic diversity of the symbiont itself. However, some evidence suggests that this could be the case: 1) some defensive symbiont populations display genetic and phenotypic variability [12-18]. 2) variants or strains of endosymbionts change in frequency in natural populations or during experimental evolution [19-21]. Nonetheless, a clear link between the selective pressure exerted on hosts and the genetic changes observed in the symbionts has been missing. In this study, we establish a relation between host adaptation to parasites and changes in the genetic composition of endosymbiont populations.

Wolbachia is a maternally-transmitted bacterial endosymbiont widespread in arthropods [22]. In some natural hosts it induces strong protection against infection with several RNA viruses [3,4,23,24]. Importantly, genetic variation in the *Wolbachia* strain of *Drosophila melanogaster* (*w*Mel), can be linked to the strength of antiviral protection [14-15]. Using experimental evolution, we have previously shown that *D. melanogaster* populations adapt to *Drosophila* C virus (DCV) challenge [25]. Resistance to this pathogen increases over twenty generations and we identified the genetic bases of this adaptation at the host level [25]. However, all individuals of the outbred founder population carried *Wolbachia* [26]. Therefore, we used this unique setup to ask if the genetic composition of the *Wolbachia w*Mel populations also changed during host adaptation to DCV challenge and whether this change could impact on *Drosophila* fitness.

Results

We performed experimental evolution on four replicate populations of *D. melanogaster* under selection with systemic DCV infection (Virus-Selected) and four replicates with mock infection (Control) [26]. DCV infection was performed at every generation using the same virus strain, at the same dose. As previously described [25], we performed genome-wide sequencing of DNA from pools of each population (Pool-seq) [27-28]. Using Pool-Seq on the Ancestral populations and on the Control and Virus-Selected populations after 20 generations [25], we determined the genetic diversity of *Wolbachia* in these populations. We found statistically significant changes in the frequency of 125 single nucleotide polymorphisms (SNPs) between the Ancestral and the Virus-Selected populations (Figure 3.2.1A, Figure S1, Figure S2, Dataset S1). Of these, 111 were also significantly different between Control and Virus-Selected

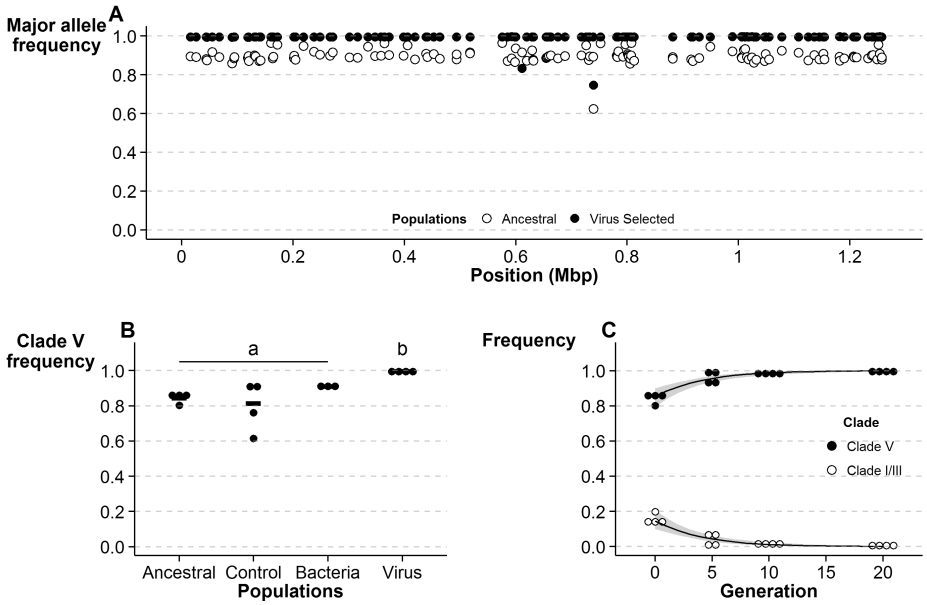


Figure 3.2.1 – Selection of *Wolbachia* Clade V variants after experimental evolution of *Drosophila melanogaster* with DCV. (**A**) Frequencies of the major allele of *Wolbachia* single nucleotide polymorphisms (SNPs) in Ancestral and Virus-Selected populations, determined by Pool-Seq. Shown are all SNPs with significantly different frequencies at generation 20 between Ancestral (open circles) and Virus-Selected populations (closed circles). (**B**) Frequencies of flies carrying Clade V *w*Mel variants in Ancestral, Control, Bacteria-Selected, and Virus-Selected populations (last three at generation 20). 96 individual flies from each population were tested for a clade V diagnostic SNP at position 805,011. Each data point represents the proportion of flies carrying clade V *w*Mel in a population. Letters (a,b) refer to statistically homogenous groups of mean Clade V frequencies, based on Tukey's pairwise comparisons between all populations ($p > 0.23$ within all group "a" populations, $p < 0.003$ for all comparisons with Virus-Selected populations). (**C**) Frequency of flies carrying Clade V (closed circles) or Clade I/III (open circles) variants in Ancestral (generation 0) and Virus-Selected populations at generations 5, 10 and 20. These frequencies were determined from 96 individuals from each replicate population, as in (B). Black solid line and gray shading represents the best fit for the logistic regression and 95% Confidence interval (CI), respectively.

populations, but not between Control and Ancestral populations, showing that these changes in the genetic composition of the *Wolbachia* populations are mostly specific to the response to viral infection.

Phylogenetic analysis, based on whole genome sequencing of *Wolbachia* and mitochondria, indicate that in the recent past *w*Mel has been strictly vertically transmitted [13,29,30]. Moreover, there is no evidence of fly lines simultaneously carrying *w*Mel variants from distant haplotypes or recombination between these [29]. Therefore we inferred *Wolbachia* haplotypes in the Ancestral, Control and Virus-Selected populations from the Pool-Seq data (S1 Text). Overall, we identified diagnostic SNPs (i.e. SNPs present in all variants, and only in variants, of a specific clade) for three of the major clades of *w*Mel (S1 Dataset) [13,14,21]. The Ancestral *Wolbachia* populations consisted of approximately 88% clade V variants and 12% of variants of clades I and III. In the Virus-Selected populations all these diagnostic SNPs became fixed with the nucleotide that matches clade V (S1 Dataset). In fact, in all the 123 SNPs that became fixed between Ancestral and Virus-Selected populations the fixed nucleotides match clade V. Moreover, between Ancestral and Control populations, the 8 SNPs that significantly changed have only been detected before in clades I and III variants, whereas Clade V specific SNPs did not significantly change in frequency between these populations (S1 Text, S1 Dataset). Therefore, we conclude that selection of *D. melanogaster* with a viral challenge changed the frequencies of *w*Mel variants in the host populations and led to fixation of clade V *w*Mel variants.

To confirm that the fixation of clade V variants was specific to the Virus-Selected populations, we analyzed individual flies from the Ancestral, Control and Virus-Selected populations as well as from a parallel selection regime in which *Drosophila* was challenged with systemic bacterial

infection [26] (Figure 3.2.1B). We determined the *w*Mel variant carried by 96 individual flies from each replicate population through restriction analysis of a PCR fragment containing a clade V diagnostic SNP. This analysis distinguishes flies carrying *w*Mel variants of clades I/III or clade V. The frequencies of flies carrying clade V *w*Mel variants in Ancestral, Control and Virus-Selected populations are in agreement with the Pool-Seq data (S1 Text) and clade V variants are only fixed in the Virus-Selected populations. We observed significant differences in frequencies between the Virus-selected populations and the other tested populations but not between any other regimes (GLMM, Selection Regime effect, $\chi^2_3 = 31.648$, $p < 0.001$, Tukey HSD, $|z| > 3.437$, $p < 0.005$ for all comparisons with the Virus-Selected populations, $|z| < 2.067$, $p > 0.23$ for all other comparisons). These data argue against drift being responsible for the fixation of clade V variants since Bacteria-Selected populations had fewer surviving individuals for a larger number of generations than Virus-Selected populations (Suppl. Text, S2 Dataset) and *w*Mel variants of clade V did not reach fixation in any of the Bacteria-Selected populations. Moreover, a time-course analysis of *w*Mel clade V frequencies in the Virus-Selected regime, based on individual genotyping, also shows that changes in frequencies were parallel in all four replicates (Figure 3.2.1C). Finally, based on the frequencies of clade I/III and clade V variants in generations 0, 5, 10 and 20 we estimated a strong selection coefficient against clade I/III variants of 0.263 (0.177-0.349) (estimated log-linear slope using a generalized linear mixed model (GLMM), Generation effect, $\chi^2_1 = 42.466$, $p < 0.001$) [31]. Therefore, fixation of clade V variants in all the Virus-Selected populations is unlikely due to drift, injury or a generic immune challenge, but the consequence of the specific adaptation to viral challenge.

To analyze the phenotype of clade V *w*Mel variants against clade III variants we established eleven different isofemale lines carrying *w*Mel from

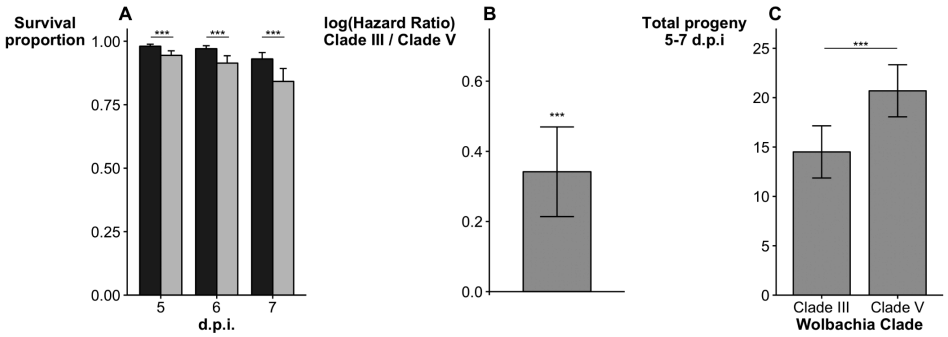


Figure 3.2.2 – *Wolbachia* Clade V variants confer higher protection to viral infection when compared with clade III variants. (**A**) Survival of flies carrying clade V and clade III *w*Mel variants five, six and seven days post infection with DCV (d.p.i). (**B**) Cox hazard ratio of flies carrying clade III *w*Mel variants compared with flies carrying clade V, calculated from survival data until 20 d.p.i. (**C**) Reproductive output of parents 5-7 d.p.i. In all assays the female progeny of eleven independent reciprocal crosses between isofemale flies, carrying Clade V and Clade III *w*Mel variants, were analyzed after systemic infection with DCV (2×10^7 $TCID_{50}$/mL). *** - $p < 0.001$. Means (± 95% confidence intervals) are shown in all panels.

---

clade V and eleven different isofemale lines carrying *w*Mel from clade III. These lines were established from the Control populations. To directly compare the differences between *w*Mel from the different clades we set up reciprocal crosses between eleven independent pairs of clade III and clade V isofemale lines. Since *w*Mel is only maternally transmitted, the female progeny of each of these paired crosses differed in the *w*Mel variant, but had the same host genotype. During the virus-selection protocol, reproduction of surviving adults took place five to seven days after DCV infection [25]. At five, six and seven days after DCV infection, flies carrying *w*Mel clade III variants had lower survival than flies with clade V variants (Figure 3.2.2A, GLMM, *w*Mel clade effect, $\chi^2_1 > 16.44$, $p < 0.001$ in all daily comparisons, see also analysis of Figure S3A, below). Analysis of the

survival data until 20 days post-infection confirms an overall lower susceptibility upon viral infection of flies with *w*Mel variants of clade V compared with flies carrying clade III variants (Figure 3.2.2B, mixed effect Cox model, *w*Mel clade effect, $\chi^2_1 = 25.817$, $p < 0.001$, see also analysis of Figure S3B, below).

We also analyzed the reproductive output of flies with the different *w*Mel variants (from the same reciprocal crosses), five to seven days post-infection with DCV (Figure 3.2.2C). Flies with clade III variants had fewer progeny than flies carrying clade V variants (linear mixed model (LMM), *w*Mel clade effect, $\chi^2_1 = 39.217$, $p < 0.001$). This difference between variants is contingent on viral infection, since their reproductive output is not significantly different in the absence of infection (LMM, $\chi^2_1 = 2.321$, $p = 0.128$, Figure S4). The differences between flies carrying *w*Mel clade III variants and flies carrying clade V based on reproductive output and survival at five to seven days post-infection could explain the relative fitness of 0.723 (0.651-0.823) calculated from the above estimated selection coefficient ($w = 1-s$).

Mitochondria are co-inherited with *Wolbachia*. Therefore, the phenotypic differences we observed between flies carrying different *w*Mel variants could, hypothetically, be due to phenotypic differences of their associated mitochondria variants. If this were the case, selection could have acted on the mitochondria and indirectly affected frequencies of *Wolbachia* variants. To test for the contribution of mitochondria to the phenotypic differences observed, we repeated these assays with the same isofemale lines and matching isofemale lines from which *w*Mel was removed by tetracycline treatment. We found a significant interaction between *w*Mel/mitochondria clade (cytotype) and *Wolbachia* presence, both in survival 5, 6 or 7 days after infection and in overall survival (Figure S3A and

Figure S3B – clade by presence of *Wolbachia*, GLMM and Mixed effect Cox model, $p < 0.001$). Importantly, in both models, there is a significant difference in survival between flies carrying *w*Mel clade V or *w*Mel clade III, but not between flies of the same cytotypes without *Wolbachia* (pairwise comparisons between clades in Mixed effect Cox Model, $|z| = 5.739$, $p < 0.001$ and $|z| = 0.868$, $p = 0.385$, for flies with and without *Wolbachia*, respectively and in pairwise comparisons between clades using GLMM at 5, 6 or 7 days post-infection $|z| > 3.794$, $p < 0.001$ and $|z| < 1.678$, $p > 0.093$, for flies with and without *Wolbachia*, respectively). Analysis of differential reproductive output had similar results. There was a significant interaction between cytotype and *Wolbachia* presence (Figure S3C, LMM, clade by presence of *Wolbachia*, $\chi^2_1 = 4.2$, $p = 0.040$). Pairwise comparisons of reproductive output between cytotypes with *Wolbachi*a show a significant difference ($t = 4.27$, $p < 0.001$) but not between cytotypes in the absence of *Wolbachia* ($t = 1.2$, $p = 0.087$). Overall, these data indicate that there is no significant difference in survival or reproductive output, upon viral infection, between flies only carrying different mitochondria. Therefore, the phenotypic differences we observe are due to differences between *w*Mel variants and not between mitochondria variants.

Finally, we tested if lower fitness upon viral infection of flies carrying *w*Mel clade III variants was associated with higher DCV load as different *w*Mel variants have been shown to confer differential resistance to DCV infection [14] (Figure S5A). Flies carrying these variants had 5.4 fold higher levels of DCV compared with flies carrying clade V variants (log-LMM, *w*Mel variant effect, $\chi^2_1 = 11.479$, $p < 0.001$). The lower resistance to viruses of flies carrying Clade III variants, compare to Clade V, may explain their lower survival and fertility upon infection. Flies with Clade III variants also had lower *Wolbachia* levels when compared with flies carrying clade V variants

(Figure S5B, LMM, $\chi^2_1 = 16.292$, $p < 0.001$). This may explain lower antiviral resistance of these variants, in line with previous findings [14,15,32,33].

Discussion

Our data show that (a) the frequency of *Wolbachia* variants specifically changed when *Drosophila* populations evolve in the presence of viruses, (b) this exposure to DCV lead to fixation of clade V *w*Mel variants, and (c) genetically identical individuals are more protected against DCV infection and display lower viral loads when they harbor these Clade V variants, relative to when they harbor other variants still present in the Control (and Ancestral) population. Moreover, the selection coefficient inferred from the evolutionary dynamics of clade V in DCV-exposed populations could be explained by the fitness advantage of clade V over clade III *w*Mel variants in isofemale lines subjected to DCV infection. These results demonstrate that host infection by parasites can be a selective force leading to genetic changes in the endosymbiont population such that the most protective variants become fixed. In turn, this evolution can contribute to host adaptation to pathogens.

We have previously identified two regions in the *D. melanogaster* genome that mediate this population adaptation to DCV infection [25]. Here we show that this adaptation also leads to change in *w*Mel genetic diversity. There may be interactions between selection on the genomes of the symbiont and the host, which we did not test here. We have demonstrated before that the Virus-selected population had a higher survival upon DCV infection than the Control populations even when *Wolbachia* was removed from these populations [25]. This indicates that, overall, the selected alleles confer an advantage in the presence of viruses independently on the presence of *Wolbachia*. However, it was recently shown that the strength of

selection on host genetic variation is decreased in the presence of these protective symbionts [34]. Therefore, the presence or absence of *Wolbachia* interacts with the selection at the host level. However, this does not address interactions between the genetic selection at the level of the symbiont and the host. We show differences between the *w*Mel variants using isofemale lines established from the Control populations, and therefore not evolved under Virus challenge. This indicates that the virus susceptibility phenotypes associated with the *w*Mel variants are independent of selection at the level of the host genome. Moreover, our assays compare the phenotypes of the progeny of several independent reciprocal crosses between lines carrying different *w*Mel variants. This setup controls for differences in host genetic background. It will be interesting in the future to investigate how genetic variation in the host impacts on the phenotypes of *Wolbachia* variants, and vice-versa.

Other *w*Mel variants were shown to differ in survival upon viral infection [14]. *w*Mel variants from clade VI confer more protection to viruses than variants from clade III or clade VIII [14]. Here clade V variants are more protective than clade III variants (and clade I variants are also counter-selected in the Virus-selected populations). These results indicate that clade V and VI are more protective against viral infections and clade I, III and VIII, less protective. However, it will be important in the future to make a direct comparison of the antiviral protection conferred by these different variants and understand their dynamics in natural populations.

Previous work showed that variants that differ in protection to viruses also differed in the cost to the host in the absence of infection, indicating a trade-off between the two traits [14,15]. This led to the suggestion that the frequencies of different variants in natural populations might depend on the prevalence of viruses [4]. Here we demonstrate that an increase in viral burden does lead to changes in *w*Mel variant frequencies.

Moreover, the selection coefficient for specific *w*Mel variants can be very strong and promote their rapid fixation. *w*Mel variants are strictly maternally transmitted and show no sign of recombination [13,29,30]. Therefore, as in these conditions specific haplotypes are fixed, the overall genetic diversity of *w*Mel is strongly reduced (since mitochondria are co-inherited with *Wolbachia* this selection may also impact on their genetic diversity).

Viruses seem to be strong natural selective pressures, as demonstrated by the fast evolutionary rates and signatures of positive selection in *D. melanogaster* genes involved in antiviral resistance [35]. And *Wolbachia* can protect hosts against several positive sense single-stranded RNA viruses [3,4,23,24], including DCV, a natural pathogen of *D. melanogaster* [36-39]. However, approximately 25 different viruses have been found to infect natural populations of *D. melanogaster* [38,40,41]. Although most of them are positive sense single-stranded RNA viruses we do not know which represent the biggest burden to natural populations. Moreover, the effect of *Wolbachia* against most of these viruses is unknown, although it protects against the few that were tested (DCV, Cricket Paralysis virus, and Nora virus [3,4]. Different *w*Mel variants also have different costs in the absence of infection and this is most probably an important factor in the dynamics of *w*Mel in natural populations [14]. Our particular experimental evolution setup, with all the individuals being infected with DCV at every generation before reproduction, demonstrates that *w*Mel selection upon viral infection is possible. In which conditions and to which degree this occurs in natural populations remains to be determined.

We can explain the strong selection coefficient for clade V over clade III *w*Mel variants with the differences in the protection to viruses they confer to their hosts. Previous analyses of virus-infected host carrying different *w*Mel variants or *Wolbachia* strains have shown differences in

viral titers and survival [14,15,23,32,33,42]. Here we also show that flies carrying clade V variants have lower viral titers and higher survival when compared to flies carrying clade III variants. This higher survival most probably contributes to the selection of clade V variants. However, the much higher fertility upon viral infection, of flies carrying clade V *w*Mel variants may be the main fitness difference that determines the strong selection coefficient. This fact suggests that in natural populations this parameter might be more important for the protective effect of *Wolbachia* against viruses and the differential selection of *w*Mel variants, than the effect on host survival.

Here, using experimental evolution, we provide direct proof that endosymbiont and host can form an evolutionary unit with adaptation relying on the evolution of both genomes. It is straightforward to extrapolate our results with maternally transmitted *Wolbachia* to interactions involving other defensive endosymbionts such as *Spiroplasma*, *Regiella*, and *Hamiltonella* [6,7,16]. The tight association between endosymbionts and their hosts make it probable that it is common for selection at the host phenotypic level to impact symbiont population genetics. It will be interesting in the future to assess to which degree this phenomenon occurs in interactions between hosts and microbes with different modes of transmission. One obvious example is the gut microbiota of mammals, which can protect the host against gut pathogens [8]and show some degree of vertical transmission [43]. As research on microbiota-induced phenotypes and potential co-evolution with hosts increases a central question arises on how selection on the microbiota-induced phenotypes impacts the population genetics of the microbes.

Materials and methods

Foundation, maintenance, and selection of populations

We used an outbred population of *D. melanogaster* established in 2006 from 160 fertilized females, as described in [25,26]. The population was kept in laboratory conditions for more than 50 non-overlapping generations at high census. Before the initiation of experimental evolution, this population was serially expanded for two generations to allow the establishment of 36 new populations of which 12 were used in this work. All individual founders were naturally infected with *Wolbachia w*Mel and the initial populations were 100% infected with *Wolbachia* (checked individually by PCR with *wsp* primers, as described in [44]).

Flies were kept in laboratory cages at constant temperature (25°C) and humidity (70%) in a light-darkness cycle (12h:12h). Flies were raised in standard cornmeal-agar medium. Each generation took three weeks and egg density per food cup was controlled.

Virus-Selected populations were infected every generation by pricking flies in the thorax with DCV (2×10^7 median tissue culture infective dose ($TCID_{50}$/mL)) [25]. *Drosophila* C virus was grown and titrated as described in [3]. This dose caused in the initial population an average mortality of 66% 10 days after infection. Three-hundred and ten males and 310 females were infected with DCV at every generation. Surviving individuals mated randomly in population cages and eggs were collected five to seven days post-infection. This selection protocol proceeded for 20 generations before Pool-Seq analysis.

Control populations were pricked at every generation with sterile solution. These populations were controlled to 600 adults at every generation.

Bacteria-Selected populations infection and selection protocol at every generation was the same as for the Virus-Selected populations. Flies were infected by pricking with *Pseudomonas entomophila* at a dose that

causes an average mortality of 66% in the initial populations (OD600 = 0.01) [26].

Whole-Genome Sequencing of Populations (Pool-seq)

DNA extraction, library preparation and whole genome sequencing of pools of individuals was described in [25]. Briefly, 12 populations were sequenced (four per regime): Ancestral (generation 0), Control and Virus-Selected populations, the latter two at generation 20. Genomic DNA was extracted from a homogenate pool of 200 individuals of each population using a high-salt extraction protocol. Genomic DNA was sheared using a Covaris S2 device (Covaris, Inc.) and paired-end 100bp libraries were prepared using the TruSeq v2 DNA Sample Prep Lit (Illumina). Libraries were sequenced on a HiSeq 2000 (Illumina).

Raw reads were trimmed using Trimmomatic [45] (leading and trailing bases clipped if quality < 20, 3' clipped if average quality of a window (4 bp) dropped below 20, minimum read length = 50) and then realigned to the reference *Wolbachia* genome (NC_002978.6 [46]) using bwa 0.6.2 [47], with the following parameters: maximum differences = 1%, maximum number of gaps = 2, maximum gap or deletion size = 12, seeding disabled. Alignments were converted to the sam/bam format using samtools [48] and sorted, filtered for quality, proper pairs and duplicate reads using bamtools [49]. Afterwards, SNPs were called simultaneously in all populations using freebayes (v 9.9.2) [50], in positions with a minimum count of the alternate allele of 2 and a minimum global alternate allele frequency of 2%. Only biallelic SNPs were considered.

Effects of the polymorphisms on putative coding sequences were predicted using SnpEff (v 4.11) [51], based on the ENSEMBL GCA_000008025.1.26 genome annotation.

Determination of frequencies of clade V *w*Mel variants

We analysed the frequency of clade V *w*Mel variants by testing individual flies in Ancestral, Virus-Selected (at generations 5, 10, and 20), Control (at generation 20), and Bacteria-Selected (evolved against *Pseudomonas entomophila*, at generation 20) populations.

We extracted DNA from 96 individual female flies of each replicate population following the protocol in (http://www.drosdel.org.uk/molecular_methods.php#prep) [52]. Briefly, single flies were squashed in 100 mM Tris-EDTA-NaCl buffer (pH 7.7), 0.5% SDS and incubated at 65 ºC for 30 minutes. After protein and RNA precipitation with 6M LiCl/ 5M KAc, DNA was precipitated using ice-cold isopropanol followed by ethanol cleaning. PCR amplification of the genomic region surrounding position 805,011 was performed using the primers 805011F (5'-AGTCGGGAGCATGAGGGAAAAGT-3') and 805011R (5'-TTTCAGCATCAGTCGCCTCCGC-3'). The polymorphism was detected by differential cleavage of amplified product with the enzyme *Bts*CI. Digestion was performed at 50 °C for 60 minutes and the digestion product visualized in an agarose gel. The polymorphism at this position distinguishes *w*Mel variants of clades I, II, III and IV from variants of clades V and VI. In our populations this SNP allows distinguishing clade V variants from clade I/III variants.

Establishment of isofemale lines carrying *w*Mel of clades III and V

Ninety-six isofemale lines were founded from Control populations. The Pool-Seq data show that these populations only had *w*Mel variants from clades III and V.

Each line was tested for three different *w*Mel SNPs. Position 805,011 was tested as above. The SNPs at positions 655,839 and 1,027,577 distinguish clades I, II and III from clades IV, V, VI and VIII. PCR

amplification of the genomic regions surrounding these positions were performed using the primers 655839F (5'-AGCAGCTCTAGCAATCGCAGCA-3'), 655839R (5'-GGCGTTTTAGGGGTGTGGTTGGT-3'), 1027577F (5'-TCCTGCATCAGTCCTGCCACCA-3'), and 1027577R (5'-GGCAGCACTGTAGGCTTGACCA-3'). The PCR products were digested at 37 °C for 60 minutes using the restriction enzymes *Msc*I and *Hin*dIII for positions 655,839 and 1,027,577, respectively. The results of the three enzymes were congruent allowing us to identify isofemale lines carrying clade V or clade III *w*Mel variants.

We also tested for the insertion IS5-WD1310 by PCR, as described in [12]. This insertion is present in clade VI variants, absent in clade III and VIII variants, but unknown for variants of other clades, including clade V [12,14]. All flies were negative for this insertion. After this analysis we selected eleven independent isofemale lines carrying clade V *w*Mel variants and eleven independent isofemale lines carrying clade III *w*Mel variants. Isofemale lines were kept in vials in similar conditions to the *D. melanogaster* populations.

Generation of flies carrying different *w*Mel variants for phenotypic characterization

Eleven independent pairs of isofemale lines with *w*Mel variants of clades III and V were crossed in a reciprocal scheme (female clade V x male clade III and female clade III x male clade V). The female progeny of these two crosses have an equivalent genetic background but different *w*Mel variants (which is maternally transmitted). This female progeny was used for the phenotypic characterization and each reciprocal pair was considered a random effect in the statistical analysis ("cross genotype", see below).

Reproductive time-window and general husbandry conditions of these crosses were the same as for the experimental evolution protocol.

Establishment of isofemale lines and generation of flies for the analysis of mitochondrial contribution to different phenotypes

To analyze the contribution of mitochondria associated with different *w*Mel clades to the fitness related phenotypes we established *Wolbachia*-free lines derived from the above selected isofemale lines carrying different *w*Mel variants.

We treated ten clade III isofemale lines and ten clade V isofemale lines with tetracycline (as in [14]). Lines were raised in fly food with 0.05mg/mL of tetracycline hydrochloride (Sigma) for two generations. After antibiotic treatment each treated line had their microbiota reconstituted with the microbiota associated with their original line. 150µL of a bacterial inoculum of each of the original lines was added to each tetracycline-treated lines. Each inoculum was constituted of 5mL of sterile water mixed with 2g of food from a 10 days old vial of the original stock, filtered to remove eggs and larvae.

All stocks were confirmed to be free of *Wolbachia* by PCR using primers specific for the *Wolbachia* gene *wsp* (81F (5'-TGGTCCAA-TAAGTGATGAAGAAAC-3') and 691R (5'-AAAAATTAAACGCTACTCCA-3')) as in [3]. Flies were raised without antibiotics for two generations before assays.

To compare the phenotype of different cytotypes in the presence or absence of *Wolbachia* we setup reciprocal crosses between lines carrying different *w*Mel variants and reciprocal crosses between their matching isofemales lines after tetracycline treatment. Only ten reciprocal crosses of each kind were performed in this assay. The phenotypic assays were performed on the progeny of these crosses.

Fitness assays

For the survival assays, 100 females (3-6 days old) from each reciprocal cross, infected with DCV, were placed in vials (10 vials with 10 individuals each), at 25 °C. The mortality was monitored daily for 20 days.

For the progeny assays, 20 couples (3-6 days old) from each reciprocal cross were infected with DCV and placed in vials 5 days after infection (1 couple per vial). Flies were allowed to lay eggs for two days and then removed (this protocol matches that of the experimental evolution). The progeny of each female corresponds to the number of pupae per vial. The same protocol was used for progeny quantification with females not exposed to DCV.

***Wolbachia* levels and viral titers**

For the quantification of *Wolbachia* and viral titers in the progeny of the reciprocal crosses, we used three DCV-infected females of the progeny of each matched pair. Seven days post-infection total nucleic acid was extracted using MasterPure Complete DNA and RNA Purification Kit (Epicentre), according to manufacturers' protocol, with some modifications. To purify DNA, 10µL of each sample was treated with 1µL of 10mg/mL RNAse A (Roche). To purify RNA, samples were treated with 1U DNAse per µg of total nucleic acid, in a total volume of 10µL, at 37º C for 30 min; the reaction was stopped by adding 1µL of RQ1 DNAse stop solution, and incubated at 65 ºC to inactivate the DNAse. RNA samples were then reverse transcribed to cDNA using M-MLV Reverse Transcriptase (Promega), according to manufacturers' instructions. DNA and cDNA samples were used to quantify *Wolbachia* and DCV levels, respectively.

Quantification of *Wolbachia* levels and viral titers was performed by qPCR as described in [14]. For each reaction we used 6µL of iQTM SYBR Green supermix (Bio Rad), 0.5µL of each primer solution at 3.6 µM and 5µL

of diluted DNA. Each plate contained three technical replicates of every sample for each set of primers. Relative amounts of *wsp* and DCV were calculated using the Pfaffl method [53] and *Drosophila Rpl32* as a reference. Levels of *wsp* and DCV are relative to the *w*Mel clade V samples.

Statistical Analysis

Allele frequency comparisons

Allele frequencies were compared using a weighted binomial model. Let v_i be the frequency of the major allele in population *i* of a given selection regime:

$$v_i = \text{logit}^{-1}\left(X_i^{\mathrm{T}}\beta + \varepsilon_i\right)$$

Where β is the vector of the Selection regime fixed effect and *Xi* is a row vector relating this fixed effect to population *i*, weighted by the read depth or number of genotyped individuals. ε_i is the residual error that captures overdispersion in the estimate of frequencies in each population.

In the pooled sequencing analysis, only positions in any of the populations with minor allele frequency > 2% were considered, and Benjamini & Hochberg adjusted *p* values (q-values) were considered significant if below a false discovery rate threshold of 0.1%.

In the comparisons of *w*Mel variant frequencies between selection regimes, *p* values for multiple comparisons were adjusted using sequential Bonferroni correction.

In generation 20 all reads or all sampled individuals in the Virus-Selected populations were fixed to one of the alleles, leading to problems of convergence in the models. To correct for that, we assigned one read or one individual to the alternative allele.

Estimation of selection coefficient

Since *Wolbachia* is maternally transmitted, selection acts as in a haploid organism. The estimate for the fitness differential between the *Wolbachia* clades in the Virus-Selected populations was then calculated according to [31] (eqn 6.3):

$$\log\left(\frac{p_t}{q_t}\right) = \log\left(\frac{p_0}{q_0}\right) + \log(w) * t$$

Where w is the relative fitness (1-s) of genotype p over genotype q.

Assuming a small s (<0.5), $(1+s)^t \sim e^{st}$. Therefore we assessed statistical significance of the coefficient using mixed logistic regression. Let $\nu_{i,t}$ be the frequency of a given *Wolbachia* genotype at generation t in population i,

$$\nu_{i,t} = \text{logit}^{-1}(s * t + \nu_{i,0} + \varepsilon_{i,t})$$

the selection coefficient (s) is the slope of the regression coefficient given the initial frequencies ($\nu_{i,0}$), $\varepsilon_{i,t}$ is the residual error that captures overdispersion in the estimate of frequencies in the populations at each time point. Using the frequencies of *w*Mel variants at generations 0, 5, 10 and 20 the selection coefficient against *w*MelI/III is 0.263 (0.177-0.349). This relatively high value is independent of the data at generation 20, when there is fixation of clade V, since the selection coefficient calculated with data from generations 0, 5, and 10 is 0.287(0.183-0.391).

We tested presence of *w*Mel in the progeny (96 individuals) of five females from different isofemale lines carrying clade III variants, and in the progeny (100 individuals) of five females from different isofemale lines carrying clade V variants. All individuals were positive for *w*Mel showing that vertical transmission is virtually 100% and similar for variants of both clades. Therefore we can compare these variants fitness using this multigenerational equation.

Survival analysis

To compare survival at days 5, 6 and 7 of flies with each *Wolbachia* variant after infection, we fitted a generalized linear mixed effects model. Let $v_{i,j}$ be the proportion of surviving flies in vial i of individuals of a given *w*Mel variant, resulting from cross j, at 5, 6 or 7 d.p.i.:

$$v_{i,j} = \text{logit}^{-1}\left(X_i^{\mathrm{T}}\beta + c_j + \varepsilon_{i,j}\right)$$

Where β is the vector of fixed effects of *w*Mel variant, X_i is the row vector relating the fixed effects of variant with vial, c_j is the random effect of fly cross genotype and $\varepsilon_{i,j}$ is the residual error that captures overdispersion for each vial.

We also compared the full survival dynamics, until 20 days post-infection, using a mixed effects Cox model. This model accounted for both parental cross and between-vial variation in survival rates. The hazard of the ith individual of a given *w*Mel strain, resulting from cross j in vial k was modeled as:

$$H_{i,j,k}(t) = H_0(t)e^{\mathrm{X_i}\beta + c_j + \varepsilon_{i,j,k}}$$

Where H_0 is the baseline hazard at time t, β is the vector of fixed effects of *w*Mel variant, X_i is the row vector relating the fixed effects of variant with the individual fly, c_j is the random effect of cross genotype and $\varepsilon_{i,j,k}$ is the random effect of vial.

In both analyses, the effect of the *w*Mel variant was compared using likelihood-ratio tests, with a model without the fixed effect term as the null model.

To analyze the effect of the mitochondria variants in the survival upon viral infection equivalent models were used taking into account the fixed effect of presence or absence of *Wolbachia*, and its interaction with the fixed effect variant.

Reproduction tests

To compare reproductive output of flies with different *w*Mel variants after infection, we fitted a linear mixed model, where $\nu_{i,j}$ is the number of pupae after a 48h oviposition period by female i resulting from cross j, with a particular *w*Mel variant.

$$v_{i,j} = \mathrm{X_i}\beta + c_j + \varepsilon_{i,j}$$

As above, β is the vector of fixed effects of *w*Mel variant, X_i is the row vector relating the fixed effects of *w*Mel variant with female *i*, c_j is a random variable representing the deviation of the cross genotype (reciprocal cross pair) from the overall mean and $\varepsilon_{i,j}$ is the random term that captures heterogeneity between different females of the same cross genotype.

The effect of the fixed factor was compared using likelihood-ratio tests.

To analyze the effect of the mitochondria variants in the reproductive output upon viral infection, a similar model was used taking into account the fixed effect of presence or absence of *Wolbachia*, and the interaction of this with the fixed effect variant.

In the second experiment (designed to test for the effect of mitochondria) there was a high number of females that did not reproduce. Therefore, we also analyzed these data using a hurdle model for count data in which two equations were used. One to compare the number of zero vs non-zero counts between the groups with a binomial model, and another to analyze the non-zero counts, assuming that these follow a zero-truncated negative binomial distribution. This analysis gave a similar result to the linear mixed model used above. In the non-zero counts data there is an interaction between cytotype and *Wolbachia* presence (χ^2_1 = 9.59, p = 0.002). There is a significant difference in reproductive output between flies carrying *w*Mel clade V or *w*Mel clade III, but not between flies of the same cytotypes without *Wolbachia* (pairwise comparisons between clades in

generalized linear mixed model, $t = 5.23$, $p < 0.001$ and $t = 1.71$, $p = 0.087$, for flies with and without *Wolbachia*, respectively).

Wolbachia and DCV titer quantification

To compare *Wolbachia* or DCV titers after infection in flies with different *w*Mel variants, we fitted a linear mixed model similar to the equation described above, with $\nu_{i,j}$, being the log(*wsp*) or log(DCV) levels.

All statistical analyses were done in R (version 3.1.2) [54]. Linear mixed models were fitted using the *lmer* function and generalized linear mixed models with the *glmer* function, both in the "lme4" package. Hurdle models were done with the *glmmADMB* function of the "glmmADMB" package. Multiple comparisons were done using the *lsmeans* function in the "lsmeans" package. Survival data were compared using the *coxme* function in "coxme" package.

Acession numbers

Trimmed fastq and assembled bam files are available via the European Nucleotide Archive (http://www.ebi.ac.uk/ena/about/search_and_browse), as project PRJEB8815, with reads accession nos. ERS684186-ERS684197 and ERS764859 – ERS764870, respectively.

Acknowledgements

We would to thank Instituto Gulbenkian de Ciência for support for TFP. We are also grateful to Miguel Landum for help in removing *Wolbachia* from isofemale lines.

References

1. Moran NA, McCutcheon JP, Nakabachi A: **Genomics and evolution of heritable bacterial symbionts**. *Annual Review of Genetics* 2008, **42**:165–190.

2. Jaenike J: **Population genetics of beneficial heritable symbionts**. *Trends in Ecology and Evolution* 2012, **27**:226–232.

3. Teixeira L, Ferreira A, Ashburner M: **The bacterial symbiont *Wolbachia* induces resistance to RNA viral infections in *Drosophila melanogaster***. *PLoS Biology* 2008, **6**:e2.

4. Hedges LM, Brownlie JC, O'Neill SL, Johnson KN: ***Wolbachia* and virus protection in insects**. *Science* 2008, **322**:702.

5. Oliver KM, Russell JA, Moran NA, Hunter MS: **Facultative bacterial symbionts in aphids confer resistance to parasitic wasps**. *Proceedings of the National Academy of Sciences of the United States of America* 2003, **100:**1803–1807.

6. Scarborough CL, Ferrari J, Godfray HC: **Aphid protected from pathogen by endosymbiont**. *Science* 2005, **310**:1781.

7. Jaenike J, Unckless R, Cockburn SN, Boelio LM, Perlman SJ: **Adaptation via symbiosis: recent spread of a *Drosophila* defensive symbiont**. *Science* 2010, **329**:212–215.

8. Kamada N, Chen GY, Inohara N, Núñez G: **Control of pathogens and pathobionts by the gut microbiota**. *Nature Immunology* 2013, **14**:685–690.

9. Oliver KM, Campos J, Moran NA, Hunter MS: **Population dynamics of defensive symbionts in aphids**. *Proceedings Biological Sciences / The Royal Society* 2008, **275**:293–299.

10. Xie J, Winter C, Winter L, Mateos M: **Rapid spread of the defensive endosymbiont *Spiroplasma* in *Drosophila hydei* under high parasitoid wasp pressure**. *FEMS Microbiology Ecology* 2015, **91**:1–11.

11. Jaenike J, Brekke TD: **Defensive endosymbionts: a cryptic trophic level in community ecology**. *Ecology Letters* 2011, **14**:150–155.

12. Riegler M, Sidhu M, Miller WJ, O'Neill SL: **Evidence for a global *Wolbachia* replacement in *Drosophila melanogaster***. *Current Biology* 2005, **15**:1428–1433.

13. Richardson MF, Weinert LA, Welch JJ, Linheiro RS, Magwire MM, Jiggins FM, Bergman CM: **Population genomics of the *Wolbachia* endosymbiont in *Drosophila melanogaster***. *PLoS Genetics* 2012, **8**:e1003129.

14. Chrostek E, Marialva MSP, Esteves SS, Weinert LA, Martinez J, Jiggins FM, Teixeira L: ***Wolbachia* variants induce differential protection to viruses in *Drosophila melanogaster*: a phenotypic and phylogenomic analysis**. *PLoS Genetics* 2013, **9**:e1003896.

15. Chrostek E, Teixeira L: **Mutualism breakdown by amplification of *Wolbachia* genes**. *PLoS Biology* 2015, **13**:e1002065.

16. Oliver KM, Moran NA, Hunter MS: **Variation in resistance to parasitism in aphids is due to symbionts not host genotype**. *Proceedings of the National Academy of Sciences of the United States of America* 2005, **102**:12795–12800.

17. Hansen AK, Vorburger C, Moran NA: **Genomic basis of endosymbiont-conferred protection against an insect parasitoid**. *Genome Research* 2012, **22**:106–114.

18. Oliver KM, Degnan PH, Hunter MS, Moran NA: **Bacteriophages encode factors required for protection in a symbiotic mutualism**. *Science* 2009, **325**:992–994.

19. Weeks AR, Turelli M, Harcombe WR, Reynolds KT, Hoffmann AA: **From parasite to mutualist: rapid evolution of *Wolbachia* in natural populations of *Drosophila***. *PLoS Biology* 2007, **5**:e114.

20. Kriesner P, Hoffmann AA, Lee SF, Turelli M, Weeks AR: **Rapid sequential spread of two *Wolbachia* variants in *Drosophila simulans***. *PLoS Pathogens* 2013, **9**:e1003607.

21. Versace E, Nolte V, Pandey RV, Tobler R, Schlötterer C: **Experimental evolution reveals habitat-specific fitness dynamics among *Wolbachia* clades in *Drosophila melanogaster***. *Molecular Ecology* 2014, **23**:802–814.

22. Werren JH: **Biology of *Wolbachia***. *Annual Review of Entomology* 1997, **42**:587–609.

23. Osborne SE, Leong YS, O'Neill SL, Johnson KN: **Variation in antiviral protection mediated by different *Wolbachia* strains in *Drosophila simulans***. *PLoS Pathogens* 2009, **5**:e1000656.

24. Glaser RL, Meola MA: **The native *Wolbachia* endosymbionts of *Drosophila melanogaster* and *Culex quinquefasciatus* increase host resistance to West Nile virus infection**. *PLoS ONE* 2010, **5**:e11977.

25. Martins NE, Faria VG, Nolte V, Schlötterer C, Teixeira L, Sucena É, Magalhães S: **Host adaptation to viruses relies on few genes with different cross-resistance properties**. *Proceedings of the National Academy of Sciences of the United States of America* 2014, **111**:5938–5943.

26. Martins NE, Faria VG, Teixeira L, Magalhães S, Sucena É: **Host adaptation is contingent upon the infection route taken by pathogens**. *PLoS Pathogens* 2013, **9**:e1003601.

27. Schlötterer C, Tobler R, Kofler R, Nolte V: **Sequencing pools of individuals - mining genome-wide polymorphism data without big funding**. *Nature Reviews Genetics* 2014, **15**:749–763.

28. Kofler R, Pandey RV, Schlötterer C: **PoPoolation2: identifying differentiation between populations using sequencing of pooled DNA samples (Pool-Seq)**. *Bioinformatics* 2011, **27**:3435–3436.

29. Early AM, Clark AG: **Monophyly of *Wolbachia pipientis* genomes within *Drosophila melanogaster*: geographic structuring, titre variation and host effects across five populations**. *Molecular Ecology* 2013, **22**:5765–5778.

30. Ilinsky Y: **Coevolution of *Drosophila melanogaster* mtDNA and *Wolbachia* Genotypes**. *PLoS ONE* 2013, **8**:e54373.

31. Hartl DL, Clark AG: **Principles of Population Genetics**. Sunderland. MA: Sinauer Associates; 1997.

32. Osborne SE, Iturbe-Ormaetxe I, Brownlie JC, O'Neill SL, Johnson KN: **Antiviral protection and the importance of *Wolbachia* density and tissue tropism in *Drosophila simulans***. *Applied and Environmental Microbiology* 2012, **78**:6922–6929.

33. Martinez J, Longdon B, Bauer S, Chan Y-S, Miller WJ, Bourtzis K, Teixeira L, Jiggins FM: **Symbionts commonly provide broad spectrum resistance to viruses in insects: a comparative analysis of *Wolbachia* strains**. *PLoS Pathogens* 2014, **10**:e1004369.

34. Martinez J, Cogni R, Cao C, Smith S, Illingworth CJR, Jiggins FM: **Addicted? Reduced host resistance in populations with defensive symbionts.** *Proceedings Biological Sciences / The Royal Society* 2016, **283**.

35. Obbard DJ, Jiggins FM, Halligan DL, Little TJ: **Natural selection drives extremely rapid evolution in antiviral RNAi genes**. *Current Biology* 2006, **16**:580–585.

36. Jousset FX, Plus N, Croizier G, Thomas M: **Existence in *Drosophila* of 2 groups of picornavirus with different biological and serological properties**. *CR Hebd Seances Acad Sci, Ser D, Sci Nat* 1972, **275**:3043–3046.

37. Plus N, Croizier G, Jousset FX, David J: **Picornaviruses of laboratory and wild *Drosophila melanogaster*: geographical distribution and serotypic composition**. *Annals of Microbiology (Paris)* 1975, **126**:107–117.

38. Brun G, Plus N: **The viruses of *Drosophila melanogaster***. In *The Genetics and Biology of Drosophila*. Edited by Ashburner M, Wright T. New York: Academic Press; 1978:625–702.

39. Johnson KN, Christian PD: **Molecular characterization of *Drosophila* C virus isolates**. *Journal of Invertebrate Pathology* 1999, **73**:248–254.

40. Habayeb M, Cantera R, Casanova G, Ekström J, Albright S, Hultmark D: **The *Drosophila* Nora virus is an enteric virus, transmitted via feces**. *Journal of Invertebrate Pathology* 2009, **101**:29–33.

41. Webster CL, Waldron FM, Robertson S, Crowson D, Ferrari G, Quintana JF, Brouqui JM, Bayne EH, Longdon B, Buck AH, Lazzaro BP, Akorli J, Haddrill PR, Obbard DJ: **The discovery, distribution, and evolution of viruses associated with *Drosophila melanogaster***. *PLoS Biology* 2015, **13**:e1002210.

42. Chrostek E, Marialva MSP, Yamada R, O'Neill SL, Teixeira L: **High anti-viral protection without immune upregulation after interspecies *Wolbachia* transfer**. *PLoS ONE* 2014, **9**:e99025.

43. Funkhouser LJ, Bordenstein SR: **Mom knows best: the universality of maternal microbial transmission**. *PLoS Biology* 2013, **11**:e1001631.

44. Zhou W, Rousset F, O'Neil S: **Phylogeny and PCR-based classification of *Wolbachia* strains using *wsp* gene sequences**. *Proceedings Biological Sciences / The Royal Society* 1998, **265**:509–515.

45. Bolger AM, Lohse M, Usadel B: **Trimmomatic: a flexible trimmer for Illumina sequence data**. *Bioinformatics* 2014, **30**:2114–2120.

46. Wu M, Sun LV, Vamathevan J, Riegler M, Deboy R, Brownlie JC, McGraw EA, Martin W, Esser C, Ahmadinejad N, Wiegand C, Madupu R, Beanan MJ, Brinkac LM, Daugherty SC, Durkin AS, Kolonay JF, Nelson WC, Mohamoud Y, Lee P, Berry K, Young MB, Utterback T, Weidman J, Nierman WC, Paulsen IT, Nelson KE, Tettelin H, O'NeillSL, Eisn JA: **Phylogenomics of the reproductive parasite *Wolbachia pipientis w*Mel: a streamlined genome overrun by mobile genetic elements**. *PLoS Biology* 2004, **2**: e69.

47. Li H, Durbin R: **Fast and accurate short read alignment with Burrows-Wheeler transform**. *Bioinformatics* 2009, **25**:1754–1760.

48. Li H, Handsaker B, Wysoker A, Fennell T, Ruan J, Homer N, Marth G, Abecasis G, Durbin R, 1000 Genome Project Data Processing Subgroup: **The sequence alignment/map format and SAMtools**. *Bioinformatics* 2009, **25**:2078–2079.

49. Barnett DW, Garrison EK, Quinlan AR, Strömberg MP, Marth GT: **BamTools: a C++ API and toolkit for analyzing and managing BAM files**. *Bioinformatics* 2011, **27**:1691–1692.

50. Garrison E, Marth G: **Haplotype-based variant detection from short-read sequencing**. *arXiv.org > q-bio* 2012.

51. Cingolani P, Platts A, Wang LL, Coon M, Nguyen T, Wang L, Land SJ, Lu X, Ruden DM: **A program for annotating and predicting the effects of single nucleotide polymorphisms, SnpEff: SNPs in the genome of *Drosophila melanogaster* strain w1118; iso-2; iso-3**. *Fly* 2012, **6**:80–92.

52. Ryder E, Blows F, Ashburner M, Bautista-Llacer R, Coulson D, Drummond J, Webster J, Gubb D, Gunton N, Johnson G, O'Kane CJ, Huen D, Sharma P, Asztalos Z, Baisch H, Schulze J, Kube M, Kittlaus K, Reuter G, Maroy P, Szidonya J, Rasmuson-Lestander A, Ekström K, Dickson B, Hugentobler C, Stocker H, Hafen E, Lepesant JA, Pflugfelder G, Heisenberg M, et al.: **The DrosDel collection: a set of P-element insertions for generating custom chromosomal aberrations in *Drosophila melanogaster***. *Genetics* 2004, **167**:797–813.

53. Pfaffl MW: **A new mathematical model for relative quantification in real-time RT-PCR**. *Nucleic Acids Research* 2001, **29**:e45.

54. R Core Team: **R: A language and environment for statistical computing. R Foundation for Statistical Computing**. 2015.

3.3. (Re)Adapting to viral infection without *Wolbachia*

Vitor G. Faria[1#], Nelson E. Martins[1], Viola Nolte[3], Christian Schlötterer[3], Élio Sucena[1, 4#]

1 – Instituto Gulbenkian de Ciências, Apartado 14, 2780-901 Oeiras, Portugal.

2 – cE3c: Centre for Ecology, Evolution and Environmental Changes, Faculdade de Ciências, Universidade de Lisboa, Campo Grande, 1749-016 Lisboa, Portugal.

3 – Institut für Populationsgenetik, Vetmeduni Vienna, 1210 Wien, Austria.

4 – Universidade de Lisboa, Faculdade de Ciências, Departamento de Biologia Animal, edifício C2, Campo Grande, 1749-016 Lisboa, Portugal.

- vfaria@igc.gulbenkian.pt; esucena@igc.gulbenkian.pt

Keywords: Selection, defensive symbiosis, host-pathogen interactions, *Wolbachia*-free, virus

Article in preparation for publication

Author contributions: V.G.F., É.S. designed research; V.G.F., and N.E.M., performed research; C.S. and É.S. contributed new reagents/analytic tools; V.G.F., N.E.M., V.N., and É.S., analyzed data; and V.G.F., and É.S., wrote the paper.

The authors declare no conflict of interest.

Funding: Fundação para a Ciência e a Tecnologia (FCT, Portugal) supports V.G.F (#SFRH/BD/82299/2011) and N.E.M (#SFRH/BPD/62964/2009). C.S. is funded by AustrianScience Fund (FWF) (P22725 and P19467) and European Research Council (ArchAdapt). This work was supported by Instituto Gulbenkian de Ciência/Fundação Calouste Gulbenkian, FCT (PTDC/SAU -IMU/120673/2010), and Vetmeduni funding. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Abstract

The presence of endosymbionts in individuals of host populations can drastically condition the evolution of both species. In *Drosophila*, *Wolbachia* has been shown to confer protection to RNA virus infection in a selectable strain-dependent manner. This supports the concept that host and symbiont may form an evolutionary unit under selection and raises the question of whether the symbiont is strictly necessary for host adaptation. Using our previously adapted populations from which *Wolbachia* was removed, and after an initial severe decay in survival to DCV infection, experimental evolution populations exposed to DCV recovered survival frequencies comparable to those attained previously in the presence of *Wolbachia*. Whole-genome sequencing upon 20 generations of selection revealed that the major genes involved in the first selection experiment, *pastrel* and *Ubc-E2H*, were kept under selection in the second round of *Wolbachia*-free adaptation with their frequencies increasing significantly. However, the genomic profile obtained in the two rounds of adaptation is not the same, showing that the physiological basis for protection against DCV is not strictly equivalent with or without *Wolbachia*. Indeed, other genes located on the two highly differentiated loci can also play smaller roles in adaptation without *Wolbachia*. These findings demonstrate how few genes, balanced with the presence/absence dynamics of a protective-endosymbiont, can drive host adaptation.

Introduction

Endosymbiotic relationships have strong implications in the adaptation processes of both partners, thus constituting a putatively important factor in the evolution of species [1–5]. In particular, facultative endosymbionts can have a huge impact on host's physiology and adaptation, triggered by a vast range of reproductive manipulations [6]. Indeed, the presence of these heritable endosymbionts can increase host fitness through protection against pathogens [7–10] or nutritional supplies [11,12]. These effects, alone or combined, may play a determinant role in the invasion and spreading of facultative endosymbionts in host populations [1,3,5].

In arthropods, the endosymbiont *Wolbachia* is widespread, albeit with varying incidence in natural populations [13]. The cause of these frequency variations of host infection, both within and between populations, is still unclear. Yet, the discovery that *Wolbachia* protects the *Drosophila* host against viral infection [9,14–16] raises the possibility that this mechanism may contribute to this pattern of ecological and evolutionary heterogeneity.

Drosophila melanogaster is infected with one *Wolbachia* strain, *w*Mel, for which several variants have been described [17]. These variants can be clustered in two major monophyletic groups: *w*Mel-like and *w*MelCS-like, which may be distinguished through variation in 8 indels and 108 SNPs [18]. Interestingly, the two *Wolbachia* clades confer different levels of protection against DCV and FHV infection, with *w*MelCS-like showing a stronger effect, directly correlating with *Wolbachia* load inside the host (*18*)(Subchapter 3.2).

Studies have demonstrated that the increase in host fitness favours endosymbiont spreading [19–21]. Also, endosymbiont variants of the same strain can fluctuate in frequency inside populations throughout time or

during evolution experiments [22,23]. Yet, thus far no study contrasted the consequences to an under-selection and pre-adapted host population after protective-endosymbiont removal.

In our previous work, we selected 4 replicates of *D. melanogaster* outbred populations against DCV and treated another 4 replicates with a systemic mock-infection, as control. After 20 generations of selection, we observed, in populations selected against DCV, an increase in survival 10 days after infection from 33% to almost 80%. We showed by whole-genome sequencing of these populations that i) *Drosophila* adaptation to DCV relied on two major loci (containing three functionally-validated genes: *pastrel*, *CG8492* and *Ubc-E2H*) with different cross-resistance properties [24] (Subchapter 2.5) and ii) the most protective *Wolbachia* substrain, *w*MelCS, was fixed by selection (Subchapter 3.2).

Here, and after 35 generations of selection (and survival after infection up to 90% at day 10), we founded 4 new derived-replicates of each evolved population (VirSys) and respective control (ContSys). We removed *Wolbachia* infection from both new selection regimes (VirSys-tet and ContSys-tet, respectively) and kept the respective viral infection treatment. We found that these pre-adapted outbred populations of *Drosophila* can increase their immunocompetence against DCV without *Wolbachia* to levels comparable to those achieved in the presence of the symbiont. Re-sequencing of those *Wolbachia*-free populations after 20 generations indicated that the new protective phenotype was based on the increase of frequency of the viral-protective alleles in the major genes revealed in the previously adaptive process: *pastrel* and *Ubc-E2H*. Moreover, other relevant gene(s) located in the differentiated peaks may also have a role in this novel adaptation.

Results

We have performed experimental evolution of a DCV-adapted outbred population of *Drosophila melanogaster*. Previously, for 34 generations, four population replicates were selected in each generation with DCV systemic infection (VirSys) and four other replicates were used as control, pricked with a buffer solution (ContSys). In generation 35, those populations were further replicated, resulting in 8 new populations, 4 adapted and 4 controls. For 5 generations (from 35 to 39), these novel populations were exposed first, for 2 generations, to a tetracycline treatment to remove *Wolbachia* infection. We further waited additional three generations to allow mitochondrial recovery of treated populations, as well as to performed microbiota standardization between infected and non-infected populations. At generation 40, we started a new set of experimental evolution, thereafter considered as generation 0 to these *Wolbachia*-free populations, referred to as VirSys-tet and ContSys-tet.

To perform this new set of experimental evolution, the same protocol of selection was performed [24], where DCV infection was imposed at every generation using the same (not coevolved) ancestral virus strain and survival measured at days 6 and 10 after infection.

At generation 0, resistance of new populations dropped significantly. In females, survival decreased from 92,8% (VirSys) to 29,4% (VirSys-tet) and from 42,6 (ContSys) to 7,0% (ContSys-tet); in males, from 83,3% (VirSys) to 22,0% (VirSys-tet) and from 22,0% (ContSys) to 8,3% (ContSys-tet) (Figure 3.2.1).

When exposed to DCV, VirSys-tet populations showed a progressive increase in survival when compared to individuals from control lines throughout the ensuing 20 generations ($p < 0.0001$). Changes in survival in the VirSys-tet selection regime were consistent among replicate populations. In females, the increase of survival started early in the new

selective process, both at day 6 and day 10 (Figure 3.2.1 A and B, left). In males, the difference in survival was absent in the early generations and increased with time, and showed a significant interaction between generation and selection regime ($p < 0.0001$). The response became significantly different at generation 11 (day 6) and at generation 18 (day 10) (Figure 3.2.1 A and B, right). At day 6 (panel A), both males and females of VirSys-tet populations responded very closely to VirSys populations after

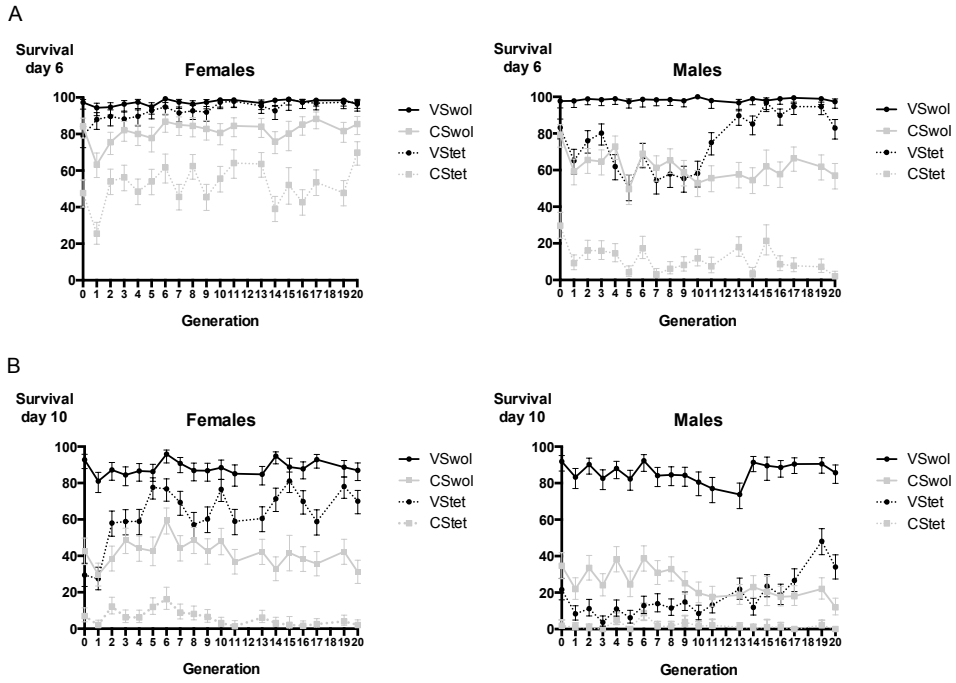


Figure **3.3.1 – Evolution of increased resistance to DCV in *Wolbachia*-free population.** Experimental evolution trajectories over 20 generations (after previous 40) of *Wolbachia*-positive populations VirSys (VS) and ContSys (CS); and *Wolbachia*-free populations VirSys-tet (VStet) and ContSys-tet (CStet) at day 6 (**A**) and day 10 (**B**). Circles represent populations exposed to the virus. Squares represent control lines. Solid lines represent populations *Wolbachia*-positive and dotted lines *Wolbachia*-free. Vertical bars correspond to the SEM survival among the four replicates populations.

20 generations. On the other hand, at day 10, while females also presented a similar response, a profound difference between males with or without *Wolbachia* was evident.

Throughout this new selection, VirSys and ContSys kept the same response profile, where VirSys continued with a very high resistance whereas ContSys kept the same survival levels when challenged with DCV, running around the values defined as the baseline before the start of the first adaptation experiment (2/3 of mortality in average among males and females).

To uncover the genetic basis responsible for the increased resistance of *Drosophila Wolbachia*-free populations evolving in presence of DCV, we performed genome-wide sequencing of DNA pools (Pool-Seq) of all replicates of both treatments, VirSys-tet and ContSys-tet, at generations 0 and 20 (Figure 3.3.2).

Using a chromosome-wide cutoff (see M&M), we observed consistent significant changes in allele frequencies over a region that spans ~500 kb on the X chromosome (*X*) (most 5′ SNP, X: 7763190 and most 3′ SNP, X:8269370) and across a 4 Mb region on the *3L* chromosome (Figure 3.3.2C). The most significantly differentiated SNPs in the *X* region corresponded to position X:7862410 and mapped to 3 different genes, CG1409, Ir7b and dec-1. Initial and final frequencies of the most significantly differentiated SNPs were 0.558 and 0.9 for X:7862410 (*CG1409*/*Ir7b*/*dec-1*). Presenting virtually the same probability of differentiation, the 4th highest differentiated SNP is on the *Ubc-E2H* gene (X: 8090522), which increased in frequency from 0.667 to 0.917.

When comparing panel C with B, we could observe that the most significantly differentiated SNPs in 3L were located in *pastrel*, where the protective allele (commonly characterized by the SNP 3L:7350895) increased in frequency, from a initial 0.733 to a final 0.875.

As shown in panels A e D, we could see chromosomal profile modifications between initial and final populations of the same treatment (ContSys-tet and VirSys-tet, respectively). Small significant alterations were registered, being the majority similar in both treatments in chromosome 3L, 3R and X.

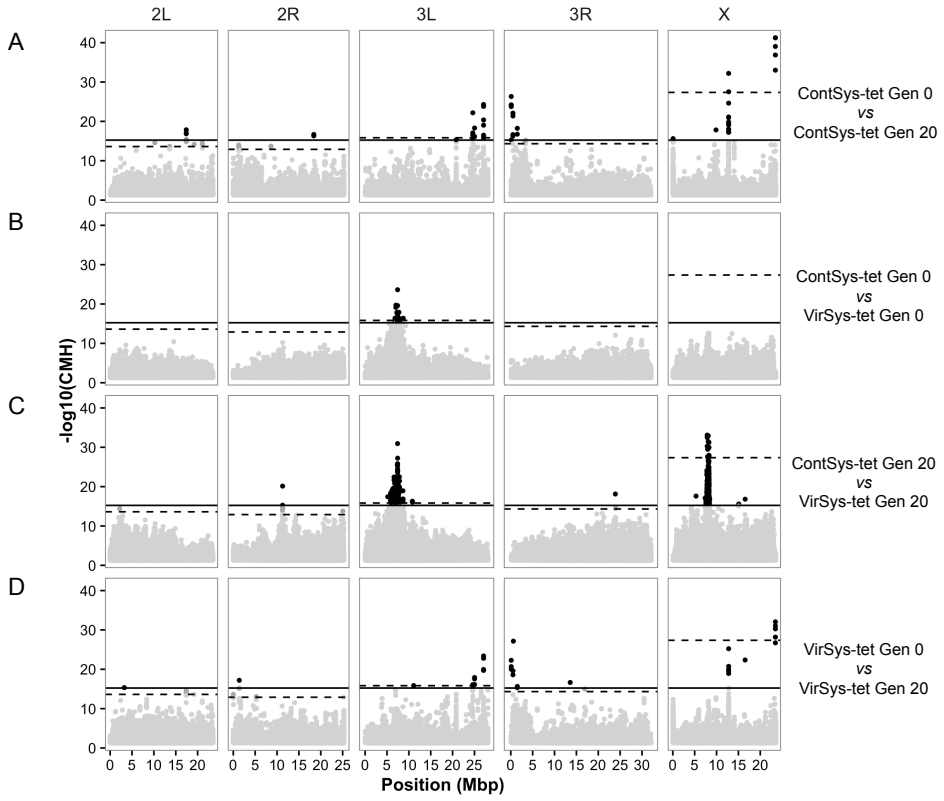


Figure 3.3.2 – Differentiation between *Wolbachia*-free selection regimes. -log10 values of the CMH statistic for every polymorphic SNP, across the five major chromosomal arms through pairwise comparison of allele frequencies between (**A**) ContSys-tet populations at generation 0 and 20, (**B**) ContSys-tet and VirSys-tet populations at generation 0, (**C**) ContSys-tet and VirSys-tet populations at generation 40, and (**D**) between VirSys-tet populations at generation 0 and 20. The solid and dotted lines represent the 99.95% quantile of the *P* values in the controls comparison at genome-wide and chromosome-wide levels, respectively.

Discussion

In this work, we have shown that a *D. melanogaster* outbred population can achieve survival levels upon DCV infection comparable to those reached with the contribution of *Wolbachia* after the removal of this protective-endosymbiont. Using Pool-Seq, we found SNPs associated with this process. We revealed the previously described genes *pastrel* and *Ubc-E2H* as the most promising players driving this second turn of anti-viral adaptation. Moreover, it is possibly that this second adaptation involved the selection of alleles that were not required, totally or partially, in the first adaptation.

As expected, treatment with tetracycline (and consequent elimination of *Wolbachia*) strongly reduced survival upon DCV infection in both adapted and control populations. The average survival of males and females at day 10 dropped 71.8% in VirSys-tet and 76.25% in ContSys-tet populations. However, the pre-adapted population VirSys-tet kept a higher survival in absolute values (Figure 3.3.1, generation 0), reflecting the changes in frequency of host's protector alleles selected in the first adaptation [24] (Subchapter 2.5). This result reiterates in our adapted outbred populations, the described strong antiviral protection conferred by *Wolbachia*.

Moreover, an important general feature to take into account when comparing antiviral protection provided by *Wolbachia* is a difference in the degree conferred by different strains [18]. As seen in Subchapter 3.2, the most protective strain present in the ancestral population, *w*MelCS-like (Clade V) was fixed in the evolved populations VirSys. However, the strain-factor, although important, is not a key point in this circumstance. As both populations VirSys and ContSys only differed between 100% and 85% at the time of treatment with tetracycline, the strain-factor did not influence significantly the decrease of DCV-protection in both *Wolbachia*-free

populations. Consequently, this effect was diluted in the striking viral-susceptibility phenotype that resulted from the absence of *Wolbachia* itself.

After this reduction in anti-viral protection, we raised the question of whether the population with its current genetic pool and in the absence of the protective endosymbiont would adapt to this challenge. If so, how long it would take to start and how far could it go?

As shown in Figure 3.3.1, the same selective pressure triggered a new adaptive process, impacting almost immediately survival in females. In males, the difference is significant from generation 11 onwards (when we consider day 6, which is the reproductive period) and at generation 18 (considering day 10, end of survival assessment). The difference in response between females and males may reflect the role of age on reproductive dynamics, or distinct sex-dependent consequences of viral infection itself (for example, the difference in size and/or in nutrition between sexes). At generation 20, VirSys-tet males were close to the VirSys response at day 6 but remained different from day 10, showing that whatever feature was selected, it was sufficient to delay death until after the age of reproduction but not conferring a longer-term protection, as previously provided by *Wolbachia* presence. In the case of females, it was expected that survival at day 10 also increased because we had established that infected females that die before day 10 have not laid eggs during the reproductive window of our protocol (day 5-7), thus having the same fitness in this period.

It is also essential to reflect on the consequences of two scenarios: adapting against virus in one population without *Wolbachia* from the beginning of the selective pressure or, as done here, removing *Wolbachia* after the population is already well adapted. These are two different questions, perhaps with different and potentially complementary answers. However, there is one specific question that can only be addressed with our

protocol: which antiviral alleles are not recruited for antiviral response because of the presence of *Wolbachia*?

Comparing whole genome sequences from the initial (0) and final (20) generations shows that the biggest difference between ContSys-tet and VirSys-tet resides in the high peak of differentiated SNPs on the 3L and X chromosomes. Both are located on the previously reported loci. This peak spanned 500kb with its highest point at position X:7862410. This SNP (as well as the 3rd highest differentiated SNP, X:7862409) was downstream of 3 different genes, *CG1409*, *Ir7b* and *dec-1*, genes not previously directly related with immune functions. Also in the top of the peak, with almost the same probability of differentiation, the 4th highest differentiated SNP on *Ubc-E2H* gene, was previously described by us as a major player in the anti-DCV response (subchapter 2.5). The SNP (X: 8090522) increased in frequency from 0.667 to 0.917. Similarly, the SNP that indicated the first adaptation (X:7984325) increased its frequency from 0.658 at generation 0 to 0.858 at generation 20.

It would be interesting to investigate whether this major frequency change in X chromosome could also explain, at least partially, the different response profiles between males and females.

Still, other changes are also present in this comparison. First, an increase in the frequency of the SNPs mapping to *pastrel.* Although the previously reported protective allele of *pastrel* had a high frequency in the end of the first round of adaptation (0.733 vs 0.158 of ContSys), this new sequencing revealed that the gene was likely kept under selection in the second round. *Pastrel* is one major player in DCV response (as determined both through GWAS [25] and Evolve and Re-sequence approaches [24]) allowing a scenario in which, after an eventual decrease in viral immunocompetence (for instance the removal of *Wolbachia*), any margin to increase the frequency of a protective allele is a quick tool for an adaptation

against viral infection. Second, there are other minor differentiating SNPs (as is the case of SNP 2R:112744239) that, if relevant, had no time to increase significantly in frequency. Therefore, although there may be other promising candidates as genes with an anti-viral function, they are not presented here as potential main drives of this second round of *Wolbachia*-free adaptation.

When looking at the comparison between ContSys-tet (0-20) and VirSys-tet (0-20), we could also observe some differentiation peaks (Figure 3.2.2 A and D). The intersection between these two panels shows the changes which have occurred in both treatments. This signature probably reflects the consequence of cleaning with tetracycline. It could thus be caused by adapting to i) the absence of *Wolbachia* itself, ii) the microbiota changes through treatment, iii) mitochondrial destabilization after tetracycline or iv) this is an artefact of the telomeric and centromeric regions. These hypotheses evidence open questions regarding how *Wolbachia* influences populations, however does not influence the interpretation of our main comparison.

The sequencing comparison of the new evolved population without *Wolbachia* (VirSys-tet at generation 20) with the symmetrical *Wolbachia*-infected population (VirSys at generation 60) is also an interesting focus for future studies. This comparison will allow clarification if any of the differentiated SNPs here uncovered also changed in VirSys though the last 20 generations. Although it is expected that few differences will be present between VirSys at generation 40 and 60 (indicated by the maintenance of DCV-response phenotype), any similarity in SNP differentiation would be excluded as responsible to increase viral resistance by the absence of *Wolbachia* itself.

It will be necessary to test the new possible relevant candidates and dissect in detail if the basis of this new round of adaptation against virus

involved more genes other than *pastrel* and *Ubc-E2H*. Clearly, genes associated to the high-differentiated SNPs of the 3L and X chromosomes are potential players. The increase in frequency of some new SNPs of previously reported protective genes could also help to explain this phenomenon. However, interestingly, the protective-allele frequency of the previously reported anti-viral gene *CG8492* dropped in this new selection from 0.642 to 0.417, no longer being under positive selection. Moreover, novel candidates also emerged in other chromosomes, being certainly justified that they are tested for anti-viral protection in the presence and in the absence of *Wolbachia*. Nevertheless, considering the residual frequency change, they are not primary candidates to explain the adaptation in our populations. This possibility opens a new range of candidates for genes involved in the response against virus, as well as genes which mediated the interaction with *Wolbachia*.

Finally, it would be interesting to explore if there are genes involved in the interaction between *Drosophila* and *Wolbachia* itself. In the other words, are there genes dependent on the presence/absence of *Wolbachia* to exert their antiviral protective function? One of them may be the *Ubc-E2H* itself. To test this interaction, it will be necessary to challenge mutants *Ubc-E2H* with and without *Wolbachia* and compare the response of each phenotype as well as test both alleles under those conditions.

Materials and Methods

Fly Populations

We used an outbred population of *D. melanogaster* founded, expanded and maintained as described in Martins et al. (2013, 2014).

Wolbachia-free populations

Wolbachia-free populations VirSys-tet and ContSys-tet, generated from of the VirSys and ContSys, respectively, were derived at generation 35, by raising the progeny for two generations on food with tetracycline (0.05mg/mL). Two generations after tetracycline treatment, flora standardization was performed by direct contact of non-treated males with the food of treated populations: 100 males of each populational replicate stayed over 24 hours in contact with the food (inside the population cages) that posteriorly received the first generation of correspondent treated-populations, at generation 39 to 40. Each replicate population of VirSys-tet was systemically infected with DCV and ContSys-tet selection regimes kept as control.

Experimental Evolution

Starting from the base population, we derived 12 lines evolving under 3 different regimes (4 replicates per treatment). In the VirSys treatment, adult flies were pricked in the thoracic region with DCV [2 × 107 tissue culture ID50 (TCID50)] at each generation. A second treatment consisted of a control for pricking, in which the needle was dipped in sterile medium (ContSys). Finally, a second group of control lines consisted of flies kept in standard food without being pricked (control). The dose of DCV that was used caused an average mortality of 66% in the initial population 10 days after infection.

These treatments were administrated to 310 males and 310 females (4–6 d after eclosion). Selection lines were kept in large population cages and surviving individuals mated randomly; reproduction took place at days 5–7 after infection by providing fresh oviposition substrate. The number of individuals in the control populations was always reduced to the initial number of infected individuals (i.e., 600).

Egg density was limited to 400 per cup, a density determined experimentally to enable optimal larval development. Each generation cycle

lasted 3 weeks. Before the beginning of the experiment, absence of vertical transmission of the parasite to the progeny was verified.

To monitor survival across generations, we infected at each generation additional sample males and female flies from each replicate of each treatment and monitored their survival in vials for at least 10 days.

Whole-Genome Sequencing

Genomic DNA preparation and sequencing were done as in Orozco-terWengel et al. [26]. Briefly, a pool of 200 individuals of each selection line was homogenized with an Ultraturrax T10 (IKA-Werke), and DNA was extracted from the homogenate using a high-salt extraction protocol. Genomic DNA was sheared using a Covaris S2 device (Covaris, Inc.) and paired-end libraries were prepared using the TruSeq v2 DNA Sample Prep Kit (Illumina). Libraries were size-selected for a mean insert size of 300 bp on agarose gels and amplified with 10 PCR cycles, and 2× 100-bp paired-end reads were sequenced on a HiSeq 2000 (Illumina). 16 groups of populations were sequenced: four replicates of ContSys-tet and VirSys-tet, both at generation 0 and 20.

Read Quality Control and Mapping

Reads were mapped following the previously described pipeline for Pool-Seq analysis. Briefly, 125-bp paired-end reads were filtered for a minimum average base quality score of 18 and trimmed using PoPoolation [27]. Reads with a minimum length ≥50 bp were then mapped against a reference containing the FlyBase *D. melanogaster* genome r6.01 (http://flybase.org). We used the following parameters: seeding of the reads disabled (–l 110), 1% missing alignments assuming an error rate of 2% (–n 0.01), maximum number of two gap openings (–o 2) and a maximum gap extension of 12 bases (–e 12, –d 12). Paired-end data were

merged to single files in SAM format with the 'sampe' option of bwa. Files were converted to BAM format with SAMtools v1.3 [28] and filtered for a minimum mapping quality of 20 and properly paired reads. BAM files were transformed to pileup files using SAMtools. We identified repetitive elements in the reference genome using RepeatMasker (http://www.repeatmasker.org) and removed them from the pileup files using PoPoolation. The average sequence coverage for the genome of the analysed populations ranged from 32 to 82-fold. In subsequent analyses, allele counts were normalized to 30 (to allow comparisons with previously published results), by scaling the raw allele frequencies. The minimum allowed coverage per position was 15, and coverages below 30 were left unscaled.

SNP Calling

Only SNPs that met the following quality criteria were considered: (*i*) occurrence in at least 2 replicate populations, (*ii*) the minor allele was covered by at least 10 reads across all populations analyzed, and (*iii*) the maximum coverage did not exceed 500.

Identification of Candidate SNPs

We used the Cochran–Mantel–Haenszel (CMH) test, as implemented in PoPoolation2 [27] to identify SNPs with changes in allele frequencies between the different regimes that were consistent among replicates as described in Orozco-terWengel et al. [26]. The CMH test is used to test 2 x 2 x k contingency tables (where k is the number of independent replicates) for independence of marginal sums across k replicates. Under the null hypothesis, odds ratios for each replicate are not different from one (i.e. if the allele frequencies between two regimes, are the same). The statistic asymptotically follows a $\chi 2$ distribution with one degree of freedom. CMH

tests were performed on a SNP-wise basis for the comparisons across groups of populations. The 99.95 percentile of the *p*-value of this statistics, both at chromosome-wide and genome-wide levels, was used as an empirical false discovery rate for calling a significant SNP.

References

1. Engelstädter J, Hurst GDD: **The ecology and evolution of microbes that manipulate host reproduction**. *Annual Review of Ecology Evolution and Systematics* 2009, **40**:127–149.

2. Moran NA, McCutcheon JP, Nakabachi A: **Genomics and evolution of heritable bacterial symbionts.** *Annual Review of Genetics* 2008, **42**:165–190.

3. Brucker RM, Bordenstein SR: **Speciation by symbiosis**. *Trends in Ecology and Evolution* 2012, **27**:443–451.

4. Martins NE, Faria VG, Teixeira L, Magalhães S, Sucena É: **Host adaptation is contingent upon the infection route taken by pathogens**. *PLoS Pathogens*. 2013, **9:**e100.

5. Faria VG, Sucena É: **Novel endosymbioses as a catalyst of fast speciation**. In *Reticulate Evolution - Symbiogenesis, Lateral Gene Transfer, Hybridization and Infectious Heredity*. Edited by Gontier N. 2015:107–120.

6. Werren JH, Baldo L, Clark ME: ***Wolbachia*: master manipulators of invertebrate biology**. *Nature Reviews Microbiologyl* 2008, **6**:741–751.

7. Scarborough CL, Ferrari J, Godfray HC: **Aphid protected from pathogen by endosymbiont**. *Science* 2005, **310**:1781.

8. Oliver KM, Russell JA, Moran NA, Hunter MS: **Facultative bacterial symbionts in aphids confer resistance to parasitic wasps.** *Proceedings of the National Academy of Sciences of the United States of America* 2003, **100**:1803–1807.

9. Teixeira L, Ferreira Á, Ashburner M: **The bacterial symbiont *Wolbachia* induces resistance to rna viral infections in *Drosophila melanogaster***. *PLoS Biology* 2009, **6:**e2.

10. Kaltenpoth M, Göttler W, Herzner G, Strohm E: **Symbiotic bacteria protect wasp larvae from fungal infestation**. *Current Biology* 2005, **15**:475–479.

11. Sabree ZL, Kambhampati S, Moran NA: **Nitrogen recycling and nutritional provisioning by blattabacterium, the cockroach endosymbiont**. *Proceedings of the National Academy of Sciences of the United States of America* 2009, **106**:19521–19526.

12. Hosokawa T, Koga R, Kikuchi Y, Meng XY, Fukatsu T: ***Wolbachia* as a**

bacteriocyte-associated nutritional mutualist. *Proceedings of the National Academy of Sciences of the United States of America* 2010, **107**:769–774.

13. Jeyaprakash A, Hoy MA: **Long pcr improves *Wolbachia* DNA amplification: *wsp* sequences found in 76% of sixty-three arthropod species**. *Insect Molecular Biology* 2000, **9**:393–405.

14. Hedges LM, Brownlie JC, O'Neill SL, Johnson KN: ***Wolbachia* and virus protection in insects.** *Science* 2008, **322**:702.

15. Moreira LA, Iturbe-Ormaetxe I, Jeffery JA, Lu G, Pyke AT, Hedges LM, Rocha BC, Hall-Mendelin S, Day A, Riegler M, Hugo LE, Johnson KN, Kay BH, McGraw EA, van den Hurk AF, Ryan PA, O'Neill SL: **A *Wolbachia* symbiont in *Aedes aegypti* limits infection with dengue, chikungunya, and plasmodium.** *Cell* 2009, **139**:1268–1278.

16. Hughes GL, Koga R, Xue P, Fukatsu T, Rasgon JL: ***Wolbachia* infections are virulent and inhibit the human malaria parasite *Plasmodium falciparum* in *Anopheles gambiae*.** *Plos Pathogens* 2011, **7**:e1002043.

17. Riegler M, Sidhu M, Miller WJ, O'Neill SL: **Evidence for a global *Wolbachia* replacement in *Drosophila melanogaster*.** *Current Biology* 2005, **15**:1428–1433.

18. Chrostek E, Marialva MS, Esteves SS, Weinert LA, Martinez J, Jiggins FM, Teixeira L: ***Wolbachia* variants induce differential protection to viruses in *Drosophila melanogaster*: a phenotypic and phylogenomic analysis**. *PLoS Genetics*. 2013, **9**:e1003896.

19. Oliver KM, Campos J, Moran NA, Hunter MS: **Population dynamics of defensive symbionts in aphids.** *Proceedings Biological Sciences / The Royal Society* 2008, **275**:293–299.

20. Jaenike J, Unckless R, Cockburn SN, Boelio LM, Perlman SJ: **Adaptation via symbiosis: recent spread of a *Drosophila* defensive symbiont**. *Science* 2010, **329**:212–215.

21. Himler AG, Adachi-Hagimori T, Bergen JE, Kozuch A, Kelly SE, Tabashnik BE, Chiel E, Duckworth VE, Dennehy TJ, Zchori-Fein E, Hunter MS: **Rapid spread of a bacterial symbiont in an invasive whitefly is driven by fitness benefits and female bias.** *Science* 2011, **332**:254–256.

22. Kriesner P, Hoffmann A a, Lee SF, Turelli M, Weeks AR: **Rapid sequential spread of two *Wolbachia* variants in *Drosophila simulans*.** *PLoS*

Pathogens 2013, **9**:e1003607.

23. Versace E, Nolte V, Pandey RV, Tobler R, Schlötterer C: **Experimental evolution reveals habitat specific fitness dynamics among *Wolbachia* clades in *Drosophila melanogaster***. *Molecular Ecology* 2014, **23**:802-814.

24. Martins NE, Faria VG, Nolte V, Schlötterer C, Teixeira L, Sucena É, Magalhães S: **Host adaptation to viruses relies on few genes with different cross-resistance properties.** *Proceedings of the National Academy of Sciences of the United States of America* 2014, **111**:5938–5943.

25. Magwire MM, Fabian DK, Schweyen H, Cao C, Longdon B, Bayer F, Jiggins FM: **Genome-wide association studies reveal a simple genetic basis of resistance to naturally coevolving viruses in *Drosophila melanogaster*.** *PLoS Genetics* 2012, **8**:e1003057.

26. Orozco-terWengel P, Kapun M, Nolte V, Kofler R, Flatt T, Schlötterer C: **Adaptation of *Drosophila* to a novel laboratory environment reveals temporally heterogeneous trajectories of selected alleles.** *Molecular Ecology* 2012, **21**:4931–4941.

27. Kofler R, Pandey RV, Schlötterer C: **Popoolation2: identifying differentiation between populations using sequencing of pooled dna samples (pool-seq)**. *Bioinformatics* 2011, **27**:3435–3436.

28. Li H, Handsaker B, Wysoker A, Fennell T, Ruan J, Homer N, Marth G, Abecasis G, Durbin R: **The sequence alignment/map format and SAMtools**. *Bioinformatics* 2009, **25**:2078–2079.

3.4. Concluding remarks

Animals live in close association with microbial partners that can shape many aspects of their lives. For instance, several insects carry bacteria that defend them against parasites and infectious diseases. As we previously saw, the intracellular bacterium *Wolbachia* protects the fruit fly *Drosophila melanogaster* against viral infection. Moreover, natural populations of *Drosophila* carry different variants of *Wolbachia*, which differ from one another in the strength of this protection.

In this Chapter, using experimental evolution and Poolseq technology, we showed that a population of *Drosophila* systematically infected with viruses for 20 generations adapts to this challenge also through changes in the *Wolbachia* it carries. The *Wolbachia* variants that provide higher protection to viruses, by increasing fly survival and fecundity upon infection, are strongly selected. This work shows that adaptation to pathogens can be achieved not only through selection of resistance on the host itself but also through the evolutionary shaping of its microbial community.

Later, we replicated both evolved and control populations and eliminated *Wolbachia* infection by performing a tetracycline treatment. We kept evolved *Wolbachia*-free populations under viral selection during 20 generations. Although immunocompetence dropped significantly after having removed *Wolbachia* infection, we find that those populations were able engage in a new adaptive process and increase again the resistance against viral infection. The dynamics of adaptation were different between males and females, as well as the survival profile in each generation. When we compared the genomes of initial and final *Wolbachia*-free populations, we find that main clusters of differentiation (where allelic frequency changes significantly) were located at the same regions of the 3L and X

chromosome, previously reported to include the genes *pastrel* and *Ubc-E2H*, two of the major responsible genes for viral protection. The increase of protective-allele frequency in both genes clearly suggests these variations as the drivers that compensate for the loss of protection triggered by the removal of *Wolbachia*. However, apart from *pastrel* and *Ubc-E2H*, other genes are now also candidates to participate in this new adaptation. Additionally, one of the DNA regions responding to selection is located at X chromosome, which may justify the disparities in the dynamics of adaptation between sexes.

In this Chapter, we showed the great influence that different haplotypes of protective-endosymbionts can have on hosts subjected to selective pressure. Likewise, we also demonstrated that the adaptive processes of hosts can drastically change and outline the genetic pool of endosymbiont populations. When *Wolbachia* was removed from populations, we could unveil new roads taken by hosts to respond and increase their resistance against viral infection. Altogether, we believe that our results help to understand the real influence of hosts and microbes in the evolution of each other.

4

Endosymbioses as engines of adaptation and speciation

4.1. Prologue

In the previous Chapter, we demonstrated the relevance of endosymbionts in the adaptation processes of the host and vice-versa, specifically showing that *Wolbachia* drives and is driven by host evolution.

These endosymbionts are normally maintained by vertical transmission. However, for many years, it has been acknowledged that horizontal transmission must also occur. In recent years, evidence has accumulated showing, in insects mostly, that horizontal transfer of secondary endosymbionts is pervasive and may entail significant morphological, physiological and ecological consequences to the novel host. Strikingly, this phenomenon could even aid speciation, not only in unicellular organisms, but also in a wide variety of metazoans, namely arthropods.

Many recent revisions in the literature have focused on specific evidence for these processes in aphids or specific *Drosophila* species and their respective endosymbiotic partners. However, this old idea, although revisited, had not been itself the focus of systematic coverage of the experimental data under a unifying conceptual framework. Thus, it was one of our objectives to revisit and systematize the available information, putting together a coherent and testable path for a mechanistic understanding of this phenomenon.

Moreover, in arthropods, many intracellular bacteria can be established and perpetuated, colonizing the germline in the course of embryogenesis. Later in this chapter, we will address what may be the evolutionary meaning a large number of endosymbionts being located in numerous somatic tissues, namely malpighian tubules. A key finding that provides hints to answer this question is the capacity of the facultative endosymbiont *Wolbachia* to colonize the germline in female flies, transiting

from the haemolymph. In the literature there is several indirect evidence to support the theoretical potential of somatic *Wolbachia* to be vertically transmitted by accessing the reproductive system. Thus, in this Chapter, we will also explore and highlight the phenotypic evidence and possible evolutionary consequences of the presence of endosymbionts in somatic tissues of hosts, namely the relationship between *Wolbachia* and *Drosophila*.

Finally, our objective in this work was also to approach, in a practical way, the question on horizontal transfer of the endosymbiont *Wolbachia*, namely by identifying and dissecting the mechanisms through which this (and other) symbionts breach the species barrier. To this aim, we tested cannibalism as a candidate mechanism to link a transient new infection with the stabilization of vertical transmission in a novel host, intra- or interspecifically.

We believe that this approach, revisiting and generating both data and ideas, contribute to a new perspective on the mechanisms and consequences of horizontal transmission of endosymbionts. Furthermore, a deeper understanding of these processes is of the essence in coming to a full understanding of the role that symbionts may have in population diversification and speciation.

4.2. Novel endosymbioses as a catalyst of fast speciation

Vitor G. Faria[1#], Élio Sucena[1,2#]

1 - Instituto Gulbenkian de Ciência, Apartado 14, 2781-901 Oeiras, Portugal

2 - Departamento de Biologia Animal, Faculdade de Ciências, Universidade de Lisboa, edifício C2, Campo Grande, 1749-016 Lisboa, Portugal

- Email: vfaria@igc.gulbenkian.pt, esucena@igc.gulbenkian.pt

keywords: endosymbiosis, speciation, arthropod evolution, *Wolbachia*, vertical transmission, horizontal transmission

This article was published in **Reticulate Evolution** Book (Symbiogenesis, Lateral Gene Transfer, Hybridization and Infectious Heredity –pp.107–120). Peer-Reviewed. Springer 2015.
DOI - 10.1007/978-3-319-16345-1_4.

This article is available online through the **Springer** webpage (http://www.springer.com/de/book/9783319163444).

Author Contributions: Conceived and designed: V.G.F., É.S. Contributed reagents/ materials/analysis tools: É.S. Wrote the paper: V.G.F., É.S.

The authors declare no conflict of interest.

Funding**:** Fundação para a Ciência e a Tecnologia (FCT, Portugal) supports V.G.F (#SFRH/BD/82299/2011). This work was supported by Instituto Gulbenkian de Ciência/Fundação Calouste Gulbenkian and FCT (#POCTI/BIA -BDE/60950/2004) to É.S. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Abstract

Many symbiotic bacteria complete their life cycle inside eukaryotic cells. In arthropods, facultative endobacteria such as *Wolbachia* and *Spiroplasma,* influence enormously the ecology and evolution of their hosts. In the last decades, the idea that endosymbiotic coevolution can lead to host speciation has been proposed and, in some instances, verified. However, although usually transmitted vertically, these bacteria can also change host through horizontal transmission. After this transfer and in a virtually instantaneous fashion, endobacteria can alter the fitness of their new host by modifying its response to the environment and/or manipulating its reproduction. In this light, horizontally transmitted endosymbionts could direct the evolutionary path taken by their new hosts. Here, we argue that from this evidence emerges a five-step scenario for the appearance of novel host lineage, in a systematic and testable manner.

Endosymbiosis in a symbiotic world

Symbiosis is the generic terminology to classify close, and in general long-term biological interactions between organisms of different species, conferring a benefit or disadvantage to at least one of them [1]. Many symbiotic relationships have been reported, revealing dynamic interactions pivotal to the evolution of species and their ecology [2, 3]. Both within and between Kingdoms, permanent or sporadic associations can be found linking organisms of different species in a range of habitats and environments [4, 5]. Unlike ectosymbionts, which establish themselves in the host's body surface, endosymbionts are lodged in the host's tissues or organs, intra or extracellularly [2]. These associations persist across generations by vertical transmission (maternal and/or paternal) which is directly inherited (like endobacteria in fungi [6] and a number of endosymbionts in invertebrates [7]) or by horizontal indirect transmission, where associations are formed *de novo* (as mycorrhization and root nodulation [8]). Yet, endosymbiosis can be obligate or facultative (for one or both partners), according to the necessity for presence of the symbiotic partner to the completion of the host's life cycle completion [4].

Here, we will focus on intracellular symbiotic relationships between different multicellular hosts and facultative endobacteria, which can be horizontally transmitted to individuals of other populations or species. We will look in detail, mostly in insects, at the potential of endosymbiosis of facultative intracellular bacteria to enable sudden phenotypic change in novel multicellular hosts [9] as well as to potentially facilitate rapid speciation processes through reproductive manipulations of the host [10].

Below, we will systematize a large yet disperse body of evidence, which in principle supports a five-step scenario where the establishment of new endosymbioses can lead to the emergence of new host (incipient) species. We will review the literature and show that i) endosymbiosis is a

common phenomenon, ii) the presence of the endosymbiont in the host frequently affects its fitness; iii) horizontal transmission of endosymbionts is likely; iv) vertical transmission may ensue and thus leading to a stable phenotype; and v) the presence of endobacteria in the new host induces significant phenotypic change with fitness consequences that may promote directional or disruptive selection. We will organize and link evidence, which support this five-step scenario and argue that the establishment of endosymbioses may culminate in fast speciation events.

1- Widespread coevolution between endosymbionts and multicellular hosts

When looking at the three most well-defined Kingdoms of the Eukarya Domain, we can relate them directly to the establishment of bacterial endosymbiosis [11, 12]. Organelles that have co-evolved intracellularly until becoming mutually obligate from free-living prokaryotes, ensure the main oxidative metabolism of Fungi, Plants and Animals [13, 14].

In addition to the bacterial endosymbionts which gave rise to organelles of eukaryotic cells, endosymbiotic relationships can be found in all kingdoms of the Eukarya Domain (in higher or lower frequency) and it is likely that many are yet to be discovered, together with the full-range of phenotypic changes they may cause [4, 15, 16]. In Fungi, we can find many symbiotic relationships with intracellular bacteria [5]. The majority of them include species of the Phylum Glomeromycota, which is composed of arbuscular mycorrhizal fungi [17] and Geosiphon [18]. In other Phyla, there are at least two other associations described: the basidiomycete *Laccaria bicolor* [19] and *Rhizopus microsporus* of the Phylum Zygomicota [20]. In Plants, most relationships established with bacteria occur via nodulation. The bacteria *Frankia* and *Rhizobia* are the most recurrent ones, establishing

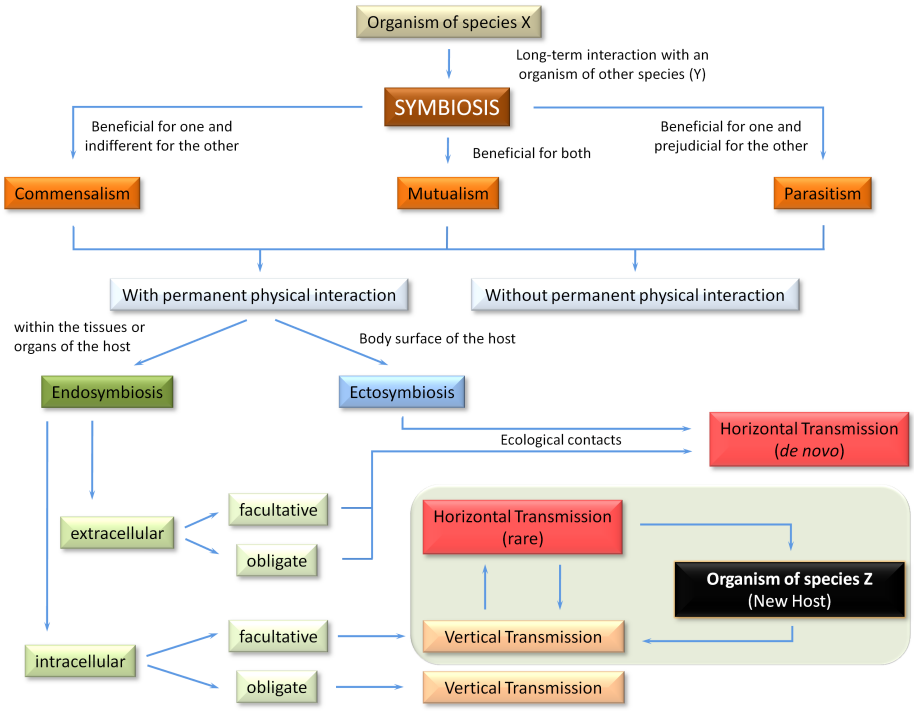


Figure 4.2.1 – Symbiotic relationships and the potential emergence of a novel host lineage. When two organisms of different species are stably related in nature, we are in the presence of a symbiotic relationship. These can exist in several combinatorial outputs between the interacting agents, with or without permanent physical interactions. Both ectosymbiosis and extracellular endosymbiosis are indirectly maintained by intraspecific horizontal transmission, where symbiosis occurs *de novo* in future generations through intimate environmental contacts. In intracellular endosymbiosis we have maternal and/or paternal vertical transmission of obligate endosymbionts, which are necessary partners for the host's development and reproduction. Here, the partners have strong co-evolution and the endosymbionts' diversification is consistent with the diversification of host populations. Facultative endosymbionts, despite being transmitted by vertical transmission, present occasional horizontal transmission within and between host species. This horizontal transmission of intracellular endosymbionts to a new host may create an organism bearing an immediate novelty, which is now subjected to new environmental selective pressures and may strive in a new lineage.

themselves in the root and forming nodules, mutualistically exchanging nutrients with plants [21]. Moreover, several bacteria species are responsible for follicular nodulation in plants of the Myrsinaceae and Rubiaceae families, a type of association less frequent and less studied than those of the root [22]. In animals, many of the endosymbiotic relationships involve bacteria as illustrated by the enormous abundance of reported associations between endobacteria and invertebrates [23]. Within this group, the most studied are the endosymbionts of arthropods, mainly insects.

Some endobacteria are presented as obligate for the host, resulting from close co-evolution with the host species and, thereafter, a corresponding diversification [24]. Usually, these endosymbionts, also called primary endosymbionts, lodge in a bacteriome and produce essential nutrients for the host [25]. On the other hand, facultative (or secondary) endobacteria are found in several cells of various host tissues, being able to infect organisms that already have obligate endobacteria [7]. In addition to being transmitted vertically, and unlike obligate endosymbionts that are entirely dependent on the host for perpetuation, facultative bacteria are also occasionally transmitted horizontally within and between host species and typically show a short evolutionary history with the current host (for review see [7]). We argue that this horizontal transmission phenomenon can bring new hosts into a process of rapid speciation with high impact in the evolution of host species (Figure 4.2.1).

2 - Fitness consequences of the presence of facultative endosymbionts

The many bacteria that complete their life cycle within eukaryotic cells constitute a fully polyphyletic group that exerts a wide range of effects on their hosts [15]. One of the most extreme consequences of this symbiotic

interaction is the manipulation of the host's reproduction, an important factor in several evolutionary processes, namely in ecologic specialization and speciation [26, 27].

The intracellular bacterium *Wolbachia* is the most pandemic symbiont in arthropods and is predominantly transmitted through the female germline. *Wolbachia* exhibits an extraordinary ability to alter the host's reproduction to selectively favour infected females, thus facilitating its maternal transmission. *Wolbachia* causes four distinct reproductive phenotypes in a range of arthropod orders: feminization, where genetic males develop as females through *Wolbachia*'s interference with the sex-determination pathway; parthenogenesis, where males are no longer required for reproduction through disruption the host's cell cycle by the bacterium; male killing, where infected males are eliminated to the advantage of surviving *Wolbachia*-infected female siblings; cytoplasmic incompatibility (CI), that reduces or prevents infected males from producing viable zygotes with females with the same infection *status* (for review see [28]). CI manipulation, the most frequently found *Wolbachia*-induced phenotype, creates an incompatibility between sperm and egg by alteration of the pronuclear envelope breakdown speed, resulting in loss of sperm chromosomes following fertilization [29]. In *Aedes albopictus* mosquitos, *Wolbachia*-infected females are at a reproductive advantage relative to uninfected females due to both CI and a fitness increase (longevity, fecundity and egg hatch) associated with *Wolbachia* infection [30]. In *D. mauritiana* infection with *Wolbachia* increases fecundity substantially through a boost of cell division and decrease of apoptosis of germline stem cells [31]. In others bacterial groups, the helical gram-positive bacterium *Spiroplasma* or the bacterium *Cardinium* can also confer a variety of fitness effects and induce host phenotypic alterations by reproductive manipulation [26, 32].

Facultative endosymbionts can also influence their hosts' defences against natural enemies [33, 34] and specialization to different plant species (for review see [35]). In the pea aphid, *Acyrthosiphon pisum*, the endosymbiotic association with facultative bacteria confers resistance to attack by the parasitoid wasp, *Aphidius ervi*, causing high mortality of developing parasitoid larvae [36]. Subsequently, it was shown that one of the common facultative symbionts of *A. pisum*, the bacterium *Regiella insecticola*, has a major effect on the resistance of the host to a fungal pathogen and lowers its rate of transmission [37]. Recently, some studies have demonstrated that the presence of *Wolbachia* can also increase the fitness of the host. In *Drosophila melanogaster*, infection with *Wolbachia* increases resistance to RNA viruses such as *Drosophila* C virus, a natural pathogen of *Drosophila* [38, 39]. Furthermore, it was shown that *Spiroplasma* protects *Drosophila neotestacea* against the sterilizing effects of a parasitic nematode, underscoring the potential impact of facultative endosymbioses in the ecological distribution and population dynamics of the host species [40, 41]. These data support the notion that the response of a host to environmental conditions also depends on its resident endobacteria.

3 - Secondary endobacteria are horizontally transmitted between hosts

As stated above, facultative endobacteria are mostly vertically transmitted to the progeny. However, since in many cases there is no concordance between the phylogeny of bacteria and their hosts, there is indication of horizontal transmission [42–45]. It is conceivable that in an environment inhabited by organisms infected and non-infected with bacteria, given enough time, high densities and reiterated contacts, the probability of horizontal transmission of symbionts is not negligible [46,

47]. Moreover, several studies have demonstrated that some microbial symbionts retain a generalized ability to infect multiple hosts [48–51].

In *Drosophila*, the only heritable endosymbionts described thus far are *Wolbachia* and *Spiroplasma* [52]. Recently, a phylogenetic analysis of *Spiroplasma* from several *Drosophila* species revealed at least five independent introductions of four phylogenetically distinct *Spiroplasma* haplotypes, indicating imperfect vertical transmission in host populations and likely horizontal transmission [53]. Likewise, *Wolbachia* molecular phylogenies are not concordant with those of their hosts, supporting occasional events of horizontal transmission [25, 54, 55]. Additionally, it has been demonstrated that *Wolbachia* is able to establish itself as a stable and vertically transmitted infection upon transfer into the hemolymph of uninfected *D. melanogaster* females [56].

Parasitoid insects constitute a prime candidate for acting as vectors of *Wolbachia* horizontal transmission. Some studies revealed extensive similarities between the *Wolbachia* strains found in parasitoids and their hosts [42], strongly supporting the hypothesis of natural *Wolbachia* transfer into other species. Another putative vector for horizontal transmission of endosymbionts are parasitic mites. Indeed, ectoparasitic mites have been shown to transfer *Spiroplasma poulsonni* from infected *D. nebulosa* to *D. willistoni* whose females will, subsequently, transmit the infection to their offspring [57]. Thus, endosymbiotic facultative bacteria show a clear propensity to establish promiscuous relationships with various intra and interspecific hosts.

4 – Endosymbiont-associated traits are transferred to the new host and maintained by bacterial vertical transmission

As we have seen, even though we currently do not fully understand the ecological mechanisms for horizontal transmission of facultative

endosymbionts, there is ample evidence that it occurs. In this section, we will provide evidence that these endosymbionts may bring instant metabolic or internal morphological novelty to their novel host, usually the same phenotypic alteration that was induced in the previous host [50, 58, 59]. Additionally, the endobacteria which change host species, undergoing strong selection for their permanence in the new host [60], can ensure the evolutionary sustainability by maintenance or acquisition of stable vertical transmission [61, 62]. Multiple independent lines of evidence support this scenario.

Three species of vertically transmitted Gammaproteobacteria from different aphid host species can infect, spread and induce variation in fitness of the host, when microinjected into a new aphid host (the pea aphid *Acyrthosiphon pisum*), as well as sustain stable vertical transmission to its offspring [63]. Recent data reinforce the potential of facultative endosymbioses in modifying the aphid phenotype. Leonardo and Mondor have shown that the endosymbiont *Regiella insecticola* can manipulate polyphenic development by changing the number of winged vs non-winged individuals under crowding, as well as the time of sexual maturation [64]. More recently, an interspecific transfection of this endosymbiont from the pea aphid to the vetch aphid *Megoura crassicauda*, has proven sufficient to confer the ability to utilize clover as a host plant [65]. In yet another example with the pea aphid, it has been shown that the presence of an endosymbiont of the genus *Rickettsiella* is sufficient to change body colour and may affect host fitness by influencing interactions with both predators and other endosymbionts [66].

Further, when male-killing *Spiroplasma* from coccinellid beetles was artificially injected into a series of naive arthropod species, this bacterium colonized host tissues and was vertically transmitted in all cases tested. Moreover, both the bacteria's efficiency of transmission and its ability to

distort offspring sex ratios in novel hosts were unaffected in the case of transfers to the native genus and reduced or incomplete in more distantly related species [67].

In *Wolbachia*, in yet another case of putative reiterated horizontal transmission, it was shown that a male-killing *Wolbachia* strain has consistent phenotypic effects in *Drosophila borealis* and its closely related species [68]. In other examples, as in the butterfly *Hypolimnas bolina*, the male killing effect of *Wolbachia* presence is suppressed without significant reduction in bacterial load. In this case, *Wolbachia* induces CI in the surviving males [69]. Similarly, the wCauA strain of *Wolbachia*, which induces CI in the lepidopteran *Cadra cautella*, causes male killing upon transfer to *Ephestia kuhniella* [70, 71]. Another example comes from the interaction between *Wolbachia* and *Trichogramma* where uninfected immature wasps that acquired *Wolbachia* while inside the host egg displayed a parthenogenetic phenotype [50]. These examples suggest plasticity in the deployment of *Wolbachia´*s large arsenal of host reproduction manipulation strategies upon horizontal transfer, and consequently in its adaptation to novel hosts.

In summary, evidence is abundant for the stabilization of *de novo* endosymbionts through vertical transmission upon seemingly rare episodes of horizontal transfer. Furthermore, in many of these instances such newly established relationships will have instantaneous phenotypic effects with impact in the fitness of the host, thus having the potential to drive evolutionary change.

5 - Host speciation and endosymbiont-induced novelties

As seen above, when the genetic system of a bacterial species is combined with that of another (arthropod) species through horizontal transmission, the new symbiotic partnership may create novel forms of

coping with selective pressures in the environment. In particular, when a mechanism of reproductive manipulation is brought by endobacteria from a former host, the endosymbiont may trigger a rapid speciation in the new host [72, 73].

The presence of *Wolbachia* in two closely related species of parasitic wasps severely reduces the frequency of hybrid offspring through bidirectional CI in interspecific crosses, and preceded the evolution of other postmating reproductive barriers [74]. In addition, bidirectional CI in host populations may: i) substantially reduce gene flow [75]; ii) reinforce genetic divergence by association between nuclear alleles and respective microbe infection state [76]; iii) increase behavioural isolation from the *Wolbachia*-infected species and/or lead to behavioural isolation between populations of the uninfected species [77]. Yet, other mechanisms may contribute to gene flow reduction between infected and uninfected individuals such as assortative mating and oviposition site preference [78]. Also incipient isolation is observed between the sister species *Drosophila recens* and *Drosophila subquinaria*, via the combined action of CI, prezygotic isolation and hybrid sterility [79, 80]. Taken together, these results support the view that facultative endosymbionts may, directly and indirectly, contribute to reproductive isolation and promote speciation of their hosts (for review, see [26, 73, 81, 82]).

Closing the circle: from a different organism to a new lineage

In intracellular bacteria, the mechanism of vertical transmission is essential to the unity of the symbiotic complex and for the co-evolution of increased benefits for both species (which ultimately may transform the bacterium into an organelle). Prior to this, ecological interactions may foster the transfer of these bacteria between hosts, within or across species. This horizontal transmission creates points of contact between

evolutionary paths and produces new synergistic combinations of phenotypic variation between organisms (morphological and/or metabolic) with direct fitness impacts and adaptive potential. Indeed, it is reasonable to assume that, as in many of the examples presented above, in some cases the mere presence of the endosymbiont will contribute to reproductive isolation and promote speciation of its host. In these circumstances, this cyclic chain of bacterial transmission would contribute to catalyze evolution by creating organisms with new phenotypes, which would be founders of new lineages (Figure 4.2.2).

A recent report has confirmed that endosymbionts can combine developmental modifications and reproductive manipulations, which translate into high fitness gains. In a six-year period in nature and in few generations in the lab, *Rickettsia* bacteria were able to increase their prevalence from a small percentage of individuals to a rampant infection in *B. tabaci* populations (sweet potato whiteflies) [83]. Although the selective pressure that causes this difference in fitness is unknown, this report illustrates that an endosymbiotic partner (and its associated benefits) present in low frequencies can sweep through the population and, in some cases, potentially create reproductive isolation within or between species. This recent data reinforces the possibility of trying to recreate rapid selection followed by isolation in the laboratory, putting our five-step scenario into a direct test.

Moreover, much can be learned on the ecological consequences of the rapid emergence of novel lineages: for example, on the evolution of plant lineages with the appearance of new pollinator species; on rapid changes in the food chain in a particular habitat; or on the dissemination of new strategies such as evolutionary induction of parthenogenesis in new species. Nonetheless, and despite the fact that each of the steps necessary for the formation of new lineages can happen quickly, the minimum time

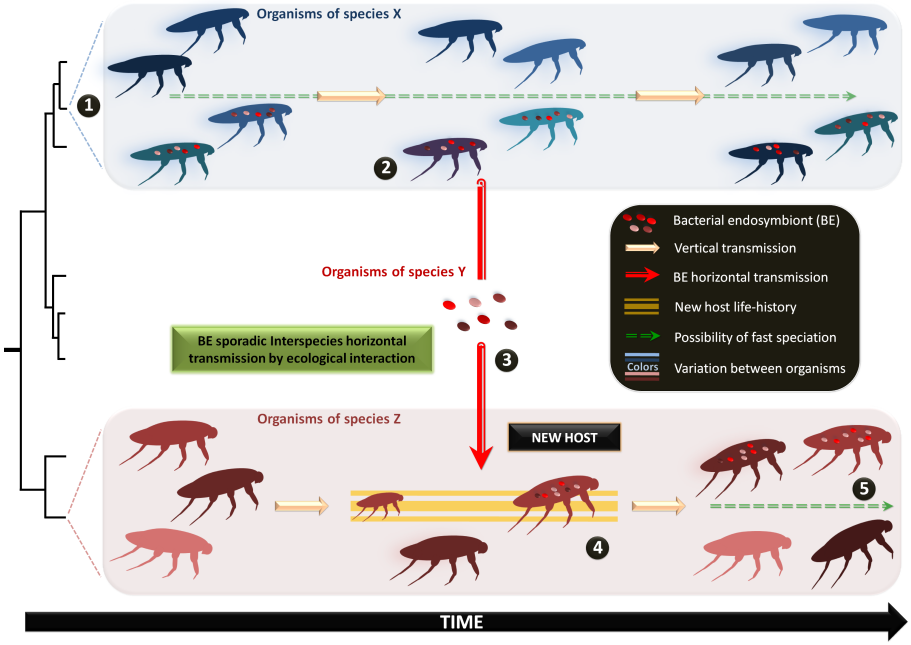


Figure 4.2.2 – Five-step scenario for the fast emergence of new lineages. (**1**) A population of species X contains infected and uninfected individuals that inherit bacterial endosymbionts (BE) of the Y species by vertical transmission (sexual - maternal and/or paternal - or asexual). (**2**) The presence of BE can bring reproductive modifications and/or metabolic advantages to the host. (**3**) Ecological interactions (for example predation, cannibalism or parasitic vectors, such as wasps and mites) may facilitate the horizontal transmission of the BE to a new species. (**4**) The endosymbiont will impact the phenotype and fitness of its new host and, if this transmission takes place during the reproductive age of the host, there may be stable vertical transmission to the next generation. (**5**) Through a mechanism of sexual manipulation (or others), which may have co-evolved with the previous host, BE may induce reproductive modifications on its new host, leading to rapid speciation.

required to complete this sequential scenario is not known. Thus, the real impact this mechanism has in driving evolutionary change and speciation in nature remains to be determined. An unexplored way to approach this

question is looking at a vast range of endosymbionts that have no phylogenetic concordance with their hosts and map onto the phylogenies the events of horizontal transmission. Thus, it could be possible to compare the speciation rates throughout the evolution of the hosts' lineages before and after the transmission of endosymbionts.

Our current state of knowledge on some of the underlying mechanisms of reproductive manipulation, developmental change and behavioural modulation by facultative endosymbionts is paving the way to approach putative processes of rapid speciation in the laboratory upon endosymbiont horizontal transmission. We argue that the time is right to test experimentally the real potential of the role of facultative endosymbionts in speciation through the controlled manipulation of partners and their relationships in customized novel endosymbioses.

Acknowledgements

The authors wish to thank Filipa Vala, Margarida Matos, Sara Magalhães, Thiago Carvalho, Patrícia Beldade, Pedro Lima, Inês Trancoso and Alexandre Leitão for discussions and the critical reading of the manuscript.

References

1. de Bary A: **The Phenomenon of Symbiosis**. Strasbourg; 1879.
2. Douglas AE: **Symbiotic Interactions**. New York: Oxford University Press; 1994.
3. Nieberding CM, Olivieri I: **Parasites: proxies for host genealogy and ecology?** *Trends in Ecology and Evolution* 2007, **22**:156–165.
4. Moran NA: **Symbiosis**. *Current Biology* 2006, **16**:866–871.
5. Tarkka MT, Sarniguet A, Frey-Klett P: **Inter-kingdom encounters: recent advances in molecular bacterium-fungus interactions**. *Current Genetics* 2009, **55**:233–243.
6. Bianciotto V, Genre A, Jargeat P, Lumini E, Becard G, Bonfante P: **Vertical transmission of endobacteria in the arbuscular mycorrhizal fungus *Gigaspora margarita* through generation of vegetative spores**. *Applied and Environmental Microbiology* 2004, **70**:3600–3608.
7. Moran NA, McCutcheon JP, Nakabachi A: **Genomics and evolution.of heritable bacterial symbionts**. *Annual Review of Genetics* 2008, **42**:165–190.
8. Lima PT, Faria VG, Patraquim P, Ramos AC, Feijo JA, Sucena É: **Plant-microbe symbioses: new insights into common roots**. *Bioessays* 2009, **31**:1233–1244.
9. White JA: **Caught in the act: rapid, symbiont-driven evolution: endosymbiont infection is a mechanism generating rapid evolution in some arthropods-but how widespread is the phenomenon?** *Bioessays* 2011, **33**:823–829.
10. Hurst GDD, Schilthuizen M: **Selfish genetic elements and speciation**. *Heredity* 1998, **80**:2–8.
11. Sapp J: **Evolution by association : a history of symbiosis**. New York: Oxford University Press; 1994.
12. de Duve C: **The origin of eukaryotes: a reappraisal**. *Nature Reviews Genetics* 2007, **8**:395–403.
13. Osteryoung KW, Nunnari J: **The division of endosymbiotic organelles**. *Science* 2003, **302**:1698–1704.

14. Dyall SD, Brown MT, Johnson PJ: **Ancient invasions: from endosymbionts to organelles**. *Science* 2004, **304**:253–257.

15. Moran NA, Wernegreen JJ: **Lifestyle evolution in symbiotic bacteria: insights from genomics**. *Trends in Ecology and Evolution* 2000, **15**:321–326.

16. Faria VG, Sucena É: ***Wolbachia* in the malpighian tubules: evolutionary dead-end or adaptation?** *Journal of Experimental Zoology Part B: Molecular and Developmental Evolution* 2013, **320**:195–199.

17. Parniske M: **Arbuscular mycorrhiza: the mother of plant root endosymbioses**. *Nature Reviews Microbiology* 2008, **6**:763–775.

18. Gehrig H, Schussler A, Kluge M: **Geosiphon pyriforme, a fungus forming endocytobiosis with Nostoc (cyanobacteria), is an ancestral member of the Glomales: evidence by SSU rRNA analysis**. *Journal of Molecular Evolution* 1996, **43**:71–81.

19. Bertaux J, Schmid M, Prevost-Boure NC, Churin JL, Hartmann A, Garbaye J, Frey-Klett P: **In situ identification of intracellular bacteria related to *Paenibacillus* spp. in the mycelium of the ectomycorrhizal fungus *Laccaria bicolor* S238N**. *Applied and Environmental Microbiology* 2003, **69**:4243–4248.

20. Partida-Martinez LP, Hertweck C: **Pathogenic fungus harbours endosymbiotic bacteria for toxin production**. *Nature* 2005, **437**:884–888.

21. Oldroyd GE, Downie JA: **Coordinating nodule morphogenesis with rhizobial infection in legumes**. *Annual Review of Plant Biology* 2008, **59**:519–546.

22. Lersten NR, Horner HT: **Bacterial leaf nodule symbiosis in amgiosperms with emphasis on Rubiaceae and Myrsinaceae**. *Botanical Review* 1976, **42**:145–214.

23. Ruby EG: **Symbiotic conversations are revealed under genetic interrogation**. *Nature Reviews Microbiology* 2008, **6**:752–762.

24. Baumann P: **Biology bacteriocyte-associated endosymbionts of plant sap-sucking insects**. *Nature Reviews Microbiology* 2005, **59**:155–189.

25. Werren JH, O'Neill SL: **The evolution of heritable symbionts**. In *Influential passengers: inherited microorganisms and arthropods*

reproduction. Edited by O'Neill SL, Hoffman AA, Werren JH. Oxford: Oxford University Press; 1997:1–41.

26. Engelstadter J, Hurst GDD: **The ecology and evolution of microbes that manipulate host reproduction**. *Annual Review of Ecology, Evolution and Systematics* 2009, **40**:127–149.

27. Tsuchida T, Koga R, Fukatsu T: **Host plant specialization governed by facultative symbiont**. *Science* 2004, **303**:1989.

28. Werren JH, Baldo L, Clark ME: ***Wolbachia*: master manipulators of invertebrate biology**. *Nature Reviews Microbiology* 2008, **6**:741–751.

29. Tram U, Sullivan W: **Role of delayed nuclear envelope breakdown and mitosis in *Wolbachia*-induced cytoplasmic incompatibility**. *Science* 2002, **296**:1124–1126.

30. Dobson SL, Rattanadechakul W, Marsland EJ: **Fitness advantage and cytoplasmic incompatibility in *Wolbachia* single- and superinfected *Aedes albopictus***. *Heredity* 2004, **93**:135–142.

31. Fast EM, Toomey ME, Panaram K, Desjardins D, Kolaczyk ED, Frydman HM: ***Wolbachia* enhance *Drosophila* stem cell proliferation and target the germline stem cell niche**. *Science* 2011, **334**:990–992.

32. Zchori-Fein E, Gottlieb Y, Kelly SE, Brown JK, Wilson JM, Karr TL, Hunter MS: **A newly discovered bacterium associated with parthenogenesis and a change in host selection behavior in parasitoid wasps**. *Proceedings of the National Academy of Sciences of the United States of America* 2001, **98**:12555–12560.

33. Gil-Turnes MS, Hay ME, Fenical W: **Symbiotic marine bacteria chemically defend crustacean embryos from a pathogenic fungus**. *Science* 1989, **246**:116–118.

34. Hurst GDD, Hutchence KJ: **Host Defence: getting by with a little help from our friends**. *Current Biology* 2010, **20**:806–808.

35. Oliver KM, Degnan PH, Burke GR, Moran NA: **Facultative symbionts in aphids and the horizontal transfer of ecologically important traits**. *Annual Review of Entomology* 2010, **55**:247–266.

36. Oliver KM, Russell JA, Moran NA, Hunter MS: **Facultative bacterial symbionts in aphids confer resistance to parasitic wasps**. *Proceedings of the National Academy of Sciences of the United States of America* 2003, **100**:1803–1807.

37. Scarborough CL, Ferrari J, Godfray HC: **Aphid protected from pathogen by endosymbiont**. *Science* 2005, **310**:1781.

38. Teixeira L, Ferreira A, Ashburner M: **The bacterial symbiont *Wolbachia* induces resistance to RNA viral infections in *Drosophila melanogaster***. *PLoS Biology* 2008, **6**:e2.

39. Hedges LM, Brownlie JC, O'Neill SL, Johnson KN: ***Wolbachia* and virus protection in insects**. *Science* 2008, **322**:702.

40. Jaenike J, Unckless R, Cockburn SN, Boelio LM, Perlman SJ: **Adaptation via symbiosis: recent spread of a *Drosophila* defensive symbiont**. *Science* 2010, **329**:212–215.

41. Jaenike J, Brekke TD: **Defensive endosymbionts: a cryptic trophic level in community ecology**. *Ecology Letters* 2011, **14**:150–155.

42. Vavre F, Fleury F, Lepetit D, Fouillet P, Bouletreau M: **Phylogenetic evidence for horizontal transmission of *Wolbachia* in host-parasitoid associations**. *Molecular Biology and Evolution* 1999, **16**:1711–1723.

43. Thao ML, Clark MA, Baumann L, Brennan EB, Moran NA, Baumann P: **Secondary endosymbionts of psyllids have been acquired multiple times**. *Current Microbiology* 2000, **41**:300–304.

44. Russell JA, Latorre A, Sabater-Munoz B, Moya A, Moran NA: **Side-stepping secondary symbionts: widespread horizontal transfer across and beyond the Aphidoidea**. *Molecular Ecology* 2003, **12**:1061–1075.

45. Ahmed MZ, De Barro PJ, Ren SX, Greeff JM, Qiu BL: **Evidence for horizontal transmission of secondary endosymbionts in the *Bemisia tabaci* cryptic species complex**. *PLoS ONE* 2013, **8**:e53084.

46. Gehrer L, Vorburger C: **Parasitoids as vectors of facultative bacterial endosymbionts in aphids**. *Biology Letters* 2012, **8**:613–615.

47. Le Clec'h W, Chevalier FD, Genty L, Bertaux J, Bouchon D, Sicard M: **Cannibalism and predation as paths for horizontal passage of *Wolbachia* between terrestrial isopods**. *PLoS ONE* 2013, **8**:e60232.

48. Schilthuizen M, Stouthamer R: **Horizontal transmission of parthenogenesis-inducing microbes in *Trichogramma* wasps**. *Proceedings Biological Sciences / The Royal Society* 1997, **264**:361–366.

49. Heath BD, Butcher RD, Whitfield WG, Hubbard SF: **Horizontal transfer of *Wolbachia* between phylogenetically distant insect species by a naturally occurring mechanism**. *Current Biology* 1999, **9**:313–316.

50. Huigens ME, de Almeida RP, Boons PA, Luck RF, Stouthamer R: **Natural interspecific and intraspecific horizontal transfer of parthenogenesis-inducing *Wolbachia* in *Trichogramma* wasps**. *Proceedings Biological Sciences / The Royal Society* 2004, **271**:509–515.

51. Duron O, Wilkes TE, Hurst GDD: **Interspecific transmission of a male-killing bacterium on an ecological timescale**. *Ecology Letters* 2010, **13**:1139–1148.

52. Mateos M, Castrezana SJ, Nankivell BJ, Estes AM, Markow TA, Moran NA: **Heritable endosymbionts of *Drosophila***. *Genetics* 2006, **174**:363–376.

53. Haselkorn TS, Markow TA, Moran NA: **Multiple introductions of the *Spiroplasma* bacterial endosymbiont into *Drosophila***. *Molecular Ecology* 2009, **18**:1294–1305.

54. Jiggins FM, Bentley JK, Majerus ME, Hurst GD: **Recent changes in phenotype and patterns of host specialization in *Wolbachia* bacteria**. *Molecular Ecology* 2002, **11**:1275–1283.

55. Baldo L, Ayoub NA, Hayashi CY, Russell JA, Stahlhut JK, Werren JH: **Insight into the routes of *Wolbachia* invasion: high levels of horizontal transfer in the spider genus *Agelenopsis* revealed by *Wolbachia* strain and mitochondrial DNA diversity**. *Molecular Ecology* 2008, **17**:557–569.

56. Frydman HM, Li JM, Robson DN, Wieschaus E: **Somatic stem cell niche tropism in *Wolbachia***. *Nature* 2006, **441**:509–512.

57. Jaenike J, Polak M, Fiskin A, Helou M, Minhas M: **Interspecific transmission of endosymbiotic *Spiroplasma* by mites**. *Biology Letters* 2007, **3**:23–25.

58. Braig HR, Guzman H, Tesh RB, O'Neill SL: **Replacement of the natural *Wolbachia* symbiont of *Drosophila simulans* with a mosquito counterpart**. *Nature* 1994, **367**:453–455.

59. Veneti Z, Toda MJ, Hurst GD: **Host resistance does not explain variation in incidence of male-killing bacteria in *Drosophila bifasciata***. *BMC Evolutionary Biology* 2004, **4**:52.

60. Vallet-Gely I, Lemaitre B, Boccard F: **Bacterial strategies to overcome insect defences**. *Nature Reviews Microbiology* 2008, **6**:302–313.

61. McGraw EA, Merritt DJ, Droller JN, O'Neill SL: ***Wolbachia* density and virulence attenuation after transfer into a novel host**. *Proceedings of the National Academy of Sciences of the United States of America* 2002, **99**:2918–2923.

62. Mira A, Moran NA: **Estimating population size and transmission bottlenecks in maternally transmitted endosymbiotic bacteria**. *Microbial Ecology* 2002, **44**:137–143.

63. Russell JA, Moran NA: **Horizontal transfer of bacterial symbionts: heritability and fitness effects in a novel aphid host**. *Applied and Environmental Microbiology* 2005, **71**:7987–7994.

64. Leonardo TE, Mondor EB: **Symbiont modifies host life-history traits that affect gene flow**. *Proceedings Biological Sciences / The Royal Society* 2006, **273**:1079–1084.

65. Tsuchida T, Koga R, Matsumoto S, Fukatsu T: **Interspecific symbiont transfection confers a novel ecological trait to the recipient insect**. *Biology Letters* 2011, **7**:245–248.

66. Tsuchida T, Koga R, Horikawa M, Tsunoda T, Maoka T, Matsumoto S, Simon JC, Fukatsu T: **Symbiotic bacterium modifies aphid body color**. *Science* 2010, **330**:1102–1104.

67. Tinsley MC, Majerus ME: **Small steps or giant leaps for male-killers? Phylogenetic constraints to male-killer host shifts**. *BMC Evolutionary Biology* 2007, **7**:238.

68. Sheeley SL, McAllister BF: **Mobile male-killer: similar *Wolbachia* strains kill males of divergent *Drosophila* hosts**. *Heredity* 2009, **102**:286–292.

69. Hornett EA, Duplouy AM, Davies N, Roderick GK, Wedell N, Hurst GD, Charlat S: **You can't keep a good parasite down: evolution of a male-killer suppressor uncovers cytoplasmic incompatibility**. *Evolution* 2008, **62**:1258–1263.

70. Sasaki T, Kubo T, Ishikawa H: **Interspecific transfer of *Wolbachia* between two lepidopteran insects expressing cytoplasmic incompatibility: a *Wolbachia* variant naturally infecting *Cadra***

cautella **causes male killing in** ***Ephestia kuehniella***. *Genetics* 2002, **162**:1313–1319.

71. Sasaki T, Massaki N, Kubo T: ***Wolbachia*** **variant that induces two distinct reproductive phenotypes in different hosts**. *Heredity* 2005, **95**:389–393.

72. Bordenstein SR: **Symbiosis and the origin of species**. In *Insect Symbiosis*. Edited by Bourtzis K, Miller T. New York, CRC Press; 2003:283–304.

73. Coyne JA, Orr HA: *Speciation*. Sunderland, MA: Sinauer Associates; 2004.

74. Bordenstein SR, O'Hara FP, Werren JH: ***Wolbachia*****-induced incompatibility precedes other hybrid incompatibilities in** ***Nasonia***. *Nature* 2001, **409**:707–710.

75. Telschow A, Hammerstein P, Werren JH: **The effect of** ***Wolbachia*** **on genetic divergence between populations: models with two-way migration**. *The American Naturalist* 2002, **160 Suppl 4**:S54–66.

76. Telschow A, Hammerstein P, Werren JH: **The effect of** ***Wolbachia*** **versus genetic incompatibilities on reinforcement and speciation**. *Evolution* 2005, **59**:1607–1619.

77. Jaenike J, Dyer KA, Cornish C, Minhas MS: **Asymmetrical reinforcement and** ***Wolbachia*** **infection in** ***Drosophila***. *PLoS Biology* 2006, **4**:e325.

78. Vala F, Egas M, Breeuwer JA, Sabelis MW: ***Wolbachia*** **affects oviposition and mating behaviour of its spider mite host**. *Journal of Evolutionary Biology* 2004, **17**:692–700.

79. Shoemaker DD, Katju V, Jaenike J: ***Wolbachia*** **and the evolution of reproductive isolation between** ***Drosophila recens*** **and** ***Drosophila subquinaria*****.** *Evolution* 1999, **53**:1157–1164.

80. Jaenike J: **Spontaneous emergence of a new** ***Wolbachia*** **phenotype**. *Evolution* 2007, **61**:2244–2252.

81. Thompson JN: **Symbiont-Induced Speciation**. *Biological Journal of the Linnean Society* 1987, **32**:385–393.

82. Brucker RM, Bordenstein SR: **Speciation by symbiosis**. *Trends in Ecology and Evolution* 2012, **27**:443–451.

83. Himler AG, Adachi-Hagimori T, Bergen JE, Kozuch A, Kelly SE, Tabashnik BE, Chiel E, Duckworth VE, Dennehy TJ, Zchori-Fein E, Hunter MS: **Rapid**

spread of a bacterial symbiont in an invasive whitefly is driven by fitness benefits and female bias. *Science* 2011, **332**:254–256.

4.3. *Wolbachia* in the malpighian tubules: evolutionary dead-end or adaptation?

Vitor G. Faria[1#], Élio Sucena[1,2#]

1 - Instituto Gulbenkian de Ciência, Apartado 14, 2781-901 Oeiras, Portugal

2 - Departamento de Biologia Animal, Faculdade de Ciências, Universidade de Lisboa, edifício C2, Campo Grande, 1749-016 Lisboa, Portugal

- Email: vfaria@igc.gulbenkian.pt, esucena@igc.gulbenkian.pt

keywords: *Wolbachia*, malpighian tubules, Facultative endosymbionts, Horizontal transmission

This article was published in **Journal of Experimental Zoology. Part B, Molecular and Developmental Evolution** (vol. 320, issue. 4, pp195–199). Peer-Reviewed. June 2013.
DOI - 10.1002/jez.b.22498.
PubMed ID - 23671034.

This article is available online through the JEZ webpage (http://onlinelibrary.wiley.com/doi/10.1002/jez.b.22498/abstract).

Author Contributions: Conceived and designed: V.G.F., É.S. Contributed reagents/ materials/analysis tools: É.S. Wrote the paper: V.G.F., É.S.

The authors declare no conflict of interest.

Funding**:** Fundação para a Ciência e a Tecnologia (FCT, Portugal) supports V.G.F (#SFRH/BD/82299/2011). This work was supported by Instituto Gulbenkian de Ciência/Fundação Calouste Gulbenkian and FCT (#POCTI/BIA-BDE/60950/2004) to É.S. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Abstract

Facultative endosymbionts, such as *Wolbachia*, perpetuate by vertical transmission mostly through colonization of the germline during embryogenesis. The remaining *Wolbachia* inside the embryo are internalized in progenitor cells of the somatic tissue. This perpetuation strategy triggers a cyclic bacterial bottleneck across host generations. However, throughout the host's life history (*Drosophila*, for example), some somatic tissues such as the malpighian tubules (MTs) show large numbers of *Wolbachia*. It is assumed that *Wolbachia* present in the progenitor cells of the MTs are confined to this somatic tissue, implicitly considering MTs as an evolutionary dead-end for these bacteria. Nevertheless, the fact that bacteria can survive and proliferate inside MTs suggests a different fate as they may access the host's reproductive system and persist in the host population through vertical transmission. Indeed, based on the particular physiological and developmental characteristics of MT, as well as of *Wolbachia*, we argue the bacteria present in the MTs may constitute a secondary pool of vertically transmitted bacteria. Moreover, somatic pools of *Wolbachia* capable of reaching the gonads and insure vertical transmission may also provide an interesting element to the elucidation of horizontal transmission mechanisms. Finally, we also speculate that somatic pools of *Wolbachia* may play an important role in host fitness, namely during viral infections. In brief, we argue that the somatic pools of *Wolbachia*, with special emphasis on the MT subset, deserve experimental attention as putative players in the physiology and evolution of both bacteria and hosts.

In arthropods, many intracellular bacteria, either obligatory or facultative for host viability, can be established and perpetuated [1, 2]. Among these, facultative endobacteria such as *Wolbachia* colonize the germline in the course of embryogenesis insuring their vertical transmission [3, 4]. Importantly, *Wolbachia* can directly manipulate the host's reproduction in ways that favour its own rate of transmission [5–7]. Nonetheless, there are also large amounts of endobacteria in numerous somatic tissues [8–12]. Yet, this aspect of *Wolbachia*-host interactions has been repeatedly overlooked. Here, using the available disperse data on this interaction between *Wolbachia* and its hosts (particularly *Drosophila*), we speculate on the physiological and evolutionary roles these bacterial populations may have, with particular emphasis on the bacteria present in the malpighian tubules (MTs).

MTs have been mostly studied developmentally and for their vital physiological roles in the maintenance of homeostasis (including osmoregulation, detoxification and ion transport) [13, 14]. Yet, and of particular relevance to our hypothesis, the MT (Figure 4.3.1, left panel) has been described repeatedly as a site for somatic intracellular storage of endosymbionts [11, 16–21]. Also, in the *Drosophila* holometabolous context and unlike most tissues, MTs are peculiar in that they maintain their integrity throughout metamorphosis [14, 22]. Below, we will expand further these considerations and discuss adaptive scenarios for both symbiotic partners.

It is consensual that across host generations there is a cyclic bacterial bottleneck whereby only endobacteria located in the germinal tissue will contribute to the next generation [23, 24]. That is, it has been assumed that only *Wolbachia* established in the germline progenitor cell niches will colonize the progeny in the course of embryogenesis. Under this assumption, it stands to reason that bacteria in the MTs would be confined

to this somatic tissue throughout the life of the fly. As a consequence, and despite the large numbers of *Wolbachia* in the MTs [11] possibly caused by the abundance of resources in this tissue, current views have implicitly considered MTs as an evolutionary dead-end for its resident endosymbiotic bacteria.

It is known that the introduction of *Wolbachia* into an uninfected female can cause *de novo* colonization of the ovaries [18, 25]. In line with these observations, although in an in vitro setting, it has been shown that *Wolbachia* can endure prolonged periods in an extracellular environment whilst maintaining its capacity to penetrate novel cellular hosts [26]. More importantly, and despite the few *Wolbachia* present in the hemolymph [11], an uninfected germarium introduced into the abdomen of a *Wolbachia*-infected female adult will be colonized by bacteria present in the body cavity [25]. This means that there are *Wolbachia* pools, of unknown origin, outside of the reproductive tissues and that they can constitute the source of germline infection and vertical transmission. Despite the putative action of the immune system through hemocytes and antimicrobial peptide (AMPs) activity [27] decreasing the *Wolbachia* circulating pool, we may expect a steady release rate of *Wolbachia* into the hemolymph (after, e.g., somatic cell death or active exit of bacteria). Indeed, MTs have been shown to contain stem cell niches which endure high turnover rates [28] in tune with a potential to provide significant numbers of free *Wolbachia* by frequent cell death. The fact that *Wolbachia* can be housed in high numbers within the MTs of larvae and throughout adult life (Figure 4.3.1, right panel) opens the possibility that these bacteria may have the potential to access the reproductive system and be vertically transmitted.

Also, as previously mentioned, *Drosophila* MTs maintain their integrity through metamorphosis [29]. Moreover, this (at least partial) integrity maintenance of MTs throughout the entire insect life cycle has

been established in several Holometabola, namely dipterans [14], coleopterans [30] and lepidopterans [31]. Thus, this seemingly general feature would allow the bacteria lodged intracellularly in the MT cells to transit from the embryo stage to adulthood, avoiding the immune response of *Drosophila*, both phagocytosis and AMPs. In this light, MTs can represent an important somatic source for *Wolbachia* that will secondarily colonize the gonads and germline after embryogenesis.

Taken together the facts listed above lead us to hypothesize that in addition to *Wolbachia* originating from germline colonization during oogenesis, a part of the bacteria incorporated into the germarium may derive from MTs and other somatic tissues, disputing the notion that somatic *Wolbachia* face an evolutionary dead-end. This hypothesis is reminiscent of mechanisms present in other arthropod hosts of obligatory endobacteria, where certain somatic cell types or organs (bacteriocytes) have evolved to act as intermediary safe havens for a later passage of

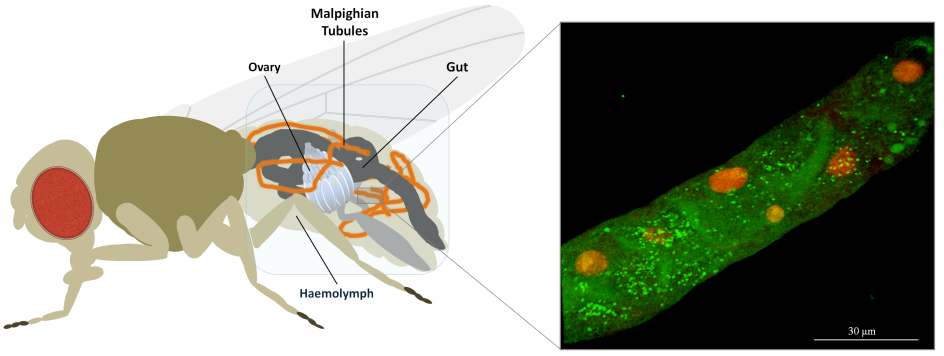


Figure 4.3.1 – *Wolbachia* in Malpighian Tubules (MTs). Left, a schematic representation of MTs in *Drosophila*. Right, under magnification, fluorescence image of MTs showing high numbers of intracellular *Wolbachia* (green dots) relative to uninfected flies (not shown). *Wolbachia* labelling was performed using BacLight, a bacterial viability dye (as in [15]). The flies used have been tested and certified as *Spiroplasma* free (as in [44].

bacteria into the germinal tissue [1, 32]. This point of view was recently explored in the relationship between the bedbug *Cimex lectularius* and *Wolbachia* [33]. Extending this reasoning, it follows that the effective population size of these bacteria increases significantly thereby augmenting the chance that more bacteria (with a putative adaptive value) will be represented in the host's next generation.

Finally, our hypothesis that somatic tissues, particularly the MTs, may have a non-negligible contribution to the vertically transmitted *Wolbachia*, may also provide an interesting element to the elucidation of horizontal transmission mechanisms. It is well established that *Wolbachia* has been transmitted horizontally multiple times throughout arthropod evolution [34, 35] but its underlying mechanisms remain mostly unknown [1]. Yet, it is likely that, whatever mechanism(s) may mediate horizontal transmission, parasitoid wasp infection, mite parasitism or cannibalism, for example, a step that does not involve the germline must occur. Indeed, in most instances eggs and germline are virtually inaccessible, and it is primarily between adults and/or larvae that interspecific interactions that foster *Wolbachia* transfer may occur [36, 37]. In this context, MTs come across as a privileged niche for bacterial growth and persistence. Each tubule has one extremity directly connected to the digestive system (between the intersection of the midgut with the hindgut), while the other end is closed and in contact with the hemolymph (Figure 4.3.1, left panel). The digestive system, in turn, is an important host-environment interface and a recurring entry for microorganisms that are transported passively through the gut after ingestion [38]. Thus, after an eventual *Wolbachia* ingestion by a non-infected individual, larvae or adult, the bacteria will have access not only to the digestive system but also to the interior of the MTs. To perform the functions of maintaining physiological homeostasis of the organism, the 4 MTs of *Drosophila* are extended along the coelomic cavity,

where the cells have a constant activity of endocytosis and exocytosis [39, 40]. This feature may promote *Wolbachia* internalization into the lumen of MTs. Yet, it is still unknown if *Wolbachia* can survive the local and autonomous immune response of the gut epithelium and MTs [41, 42]. All things considered, it is reasonable to consider that somatic pools of *Wolbachia* capable of reaching the gonads and insure vertical transmission can play a pivotal role in turning an improbable horizontal transfer event into a stable vertically transmitted infection.

From the host's perspective, no specific physiological role for this somatic bacterial colony, or others, has been established. Yet, it is conceivable that the host itself, *Drosophila* or other, can provide an opportunistic leverage for *Wolbachia* proliferation. For example, the established protection against stress and natural enemies provided by *Wolbachia* [43–45] may depend on a minimum bacterial population size. If the reproductive tissue cannot accommodate a sufficient number of *Wolbachia* to confer systemic protection, selection would favour somatic niches of bacteria [46] of which MTs stands as a prime candidate. In sum, the large number of *Wolbachia* in MTs could constitute an adaptive feature of the host.

Recent advances on the fitness benefits for hosts provided by *Wolbachia* raise important physiological and evolutionary questions on the nature and dynamics of this relationship [44, 47, 48] and can be extended to other host-endosymbiont associations [49–52]. Yet, the putative role(s) of the somatic populations of *Wolbachia*, particularly the one housed in the MTs throughout development as during adulthood, has been largely overlooked. In other cases and species, different somatic tissues could play a similar function by representing a preferential region for endosymbiont proliferation, thus assuming comparable importance in the evolutionary process.

The hypotheses here presented may serve as a foundation for new integrative studies towards the clarification of the immediate and ultimate causes for the establishment of endosymbiotic stable evolutionary strategies, both in single and multiple infections. In this research program, an important line of enquiry consists on the role(s) played by somatic endosymbiont pools, of *Wolbachia* in particular, and the putative importance of the MTs in this process.

Acknowledgements

The authors wish to thank Luís Teixeira, Inês Trancoso and Alexandre Leitão for discussions and the critical reading of the manuscript.

References

1. Moran NA, McCutcheon JP, Nakabachi A: **Genomics and evolution of heritable bacterial symbionts**. *Annual Review of Genetics* 2008, **42**:165–190.

2. Oliver KM, Degnan PH, Burke GR, Moran NA:. **Facultative symbionts in aphids and the horizontal transfer of ecologically important traits**. *Annual Review of Entomology* 2010, **55**:247–266.

3. Ferree PM, Frydman HM, Li JM, Cao J, Wieschaus E, Sullivan W: ***Wolbachia* utilizes host microtubules and dynein for anterior localization in the *Drosophila* oocyte**. *PLoS Pathogens* 2005, **1**:e14.

4. Serbus LR, Sullivan W: **A cellular basis for *Wolbachia* recruitment to the host germline**. *PLoS Pathogens* 2007, **3**:e190.

5. Charlat S, Hurst GD, Mercot H: **Evolutionary consequences of *Wolbachia* infections**. *Trends in Genetics* 2003, **19**:217–223.

6. Werren JH, Baldo L, Clark ME: ***Wolbachia*: master manipulators of invertebrate biology**. *Nature Review Microbiol*ogy 2008, **6**:741–751.

7. Engelstadter J, Hurst GD: **The ecology and evolution of microbes that manipulate host reproduction**. *Annual Review of Ecology, Evolution and Systematics* 2009, **40**:127–149.

8. Dobson SL, Bourtzis K, Braig HR, Jones BF, Zhou W, Rousset F, O'Neill SL: ***Wolbachia* infections are distributed throughout insect somatic and germ line tissues**. *Insect Biochemistry and Molecular Biology* 1999, **29**:153–160.

9. Cheng Q, Ruel TD, Zhou W, Moloo SK, Majiwa P, O'Neill SL, Aksoy S: **Tissue distribution and prevalence of *Wolbachia* infections in tsetse flies, *Glossina* spp**. *Medical and Veterinary Entomology* 2000, **14**:44–50.

10. Ijichi N, Kondo N, Matsumoto R, Shimada M, Ishikawa H, Fukatsu T: **Internal spatiotemporal population dynamics of infection with three *Wolbachia* strains in the adzuki bean beetle, *Callosobruchus chinensis* (coleoptera: Bruchidae)**. *Applied and Environmental Microbiology* 2002, **68**:4074–4080.

11. Goto S, Anbutsu H, Fukatsu T: **Asymmetrical interactions between *Wolbachia* and *Spiroplasma* endosymbionts coexisting in the same**

insect host. *Applied and Environmental Microbiology* 2006, **72**:4805–4810.

12. Narita S, Nomura M, Kageyama D: **Naturally occurring single and double infection with *Wolbachia* strains in the butterfly *Eurema hecabe*: transmission efficiencies and population density dynamics of each *Wolbachia* strain**. *FEMS Microbiology Ecology* 2007, **61**:235–245.

13. O'Donnell MJ, Maddrell SHP: **Fluid reabsorption and ion transport by the lower malpighian tubules of adult female *Drosophila***. *Journal of Experimental Biology* 1995, **198**:1647–1653.

14. Beyenbach KW, Skaer H, Dow JAT: **The developmental, molecular, and transport biology of malpighian tubules**. *Annual Review of Entomology* 2010, **55**:351–374.

15. Casper-Lindley C, Kimura S, Saxton DS, Essaw Y, Simpson I, Tan V, Sullivan W: **Rapid fluorescence-based screening for *Wolbachia* endosymbionts in *Drosophila* germ line and somatic tissues**. *Applied and Environmental Microbiology* 2011, **77**:4788–4794.

16. Noda H, Munderloh UG, Kurtti TJ: **Endosymbionts of ticks and their relationship to *Wolbachia* spp. and tick-borne pathogens of humans and animals**. *Applied and Environmental Microbiology* 1997, **63**:3926–3932.

17. Kurtti TJ, Palmer AT, Oliver JH: **Rickettsiella-like bacteria in *Ixodes woodi* (Acari: Ixodidae)**. *Journal of Medical Entomology* 2002, **39**:534–540.

18. Ruang-Areerate T, Kittayapong P: ***Wolbachia* transinfection in *Aedes aegypti*: a potential gene driver of dengue vectors**. *Proceedings of the National Academy of Sciences of the United States of America* 2006, **103**:12534–12539.

19. Klyachko O, Stein BD, Grindle N, Clay K, Fuqua C: **Localization and visualization of a coxiella-type symbiont within the lone star tick, *Amblyomma americanum***. *Applied and Environmental Microbiology* 2007, **73**:6584–6594.

20. Bution ML, Caetano FH, Zara FJ: **Contribution of the malpighian tubules for the maintenance of symbiotic microorganisms in cephalotes ants**. *Micron* 2008, **39**:1179–1183.

21. Kleinschmidt B, Kölsch G: **Adopting bacteria in order to adapt to water—how reed beetles colonized the wetlands (coleoptera, chrysomelidae, donaciinae)**. *Insects* 2011, **2**:540–554.

22. Tapadia MG, Shukla A: **Differential localization and processing of apoptotic proteins in malpighian tubules of *Drosophila* during metamorphosis**. *European Journal of Cell Biology* 2011, **90**:72–80.

23. Mira A, Moran NA: **Estimating population size and transmission bottlenecks in maternally transmitted endosymbiotic bacteria**. *Microbial Ecology* 2002, **44**:137–143.

24. Jaenike J, Dyer KA, Reed LK: **Within-population structure of competition and the dynamics of male-killing *Wolbachia***. *Evolutionary Ecology Research* 2003, **5**:1023–1036.

25. Frydman HM, Li JM, Robson DN, Wieschaus E: **Somatic stem cell niche tropism in *Wolbachia***. *Nature* 2006, **441**:509–512.

26. Rasgon JL, Gamston CE, Ren X: **Survival of *Wolbachia pipientis* in cell-free medium**. *Applied and Environmental Microbiology* 2006, **72**:6934–6937.

27. Lemaitre B, Hoffmann J: **The host defense of *Drosophila melanogaster***. *Annual Review of Immunology* 2007, **25**:697–743.

28. Singh SR, Liu W, Hou SX: **The adult *Drosophila* malpighian tubules are maintained by multipotent stem cells**. *Cell Stem Cell* 2007, **1**:191–203.

29. Skaer HB: **Development of the alimentary canal**. In *The development of Drosophila*. Edited by Bate CM, Martinez AA. Cold Spring Harbor, NY: Cold Spring Harbor Lab. Press; 1993:941–1112.

30. Byers JR: **Metamorphosis of the perirectal malpighian tubules in the mealworm *Tenebrio molitor* L. (Coleoptera, Tenebrionidae). II. Ultrastructure and role of autophagic vacuoles**. *Canadian Journal of Zoology* 1971, **49**:1185–1191.

31. Ryerse JS: **Developmental changes in malpighian tubules cell structure**. *Tissue and Cell* 1979, **11**:533–551.

32. Werren JH, O'Neill SL: **The evolution of heritable symbionts**. In *Influential passengers: inherited microorganisms and arthropods reproduction*. Edited by O'Neill SL, Hoffman AA, Werren JH. Oxford: Oxford University Press; 1997:1–41.

33. Hosokawa T, Koga R, Kikuchi Y, Meng XY, Fukatsu T: ***Wolbachia*** **as a bacteriocyte-associated nutritional mutualist**. *Proceedings of the National Academy of Sciences of the United States of America* 2010, **107**:769–774

34. Vavre F, Fleury F, Lepetit D, Fouillet P, Bouletreau M: **Phylogenetic evidence for horizontal transmission of *Wolbachia* in host-parasitoid associations**. *Molecular Biology and Evolution* 1999, **16**:1711–1723.

35. Baldo L, Ayoub NA, Hayashi CY, Russell JA, Stahlhut JK, Werren JH: **Insight into the routes of *Wolbachia* invasion: High levels of horizontal transfer in the spider genus *Agelenopsis* revealed by *Wolbachia* strain and mitochondrial DNA diversity**. *Molecular Ecology* 2008, **17**:557–569.

36. Heath BD, Butcher RD, Whitfield WG, Hubbard SF: **Horizontal transfer of *Wolbachia* between phylogenetically distant insect species by a naturally occurring mechanism**. *Current Biology* 1999, **9**:313–316.

37. Huigens ME, de Almeida RP, Boons PA, Luck RF, Stouthamer R: **Natural interspecific and intraspecific horizontal transfer of parthenogenesis-inducing *Wolbachia* in *Trichogramma* wasps**. *Proceedings Biological Sciences / The Royal Society* 2004, **271**:509–515.

38. Vallet-Gely I, Lemaitre B, Boccard F: **Bacterial strategies to overcome insect defences**. *Nature Reviews Microbiology* 2008, **6**:302–313.

39. Wessing A, Zierold K: **The formation of type-I concretions in *Drosophila* malpighian tubules studied by electron microscopy and X-ray microanalysis**. *Journal of Insect Physiology* 1999, **45**:39–44.

40. O'Donnell MJ, Linton SM: **Novel aspects of the transport of organic anions by the malpighian tubules of *Drosophila melanogaster***. *Journal of Experimental Biology* 2000, **203**:3575–3584.

41. McGettigan J, McLennan RK, Broderick KE, Kean L, Allan AK, Cabrero P, Regulski MR, Pollock VP, Gould GW, Davies SA, Dow JAT: **Insect renal tubules constitute a cell-autonomous immune system that protects the organism against bacterial infection**. *Insect Biochemistry and Molecular Biology* 2005, **35**:741–754.

42. Davies SA, Dow JAT: **Modulation of epithelial innate immunity by autocrine production of nitric oxide**. *General and Comparative Endocrinology* 2009, **162**:113–121.

43. Hedges LM, Brownlie JC, O'Neill SL, Johnson KN: ***Wolbachia*** **and virus protection in insects**. *Science* 2008, **322**:702.

44. Teixeira L, Ferreira A, Ashburner M: **The bacterial symbiont *Wolbachia* induces resistance to RNA viral infections in *Drosophila melanogaster***. *PLoS Biology* 2008, **6**:e2.

45. Brownlie JC, Cass BN, Riegler M, Witsenburg JJ, Iturbe-Ormaetxe I, McGraw EA, O'Neill SL: **Evidence for metabolic provisioning by a common invertebrate endosymbiont, *Wolbachia pipientis*, during periods of nutritional stress**. *PLoS Pathogens* 2009, **5**:e1000368.

46. Osborne SE, Iturbe-Ormaetxe I, Brownlie JC, O'Neill SL, Johnson KN: **Antiviral protection and the importance of *Wolbachia* density and tissue tropism in *Drosophila***. *Applied and Environmental Microbiology* 2012, **78**:6922–6929.

47. Moreira LA, Iturbe-Ormaetxe I, Jeffery JA, Lu G, Pyke AT, Hedges LM, Rocha BC, Hall-Mendelin S, Day A, Riegler M, Hugo LE, Johnson KN, Kay BH, McGraw EA, van den Hurk AF, Ryan PA, O'Neill SL: **A *Wolbachia* symbiont in *Aedes aegypti* limits infection with dengue, *Chikungunya*, and *Plasmodium***. *Cell* 2009, **139**:1268–1278.

48. Fast EM, Toomey ME, Panaram K, Desjardins D, Kolaczyk ED, Frydman HM: ***Wolbachia*** **enhance *Drosophila* stem cell proliferation and target the germline stem cell niche**. *Science* 2011, **334**:990–992.

49. Scarborough CL, Ferrari J, Godfray HC.: **Aphid protected from pathogen by endosymbiont**. *Science* 2005, **310**:1781.

50. Oliver KM, Degnan PH, Hunter MS, Moran NA: **Bacteriophages encode factors required for protection in a symbiotic mutualism**. *Science* 2009, **325**:992–994.

51. Jaenike J, Unckless R, Cockburn SN, Boelio LM, Perlman SJ: **Adaptation via symbiosis: Recent spread of a *Drosophila* defensive symbiont**. *Science* 2010, **329**:212–215.

52. Xie J, Vilchez I, Mateos M. 2010. ***Spiroplasma*** **bacteria enhance survival of *Drosophila hydei* attacked by the parasitic wasp *Leptopilina heterotoma***. *PLoS ONE* 2010, **5**:e12149.

4.4. Testing cannibalism as a mechanism for horizontal transmission of *Wolbachia* in *Drosophila*

Vitor G. Faria[1#], Tânia F. Paulo[1], Élio Sucena[1,2#]

1 - Instituto Gulbenkian de Ciência, Apartado 14, 2781-901 Oeiras, Portugal

2 - Departamento de Biologia Animal, Faculdade de Ciências, Universidade de Lisboa, edifício C2, Campo Grande, 1749-016 Lisboa, Portugal

- Email: vfaria@igc.gulbenkian.pt, esucena@igc.gulbenkian.pt

keywords: *Wolbachia*, horizontal transmission, cannibalism, *Drosophila*, ingestion, endosymbiosis

This article was published in the journal **Symbiosis** (vol. 68, issue. 1, pp79-85). Peer-Reviewed. November 2015.
DOI - 10.1007/s13199-015-0354-y.

This article is available online through the Symbiosis webpage (http://link.springer.com/article/10.1007%2Fs13199-015-0354-y).

Author Contributions: Conceived and designed the experiments: V.G.F., É.S. Performed the experiments: V.G.F., T.F.P., Analyzed the data: V.G.F. Contributed reagents/ materials/analysis tools: É.S. Wrote the paper: V.G.F., É.S.

The authors declare no conflict of interest.

Funding**:** This work was supported by the Fundação para a Ciência e a Tecnologia funds VF (#SFRH/BD/ 82299/2011). This work was supported by Instituto Gulbenkian de Ciência/Fundação Calouste Gulbenkian and FCT (#POCTI/BIA -BDE/60950/2004) to É.S. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Abstract

Wolbachia are intracellular symbionts of many species of animals, mostly arthropods. Vertical transmission of *Wolbachia* is exclusively maternal and this endobacterium promotes reproductive manipulations of its hosts, increasing the fitness of infected females. Moreover, *Wolbachia* provides its hosts with a wide range of adaptive features ranging from protection against viral infections to dietary niche occupancy. Therefore, *Wolbachia* can potentially contribute to the evolutionary processes of sexual selection and speciation. The horizontal transmission of *Wolbachia* is strongly suggested by the non-concordant phylogeny of this endosymbiont and that of its hosts. However, the ecological mechanism(s) responsible for endosymbiont transmission between different hosts is still largely unknown. In the present study, we look at ingestion as a possible natural form of *Wolbachia* horizontal transmission. To this aim, we tested cannibalism between infected and uninfected *Drosophila* hosts, under different conditions of nutrition and gut integrity. Although ingestion represents a general and incontestable portal of entry for microorganisms, we did not find infection by *Wolbachia* in the progeny of cannibal individuals fed on infected flies. Our study suggests that if ingestion is a vehicle for horizontal transmission of *Wolbachia* in nature, either it happens very rarely or it requires other factors or conditions to be effective. We discuss the likeliness of this mechanism with respect to the likelihood of each step necessary for horizontal transmission.

Introduction

The α-proteobacteria of the Genus *Wolbachia* live intracellularly in a variety of animals, including arthropods and nematodes [1, 2]. In arthropods, *Wolbachia* is typically transmitted vertically from mother to offspring. It causes a wide range of reproductive manipulations in different host species whereby increasing the fitness of infected females and, consequently, also increasing its own transmission rate [3]. These mechanisms include: (i) the induction of cytoplasmic incompatibility between individuals that do not share infection status, (ii) the induction of parthenogenesis in diploid females and (iii) the feminization or death of infected males (for revision see [4]). Additionally, recent studies have shown that in *Drosophila melanogaster*, *Wolbachia* infection may also confer an advantage to its host through an increased resistance to RNA virus infection [5, 6].

It is estimated that *Wolbachia* infects 20-80% of insect species [7] possibly making it the most recurrent endosymbiont on the planet. The wide distribution of these bacteria is attributed to the high efficacy of vertical transmission. This efficacy may rely on the usage *Wolbachia* makes of the host's cytoskeleton and intracellular transport system to migrate and ensure its presence within the future embryos [8, 9]. In addition to the colonization of the germline during embryogenesis, *Wolbachia* remaining inside the embryo are internalized in progenitor cells of the somatic tissue [10, 11], with potential physiological and evolutionary consequences [12].

The widespread presence of *Wolbachia* must also rely on horizontal transmission, which can be attested by the presence of close strains of *Wolbachia* in phylogenetically distant hosts [13, 14]. Indeed, unlike mitochondria or obligatory bacterial endosymbionts, the molecular phylogeny of *Wolbachia* is not always concordant with that of its hosts [15, 16]. These well-established patterns raise two important questions: i)

which ecological conditions and mechanisms mediate horizontal transmission and ii) how does a transient horizontal transfer turn into a stable vertical transmission? Regarding this problem Frydman and colleagues reported that when haemolymph of an infected *D. melanogaster* fly is microinjected into adult uninfected females, *Wolbachia* could be transmitted vertically [10]. After 15 days upon haemolymph microinjection into uninfected female flies, *Wolbachia* could be detected in their offspring after preferentially establishing itself in the ovaries somatic stem cell niches. Also, it has been shown that *Wolbachia* is viable for several days outside the host's cell, thus allowing for a possible transfer across cells [17]. Together these reports provide a link between horizontal and vertical transmission, indicating that any mechanism capable of introducing *Wolbachia* into the female's haemolymph may permit the establishment and perpetuation of *Wolbachia* in new hosts.

Despite their importance for understanding the epidemiological and evolutionary dynamics of *Wolbachia* infection, the ecological mechanisms responsible for the transfer of bacteria to new hosts in nature are still largely unknown [18]. One strong candidate mechanism consists of parasitoid wasps acting as *Wolbachia* vectors. This is based on different evidence: i) the extensive similarities between *Wolbachia* strains found in parasitoids and their hosts [13, 19]; ii) *Wolbachia* can be transmitted to a parasitic wasp from its infected host [21, 22]; iii) when infected and uninfected parasitoid wasp larvae share the same host egg, intra- and interspecific horizontal transfer of parthenogenesis-inducing *Wolbachia* may occur [22–24]. Another hypothetical vector for horizontal transmission of *Wolbachia* are ectoparasitic mites, known to transfer the *Drosophila* endosymbiont, *Spiroplasma poulsonni*, from infected *D. nebulosa* to *D. willistoni* [25]. Based on our observations of *Drosophila* larval and adult behaviour in crowded environments, we reasoned that cannibalism or

scavenging, often witnessed not only in the laboratory but also in nature, could constitute a route for horizontal *Wolbachia* transfer. Moreover, occasional horizontal transmission via the oral route has been reported for the pea aphid *Bemisia*-like symbiont [26]. Indeed, the digestive system is considered to be the major interface between the insect host and the microbial environment, constituting a privileged gateway for microorganism invasion [27]. However, as most ingested bacteria are eliminated by the immune system or by peristalsis, few bacteria can persist in large numbers in the digestive tract of insects [28]. Nonetheless it is important to note that some bacterial species ensure their proliferation in recent hosts by passing through the digestive tract to other organs or cavities [29, 30].

Recent studies have demonstrated that, after predation of infected hosts, previously uninfected isopods, *Armadillidium vulgare* and *Porcellio dilatatus dilatatus*, would become infected with *Wolbachia* [31]. Also, in the ant *Acromyrmex echinatior*, it has been hypothesized that the faecal-oral route could constitute a means for horizontal transmission of *Wolbachia* [32].

In this work, we have tested if upon ingestion, *Wolbachia* could be transmitted stably to the offspring of a *Drosophila* host. For this, several ingestion experiments were performed using infected and uninfected hosts of *D. melanogaster* and *D. simulans*, at different developmental stages. Nutritional variation, dehydration and intestinal injury were used in an attempt to mimic naturally-occurring potentiating factors for the passage of *Wolbachia* into the body cavity of the fly and the subsequent establishment of a symbiotic relationship with this new host. Through a PCR-based analysis of the offspring we were unable to find any infection by *Wolbachia*, both in early and late progeny. This result suggests that the ingestion of *Wolbachia* by a non-infected new host is not sufficient in itself to establish a

stable infection horizontally or is too rare to be detected within the limits of our experiment.

Materials and Methods

Foundation and maintenance of *Drosophila* outbred populations

Outbred populations of *Drosophila melanogaster* and *Drosophila simulans* were established in the laboratory [33]. Respectively, 160 and 90 females *Wolbachia*-infected *D. melanogaster* and *D. simulans*, collected from the southwest of Portugal (Azeitão) were used to establish two laboratory populations ($MelO^+$ and $SimO^+$, respectively). After over 50 generations in the laboratory, both populations ($MelO^+$ and $SimO^+$) were replicated for the establishment of four new populations: two infected with *Wolbachia* as the founding populations (mel^+ and sim^+) and two treated with tetracycline during four generations for total *Wolbachia* elimination (mel^- and sim^-). We confirmed the absence of *Spiroplasma* in all populations. For the *Serratia* assays, the *D. simulans* populations were established using two isofemale lines from the Drosophila Species Stock Centre (UC San Diego, California, US) sim^+ (14021-0251.138) and sim^- (14021-0251.01). All populations were kept in cages with an effective size between 1500 and 2000 individuals with non-overlapping generations, in a day/night cycle of 12 hours, constant temperature of 25º C, standard level of relative humidity (70%) and fed on standard cornmeal-agar medium. The infection status of populations was monitored regularly through PCR (see below).

Wolbachia extraction

Wolbachia bacteria were extracted by crushing 100 infected adults or approximately 500 embryos of *D. melanogaster* or *D. simulans*, previously washed in 70% ethanol, and transferred to 1mL of ice-cold PBS (adapted

from [10]). For adult co-infected ingestion assays and adapting a protocol described previously [17], *Wolbachia* bacteria were extracted by smashing approximately 500 infected flies in 10mL of Schneider's medium. The confirmation of bacterial viability after extraction was also performed as described by Rasgon and colleagues [17]. As a control for both extractions, the same procedure of extractions was performed in parallel, but with non-infected females from treated *D. melanogaster* and *D. simulans* populations (mel^- and sim^-). In all cases, the homogenate was used entirely.

Adult Ingestion assay

For ingestion experiments with adults, 4-7 day old females were used from the mel^- population. From the regular stock of flies (which were maintained in rich medium), 20 replicates of 20 adult females were used to exclusively ingest 250µL of a *Wolbachia*-containing suspension homogenized in PBS (coming from infected adults of mel^+ populations) for a period of 48-hours. These experiments were also undertaken with a previous 72-hour treatment either with a poor medium (rich medium diluted 1:10 in water) or in a condition of starvation, where the females spent a 48-hour period in total absence of nutritional resources until the beginning of the ingestion treatment.

Larval ingestion assay

For the ingestion experiments with larvae, we used mel^- larvae from the three larval stages. Larvae ingested a homogenate, containing adults (or embryos), from mel^+ or sim^+ populations infected with *Wolbachia* for a period of 24 hours. In each of the experiments, 5 replicates of 50 larvae were fed on 500µL of homogenate from 40 flies.

Adult co-infected ingestion assays

For ingestion experiments with adults, 4-7 day old females were used from the mel$^-$ population. From the regular stock of flies, 10 adult females were used per replicate to exclusively ingest i) 250µL of *Serratia marcescens* (a kind gift from B. Lemaitre) for a period of 24 hours ii) 250µL of a *Wolbachia*-containing suspension for a period of 24 hours. The food solution containing *Serratia* bacteria was prepared from an overnight culture grown exponentially at 37 ºC and was diluted with a sterile 50-mM sucrose solution to a final OD_{600} = 15. These experiments were also undertaken either with *Wolbachia* with a previous 24-hour ingestion treatment with LB or with *Serratia* and posterior treatment with sim$^-$ and mel$^-$.

Diagnostic PCR

In all procedures, tested females gave rise to the adult F1 from which genomic DNA was extracted from pools of 10 adult females and screened for *Wolbachia* infection by PCR through the amplification of *wsp* gene fragment using primers *wsp*81F 5´TGG TCC AAT AAG TGA TGA AGA AAC 3´ and *wsp*691R 5´AAA AAT TAA ACG CTA CTC CA 3´ [34]. *Wolbachia* strains of *D. melanogaster* and *D. simulans* generate PCR amplicons of different sizes, 632bp and 611bp, respectively. This diagnostic PCR was further confirmed in 10% of samples chosen randomly by sequencing the respective PCR products.

Results and Discussion

We fed *D. melanogaster* larvae and adults of the *Wolbachia* negative outbred population (mel$^-$) with embryo or adult fly homogenates from *Wolbachia* infected populations of *D. melanogaster* (mel$^+$) and *D. simulans* (sim$^+$). As controls we applied the same procedures using homogenates from uninfected populations referred to as mel$^-$ and sim$^-$. The status of

Wolbachia infection of the populations used in these experiments is shown in Figure 4.4.1A, also illustrating the size difference between *wsp* gene amplification products of *Wolbachia* strains from *D. melanogaster* and *D. simulans*. Confirmation of the different strains was obtained by sequencing the *wsp* gene fragment (Figure 4.4.1B). These results validate our procedure for the simultaneous determination of the infection status and *Wolbachia* strain present in individual or pooled adult flies (as to ascertain instances of intra- or interspecific transmission). We tested the F1 of fed females at two time points: early F1 (8 to 10 days) and in late F1 (more than 15 days), determined by the description of *Wolbachia* dynamics upon entry into the haemolymph and subsequent stable establishment in the germline [10]. A representative gel of the PCR-based screen for *Wolbachia* infection is presented in Figure 4.4.1C.

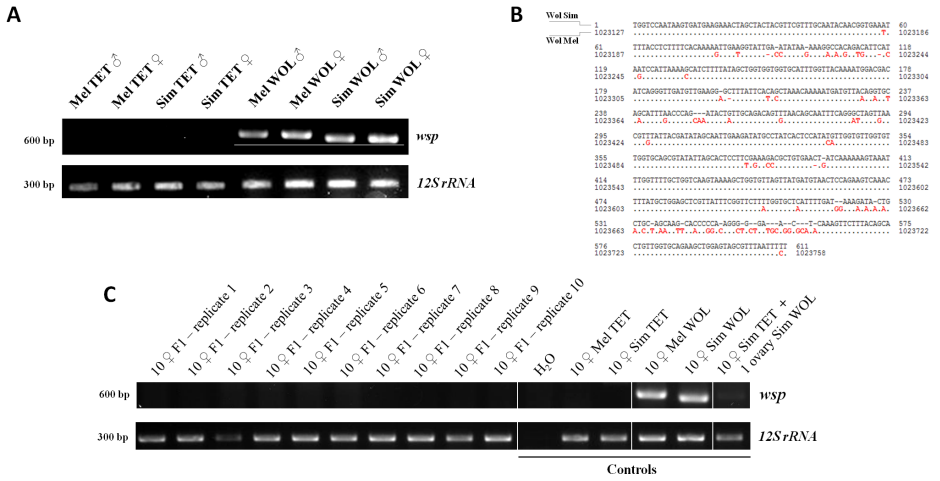


Figure 4.4.1 – Screen for *Wolbachia* in the initial and tested populations. (**A**) Infection status in males and females of initial populations – F0; (**B**) Differentiation of *Wolbachia* strains of *D. melanogaster* and *D. simulans* by *wsp* gene sequencing; (**C**) Representative PCR for *Wolbachia wsp* gene in tested females progeny, indicating *Wolbachia* absence in F1 (10 replicates + controls).

TABLE 4.4.1 – Summary of larval ingestion assays and respective female progeny analyses

Stage	Condition	N test	Wol ext	Analyzed	Wol F1e	Wol F1l
Larvae	**Rich food**	**50 (5)²**	**Adults Sim⁺**	**10**	**–**	**–**
Larvae	Rich food	**50 (5)²**	Adults Sim⁻	10	**–**	**–**
Larvae	**Rich food**	**50 (5)⁴**	**Adults Mel⁺**	**10**	**–**	**–**
Larvae	Rich food	**50 (5)⁴**	Adults Mel⁻	10	**–**	**–**
Larvae	**Rich food**	**50 (10)²**	**Embryo Mel⁺**	**10**	**–**	**–**
Larvae	Rich food	**50 (10)²**	Embryo Mel⁻	10	**–**	**–**

Stage – Developmental stage of tested individuals; **Condition** – Previous treatment; **N test –** number of tested individuals, **( )** number of replicates, **Superscript –** number of independent experimental sets; **Wol ext –** *Wolbachia* extraction; **Analyzed** – number of F1 females analyzed (per replicate); **Wol F1e** – presence (+) or absence (-) of *Wolbachia* in early F1; **Wol F1l** – presence (+) or absence (-) of *Wolbachia* in late F1.

Larval ingestion could lead to the stable transmission of *Wolbachia* by one of two ways: i) establishing itself in cells of somatic tissue, surviving the metamorphosis stage of the host and colonizing the ovaries of adult females, or ii) crossing the epithelium of the digestive system and colonizing the stem cells of the future ovary. To validate these findings, we fed *D. melanogaster* larvae of different stages, previously maintained in normal medium, a homogenate of mel⁺ and sim⁺ infected embryos or adults for 24 hours (Table 4.4.1). In a second set of experiments, we placed mel⁻ adult flies on a diet composed of a mel⁺ adult homogenate for 48 hours (Table 4.4.2A). If ingestion of *Wolbachia* occurs in the adult stage, it should be enough for a successful transmission that the endosymbiont crosses the midgut and passes to the haemolymph [10]. Yet, it should be stressed that it is unclear what is the necessary concentration of haemolymph *Wolbachia* for the establishment of these bacteria in the ovaries.

Both in the larvae and adult ingestion experiments, the early and late F1 flies tested did not show the presence of *Wolbachia* (Table 4.4.1 and Table 4.4.2A, "Wol F1e and Wol F1l"). This negative result holds true even when varying the *Wolbachia* source, both *D. melanogaster* and *D. simulans* (intra- or interspecific), and the stage at which the *Wolbachia* homogenate

was extracted, embryos or adults. Our findings indicate that if horizontal transmission by ingestion occurs in nature, within or between *Drosophila* species, it is a rare event.

TABLE 4.4.2 – Summary of adult ingestion assays and respective female progeny analyses

Stage	Condition	N test	Wol ext	Analyzed	Wol F1e	Wol F1l
A						
Adults	**Rich food**	**20 (20)²**	**Adults Mel⁺**	**10**	**–**	**–**
Adults	Rich food	**20 (20)²**	Adults Mel⁻	10	**–**	**–**
B						
Adults	**Poor food**	**20 (10)**	**Adults Mel⁺**	**10**	**–**	**–**
Adults	Poor food	**20 (10)**	Adults Mel⁻	10	**–**	**–**
Adults	**Starvation**	**20 (10)**	**Adults Mel⁺**	**10**	**–**	**–**
Adults	Starvation	**20 (10)**	Adults Mel⁻	10	**–**	**–**

Stage – Developmental stage of tested individuals; **Condition** – Previous treatment; **N test –** number of tested individuals, **( )** number of replicates, **Superscript –** number of independent experimental sets; **Wol ext –** *Wolbachia* extraction; **Analyzed** – number of F1 females analyzed (per replicate); **Wol F1e** – presence (+) or absence (-) of *Wolbachia* in early F1; **Wol F1l** – presence (+) or absence (-) of *Wolbachia* in late F1.

Another aspect to consider is that our progeny analysis treats the whole putative process of infection as a binary outcome (F_1 infected or non-infected) and cannot pinpoint the critical step at which the infection fails to progress. We may consider the absence of *Wolbachia* in the *D. melanogaster* F_1 flies as the product of low probability events, each one necessary for the occurrence of horizontal transmission. We can formalize this idea through the equation:

$$\mathbf{P_{HT(\mathit{w})}} = P_{EI}\,(\alpha)\ X\ P_{AH}\,(\beta)\ X\ P_{BS}\,(\gamma)\ X\ P_{OC}\,(\delta)\ X\ P_{VT}\,(\varepsilon)$$

where the probability of any horizontal transmission of *Wolbachia* ($\mathbf{P_{HT\ (\mathit{w})}}$) is equal to multiplying the probabilities of all the independent steps required for its occurrence: the environmental interaction between *Wolbachia* infected and non-infected individuals (P_{EI}), here tested as ingestion; the access of *Wolbachia* to the haemolymph (P_{AH}); the bacterial

survival in the new host (P_{BS}); the colonization of ovaries (P_{OC}); and the vertical transmission (P_{VT}). Each of these steps can still be associated with a correction factor (α, β, γ, δ and ε) linked to specific ecological conditions.

Wolbachia ingestion by a non-infected new host is not in itself sufficient to establish a stable infection in *Drosophila* but specific ecological conditions may favour this process (here, formalized as α, β, γ, δ and ε). Indeed, there is ample evidence that several aspects of host life-history have a significant impact on the transmission of *Wolbachia* [35–37]. Thus, we have manipulated some of these factors in order to favour horizontal transmission via ingestion, namely starvation and infection with a known natural bacterial pathogen. Interestingly, under nutritional restriction, the apoptotic region present in the ovaries (region 2a/2b of the germarium) [38] overlaps with the region of *Wolbachia* entrance into the germinal tissue [10], raising the hypothesis that the invasion of the germinal tissue by *Wolbachia* is opportunistic (δ). Additionally, the absence of nutritional resources in nature could also trigger an increase in cannibalism (α) and in bacterial infections due to the weakening of the host's tissue barrier by cell

TABLE 4.4.3 – Summary of adult co-infection ingestion assays and respective female progeny analyses

Stage	Condition	N test	Wol ext	Analyzed	Wol F1e	Wol F1l
					F0 Mortality	
Adults	**LB**	**10 (6)**	**Adults Mel$^+$**	**10**	**0**	–
Adults	LB	**10 (6)**	Adults Mel$^-$	10	0	–
Adults	**LB**	**10 (12)**	**Adults Sim$^+$**	**10**	**0**	–
Adults	LB	**10 (12)**	Adults Sim$^-$	10	1	–
Adults	***Serratia***	**10 (24)**	**Adults Mel$^+$**	**10**	**0**	–
Adults	*Serratia*	**10 (6)**	Adults Mel$^-$	10	15	–
Adults	***Serratia***	**10 (24)**	**Adults Sim$^+$**	**10**	**45**	–
Adults	*Serratia*	**10 (6)**	Adults Sim$^-$	10	24	–

Stage – Developmental stage of tested individuals; **Condition** – Previous treatment; **N test –** number of tested individuals, **( )** number of replicates, **Superscript –** number of independent experimental sets; **Wol ext –** *Wolbachia* extraction; **Analyzed** – number of F1 females analyzed (per replicate); **Wol F1e** – presence (+) or absence (-) of *Wolbachia* in early F1; **Wol F1l** – presence (+) or absence (-) of *Wolbachia* in late F1; **F0 Mortality** - % of dead females 3 days after treatments.

death (β). With this aim, we placed mel$^-$ adult females, previously maintained in nutritionally poor medium or under starvation, on a diet composed of a mel$^+$ adult homogenate for 48 hours (Table 4.4.2B). Under these conditions we observed a total absence of *Wolbachia* in F_1 tested females. Next, we used an oral infection model by previous infection with *Serratia marcescens* as an enhancer of secondary infection with ingested *Wolbachia* (β). Indeed, it has been shown that severe intestinal injury produced by *S. marcescens* promotes its crossing from the gut to the fly's body cavity [39]. The subsequent ingestion of *Wolbachia* could follow the same route, increasing the probability of *Wolbachia* entry into the *Drosophila* haemolymph. In this experiment, adult females ingested a suspension of the entomobacterium *S. marcescens* and, subsequently, ingested *Wolbachia* extracted from infected adults of *D. melanogaster* and *D. simulans* (mel$^+$ and sim$^+$) (Table 4.4.3). Here, only the late progeny of female flies was analyzed and the percentage of female mortality three days after ingestion of *S. marcescens* is shown (Table 4.4.3 – "F0 Mortality"). Regardless of a previous exposure to injury stress, these females did not give rise to *Wolbachia* infected F_1s, indicating the absence of *Wolbachia* transmission (Table 4.4.3 – "Wol F1l"). Despite the absence of *Wolbachia* in late progeny of tested females, this co-infection scenario presents itself as an excellent model to study the horizontal transmission of several endosymbionts to different potential new hosts. Indeed, recently it has been proposed that the ingestion of mushrooms could constitute the gateway for *Wolbachia* transmission between species [40].

After an ingestion episode and once inside a potential new host, bacteria must endure the local defence deployed by the digestive system, such as low pH, the production of Reactive Oxygen Species (ROS) and Anti-Microbial Peptides (AMPs). Insect parasitoids, mites or wounding can avoid this immune local challenge by providing a more direct path for bacteria to

penetrate the body cavity of the new host. This route is not without danger as invading *Wolbachia* must survive the host melanization reaction triggered by injury. Finally, for *Wolbachia* to establish a viable horizontal infection once in the haemolymph [10], it must overcome the systemic action of AMPs and phagocytosis by haemocytes. As a result, it is still unclear if the individual frequencies or efficiencies of each one of these potential mechanisms would be enough to explain all the evidence for horizontal transmission. An additional important element consists on the effects that ecological co-factors (such as those studied here: resource limitation and co-infection) have on *Drosophila* immune response translating into changes in the success of bacteria to invade and establish (γ) [41].

Thus, the mechanisms governing horizontal transmission of facultative endobacteria, particularly of *Wolbachia*, remain unknown. As mentioned above, insect parasitoids and parasitic mites may promote some of these symbiotic exchanges; however, other mechanisms that complete the puzzle of the pathways that facultative endobacterial species utilize to accomplish a new invasion, have yet to be explained. Although *Wolbachia* has been specializing throughout evolution in the vertical transmission strategy, we do not know the true horizontal transmission capacity of this endobacterium, a feature which is an ancestral characteristic of rickettsial bacteria and is still conserved in close related Genera [42]. Therefore, it is essential to continue the study of the mechanisms responsible for horizontal transmission phenomena that associated with several phenotypic and reproductive manipulations and may play an important role in the enormous diversity of arthropods [43].

Acknowledgements

The authors wish to thank Alexandre Leitão, Nelson Martins, Inês Trancoso and Luis Teixeira for discussions and Patrícia Beldade for the critical reading of the manuscript.

References

1. Werren JH: **Biology of *Wolbachia***. *Annual Review of Entomology* 1997, **42**:587–609.

2. Harris HL, Brennan LJ, Keddie BA, Braig HR: **Bacterial symbionts in insects: balancing life and death**. *Symbiosis* 2010, **51**:37–53.

3. Charlat S, Hurst GD, Mercot H: **Evolutionary consequences of *Wolbachia* infections**. *Trends in Genetics* 2003, **19**:217–223.

4. Werren JH, Baldo L, Clark ME: ***Wolbachia***: **master manipulators of invertebrate biology**. *Nature Reviews Microbiology* 2008, **6**:741–751.

5. Hedges LM, Brownlie JC, O'Neill SL, Johnson KN: ***Wolbachia* and virus protection in insects**. *Science* 2008, **322**:702.

6. Teixeira L, Ferreira A, Ashburner M: **The bacterial symbiont *Wolbachia* induces resistance to RNA viral infections in *Drosophila melanogaster***. *PLoS Biology* 2008, **6**:e2.

7. Jeyaprakash A, Hoy MA: **Long PCR improves *Wolbachia* DNA amplification: *wsp* sequences found in 76% of sixty-three arthropod species**. *Insect Molecular Biology* 2000, **9**:393–405.

8. Ferree PM, Frydman HM, Li JM, Cao J, Wieschaus E, Sullivan W: ***Wolbachia* utilizes host microtubules and dynein for anterior localization in the *Drosophila* oocyte**. *PLoS Pathogens* 2005, **1**:e14.

9. Serbus LR, Sullivan W: **A cellular basis for *Wolbachia* recruitment to the host germline**. *PLoS Pathogens* 2007, **3**:e190.

10. Frydman HM, Li JM, Robson DN, Wieschaus E: **Somatic stem cell niche tropism in *Wolbachia***. *Nature* 2006, **441**:509–512.

11. Goto S, Anbutsu H, Fukatsu T: **Asymmetrical interactions between *Wolbachia* and *Spiroplasma* endosymbionts coexisting in the same insect host**. *Applied and Environmental Microbiology* 2006, **72**:4805–4810.

12. Faria VG, Sucena É: ***Wolbachia* in the malpighian tubules: evolutionary dead-End or adaptation?** *Journal of Experimental Zoology Part B: Molecular and Developmental Evolution* 2013, **320**:195–199.

13. Vavre F, Fleury F, Lepetit D, Fouillet P, Bouletreau M: **Phylogenetic evidence for horizontal transmission of *Wolbachia* in host-parasitoid associations**. *Molecular Biology and Evolution* 1999, **16**:1711–1723.

14. Baldo L, Ayoub NA, Hayashi CY, Russell JA, Stahlhut JK, Werren JH: **Insight into the routes of *Wolbachia* invasion: high levels of horizontal transfer in the spider genus *Agelenopsis* revealed by *Wolbachia* strain and mitochondrial DNA diversity**. *Molecular Ecology* 2008, **17**:557–569.

15. Werren JH, O'Neill SL: **The evolution of heritable symbionts**. In *Influential passengers: inherited microorganisms and arthropods reproduction*. Edited by O'Neill SL, Hoffman AA, Werren JH. Oxford: Oxford University Press; 1997:1–41.

16. Jiggins FM, Bentley JK, Majerus ME, Hurst GD: **Recent changes in phenotype and patterns of host specialization in *Wolbachia* bacteria**. *Molecular Ecology* 2002, **11**:1275–1283.

17. Rasgon JL, Gamston CE, Ren X: **Survival of *Wolbachia pipientis* in cell-free medium**. *Applied and Environmental Microbiology* 2006, **72**:6934–6937.

18. Haine ER, Pickup NJ, Cook JM: **Horizontal transmission of *Wolbachia* in a *Drosophila* community**. *Ecological Entomology* 2005, **30**:464–472.

19. Li J, Wang Z, Bourguet D, He K: ***Wolbachia* infection in populations of *Ostrinia furnacalis*: diversity, prevalence, phylogeny and evidence for horizontal transmission**. *Journal of Integrative Agriculture* 2013, **12**:283–295.

20. Heath BD, Butcher RD, Whitfield WG, Hubbard SF: **Horizontal transfer of *Wolbachia* between phylogenetically distant insect species by a naturally occurring mechanism**. *Current Biology* 1999, **9**:313–316.

21. Morrow JL, Frommer M, Shearman DCA, Riegler M: **Tropical tephritid fruit fly community with high incidence of shared *Wolbachia* strains as platform for horizontal transmission of endosymbionts**. *Environmental Microbiology* 2014, **16**:3622–3637.

22. Schilthuizen M, Stouthamer R: **Horizontal transmission of parthenogenesis-inducing microbes in *Trichogramma* wasps**. *Proceedings Biological Sciences / The Royal Society* 1997, **264**:361–366.

23. Huigens ME, Luck RF, Klaassen RH, Maas MF, Timmermans MJ, Stouthamer R: **Infectious parthenogenesis**. *Nature* 2000, **405**:178–179.

24. Huigens ME, de Almeida RP, Boons PA, Luck RF, Stouthamer R: **Natural interspecific and intraspecific horizontal transfer of**

parthenogenesis-inducing *Wolbachia* in *Trichogramma* wasps. *Proceedings Biological Sciences / The Royal Society* 2004, **271**:509–515.

25. Jaenike J, Polak M, Fiskin A, Helou M, Minhas M: **Interspecific transmission of endosymbiotic *Spiroplasma* by mites**. *Biology Letters* 2007, **3**:23–25.

26. Darby AC, Douglas AE: **Elucidation of the transmission patterns of an insect-borne bacterium**. *Applied and Environmental Microbiology* 2003, **69**:4403–4407.

27. Douglas AE, Beard CB: **Microbial symbiosis in the midgut of insects**. In *Biology of the Insect Midgut*. Edited by Lehane MJ, Billingsley PF. London: Chapman and Hall; 1996:419–431.

28. Vallet-Gely I, Lemaitre B, Boccard F: **Bacterial strategies to overcome insect defences**. *Nature Reviews Microbiology* 2008, **6**:302–313.

29. Marsollier L, Aubry J, Coutanceau E, Andre JP, Small PL, Milon G, Legras P, Guadagnini S, Carbonnelle B, Cole ST: **Colonization of the salivary glands of *Naucoris cimicoides* by *Mycobacterium ulcerans* requires host plasmatocytes and a macrolide toxin, mycolactone**. *Cellular Microbiology* 2005, **7**:935–943.

30. Chiel E, Zchori-Fein E, Inbar M, Gottlieb Y, Adachi-Hagimori T, Kelly SE, Asplen MK, Hunter MS: **Almost there: transmission routes of bacterial symbionts between trophic levels**. *PLoS ONE* 2009, **4**:e4767.

31. Le Clec'h W, Chevalier FD, Genty L, Bertaux J, Bouchon D, Sicard M: **Cannibalism and predation as paths for horizontal passage of *Wolbachia* between terrestrial isopods**. *PLoS ONE* 2013, **8**:e60232.

32. Frost CL, Pollock SW, Smith JE, Hughes WOH: ***Wolbachia* in the flesh: symbiont intensities in germ-line and somatic tissues challenge the conventional view of *Wolbachia* transmission routes**. *PLoS ONE* 2014, **9**:e95122.

33. Martins NE, Faria VG, Teixeira L, Magalhães S, Sucena É: **Host adaptation is contingent upon the infection route taken by pathogens**. *PLoS Pathogens* 2013, **9**:e1003601.

34. Zhou W, Rousset F, O'Neil S: **Phylogeny and PCR-based classification of *Wolbachia* strains using *wsp* gene sequences**. *Proceedings Biological Sciences / The Royal Society* 1998, **265**:509–515.

35. McGraw EA, O'Neill SL: **Evolution of *Wolbachia pipientis* transmission dynamics in insects**. *Trends in Microbiology* 1999, **7**:297–302.

36. Hurst GD, Jiggins FM, Robinson SJ: **What causes inefficient transmission of male-killing *Wolbachia* in *Drosophila*?** *Heredity* 2001, **87**:220–226.

37. Mouton L, Henri H, Charif D, Boulétreau M, Vavre F: **Interaction between host genotype and environmental conditions affects bacterial density in *Wolbachia* symbiosis**. *Biology Letters* 2007, **3**:210–213.

38. Drummond-Barbosa D, Spradling AC: **Stem cells and their progeny respond to nutritional changes during *Drosophila* oogenesis**. *Developmental Biology* 2001, **231**:265–278.

39. Nehme NT, Liegeois S, Kele B, Giammarinaro P, Pradel E, Hoffmann JA, Ewbank JJ, Ferrandon D: **A model of bacterial intestinal infections in *Drosophila melanogaster***. *PLoS Pathogens* 2007, **3**:e173.

40. Stahlhut JK, Desjardins CA, Clark ME, Baldo L, Russell JA, Werren JH, Jaenike J: **The mushroom habitat as an ecological arena for global exchange of *Wolbachia***. *Molecular Ecology* 2010, **19**:1940–1952.

41. Schneider DS: **Physiological integration of innate immunity**. In *Insect infection and immunity: evolution, ecology, and mechanisms*. Edited by Rolff J, Reynolds SE. Oxford: Oxford University Press; 2009:106–118.

42. Anderson CL, Karr TL: ***Wolbachia*: evolutionary novelty in a rickettsial bacteria**. *BMC Evolutionary Biol*ogy 2001, **1**:10–15.

43. Faria VG, Sucena É: **Novel endosymbioses as a catalyst of fast speciation**. In *Reticulate evolution: symbiogenesis and lateral gene transfer*. Edited by Gontier ND. Springer International Publishing; 2015: 107–120.

4.5. Concluding remarks

In this Chapter, we started reviewing the literature across arthropods showing that i) endosymbiosis is a common phenomenon, ii) the presence of the endosymbiont in the host may affect its fitness, iii) horizontal transmission of endosymbionts is likely and, that it is possible that iv) vertical transmission will ensue leading to a stable phenotype in which v) the presence of endobacteria in the new host induces significant phenotypic change with fitness consequences promoting directional or disruptive selection. We organized disperse evidence which supports this five-step scenario and argue that the establishment of new endosymbioses can lead, rapidly and cyclically, to the emergence of new hosts species. Furthermore, we argue that this proposed concept and framework can be put to experimental test through the controlled manipulation of the partners and their relationships in customized novel endosymbioses, perhaps helping to shed light over a mechanism with a putative role in the generation of the enormous biodiversity of arthropods.

Next, we looked more specifically at a new case study, *Wolbachia* in the malpighian tubules (MTs) of *Drosophila*, and pinpointed the evolutionary consequences of this specific association for both partners. Altogether the facts presented in Subchapter 4.3 lead us to hypothesize that part of the bacteria incorporated into the germarium may derive from MTs and other somatic tissues, challenging the notion that somatic *Wolbachia* face an evolutionary dead-end. We have set clear testable hypotheses and driven attention to an unexplored role of endosymbionts in the physiology and evolution of host-symbiont and particularly *Drosophila*/*Wolbachia* relationships.

An additional novel finding present in our hypothesis is that somatic tissues, particularly the MTs, can have a non-negligible contribution to the vertically transmitted *Wolbachia*. This assumption may also provide an

interesting element to the elucidation of horizontal transmission mechanisms, where an intermediate step that does not involve the germline must occur. A point of view also discussed in our work is the physiological and evolutionary role of this somatic bacterial colony that, from the host´s perspective, may even be actively controlled to increase its own fitness.

To push forward the study of horizontal transmission and in order to develop these ideas in a practical way, we then tested ingestion by cannibalism as a mechanism of horizontal transmission of the endosymbiont *Wolbachia* in *Drosophila*. Ingestion clearly represents an obvious path for microorganisms, however we did not find the presence of *Wolbachia* in the progeny of non-infected flies. Our study thus points to a phenomenon that even if possible, happens very rarely or requires other accessory conditions to be triggered.

We hope to have contributed to helping pointing the way towards the dissection of the mechanisms through which this (and other) symbionts break the species barrier. This is a central point in the pursuit of an understanding of the pervasive pattern of symbiosis seen across taxa.

5

General discussion

Final considerations and ideas for future studies

This Thesis aimed to contribute to the understanding of host-microbe interactions. Several aspects of these relationships were approached, from pathogenicity to mutualism, from vertical to horizontal transmission. For achieving that goal, we used *D.melanogaster* as a model organism, both in outbred populations and in diverse genetic lines. These biological tools, when challenged with natural pathogens allowed the capture and analysis of many factors, answering a series of outstanding questions. Moreover, this Thesis did not only approach the side of pathogenic microorganisms, but also, and simultaneously, on the host's relationship with its intracellular endosymbiont *Wolbachia*. In this complex circuit, we had the opportunity to address a vast range of consequences for the different sides and how each part was influenced by the presence of the other, reinforcing the comprehension of the selective and adaptive dynamics of host-microorganisms relationships.

In Chapter 1 – **General Introduction: an overview on host-microbe interaction and evolution** – the literature was reviewed, addressing the main topics relevant to the framework of our stories. The goal was to provide a comprehensive review, referring when appropriate to the introductions of the Subchapters. Looking at the citations in Chapter 1, the enormous recent progress of our knowledge on several aspects of host-microbe interactions (such as adaptation, co-evolution, immunity or even behaviour) is evident. With i) the explosion of genomics, ii) the increase of our capacity to manipulate microorganisms and iii) the numerous genetic tools of model organisms (where *Drosophila* certainly stands out), we now started to unravel the strategies found by species to survive and perpetuate.

For all who are dedicated to this field, it is exciting to see that reality repeatedly exceeds our imagination, showing that nature endlessly finds

new answers to solve old and new problems. A limiting selective pressure today may no longer be tomorrow's, making available a new range of unusual solutions. This fact will certainly influence the future of this population and thus trigger a domino effect on other species ecologically interconnected.

This substantial expansion in production of knowledge is only possible with the increasing research community working on these subjects. Both in theoretical and applied areas, the many aspects of host-microbe interactions are being explored. Of them, several relevant works were referred in this Thesis, although taking into consideration redundancy and space limitations.

In Chapter 2 – ***Wolbachia*-positive host adaptation against pathogens** – we approached several questions regarding host-pathogen interactions using different methodologies, trying to answer a large range of relevant questions for the field.

In the first segment of results, **Subchapter 2.2**, we deepened and dissected the protocol used in the laboratory to generate *Drosophila* resources for many areas of biological research. Systematizing and disseminating this procedure, we intended to contribute to standardize, and also maximize, the data production in studies with new biological tools collected from the wild. This type of parallelism would be a hugely added value to numerous topics, making it possible to address multiple questions by combining and varying the selective pressures, as well as the place and time of collected populations.

For example, in our laboratory, populations were founded under a uniform protocol, originated from the same location but in two different times, 2007 and 2013. We can therefore think what would be the result of

the repetition of the work presented in this Thesis using the population created in 2013. Certainly the basis of a different adaptive response will be contained in the initial genetic pool of the population, not only the eventual presence or absence of causative alleles but also the allelic frequency when the selection starts. Moreover, not only the population under test will be influencing the response, but also the microbiome.

If we sequence the newest population, we will know the genetic composition as well as the frequencies of the present alleles, being possible to theoretically simulate the time required for the pre-diagnosed causative agents to respond against the selective pressure imposed. But this exercise, although feasible, will probably not reflect reality. The potential absence of one or more causative alleles, and, even more unpredictable, the presence of relevant new players (in the same or other genes) will alter undoubtingly the profile of response and the dynamics of adaptation.

With this in mind, and looking forward to overcome these limitations, we believe that the time is right to create a platform of outbred populations exchange. A platform to share, or even an international bank to maintain the populations, would be very valuable to compare results with parallelism, accelerating thus our understanding of the evolution of species. However, this only makes sense putting into practice the standardization of foundation and maintenance of populations, as well as the maximization of biological resources generated from each foundation. Several issues can be addressed with this implementation, such as: what is the real influence of the effective population size for the adaptation of some feature? What is the actual difference in the adaptive dynamics of unidirectional evolution and co-evolution? What is the impact of selective pressures on populations from different locations, at different times and under different conditions? Or even, which is the real relationship between the variance of the initial

isofemale lines and the population's adaptive dynamics? We hope that this dissertation, and the work here contained, contributes to accelerate that discussion and build up this idea.

In **Subchapter 2.3**, we started using our outbred populations to approach a vast range of questions regarding host-pathogen relationship. We started based on the fact that pathogens enter their hosts through several routes, the most common being ingestion (oral infection) and breaches in the cuticle (systemic infection). As stated in Chapter 1, several studies have shown that these infection routes strongly affect the evolution of pathogen virulence, though little attention has been given to the role of host evolution in this process. Thus, we studied the effect of infection routes on the evolution of host defenses, using *Drosophila melanogaster* and its natural pathogen *Pseudomonas entomophila*. Profiting from the power of experimental evolution, in which the evolution of populations is followed in real-time, we showed that the evolved response is specific to the route of infection and to pathogen. Indeed, flies that resist bacteria through ingestion are not protected from systemic infection with the same bacteria species, and vice-versa.

Since our infection protocol only allows the adaptation of the host population, these results made us consider what are the consequences for the real process of co-evolution of both species. When hosts and bacteria share the same environment, it is natural to consider that both routes of infection are possible (and even likely), being certainly interesting to see what would happen with a possible simultaneous infection through both routes. Knowing that mortality has different intervals according to the route of infection, it will be exciting to investigate how these facts will condition the adaptation in both host and bacteria populations.

Furthermore, we showed that evolution of resistance to one pathogen does not extend to infections with bacteria of different genera via the same infection route. This result opens new perspectives and identifies new targets of research, but we are still lacking comparison points to realize how often this pattern occurs. Thus, although this work certainly contributes to the understanding of pathogenic infection dynamics and the respective host's immune response, this degree of specificity calls for more attention onto pathogen infection routes in other host-parasite complexes.

In **Subchapter 2.4**, we went one step further, searching for the genetic basis responsible for the population's adaptation against *P. entomophila*. As revealed in Subchapter 2.3, survival of *D. melanogaster* to an oral infection increases within the first three generations of selection, whereas the response to systemic infection is slower. To try to discover where the genetic origin of this drastic difference of response was located, we used pool-seq technology in evolved and control populations.

While in the systemically evolved populations we found several differentiated genomic regions with numerous SNPs changing in frequency, in the population evolved orally, we find an unexpected small number of SNP frequency changes. As discussed in this subchapter, many questions are raised regarding the driving forces for such fast adaptation, because, as a consequence, a strong genetic signature was expected. This signature, if not present in the host, could be expected in the microbiome. However, this hypothesis was experimentally discarded.

Also, we tested the genes where the highest differentiated SNPs were located. By performing RNAi tests, we found that several of these genes revealed by Pool-seq are actually causative of different immune responsiveness. Thus, the dimly selected alleles may represent uncharacterized bacterial-protective variants of these genes. Now, it is

necessary to expand the assays to the others genes in peaks where non-causative genes were found, as well as, although less relevant, to other small peaks not yet tested. Moreover, to go further in the characterization of the causative genes, it is important to clarify if the alleles that decrease in frequencies are non-protective or just less protective. To approach that, performing mendelian substitutions or using the CRISPR system could be very effective options.

In another perspective, to approach the heritability of each evolved phenotype, it would be interesting to perform crosses between both populations and discover whether or not the phenotypes of resistance against each route are maintained in the progeny. It will help to understand the basis of heredity of these traits.

We also determined, in **Subchapter 2.5,** the phenotypic and genetic changes underlying adaptation upon experimental evolution of a *Drosophila melanogaster* population under viral infection [*Drosophila* C virus (DCV)]. After 20 generations, selected flies showed increased survival upon infection with DCV and two other viruses. Using the same approach, whole-genome sequencing and through RNAi, we identified and functionally validated three genes responsible for the adaptive process and revealed their differential roles in the correlated responses observed.

The protective allele of *pastrel* was revealed as the major player in the adaptive response of the population. In addition, two genes not previously identified as important in immunity, *CG8492* and *Ubc-E2H*, strikingly showed that the defense against virus can evolve in a simultaneous combination of efforts between generic and specific responses.

Although several studies address the genetics of this interaction using different methodologies (e.g., gene expression profiles, isogenic lines,

mutants), no study so far has used the powerful combination of experimental evolution and genomics to tackle this issue, as we did in Subchapters 2.3, 2.4 and 2.5. It has recently been acknowledged that this methodology allows for a nearly unbiased estimate of the loci underlying adaptation of a population to a given selection pressure. Curiously, it had never been used to tackle host-parasite interactions.

It would be interesting to compare this kind of studies with other experimental evolution experiments performed with populations founded in accordance with Subchapter 2.2. Additionally, the use of the same population with other viruses or cross-infection challenges would also be an important method to reveal the similarities and specificities of each one of the many host-pathogen relationships.

In **Subchapter 2.6**, we set out to test if, as usually expected and frequently found, the increase of immunocompetence has a cost in the absence of activation of the immune system. We focused on costs of the population in returning to the ancestral environment, primarily relaxing the adapted populations and testing fitness-traits in those conditions, but we also tested other life-history traits without infection. Interestingly, we found no cost in the ancestral environment (maintenance conditions), as for several generations without selective pressure, we observed the maintenance of the evolved immunocompetence level. Likewise, no trade-offs were revealed in the other tested parameters when the populations were put under stress.

Obviously, we have to take into account that it is possible that an eventual cost will only appear in other conditions or in other traits. Therefore, in the absence of the costs found by us, only the ancestral environment is a categorical statement because the maintenance conditions are fixed, consequently allowing a precise test on the fitness parameters of

populations. All other tested parameters of life-history traits cannot be generalized because, as mentioned above, costs can appear in specific circumstances.

It is also important to note that costs after the activation of the immune system are relevant, perhaps central, to the evolution of species. One example is the BactSys populations in Subchapter 2.3, which respond worse when challenged against DCV. Since both species of microorganisms are natural pathogens of *Drosophila*, it becomes essential to investigate the possible consequences of a trade-off in the resistance against both.

In this chapter, we tried to show the potential of combining a vast range of biological resources and different methods and technologies. We believe that this objective was achieved, and that the findings here presented demonstrate the advantage of this high-scoped approach.

In Chapter 3 – **Host-endosymbiont evolution: selection and adaptation** – we dedicated particular attention to the influence of *Wolbachia* on the adaptation of *Drosophila* against viruses. To do that, in the lab, we used the same setup as in the previous Chapter.

We showed in **Subchapter 3.2** that after 20 generations of viral selection (previously described in Subchapter 2.5) *Wolbachia* genetic diversity changed and particular variants reached fixation with a strong selection coefficient. We further demonstrated that flies carrying the *Wolbachia* variants subjected to selection showed differential survival and reproductive outputs upon viral infection, which can fully explain the selection coefficient calculated. Therefore, we provided the first evidence that host and symbiont genomes are acting as a single evolutionary unit in the response to a pathogen infection.

The next step will be to uncover the reason of the observed fluctuations in the frequency of endosymbiont strains in nature, and whether the presence/absence dynamics are due to selection directly on endosymbionts (as bacteriophages) or the result of indirect selection through the host (for example virus, as demonstrated in this Thesis). Further studies are needed to understand what are the selective pressures are that drive these fluctuations and the relative importance of each in different host-endosymbiont populations.

Several previous studies have explored the consequences of the presence of *Wolbachia* in hosts selected or challenged against viral infection. In contrast, our work also shows the symmetric consequence, the effect of host adaptation on the *Wolbachia* population. Our findings revealed thus direct evidence of selection for increased host adaptation to pathogens through selection at the level of the microbial symbionts, relying on the evolution of both genomes.

In **Subchapter 3.3**, we tested the consequences of the removal of *Wolbachia* (and consequently its viral protection) in the evolved population against DCV. As expected, after antibiotic treatment in sister-replicates, the viral protection dropped drastically in both evolved and control populations. We kept the previously adapted population under the same selective pressure and after 20 generations we were able to show that a new adaptive process had occurred. Whole genome sequencing showed that the same regions of the 3L and X chromosome identified before are the primary regions to explain this increase in immunocompetence. The increase of frequency of viral-protective alleles of *Ubc-E2H* and *pastrel* strongly indicate that, one more time, these genes are responsible for the increase in resistance to DCV infection, now compensating fully for the viral-protection lost by the removal of *Wolbachia*.

Several new questions emerged from these findings, such as: How recurring in nature is this phenomenon of genetic pool alteration of the host population by loss of endosymbiont protection? Would the non-fixation of protective endosymbionts, such as that of *Wolbachia* against viruses, indicate that this is not a too strong (or long-lasting) selective pressure in nature? Being the most protective *Wolbachia* variants those that promote infections with highest bacterial loads, it is conceivable that different trade-offs trigger the variation of strains frequencies, according to the fluctuation of the intensity of each selective pressure at different moments.

Moreover, once again, we showed that the *Evolve and Re-sequence* methodology is considerably efficient to reveal the genetic modifications at the basis of adaptation.

In brief, we showed that the interaction of an animal with a pathogen can shape its associated microbial populations, which in turn constrains host adaptation. When the protective endosymbiont is removed from the equation, we demonstrated that hosts can find quickly new genetic arguments to compensate for the lost protection. We consider that these findings add additional layers of complexity onto the perception and understanding of host-microbe interactions. Moreover, this Chapter reinforces the achievement of this combined methodology, already highlighted in Chapter 2.

In Chapter 4 – **Endosymbioses as engines of adaptation and speciation** – and as highlighted in **Subchapter 4.1**, we explored the consequences to both partners of the endosymbiotic relationship during coevolution but also after a horizontal transmission event to another host.

First, in **Subchapter 4.2**, we took into account the several evidence and characteristics of endosymbionts (mostly facultative) and we

elaborated a parsimonious explanation that supports a fast and cyclic speciation of host lineages after the horizontal transmission of endosymbionts that orchestrate reproductive or behavioural manipulation. Although the subject of speciation is a topic of practical study in some model organisms, it will always be a great challenge to have the real perception of the minimum time required for this event to happen, even if incipient.

Speciation tests are normally observed and tested when they are already in progress and when there is evidence of some sort of reproductive isolation, without having certainty when speciation really began or for how long it will take. Thus, although we think that a particular mechanism is plausible to enable the creation of new species at a certain speed, we do not know which is the real contribution to the speciation process, and consequently to biodiversity.

Furthermore, testing complete speciation processes is clearly a risky task due the possibility that even a mechanism estimated as rapid, is expected not to be on the laboratorial time scale. However, speciation by endosymbiosis may sometimes contribute to overcome this issue and to understand its importance in the diversity of species, principally in Arthropods. When we know exactly how fast and frequent this phenomenon may be, we can begin to uncover which are the true ecological consequences for the emergence of novel lineages through host-microbe interactions. Perhaps a study of experimental evolution with different populations that start to suffer from cytoplasmic incompatibility or behavioural changes, triggered by endosymbionts after horizontal transmission, is an elegant and informative way to see the real effect on the host populational structure and perhaps speciation rates.

However, until the moment of any horizontal transmission, the previous host and the endosymbiont have to live together and possibly co-evolve. Although only the endosymbionts present in the reproductive germinal tissue ensure the vertical transmission to the next generation, it is not certain that this population is static.

With this in mind, in **Subchapter 4.3**, we approached this problem and its possible consequences, both physiologically and evolutionary. We explored many points of view and we produced the hypothesis that intracellular endobacteria stored in somatic tissues may have a chance of reaching the germ line. This possibility leads to an evolutionary strategy, where bacteria allocated in superinfected somatic tissues may be represented in the next generation.

Another important question is whether these different amounts of bacteria in different tissues can be the consequence of an active control by the host. Since the presence of this endosymbionts can bring a considerable increase in fitness to host, maybe they are actively monitored for example to respond better against pathogens.

A first query that remains unexplained is whether the difference between the amount of endobacteria in somatic tissues is a consequence of variance in the initial colonization or differential bacterial multiplication. To answer this question, a necessary step will be a detailed characterization of the quantities of bacteria and to know whether they change during the host's lifetime or according to the environment.

This differential growth can also be controlled by the bacterial population, taking advantage of the favourable conditions of certain tissues to use nutrients and proliferate. In this case, we may be facing the opposite case, a reduction of host's fitness. To uncover this issue, some experiments can be performed, such as transplantation of superinfected tissues to other

individuals or also identification of lines with different *Wolbachia*-infected phenotypes and posterior testing of their response capabilities for relevant factors.

To experiment in a practical way the potential for *Wolbachia* to perform horizontal transmission we opted to test, in **Subchapter 4.4**, cannibalism as a mechanism behind this phenomenon. The characteristics of intracellular endosymbionts made us consider that ingestion was the best candidate for a transmission process that does not involve another parasitoid species as a vehicle, a path already described as possible but still with an unclear efficiency.

If the limiting factor for the horizontal transmission was the probability of a non-infected individual feeding from an infected one, then our experiment could overcome this environmental limitation, raising the probability of intaking *Wolbachia* to virtually 1. However, in this type of approaches with binary phenomena, possibly with low probability, only a positive result would be categorical. In our case, we still do not know what is the likeliness of this phenomenon, as it may fall orders of magnitude away from the tested scale. Nevertheless, the absence of transmission with the tested numbers shows that in the crowded larval environment of most species of flies, a promiscuous sharing of endosymbionts should rarely occur.

Another point of interest raised in **Subchapter 4.4**, is the possible need of one or more co-factors, in one or more steps taken by endobacteria, that potentially trigger horizontal transmission in a effective way. Perhaps these will only happen in certain circumstances, when the host (or the endosymbiont, depending on who drives the process) are faced with a specific selective pressure or a particular context, being therefore key to understanding this phenomenon.

Putting all together, now it is important go further and search what protective phenotypes are conferred by *Wolbachia* immediately after transmission to a new host species, where ecological dynamics (namely the sharing of the same habitat) indicate that the same pathogen can be a selective pressure for both the former and new host.

Despite ample knowledge on the genetics and physiology of host responses to parasites, much is still unknown about the genetic basis of host adaptation to parasites. Moreover, adaptation to one parasite is likely to impact the outcome of different infections. Yet these correlated responses, seminal to the understanding of host evolution in multiparasite environments, remain still poorly studied. Consequently, many others studies have to be conducted to fill this gap and robustly distinguish the rules from the exceptions.

With this in mind, this Thesis aimed to contribute in different ways to a better understanding of host-microbe relationships. Using different techniques and methodologies, as well as practical and theoretical approaches, we added some pieces in this huge under-construction puzzle that is the never-ending battle between species: a war made of broken alliances and old enemies, because in nature there is no forgiveness, but there is also no resentment.