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Testing endosymbiont-mediated immune protection in a novel host species

Mestrado em Biologia Evolutiva e do Desenvolvimento

Tânia Filipa Teixeira Paulo

Dissertação orientada por:

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Abstract

The complexity of life forms cannot be explained without the concept of symbiosis. Symbiotic relations exist abundantly in nature, particularly the ones between bacteria and eukaryotic hosts. One of the most widespread endosymbiotic bacteria described belongs to the genus *Wolbachia*, naturally infecting arthropods and nematodes. In arthropods, *Wolbachia* induces reproductive manipulations, to promote infected female's fitness as they are vertically transmitted, maternally. Additionally, in *Drosophila melanogaster*, they have been shown to confer protection against viral infections.

Being an obligatory vertically-transmitted endosymbiont, *Wolbachia* phylogeny should recapitulate that of its hosts. However, several studies show that host and symbiont phylogenies are not concordant, suggesting that horizontal transfers must have occurred throughout time. This has been confirmed, namely between parasitoid wasps and their hosts.

Considering these observations, the present project aimed at uncovering if and at which rate horizontal transmission of *Wolbachia* could occur between *Drosophila melanogaster* and its natural parasitoid *Leptopilina boulardi*. Also, we tested if the viral protection phenotype induced in *Drosophila* would be passed on to the novel host, after this horizontal transfer event.

Simultaneously we screened a wild-caught population of *Leptopilina heterotoma* for *Wolbachia* presence. We obtained infected individuals with which we established an isofemale line and derived *Wolbachia*-negative counterparts using antibiotic treatment. With this tool, we characterized the effects of *Wolbachia* presence upon viral systemic infection.

Our results show that horizontal transmission of *Wolbachia* happens between *Leptopilina boulardi* and its host, but the infection is not stably maintained. We also see that these wasps do not appear to be susceptible to viruses that are pathogenic in *Drosophila* (specifically DCV and FHV) and appear to be mildly detrimental to *Wolbachia*-infected *Leptopilina heterotoma*. Finally, we have verified that a natural endosymbiont infection appears to delay full development time in *Leptopilina heterotoma*, although no effect is detected for longevity.

With this work we have established an experimental system for the controlled and systematic study of the complex interactions between *Drosophila*, parasitoid wasps, *Wolbachia* and viruses.

Key-words:

Parasitoid wasp, *Drosophila melanogaster*, horizontal transmission, *Wolbachia*, immune protection

Resumo

A diversidade de formas de vida dificilmente é explicada sem ser tido em conta o conceito de simbiose. Relações simbióticas são abundantemente encontradas em ambientes naturais. Os primeiros eventos de endossimbiose conhecidos terão ocorrido há cerca de 1,5 milhares de milhão de anos atrás. Estes acontecimentos descrevem a incorporação de cianobactérias e proteobactérias ancestrais em organismos procariotas. Quando a simbiose se tornou mutualista obrigatória, estes organismos procariotas associados a endossimbiontes tornaram-se nos primeiros eucariotas do planeta, e os seus anteriormente designados endossimbiontes tornaram-se organelos, nomeadamente cloroplastos e mitocôndrias.

Atualmente existem incontáveis exemplos de organismos vivos que não existiriam se não se estabelecessem relações simbióticas entre diferentes entidades biológicas, constituindo a base para a enorme diversidade de formas de vida existentes. Alguns dos casos mais frequentes de interações entre diferentes organismos que moldam inquestionável e determinantemente a história evolutiva do planeta são os que ocorrem entre procariotas e hospedeiros eucariotas. Estes sistemas podem ser encontrados em todas as circunstâncias, quer em metazoários como plantas e animais, quer em organismos unicelulares. Por exemplo, o filo Porífera realiza incontáveis associações com microrganismos bacterianos que lhe permitem obter nutrientes mais eficazmente ou ainda os vários casos de plantas que formam associações com bactérias fixadoras de azoto. Numa tentativa de classificar os diferentes tipos de interações que podem ocorrer entre dois ou mais organismos, foram atribuídos nomes a categorias discretas que ocorrem no espectro de interações naturais possíveis. Relações simbióticas podem ser comensais, mutualistas ou parasíticas.

Comensalismo descreve o tipo de interações em que um organismo beneficia da relação que desenvolve com outro, enquanto o segundo permanece indiferente. Relações mutualistas englobam situações em que a associação é mutualmente benéfica para ambos os envolvidos. Parasitismo implica que um dos envolvidos seja prejudicado, para benefício do outro. Estas duas últimas formas de interação estão intimamente relacionadas, na medida em que as medidas de adaptação que um endossimbionte tem de sofrer para poder invadir e colonizar um hospedeiro, qualquer que seja a interações que acabe por vingar, são

semelhantes para uma relação mutualista ou parasita, especialmente se considerarmos as relações abundantes que se estabelecem entre bactérias e hospedeiros eucariotas. No entanto, e apesar das referidas classificações existirem e serem relevantes, é difícil atribuir qualidades discretas a endossimbiontes, uma vez que os efeitos que induzem no seu hospedeiro podem variar entre mutualistas ou patogénicos, consoante vários aspectos (como factores ambientais).

Um exemplo privilegiado deste tipo de comportamento variável é o das bactérias do género *Wolbachia*. Estas são alfa-proteobactérias, membros do grupo Rickettsiales (que engloba todos as bactérias endossimbióticas obrigatórias conhecidas), que infectam artrópodes e algumas espécies de nemátodes filariais. Este endossimbionte é um dos mais representados na natureza, infectando mais de 60% de todos os insectos conhecidos, para além de presente em ácaros, aranhas, escorpiões e isópodes. Sendo um dos organismos mais bem estudados atualmente no que diz respeito a relações de simbiose, existem vários estudos que descrevem algumas das adaptações que sofreu de modo a proliferar nas células dos hospedeiros. Nomeadamente, a bactéria utiliza mecanismos de transporte vesicular para viajar dentro das células do hospedeiro. No entanto, a característica deste género que é indubitavelmente mais estudada é a sua capacidade de manipular o sistema reprodutivo do hospedeiro em seu proveito.

Existem diferentes formas segundo as quais *Wolbachia* consegue manipular a reprodução dos seus hospedeiros, nomeadamente: feminização, morte de machos, partenogénese e incompatibilidade citoplasmática (IC). Feminização descreve a transformação fenotípica em fêmeas de organismos geneticamente masculinos; morte de machos acontece quando machos infectados são inviabilizados, disponibilizando mais recursos para as irmãs que possam transmitir a infecção à geração seguinte; partenogénese descreve a produção de prole unicamente feminina contribuição parental masculina; por fim, a manipulação reprodutiva mais comum, a IC descreve o processo segundo o qual fêmeas infetadas geram menos prole viável quando fertilizadas por machos não infetados (ou se ambos hospedarem estirpes incompatíveis). Todos estes processos de manipulação reprodutiva têm como objectivo maximizar a dispersão e colonização de *Wolbachia* pelo maior número de indivíduos possível, o que por sua vez é conseguido através do favorecimento da descendência feminina. O principal motivo que explica estes processos

prende-se com a forma canónica de transmissão de *Wolbachia* entre hospedeiros, que é feita verticalmente por via materna.

Uma outra influência que *Wolbachia* exerce sobre os seus hospedeiros prende-se com a capacidade de proteger *Drosophila melanogaster* contra (algumas) infeções por vírus de RNA. Esta capacidade foi descrita recentemente (não só para *Drosophila* mas também para o mosquito *Culex pipiens*) e desde então múltiplos trabalhos têm sido desenvolvidos na tentativa de caracterizar e determinar os mecanismos subjacentes. É sabido que *Wolbachia* protege contra Drosophila C Virus (DCV) e Flock House Virus (FHV), entre outros vírus de RNA, mas que a proteção não se estende para vírus de DNA (que não se conhece infectarem naturalmente espécies de *Drosophila*) e que, inclusive, diferentes níveis de proteção estão associados a diferentes estirpes da bactéria.

Outra peculiaridade deste género de endossimbiontes prende-se com a filogenia discordante que apresentam relativamente à dos seus hospedeiros. Tendo em conta que são verticalmente transmitidas, seria de esperar que a árvore filogenética das estirpes de *Wolbachia* espelhasse, com alguma exatidão, a árvore filogenética dos seus respetivos hospedeiros. Isto não se verifica sugerindo que, além da transmissão vertical, eventos de transmissão horizontal entre hospedeiros têm de ter ocorrido ao longo do tempo. Adicionalmente, estudos baseados nestas filogenias de *Wolbachia* permitem inferir que existem enormes semelhanças entre as estirpes albergadas por certos insectos filogeneticamente distantes, nomeadamente entre vespas parasitóides e os seus respectivos hospedeiros. Tendo isto em conta, foram conduzidos estudos que determinaram que a bactéria pode ser transmitida horizontalmente entre diferentes hospedeiros por canibalismo de animais infectados, por partilha próxima de nichos ecológicos e através de um vector como uma vespa parasitóide.

Reunindo toda a informação apresentada acima, das características da *Wolbachia*, especificamente da sua capacidade de induzir proteção viral em *Drosophila*, da sua filogenia discordante (indicativa de eventos de transmissão horizontal) e da existência de casos reportados em que vespas parasitóides atuam como vector transportador de endossimbiontes, formulámos as questões que apresentamos de seguida.

1- Consegue a *Wolbachia* conferir proteção viral num hospedeiro parasitóide recém-adquirido, fruto de um evento de transmissão horizontal?

2- Está uma população natural de vespas parasitóides protegida contra infecções virais, pela sua estirpe nativa de *Wolbachia*?

    2.1- Como responde esta população natural, em comparação com as vespas mantidas no laboratório?

    2.2- Existem custos associados à manutenção de *Wolbachia*?

Começámos por tentar responder à primeira questão estabelecendo linhas isogénicas da vespa *Leptopilina boulardi* não infectadas por *Wolbachia* e sujeitando-as a hospedeiros de *Drosophila melanogaster* contendo *Wolbachia*. Utilizando duas linhas de moscas infectadas com estirpes individuais diferentes, sujeitámos as vespas a estes hospedeiros infectados e recolhemos toda a descendência após este evento de parasitação. Com estas fêmeas estabelecemos linhas isogénicas, das quais recolhemos, na geração seguinte, indivíduos suficientes que nos permitisse extrair DNA e testar a presença de *Wolbachia* por PCR. Com estes dados pudemos inferir a taxa de transmissão horizontal entre *Leptopilina boulardi* e *Drosophila melanogaster*. Numa tentativa de avaliar se a proteção viral conferida em mosca era transmitida com o endossimbionte para o novo hospedeiro estabelecemos um protocolo de infecção viral sistémica em vespa. No entanto, verificámos que a espécie *Leptopilina boulardi* não parece ser susceptível aos vírus de RNA que são canonicamente testados em *Drosophila*. Atestámos também que, nesta espécie, uma transmissão horizontal de *Wolbachia* não origina uma infecção estável, que seja em última análise, verticalmente mantida.

Para responder à questão 2, recolhemos indivíduos da natureza e testámos a presença de *Wolbachia* assim como determinámos a espécie em questão. Deparámo-nos com uma linha de *Leptopilina heterotoma*, infectada com *Wolbachia*. Para estabelecermos um controlo negativo, usámos um tratamento de antibiótico que tratou a infecção, proporcionando duas linhas semelhantes de vespa, uma com e outra sem *Wolbachia*. Posteriormente, infecção viral foi realizada para esta espécie, onde vimos um efeito pequeno da ação de DCV, apenas detectável na linha que continha a infecção bacteriana de origem mas não na linha tratada.

Para determinar custos, realizámos ainda medições quanto ao tempo total de desenvolvimento e longevidade de ambas as linhas de *Leptopilina heterotoma*, onde detectámos um possível efeito da *Wolbachia* a atrasar ligeiramente o desenvolvimento de ovo até adulto.

Sumariamente, os nossos resultados indicam que a transmissão horizontal de *Wolbachia* ocorre, embora o estabelecimento de uma infeção vertical estável seja seja, neste caso, indetectável. Concluímos também acerca do efeito que uma infecção viral pode ter (ou não) sobre uma espécie com a qual provavelmente partilha nichos ecológicos na natureza (considerando que tanto *Leptopilina boulardi* como *Leptopilina heterotoma* são parasitóides naturais de *Drosophila melanogaster*, o hospedeiro por excelência de DCV). Podemos ainda verificar que a presença deste endossimbionte pode induzir custos num hospedeiro nativo, como de resto é verificado noutras espécies.

Palavras-chave:

Vespa parasitóide, *Drosophila melanogaster*, *Wolbachia*, transmissão horizontal, proteção viral

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Introduction

"In the beginning the Universe was created. This has made a lot of people very angry and has been widely regarded as a bad move." – Douglas Adams in *The Hitchhiker's Guide to the Galaxy*

Symbionts and pathogens

Throughout evolutionary time, diversification of molecules and consequent rearrangements and establishment of the first forms of life implied deep levels of primary interaction and formation of connections[1]. The organelles present in current eukaryotic cells, such as mitochondria (descendants of ancestral alfa-proteobacteria) and chloroplasts (descendants of ancestral cyanobacteria), have been proven to be probably the most ancient recorded symbiotic event, dating back to 1,5 billion years ago[2]. Upon the leap of microorganism diversification, with the motor of the recently evolved photosynthesis, oxygen became a major influence on the establishment of novel organismal relations. In general, these interactions that are established between prokaryotes and eukaryotic hosts imply specific binding of the "invader" to the host's cell surface, uptake by constitutive or triggered phagocytosis and the utmost important posterior survival and active replication of the internalized prokaryote (either in specialized structures or the cytoplasm of the new host)[3].

Countless examples exist in nature of two organisms organizing and forming associations (ref). For instance, the Porifera phylum and their associated microorganisms, Cnidaria and CO_2 fixing algae, plants and nitrogen fixing bacteria, to name a few[1]. There is a spectrum of classifications that can be attributed to these symbiotic associations, but the vast majority seems to fall on one of the categories: mutualism, commensalism or parasitism. Commensalism is the specific case of when one of the involved parties takes an advantage of the interaction, while the other remains neutral to it. The other two types of interaction, mutualism and parasitism, will be more thoroughly discussed below.

Mutualistic symbiosis encompasses the mutually beneficial interaction between organisms[4]. Many of the described mutualistic interactions are based on nutritional transfers[5] from symbiont to host, through exchange of low molecular weight compounds or

by digestion. Both host and symbiont are extremely well-adapted to each other, ensuring maximum balance and intracellular survival of the colonizing symbiont.

Another well-studied type of interaction between bacteria and eukaryotic hosts is parasitism. Parasitic interactions describe associations between organisms where one benefits at the expense of the other. This prejudicial effect can range from cell and tissue damage to the death of the host. In this context, another important aspect of the interaction between host and symbiont, concerns the way host immune system responds to bacterial invasion. Interestingly, to date, similar mechanisms of colonization have been described for bacteria that are parasitic, commensal or mutualistic.

The most well described examples of symbiont impact on host tissues come from arthropods. It has been estimated that more than half of the insects have a bacterial symbiont that initiated this relationship by allowing hosts to explore nutritionally low food sources[6]. These bacteria-host interactions have evolved many times throughout evolutionary time and are widespread in plants and animals. Because bacteria usually have lower generation times and generate larger populations than eukaryotic organisms, they are more prone to genetic change and, consequently, phenotypic change. This intimate connection ultimately results in the acquisition of novel metabolic traits or even formation of specialized tissues and creation of defense mechanisms by hosts[7,8]. Despite the countless examples of established parasitic and mutualistic relations between arthropod hosts and bacteria, it remains debatable if bacteria have an *a priori* mutualistic or pathogenic nature, or whether this is dictated by the host or environmental cues[7,8]. Interestingly, in more recent studies, there are reported cases where the same organism shows both mutualistic and pathogenic phenotypes[3], whether on the same host or on different hosts[9], depending on a number of factors (for instance, endosymbiont load).

Wolbachia spp.: a prime endosymbiotic example

One prime example of this behavioral plasticity is provided by bacteria of the genus *Wolbachia*. These are Gram-negative α-proteobacteria members of the Rickettsiales[10]. This order contains all known species of obligatory endosymbiotic bacteria, whose effects on hosts cover the entire mutualistic-to-parasitic spectrum. Unlike related genera (for instance

Rickettsia and *Anaplasma*), *Wolbachia*'s life cycle does not include a mammalian host and is only, until now, known to infect arthropods and nematodes[11]. Although it seems at first glance that this is limiting in terms of number of hosts, *Wolbachia* is one of the most widespread endosymbionts known to date, infecting about 66% of all insect species, according to a recent meta-analysis study[12]. The first ever described case of *Wolbachia* infection was in the mosquito *Culex pipiens* (hence the "type" species named *Wolbachia pipientis*), but currently it is known to infect all kinds of arthropods, from insects to mites, spiders, scorpions and isopods, as well as filarial nematodes[13].

There are several reasons for the incredibly wide distribution of this endosymbiont in natural populations, one of which being their remarkable adaptation to endure intracellular life inside arthropod cells. They have been reported to use the host's spindle apparatus during cell division and cytoskeletal motors in order to travel within cells[14–16]. Another important feature of *Wolbachia* in this respect may consist of the high number of ANK domains in its genome, important in mediating host-pathogen interactions in eukaryotes[17], associated to the presence of viral like elements and of a bacteriophage (WO)[18,19]. Another factor that significantly influences the wide distribution of this endosymbiont is its reproductive manipulation of the host, which includes feminization, male-killing, induction of parthenogenesis and Cytoplasmic incompatibility (CI). Because bacteria of this genus are vertically transmitted by females, these types of reproductive phenotypes induced by *Wolbachia* increase transmission rate substantially.

Reproductive manipulation: Feminization

Feminization consists of the transformation of genetic males into females. This phenomenon is known to take place in isopods and in some insects. It is not known what are the exact mechanisms behind this *Wolbachia*-induced phenotype, although in isopods it has been shown that hypertrophy of the androgenic gland relates to the high proliferation of the bacteria[20]. In insects, feminization is known to occur in the Lepidoptera and Hemiptera orders through undetermined mechanisms[21,22].

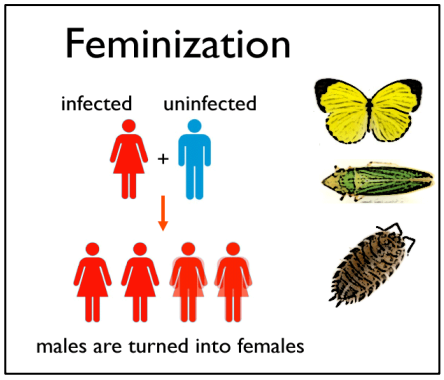

Figure 1 - *Wolbachia*-induced feminization

Reproductive manipulation: Male-killing

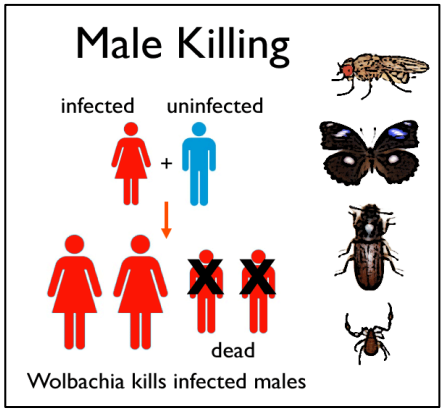

Figure 2 - *Wolbachia*-induced male killing

Male-killing has been found in a number of different insect orders, namely in Diptera[23], Coleoptera[24], Pseudoscorpiones[25,26] and Lepidoptera[27,28]. In the pseudoscorpion order it has been shown also that *Wolbachia* induced male-killing has benefits for the host, specifically more and bigger daughters[25], without the cost of brood abortion. In lepidopterans, two different species (*Hypolimnas bolina*[27] and *Acrea encedon*[28]) have been found infected each by two similar strains that induce the same male-killing phenotype. Male-killing has also proven to be a stable strategy for *Wolbachia* infection in *Drosophila borealis* and *Drosophila innubila*[29].

Reproductive manipulation: Parthenogenesis

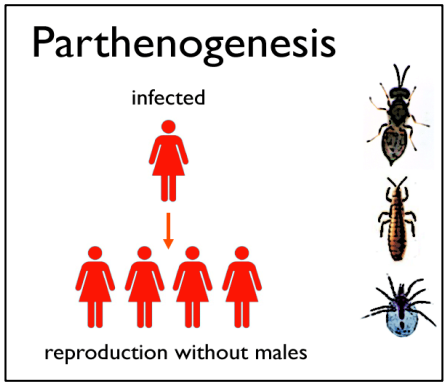

Figure 3 - *Wolbachia*-induced parthenogenesis

Wolbachia-induced parthenogenesis has been described in many different arthropods species, all of them with an arrhenotokous development (males originating from unfertilized eggs). Examples include, thrips, mites and some hymenoptera, such as parasitoid wasps. One of the first described cases of this phenomenon was published in 1990, when an antibiotic treatment appeared to induce parthenogenesis in *Trichogramma* spp.[30]. Later studies show that parthenogenesis in these individuals is provoked in the early stages of embryonic development, where the cell cycle is disrupted, leading to the diploid development of unfertilized eggs (ref). One example of the type of disruptions that can occur is described for *Trichogramma* spp.[31] and *Leptopilina clavipes*, where the first anaphase of embryonic development is abortive, resulting in one diploid embryo instead of two haploid ones[32]. Differently, in *Bryobia praetiosa*, what seems to be disrupted is meiosis, because diploid gametes are produced. Overall, this process of *Wolbachia* induced parthenogenesis is specific and the mechanism through which it occurs is hard to generalize.

Reproductive manipulation: Cytoplasmic incompatibility

The most common reproductive phenotype induced by *Wolbachia* is cytoplasmic incompatibility (henceforth referred to as CI). CI entails two distinct ways of action: sperm modification during spermatogenesis (a still uncharacterized effect is induced in sperm during its production) and rescue of the modification in crosses of incompatible gametes (if fertilization occurs with a *Wolbachia*-compatible egg, the viability phenotype is rescued). It can also be defined as uni- or bi-directional, when sperm from an infected male fertilizes uninfected eggs or when male and female gametes are infected by different *Wolbachia* strains, respectively[11]. The mechanisms that underlie these reproductive changes are mostly undetermined to date, but there are some hints that point to the processes that are disrupted in the host's reproductive tissues[33]. Some cytogenetic mechanisms behind CI have been studied in mosquitoes[34], Drosophila[35] and parasitoid wasps[36] and they all seem to point to the induction of early mitotic defects in the fertilized eggs, whether by impediment of fusion of the paternal pronuclei[34,37] or by incorrect segregation and organization of paternal chromosomes in early mitosis [35,36]. An additional level of complexity is attributed to CI when we consider that its expression is influenced not only by the presence of endosymbiotic bacteria, but by its load, strain and even host genotype[38]. The interaction between all of these factors is also relevant because, if transfected, not all strains maintain their original tendencies and are not capable of completely rescuing modifications induced by different ones[39].

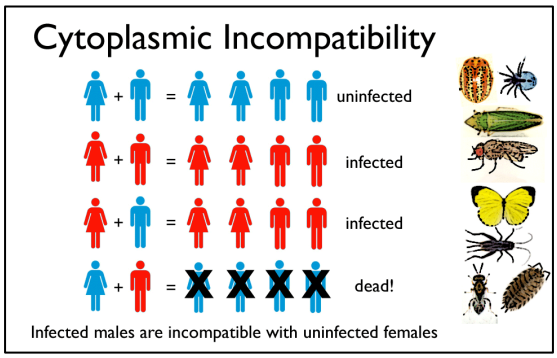

Figure 4 - *Wolbachia*-induced cytoplasmic incompatibility

Lastly it is important to consider that the reproductive manipulation processes described above are not necessarily host or strain-specific. There are several reported cases of bacterial transfer between different hosts that lead to the induction of unexpected phenotypes in the novel host. *Wolbachia* transfer between lepidopterans leads to an induction of male-killing in the novel host *Ephestia kuehniella,* whether from a strain that in its natural host (*Cadra cautella*) induces CI[40] or feminization (from an *Ostrinia scapulalis* host)[9].

Alternative influences of *Wolbachia* infection

As described above, *Wolbachia* can influence the host's reproductive system in order to increase its own transmission rates and spread rapidly in a population[41]. These manipulations allow for a faster spreading of *Wolbachia* infection at the expense of the host's reproductive potencial, by decreasing the number of possible offspring. This big range of deleterious effects would in theory imply that, unless these phenotypes of reproductive parasitism were strong enough, then some other kind of beneficial effect would have to be conferred by the presence of these endosymbiotic bacteria in host tissues, to allow for such a prevalent presence[42]. This has been reported not to be the case in *Drosophila melanogaster* infected by *Wolbachia*[43,44]. A few studies have shown that harboring *Wolbachia* can have some beneficial effects on hosts[45–47], although those effects didn't seem to be sufficient to justify the massive invasion of these bacteria in natural populations of *Drosophila*[48]. In more recent studies, however, a beneficial fitness effect of *Wolbachia* infection in *D. melanogaster* has been described. In 2008 two independent research groups described that the presence of *Wolbachia* in *D. melanogaster* conferred protection against RNA-viruses systemic infection[49,50]. Posteriorly, this protection was described for oral infection[51,52] as well.

The first virus that was shown to have its pathogenicity affected by these endosymbionts was Drosophila C Virus (DCV). DCV is a horizontally transmitted pathogen, first discovered in *D. melanogaster*[53,54]. It is a non-enveloped, positive-sense, single-stranded RNA virus that belongs to the *Dicistroviridae*[53] family (genus *Cripavirus*). DCV can be highly pathogenic to flies, specifically if it is systemically introduced into the haemolymph[55,56], although there is a high variability in the phenotypes induced by this pathogen[57]. The protective effect conferred by *Wolbachia* in *D. melanogaster* is not DCV-specific. It was demonstrated that the viral protective effects included similar viruses (Cricket Paralysis Virus [CrPV]) and viruses that belong to other families (such as Flock House Virus [FHV] and Nora virus), although it didn't comprise DNA viruses, that usually don't infect *Drosophila*[49]. The mechanisms through which *Wolbachia* confers this protection are still mostly unknown, but there seems to be indication that the level of protection they confer correlates with titer[51,58]and with production of reactive oxygen species (ROS)[59]. Moreover, there is evidence that points to this endosymbiont being involved in other kinds of immune protection in arthropods hosts, although these are still scarcely described and uncharacterized[60,61].

Taken together, the phenotypic effects that are induced in hosts by *Wolbachia* infection, whether they are of reproductive manipulation, whether of viral protection in *Drosophila*, contribute to an understanding of the wide abundance of this endosymbiont in natural arthropod populations.

Routes of transmission: Vertical *versus* Horizontal transmission

As previously mentioned, the canonical route of *Wolbachia* transmission is vertical, from mother to offspring. Considering this, it would be expected that the phylogeny of this bacteria would be in agreement with that of its hosts (meaning that the phylogenetic trees could be inferred from each other) having consistently evolved in parallel and close association. However, several studies have shown that this is not the case and that the phylogeny of *Wolbachia* cannot be explained by exclusive vertical transmission events[62–67]. Further research in this area led to the confirmation that horizontal transmission could be behind this disparity between host and endosymbiont phylogenies and showed the occurrence of these events in a number of different species[27,68–73]. It could be considered that this incongruence between bacterial phylogenies could be explained by recombination events in the sequences that are usually used to classify *Wolbachia* strains (16S[66], *wsp*[64], MLST[74]) but this would not be sufficient to explain why closely related strains infect hosts that, although phylogenetically distant, share ecological niches[62] and could not be representative of real recombination rates happening in *Wolbachia*, recently described to be virtually absent[75].

Some of the studied cases that describe horizontal transmission of *Wolbachia* in natural conditions involve direct passage of the endosymbiont between hosts (also made possible by its ability to survive outside host cells for a limited period of time[76]). For instance, in woodlice, horizontal transmission occurs but is dependent of blood-to-blood contact[77], or requires cannibalism or predation[78], whilst in *Drosophila melanogaster* this route of horizontal transmission of *Wolbachia* has been proven not to be efficient[79].Evidence also shows that horizontal transmission has occurred between isopods and their spider predators, parasitic phoretic mites and specialist parasitoid dipterans[73]. *Wolbachia* has also been proven to use a plant substrate in order to spread to different novel hosts that share it as a food source or that inhabit it (whiteflies, planthoppers and flea beetles in pumpkin

leaves for instance[80], or hoppers and parasitoid flies in rice-fields[72]). Phylogenetic evidence also points to events of recent transfer in the spider *Agelenopsis* genus[68] and the predator-prey system of Acari mites *Metaseiulus occidentalis* and *Tetranychus urticae*[81]. A horizontal transmission route has been observed also for intra- and inter-specific transmission of *Wolbachia* between two individuals of *Trichogramma* spp. sharing the same host egg[82].

In the above-mentioned cases it is implied that a newly acquired endosymbiont invades host tissues and colonizes them successfully, namely the germ line, in order to give rise to a stable *Wolbachia* infection. However, in order to effectively colonize its new host the bacteria has to overcome the local immune defense mechanisms (like the deployment of AMPs [Anti-Microbial Peptides] and increased levels of ROS)[83], not to mention the migration through host tissues and reaching the germline. A possible way to minimize these defense mechanisms would be for the *Wolbachia* to reach the haemolymph directly. Several studies have proven that this can occur by artificially injecting *Wolbachia* into new hosts. These tests have been performed with the intent of trying to manipulate viral loads by inserting a more protective strain of *Wolbachia* than the one naturally carried in mosquitoes [84], between lepidopterans[9,85], between isopods[86] and between dipterans (from *Rhagoletis cerasi* to *Ceratitis capitata* and *Drosophila simulans*)[87], including the thoroughly studied transfers within the *Drosophila* genus[88–90]. Although some of these transfers show that a newly acquired *Wolbachia* infection can be stably maintained after a systemic insertion in the new host, this still remains an artificial route of transmission and thus fails to have, to an extent, ecological relevance. Meanwhile, and supporting the view that strengthens the systemic route as less detrimental for establishment of a novel infection, there are reported several cases where parasitoid wasps serve as vectors for *Wolbachia* horizontal transmission.

Vectors

Parasitoid wasps are hymenopterans that parasitize invertebrates, mainly insects and that, end up killing their host in order to complete their life-cycle[83,91]. Because they intimately share development with hosts, growing either on their surface within them, the host-parasitoid system offers a great opportunity for endosymbionts to be passed horizontally. This is revealed in a number of species where similar strains of *Wolbachia* have been described for parasitoids and arthropods these wasps are known to infect. There are

described examples for *Trichogramma bourarache* and the host moth *Ephestia kuehniella*[64], for the *Nasonia-Sarcophaga* system[62], for the *Solenopsis* spp. ants complex (with a *Pseudoacteon* spp. wasp as vector)[92] , for the *Bemisia* tabacci whitefly[93] and in the model system *Drosophila melanogaster* and its parasitoids[63,69].

In 1999, Vavre et. al[63] determined the phylogeny of *Wolbachia* based on the *wsp* gene and contrasted it to the phylogeny of the hosts that harbored the respective strains. This allowed the acknowledgement that the phylogeny of *Wolbachia* did not comply with strict vertical transmission in the *Drosophila*-parasitoids complex. Besides, this study enabled us to infer that the close proximity that exists between certain strains of *Wolbachia* can be explained by horizontal transfers between *Drosophila* hosts and parasitoid wasps (namely, the closely related strains of *Drosophila simulans* and *Leptopilina heterotoma* or of *Drosophila melanogaster* and *Asobara tabida*). Another study stated that these horizontal transmission events could occur by natural mechanisms and be observed in real time, in laboratory conditions, by performing horizontal transmission of *Wolbachia* from a *Drosophila simulans* host to a *Leptopilina boulardi* wasp[69].

With these studies in mind and considering what had already been demonstrated for *Wolbachia*, we set out to address some outstanding questions. By analyzing the phylogeny of this endosymbiont it is clear that horizontal transmission occurs and that parasitoid wasps are in a privileged position to enable these transmission events. Additionally, it has been described that interspecific transmission of *Wolbachia* can induce similar phenotypes in the novel host as the ones describe in a native environment. Lastly, considering the aforementioned ability to provide viral protection to their *Drosophila melanogaster* hosts, we hypothesized about the maintenance of these specific phenotypes after horizontal transmission from a native host to a novel one (see below). To this aim, we established populations of *Drosophila melanogaster*, *Leptopilina boulardi* and *Leptopilina heterotoma*.

We started by performing horizontal transmission of *Wolbachia* between *Drosophila melanogaster* and *Leptopilina boulardi*. Subsequently we attempted to establish an isogenic line of wasps in which we could test the effects that the newly acquired endosymbiont could be inducing, with emphasis in possible viral protective abilities. In parallel, we tested a wild-caught line of *Leptopilina heterotoma* and verified it was infected by a native strain of *Wolbachia*. In order to perform further tests on the effects that the bacteria could be

inducing, we established a negative control for the infection by curing the wasps with an antibiotic treatment. Lastly, we compared ecologically relevant life-history traits of *Wolbachia*-infected and uninfected wasps of *Leptopilina heterotoma*, as well as inferred about possible viral protective effects that could be induced by this native strain of this endosymbiotic bacterium.

Main questions

By considering all the information mentioned before and the established model systems maintained in the laboratory (*Drosophila melanogaster*, *Leptopilina boulardi* and *Leptopilina heterotoma*) the following questions were addressed:

Question 1: Does *Wolbachia* confer viral protection in a novel parasitoid wasp host, after an event of horizontal transmission?

Question 2: Is a wild wasp population naturally infected with Wolbachia protected from viral infections?

Question 2.1: How do these wasps respond, in comparison with the ones being kept in the lab?

Question 2.2: Are there costs in harboring *Wolbachia*, for this particular wild-caught wasp?

Materials and Methods

Populations

Drosophila melanogaster - For the horizontal transmission experiments with *Leptopilina boulardi* we used two *Wolbachia*-positive lines of *Drosophila melanogaster* (carrying the *w*Mel_like and wMelCS_b strains) and an isogenic *Wolbachia*-negative line for control (*iso*), as described previously [49,58]. For maintenance of wasp stocks, we used a line of *Dif* mutants, *Dif* being a central transcription factor of the Toll pathway involved in immune response [83,94]. *These* mutants are more susceptible to wasp parasitation thus ensuring a maximization of wasp progeny, ideal for maintenance conditions. All fly stocks were kept in standard food at 25º C, 60-80% humidity and 12:12 light/dark cycles.

Leptopilina spp. - The *Leptopilina boulardi-G486* [95] strain was maintained in the lab at room temperature. All tests were performed at 25º C until adults eclosed, at which point they were transferred to 18º C in fly food vials with honey-soaked lids.

We collected decaying figs from the wild, colonized by *Drosophila* spp. larvae. After 48 hours in quarantine conditions, adult wasps began to emerge from the rotten fruits and 8 females were retrieved. These 8 females were exposed separately to L2 *Dif* larvae to oviposit. From these females we could establish successfully 5 lines that were designated w3, w4, w6, w7 and w8.

Through sequencing of a cytochrome-oxidase subunit I (COI) fragment (see below) and phenotypic analysis we determined that w3, w6, w7 and w8 were *Leptopilina boulardi* and w4 was *Leptopilina heterotoma*. Also, PCR with specific primers (see below) showed that only the w4 line was infected with *Wolbachia* and no line was infected with *Spiroplasma*.

The outbred lines (both *Leptopilina boulardi* and *Leptopilina heterotoma*) were maintained in *Dif* flies at 25º C during development and at 18º C as adults. Both temperatures featured 60-80% humidity and 12:12 light/dark cycles.

Horizontal transmission

For both *Drosophila melanogaster Wolbachia*-positive lines, 8-12 hours egg-lays were done in bottles. After 48 hours we introduced *Leptopilina boulardi* females and allowed

them to oviposit. After 10 days, emerging flies resulting from successful host immune responses or from unsuccessful wasp infection, were discarded.

After 20 days, every female wasp that emerged was allowed to oviposit in L2 *Dif* larvae individually and establish an isogenic line. Pools of 30 males that were not used to fertilize these females were collected and stored at -20º C. When the offspring emerged after approximately 20 days, 20 to 30 individuals from each isogenic line were collected and tested for the presence of *Wolbachia*. DNA extraction and PCR testing with *Wolbachia*-specific primers (see below) was conducted on F1 males and on all of the isogenic lines (F2) to assess horizontal transmission events. Given that only 90 lines originated females, they were prioritized in PCR testing.

Wolbachia removal from *Leptopilina heterotoma*

To generate *Wolbachia*-negative (wol-) controls for the tests in *Leptopilina heterotoma*, we performed an antibiotic treatment as previously described[96].

Tetracycline was administrated orally to *Drosophila melanogaster* larvae from egg to L2 larval stage by mixing tetracycline to standard food at 0.05 mg/mL. *Leptopilina heterotoma* adults were fed a solution of tetracycline mixed with honey at 6%, for at least 48h prior to *Drosophila* larvae infection. Filter papers were embedded in this solution and given to the adults where they had no other source of nutrition.

Leptopilina heterotoma was free of *Wolbachia* after 5 generations of treatment as confirmed through PCR (see below).

Staining and Imaging

Female wasps of all lines were collected and their ovaries dissected in PBS. For staining with Propidium Iodide (PI), phalloidin or DAPI, tissues were fixed for 20 minutes rotating in a solution containing 3:1 of heptane to fixative (for 500µL: 94µL of 16% HCOH, 50µL of 10X PBS, 2,5µL of IGEPAL or NP40 (100%) and 353µL of MiliQ), and subsequently washed in PBT. For PI staining, an extra RNA-ase treatment step was performed (400µg/mL for 2 hours) to minimize confounding RNA staining.

Propidium Iodide was used at 10µg/mL and DAPI at 1:1000, both incubated for 20 minutes in the dark and with rotation. For phalloidin staining samples were permeabilized for 1 hour with block buffer (for 4mL: 400µL of 10 % BSA, 400µL of 10X PBS, 44µL of TritonX

and 3156μL of MiliQ) and incubated for 2 hours in the dark and with rocking using 1:1000 phalloidin dilution. After each incubation, samples were washed twice in PBS and/or PBT. Mounting was done in a drop of Vectashield®.

For the SYTO9 staining, freshly dissected ovaries were placed on a drop of mounting medium on a slide and incubated as they were imaged.

Imaging was done using a Leica SP5 Live confocal microscope.

Life-history traits assays

To infer about possible effects of *Wolbachia* in our line of *Leptopilina heterotoma*, full development time, offspring sex ratio and longevity were measured in Wol- and Wol+ lines. 4 replicates with 60 L1 *Dif* larvae were done in standard food vials, for each line of *Leptopilina heterotoma*. After 24 hours, female wasps were introduced and left to oviposit. Adult eclosions were checked every morning and developmental time and offspring sex ratio were scored.

Individuals were kept with access to honey, in fly food vials at 18º C under controlled conditions, according to their replicate number and day they had eclosed at. Mortality was checked daily.

Survival assays and parasite stocks

Viral infection experiments were conducted inside empty standard drosophila vials, with honey and filter paper watered daily. Each replicate contained 10 to 20 individuals, depending on the assay. All of infection assays were performed with male wasps.

Viral infections were performed by pricking the wasps in the anterior thoracic region with a 0.10 mm Minutien Insect Pin by Austerlitz dipped in a virus solution (10^9 $TCID_{50}$/mL) [58,97]. Controls were performed similarly using MiliQ sterilized water. Survival was followed daily.

Drosophila C Virus (DCV) was kept in aliquots at -80º C and thawed for tests. They were previously prepared and titrated as described before[49,98].

DNA extractions and Polymerase Chain Reactions (PCR)

DNA extractions were performed using an adapted version of the DrosDel DNA extraction kit for 96 well plates (see Annex I).

PCR reactions for *Wolbachia* detection were performed under the following conditions: 2 minutes at 94º C followed by 35 cycles (30 seconds at 94º C, 1 minute at 60º C, 1 minute at 72º C) and finally 10 minutes at 72º C. The amplification of the *Wolbachia*-specific *wsp* fragment was done using the primers wsp81F (5' TGGTCCAATAAGTGATGAAGAAAC 3') and wsp691R (5' AAAAAT TAAACGCTACTCCA 3') [99]

For detection of possible *Spiroplasma* infections, specific primers were used, namely SpoulF (5' GCTTAACTCCAGTTCGCC 3') and SpoulR (5' CCTGTCTCAATGTTAACCTC 3')[100], with the same reaction protocol.

To control for the DNA extraction quality and PCR viability COI was always ran in parallel, with the primers COI F (5' GGTCAACAAATCATAAAGATATTGG 3') and COI R (5' TAAACTTCAGGGTGA CCAAAAAATCA 3') using the same reaction protocol as above. For the outbred *Leptopilina* spp. lines, COI fragments were sequenced and ran through BLAST®, to determine the species.

PCRs were done using GoTaq® G2 Flexi reagents: 5X (Green or Colorless) GoTaq® Flexi Buffer, $MgCl_2$ 25mM, primers (forward and reverse) 10 mM, dNTPs 0,2 mM, GoTaq® G2 Flexi DNA Polymerase 5u/µL and 100 ng of template DNA. PCR products were ran on a 1.5% agarose gel.

Sequencing was done using the BigDye Terminator protocol v1.1 established in the lab, and the subsequent DNA precipitation protocol, both in Annex II.

Statistical analysis

All statistical analysis was conducted using R, version 3.0.2. The statistical tests used are mentioned throughout the manuscript.

Results

Interspecific horizontal transmission of *Wolbachia*

The main aim of this experiment was to see if and to which extent interspecific horizontal transmission of *Wolbachia* by means of a natural route, occurred between our two chosen species, *Drosophila melanogaster* and *Leptopilina boulardi*. Previous work[69] reported that horizontal transmission of this endosymbiont did occur between *Leptopilina boulardi* and *Drosophila simulans* (Riverside), at a low rate (approximately 0,7%) and phylogenetic evidence[63] points to the similarities existing between the strains of *Wolbachia* found in parasitoid wasps and their hosts.

With this information we hypothesized that, being a natural host of *Leptopilina boulardi*, *Drosophila melanogaster* would also be prone to transmit its own endosymbionts to a parasitoid wasp infecting it. In order to test this we established isogenic wasp lines (that would allow us to pinpoint individual events of transmission between host and parasitoid) from single females that eclosed from a *Wolbachia*-positive *Drosophila* host. We used two strains of *Wolbachia* and one isogenic line as control (see Materials and Methods).

Our initial setup aimed to maximize the chances of horizontal transmission by having a large number of target larvae for wasp infection. We carried out egg-lays of *Wolbachia*-positive flies in bottles and let female wasps oviposit freely. We then collected all of the progeny and used the F1 females to establish isogenic lines. Out of the starting 326 females, 186 had developed in wMel_like-positive larvae and 116 in wMel_CS-positive larvae, while the remaining 24 lines developed in the iso population. Not all of these females, however, were successful in generating progeny and we were only able to establish an F2 from 201 lines. Out of these 201 lines, 34 had inconclusive PCR results, which left us with the remaining 167 from which we could determine the *Wolbachia* infection status (Figure 5). From the 201 lines that originated testable progeny, only 90 gave rise to female offspring.

In the initial PCR testing (see Materials and Methods) one of the isogenic lines was positive for *Wolbachia* wMel_CS strain simultaneously producing F2 female offspring. Unfortunately, it eventually lost the bacterial infection before we were able to image it using fluorescence microscopy (see below), sometime around the fourth generation. In agreement with the work of Heath et. al (1999), *Wolbachia* horizontal transmission did not, in

laboratory conditions, give rise to a stable vertical transmission in *Leptopilina boulardi*. This stopped us from testing its possible immunological protective effects in a newly acquired host. The reasons and possible implications of this observation will be more thoroughly discussed ahead.

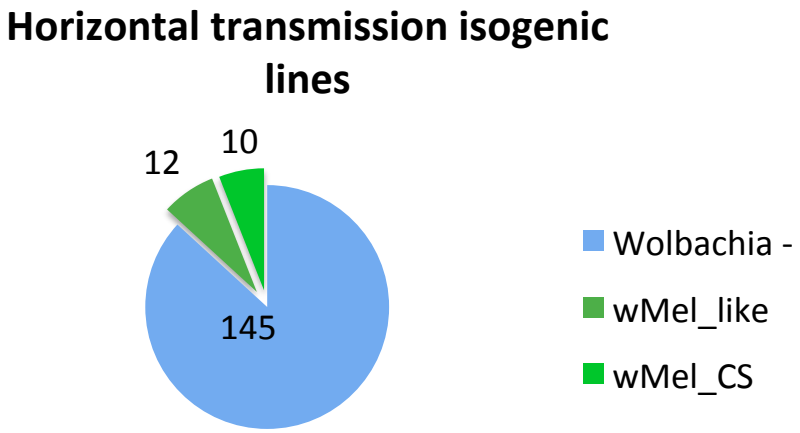

Figure 5 – Interspecific horizontal transmission of *Wolbachia*. Numbers represent the number of *L. boulardi* isogenic lines that were *Wolbachia*-positive (wMel_like and wMel_CS) in F2 after horizontal transmission event (confirmed through PCR). Wolbachia- represents the number of lines that were *Wolbachia*-negative in the F2.

Mortality assays

- *Leptopilina heterotoma*

In order to see what was the effect of DCV infection in *Leptopilina heterotoma* wasps, we systemically infected males and measured their survival daily. Figure 6-A shows survival during the first 25 days after the viral systemic infection. Cox mixed-effects model was fitted to the data and a type II ANOVA shows that there was no significant effect of population (Wol+ and Wol-) (p-value: 0.145). However, treatment (Control or DCV infection) was relevant for survival (p-value: 0.046) as well as the interaction between treatment and population (p-value: 0.023). The model also showed a significant difference between control and viral infection in the Wol+ population (p-value: 0.006) but not in Wol- (p-value: 0.660). When using multiple pairwise comparison analysis, the difference between treatments within the *Wolbachia*-positive population is strengthened (p-value:0.012). This analysis also shows a difference between populations for DCV infection (p-value: 0.032). In Figure 6-B the hazard ratios illustrate the aforementioned comparison (showing the higher probability of dying by DCV for Wol+).

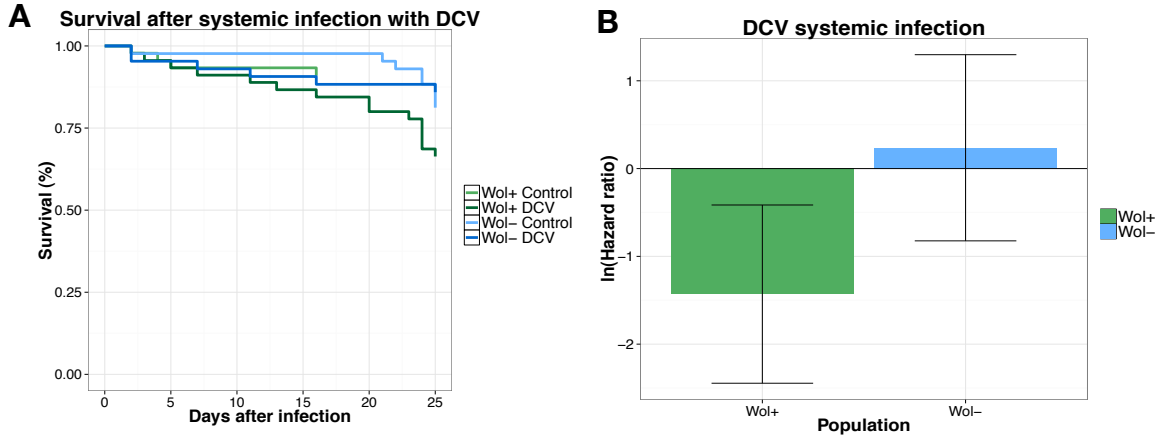

Figure 6 - Survival of Wol+ and Wol- *L. heterotoma* after systemic infection with DCV. A) Survival plot for both populations, either with Control treatment (water prick) or DCV infection. B) Hazard ratios for both populations infected with DCV, relative to Control. Vertical bars correspond to the standard error of the estimated ratio between both treatments

- *Leptopilina boulardi*

A similar systemic infection protocol was conducted for *Leptopilina boulardi* and the respective survival curve is shown in Figure 7-A. Cox proportional hazard model was fitted to the data and shows no significant effect of treatment (Control without prick, Control pricked with water, prick with DCV and prick with FHV) in survival (p-value > 0,05). When using multiple comparison analysis, this same result is verified, and none of the treatments is significantly different from the water Control (p-values > 0,05). This lack of effect is shown in the hazard plots of Figure 7-B. There are several possible explanations for these observations, and they will be more thoroughly discussed ahead, although we cannot find an effect of infection with the chosen viruses in this species of parasitoid wasps.

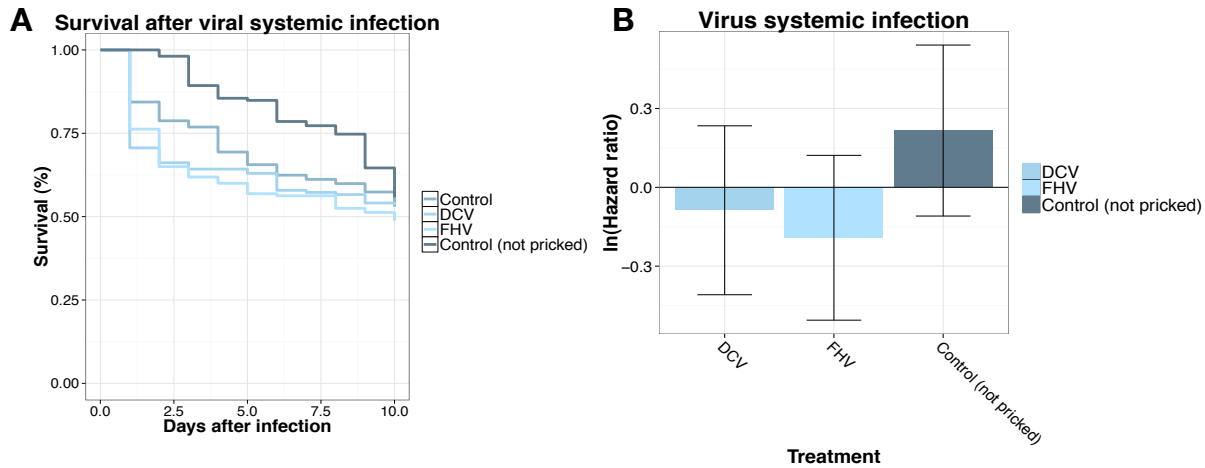

Figure 7 - Survival of *L. boulardi* after viral systemic infection. A) Survival plot for all treatments (Control without prick, Control with water prick, DCV prick and FHV prick). B) Hazard ratio for all treatments, relative to the water Control. Vertical bars correspond to the standard error between both treatments

Confirmation of *Wolbachia* presence

- *Leptopilina boulardi*

In order to definitely dismiss or confirm the presence of *Wolbachia* in the *Leptopilina boulardi* isogenic line that was positively infected after horizontal transmission (confirmed through PCR), wasp ovaries were stained and imaged. As there was no successfully established antibody staining protocol for *Wolbachia*, unspecific DNA dyes were chosen[15,101]. With this method it was straightforward to separate the host DNA staining from the endosymbiont's DNA, not only because of the size of the coloured clusters, but also from their localization (endosymbiont DNA is intracellular and so, is found characteristically surrounding the host cell nucleus).

In order to maximize the chance of staining endosymbiont DNA (because, if present, they would be in low amounts in the new host's tissues) the ovaries were chosen as a premium location for *Wolbachia*. Seen as it is maternally transmitted, the ovaries would be a preferred place for the bacterial cells to make their way to the germ line and eventually to the offspring.

Our negative control for *Wolbachia* presence consisted of the *Leptopilina boulardi* line that never came in contact with infected hosts, that is, the *G486* strain that has been continuously kept in *Dif* mutants.

Figure 8 shows stainings of the *G486* strain. The phalloidin staining allows for a clear distinction between different types of tissues, namely stripes typical of muscle, part of the supporting structures for the ovarioles. If *Wolbachia* were present, it would be expected to find small PI clusters surrounding the host's cellular nuclei, which is not the case. In contrast, as expected, the cytoplasm appears devoid of staining for DNA, confirming the absence of endosymbionts. This is also true for Figure 9 (which shows the ovaries of wasps at the 9th generation after horizontal transmission) where no endosymbiotic DNA is visible in the host's cytoplasm. These results allowed us to confirm what was being verified by PCR, which was that the *Wolbachia* was no longer infecting its transient new host, and that the event of horizontal transmission didn't successfully give place to a stable vertical transmission.

Considering these results, we conclude that, as expected, our lab-kept line of *Leptopilina boulardi* is negative for the presence of *Wolbachia*. We were also able to confirm that, although *Wolbachia* was transmitted horizontally between different hosts (as checked

by PCR), it did not colonize host tissues in a stable manner, and so, was lost 9 generations after the initial transmission event.

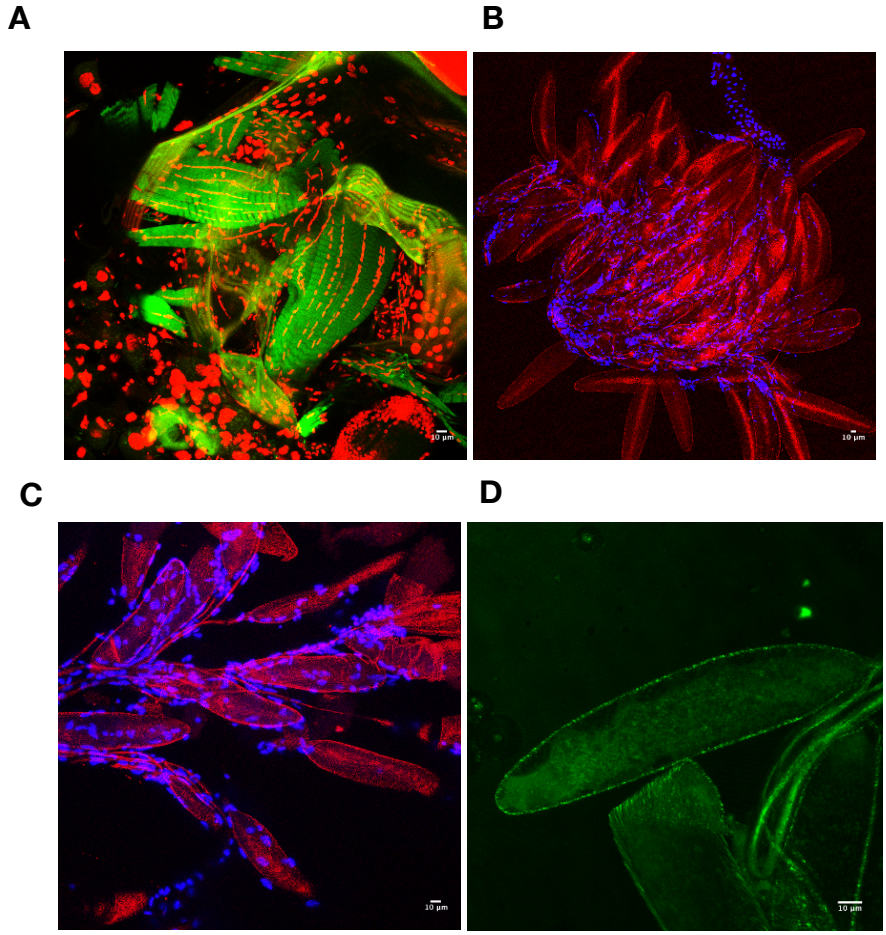

Figure 8 - Stained ovaries of L. *boulardi.* A) Ovaries stained with phalloidin (green) and propidium iodide (PI, red). 20X magnification. B) and C) Ovaries stained with DAPI (blue) and phalloidin (red). B) is 20X and C) is 40X magnification. D) Ovaries stained with SYTO9, 20X magnification. Scale bars = 10 µm.

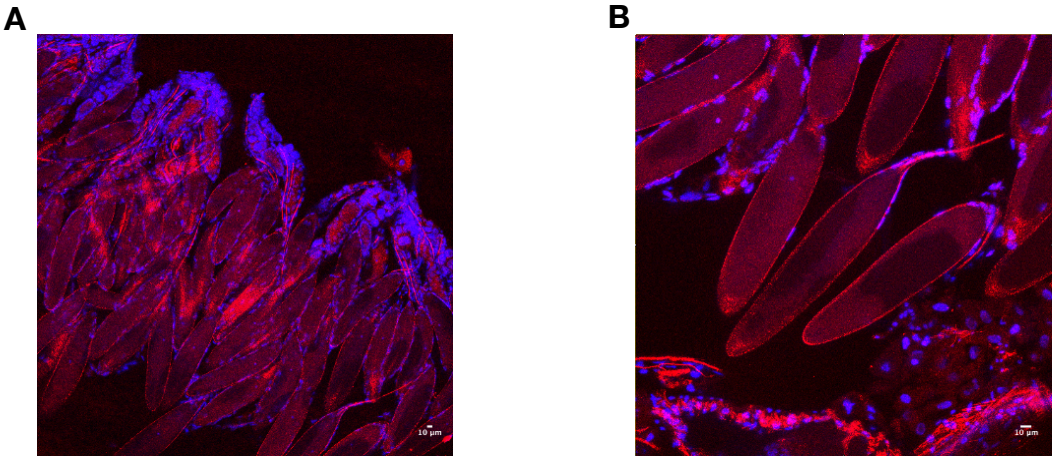

Figure 9 - Ovaries of *L. boulardi*, 9 generations after horizontal transmission of *Wolbachia*. Staining with DAPI (blue) and phalloidin (red). A) 20X and B) 40X magnification. Scale bars = 10 µm.

-Leptopilina heterotoma

Similar staining protocols as for *Leptopilina boulardi* were applied to ovaries of *Leptopilina heterotoma*. In Figure 10 – A), B) and C) there is a clear signal both on host DNA and on clusters of bacterial DNA spread throughout the cytoplasm. *Leptopilina* DNA is organized in big round nuclei and surrounding them are small indistinct agglomerates of DNA that are characteristic of *Wolbachia* infection.

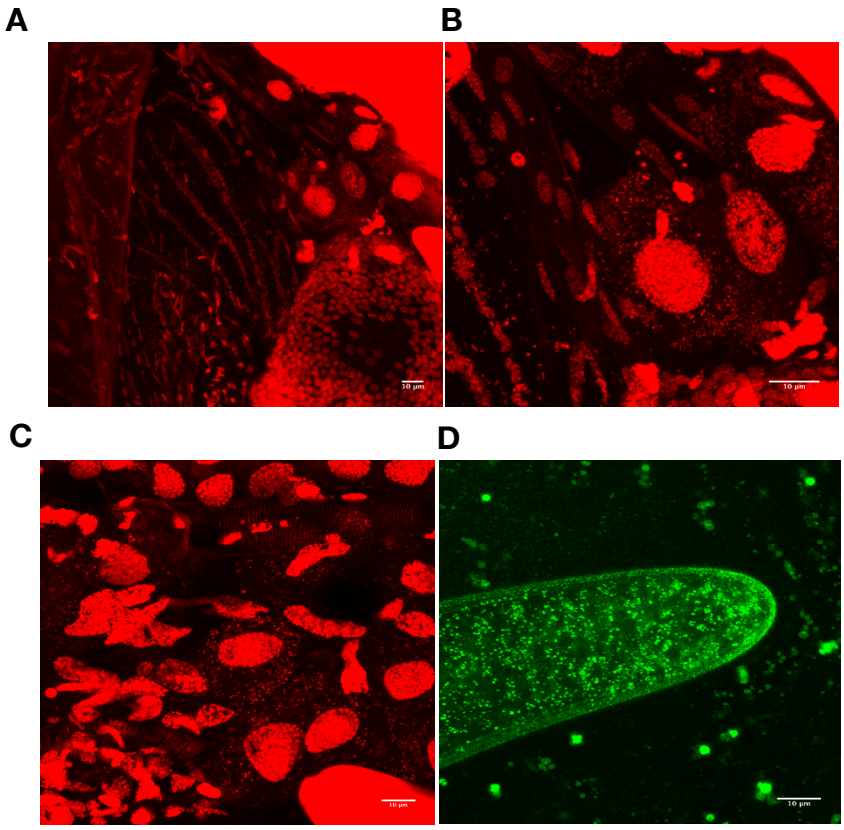

Figure 10 – Stained ovaries of *L. heterotoma*. A), B) and C) have PI staining (red) and D) has SYTO9. A) is 20X magnification. B) , C) and D) are 40x magnification. (B) is a close-up of A)). Scale bars = 10 µm.

Because we wanted to study the effects of the endosymbiont in a wasp host, it was necessary to create in this species a negative control for the infection. To this end we performed an antibiotic treatment inspired in procedures used in other insects (see Materials and Methods). After 5 generations of treatment it was no longer possible to detect the presence of *Wolbachia* through PCR and in order to get a more resilient result, we dissected and imaged treated wasp ovaries.

In Figure 11 – A) and B) no fluorescence is detected in the cytoplasm. The host's cell nucleus is brightly stained, as is in Figure 10 – A), B) and C), with the difference of being

surrounded by a clear cytoplasm, indicating the absence of *Wolbachia* in the host cell. There is a clear distinction between the cytoplasm of untreated and treated individuals. With this corroborating result, we can conclude that the treated line of *Leptopilina heterotoma* was *Wolbachia*-free and may be used to test the influence of *Wolbachia* on host traits.

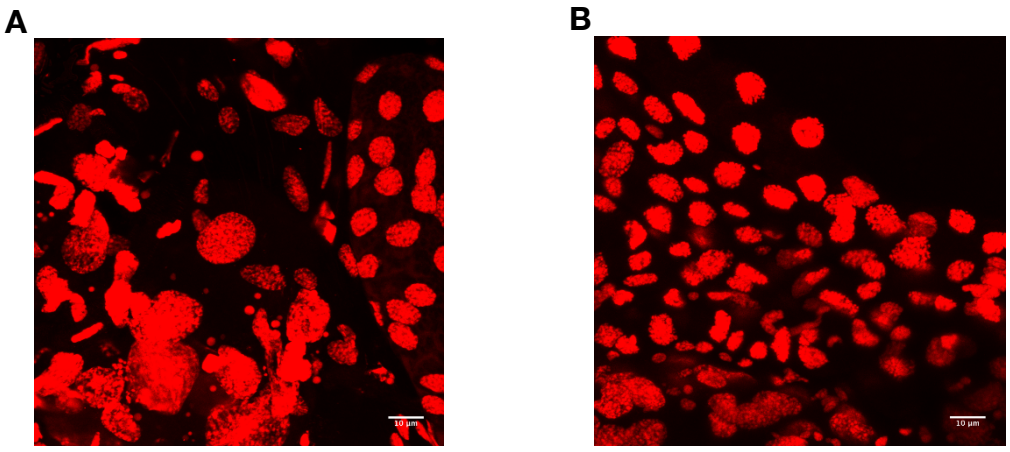

Figure 11 - Stained ovaries of *L. heterotoma*, after 5 generations of antibiotic treatment. A) and B) have PI staining and 40X magnification. Scale bars = 10 µm.

Influence of *Wolbachia* on life-history traits

Development time

In an attempt to learn more about the possible implications of removing *Wolbachia* from our outbred line of *Leptopilina heterotoma*, we analyzed the differences between Wol+ and Wol- lines in developmental time (egg to adult). We controlled for density-related differences that could interfere with the outcome of the experiment by counting host *Drosophila* larvae into individual tubes where female wasps were placed to oviposit.

Eclosion time of adult wasps was measured daily (see Annex III) and sex ratios counted. A type II ANOVA was ran on the dataset and fitted to a Cox mixed-effects model. This analysis shows that there is an effect of population (p-value: $1{,}118 \times 10^{-10}$) and sex (p-value: $2{,}2 \times 10^{-16}$) on development time, although there is only a mild effect of the interactions of these two factors (p-value: 0,095). By doing multi-comparison analysis, we are able to confirm that there is indeed a difference between populations and between sexes (within and between populations) (p-value of difference between sexes in Wol+: $1{,}94 \times 10^{-11}$; and in Wol-: ~ 0; p-value of the difference between populations by averaging the

sexes: 1,33 × 10^{-10}). We can conclude from these results that there seems to be a significant influence of sex and presence of *Wolbachia* in development time. In the Discussion, possible implications and ecological relevance of eclosing earlier will be explored.

Longevity

Leptopilina heterotoma Wol+ and Wol- individuals were collected right after eclosion and their survival scored throughout 70 days (Figure 12 – A). A Cox mixed-effects model showed that the overall longevity of Wol+ and Wol- wasps was not significantly different (p-value: 0,671). Also, no detectable effect of the interaction between the wasp line and sex (p-value: 0,189) could be detected. The sex factor, proved to be significant for the overall longevity, with males dying earlier than females (p-value: 2×10^{-16}) in both populations (Figure 12 – B)). Multiple comparison analysis shows that there are no significant differences between populations, for both sexes (Females – p.value: 0,406; Males – p.value: 0,585), or averaging them (p.value: 0,570) and it reinforces the difference existing between sexes, for Wol+ (p.value: 2,26 × 10^{-6}) and for Wol- (p-value: 4,44 × 10^{-15}).

These results clearly show that females of *Leptopilina heterotoma* live longer than their male counterparts, and that this difference happens independently of the *Wolbachia* infection status. No detectable difference between longevity of both populations was seen, contrary to published works[96] which can be explained by a number of factors, (from maintenance conditions, to strain establishment) that will be explored ahead.

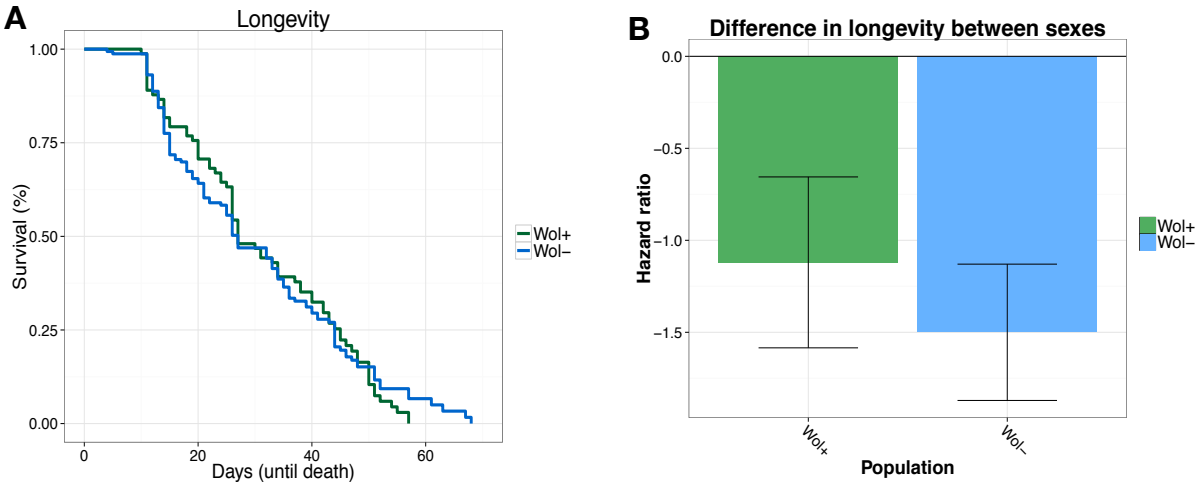

Figure 12 - Longevity of *L. heterotoma* Wol+ and Wol- lines. A) Full adult longevity curve (number of days until death). B) Relative probability of males dying compared to females, for both lines.

Discussion

Interspecific horizontal transmission of *Wolbachia* occurs but is not stable in the new host

The horizontal transmission assay was conducted to explore to what extent could the interspecific transmission of *Wolbachia* occur between our two chosen species. In nature, *Drosophila melanogaster* and *Leptopilina boulardi* share the same habitat and develop in close proximity, being this species of parasitic wasp a specialist of this fly host [102,103]. Having had similar work developed in a closely-related host species[69], we aimed at determining the capacity of *Wolbachia* to be transmitted to *Drosophila melanogaster*. In an effort to characterize possible transmission events we used two strains of *Wolbachia* that are known to exist at different titers in the host's tissues[58], so that we could assess the importance of this trait on horizontal transmission.

As shown in Figure 5, there was a 14,7% rate of horizontal transmission of *Wolbachia* between *Drosophila melanogaster* and isogenic lines of *Leptopilina boulardi,* which is a much higher rate of transmission than the one we were initially expecting to see. If we analyze both strains individually, we see that there was a similar transmission rate between the two (6,7% and 8% for wMel_CS and wMel_like, respectively). Because wMel_CS exists in host tissues in higher quantities than wMel, we reasoned that this strain would maybe be transmitted more frequently than its counterpart. We assumed that having more bacteria would make colonization of new uninfected tissues easier and more efficient. However this doesn't seem to be the case here, since wMel and wMel_CS have transmission rates that are indistinguishable from one another.

Given that this experiment had never been conducted for our two chosen species, we believe to have established a good starting point in assessing the percentage of horizontal transmission of *Wolbachia* that can occur in a natural environment, between *Drosophila melanogaster* and *Leptopilina boulardi*. We must consider, however, that experimental errors might have occurred at (for instance, unforeseen sample contaminations) that made us overestimate these transmission rates.

In other species of arthropods, horizontal transmission of endosymbionts has been demonstrated in a wide variety of different scenarios, including through a natural vector or

by artificially transplanting *Wolbachia* between hosts. Artificial horizontal transmission has been performed between *Drosophila* spp. species[90,88], between tephritids and *Drosophila* spp.[87], between isopods[86] and in lepidopterans[9,40]. Cases where natural transmission has been experimentally verified include *Solenopsis* spp. ants[92], within-cast transmission in *Acromyrmex echinatior*[104], *Trichogramma* wasps[70,82], rice-field insects[72], crustaceans[73], within the pumpkin-insect community[80], amongst others.

In some of the above-mentioned cases, parasitoid wasps have been reported (or speculated) to act as transmission vectors of *Wolbachia* between the different hosts, becoming themselves infected or not in the process[93]. This would be in agreement with the contradictory phylogeny of this endosymbiont, in the cases where the parasitoid incorporates the bacteria instead of simply carrying it from host to host.

We believe that the cases where wasps act as phoretic vectors are likely to be more frequent in nature than the ones where *Wolbachia* fixes on wasp tissues first and is subsequently transmitted to a new host. These transient infections (that can later result in horizontal transmission between hosts) are difficult to calculate and consider, since there is no direct phylogenetic trace that can be followed. This implies speculation as to the methods of horizontal transmission that explain the presence of similar strains of *Wolbachia* in phylogenetically distant hosts. Also, this mechanism allows a way for bacteria to be transmitted without undergoing the selective pressure imposed by infecting and fixing in an added number of hosts. To some degree, it would also explain why no successful infection by *Wolbachia* has been found in *Leptopilina boulardi*, although it is ecologically immersed in an environment that would be highly prone to infection. Indeed, *Leptopilina boulardi* parasitizes infected *Drosophila melanogaster* and *Drosophila simulans* hosts and shares them with infected *Leptopilina heterotoma*. We hypothesize that this species is more prone to phoretic transmission of *Wolbachia* instead of harboring it first, since fixation is simply not observed. It has been proposed[105] that *Leptopilina boulardi* being sometimes infected by a specific virus (LbFV)[61] could be related to its *Wolbachia*-negative infection status, although no evidence exists to support this. There is indication that this virus forces female wasps to superparasitize hosts, a behavior not characteristic of this species[106,107], which ultimately maximizes its own transmission. However, we cannot assume that LbFV was involved in the lack of vertical transmission of *Wolbachia* observed in our experiments. In fact, *Wolbachia*

was never found in wild-caught wasps of *Leptopilina boulardi*, independently of their virus-infection status, but it is undoubtedly a hypothesis that needs testing and further characterization.

In the future we aim to deepen our work on the rate of horizontal transmission between *D. melanogaster* and *Leptopilina* spp., by repeating and adding to the number of isogenic lines and/or by including more strains of *Wolbachia* (and even testing possible interactions between strains). We would like to try using a strain of *Wolbachia* that exists in extremely high titers in host's tissues (for instance, the Popcorn strain) and see if it has an influence on overall horizontal transmission rate. We also intend to redo the horizontal transmission experiment with both infected and uninfected lines of *Leptopilina heterotoma* and confirm if the lack of a stable infection by a newly acquired endosymbiont is genus-specific.

Furthermore, we plan to optimize our *Wolbachia* detection protocol using microscopy. In this study no specific markers were used because no successful antibody staining was available to us at the time. Although DNA-dyes were adequate to identify relatively high concentrations of *Wolbachia*, we can speculate that small quantities (such as the ones we expect will be present in novel host tissues after horizontal transmission) would go undetected. Also, we intend to mark germline cells (with a VASA antibody, for example) to locate with precision the endosymbiont in the wasp's gonadal tissues. Maybe by characterizing one of the lines that were positive for infection after horizontal transmission we will determine that the lack of stable vertical transmission is a product of mislocalization of the new endosymbiont to places with low probability of being transmitted to the offspring.

DCV has small or no impact on a parasitoid of its native host

An important objective of this project was to assess if *Wolbachia* conferred protection against RNA viruses to a novel host as it is known to do in *Drosophila* spp.[49,50].

To test this hypothesis we began by adapting a systemic viral infection protocol to a wasp recipient. This infection protocol took a long time to be optimized so that consistent results could be obtained without the confounding effects of experimental error (namely by reducing the pricking-associated mortality of the tested individuals). However, and despite

being in all regards similar to the well-established protocol used for *Drosophila melanogaster*[97], there are still undetermined aspects about the infection that we consider of vital importance to discuss here.

What our data seems to indicate is that DCV is of little to no consequence to a *Leptopilina* host when injected. Results point to a statistically significant effect of treatment when we test *Leptopilina heterotoma*, although *Wolbachia* presence seems to have an effect. What is also apparent is that the *Wolbachia*-infected line shows some susceptibility to the treatment (there is a slightly higher mortality when this line is pricked with DCV) relative to the *Wolbachia*-negative one. This result went against our initial prediction in that no visible protective effect was detected in the test. This can be explained by a number of factors, starting with the experimental design used. One of the first questions that arises is to which extent the difference can be considered relevant (considering that the p-value is on the edge of significance) and not just an artifact of sample size. Another aspect, in our perspective more relevant, is to which extent does the virus actually infect the wasp's tissues upon pricking. Although we know that the used method assures virus proliferation in tissues of *Drosophila melanogaster*[108], we did not test viral loads upon infection of *Leptopilina* spp. Because the experimental infection protocol was slightly changed and adapted to the new species, it would be incorrect not to consider that the changes could have an impact in the quantity and access that DCV (or FHV) have to new tissues.

Considering that one of the changes was that we reduced the size of the needle used for the pricking, it is reasonable to assume that the amount of viral particles being inserted in the wasp is smaller than the expected and necessary for invasion and proliferation. The fact that the wasp's exoskeleton is much harder to perforate than that of *Drosophila* spp., also makes us wonder about how much of the virus is actually lost in the whole procedure. To tackle this we want to perform a quantitative analysis on the pricked individuals and assess the amount of viral load present inside the tissues throughout the days after infection.

In case we observe the absence of virus in pricked wasps, then the effect we see in the *Wolbachia*-positive population has to be a byproduct of the experimental setup. If not, we must conclude that DCV has indeed a subtle effect on survival in *Leptopilina heterotoma* and that somehow the presence of *Wolbachia* can be exacerbating that effect (considering

that the *Wolbachia*-negative line doesn't show any significant mortality by DCV infection). We can speculate that in this species of wasp, the endosymbiont could be inducing a cost to the host by interacting with the virus and ending up damaging the tissues it parasitizes in the process (considering that the complete way through which *Wolbachia* establishes itself and confers protection is still undetermined[49]). This would in the end be the exact opposite of what is described for *Drosophila* spp. but considering that the mechanisms are still unknown, it cannot be discarded.

Another important factor that causes us to question the overall action of the chosen viruses in wasps is the total lack of detectable effect of DCV or FHV in *L. boulardi*. Again we can question whether the absence of an effect is a product of experimental procedures, leading for example to a lack of viruses in host tissues. Alternatively, we can speculate that the presence of *Wolbachia* is in fact what is triggering the slightly deleterious effect of the viral infection that was observed in the sister species *L. heterotoma*, considering that the *L. boulardi* line used is *Wolbachia*-free.

The importance of *Wolbachia* influence for the final survival is suggested by the tendencies of the curves and hazard plots of Figure 7, where both DCV and FHV-infected individuals die slightly more than the control (seen by the negative hazard ratios), although not to a statistically significant degree.

It could also be that the differences observed between both tests of the different *Leptopilina* species are constraints of the host species themselves, that although closely related, have many different physiological traits[95]. To narrow down our possibilities we would ideally use the same protocol and test a *Wolbachia*-positive line of *Leptopilina boulardi*. As thoroughly mentioned in this thesis, that is a feat not yet accomplished.

Another possible explanation for our viral infection data relates do the viral action itself. The specific characteristic that leads to viral proliferation and consequent deleterious effect in *Drosophila* spp. host is still mostly undetermined, although several hypotheses have been put forward[56]. In *Drosophila melanogaster*, the JAK/STAT and RNA-interference pathways have been involved in the immune response against these pathogens[83], but the viral trigger of these responses is still unknown. It has been reported that endocytosis is important for infection and pathogenesis[109]. This is one of the first steps in the replication and infection cycle of DCV[110], bringing us to question of at which point in this cycle does the

infection and pathogenicity differ between *Drosophila* and *Leptopilina*. If we were able to pinpoint this difference we would be capable of maybe determining where and why does DCV (and to some extent FHV) not affect overall survival of *Wolbachia*-negative lines of both *Leptopilina* species.

In order to conclude with more certainty about these viral infection experiments, the first step will be to quantify viral loads in wasp tissues after systemic infection, by qPCR for instance. In these future tests it is also necessary to ensure a big enough sample size that allows us to conclude with certainty about possible differences between treatments, without the confounding effects of protocol associated mortality, for instance. Then, according to those results determine if the observed action in survival is *Wolbachia*-related or not. Lastly, test if the lack of viral action is the product of faulty proliferation and pathogenicity in "unexpected" host tissues. For this, ideally we would have complete characterization of the action of DCV in host tissues and another virus that proved to be more pathogenic in *Leptopilina* spp. .

Wolbachia appears to be influencing development time, but not longevity

Our data suggests that there is a cost in harboring *Wolbachia*, namely that it affects development time although it doesn't seem to affect longevity. We saw a decrease in full development time in the *Leptopilina heterotoma* line from which *Wolbachia* was removed, in comparison with the wild type oubred line, but not in overall adult longevity (although the difference between sexes was always significant, with males developing at least one day before, as described[103]). This result is not in agreement with what was previously described for this species[111], although differences in experimental setup could make these two works not comparable. In Fleury et. al (2000) wasps were reared at 22 ºC, which explains why their (lower) development time is 24,9 days (for uninfected males), and the one we tested was 19 days (for uninfected males as well). There is also a difference between the two studies concerning the statistical approach used to measure the differences between infected and uninfected individuals, seen as they used a two-way ANOVA on the raw data and we fitted our data to a Cox mixed-effects model and only then analyzed possible differences (either with ANOVA or multiple comparisons analysis), which could explain to a limited extent the

differences between significant results. What can also be influencing the observed differences is the disparity between the origins of *Leptopilina heterotoma* lines and the respective endosymbiont strains they both harbor.

Although further corroboration is necessary, we see a tendency towards the induction of a cost by the presence of *Wolbachia* in a wild population of *Leptopilina heterotoma*.

There are several studies that report beneficial, deleterious or neutral effects in hosting *Wolbachia* and these contradictory effects seem to be directly related to the host species and/or the *Wolbachia* strain in question[88]. As previously mentioned and explored in the present work, a number of studies have established beneficial effects, namely in providing resistance against RNA-viruses[49,50] in *Drosophila* spp.. In these cases, protection relates to the strain-specific titers of *Wolbachia* in host tissues that in turn correlate with viral protection levels. In an environment where there is no immunological stimulus, however, the presence of some of these strains of *Wolbachia* induces a physiological cost (as for example, the wMelPop strain[58]). Other studies show a negative impact of harboring, either a native strain (in *Leptopilina heterotoma*, according to our results, cytoplasmic incompatibility in *Drosophila simulans*[88] or feminization in isopods[20]) or a horizontally transmitted one (host mortality after *Wolbachia* transfection in *Porcellio dilatatus*[86], for example).

In the specific case of parasitoid wasps it is difficult to state with certainty the definite effects that *Wolbachia* causes. However these are clear in the mentioned case of *Leptopilina heterotoma*, the reported embryonic mortality in *Trichogramma* spp.[112] and in *Trichogramma brassicae* where *Wolbachia* infection increases host handling time[113]. An example of beneficial effect of *Wolbachia* infection was reported recently in *Asobara tabida,* where bacteria presence has been shown to enhance the host-searching ability of females[114].

The type of influence that *Wolbachia* has on its hosts is not necessarily immutable. As previously mentioned, infection by this endosymbiont can lead to both costs and benefits for the host, and these effects can even be interchangeable, within a particular *Wolbachia* strain-host pair. In order to ascertain the type of interaction that is established between *Wolbachia* and its different hosts, it is necessary to take into account aspects concerning the

intrinsic characteristics of the strain, as well as the host mechanisms that mediate their prevalence.

Wolbachia does not induce always the same phenotypes when it infects hosts. However, and although it is hard to establish a definite rule, in each bacteria-host case, the co-evolution process will have ensured that the interaction is evolutionarily stable, if it has been established some time ago and is therefore fixed; or, that it is still mostly undetermined (which can reflect high cost for the host), if it is recent or is in a transient state. Mostly, what this implies is that it is extremely demanding to make general conclusions about a system that is in itself, constantly changing and adapting, such as the *Wolbachia*-host relationship.

It is necessary to further characterize the *Wolbachia* strain (or strains) that are infecting our line of *Leptopilina heterotoma* in order to better understand the influence that this infection may be having on the host's physiology (since it is known that different strains can induce different effects[58]).

Our line hosts a strain that shares 99% identity with variant 3 of the Lhet[63] strain and with the strains found in *Spalangia nigroaenea*, in *Trichogramma cacoeciae* and in *Ephestia kuehniella*[64]. In these species, the effect of *Wolbachia* is described as mostly parthenogenetic in *Trichogramma* spp.[11282] wasps and still unknown in *Spalangia nigroanea*[115,116] (although it is known to induce partial CI in the closely related species *Spalangia cameroni*). *Ephestia kuehniella* seems to equally harbour CI-inducing strains[117,118]. With this knowledge we may speculate that the *Wolbachia* harbored by our wasp line would be able to induce similar phenotypes in its native host. However, considering that such a high sequence similarity between *Wolbachia* strains leads to such different influences on the host's reproductive system (as mentioned above), a definitive model should be hard to establish without experimental tests.

As it appears, these reproductive manipulations can either be a product of that small difference between bacterial strains, of the host's background response to the presence of *Wolbachia* on its tissues, or lastly, of the interaction between these two factors. In order to test which of these factors define the reproductive manipulations that take place in nature, it would be necessary to test an array of host-symbiont strain combinations and see what would be the phenotypic outcome.

In the case of our *Leptopilina heterotoma* line we have not yet been able to characterize the exact effects that the native *Wolbachia* has on the host's reproduction. We can speculate, however, considering what was mentioned above, that the effects will range from parthenogenesis and CI (as described for *Trichogramma* and *Spalangia*) to male-killing or feminization, considering that the effect is apparently far from static. The proposed more detailed characterization of the endosymbiont will shed light onto this problem.

Final remarks

Despite its shortcomings, this thesis has shed some light onto the requirements surrounding *Wolbachia*–mediated immune protection in a newly-acquired host. Having started by characterizing to which extent and rate *Wolbachia* is horizontally transmitted between a *Drosophila melanogaster* host and a *Leptopilina boulardi* parasitoid, we were able to determine that these events can occur naturally and consequently that this specific route of transmission may have ecological relevance. At this point in the project we unfortunately had a setback when we verified that one horizontal transmission event did not give rise to a stable *Wolbachia* infection in our novel wasp host. What this entailed was that the initial question remained partially unanswered, because a *Wolbachia*-induced phenotype could not be tested in the new recipient.

In the viral infection experiments we did not detect an effect of the tested RNA-viruses in *Leptopilina* wasps hosts, except when *Wolbachia* was present. This also constituted an important drawback concerning our proposed main question. However, this work has generated relevant new knowledge concerning this host-parasitoid-endosymbiont-virus complex system.

We hypothesize that the lack of *Wolbachia* fixation after horizontal transmission in *Leptopilina boulardi* is probably a Boulardi-clade acquired specificity considering that to date no naturally infected individuals of this species have been found. The phylogeny of *Leptopilina* suggests one of two scenarios: 1) upon the split of the Heterotoma and Boulardi clades, *Wolbachia* infection was lost only in the Boulardi species and independently in the Clavipes clade; 2) the acquisition of *Wolbachia* is relatively new, having only occurred in the Heterotoma clade and in some species of Clavipes. Further phylogenetic studies on these wasps and their strains of

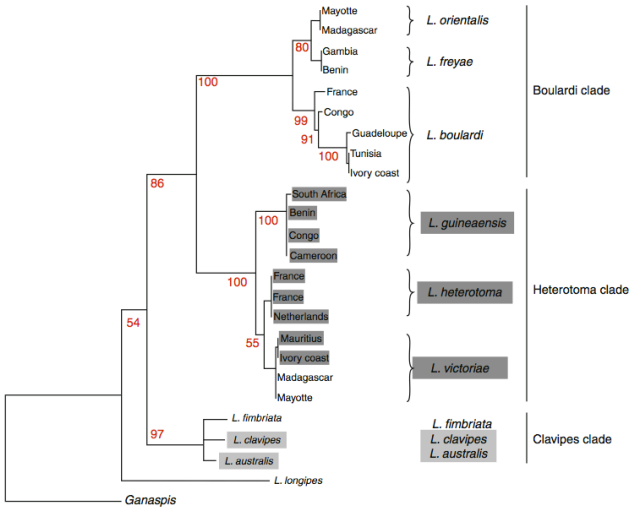

Figure 13 - Phylogenetic tree of the *Leptopilina* genus reconstructed by maximum likelihood (using ITS1). In dark grey, populations infected with CI-inducing *Wolbachia* and in light grey with parthenogenesis-inducing *Wolbachia*. Origin of the individuals is indicated. Figure taken from[105]

Wolbachia are necessary in order to ascertain, which of these scenarios is the most likely. According to our data, it is reasonable to assume that the *Wolbachia* infection in *Leptopilina heterotoma* is a relatively new occurrence, considering the fact that it appears to induce costs on the host (and admitting that a stable infection should not be prejudicial for one of the participants). This physiological cost is apparent in our work in two different instances. Firstly, in the slight delay in development time observed in infected individuals. Secondly, in the effect on survival after viral infection conferred by the presence of *Wolbachia*. We speculate that some type of interaction is happening in host tissues between endosymbiont and virus that induces an overall cost, instead of the beneficial protective effect that has been described for the well-established *Drosophila melanogaster*-*Wolbachia* association. Although this does not agree with our starting premise it seems to indicate that at some level, *Wolbachia* and RNA-viruses have a complex interaction when infecting arthropod hosts (which can end up being beneficial or not).

Overall we were able to conclude that the complex interactions that take place between endosymbionts and arthropods have a high specificity and cannot be determined by only one of their backgrounds. It is necessary to face the questions regarding these relations with care and always consider them on an evolutionary perspective. Here, we began by assuming that being a naturally occurring system, a *Drosophila*-*Leptopilina*-*Wolbachia*-DCV association would entail that all of these organisms are exposed to each other and so have developed their responses accordingly. What we did not anticipate was that *Leptopilina* would have developed mechanisms to resist (or tolerate) viral infections. Also, we overestimated the ability of *Wolbachia* to successfully colonize a new host after being horizontally transmitted by a natural mechanism.

In order to address the questions that remained unanswered after this project we aim to further study possible horizontal transmission events (maybe including a new wasp species, also a natural parasitoid of *Drosophila*) and analyze the effect of other viruses (some that may be specific of wasps and other that are more generalist) and their possible interactions with *Wolbachia* in host tissues.

"I may not have gone where I intended to go, but I think I have ended up where I needed to be." –
Douglas Adams in *The Long Dark Tea-time of the Soul*

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Annexes

Annex I

DNA extraction method for 96-well plate

1. Switch on 65°C water bath.
2. Place one grinding ball in each well and add 150ul of Buffer A. Seal the plate with PCR Sealing Film.
3. Put plate in adapters and attach to the grinder.
4. Grind for 1min at 20/s.
5. Pulse plate to 4000rpm in a centrifuge.
6. Add an additional 150ul Buffer A. Replace plate in the grinder in the opposite orientation.
7. Grind for 1min at 20/s.
8. Grind for 1min at 20/s.
9. Pulse plate to 4000rpm.
10. Incubate plate in a 65°C water bath for 30mins.
11. Pulse plate to 4000rpm.
12. Add 600ul 6M LiCl / 5MKAc (ratio 2.5:1), invert plate ~3 times and incubate on ice for 20min.
13. Spin plate 4000rpm 15min.
14. Transfer 600ul of supernatant to a new 1.2ml plate. Try to avoid carrying over any gunk.
15. Add 450ul ice-cold isopropanol (propan-2-ol) and mix.
16. Spin 4000rpm 30 min.
17. Aspirate supernatant and wash in 500ul 70% EtOH.
18. Spin 4000rpm 15min.
19. Aspirate supernatant and dry pellet at 37°C.
20. Resuspend pellet in 50ul Sigma water.

Buffer A

- 100mM Tris-HCl (pH 7.7)

- 100mM EDTA

- 100mM NaCl

- 0.5% SDS

Annex II

Sequencing and DNA precipitation protocol

Protocol for Cycle Sequencing
BigDye Terminator v1.1

1 – For each reaction, mix the following reagents:

Reagent	Quantity
Buffer	2.0µL (4ºC)
Terminator Ready Reaction Mix	2.0µL (-20ºC)
Template	
Single-stranded DNA	50-100ng (70ng)
Double-stranded DNA	200-500ng (350ng)
PCR product DNA	30-90ng (70ng)
Primer	3.2pmol
Deionized water	q.s.
Total volume	10µL

1.1 Mix well and spin briefly.

2 – Cycle Sequencing

2.1 Place the tubes in the thermal cycler, begin thermal cycling as follows:

Perform an initial denaturation.
Rapid thermal ramp to 96ºC
96ºC for 1min.

Repeat the following for 25 cycles:
- Rapid thermal ramp to 96ºC
- 96ºC for 10sec.
- Rapid thermal ramp to 50ºC
- 50ºC for 5sec.
- Rapid thermal ramp to 60ºC
- 60ºC for 1.15min.

- Rapid thermal ramp to 4ºC and hold until ready to purify.
(Rapid thermal ramp is 1ºC/sec)

These are the standard reaction conditions for cycle sequencing. Individual sequences may require optimization. Please consult the Automated DNA Sequencing CHEMISTRY GUIDE.

3 – Ethanol/NaAc Precipitation Protocol

3.1 Remove the tubes from the thermal cycler and briefly spin.

3.2 Add to each tube:
- 10µL of H_2O
- 2µL of 3M sodium acetate, pH4.6
- 50µL of 95% ethanol

3.3 Transfer to a microcentrifuge tube and mix.

3.4 Incubate at room temperature for 1hour to precipitate the extension products.

(Precipitation times < 15 minutes will result in the loss of very short extension products. Precipitation times > 24hours will increase the precipitation of unincorporated dye terminators.)

3.5 Set the centrifuge at 4ºC.

3.6 Centrifuge at maximum speed (14000rpm) for 30min.

3.7 Carefully aspirate the supernatant with a pipette tip and discard.

3.8 Rinse the pellet with 250µL of 70% ethanol.

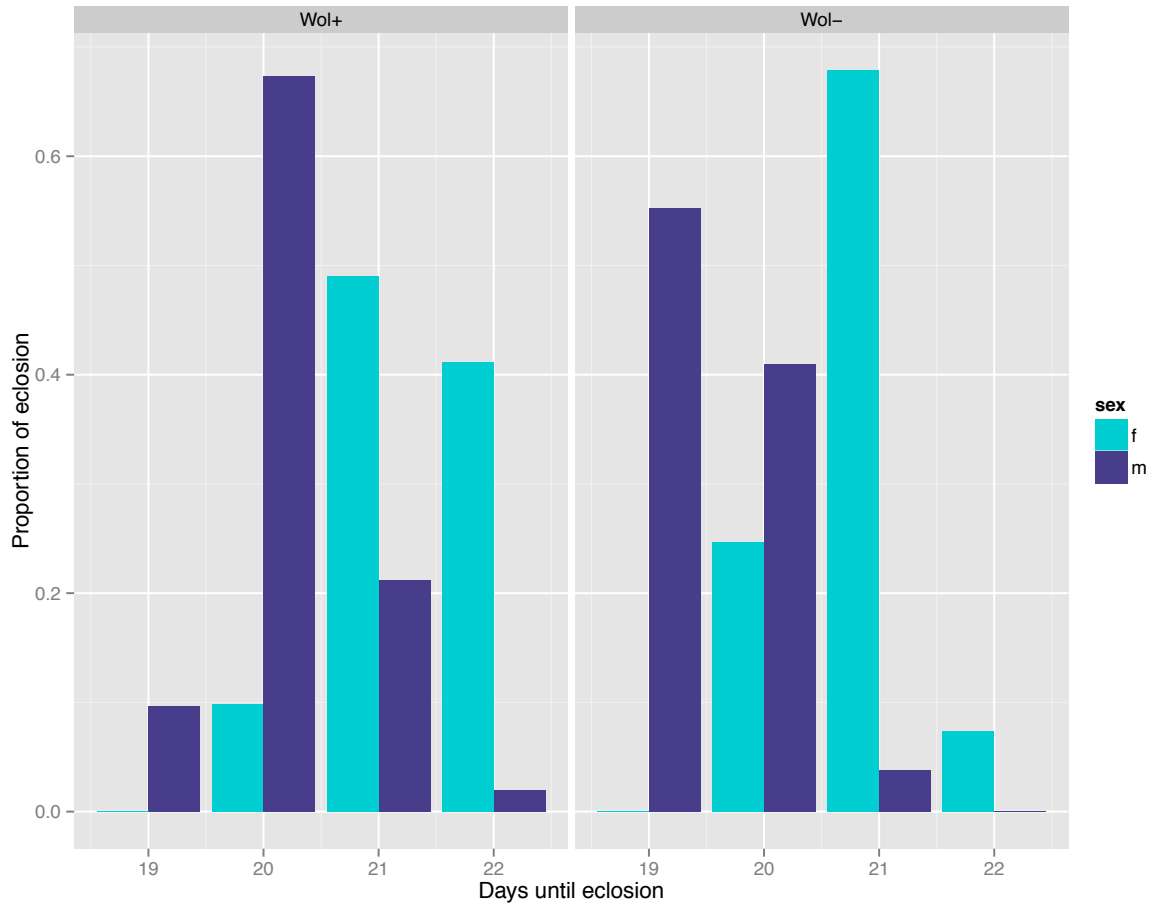
3.9 Vortex briefly.

3.10 Centrifuge at maximum speed (14000rpm) for 15min.

3.11 Carefully aspirate the supernatant with a pipette tip and discard.

3.12 Dry the pellet.

Annex III



Supplementary 1: Proportion of wasp eclosion for the *L. heterotoma* Wol+ and Wol- lines. Colors represent sex of eclosing individuals collected daily (f=females and m=males). The x axis represents number of days until adult eclosion.