The double-edged toxins: how *Spiroplasma* Ribosome-Inactivating Proteins affect *Drosophila melanogaster* life history traits

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Resume

De nombreux insectes vivent en association avec des bactéries symbiotiques hébergées dans leurs tissus, appelées endosymbiotes. Certains d'entre eux confèrent à leur hôte des avantages écologiques tels qu'une protection contre les parasites ou une meilleure tolérance à la chaleur. *Spiroplasma poulsonii* est un endosymbiote capable d'infecter la drosophile. Il prolifère dans l'hémolymphe des larves et des adultes, et colonise la lignée germinale des femelles durant la vitellogenèse, avec un mode de transmission verticale. La transmission s'accompagne d'un phénotype appelé « male-killing », qui consiste en la mort des embryons mâles. Ce processus favorise la transmission du symbiote dans les populations naturelles en augmentant la fréquence des femelles infectées. *Spiroplasma* ne possède pas de paroi, ce qui le rend indétectable pour le système immunitaire de l'hôte

Ce projet de thèse vise à mieux caractériser l'interaction *Drosophila-Spiroplasma,* en portant une attention particulière au rôle du symbiote et à ses facteurs de virulence. Dans une première partie, le développement d'un milieu de culture pour *Spiroplasma* est décrit. La possibilité de cultiver *Spiroplasma in vitro* nous a permis d'identifier des gènes dont l'expression change lorsque la bactérie vit dans l'hôte *versus* en culture. Ainsi, *Spiroplasma* exprime spécifiquement chez son hôte des gènes codant pour des toxines de la famille des Ribosome-Inactivating-Proteins (RIPs). Les RIPs de *Spiroplasma* protègent la drosophile contre des parasites tels que les guêpes parasitoïdes et les nématodes. Le fait que les gènes RIPs soient exprimés constitutivement, même en l'absence de parasites, soulève des questions quant à leur impact sur l'hôte. Dans une deuxième partie de ma thèse, j'ai étudié l'incidence des RIPs de *Spiroplasma* sur la drosophile. Les données obtenues suggèrent que ces RIPs ont des effets délétères chez leur hôte en réduisant l'espérance de vie des adultes et en augmentant la mortalité embryonnaire. La toxicité des RIPs est toutefois plus marquée envers les embryons mâles suggérant

un rôle de ces toxines dans le processus de 'male-killing'. Dans la troisième partie de ma thèse, la réaction de l'hôte à l'action des toxines RIPs fut analysé. Une analyse transcriptomique d'embryons infectés par *Spiroplasma* a révélé que cette bactérie induit l'expression de la chaperonne Heat-Shock-Protein 70B. HSP70B participe à l'homéostasie de la cellule en assurant le repliement des protéines et en augmentant leur demi-vie. Nos résultats suggèrent que le gène *hsp70B* est induit suite à l'action des RIPs de *Spiroplasma*. Des drosophiles déficientes pour *Hsp70B* montrent une susceptibilité accrue à *Spiroplasma*, et notamment une espérance de vie plus courte. Ainsi, la chaperonne HSP70B protège la drosophile de l'action des toxines RIPs. Une conséquence inattendue de l'expression de HSP70B par *Spiroplasma* est d'augmenter la tolérance de la drosophile à la chaleur. Nous proposons que la stimulation des voies de stress de l'hôte par *Spiroplasm*a confère un avantage à la drosophile par hormèse. L'ensemble de ce travail montre que les RIPs produites par *Spiroplasma* peuvent avoir des impacts bénéfiques ou néfastes sur l'hôte en fonction du contexte écologique dans lequel ce dernier se trouve. Ainsi, la présence d'une bactérie endosymbiotique modifie des paramètres importants de la physiologie de l'hôte et ses capacités d'adaptation à son environnement.

Abstract

Many insect species are associated with endosymbiotic bacteria which have the particularity of living within host tissues. Endosymbionts benefit from this stable and nutritious environment, while providing ecological advantages to their host, such as protection against parasites or thermal tolerance. *Spiroplasma poulsonii* is an endosymbiotic bacterium that infects natural populations of *Drosophila melanogaster. Spiroplasma poulsonii* lacks a cell wall, a fact that renders it invisible to the host immune system and allows it to thrive in the host hemolymph. It invades the female germline by co-opting the host's yolk transport and uptake machinery, which ensures its vertical transmission. Its efficient transmission is associated with a phenotype called male-killing, whereby infected male embryos die during their early development while infected females survive. The mechanisms that ensure the stability of *Drosophila-Spiroplasma* symbiosis are increasingly well understood, but the bacterial genes involved remain poorly known because of the intractability of *Spiroplasma.*

This project aims at better characterizing the *Drosophila-Spiroplasma* interaction, with particular focus on the bacterial side. In the first part, I developed a method to cultivate *Spiroplasma poulsonii in vitro* by optimizing a commercially available medium. This culture method allowed comparing the transcriptome of *in vitro* grown versus host-grown *Spiroplasma*, enabling us to identify putative genes involved in the interaction with the host. Interestingly, inside its insect host, *S. poulsonii* up-regulates genes coding for toxins of the Ribosomal Inactivating Protein (RIP) family. RIPs were previously known for their role in host protection against macro-parasites, such as wasps and nematodes. Their up-regulation in unparasitized hosts compared to culture was thus peculiar, raising the question what effect these RIPs have on host biology. Thus, in the second part, I studied the function of S. *poulsonii* RIPs in the absence of parasites. I showed that two of them are constantly expressed within the host and provide evidence that these toxins

shorten host life span and increase embryonic mortality. Interestingly, the expression of RIPs was more toxic to male embryos than females, suggesting that RIPs contribute to *S. poulsonii*-induced male-killing. Last, I studied the *D. melanogaster* response to RIPs, and how the host mitigates the deleterious effects of these toxins by up-regulating the cytosolic chaperone Heat-Shock-Protein 70B (HSP70B). This protein carries out essential functions in protein homeostasis under normal and stressful conditions such as folding, refolding, or increasing the half-life of proteins. Interestingly, up-regulation of *Hsp70B* in the presence of RIPs results in an increased lifespan and in a better tolerance to heat stress, which may be ecologically advantageous. Altogether, this work illustrates how *Spiroplasma*-derived RIP toxins can differentially affect *Drosophila* depending on the ecological context, ranging from beneficial upon parasite infection or heat shock to detrimental in the absence of such environmental pressures.

Keywords: *Spiroplasma*, *Drosophila*, endosymbiosis, Ribosome Inactivating Proteins, male-killing, Heat-Shock-Proteins

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Abbreviations:

RNAi: RNA (RiboNucleic Acid) interference

Def: Deficiency

ARP: Adhesion Related Proteins

RIP: Ribosome Inactivating Protein

*Sp*RIP: *S. poulsonii* Ribosome Inactivating Protein

Spaid: *S. poulsonii androcidin*

REP: Rough Eye Phenotypes

TUNEL: Terminal deoxynucleotidyl transferase dUTP Nick End Labeling

qPCR: quantitative Polymerase Chain Reaction

RT: Reverse Transcription

BSK medium: Barbour-Stoenner-Kelly medium

HSP: Heat Shock Protein

Chapter I: Introduction:

1. Symbiosis

"Symbiosis" originates from the Greek word for "living together" and was first introduced in 1877 by Albert Frank to describe lichens (Frank, 1877). Symbiosis was more formally defined a few years later by Anton De Bary as an association between organisms belonging to different species, regardless of the duration or the potential benefit or hindrance for the partners (De Bary, 1879). In 1909, Eugene Warming used the term symbiosis to describe an association where all partners benefit from such interactions, creating a debate around whether mutualism should be included in the definition of symbiosis (Warming E., 1909). This debate lasted until the 1990s, when scientists agreed that symbiosis actually includes a continuum of interactions ranging from parasitism to mutualism and that both ends of the continuum share a common basis of molecular mechanisms (Heddi et al., 1999). Nowadays most authors agree on the original definition proposed by Frank and De Bary and do not consider symbiosis as necessarily mutualistic (Hammerstein and Noë, 2016; Heddi et al., 1999; Hosokawa et al., 2010; Kremer et al., 2009; Mandyam and Jumpponen, 2015; Moran and Wernegreen, 2000).

In 1993, Nardon and Grenier classified symbioses based on the location of the symbiont (the smallest organism in the symbiosis) with respect to the host, defining ecto- and endosymbiosis (Nardon and Grenier, 1993).

1.1 Ectosymbiosis

Ectosymbiotic relations are defined as those where the symbiont resides on the surface of the host, including internal surfaces such as the epithelial tissues of the digestive tube and the ducts of glands. Ectosymbionts can be macroscopic, such as oxpeckers feeding from rhinoceros' parasites (McElligott et al., 2004), or

microscopic like sulfur-fixing bacteria on the cuticle of the Pompeii worm (Cary et al., 1997).

The most representative ectosymbiotic association is the intestinal microbiota, which is present in almost all animals (with varying composition) and has been described to support the host in a broad range of biological functions, such as nutrition, immunity, and physical protection against infections (Lemaitre and Miguel-Aliaga, 2013; Martino et al., 2017; Masson and Lemaitre, 2017; Thursby and Juge, 2017). The gut microbiota also benefits from symbiosis, as recently demonstrated by the growth of the symbiont *Lactobacillus plantarum* that is fostered by metabolites actively produced by the gut of *Drosophila melanogaster* (Storelli et al., 2018).

1.2 Endosymbiosis

Endosymbiotic relations are defined as those where the symbiont lives inside the host. As a consequence of living in such stable environments, some endosymbiotic bacteria have diverged from their pathogenic microbial counterparts, leading them to lose metabolic capacities and consequently rely on the host to survive (McCutcheon, John and Moran, Nancy, 2012; Mira and Moran, 2002; Moya et al., 2009) .

Most endosymbionts are either extracellular or intracellular, however some endosymbionts can carry out both levels of integration at different moments of their host life cycle. To designate the latter, Schwemmler proposed in 1980 the term “endocytobiosis"; this term however is used very little in the scientific literature (Schwemmler, 1980). Endosymbionts are divided into two groups based on the host perspective: “obligate” if the host cannot live without the symbionts and “facultative” otherwise.

1.2.1 Obligate (or primary) endosymbiosis

Obligate endosymbiosis refers to an association whereby a host and an intracellular endosymbiont require each other to survive and reproduce. Thus, the endosymbiont cannot be found as free-living bacteria and 100% of wild host individuals bear symbionts. Most obligate endosymbionts are housed inside specialized host cells called bacteriocytes that are sometimes grouped together, forming an organ called a bacteriome. Some obligate endosymbionts can be eliminated in laboratory conditions by using antibiotics or heat treatment resulting in a major decrease in host fitness, including severe impairments to fertility (Douglas et al., 1989; Nardon, 1973; Nogge, 1976; Wilkinson, 1998).

Obligate endosymbionts are particularly frequent in insects. 10% of known species rely on such interactions for their development or reproduction (Buchner, 1965; Douglas, 1989; Moran and Telang, 1998). Endosymbionts provide their host with nutrients that are absent from its nutritional niche. Obligate endosymbiosis is thus a feature of insects living on unbalanced diets, such as aphids living on phloem sap, weevils on cereals, or tsetse flies on mammalian blood.

Obligate endosymbionts are vertically transmitted by females, leading to co-evolution and even to co-speciation between the host and the endosymbiont (Baumann and Paul, 2005; Degnan et al., 2004; Hosokawa et al., 2012; Lefrèvre et al., 2004; Mazzon et al., 2010).

The symbiotic lifestyle has consequences on the dynamics of symbiont genome evolution, and it becomes highly relevant in obligate endosymbionts. Symbiotic lifestyle leads to a relief of the selective pressure on genes that are redundant with the host's or that are detrimental for the association. An example is the obligate endosymbiont of pea aphids, *Buchnera aphidicola*, whose genome has suffered wide-ranging erosion as a consequence of large deletions and chromosome

rearrangements as well as accumulation of point mutations (Moran and Mira 2001).

The most frequently deleted genes are those coding for metabolic pathways encoded in the host genome (Shigenobu, 2000; Latorre, 2005), along with genes encoding for virulence factors that could harm host tissues, such as secretion systems (Dale, 2002; 2003). In the oldest associations, genes encoding for peptidoglycan and lipopolysaccharide synthesis proteins also tend to be lost, rendering obligate endosymbionts less immunogenic for insects (Gomez-Valero, 2004). Last, recombination and DNA repair genes often get pseudogenized, which in turn accelerates their genomic erosion (Shigenobu, 2000, Dale, 2003, Moran, 2008).

Genome size reduction is not linear over time. Most deletions occur early in the endosymbiotic association (Gomez-Valero et al., 2004). Globally, however, interaction age correlates well with the endosymbiont genome size: the older the interaction, the smaller the endosymbiont genome (Akman and Douglas, 2009; Conord et al., 2008; Fisher et al., 2017; Lefrèvre et al., 2004; McCutcheon and Moran, 2011; Wernegreen, 2015). For instance, the symbiosis between the leafhopper *Homalodisca coagulata* and *Candidatus sulcia* established over 260 million years ago results in an endosymbiont genome size of only 246 kb (McCutcheon and Moran, 2012), one of the smallest known bacterial genomes. On the other hand, the cereal weevil *Sitophilus oryzae* established a symbiotic association only about 28 000 years ago with *Sodalis pierantonius,* whose genome of 4.5 Mb is similar in size to that of *E. coli* (Conord et al., 2008; Lefèvre et al., 2004).

1.2.2 Facultative (or secondary) endosymbiosis

Facultative endosymbionts are not strictly necessary for host survival. Consequently, their prevalence is below 100% in wild populations. Facultative

endosymbionts are particularly widespread among insects (Ishikawa, 2003), which will be the focus of this chapter. In many cases, facultative endosymbionts provide an ecological benefit to their host, such as faster development, protection against parasites and viruses, or increased resistance to extreme environments (Montllor et al., 2002; Moreira et al., 2009; Oliver et al., 2005, 2003; Raquin et al., 2015; Weeks et al., 2007). In some cases, however, the impact of facultative endosymbionts on the host remains elusive. For example, *Spiroplasma* symbionts infect 85% of wild *Drosophila mojavensis* with no known effect on the host so far (Haselkorn et al., 2013). Given that nematode infections represent the main ecological pressure for *Drosophila mojavensis*, a hypothesis that remains to be tested is whether *Spiroplasma* protects *Drosophila mojavensis* against parasites.

1.2.2.1 Mechanisms of transmission of facultative endosymbionts

1.2.2.1.1 Vertical transmission

Some facultative endosymbionts have strict vertical transmission, mostly maternal and sometimes paternal (Bright and Bulgheresi, 2010; Kikuchi, 2009; Watanabe et al., 2014). The molecular and cellular mechanisms underlying vertical transmission of symbionts are not completely understood. One of the most studied models for vertical transmission of endosymbionts is *Drosophila* infected by *Wolbachia. Wolbachia* is a type of intracellular bacteria that is extremely widespread among insects (Werren, 1997). In adult female flies, *Wolbachia* colonizes the posterior pole of the oocyte, where pole cells (the future gonads) will be formed. It does so by interacting with the oocyte cytoskeleton (Ferree et al., 2005). *Drosophila* can also host another vertically transmitted endosymbiont called *Spiroplasma.* These bacteria reach the oocyte by passing through the intercellular space of the ovarian follicular cells and get endocytosed within yolk granules during the vitellogenic stages of oogenesis (Herren et al., 2013). Thus, *Wolbachia* and *Spiroplasma* infect

the germline of the same host at different stages and through different mechanisms, indicating that vertical transmission has evolved several times independently in the evolutionary history of insects.

Tsetse flies are an interesting example for endosymbiont transmission. These insects are viviparous, with larvae maturing in a uterus and fed by the mother with a milk-like substance produced by a specialized gland. The facultative endosymbiont *Sodalis glossinidus* is capable of infecting the milk gland and is transmitted to the developing larva through the milk, much later than most other symbionts (Balmand et al., 2012).

1.2.2.1.2 Horizontal transmission

Most facultative endosymbionts are strictly vertically transmitted, but phylogenetic analysis of *Wolbachia*, one of the most widespread endosymbionts in insects, raised the possibility of horizontal transmission (Breeuwer and Werren, 1990; Li et al., 2017; O'Neill et al., 1992; Sandström et al., 2001). Further phylogenetic studies later revealed that horizontal transmission can occur for *Wolbachia, Arsenophonus* and *Cardinium ,* all infecting the whitefly *Bemisia tabaci* (Ahmed et al., 2013). More recently, other examples of horizontal transmission have been described. Endosymbionts of whiteflies can be left on plants on which they feed, and taken up by other individuals (Li et al., 2017). Other examples involve insect ectoparasites acting as phoretic vectors, as illustrated by the horizontal transmission of *Spiroplasma* by mite bites to *Drosophila* (Jaenike et al., 2007) or the transmission of *Hamiltonella* and *Regiella* to new aphid hosts by parasitoid wasps acting as "dirty needles" (Gehrer and Vorburger, 2012).

1.2.2.2 Strategies of facultative endosymbionts to persist in nature

As facultative endosymbionts are not required for the host to survive, it has been shown that even a 95-99% successful transmission rate (rather than 100%) can

result in the loss of the symbiont (O'Neill et al., 1998). This model suggests that endosymbionts are fated to be lost over generations. However, endosymbiotic bacteria have developed several strategies to overcome this fate, to spread in insect populations and maintain themselves over long evolutionary times.

1.2.2.2.1 Minimize damage to the host

One of the main strategies facultative endosymbionts have developed to maximize their spread in nature is to minimize the damage inflicted to the host (Werren and O'Neill, 1997). For instance, *Spiroplasma* and *Wolbachia* in *Drosophila* have low fitness costs for their hosts (Martins et al., 2010; Unckless and Jaenike, 2012). However, there are a few exceptions such as a *Wolbachia* strain wMelPop that over-replicates in its insect hosts and causes severe brain damage and life shortening (Min and Benzer, 1997), suggesting that the level of damage caused to the host is strain-specific.

Another example is the loss of metabolic genes that would promote symbiont growth beyond what the host can support. For instance, *Spiroplasma poulsonii* has lost the ability to metabolize trehalose, the most abundant carbohydrate in the fly hemolymph. This prevents the overgrowth of the symbiont, and probably increases the fly lifespan (Herren et al., 2014; Paredes et al., 2015).

1.2.2.2.2 Increase host fecundity

Facultative endosymbionts are mostly spread through vertical transmission. Thus increasing the host's fecundity also increases the endosymbiont population, a strategy observed in some facultative endosymbionts (Douglas et al., 1994). For instance, *Drosophila simulans* or the mite *Metaseiulus occidentalis* have higher egg laying rates when they are infected by *Wolbachia* or by *Cytophaga*, respectively (Weeks et al., 2007; Weeks and Stouthamer, 2004). The molecular mechanism leading to increased egg laying is not yet understood, although it is probably a

direct consequence of nutritional support provided to the host by the symbiont (Douglas et al., 1994).

1.2.2.2.3 Manipulate host reproduction

A vast majority of facultative endosymbionts are exclusively transmitted through female germline cells. This has led some endosymbionts to evolve strategies to increase the percentage of females in populations. Such strategies include four known mechanisms: cytoplasmic incompatibility, male killing, feminization, and parthenogenesis (Figure 1). Relatively few bacterial endosymbiont genera can manipulate host reproduction, the best known examples so far being *Wolbachia, Rickettsia, Cardinium, Arsenophonus* and *Spiroplasma* (Bandi et al., 2001; Boutzis and O'Neil, 1998; Felsheim et al., 2009; Johanowitz and Hoy, 1998; Lawson et al., 2001; Zchori-Fein et al., 2001).

1.2.2.2.3.1 Cytoplasmic incompatibility

Cytoplasmic incompatibility has only been described in *Wolbachia* and *Cardinium* (Engelstädter et al., 2006). It denotes the phenomenon where progeny from infected males mating with uninfected females, or females infected with a different strain of bacteria, fail to develop (Engelstädter and Telschow, 2009; Yen and Barr, 1973). Uninfected females thus have fewer progeny compared to infected females. This strategy is particularly efficient in insect species whose females mate only once (e.g. mosquitoes) because infected males thus act as sterilizing agents for uninfected females. Cytoplasmic incompatibility was first described in mosquitoes (Hertig and Wolbach, 1924) and later identified in several other insects including *Drosophila* (Turelli and Hoffmann, 1995). Cytoplasmic incompatibility has also been described in non-insect arthropods, like *Crustacea* and *Arachnidae* (Breeuwer, 1997; Rousset et al., 1992).

Bacterial genes involved in this phenotype have been recently identified in *Wolbachia*. Most *Wolbachia* genomes contain a prophage with genes encoding for proteins with ankyrin-repeat domains and ubiquitinase activity, called *cif* (Beckmann, et al., 2017; Le Page et al., 2017). The current model indicates that progeny coming from *Wolbachia*-infected males mated with uninfected females die as a consequence of the ubiquitinase activity of *cif*. This is rescued when mothers are infected with the same strain of *Wolbachia* because the bacteria secrete an antitoxin with a deubiquitinase activity, called *cid* (Beckmann, et al., 2017; Le Page et al., 2017).

1.2.2.2.3.1.2 Male killing

Another frequent reproductive manipulation is male-killing, which results specifically in the death of infected male embryos, while infected females survive (Counce and Poulson, 1962; Malogolowkin and Poulson, 1957; Poulson and Sakaguchi, 1961). This phenotype can be caused by a broad range of endosymbionts including *Arsenophonus, Rickettsia, Spiroplasma, Wolbachia,* and *Hamiltonella*-related symbionts (Hurst and Frost, 2015).

Recent findings showed that male-killing *Spiroplasma* encode a toxin called Spaid that has a deubiquitinase domain similar to the cytoplasmic incompatibility factor *cif* in *Wolbachia* (Harumoto and Lemaitre, 2018). Furthermore, the male-killing *Wolbachia* genome contains a prophage sequence similar to that of the strains responsible for cytoplasmic incompatibility (Bordenstein and Vanderbilt unpublished data -*Drosophila* Research Conference 2018-). These data point to a possible shared mechanism underlying male-killing ability between different endosymbionts. Nevertheless, phylogenetic analysis of male-killing bacteria, such as *Spiroplasma, Wolbachia, Rickettsia* and *Arsenophonus*, shows that male-killing must have independently evolved (Hurst et al., 1997; Jaenike et al., 2003; Poulson and Sakaguchi, 1961; Werren et al., 1986, 1994).

It is hypothesized that male killing can be beneficial for female hosts, as it decreases feeding competition between siblings. In some instances, dead males even become an extra food source for the surviving females: symbiont-infected female ladybugs feed on their dead brothers (Hurst et al., 1993; Jaenike et al., 2003). Furthermore, male mortality reduces inbreeding by forcing surviving females to mate with non-sibling males (Dannowski et al., 2009; Hurst and Frost, 2015; Werren, 1987).

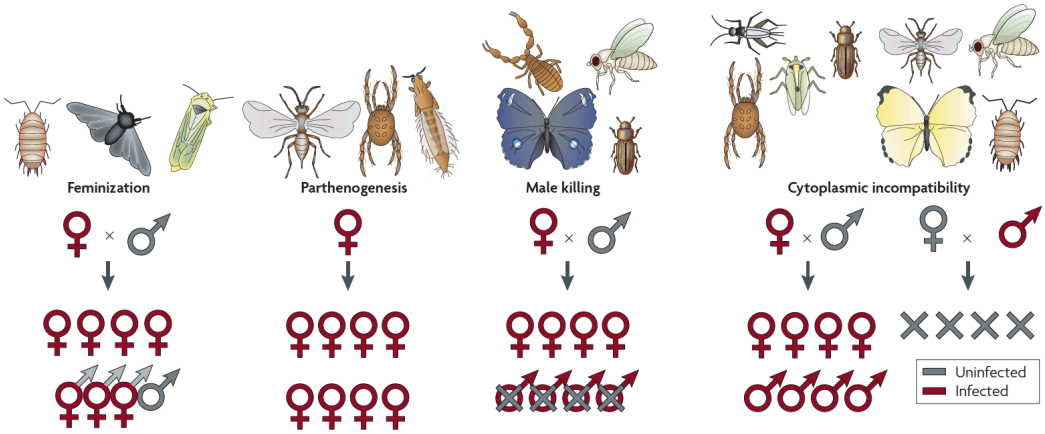
1.2.2.2.3.1.3 Parthenogenesis

Parthenogenesis is induced by *Wolbachia, Cardinium,* and *Rickettsia,* and has been described in Hymenoptera (Hurst and Frost, 2015). Genetic sexual identity in Hymenoptera is haplodiploidy: males are haploid while females are diploid. Thus unfertilized eggs develop as males, and fertilized ones as females (Rousset et al., 1992; Stouthamer et al., 1990). Some strains of *Wolbachia* have evolved to manipulate the development of unfertilized eggs, interfering with chromosome segregation at anaphase of the first mitosis. This leads to a diploid nucleus and the production of females without male fertilization (Giorgini et al., 2010; Stouthamer and Kazmer, 1994; Vavre et al., 2004).

1.2.2.2.3.4 Feminization

Feminization is the process by which genetic males are transformed into phenotypic females (Araki et al., 2001; Bouchon et al., 1998; Dyer and Jaenike, 2004). This phenotype is induced by *Cardinium, Wolbachia, Microspora* and *Nosema* (Hurst and Frost 2015). *Wolbachia* is able to induce this phenotype in the terrestrial crustacean *Armadillidium vulgare* (*Isopoda*), butterflies (*Lepidoptera*) and leafhoppers (*Homoptera*) (Kageyama et al., 2012). The mechanistic basis of feminization has been partially elucidated in *A. vulgare* and *Gammarus duebeni*. In these species *s*ex determination relies on the presence of androgenic hormone during development. *Wolbachia* infecting *A. vulgare* and *Microspora* infecting

Gammarus duebeni both prevent the differentiation of the androgenic gland, dramatically reducing the production of male hormones (Janicke et al., 2013; Juchault et al., 1992; Juchault and Legrand, 1985). A different system has been found in *Zyginidia pullula* infected with *Wolbachia,* where the bacteria affect with male DNA methylation, resulting in female patterns of methylation in the male genomes (Negri et al., 2009).



(Werren et al., 2008)

Figure 1: Graphic representation of four reproductive manipulation mechanisms induced by endosymbiotic bacteria

1.2.2.3 Conferring benefits to the host

Some endosymbionts have also evolved mechanisms to confer ecological benefits to their hosts so that infected individuals have a fitness advantage in the wild. Some species can produce metabolites that complement their hosts' diet. Others can support the host against environmental stresses such as heat, or protect the host against natural enemies.

1.2.2.3.1 Assisting against environmental stresses

As ectotherms, insects are very sensitive to environmental temperatures. Several maternally transmitted facultative endosymbionts promote heat tolerance in

aphids, such as *Serratia*, *Hamiltonella,* and *Regiella* (Russell and Moran, 2006). Aphids that harbor *Serratia symbiotica* have shorter developmental times and increased fecundity at high temperatures (Chen et al., 2000). Furthermore, pea aphids without *Rickettsia* delay their reproduction at high temperatures (Montllor et al., 2002). Some *Wolbachia* strains also modulate dopamine levels in *D. melanogaster*, which leads to thermo-tolerance (Gruntenko et al., 2017).

1.2.2.3.2 Protecting against natural enemies

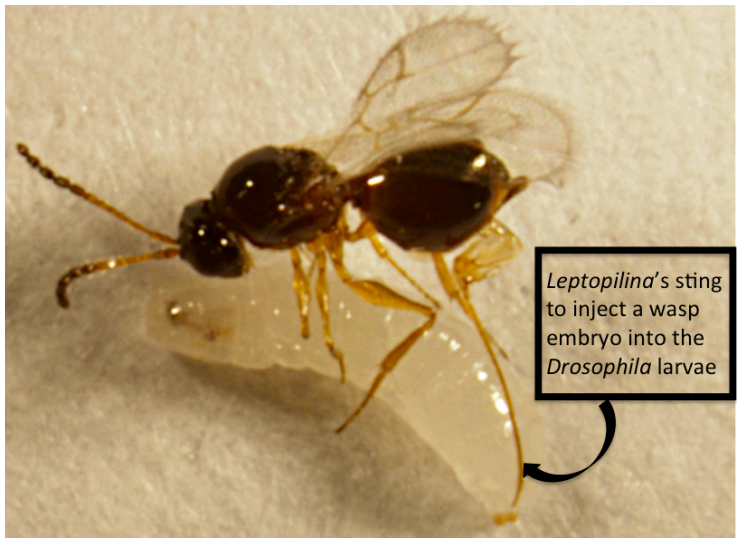
Insects face infections from intracellular (*e.g.* bacteria, viruses and microsporidia) and extracellular pathogens (*e.g.* bacteria, fungi, nematodes or parasitoid wasps). Thus, insects have evolved a potent immune system that detects and kills pathogens (Lemaitre and Hoffmann, 2007). However, multiple studies have revealed that facultative endosymbionts also protect their hosts against certain parasites (Hedges et al., 2008; Jaenike et al., 2010; Oliver et al., 2003; Rothacher et al., 2016; Scarborough et al., 2005; Teixeira et al., 2008; Vorburger, 2014; Xie et al., 2010).

1.2.2.3.2.1 Endosymbiont conferring protection against macroparasites

The main macroparasites of insects are mites, wasps and nematodes. These parasites are usually very specific in terms of host species and developmental stage they target (Truscott et al., 2017). Parasitoid wasps are a large group of hymenopterans that lay their eggs in the larva or pupa of other arthropods (Figure 2). When the wasp larva hatches, it feeds on the internal tissues of the host arthropod. Consequently, a wasp will hatch after pupation (Stireman, 2016) .

Pea aphids are highly susceptible to infections by parasitoid wasps, except when they harbor the endosymbionts *Hamiltonella defense*, *Serratia symbiotica* or *Regiella insecticola*. These endosymbionts block the parasitoid wasp's development (Oliver et al., 2003). Genome Wide Association Studies for different *H. defensa*

genomes revealed *APSE-3* as a candidate gene responsible for protection against wasps. This gene encodes a bacteriophage-associated toxin that targets eukaryotic cells (Degnan and Moran, 2008; Oliver et al., 2009). Interestingly, the phage toxin identified in *H. defensa* does not exist in *S. symbotica* and *R. insecticola*, which also protect against parasitic wasps (Oliver et al., 2003; Vorburger et al., 2010, 2009). The mechanism of how these two endosymbionts protect their hosts against wasps remains unknown (Vorburger et al., 2010). The variety of mechanisms described above suggests that different species of bacterial endosymbionts have independently evolved the capacity to protect the host against the same parasitoid wasp.



(Adapted from Bajgar et al., 2015)

Figure 2: Illustration of *Drosophila* infestation by a parasitoid wasp.

1.2.2.3.2.2 Endosymbiont conferring protection against microparasites

To date, endosymbionts have not been found to protect against other bacterial pathogens. Aphids seem to bear endosymbionts that are protective against fungi (*R. insecticola* and *Spiroplasma*) (Lukasik et al., 2013; Scarborough et al., 2005).

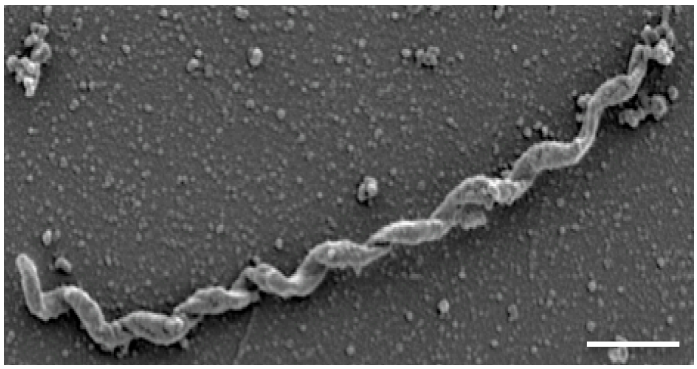
Wolbachia has been found to protect its host against viruses (Teixeira et al., 2008). However, the underlying mechanism is still under debate. One theory proposes that *Wolbachia* activates the fly immune system (Ye et al., 2013). Another theory proposes that *Wolbachia* competes with the virus for essential metabolites such as cholesterol (Caragata et al., 2013). Recent findings show a correlation between the copy number of several genes in the *Wolbachia* genome found in the Octomom region, and bacterial load and protection against viruses (Chrostek et al., 2013). *Wolbachia* can also protect mosquitos (*Aedes* and *Anopheles* genera) against Dengue Virus, *Chikungunya* Virus and *Zika* Virus (Bian et al., 2010; Frentiu et al., 2014; Raquin et al., 2015). Because of the antiviral protection it confers, *Wolbachia* has the potential to be turned into a powerful tool to fight the spread of human diseases (Hoffmann et al., 2011). Indeed, cytoplasmic incompatibility allows for the fast spread of *Wolbachia* into natural populations, suppressing virus titers in vector insects, leading to decreased transmission rates to humans (Hoffmann et al., 2011).

2. The *Spiroplasma-Drosophila* association as a model for endosymbiosis

Drosophila melanogaster has been largely used as a model organism to investigate genetic and molecular aspects of animal biology (Roberts, 2006). In contrast, symbiosis has been mostly studied with ecological approaches (Wernegreen, 2012). Thus, the use of *D. melanogaster* as host allows combining the evolutionary knowledge about symbiosis with the genetic toolkit of *D. melanogaster* to reveal the functional aspects of host-endosymbiont interactions (Harumoto et al., 2014; 2016; Le Page et al., 2017; Veneti et al., 2005). Only two endosymbionts can infect natural populations of *D. melanogaster*: *Wolbachia* and *Spiroplasma* (Mateos et al., 2006).

2.1 The *Spiroplasma* genus

Spiroplasmas are long helical Gram-positive bacteria belonging to the Mollicute clade (Figure 3). Mollicutes belong to the lineage of eubacteria (around 600 - 800 million years), however, this clade has lost several features of this lineage, the most remarkable example being that they are devoid of cell wall (Gasparich, 2002). The *Spiroplasma* genus is estimated to have diverged around 300-600 million years ago. All *Spiroplasma* species are specialized in infecting arthropods or plants, and have lost the capacity to live freely (Regassa and Gasparich, 2006).

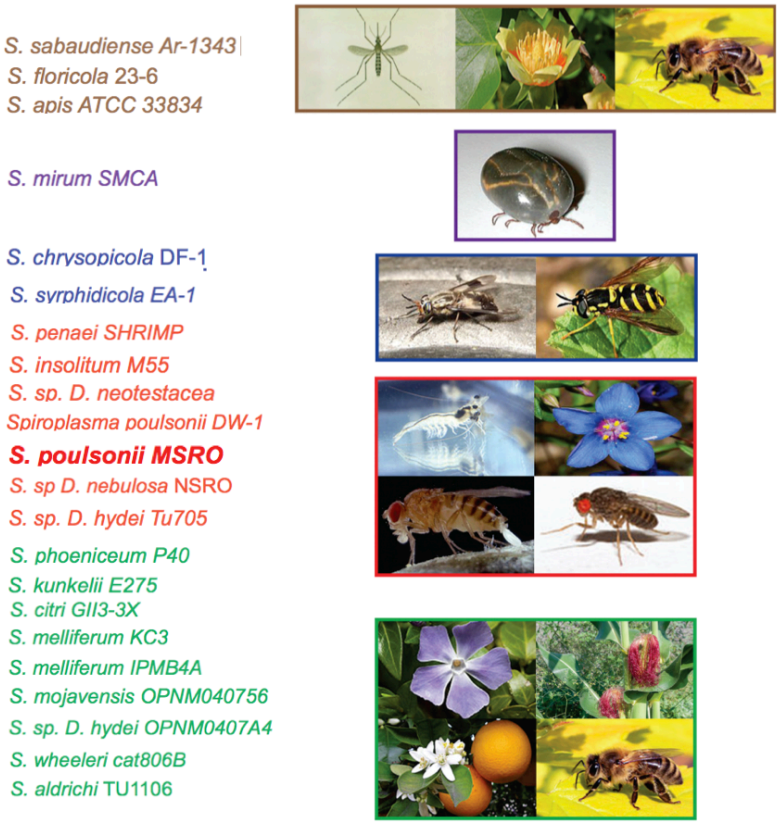


(Ramond et al., 2016)

Figure 3: Scanning electron micrograph of *S. poulsonii* extracted from one-week old *Spiroplasma*-infected female *Drosophila melanogaster*. Bar corresponds to 1 µm.

Depending on the type of interactions with the host, the *Spiroplasma* genus can be divided into four categories (Figure 4). The first category is between plant hosts, and this includes *S. citri, S. kunkelii and S. phoeniceum* (Bové et al., 2003; Saillard et al., 1987; Whitcomb et al., 1986). The second category contains strict pathogens like *Spiroplasma eriocheiris* found in crustaceans or *Spiroplasma melliferum* found in bees (Alexeev et al., 2012; Wang et al., 2005, 2003). The third category comprises mutualistic ectosymbiotic *Spiroplasma* species, found for instance in mosquitoes, such as *Spiroplasma diminutum* which live in the gut (Vorms-le Morvan et al., 1991). The last category comprises facultative endosymbiotic

Spiroplasmas. This group is characterized by vertically transmitted species which can induce reproductive manipulation such as male killing, like *Spiroplasma poulsonii* (Counce and Poulson, 1962; Goodacre et al., 2006; Haselkorn, 2010; Hurst and Jiggins, 2000). This group has successfully spread in nature among insects. Although it is difficult to precisely estimate their prevalence, several studies suggest that 5 to 15% of insects are infected by endosymbiotic *Spiroplasma* species (Bandi et al., 2001; Gasparich, 2002).



(Adapted from Paredes et al., 2015)

Figure 4: List of *Spiroplasma* species and their hosts. Color codes associate *Spiroplasma* species with their hosts.

2.2 *Spiroplasma poulsonii* and *Drosophila*

Spiroplasma was first found in flies in a population with female-biased sex-ratio (Poulson and Sakaguchi, 1961). Interestingly, electron microscopy revealed the

presence of bacteria in the fly. However, it was wrongly classified as a spirochaete because of its morphology (David and Poulson, 1979; Poulson and Sakaguchi, 1961).

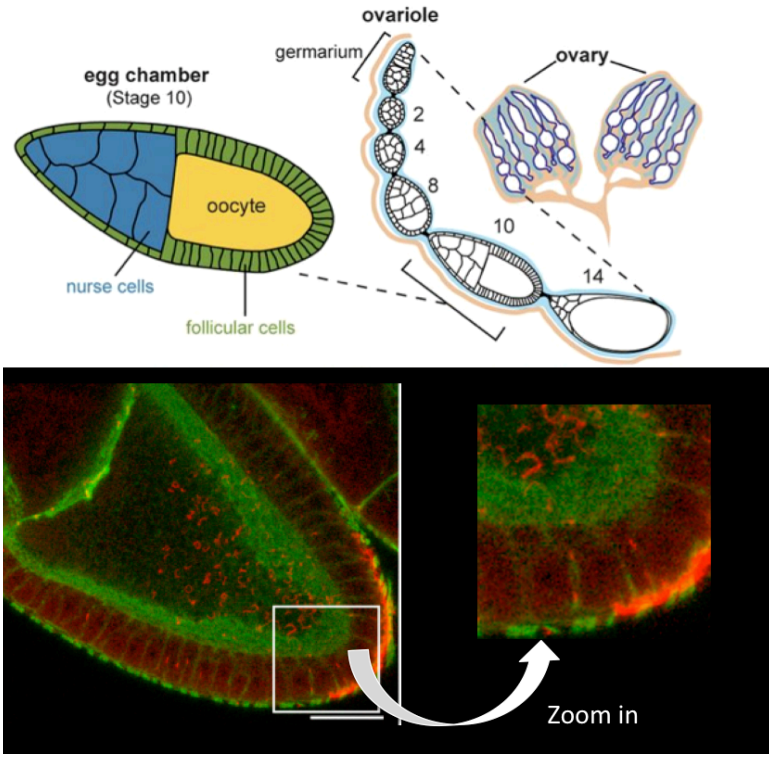
In 1973 the "male-killer spirochaetes" were reclassified as *Spiroplasma* following the identification of a new group of these pathogenic bacteria in plants named *Spiroplasma* (Davis and Worley, 1973; Saglio et al., 1973). This group of bacteria were considered strict pathogens until 1979, when *Drosophila hydei* (*D. hydei*) was found to harbor a new species of *Spiroplasma* that did not induce male-killing (Ota et al., 1979), making it, at that time, the first *Spiroplasma* species that did not harm the host.

The *Drosophila* immune system cannot detect and target *S. poulsonii* because of its lack of peptidoglycan that renders it non-immunogenic (Anbutsu and Fukatsu, 2010; Bourtzis et al., 2000; Herren and Lemaitre, 2012; Hurst et al., 2003; Lemaitre and Hoffmann, 2007; Siozios et al., 2008). As a consequence, *Spiroplasma* has little limitation to grow and its titer increases along the whole fly life cycle (Anbutsu and Fukatsu, 2003; Herren and Lemaitre, 2011). The sole titer-limiting factor identified so far is the availability of host lipids (Herren et al., 2014), although other metabolic factors might come into play (Masson et al., 2018). Interestingly, this continuous bacterial growth in adult flies was absent from a serendipitously isolated mutant strain of *Spiroplasma* in *D. nebulosa* that had lost its male-killing ability (Anbutsu and Fukatsu, 2003; Kageyama et al., 2006).

2.3 *Maternal transmission of Spiroplasma poulsonii*

Spiroplasma poulsonii vertical transmission was discovered very early on because of the male-killing phenotype that facilitates monitoring the infection through generations. However, transmission was visualized only in 1988, when electron microscopy revealed that *Spiroplasma* associates with yolk granules in the oocyte (Niki, 1988). Later, *Spiroplasma* titer was quantified in different *Drosophila* organs

and found to be particularly high in ovaries (Anbutsu and Fukatsu, 2006). These data suggested that *Spiroplasma* is vertically transmitted though transovarial transfer, which was confirmed in 2013 by molecular and genetics experiments (Figure 5) (Herren et al., 2013).



(Adapted from Herren et al., 2013)

Figure 5: Stage 10 oocytes of *Spiroplasma*-infected *Drosophila. Spiroplasma* is in red.

Spiroplasma colonize the germline in the female fly at the stage when the vitellogenic oocytes are formed (Herren et al., 2013). To be internalized by the oocyte, *Spiroplasma* hijacks the yolk transport and uptake machinery. It migrates between follicular cells of the egg chamber along with yolk proteins, reaches the oocyte membrane and is internalized through endocytosis triggered by the recognition of yolk proteins by the oocyte membrane receptor Yolkless (Herren et al., 2013).

2.4 *Spiroplasma poulsonii* as male-killing bacteria

Spiroplasma male killing was first described in the 1960s when microscopy studies observed and described which parts of infected embryos appeared damaged by *Spiroplasma* (Sakaguchi and Poulson, 1961; 1963). In the 1970s, scientists analyzed the interactions between *S. poulsonii* and the sex determination pathway in *D. melanogaster* (Miyamoto et al., 1975; Tsuchiyama et al., 1978). In *D. melanogaster,* sex is determined by the presence of the *Sex-lethal* gene (*sxl*), which is expressed in females exclusively (Cline and Meyer, 1996). Expression of *sxl* in females activates the female sex differentiation pathway, whereas its absence in males allows the activation of the dosage compensation pathway (Cline and Meyer, 1996). The Dosage Compensation Complex (DCC) is a ribonucleoprotein complex that doubles the expression of most X-linked genes in males, which compensates for the male single X chromosome copy (Kelley and Kuroda, 1995).

Flies with a deficient sex determination pathway develop as intersex: individuals are genetically uniform but all or some parts of their somatic tissues have the opposite sexual phenotype as the one predicted from their genotype. *S. poulsonii* infected female intersex flies survive, suggesting that somatic sexual identity is not involved in male killing (Miyamoto et al., 1975). Moreover, individuals with a male karyotype are killed regardless of their somatic sex and gynandromorph flies (sexual mosaic individuals that are genetically chimeric, i.e. consisting of male and female tissues) showed that the number of X chromosomes seems to be determinant for male killing (Tsuchiyama et al., 1978). It was finally demonstrated in 2005 that male embryonic lethality in *D. melanogaster* is linked to DCC and not to the sex determination pathway (Veneti et al., 2005). Male embryos deficient for DCC were able to survive until larval stage (Veneti et al., 2005), pointing to DCC as one of the targets of *Spiroplasma.*

Between 2013 and 2016, a new research approach was used to better understand male-killing by observing cytological damage using fluorescent microcopy. Male *Spiroplasma*-infected embryos at 8 to 11h after egg laying (AEL) displayed massive apoptosis and disrupted neurogenesis compared to female ones (Bentley, Joanna et al., 2007; Cheng et al., 2016; Harumoto et al., 2014; 2016; Martin et al., 2013). Interestingly, apoptosis had been correlated with chromatin bridges on the X chromosome during cell mitosis of male embryos (Harumoto et al., 2016).

Recently, a weak male-killer strain of *S. poulsonii* has been described (Harumoto and Lemaitre, 2018). This strain was derived from a serendipitously isolated mutant strain of *Spiroplasma poulsonii*, which killed males only partially. This new weak male-killer strain has a truncated gene, called Spaid, which contains an ankyrin-repeat domain and a deubiquitinase domain. The exact mode of action of Spaid remains unclear, however Spaid co-localizes with the DCC and ectopic expression of this gene in uninfected flies completely phenocopies male-killing (Harumoto and Lemaitre, 2018).

2.5 *Spiroplasma poulsonii* protects *Drosophila* against macro-parasites

Spiroplasma was initially studied in the late 60's as a male-killer bacterium, and was considered a reproductive parasite with no beneficial outcome for the host. From 2010 on, several studies revealed that *Spiroplasma* can play an important role in protecting its host against macro-parasites, revealing a mutualistic aspect of the interaction (Jaenike et al., 2010; Xie et al., 2010). In *Drosophila neotestacea, Spiroplasma* partially blocks nematode infections and restores host fecundity (which is lowered by nematode infections) (Hamilton and Perlman, 2013; Jaenike et al., 2010). *Spiroplasma* infection in *Drosophila hydei, Drosophila neotestacea* and *Drosophila melanogaster* is also correlated with an increased survival after parasitoid wasp infestation (Ballinger and Perlman, 2017; Xie et al., 2011, 2010).

Interestingly, *Spiroplasma* confers resistance against both nematodes and wasps in *Drosophila* species by synthetizing a family of toxins called Ribosomal Inactivating Proteins (RIPs), which block eukaryotic protein synthesis (Ballinger and Perlman, 2017; Hamilton et al., 2016). *Spiroplasma* RIPs have been shown to target the ribosomes of wasps and nematodes, resulting in the death of these macro-parasites. The effect of RIPs might be reinforced by nutrient competition between *Spiroplasma* and wasp larvae for host lipids (Paredes et al., 2016). Protection is ecologically crucial for the symbiosis, as it is one of the main drivers of *Spiroplasma* spreading in wild populations (Jaenicke et al., 2010).

Ribosome-inactivating proteins (RIPs) inactivate ribosomal RNA in a sequence-specific manner (Walsh et al., 2013a). They display a rRNA N-glycosidase activity that cleaves an adenine on a conserved loop structure of the 28S rRNA of eukaryotic ribosomes, called the sarcin-ricin loop (Endo and Tsurugi, 1987, 1986). Cleavage and release of adenine (referred to as “depurination”) prevents the recruitment of translation elongation factors and subsequent protein synthesis (Endo and Tsurugi, 1987, 1986).

Most RIPs are produced by plants as a defensive mechanism against parasites, for instance ricin, abrin and saporins (Barbieri et al., 2006; Walsh et al., 2013). However, RIPs have also been found in pathogenic bacteria to promote their survival and replication in the host organism (Johannes and Römer, 2010; Walsh et al., 2013). Understanding the mode of action of these toxins has thus been the focus of a large scientific effort due to their relevance in human health, both as a bacterial virulence factor and as possible therapeutic molecule (Barbieri and Stirpe, 1982; Virgilio et al., 2010). RIP toxin exposure, even at low dose, can lead to mortality for eukaryotes (Walsh et al., 2013b). Their high toxicity has resulted in some RIPs, such as ricin, being classified as potential weapons for bioterrorism (Rotz et al., 2002)

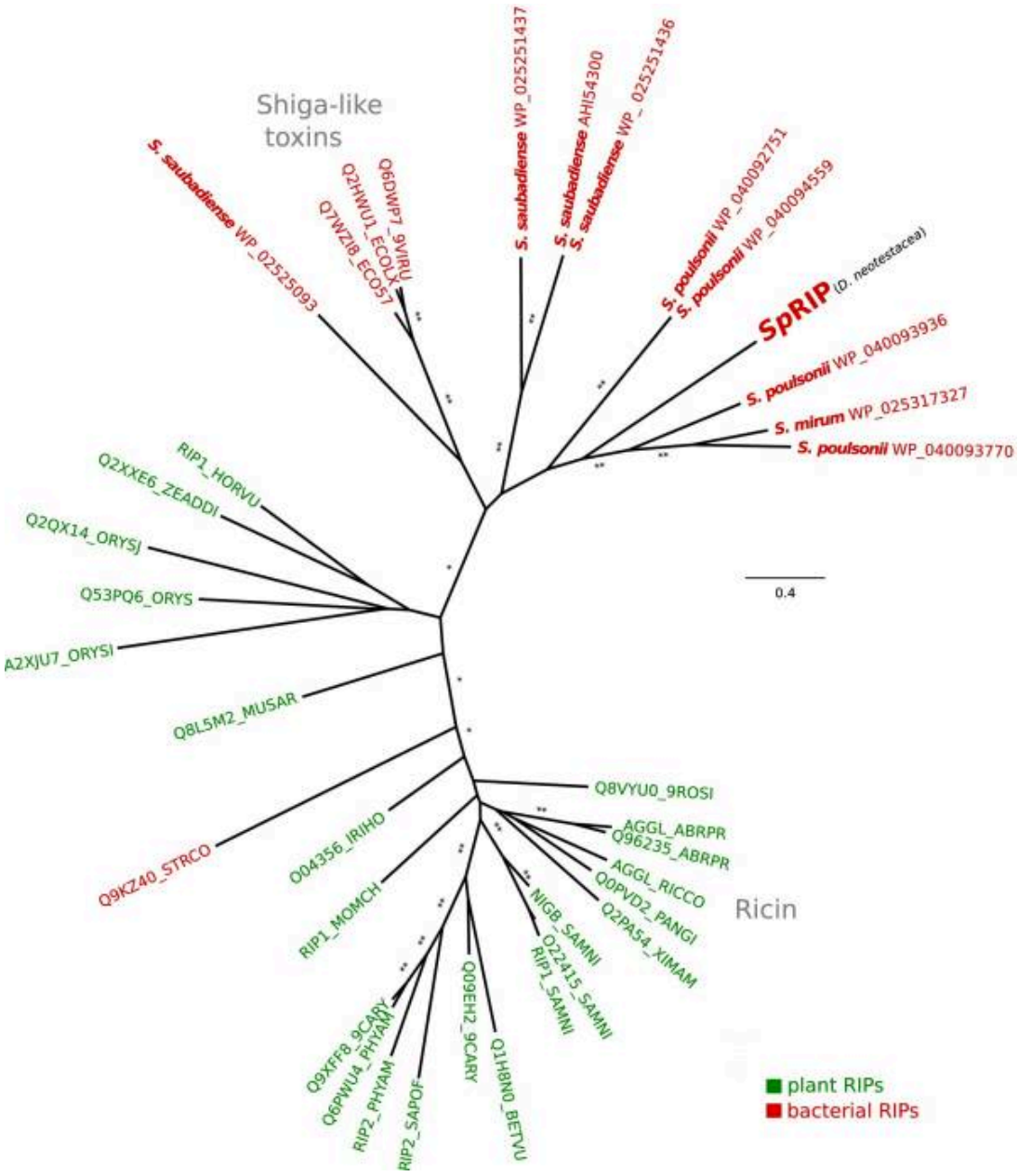
About one hundred RIPs have been described so far, grouped in two major categories depending on their activity (Figure 6) (Stirpe, 2004; Stirpe and Battelli, 2006). Type 1 RIPs are monomeric proteins with RNA N-glycosidase enzymatic activity (Bergan et al., 2012; Stirpe, 2004). Type 2 RIPs are composed of an A-subunit with RNA N-glycosidase activity associated with one or several B-subunit(s) (Bergan et al., 2012; Olsnes and Alexander, 1973; Olsnes and Pihl, 1973; Stirpe, 2004). The B-subunit is a lectin-like peptide with affinity for cell surface sugars, which facilitates the internalization of the toxin into the cell. RIPs containing A- and B-subunits display a higher toxicity than monomeric ones (Barbieri and Stirpe, 1982).

Type 1 RIPs: This group is the largest, and most of its members are produced by plants of various genera, including *Caryophyllaceae, Cucurbitaceae*, and *Euphorbiaceae* (Stirpe, 2004). Because they do not have B-subunits, these RIPs lack an efficient mechanism to enter host cells, resulting in low toxicity (Stirpe and Battelli, 2006). Plants producing type 1 RIPs such as spinach and tomato can be safely consumed (Barbieri et al., 2006; Ishizaki et al., 2002).

Type 2 RIPs: Most toxins from this group, such as ricin and abrin, are produced from plants (Olsnes, 2004), however, some Gram-negative bacteria also produce similar toxins, such as Shiga- and Shiga-like toxins (Trofa et al., 1999). Ricin and abrin are produced by the seeds of *Ricinus communis* and *Abrus precatorius,* respectively (Olsnes, 2004). Ricin was isolated for the first time in 1888 (Stillmark, 1888). Preliminary results lead to classify these toxins as agglutination factors since they induced the clumping of erythrocytes and the precipitation of serum-soluble proteins. Accordingly, agglutination was hypothesized to be the cause of toxicity until the early 70's when it was found that these toxins are potent protein synthesis inhibitors in eukaryotic cells (Lin et al., 1973; Lucio et al., 1973; Olsnes and Pihl, 1972). Shiga and Shiga-like toxins were identified in late 19th century by Kiyoshi Shiga, who was investigating the cause of dysentery during a large epidemic

with exceptionally high mortality rates (Trofa et al., 1999). Mortality was associated with a Gram-negative "bacillus" isolated from the stool of patients, *Shigella dysenteriae*, that produces large quantities of Shiga-toxins (Trofa et al., 1999). Interestingly, *Shigella* and *Escherichia* genera are closely related, and some strains of *Escherichia coli* can also produce similar toxins (so called Shiga-like toxins) (Hunt, 2010; Walsh et al., 2013).

Transcriptomic analysis from *Spiroplasma*-infected *D. neotestacea* and genome sequencing from *Spiroplasma*-infected *D. melanogaster* revealed the presence of 5 genes coding for RIP toxins in the *Spiroplasma poulsonii* genome (Ballinger and Perlman, 2017; Hamilton et al., 2016, 2014; Paredes et al., 2015). Bioinformatics analysis predicted these RIPs to belong to type I. Although this is the less toxic group, the high load of *Spiroplasma* in hemolymph may result in sufficiently high doses of RIPs to achieve efficient parasite killing.



(Hamilton et al., 2016)

Figure 6: *Spiroplasma* strains encode divergent RIP-like sequences. Amino acid phylogeny of *Sp*RIP aligned with other putative *Spiroplasma* Maximum-likelihood

3. Scope and outlines

Symbiosis between *Drosophila* and *Spiroplasma* is incredibly complex, yielding a variety of phenotypes among which the most striking are indubitably male killing and protection against parasites. However, functional studies are limited by the intractability of *Spiroplasma,* which, similar to the majority of insect endosymbionts, is neither cultivable nor transformable. Previous studies thus focused on the host side of the interaction. The bacterial determinants of symbiosis however remain elusive, with the exception of Spaid and RIPs.

The objective of this work is to further characterize *Spiroplasma* genes involved in the interaction with *Drosophila*, with a particular focus on RIPs. In a first part, I will present a collaborative project in which *Spiroplasma* has been *in-vitro* cultured, after more than twenty years of trials. This first step allowed comparing transcription rates of *Spiroplasma* genes when the bacteria are grown in culture versus in the host, leading to the identification of putative genes required for host interaction. Such transcriptome experiment was my input in the project (Figure 9).

RIPs were found up-regulated in host-grown *Spiroplasma versus* cultured *ex vivo*. This led me to a second part where I investigated the role of RIPs towards *Drosophila* beyond their well-established role in protection against macro-parasites. I used *Drosophila* genetics to produce RIPs in uninfected flies and discovered that RIPs can target and damage the host. This study reveals that *Spiroplasma* RIPs have detrimental effects on the host. In a third part, I focused on the host response to *Spiroplasma* RIPs, and found that the stress response Heat-Shock Protein 70B is necessary to mitigate the deleterious effects of RIPs.

Chapter II:

In vitro culture of the insect endosymbiont Spiroplasma poulsonii highlights bacterial genes involved in host-symbiont interaction

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Abstract

Endosymbiotic bacteria associated with eukaryotic hosts are omnipresent in nature, particularly in insects. Studying the bacterial side of host-symbiont interactions is however often limited by the unculturability and genetic intractability of the symbionts. *Spiroplasma poulsonii* is a maternally transmitted bacterial endosymbiont that is naturally associated with several *Drosophila* species. *S. poulsonii* strongly affects its host's physiology, for example by causing male-killing or by protecting it against various parasites. Despite intense work on this model since the 1950s, attempts to cultivate endosymbiotic *Spiroplasma in vitro* have failed so far. Here, we developed a method to sustain the *in vitro* culture of *S. poulsonii* by optimizing a commercially accessible medium. We also provide a complete genome assembly, including the first sequence of a natural plasmid of an endosymbiotic *Spiroplasma* species. Last, by comparing the transcriptome of the *in vitro* culture to the transcriptome of bacteria extracted from the host, we identified genes putatively involved in host-symbiont interactions. This work provides new opportunities to study the physiology of endosymbiotic *Spiroplasma*, and paves the way to dissect insect-endosymbiont interactions with two genetically tractable partners.

Importance

The discovery of insect bacterial endosymbionts (maternally transmitted bacteria) has revolutionized the study of insects, suggesting novel strategies for their control. Most endosymbiont are strongly dependent on their host to survive, making them uncultivable in artificial systems and genetically intractable. *Spiroplasma poulsonii* is an endosymbiont of *Drosophila* that affects host metabolism, reproduction and defense against parasites. By providing the first reliable culture medium that allows a long-lasting *in vitro* culture of *Spiroplasma* and its complete genome, this work lays the foundation for the development of genetic engineering tools to dissect

endosymbiosis with two partners amenable to molecular study. Furthermore, the optimization method that we describe can be used on other yet uncultivable symbionts, opening new technical opportunities in the field of host-microbes interactions.

Key words: *Spiroplasma*, endosymbiosis, axenic medium, host-symbiont interaction

Introduction

Insects frequently maintain symbiotic relationships with vertically transmitted bacterial partners that live within their body, called endosymbionts. Some endosymbionts provide a direct benefit to the host's development and fertility by complementing its diet**.** Others grant their host with a conditional benefit that arises only in given contexts, for example by providing resistance to heat, parasites or viruses (Douglas, 2016). Deciphering the molecular dialogue that underlies host-endosymbiont interactions is thus of major importance to better understand the physiology and evolution of insects. However, functional studies are often focused on the host side, because nearly all endosymbiotic bacteria are uncultivable, and thus genetically intractable. As a consequence, the bacterial determinants that affect the interaction remain largely unknown. To date, only five endosymbionts, *Arsenophonus nasoniae (*parasitic wasp*)*, *Sodalis glossidinus* (tsetse flies), *Arsenophonus arthropodicus* (louse flies), *Serratia symbiotica* and *Hamiltonella defensa* (aphids), have been cultivated in cell-free media (Brandt, et al., 2017; Dale et al., 2006; Sabri et al., 2011; Welburn et al., 1987). Systems of co-culture with insect cell lines have also been used successfully for some endosymbionts (Kikuchi, 2009), but such techniques are delicate to set up and they do not always allow genetic engineering.

The *Spiroplasma* genus comprises diverse bacteria including commensal, pathogenic and mutualistic species, most of them being obligate associates to arthropod or plant partners (Gasparich, 2002). *Spiroplasma* cells are long, helical, and devoid of a cell wall. Extensively studied species include pathogens of crustaceans (Wang et al., 2004), insects (*e.g.* the bee pathogen *S. melliferum)* (Carle et al., 1985), and plants. Plant pathogens proliferate in phloem and are vectored by phloem-feeding insects (Saglio et al., 1973; Saillard et al., 1987; Whitcomb et al., 1986). Some, notably *Spiroplasma citri*, can be grown *in vitro* and are amenable to genetic studies. In addition to strains that are infectious and transmitted horizontally between hosts, many *Spiroplasma* are facultative inherited endosymbionts of insects (*i.e.* with trans-ovarial transmission).

Along with *Wolbachia*, *Spiroplasma* are the only known inherited symbionts of *Drosophila* (Mateos et al., 2006). By far the best-studied species is *Spiroplasma poulsonii* MSRO (*Melanogaster* Sex-Ratio Organism), which infects *D. melanogaster* and is the focus of this study. As other facultative endosymbionts, *S. poulsonii* (hereafter *Spiroplasma*) is transmitted vertically with high efficiency, causes reproductive manipulation (male-killing) and confers protection to its *Drosophila* host against parasitoid wasps (Paredes et al., 2016; Xie et al., 2011).

Taking advantage of the genetic tools available in *Drosophila*, current works have started to investigate the molecular mechanisms underlying *Drosophila-Spiroplasma* symbiosis (Harumoto et al., 2014; 2016; Herren, et al., 2013; 2014). The study of the bacterial determinants, however, has been hampered by the fact that the endosymbiont was unculturable. To expand the toolbox with which to study this endosymbiosis, we designed a method to optimize the Barbour-Stoenner-Kelly H medium (BSK-H) so it allows a sustainable *in vitro* culture of *Spiroplasma.* We also re-sequenced *S. poulsonii* MSRO genome in order to provide a complete draft of the chromosome, as well as the first complete sequence of a natural plasmid in this species. By comparing the transcriptome of the bacterium *in*

vitro and in-host, we identified genes potentially involved in the interaction with the host.

Results

Design and optimization of a culture medium for *S. poulsonii*

Unlike pathogenic *Spiroplasma* species, *S. poulsonii* has a partially degenerated genome (Paredes et al., 2015), leading to a poor adaptability to environmental changes. *In vitro*, it results in the inability of *S. poulsonii* to grow in culture media designed for pathogenic *Spiroplasma,* such as SP4 medium. We thus developed a new medium using as a starting point the commercial medium Barbour-Stoenner-Kelly H (BSK-H). This standardized complex medium has been designed for the culture of the spirochete *Borrelia burgdorferi* (Pollack et al., 1993) and is enriched in nutrients that are predicted to be required by *Spiroplasma* based on its genome. The base medium allows for survival of *Spiroplasma* for several days, but does not sustain its growth. To optimize the medium composition, we elaborated an experimental design to assess the effect of four factors on the growth: pH, partial pressure in O_2 (pO_2), fly extract supplementation (FES) and lipids supplementation (LS). Three levels for each factor were cross-tested against each other following an orthogonal array of assays in BSK-H medium (Figure 7A).

To penalize factor levels that bring in high variability, we computed a growth indicator called Contribution to Reproducible Growth (cRG; mathematical details in Material and Methods section). The analyses of cRG values predicted the best medium to be BSK-H supplemented with 7.5% of fly extract, 5% of lipids mix, at pH 7.5 and under 10% pO_2 (Figure 7B) Experimental validation of this predicted best medium, named BSK-H-spiro, yielded a sustained growth of *Spiroplasma* for 6 to 7 days before the bacterial titer reaches a plateau (Figure 7C). The growth was confirmed by microscopy observations (Figure 7D and 7E). *Spiroplasma* culture in

BSK-H-spiro could be maintained for more than a year by twofold or threefold dilution in fresh medium every week. Repeated greater dilutions progressively lead to the collapse of the culture (Supplementary figure 1).

A.

Assay	Factors			
	pO_2	pH	FES	LS
1	<1%	7	2.5%	0%
2	<1%	7.5	5%	5%
3	<1%	8	7.5%	10%
4	5%	7	5%	10%
5	5%	7.5	7.5%	0%
6	5%	8	2.5%	5%
7	10%	7	7.5%	5%
8	10%	7.5	2.5%	10%
9	10%	8	5%	0%

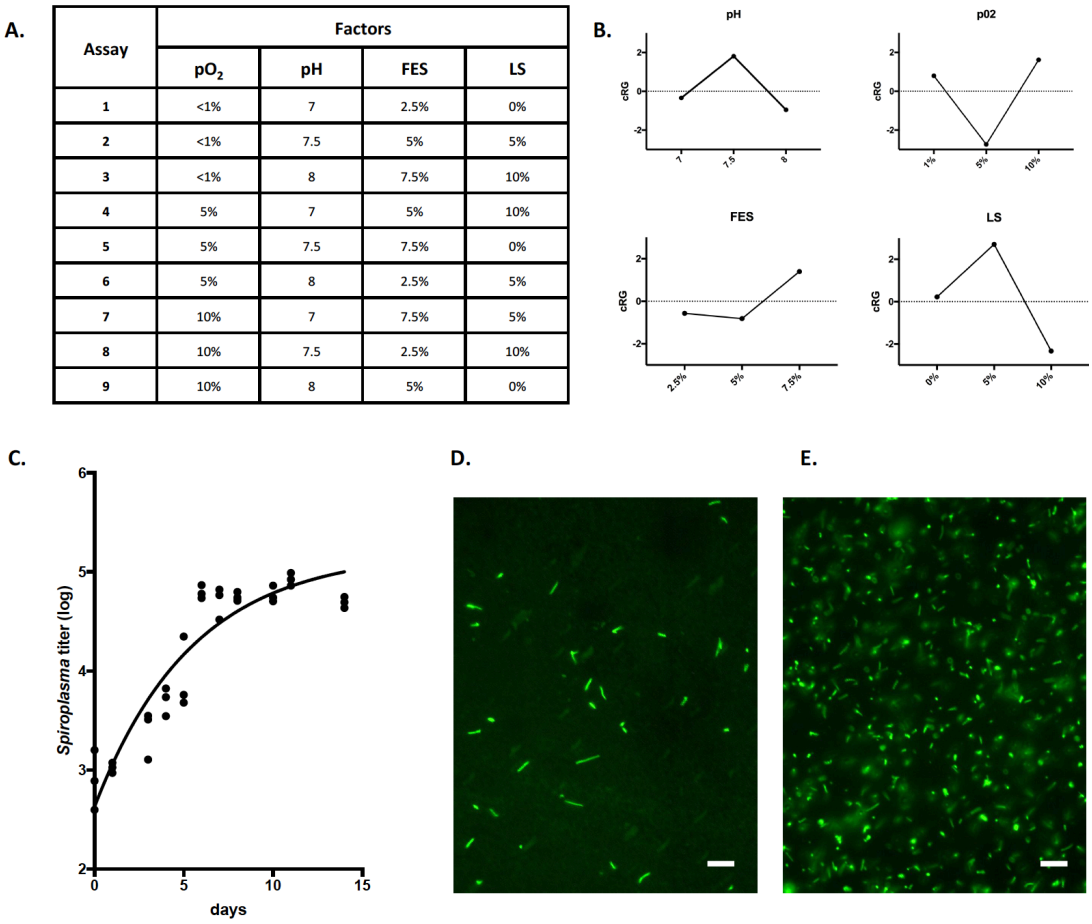

Figure 7. (A) Orthogonal matrix of assays for BSK-H-spiro optimization. Each assay has been independently repeated three times. pO2: partial pressure in O2; FES: Fly Extract Supplementation; LS: Lipids Supplementation. **(B)** cRG values computed from the assays for each factor. **(C)** Growth curve of *S. poulsonii* in BSK-H-spiro medium at 25°C under 10% O_2 and 5% CO_2. Each point represents one qPCR measurement of *Spiroplasma* titer in one repetition. The line represents a one-phase exponential fit computed on three independent repetitions. **(D)** Freshly diluted and **(E)** 2-weeks-old culture stained with Syto9. Scale bar represent 10 µm.

Interestingly, the culture can also be frozen at -80°C for more than a year and revived without adding any cryoprotectant. This singularity is likely due to their absence of cell wall that makes them more deformable, thus more resilient to freezing-induced mechanical stress.

The medium allowed a doubling time of around 30 hours with no difference between a one-month-old and a one-year-old culture. Infection of naive flies with the culture resulted in a 100% transmission success (24/24 flies) for the one-month-old culture and 96% infection success (23/24 flies) for the one-year-old, although the amount of bacteria injected (10^2/fly) is lower than the amount usually injected during hemolymph-transfer infections (10^4/fly). All culture-infected flies transmitted the bacteria to their offspring and displayed a male-killing phenotype, suggesting that a prolonged *in vitro* culture does not significantly alter the host-interaction abilities of *S. poulsonii*.

S. poulsonii genome sequence update

The *S. poulsonii* MSRO genome has been first sequenced and annotated in 2015 (Paredes et al., 2015). However, the presence of repeated sequences complicated the assembly of this draft that was covering only 93% of the estimated chromosome size. Furthermore, there was a doubt about the nature of two extrachromosomal contigs that could have been either plasmids or misassembly products. To complete the genome sequence, we took advantage of recent upgrades in PacBio technology and performed a second sequencing. The new assembly produced 8 contigs, including a large contig of 1.8 Mb corresponding to the full circular chromosome of *Spiroplasma.* 2217 coding sequences were identified, of which 1865 are identical to the first assembly prediction (Figure 8A). Seven smaller contigs were also produced, of which one (contig #7) could be circularized. We aligned this contig to the reference sequences of plasmids from *S. citri* and *S. kunkelii*, for which plasmids have been well characterized

(Supplementary figure 2). The alignment revealed two conserved synteny blocks between contig #7 and the references. We also detected a coding sequence with 87% homology to the sequence pE, proven to code for a plasmid replication protein on *S. citri* plasmids (Breton et al., 2008). The presence of those genes on the circular sequence of contig #7 strongly suggests that it is the first full sequence of a plasmid in the group IV of *Spiroplasma*, to which *S. poulsonii* belongs, and hereafter called pSMSRO (Figure 8B). The analysis of the remaining extrachromosomal contigs did not allow circularizing any of them, or detecting any conserved genes with other *Spiroplasma* plasmids, although we cannot exclude that other plasmids were present but not detected in the sequencing data.

A.

	MSRO v1	MSRO v2
Total number of contigs	12	8
Number of chromosomal contigs	10	1
Combined size of chromosomal contigs (bp)	1'757'846	1'883'005
Estimated coverage (%)	93	100
G+C content (%)	26.5	26.4
Coding density (%)	76.6	82.2
Chromosomal CDS	1'894	2'217
CDS transferred from v1	-	1'865
Extra copies of v1 CDS	-	192
Novel in gaps of v1	-	156

B.

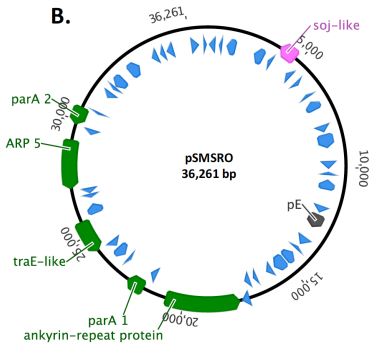

Figure 8. (A) Comparison between the first draft genome of *S. poulsonii* MSRO (Paredes et al., 2015) and this work. CDS: coding sequence. **(B)** Graphic map of contig #7 after circularization (plasmid pSMSRO). Blue unnamed arrows are hypothetical protein coding sequences without annotation. Green arrows are annotated genes. The pink arrow indicates a pseudogene and the gray arrow indicates the coding sequence of the *Spiroplasma* plasmid replication protein pE.

This new draft confirmed the metabolic landscape already described in 2015 (Paredes *et al*. 2015), with no obvious difference regarding the presence or absence of metabolic genes. However, the full coverage and the extended annotation of the new draft allowed establishing a comprehensive list of *Spiroplasma* virulence factors (Table 1). Five virulence factors were initially

reported in the first genome: two Spiralins (A and B), a chitinase, a cardiolipin synthase and a glycerol-3-phosphate oxidase. Spiralin A is found in other *Spiroplasma* species including *S. citri*, while Spiralin B is found only in *S. poulsonii* (Paredes et al., 2015). We found a third gene coding for a Spiralin-like protein, Spiralin C, which shares only 15% homology with SpiA and SpiB but has a conserved Spiralin domain. We also identified a group of five genes coding for Adhesion Related Proteins (ARPs) that were already present in the first draft but misannotated. Intriguingly, these include one sequence located on pSMSRO, but also four sequences located on the chromosome, while all *S. citri* ARPs are extrachromosomal (Saillard et al., 2008). A sixth chromosomal ARP pseudogenized by an insertion sequence was identified, as well as two shorter genes partially homologous to ARPs. We also identified a gene containing a *Clostridium* epsilon toxin (Etx) conserved domain. Etx are major toxins of *Clostridium perfringens* and cause a variety of symptoms in mammals, including brain damage (Stiles et al., 2013).

FAMILY	NAME	GENE ID	GenBank locus tag	CONTIG	COORDINATES	SIGNAL PEPTIDE	TM DOMAIN	PREDICTED ADRESSING	REFERENCE
Spiralins	SpiA	MSRO_01314	SMSRO_SF013140	1	1005468:1004767	Yes	No	Membrane	a
	SpiB	MSRO_00966	SMSRO_SF009660	1	753920:754717	Yes	No	Membrane	a
	SpiC	MSRO_01589	SMSRO_SF015890	1	1203572:1204045	No	No	Unknown	c
Adhesion Related Proteins	SpARP1	MSRO_00252	SMSRO_SF002520	1	205842:206939	Yes	1	Membrane	c
	SpARP2	MSRO_01185	SMSRO_SF011850	1	908030:909277	Yes	Unsure	Membrane	c
	SpARP3	MSRO_02268	SMSRO_SF022680	1	1731722:1730625	Yes	1	Membrane	c
	SpARP4	MSRO_02445	SMSRO_SF024450	1	1870575:1871513	Yes	1	Membrane	c
	SpARP5	MSRO_03049	SMSRO_SFP00390	7	12713:12147	Yes	1	Membrane	d
Metabolic genes	Cls	MSRO_00101	SMSRO_SF001010	1	81414:82952	No	3	Membrane	a
	ChiD1	MSRO_00845	SMSRO_SF008450	1	671704:672774	Yes	No	Secreted	a
	ChiD2	MSRO_01311	SMSRO_SF013110	1	1002344:1002357	pseudogenised			c
	GlpO	MSRO_01844	SMSRO_SF018440	1	1400479:1401657	No	No	Cytosol	a
Toxins	RIP1	MSRO_01653	SMSRO_SF016530	1	1253115:1254512	Yes	No	Secreted	b
	RIP2	MSRO_01882	SMSRO_SF018820	1	1438476:1439966	Yes	No	Secreted	b
	RIP3	MSRO_02388	SMSRO_SF023880	1	1820456:1821802	Yes	No	Secreted	b
	RIP4	MSRO_02072	SMSRO_SF020720	1	1584448:1585794	Yes	No	Secreted	b
	RIP5	MSRO_00366	SMSRO_SF003660	1	293319:294665	Yes	No	Secreted	b
	ETX-like	MSRO_02161	SMSRO_SF021610	1	Gene structure unclear				c
	Ankyrin repeat	MSRO_03010	SMSRO_SFP00290	7	6975:9461	Yes	No	Secreted	d

Table 1. Virulence factors of *S. poulsonii*. References indicate that (a) the gene has been detected and annotated by Paredes *et al.* 2015; (b) the gene has been detected by Paredes *et al.* 2015 and annotated and discussed by Hamilton *et al.* 2016; (c) the gene has been detected by Paredes *et al.* 2015 but not annotated or/and not discussed; (d) the gene has been detected and annotated in this work for the first time.

Our genome analysis confirms the presence of five sequences coding for Ribosome-Inactivating Proteins (RIPs), that were initially identified by Hamilton *et al.* (2016) (Hamilton et al., 2016). Last, the plasmid bears a coding sequence for an ankyrin-repeat protein (Ank). Ank repeats are found in many virulence effector proteins (Al-Khodor et al., 2010). Remarkably, they are widely found in the genome of *Wolbachia*, another widespread endosymbiont that manipulate insects reproduction, where their large number and diversity suggest that they could play a crucial role in host-symbiont interactions (Siozios et al., 2013).

Transcriptome analysis of *S. poulsonii* in culture versus in host

To detect genes involved in *Spiroplasma* interaction with its host, we compared the transcriptome of *Spiroplasma* collected from *Drosophila* hemolymph to the transcriptome of 2 months *in vitro* cultured *Spiroplasma*. This reference transcriptome produced by pooling transcripts detected in both conditions contains 1491 transcripts. 97.18 % of the reads mapped to the chromosome and 1.74 % to contig #7, while no significant signal was detected for any other extrachromosomal contig. This supports the hypothesis that contig #7 is a plasmid of *Spiroplasma* while other extrachromosomal contigs are misassembly products. The most expressed gene in all conditions is *spiB*, followed by housekeeping genes related to cell division, transcription and translation.

Pairwise comparison between the two experimental conditions identified 465 genes differentially expressed, 201 of them being more transcribed in the host while 264 were significantly more expressed in the culture (Figure 9A). 258 (55%) of the differentially expressed genes were only annotated as “hypothetical proteins” and were not further accounted for in the analysis. Genes that were identified by homology but whose function was very general or unclear were grouped in the category “others”. The remaining sequences have been manually clustered

according to their predicted function (Figure 9B). A majority of identified genes that were found differentially expressed was associated to metabolic pathways and metabolite transport, probably as a consequence of differences between the composition of the medium and the fly hemolymph. Aside from this, a large cluster of genes was related to ribosome assembly and translation, including ribosome structural proteins, tRNA ligases and translation regulators. Some members of this cluster were found up-regulated in the culture while others were up-regulated in the fly, suggesting that the switch of environment triggers a qualitative change in the translational activity of the bacterium. The *in vitro* culture dataset also showed enrichment in transcripts related to DNA replication and cell division, consistent with a doubling time of around 30 hours *in vitro* versus 170 hours in the adult fly (Herren and Lemaitre, 2011).

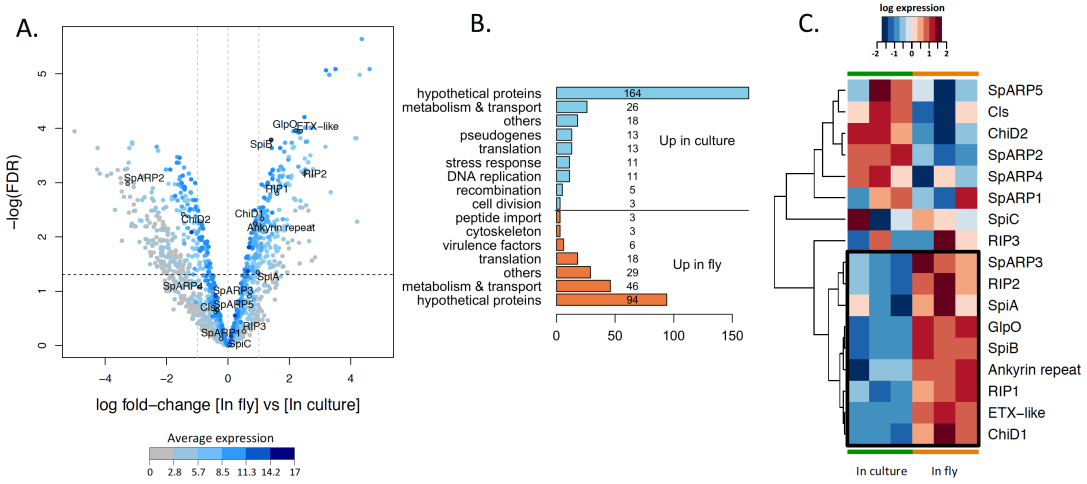

Figure 9. (A) Volcano plot of differential gene expression of *S. poulsonii* in host *versus* in culture. Each point represents the average value of one transcript within three repetitions. The expression difference is considered significant for a log2 fold-change ≥ 1 (outer dotted vertical lines) and for a p-value ≤ 0.05 (-log [FDR] ≥ 1.3, large dotted horizontal line). Points are colored according to their average expression in all datasets. Names and outlined points represent virulence factors. **(B)** Manual clustering of the transcripts differentially expressed by *S. poulsonii* in the fly versus in the culture. The number of sequences in each category is indicated on the bars. **(C)** Heatmap of *S. poulsonii* virulence gene expression. Each column

represents one repetition of the in-culture or in-fly dataset. Colors represent the log10 of the expression level in the corresponding repetition. black box framed by a black line highlights the cluster of genes that is induced when *S. poulsonii* is in the host compared to *in vitro*.

Interestingly, transcripts involved in DNA recombination were also enriched in *Spiroplasma* grown in culture, including *ruvA*, *ruvB*, *recR* and *recU*, as well as gene belonging to the *comEC* family. The comE operon contributes to the natural competence in *Bacillus subtilis,* ensuring the binding and uptake of transforming DNA (Hahn et al., 1993), which suggests that *S. poulsonii* might be naturally competent.

Last, several differentially expressed sequences were identified as pseudogenes resulting either from a frameshift mutation or from the insertion of a mobile element in the coding sequence that causes the protein to be truncated. The active transcriptional regulation of these genes suggests a recent pseudogenization, possibly as a consequence of *S. poulsonii* switching from a free-living to an endosymbiotic lifestyle.

Virulence factors could be classified in two clusters depending on their expression profile (Figure 9C). *spiA*, *spiB*, *RIP 1* and *2*, *ank*, *etx*, *glpO* and *chiD1* have a lower expression level in culture compared the host, pointing to their role in host-symbiont interaction. Other virulence genes do not display a significant change in their expression level and have a low average expression. Such genes include *spiC*, *RIP3*, *ARP 1*, *4* and *5*, *chiD2* and the *cardiolipin synthase*. *ARP2* is the only virulence gene that is up-regulated in culture compared to fly, and *RIP4* and *5* as well as *ARP3* are not detected at all in the transcriptome, implying that they might be pseudogenes resulting of a duplication of the coding sequence without the regulatory upstream sequence.

Finally, a gene encoding a ferritin, a protein involved in iron sequestration, was more expressed in *Spiroplasma* extracted from *Drosophila* compared to culture, suggesting that iron availability could be a proliferation-limiting factor along with lipids and glucose availability (Herren et al., 2014; Paredes et al., 2015).

Discussion

We developed the first reliable method to culture endosymbiotic *Spiroplasma* from *Drosophila* in a cell-free medium. This is an important step forward because cultivation is a prerequisite for addressing functional questions on the regulation of this symbiosis *via* genetic manipulation of the bacterial partner. While pathogenic *Spiroplasma*, such as *S*. *citri*, can be easily cultivated in standard growth media, no suitable medium was available to grow any endosymbiotic *Spiroplasma* outside of their hosts. This was not for a lack of trying: much work was done in the 1980s to attempt to set up a culture medium for *S*. *poulsonii*, until one paper reported in 1986 the successful cultivation of a *Drosophila Spiroplasma* in a cell free medium (Hackett et al., 1986). According to the authors, the critical factor was to supply *Spiroplasma* with growing insect cells in the course of primary isolation, followed by a succession of passages that allowed for *Spiroplasma* to adapt to an insect cell free medium. Unfortunately, this work could not be repeated despite several attempts from various laboratories. *Spiroplasma* thus remained uncultivable in practice.

An important difference between previous attempts and the present work is that crucial requirements of the symbiont were addressed based on very recent discoveries about *S. poulsonii* physiology. The need of *Spiroplasma* for host lipids to synthesize its membrane, for example, has been demonstrated only a few years ago (Herren et al., 2014; Paredes et al., 2015) and lipids supplementation turned out to be crucial to promote the growth *in vitro*. Another point was the supplementation of the medium with fly extract, which was also not undertaken in

previous works. Since the unsupplemented medium already contains glucose, essential amino-acids, lipids and vitamins needed by the bacteria, we assume that the growth improvement observed with fly extract supplementation might come either from a fly hormone or neurotransmitter, or from a non-organic growth factor available in the fly. A possible candidate would be iron, as suggested by the over-expression of a ferritin-like coding gene by *S. poulsonii in vitro*, or another metal ion, not present in sufficient quantities in the BSK-H base medium (Williamson et al., 1983). Importantly, starting with a high density of bacteria seems to be a key point for the successful establishment of the culture. Initiating with a too small amount of infected hemolymph does not allow the culture to thrive, and repeated strong dilutions lead to a collapse of the culture. This suggests the existence of a density threshold below which *Spiroplasma* growth is inhibited. The exact mechanism leading to this inhibition remains however elusive, as the genome analysis did not highlight any *quorum* sensing system.

This work also allowed a first comparison between the *in vivo* and the *in vitro* transcriptome of *S. poulsonii*, identifying genes that are overexpressed when the bacterium is in contact with its host. Membrane proteins are particularly interesting as they are associated with host infection in pathogenic *Spiroplasma*. *S. citri* Spiralin for example acts as a lectin and binds to insect host's glycoproteins to invade cells (Duret et al., 2014; Killiny et al., 2005). Its expression is down-regulated when the bacterium is in its plant hosts compared to *in vitro* culture, while it is not altered between in-insect and in-culture (Dubrana et al., 2016). In *S. poulsonii*, *spiA*, the closest homologue to *S. citri spiralin*, is only slightly up-regulated in the insects while *spiB*, only found in *S. poulsonii*, is strongly up-regulated. This points to a function of SpiB that is specific to endosymbiosis, possibly related to the bacterial entry in the oocyte during vertical transmission. ARPs are also major lipoproteins involved in *S. citri* transmission (Berho et al., 2006; Béven et al., 2012), turning out to be essential for insect cell invasion but nonessential for transmission from insect

to plants (Breton et al., 2010). In *S. poulsonii*, few predicted ARPs have a complete and functional sequence, and their expression is not differentially regulated *in vitro* compared to in-host. *S. citri*, as a strictly horizontally transmitted pathogen, could require diverse ARPs to infect new hosts efficiently. *S. poulsonii* on the other hand is mostly vertically transmitted, although horizontal transmission to new host is possible notably *via* ectoparasite vectors (Haselkorn et al., 2009; Jaenike et al., 2007). ARPs in this species could thus be less diverse because of its more limited host range. The chromosomal location of most *S. poulsonii* ARPs (rather than extrachromosomal as in *S. citri* (Saillard et al., 2008) also reflects a lesser ability of these genes to be horizontally transferred. This could reflect the fixation of this gene family during the co-evolution of vertically transmitted *Spiroplasma* with its host.

Several genes coding for toxins are overexpressed in the host compared to *in vitro*, including Ribosome-Inactivating Proteins and two yet uncharacterized toxins (Ank and Etx). RIPs are involved in the protection of *Drosophila* against nematodes and parasitoid wasps by selectively inactivating the 28S ribosomal RNA of the parasites (Ballinger and Perlman, 2017; Hamilton et al., 2016). The up-regulation of *RIP1* and *RIP2* when *S. poulsonii* is in-host compared to *in vitro* suggests that RIPs could have a function in host-symbiont interactions, regardless of parasite infections, possibly in male-killing. Etx might be involved in the neuronal symptoms, notably tremors and dopaminergic neurons degeneration observed in *Spiroplasma*-infected flies when old (Herren and Lemaitre, 2011). We also cannot exclude that it functions as a defensive agent against parasites, which would explain the transcription increase when S*. poulsonii* is in the insect compared to *in vitro,* although the toxicity of Etx has not yet been investigated on non-mammal models. Further functional studies will be necessary to assess the exact function of these toxins in the *S. poulsonii-Drosophila* interaction.

It is noteworthy that 2 out of 18 identified virulence genes (*ARP5* and *ank*) are located on a plasmid, which indicates that extrachromosomal DNA may play an important role in *S. poulsonii-Drosophila* interactions. Variability in plasmid presence and/or copy number in *S. poulsonii* strains could be accountable for variability in the phenotypes caused to the hosts, including variable male-killing penetrance.

In conclusion, the method described in this work is the first protocol to allow cultivating an endosymbiotic *Spiroplasma* in a cell-free medium in almost thirty years. The technical approach that was used to design the BSK-H-spiro medium can be adapted to optimize a medium for other uncultivable bacteria, for which a favorable physico-chemical environment can be partially predicted. The expression of *comE* indicates that *S. poulsonii* might be naturally competent and the transcriptional regulations observed with recombination-related genes suggests that knock-out mutants by homologous recombination might be possible despite *recA* pseudogenisation (Paredes et al., 2015), as in pathogenic *Spiroplasma* species (Lartigue et al., 2002; McCammon et al., 1990). Eventually, several bacterial genes were predicted to have a key function in the interaction between *S. poulsonii* and its host, including virulence factors. These genes are thus priority candidates for further investigation upon the development of genetic tools to modify *S. poulsonii*, in order to unravel their precise function. Coupled with the powerful genetic tools available on the *Drosophila* side, the development of genetic tools to modify *S. poulsonii* will be a major achievement in the field of symbiosis, as it will provide the first insect model where both the host and the endosymbiont are readily transformable.

Material and Methods

Spiroplasma stock

We used a wild-type Oregon-R (OR^R) fly stock that has been cured from *Wolbachia* by antibiotics treatment and infected by the *S. poulsonii* MSRO strain Uganda (Herren and Lemaitre, 2011; Pool et al., 2006). The stock has been maintained in the lab for several years between these treatments and the experiments.

Cell-free culture medium design

The medium basis was the Barbour-Stoenner-Kelly H medium (BSK-H) without L-Glutamine from BioSell (Feucht bei Nürnberg, Germany). BSK-H from Sigma has also been used successfully. The design of an orthogonal array of growth assays has been based on the choice of four factors (pH, partial pressure in oxygen (pO_2), fly extract supplementation (FES) and lipids supplementation (LS) that were *a priori* expected to affect *Spiroplasma* growth significantly. The use of an orthogonal array allows the extraction of relevant information from a reduced number of factor level combinations rather that from all possible combinations. For each factor, three levels were arbitrarily chosen around an expected optimal value (*e.g.* pH 7.5 as expected optimal value, 7 - 7.5 - 8 as tested levels). Cultures were started from hemolymph extracted from the thorax of one-week-old infected flies by aspiration with a nano-injector Nanoject II (Drummond Scientific). A preculture was launched one week prior to the experiment by adding 8 µL of hemolymph to 3.2 mL of BSK-H + 5% fly extract (1000 to 5000 bacteria/µL), without agitation. Aliquots of 100 µL of preculture were then frozen at -80°C before use. For each assay, aliquots were centrifuged for 40 minutes at 2'000 rcf at 18°C, and pellets were resuspended in 200 µL of medium. 10 µL aliquots were taken one day and seven days later for growth assessment by quantitative PCR. A linear regression on the log-transformed measures between day 1 and day 7 was computed for each level of each tested

factor and the slope of the regression was used as a growth rate measurement. Three independent replicates were made for each medium testing. Since some combinations of factors yielded a high variability in the growth, we analyzed the data with a statistical approach inspired from the Taguchi method (Rao et al., 2008; Taguchi, 1991). To penalize factor levels that entail a high variability in the growth, a "reproducible growth" parameter was computed, RG = $10\times\log(\bar{S}^2) - 10\times\log\left(1+3\times(\sigma/\bar{S})^2\right)$ with $\bar{S}$ the average slope with the considered level of the considered factor and σ the standard deviation. The contribution of one level to the reproducible growth (cRG) was calculated as cRG = $RG_{considered\ level} - RG_{all\ levels}$. For each factor, the level with the highest cRG was selected as the optimal value. An experimental validation was then performed in a medium bringing together the best levels for each of the four factors, hereafter designed as BSK-H-spiro, following the same protocol as for the optimization assays. Three independent replicates were made for the validation assay. Protocols for preparing the fly extract, the lipid mix and the BSK-H-spiro are detailed in Supplementary Data 1. The media of freshly started cultures were completely renewed every three to four passages until at least the twelfth passage, by centrifugation for 20 minutes at 12'000 rcf at 18°C and replacement of the used medium (supernatant) by an equivalent amount of fresh medium. These replacements are a necessary adaptation step that becomes needless for older cultures. Long-lasting cultures were maintained by a weekly 3-fold dilution in fresh culture medium. All experiments and cultures were performed at 25°C, which is the temperature at which *Drosophila* infected stocks are routinely maintained, and yet close to the 26°C predicted optimal temperature for *S. poulsonii* (Williamson et al., 1999).

Culture density measurement

For DNA extraction for quantitative PCR, bacteria were lysed by osmotic shock by adding 400 µL of distilled water to 10 µL of culture and heated at 95°C for 15 minutes. This simple method insures an efficient yield from a small amount of

initial bacterial material. DNA was then used for quantitative PCR as described before (Anbutsu and Fukatsu, 2003) with primers amplifying a 300 bp fragment of the 16S rRNA gene (for 5'-TACATGCAAGTCGAACGGGG-3' ; rev 5'-CTACTGCTGCCTCCCGTAG-3'). Microscopic observation was performed as previously described (Herren and Lemaitre, 2011).

Fly infections

One-week-old female Oregon flies were infected from the *Spiroplasma* culture by an injection of 23 nL of a dense culture (one week after the latest dilution with fresh medium) with a nano-injector Nanoject II (Drummond Scientific). Flies were allowed to recover from the injection in a tube with fresh medium in absence of males for one day. Flies were then coupled with a male and each couple was isolated in a tube in order to monitor the infection status of the progeny of single-flies. Couples were flipped onto fresh medium every two or three days. The eggs laid for the first week following mating were discarded. The progeny was screened for *Spiroplasma* infection one week after hatching by Syto9 staining as previously described (Herren and Lemaitre, 2011).

Genome sequencing and analysis

Spiroplasma DNA was extracted from fly hemolymph as previously described (Paredes et al., 2015). Processing of the samples was performed in the University of Lausanne Genomic Technologies Facility. The DNA was sheared in a Covaris g-TUBE (Covaris, Woburn, MA, USA) to obtain 20 kb fragments. After shearing the DNA size distribution was checked on a Fragment Analyzer (Advanced Analytical Technologies, Ames, IA, USA). 5 µg of the sheared DNA was used to prepare a SMRTbell library with the PacBio SMRTbell Template Prep Kit 1 (Pacific Biosciences, Menlo Park, CA, USA) according to the manufacturer's recommendations. The resulting library was size selected on a BluePippin system (Sage Science, Inc. Beverly, MA, USA) for molecules larger than 20 kb. The recovered library was

sequenced on one SMRT cell with P6/C4 chemistry and MagBeads on a PacBio RSII system (Pacific Biosciences, Menlo Park, CA, USA) at 240 min movie length. Assembly was performed with HGAP (hierarchical genome assembly process) version 2 from the PacBio smrtpipe (v2.3.0). Circularization of main contig #1 was performed using Amos (v3.1.0, Amos consortium, http://amos.sourceforge.net). Plasmid contig #7 was refined using the PacBio read data from Paredes et al. 2015 with Quiver version 1. Genome annotation was performed with Prokka v 1.11 (Seemann, 2014) using parameters --addgenes --genus *Spiroplasma* --species poulsonii --gcode 4 --rawproduct –rfam --rnammer). Nucmer tool (Mummer suite v3.23 (Kurtz et al., 2004)) was used to align CDS from annotation version JTLV01000000 to the new annotation. Some gene product annotations were refined using NCBI PSI-blast tool. Multiple alignments of *Spiroplasma* plasmids have been performed using MAUVE version 2.4.0 (Darling, et al., 2004).

RNA sequencing and analysis

RNA was extracted from (i) the hemolymph of 30 one-week-old infected flies and (ii) 20 mL of four-month-old *in vitro* culture pelleted by 30 minutes of centrifugation at 16 000 g by TRIzol method following manufacturer's instructions. Three independent replicates were prepared for each condition. Libraries were prepared using the Illumina Truseq RNA kit and sequenced on Illumina HiSeq 2000 in the University of Lausanne Genomic Technologies Facility. Purity-filtered reads were adapters and quality trimmed with Cutadapt v.1.8 (Martin, 2011). Reads matching to ribosomal RNA sequences were removed with fastq_screen v. 0.9.3 (Babraham Bioinformatics.) http://www.bioinformatics.babraham.ac.uk/projects/fastq_screen/). Remaining reads were further filtered for low complexity with reaper v. 15-065 (Mattew, et al., 2013). Reads were aligned against the *Spiroplasma poulsonii MSRO (v2)* genome using STAR v. 2.5.2b (Dobin et al., 2013). The number of read counts per gene locus was summarized with htseq-count v. 0.6.1 (Anders et al., 2015)

using *Spiroplasma poulsonii MSRO (v2)* gene annotation. Quality of the RNA-seq data alignment was assessed using RSeQC v. 2.3.7 (Wang et al., 2012). Statistical analysis was performed for genes in R version 3.4.1 (R Core Team, 2016). Genes with low counts were filtered out according to the rule of 1 count per million (cpm) in at least 1 sample. rRNA and tRNA genes counts were discarded. Library sizes were scaled using TMM normalization with EdgeR package version 3.18.1 (Robinson et al., 2010) and log-CPM transformed with limma voom function, limma package version 3.32.5 (Law et al., 2014). Differential expression was computed with limma (Ritchie et al., 2015) by fitting the samples into a linear model and performing "in-fly" versus "in-culture" comparison. Moderated t-test was used and adjusted p-value computed by the Benjamini-Hochberg method, controlling for false discovery rate.

Accession numbers

Genome under DDBJ/EMBL/GenBank accession no. JTLV00000000 was updated. The version described in this paper is version JTLV02000000. The RNAseq differential expression analysis can be found in Supplementary Data 2**.**

Acknowledgments

We thank Emmanuel Beaudoing and Chloé Jollivet for technical help regarding the transcriptome assembly and the culture maintenance, respectively. We also thank Élodie Ramond and Samuel Rommelaere for constructive discussions and Christoph Vorburger and Aurélien Vigneron for the critical review of the manuscript. This work was funded by ERC advanced grant no. 339970 and the SNF Sinergia grant no. CRSII3_154396.

Chapter III:

Contribution of Spiroplasma poulsonii RIP toxins to male-killing and life span shortening phenotypes in Drosophila melanogaster

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Background: Insects frequently live in close relationship with mutualistic symbiotic bacteria that carry out beneficial functions for their host, like protection against parasites and viruses. However, in some cases, the mutualistic nature of such associations is put into question because of detrimental phenotypes caused by the symbiont. One example is the association between the vertically transmitted facultative endosymbiont *Spiroplasma poulsonii* and its natural host *Drosophila melanogaster.* Whereas *S. poulsonii* protects its host against parasitoid wasps and nematodes by the action of toxins from the family of Ribosome Inactivating Proteins (RIPs), the presence of *S. poulsonii* has been reported to reduce host's life span and to kill male embryos by a toxin called *Spaid*. In this work, we investigate whether *Spiroplasma* RIPs have harmful effects on *Drosophila* in the absence of parasite infection.

Results: We show that only two *Spiroplasma* RIPs (*SpRIP1* and *SpRIP2*) among the five RIP genes encoded in the *S. poulsonii* genome are expressed during all the *Drosophila* life cycle. Over-expression of *SpRIP1* and *2* in uninfected flies confirms their toxicity, as indicated by a reduction of *Drosophila* lifespan and hemocyte number. We also show that expression of *SpRIPs* during embryogenesis leads to a female-biased sex-ratio in the surviving individuals. Accordingly, the ectopic expression of *SpRIPs* in uninfected embryos causes apoptosis and neural defects, as observed during bacteria-induced male-killing.

Conclusion: Our results indicate that RIPs released by *S. poulsonii* contribute to the reduction of host lifespan and could enhance male-killing. This suggests that *SpRIPs* may have important function in insect-symbiont relationships beyond protection against parasites.

Keywords: *Spiroplasma*, endosymbiosis, Ribosome Inactivating Protein, male-killing, *Drosophila.*

Introduction

Endosymbiosis refers to a persistent interaction between two partners, generally a eukaryotic host and a microbial symbiont that lives within the host's body. Such interactions are particularly frequent in insects, of which more than half of species are estimated to harbor at least one endosymbiont (Ishikawa, 2003; Kikuchi, 2009). Insect endosymbionts can affect their host in multiple ways, including beneficial effects such as metabolic complementation, heat tolerance or protection against viruses and parasites (Dunbar et al., 2007; Ferrari and Vavre, 2011; Montllor et al., 2002; Teixeira et al., 2008; Vigneron et al., 2018). However some endosymbiotic associations can also have detrimental consequences for the insect fitness, such as a decreased lifespan or fertility (Ciche et al., 2006; Raquel and David, 2016). Among the most widespread facultative endosymbionts that manipulate insect reproduction, are the genera *Wolbachia* and *Spiroplasma* (Hamilton and Perlman, 2013).

Spiroplasma poulsonii (hereafter *Spiroplasma*) is a natural symbiont of the fruit fly *Drosophila melanogaster* (Anbutsu and Fukatsu, 2011; Counce and Poulson, 1962; Mateos et al., 2006). It lives extracellularly in the fly hemolymph and gets vertically transmitted by trans-ovarial transfer. *Spiroplasma* colonizes the germline during vitellogenesis by co-opting the yolk transport and internalization machinery (Herren et al., 2013). Intriguingly, it completely lacks a cell-wall and thus immunogenic surface molecules, such as peptidoglycan, which renders it invisible for the host immune system (Herren et al., 2014; Herren and Lemaitre, 2011; Hurst et al., 2003; Lemaitre et al., 1996; Lemaitre and Hoffmann, 2007). In adult flies, *Spiroplasma* grows over time reaching a titer of 10^5-10^6 bacteria per µl of hemolymph (Masson et al., 2018). *Spiroplasma* infection shortens the life-span of *Drosophila,* suggesting that either the bacteria causes damages only at high titer, or the damages take time to kill the host (Herren et al., 2014). Interestingly, the

growth of *S. poulsonii* is limited by the availability of host lipids, preventing its overgrowth in condition of nutrient scarcity (Herren et al., 2014).

One of the most striking phenotypes caused by *Spiroplasma* is male-killing, whereby infected male embryos die during their development while infected females survive (Counce and Poulson, 1962). As *Spiroplasma* is only transmitted by female flies, male-killing is thought to favor the spread of the bacteria among host natural populations (Hurst and Frost, 2015). Recently, a *Spiroplasma* toxin containing Ankyrin-repeats, named *Spiroplasma* Androcidin (*Spaid*), has been described as a crucial male-killing agent (Harumoto and Lemaitre, 2018; Oishi, 1971). Heterologous expression of *Spaid* in uninfected flies is sufficient to kill males. Moreover, its expression during early embryogenesis induces DNA-damage-dependent apoptosis and defective neurogenesis in uninfected male embryos, which fully recapitulates male-killing phenotypes (Harumoto et al., 2014; 2016; Martin et al., 2013). *Spaid* mediates male-killing by targeting the dosage compensation machinery, a male-specific ribonucleoprotein complex that hyper-transcribes the male X chromosome (Cline and Meyer, 1996). It is, however, unclear whether *Spaid* is the only toxin coded by *Spiroplasma* that kill males (Harumoto and Lemaitre, 2018).

Studies have shown that in some contexts, *Spiroplasma* can also provide a benefit to its host as they mediate protection against parasitoid wasps and nematodes in several *Drosophila* species (Ballinger and Perlman, 2017; Hamilton et al., 2016; Paredes et al., 2016; Xie et al., 2011). Protection is a major ecological benefit that can lead to a fast spreading of *Spiroplasma* in wild populations (Jaenike et al., 2010). Two complementary mechanisms have been implicated in *Spiroplasma* protection against parasites: a metabolic competition for host lipids between *Spiroplasma* and the parasites, and *Spiroplasma* production of Ribosome-Inactivating Proteins (RIPs) that damage ribosomes of both wasp eggs and nematodes (Ballinger and Perlman, 2017; Hamilton et al., 2016; Paredes et al.,

2016). RIPs are found in plants and bacteria, where they act as a defense against eukaryotic parasites (Ballinger and Perlman, 2017; Endo and Tsurugi, 1986; Hamilton et al., 2016; Lainhart et al., 2009; Virgilio et al., 2010). They recognize a conserved region of the 28S ribosomal RNA called the Sarcin-Ricin Loop (SRL). The secondary structure of the SRL consists in a hairpin loop displaying an adenine that is necessary for protein synthesis (Bergan et al., 2012). RIPs cleave the central adenine from the SRL in a process called depurination, thus blocking protein synthesis (Szewczak and Moore, 1995).

In this article we investigated the role of RIPs produced by the facultative endosymbiont *S. poulsonii* (hereafter *Sp*RIPs) in its natural host *D. melanogaster.* Similarly to the reduced life span observed in infected flies, we show that heterologous expression of *SpRIPs* coding genes in uninfected flies shortens their life span. Furthermore, we observed that uninfected *D. melanogaster* embryos expressing *SpRIPs* have a high mortality rate and a female-biased sex-ratio among the surviving individuals (70% females), indicating that males are more sensitive to the toxin. Embryonic death caused by *Sp*RIP*s* is associated with an increase of apoptosis and neural defects, which partially phenocopies *Spiroplasma*-induced male-killing. These results suggest that *SpRIPs* could enhance *Spaid* -induced male-killing and highlight the versatile effects of symbiont-produced toxin on host insect physiology and reproduction.

Results

*Sp*RIPs depurinate the 28S rRNA of *D. melanogaster*

S. poulsonii genome contains five genes encoding RIPs (*SpRIP1-5*) (Ballinger and Perlman, 2017; Hamilton et al., 2016; Masson et al., 2018; Paredes et al., 2015). All of them have a signal peptide, suggesting a secretion of the mature protein, and a conserved N-glycosidase domain in charge of the depurination reaction (Ballinger and Perlman, 2017; Hamilton et al., 2016). Transcriptomics analysis has shown that

only two of them, *SpRIP1* and *SpRIP2*, are expressed *in vivo* and *in vitro*, pointing to a possible pseudogenization of *SpRIP3*, *4* and *5* (Ballinger and Perlman, 2017; Masson et al., 2018).

To confirm the expression pattern of *SpRIPs* in infected flies, we performed RT-qPCR analysis on each of the *SpRIPs*. We confirmed that only *SpRIP1* and *SpRIP2* are expressed by *S. poulsonii* in *D. melanogaster* with no significant changes in expression level along the fly life cycle (Figure 10A and 10B). *SpRIP 3*, *4* and *5* transcripts were not detected, reasserting that are not expressed (data not shown). We then measured RIP activity using a RT-qPCR assay. This assay relies on the ability of reverse transcriptases to incorporate a thymine in complementary DNA in place of the void position present on the depurinated RNA molecule. It is then possible to design primers that bind specifically to the intact cDNA (containing an adenine) or to the depurinated one (containing a thymine) (Hamilton et al., 2016). Comparisons between infected and uninfected flies confirmed that *S. poulsonii* depurinates the 28S rRNA of *Drosophila,* as previously shown for larvae and one week old adult flies (Ballinger and Perlman, 2017). Monitoring RIP activity all along the whole *Drosophila* lifecycle revealed particularly high levels of depurination in embryos and old adult flies (Figure 10C). A control assay using primers amplifying fragments outside of the SRL show that the total number of 28 rRNA transcripts is the same between infected and uninfected flies (Figure 10D). As the level of expression of *SpRIP* is constant, the high level of depurination in embryos and old adult flies likely results from a higher ratio of *Spiroplasma* over host tissues at these stages.

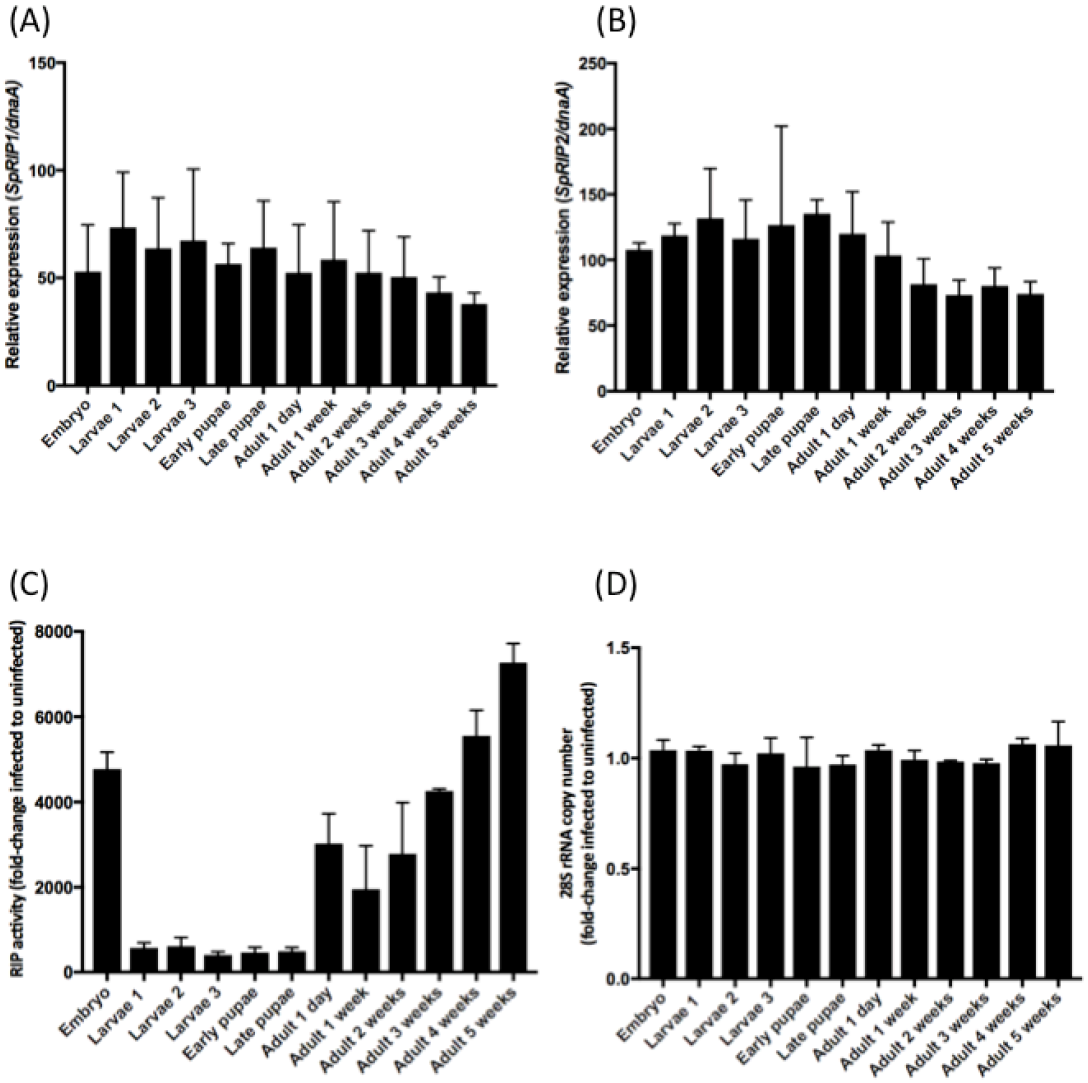

Figure 10. (A) *SpRIP1* expression level in infected flies along *Drosophila* development stages (One way ANOVA; development stage p=0.9055). **(B)** *SpRIP2* expression level in infected flies along *Drosophila* development stages (One way ANOVA; development stage p=0.5129). **(C)** RIP activity in infected flies compared to uninfected flies (Two way ANOVA; *Spiroplasma* infection p*** <0.0001; development stages p***<0.0001; interaction p***<0.0001). **(D)** Intact 28S rRNA quantification in infected versus uninfected flies along *Drosophila* development stages.

SpRIP1 and *SpRIP2* expression is toxic for *Drosophila melanogaster*

We generated four different transgenic fly lines expressing singly *SpRIP1*, *SpRIP2*, BiP+*SpRIP1* or BiP+*SpRIP2* under the control of the *GAL4/UAS* system (Duffy, 2002). BiP is a signal peptide used to trigger the secretion of proteins in *D. melanogaster* (Robert et al., 1998; Soejima et al., 2013). The toxicity of these constructs was tested using the "Rough Eye Phenotypes" (REP) assay allows the study of toxin activity driven by an eye-specific driver (*ey-GAL4*) to observe eventual deleterious effect of the protein on this organ's structure (Halder et al., 1998; Iyer et al., 2016).

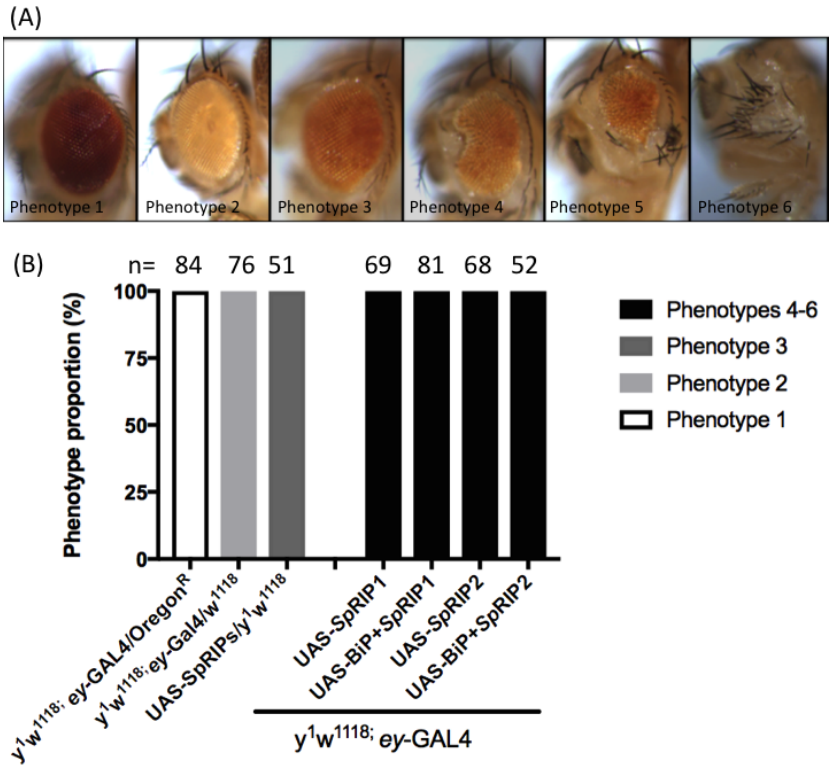

Figure 11. Rough Eye Phenotype assay. **(A)** Representative bright-field images of *Drosophila* eye phenotypes obtained during the assay. **(B)** Proportion of each phenotype. n indicates the number of flies obtained for each cross. Each cross has been repeated three independent times.

The REP assay allows to study toxin activity by monitoring defects including loss of bristles, fusion of ommatidias, necrosis, loss of pigmentation and reduced eye size (Iyer et al., 2016; Van-Vactor et al., 1991). All control flies developed a normal eye structure. On the contrary, flies expressing *UAS-SpRIPs* under *ey-GAL4* control developed a reduced eye along with severe abnormalities, and in some cases no eye at all (Figure 11). This demonstrates that both *Sp*RIP1 and 2 act as toxins on *Drosophila* cells.

Ectopic expression of *SpRIP1* and *SpRIP2* decreases uninfected flies life span

Spiroplasma-infected flies have a shorter lifespan compared to uninfected ones (Herren et al., 2014). Moreover, old infected flies have been reported to have a decreased climbing activity which suggests neurological damages (Herren et al., 2014). We first confirm this phenotype, observing that infected flies have a lifespan reduced by about 20 days (Figure 12 and Supplementary Figure 3). As *Spiroplasma* resides in the hemolymph, we hypothesized that the impact of *Spiroplasma* on host lifespan could be due to accumulation of a toxin released in the hemolymph. Accordingly, proteomics analysis of hemolymph of two weeks old *Spiroplasma*-infected flies revealed the presence of *Sp*RIP1 and *Sp*RIP2 (Rommelaere and Masson, unpublished data).

To further address a role of *Sp*RIPs on *Drosophila* viability, we tested the effect of ectopic expression of *SpRIPs* on the life span of uninfected flies. *Drosophila* expressing *SpRIP1* or *BiP+SpRIP1* constructs did not develop further than larval instars, preventing the use of these constructs for lifespan analysis. Interestingly, uninfected flies expressing *SpRIP2* and *BiP+SpRIP2* had a markedly decreased lifespan by about 30 days in average compared to uninfected flies which live about 75 days (Figure 12; Logrank test p***< 0.0001). To further test the implication of *Sp*RIPs in premature adult lethality, we generated a transgenic fly line expressing a 1492 bp fragment of the 28S rRNA under the control of a *UAS* upstream sequence

(Duffy, 2002). This fragment contains the conserved SRL targeted by RIPs and was designed to buffer RIP activity by increasing the number of targets for the toxin, thus working as an antidote. *Spiroplasma*-infected flies with ubiquitous expression of SRL fragment display an increase in their lifespan by about 5 days compared to infected wild-type flies (Figure 12; Logrank test p****< 0.0001). Collectively, these results are consistent with the implication of *Sp*RIP in shortening *Drosophila* life span.

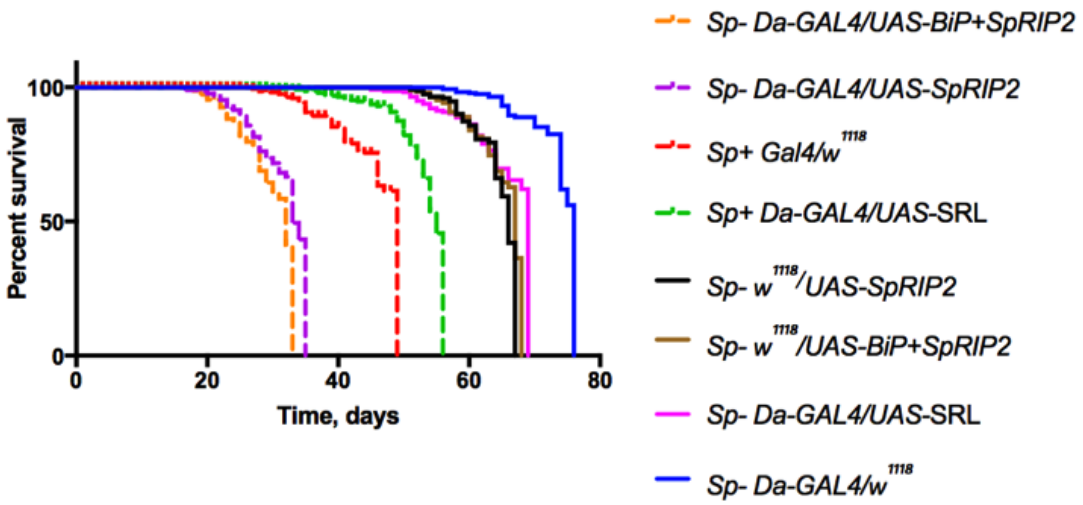

Figure 12. Effect of *SpRIP2* expression on *D. melanogaster* lifespan. *Sp-* and *Sp+* refer to uninfected or *Spiroplasma*-infected condition respectively. *UAS* constructs were driven by the ubiquitous *Da-GAL4* driver. Sample labels are ordered from the shortest to the longest lifespan. Plain lines represent uninfected stocks and controls. Dashed lines represent either infected flies or expressing *SpRIPs*. Pairwise comparison of survival fits where analyzed by Log-rank (Mantel-Cox) test.

***Spiroplasma*-infected flies and uninfected flies expressing *SpRIP2* have reduced hemocyte count**

As *S. poulsonii* is found in the fly hemolymph, we hypothesized that hemocytes should be the most affected cell type by RIP toxins. We thus visualized hemocyte in *Spiroplasma*-infected and uninfected adult flies, carrying the hemocyte marker *Hml-GAL4, UAS-GFP*. In uninfected adult flies, sessile hemocytes are found in

patches beneath the cuticle in the middle of the dorsal abdomen (Ramond et al., 2015). Interestingly, *Spiroplasma*-infected flies have reduced number of sessile patches (Figure 13A).

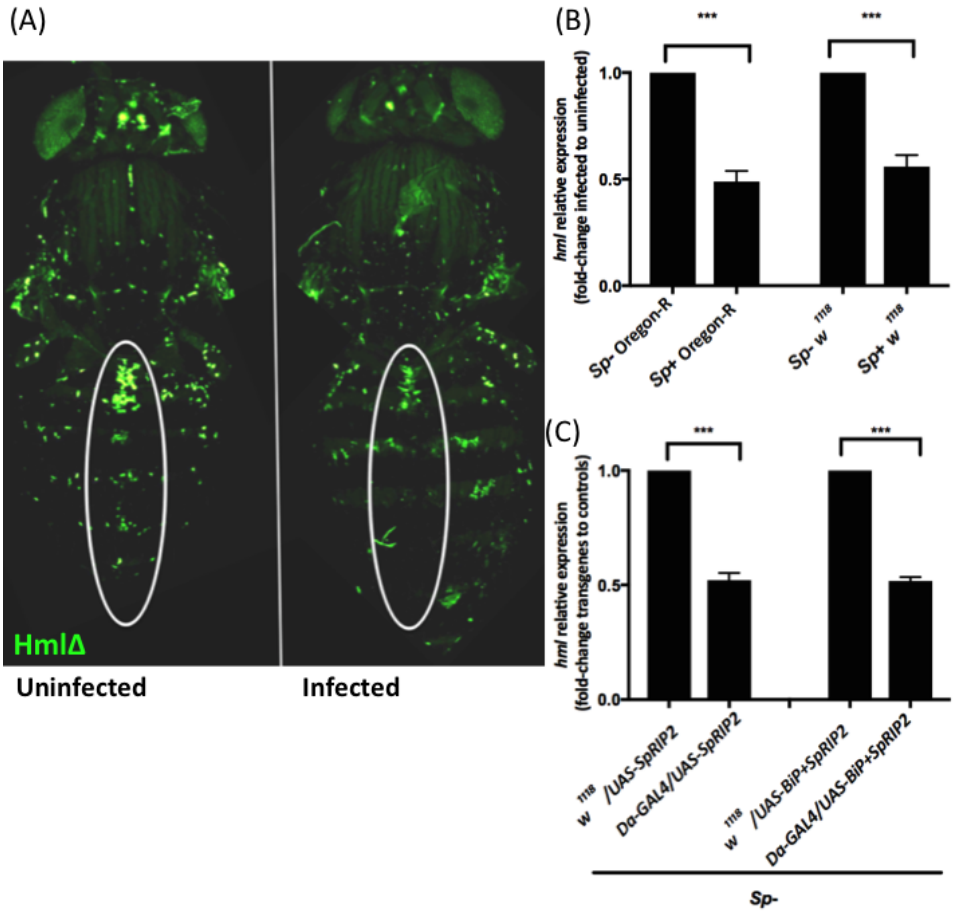

Figure 13. (A) Confocal image of *HmlΔ-GAL4/UAS-GFP* adult flies. In uninfected flies, hemocyte patches are mostly located within the white circle, following the antero-posterior axis. In infected flies only a few patches remain and have lower fluorescence intensity. **(B)** *hml* transcription level in infected and uninfected adult wild-type flies (Dunnett's multiple comparisons test p***< 0.0001). **(C)** *hml* transcription level in lines expressing *UAS-SpRIP2* and *UAS-BiP+SpRIP2* under *Da-GAL4* control. Expression of both constructs lead to a decrease in hemocyte number (Dunnett's multiple comparisons test p***< 0.0001). *Sp-* and *Sp+* refer to uninfected or *Spiroplasma*-infected condition respectively. Controls are normalized as 1.

To confirm this observation, we indirectly estimated the number of hemocytes in adult flies by monitoring the expression of *hemolectin* (*hml*), a gene which expression is hemocyte-specific. Consistent with a reduction of the number of hemocytes, the expression of *hml* was halved in *Spiroplasma*-infected flies compared to uninfected ones in two different wild-type strains (Figure 13B). We conclude that the presence of *Spiroplasma* greatly reduces the number of hemocytes. This reduction could reflect the shortening of lifespan as a decreased hemocyte count is one of the hallmark of aging in flies (Horn et al., 2014). To test whether *Sp*RIPs could mediate this effect, we monitored the level of hemocytes in adult flies expressing *SpRIP2* and *BiP+SpRIP2* under the control of two ubiquitous *GAL4* drivers. *hml* expression quantification revealed a decrease in the number of hemocytes in these flies similar to the decrease observed upon *Spiroplasma* infection (Figure 13C and Supplementary Figure 4). These results suggest that *Sp*RIPs cause hemocytes death, which in turn could contribute to aging and premature death of flies.

SpRIPs **ectopic expression causes embryo mortality with a bias toward male-killing**

We have shown that RIP activity is particularly high in *Spiroplasma*-infected embryo compared to other developmental stages (Figure 10C) raising the possibility that *Sp*RIP1 and *Sp*RIP2 could contribute to male-killing in addition to *Spaid* (Harumoto and Lemaitre, 2018). To test this possibility, we first monitored the effect of the ectopic expression of *SpRIPs* in uninfected individuals by using either the ubiquitous zygotic *Da-GAL4* driver in embryos or the maternal driver *MTD-GAL4*.

We monitored embryo mortality as the percent of embryos that do not hatch, which is about 5% in uninfected wild-type embryos (Figure 14A). All uninfected embryos with ectopic expression of *SpRIP1* or *BiP+SpRIP1* die, reflecting the high toxicity of *Sp*RIP1. However, the expression of *UASp-SpRIP2* kills about 70% of the

embryos (Dunnett's multiple comparison test against uninfected *w1118* p**<0.0074). Interestingly, over-expression of *UASp-BiP+SpRIP2* shows a lower toxicity with a mortality rate up to 30% (Dunnett's multiple comparison test against uninfected *w1118* p***<0.0001) (Figure 14A).

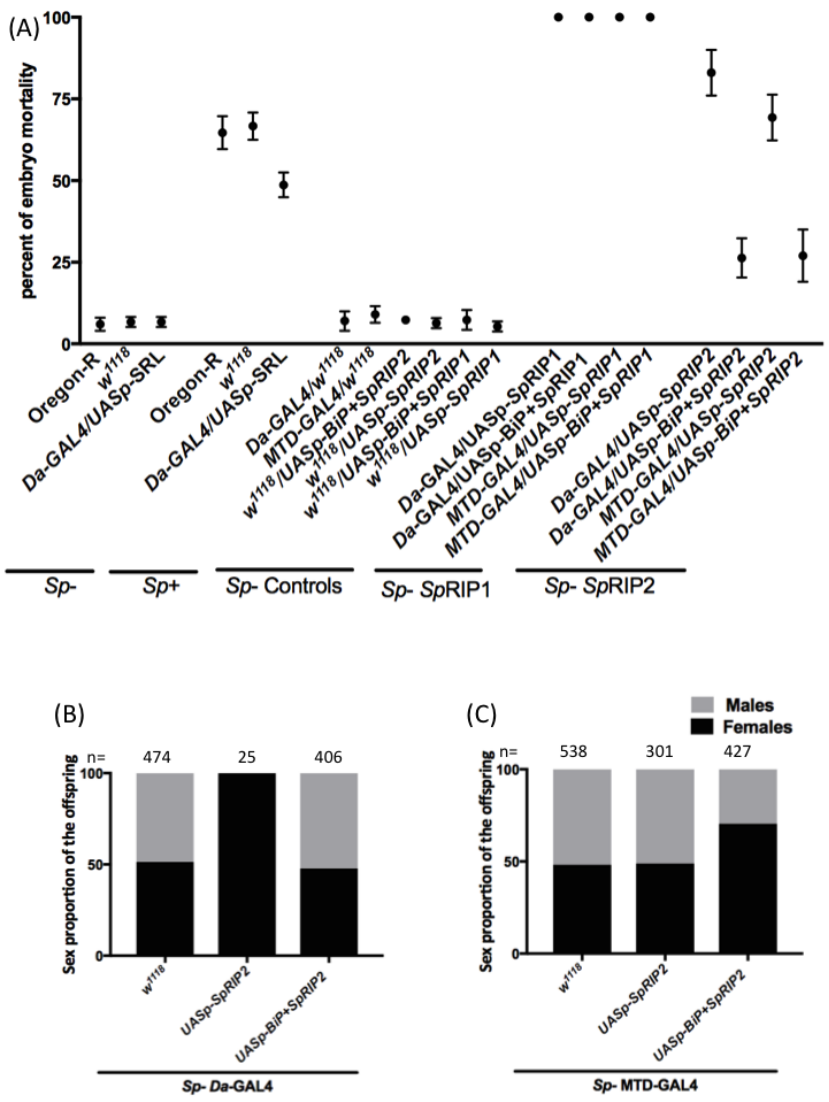

Figure 14. (A) Effect of *SpRIP* expression on embryo mortality. **(B)** Sex ratio of uninfected flies expressing *UASp-SpRIP2* under *Da-GAL4* control (ubiquitous). **(C)** Sex ratio of uninfected flies expressing *UASp-SpRIP2* under *MTD-GAL4* control (maternal specific). n indicates the number of adult flies counted for the assay. *Sp-* and *Sp+* refer to uninfected or *Spiroplasma*-infected condition respectively.

We hypothesized that the secretion of the toxin out of the embryo's cells reduces its toxicity. To reinforce the hypothesis that RIP activity is indeed responsible for embryo death, we measured RIP activity during embryogenesis for each construct. We observed a strong correlation between the level of RIP activity and the mortality (Pearson's correlation test p***<0.001) (Supplementary Figure 5), suggesting that the mortality results from RIP activity. While the sex-ratio of uninfected hatching flies is of 50% females and 50% males (Figure 14B and 14C), the sex-ratio of uninfected *D. melanogaster* hatching flies from embryos expressing *SpRIP2* were biased toward female. It ranged from 70% when the construct is under the control of the maternal driver *MTD* (Fisher's exact test p***<0.001) to 100% when the ubiquitous driver *Da-GAL4* was used (Fisher's exact test p***<0.001) (Figure 14B and 14C).

Spiroplasma-induced male-killing in *Drosophila* is associated with abnormal apoptosis and neural disorganization during embryogenesis (Cheng et al., 2016; Harumoto et al., 2014; 2016; Martin et al., 2013). We thus examined whether over-expression of *SpRIP2* could phenocopy these pathologies. Most embryos expressing *BiP+SpRIP2* under the maternal *MTD-GAL4* driver displayed a disorganized nervous system compared to control embryo (Figure 15A, $\chi 2$ test p*<0.0318) and much stronger level of apoptosis (Figure 15B, $\chi 2$ test p**<0.0039). Thus, we concluded that the cellular damages caused by ectopic expression of *SpRIPs* are similar to the *Spiroplasma* induced male-killing-like phenotypes and that male embryos are more sensitive to the *Sp*RIPs activity than females.

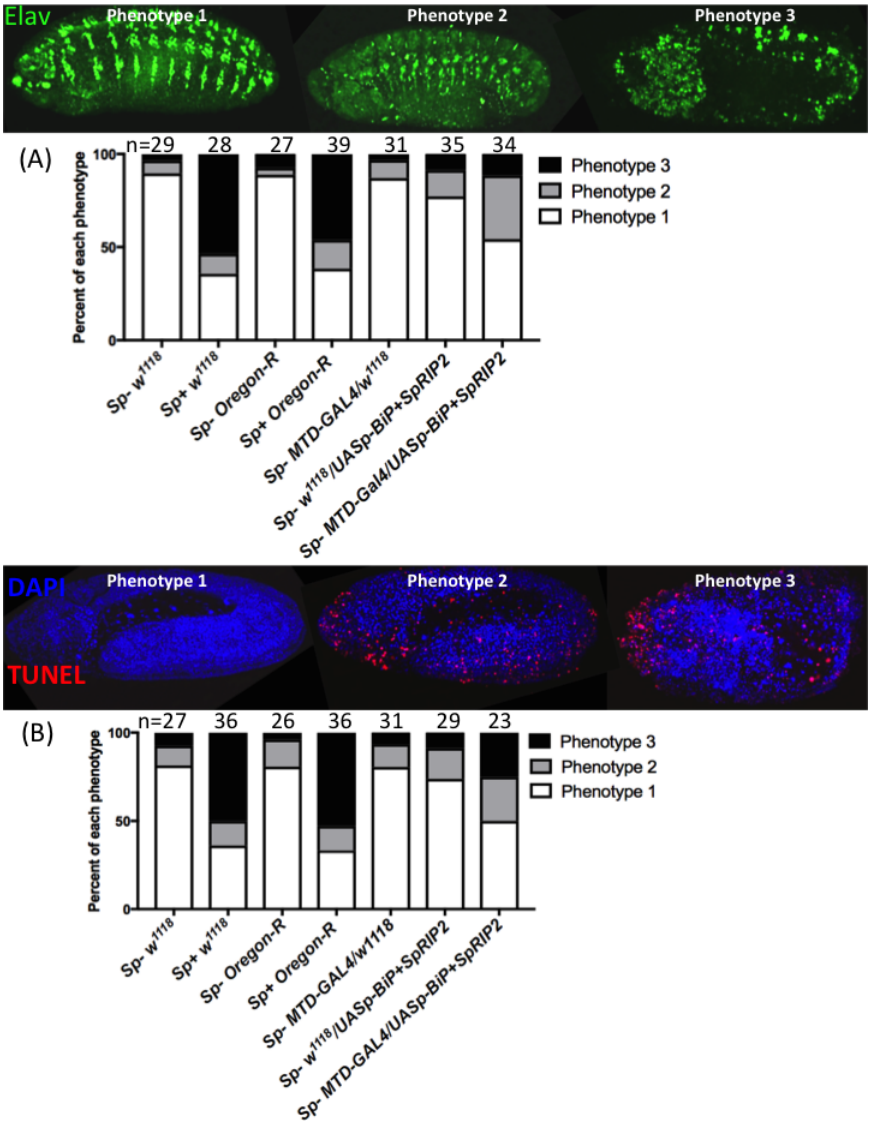

Figure 15. Scoring of male-killing-associated phenotypes in embryos expressing *BiP+SpRIP2* under the control of *MTD-GAL4* driver. **(A)** Scoring of stage 13 embryo according the apparent organization of their nervous system. Green indicates the expression of ELAV. Phenotype 1: Neurons are well defined in three patches. Phenotype 2: neuron patterns are distinguishable, but the patches are not visible. Phenotype 3: Neurons are completely disorganized. **(B)** Scoring of stage 11 embryo shape with DAPI (blue) and apoptosis by TUNEL staining (red). Phenotype 1: no or few apoptosis signal is observed. Phenotype 2: a massive apoptosis signal is detected but the embryo has a normal organization. Phenotype 3: a massive apoptosis signal is observed along with a misshaping of the embryo. n indicates the number of embryos counted for the assay. *Sp-* and *Sp+* refer to uninfected or *Spiroplasma*-infected condition respectively.

To further test the possible implication of *Sp*RIPs in embryo mortality, we took advantage of the *UASp-SRL* construct by analyzing whether buffering RIP activity with additional SRL target could rescue *Spiroplasma*-infected embryos from dying. We first observed that embryonic lethality reaches about 65% in *Spiroplasma*-infected flies, well above the expected 50% if males only were dying. This suggests that *Spiroplama* does not only kill males but also affect a small fraction of the female progeny, roughly estimated at 12.5% (Dunnett's multiple comparison test p***<0.0001). Interestingly, ectopic expression of SRL slightly decreases mortality of infected embryo by 10% (Dunnett's multiple comparison test p***<0.0001) (Figure 14A). The sex ratio of the surviving embryos was still 100% females, suggesting that the buffering of RIP activity by the *UASp-SRL* construct is sufficient to rescue females but not males, likely because of their higher sensitivity to RIP activity.

Discussion

S. poulsonii protects its host against macro-parasites such as nematodes and parasitoid wasps and RIP toxins has been suggested to play a major role in this protection (Ballinger and Perlman, 2017; Hamilton et al., 2016). In this study, we provide evidence that *Spiroplasma* RIPs could affect symbiosis beyond their traditional implication in endosymbiont-mediated protection by harming the host and contributing to male-killing.

We show that only two out of the five putative RIP genes contained in *Spiroplasma* genome are expressed all along the life cycle of *Drosophila* with peaks during embryogenesis and late adulthood. As *S. poulsonii* titer increases with time (Herren et al., 2014), we hypothesize that the peak in old adults is a consequence of the high density of *Spiroplasma* in the host hemolymph, rather than a change in the gene expression in the bacteria. Our over-expression studies confirm that *SpRIP1* and *SpRIP2* are toxins targeting the 28S rRNA of its host. They also suggest that

*Sp*RIP1 has more activity than *Sp*RIP2, although we cannot exclude that their differential activities result from differences in transgene expression. We noticed that the addition of a *Drosophila* secretion signal to the protein sequence tends to reduce its toxicity, which is consistent with *Sp*RIPs targeting 28S rRNA within the cells.

Previous studies have shown that *Spiroplasma* shortens fly lifespan but the underlying mechanism was unknown, although the synthesis of cardiolipins by the bacteria has been proposed as a cause (Herren et al., 2014). Here, we provide evidence that *Spiroplasma* RIPs contribute to the premature death of infected flies. According to this model, the increasing *Spiroplasma* titer in aging flies is accompanied by an increase of *Sp*RIP toxins in the fly hemolymph that eventually damages the host tissues. Similarly to infected flies, we also show that over-expression of *SpRIP1* and *SpRIP2* are associated with an increase in lethality, shortened life span and a decrease in hemocytes number, which is a hallmark of *Drosophila* aging (Horn et al., 2014). While *Sp*RIPs contribute to the protection against *Drosophila*'s parasites, our study suggests that these toxins have also a detrimental effect in the host, which has a tangible impact in late adulthood. This suggests that *Spiroplasma* has not developed any mechanism to shut down RIP expression at the adult stage or in absence or parasite infections. Maintaining a constitutive *Sp*RIP production could be a way to react as quickly as possible to parasite infections at a low cost for the host. It is indeed likely that the fitness cost associated with lifespan reduction in *Drosophila* is minimal, as most eggs are laid during the first two weeks (Ashburner, 1989).

Another striking phenotype associated with *SpRIPs* ectopic expression in uninfected flies is a decrease in embryo viability and a female-biased sex ratio. Male-killing in *Spiroplasma*-infected *Drosophila* has been attributed to a bacterial toxin, Spaid, which contains an ankyrin-repeat domain that allows interaction with eukaryotic proteins (Harumoto and Lemaitre, 2018). Spaid targets the male-specific dosage

compensation machinery and causes male embryo death by triggering massive apoptosis (Harumoto and Lemaitre, 2018). Ectopic expression of *Spaid* in embryos by the *UAS-GAL4* system recapitulates the pathology described in male-killing. Furthermore, a variant of *Spiroplasma* with a truncated form of *Spaid* gene displays reduced male-killing activity. Although the ectopic expression of *Spaid* in uninfected flies recapitulates markers of male-killing, the involvement of other *S. poulsonii* proteins as male-killing enhancer or as redundant factors could not be excluded. In this article, we provide evidence that RIP toxins could, in addition to *Spaid*, contribute to male-killing. While *Sp*RIPs show an overall toxicity toward both males and females, we observed that a mild expression of *SpRIPs* tends to affect more strongly male than female embryos. Consistent with a role of *Sp*RIPs in male-killing, embryonic expression of the *UASp-SRL* construct, that buffers RIP activity, reduces embryo mortality induced by *Spiroplasma*. The exact mechanism that makes male embryos more sensitive to *Sp*RIPs than females, however, remains to deciphered.

Conclusion

Insect endosymbioses encompass a continuum of interactions ranging from mutualism to parasitism. In some cases however, assessing the beneficial or detrimental nature of the interaction for the host can be delicate. The *Spiroplasma*/*Drosophila* symbiosis is a prime example of such versatile ecological outcome: the bacteria protects its host against widespread parasites, conferring a major ecological benefit, but also kills male progeny and drastically reduces the adults lifespan, reflecting a pathogenic interaction. *Sp*RIPs are involved in these two different faces of *Spiroplasma* endosymbiosis. They are directly involved in host protection against parasites, but we show that they can also damage the host to death in absence of parasite infection, making them the first described toxins from an endosymbiont to directly harm its adult host. Moreover we provide

evidences that *Sp*RIP could contribute to male-killing, suggesting that the host reproduction manipulation is achieved by redundant mechanisms.

Material and methods

Fly stocks and handling

Infected lines were obtained several years before this study by injection of *Spiroplasma*-infected hemolymph in Oregon-R females (Herren and Lemaitre, 2011). Hemocytes were observed on 4 weeks old females *w1118*; *HmlΔGAL-4, UAS-GFP* (Bretscher et al., 2015). For all the experiments flies were maintained at 25°C on standard cornmeal medium. Embryos were collected from 5 to 7 days old flies by using cages and yeasted grape juice plates. Lifespan experiments were done as described in (Herren and Lemaitre, 2011). Driver for REP assay (*ey-GAL4*) was obtained from Bloomington stock center (#8221). All experiments have been repeated three independent times.

RNA, DNA extractions and RT-qPCR

RNA, DNA extractions, and RT-qPCR were performed as described in (Herren et al., 2014; Herren and Lemaitre, 2011; Paredes et al., 2016). Reverse transcription was done using 500 ng of RNA per sample, which was isolated from 3 adult flies, 3 larvae, 3 pupae, or 100 to 300 embryos. *SpRIPs* expression and activity were measured along the whole life cycle by RT-qPCR. Expression for each *SpRIP* was analyzed individually except for *SpRIP3*, *SpRIP4* & *SpRIP5* that were measured with a single pair of primers because of their high sequence identity. qPCR calculations for the expression level of *SpRIPs* was done following the ΔCT method normalizing by *dnaA* expression level. Primers for *dnaA* are described in (Herren and Lemaitre, 2011). RIP activity assay was performed as described in (Hamilton et al., 2016). All qPCR calculations for RIP activity and hemocyte count have been done following the ΔΔCT method and these figures represent the fold change between the

experimental condition samples and the controls which are valued as 1 (Livak and Schmittgen, 2001). Primers for *hml* are: Forward: 5'-GAGCACTGCATACCCCTACC-3' Reverse: 5'-CCGTGCTGGTTACACTCCTT-3' (efficiency = 1.88). Gene expression levels were normalized to *rps17*. Figures and statistical results were obtained using GraphPad Prism 7.0b software. All experiments have been repeated three independent times.

Design and construction of *UAS-SpRIP1* and *UAS-SpRIP2* constructs

Spiroplasma has an alternative genetic code and a strong codon bias compared to *Drosophila* (Paredes et al., 2015). *SpRIP1* and *SpRIP2* gene sequences have thus been codon optimized for insect translation using Geneious v8.1.9. A BiP sequence was added at the 5' end of the RIP genes flanked by two BglII restriction sites. The optimized *BiP-SpRIP1* and *2* was fully synthesized and cloned in a pDONR221 vector for Gateway cloning by Invitrogen GeneArt gene synthesis services. Optimized *SpRIP1* and *2* were obtained from *BiP+SpRIP1* and *2* by digestion of the *BiP* sequence by BglII and re-ligation of the plasmid on itself. The fragment of 28S rRNA was amplified from Oregon-R flies and also cloned in pDONR221. All transgenes were cloned into a *UASp* and a *UASt* vector by Gateway LR reaction and injected in *D. melanogaster* w1118 embryos by Bestgene Inc, Chino Hills, USA.

Embryo mortality assay

A total of 100 embryos were collected per genotype on grape juice plates 15-20h after egg laying. After ten more hours, the number of remaining embryos that did not hatch (dead embryos) was counted. Experiments were done simultaneously with two different drivers, the ubiquitous *Da-GAL4*, and the maternal *MTD-GAL4*. All experiments have been repeated three independent times.

Fluorescence microscopy

Embryo neural system was stained using anti-Elav antibody, a protein that is specifically expressed in differentiated neural cells, allowing the visualization of the neurons structure (Robinow and White, 1991, 1988). Apoptosis was detected by Terminal deoxynucleotidyl transferase dUTP Nick End Labeling (TUNEL). This method detects DNA fragmentation by labeling the double-strand DNA breaks generated during apoptosis (Abrams et al., 1993). Both assays were done as it is described in (Harumoto et al., 2014; 2016). Pictures were acquired on a Zeiss LSM 700 confocal microscope on the Bio-Imaging and Optics Plateform (BiOP, EPFL).

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Chapter IV:

The Drosophila heat-shock-protein 70B mitigates Spiroplasma poulsonii deleterious effects and promotes endosymbiont-mediated heat tolerance

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Article in progress

Background: Many insect species harbor bacterial symbionts living within their tissues (endosymbionts). Endosymbionts benefit from a stable and nutritionally rich environment, while providing their host with ecological advantages such as nutritional complementation, protection against parasites or tolerance to heat.

Spiroplasma poulsonii is a natural endosymbiont of the fruit fly *Drosophila melanogaster*. It lives extracellularly in the host hemolymph and is vertically transmitted by trans-ovarial transfer. *Spiroplasma* causes male killing, a reproductive manipulation strategy that enhances its own transmission, whereby all infected male embryos die during early embryogenesis while females survive. The presence of *S. poulsonii* also protects *D. melanogaster* larvae against parasitoid wasp and adults against nematode infections by secreting Ribosome-Inactivating Proteins (RIPs) that target the parasites. RIPs however also harm the host by reducing its lifespan and increasing embryo mortality.

Results: We show that *S. pouslonii* infection induces the over-expression of *Heat Shock Protein 70B* (*HSP70B*) gene in *Drosophila* embryos and old adults. Uninfected transgenic flies expressing *Spiroplasma* RIPs also show high levels of *HSP70B* transcripts, suggesting that RIPs are responsible for *Hsp70B* induction. Functional studies indicate that HSP70B mitigates *Spiroplasma*-induced damages to the host tissues, promoting survival of infected embryo and extending the life span of infected flies. Our study also reveals that *Spiroplasma*-mediated induction of *Hsp70B* promotes a short-term tolerance to heat, indicating that the HSP stress response could bring an ecological benefit to infected flies.

Conclusion: Our results indicate that RIPs released by *S. poulsonii* up-regulate *Hsp70B* genes in host, which mitigate the deleterious effects of RIPs on host tissues and promotes thermo-tolerance. These results support the notion of endosymbiont-mediated protection against heat, and possibly other environmental stresses through hormesis.

Keywords: Heat Shock Protein, *Spiroplasma*, endosymbiosis, Ribosome Inactivating Protein*, Drosophila*.

Introduction

Natural populations of *Drosophila melanogaster* can harbor two facultative endosymbionts: *Wolbachia pipientis*, and *Spiroplasma poulsonii* (Mateos et al., 2006; Montenegro et al., 2005). *Wolbachia* is a widespread, vertically transmitted, intracellular bacteria present in more than 40% of arthropod species (Werren, 1997). In contrast, *S. poulsonii* is an extracellular bacterium that lives in the fly hemolymph but is also vertically transmitted colonizing the germline during oogenesis (Herren et al., 2013). Infected progeny undergoes a sex ratio distortion known as male-killing, which consists in the death of all male infected embryos while most female embryos survive (Counce and Poulson, 1962; Malogolowkin and Poulson, 1957; Poulson and Sakaguchi, 1961). Male killing is thought to promote the propagation of the endosymbiont by favoring the female over the male lineage (Hurst and Frost, 2015). *S. poulsonii* also confers protection to *D. melanogaster* against parasitoid wasps and nematodes (Jaenike et al., 2010; Xie et al., 2010). As parasitoid wasps and nematodes are among the most frequent parasites of *Drosophila* in the wild (Fleury et al., 2009), *Spiroplasma* infection thus confers a crucial ecological advantage, which can lead to *S. poulsonii* spread in natural populations of *Drosophila* across continents (Jaenike et al., 2010). Two non-mutually exclusive mechanisms have been proposed to explain *Spiroplasma* protection against macro-parasites: metabolic competition and toxin production. According to the first one, *Spiroplasma* and parasitoids compete for host's lipids during larval and pupal stages (Paredes et al., 2016). Thus, lipid depletion by *Spiroplasma* prevents the development of the wasp. Alternatively, it has been shown that *Spiroplasma* encodes for a family of toxins called Ribosomal Inactivating Protein (RIP) that target the parasitoid wasp and nematode ribosomes

(Ballinger and Perlman, 2017; Hamilton et al., 2016). RIPs belong to a group of toxins found in plants and bacteria that act against predators. They target a conserved sequence of the eukaryotic 28S ribosomal RNA called the Sarcin-Ricin Loop (SRL) (Bergan et al., 2012), of which they cleave a purine in a process called depurination, thus blocking protein synthesis (Szewczak and Moore, 1995). The *S. poulsonii* genome encodes five RIPs genes but only two of them RIP1 and RIP2 are transcribed. *RIP1* and *RIP2* are constitutively expressed, even in absence of parasite infection, along the whole fly life cycle (Garcia-Arraez et al., 2018). However, *Sp*RIPs also target *Drosophila* ribosomes to some extent, inflicting damage to its host. A recent study indicates that *Sp*RIPs increase embryonic mortality and reduce adults life span (Ballinger and Perlman, 2017; Garcia-Arraez et al., 2018).

Heat Shock Proteins (HSPs) are cytosolic chaperones that play an essential role in protein homeostasis under normal and stressful conditions. They ensure the proper folding of nascent proteins, the refolding of damaged proteins and can extend the half-life of proteins during stress (Hartl and Hayer-Hartl, 2002; Mayer and Bukau, 2005; Ramos et al., 2005; Walter and Buchner, 2002). Indeed, HSPs have been shown to enhance cell survival under various stresses, such as heat, cold, infections or exposure to toxins (Cobreros et al., 2008; Giffard et al., 2008; Giffard and Yenari, 2004; Hartl and Hayer-Hartl, 2002; Mayer and Bukau, 2005; Meimaridou et al., 2009; Nishikawa et al., 2008; Santoro, 2000; Stojadinovic et al., 1997; Young et al., 2017).

In this paper, we investigated the transcriptional changes induced by *Spiroplasma* infection in embryo, which revealed a strong induction of *HSP70B* coding genes. We showed that infected flies and non-infected transgenic flies expressing *Sp*RIPs displayed a higher level of *Hsp70B* expression compared to uninfected flies. Furthermore, infected transgenic flies over-expressing *Hsp70B* displayed a decrease in embryo mortality and an extended life span compared to infected wild-type flies; two phenotypes which have recently been associated to *Sp*RIPs (Garcia-

Arraez et al., 2018). Our results suggest that *HSP70B* induction in *Drosophila* upon *Spiroplasma* infection mitigates the deleterious effect of *Spiroplasma* RIP toxins. Interestingly, we provide evidences that *HSP70B* induction by RIP toxins increases *Drosophila* tolerance to heat, suggesting that this stress response could turn into an ecological benefit under certain environmental conditions.

Results

Early female embryos infected with *Spiroplasma* exhibit a transcriptional stress response

As male-killing occurs during early embryogenesis, we performed a RNA-seq analysis in male and female single embryos to seek for a sex-difference in the transcriptional response to *Spiroplasma* infection. Pathological phenotypes associated with male-killing, like abnormal apoptosis or neural defects take place in embryos 4-10 hours after egg laying (AEL) (Cheng et al., 2016; Harumoto et al., 2016, 2014; Martin et al., 2013). To detect potential earlier effects of male-killing, we collected embryos 3 hours AEL, at the end of the cellularization stage. We collected three single male or female embryos that were either uninfected or *Spiroplasma*-infected. Embryos were staged by collecting only those whose cephalic furrow appears, and were subsequently sexed by PCR of a *Y*-linked locus.

Comparison between infected and uninfected male single embryos led to the identification of 1207 differentially expressed genes. In female embryo however, only 20 genes were differentially expressed upon infection (Figure 16). A gene ontology analysis of genes differentially expressed in infected compared to uninfected male embryos reveals an enrichment in genes involved in splicing and metabolism (Tables 2 and 3). A comparison of the chromosomal transcription rates for all genes between infected and uninfected embryos revealed no differences in female samples, while males over-transcribe the X chromosome upon *Spiroplasma*

infection. Thus, our RNAseq analysis reveals that male-killing *Spiroplasma* has tangible effects on embryo earlier than previously thought. Among the twenty genes differently expressed between infected and uninfected female embryos, 18 are up-regulated, among which five are involved in stress response: *Hsp70Bc, Hsp70Bb*, *Hsp68*, *cy6a23* and *ir10a*. *Hsp70Ba Hsp70Bb and Hsp70Bc* are part of a cluster of highly homologous genes that have been involved in tolerance to heat and various stresses (Bettencourt et al., 2008). All genes in the cluster display high sequence similarity and they will be collectively referred to as *Hsp70B* (Bettencourt et al., 2008). This indicates that infected female embryos undergo an active stress response upon *S. poulsonii* infection that probably contributes to their survival. In this article, we further explore the role of *Hsp70* in *Drosophila–Spiroplasma* endosymbiosis.

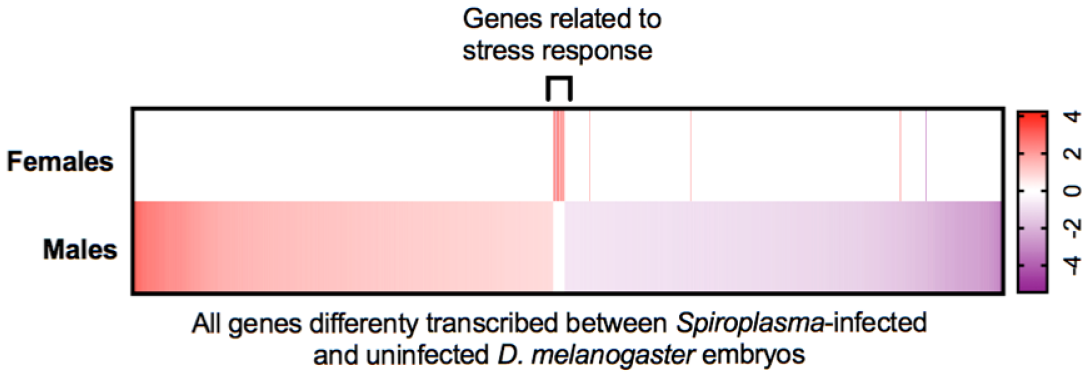


Figure 16. Differentially expressed genes in male and female *Spiroplasma*-infected embryos at the end of the cellularization stage compared to uninfected ones.

Splicing enrichment for GO analysis in male embryos	P-value
RNA splicing	1.42E-11
mRNA processing	2.15E-11
RNA processing	5.33E-11
mRNA metabolic process	7.17E-11
RNA splicing, via transesterification reactions	8.76E-11
RNA splicing, via transesterification reactions with bulged adenosine as nucleophile	4.80E-10
mRNA splicing, via spliceosome	4.80E-10
Regulation of RNA metabolic process	5.18E-07
Regulation of mRNA splicing, via spliceosome	2.19E-05
Regulation of mRNA processing	5.42E-05
Regulation of alternative mRNA splicing, via spliceosome	6.56E-05
Regulation of RNA splicing	7.39E-05

Table 2. Gene-ontology terms for the 57 genes involved in splicing differentially expressed between uninfected and infected male embryo at 3h AEL. P-value is the enrichment p-value computed according to the mHG or HG model.

Metabolism enrichment for GO analysis in male embryos	P-value
Cellular metabolic process	1.12E-16
Heterocycle metabolic process	6.39E-13
Organic cyclic compound metabolic process	1.19E-12
Cellular macromolecule metabolic process	2.78E-12
Nucleobase-containing compound metabolic process	1.17E-11
Primary metabolic process	1.87E-11
Cellular aromatic compound metabolic process	2.14E-11
mRNA metabolic process	7.17E-11
Cellular nitrogen compound metabolic process	1.03E-10
Metabolic process	1.43E-10
Organic substance metabolic process	1.75E-10
RNA metabolic process	1.90E-10
Nucleic acid metabolic process	3.55E-10
Nitrogen compound metabolic process	2.94E-09
Macromolecule metabolic process	2.73E-08
Regulation of macromolecule metabolic process	8.44E-08
Regulation of metabolic process	1.73E-07
Regulation of RNA metabolic process	5.18E-07
Regulation of primary metabolic process	9.20E-07
Regulation of cellular metabolic process	9.71E-07
Regulation of nucleobase-containing compound metabolic process	1.07E-06
Regulation of nitrogen compound metabolic process	2.10E-06
Negative regulation of macromolecule metabolic process	2.47E-05

Table 3. Gene-ontology terms for the 495 genes involved in metabolism differentially expressed between uninfected and infected male embryo at 3h AEL. P-value is the enrichment p-value computed according to the mHG or HG model.

***Spiroplasma* infection induces the expression of *Hsp68* and *Hsp70B* in old female flies.**

We then explored whether *Spiroplasma* induces the expression of *Hsp70* at later stages. For this, we monitored by RT-qPCR the expression level of *Hsp68, Hsp70B and Hsp23* along the whole life cycle of *Spiroplasma*-infected individuals. In accordance with the RNAseq results, only *Hsp68*, and *Hsp70B* were up-regulated in infected embryos (Figure 17A and 17B).

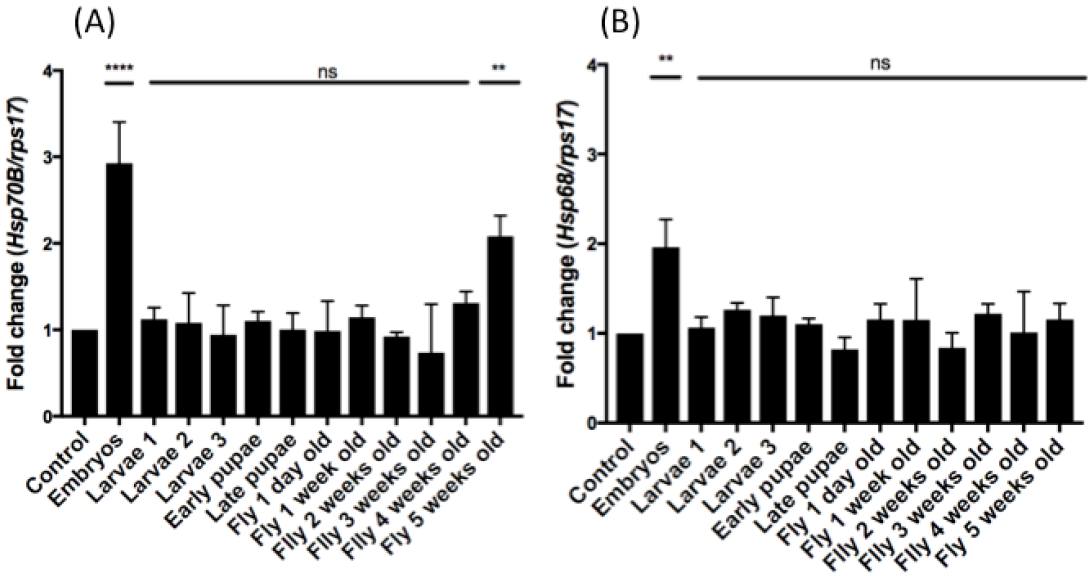


Figure 17. (A) *Hsp70B* expression levels in infected flies along *Drosophila* life cycle (Two way ANOVA followed by post-hoc Tukey HSD; *Spiroplasma* infection in embryos p****<0.0001; *Spiroplasma* infection in five weeks old flies p**<0.0076). **(B)** *Hsp68* expression level in infected animals along *Drosophila* life cycle (Two way ANOVA followed by post-hoc Tukey HSD; *Spiroplasma* infection in embryos p**<0.0081). Expression is indicated as a fold-change between infected and uninfected, uninfected being represented as 1.

Infected larva, pupa and young adult flies showed no expression difference for these two genes in infected individuals compared to their uninfected counterparts. Interestingly, infected old adult flies have a two-fold increased expression of

Hsp70B compared to uninfected old flies (Figure 17A). Consistent with this, a proteomics analysis showed that Hsp70B proteins are detected in higher amounts in the hemolymph of infected adult females compared to uninfected ones (Rommelaere and Masson, unpublished data). *Hsp23* was not up-regulated upon *Spiroplasma* infection during the whole fly life cycle (data not shown). We conclude that *Spiroplasma* infection triggers the over-expression of a subset of heat-shock proteins in embryos, female larva and female adult flies, most notably *Hsp70B*.

Hsp70B promotes survival of _Spiroplasma_-infected adult flies

The high titer of *Spiroplasma* in old flies causes lethality, indicating that *Spiroplasma* inflicts some damage to its host. We hypothesize that HSP production could be a host response to promote fly survival to *Spiroplasma*. To test the contribution of *Hsp70B* in host resistance to *Spiroplasma* infection, we compared the survival of *Spiroplasma*-infected and uninfected flies with altered expression of *Hsp70B.* For this, we used an *in vivo RNAi* strategy by expressing ubiquitously an *Hsp70B* interfering RNA construct (genotype: *Da-GAL4, UAS-Hsp70B-IR*) or the homozygous viable *Df(3R)Hsp70B* deficiency line, which carries a four-gene deletion of all *Hsp70B* variants. At 25°C, uninfected flies expressing ubiquitously the *Hsp70B*-RNAi or deficient for *Hsp70B* displayed the same lifespan (around 75 days) than their wild-type counterparts (Figure 18A). Interestingly, infected *Hsp70B RNAi* or deficient flies had a markedly decreased lifespan compared to wild-type infected flies, roughly 35-40 days in average compared to 50 days wild-type (Logrank test p***< 0.0001) (Figure 18A). In contrast, infected flies with reduced expression of two other *Hsp* genes, such as *Hsp70A* and *Hsp23*, displayed a normal lifespan upon *Spiroplasma* infection (Supplementary figure 7). To further test the implication of *Hsp70B* in fly survival upon *Spiroplasma* infection, we generated a transgenic fly line that over-expresses this gene under the control of a UAS upstream sequence (Duffy, 2002). *Spiroplasma*-infected flies with ubiquitous over-expression of *Hsp70B* displayed an increase in their lifespan by about 10 days compared to

infected wild-type flies (Figure 18A; Logrank test p****< 0.0001). We conclude that the stress responsive genes *Hsp70B* contribute to *Drosophila* survival to *Spiroplasma*.

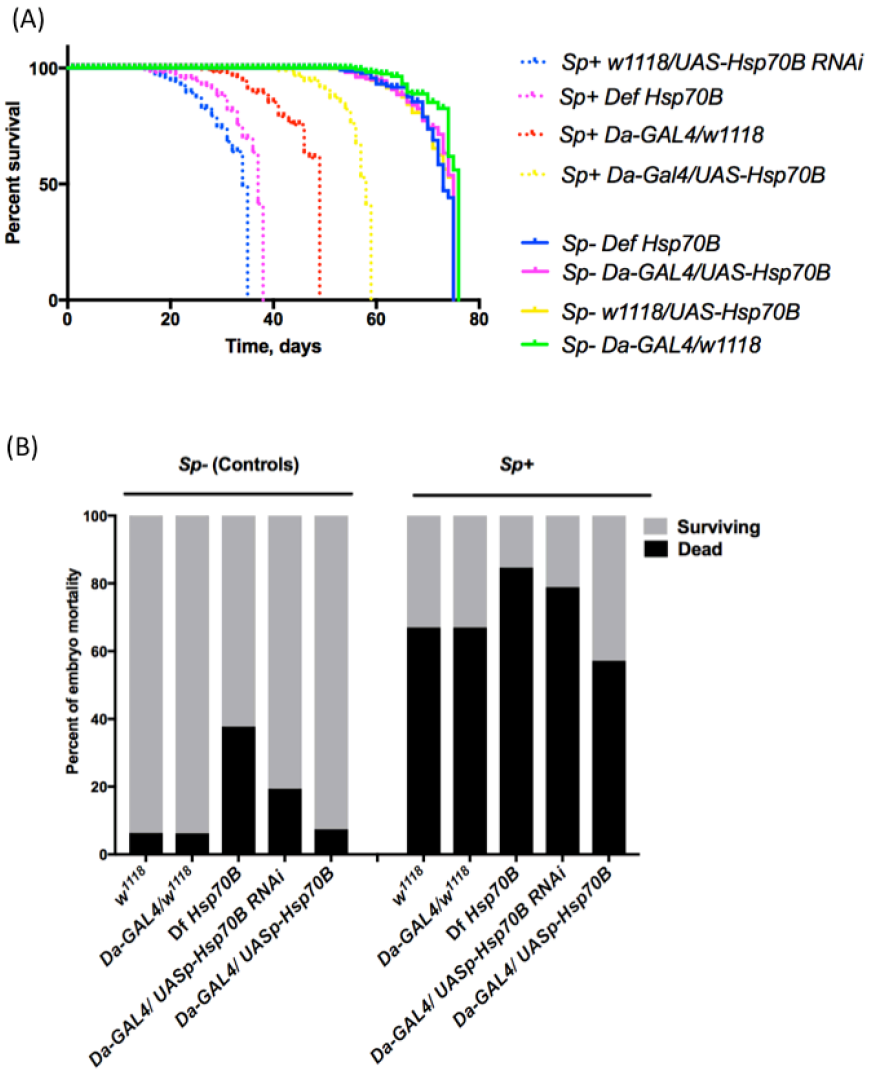


Figure 18. (A) *Hsp70B* contributes to survival of *D. melanogaster* adults upon *Spiroplasma* infection. Genotype of flies are indicated on the right, and ordered from the shortest to the longest lifespan. Plain lines: uninfected flies and controls. Dashed lines: infected flies. Pairwise comparison of survival fits where analyzed by Log-rank (Mantel-Cox) test. **(B)** Mortality rate of control and *Spiroplasma*-infected embryos with reduced (*Df(3R)Hsp70B* or *Da-GAL4; Hsp70-IR*) and increased (*Da-GAL4; UAS-Hsp70B*) expression level of *Hsp70B*. *Sp-* and *Sp+* refer to uninfected or infected condition respectively.

Hsp70B promotes survival of *Spiroplasma*-infected embryos

We then investigated whether *Hsp70B* also promotes host survival at the embryonic stage, when male killing occurs. We thus monitored the mortality of infected and uninfected embryos with higher or lower expression of *Hsp70B*. The percentage of embryonic mortality was about 5% in uninfected wild-type embryos and 65% in wild-type infected embryos as previously described (Garcia-Arraez et al., 2018). We observed that the mortality of both *Df(3R)Hsp70B* and *Hsp70B*-silenced embryos increases compared to wild-types, regardless of *Spiroplasma* infection (Figure 18B). Interestingly, infected embryos over-expressing *Hsp70B* displayed a decreased mortality rate of 8% compared to infected wild types (one-way ANOVA; $p^{*}<0.0257$) (Figure 18B). These results suggest the implication of *Hsp70B* in supporting *Spiroplasma*-infected *Drosophila* embryos.

Up-regulation of *Hsp70B* in *Spiroplasma*-infected flies promotes heat tolerance for short time exposures

Several endosymbionts increase host fitness by increasing their ability to resist to heat (Chen et al., 2000; Montllor et al., 2002). Heat shock proteins have initially been discovered because of their function in resistance to heat. This raises the intriguing hypothesis that *Spiroplasma* could indirectly promote heat tolerance by inducing a HSP response in their host. To test whether *Hsp* genes induction upon *Spiroplasma* infection leads to an increased heat tolerance, we assessed the behavior and survival of infected and uninfected wild-type flies at different temperatures. Interestingly, we found that flies exposed to a 37°C heat shock for 3 hours exhibit different behavior depending of their infection status. In normal condition during daytime, most uninfected wild-type flies appear active (i.e. either climbing on the wall of their vial, walking on the medium or flying). A heat stress of three hours induced a reduction of activity, with about 80% of flies being inactive

at the bottom of the vial. In sharp contrast, only 30% of *Spiroplasma*-infected flies stay inactive at the bottom of the vial (Figure 19A and 19B).

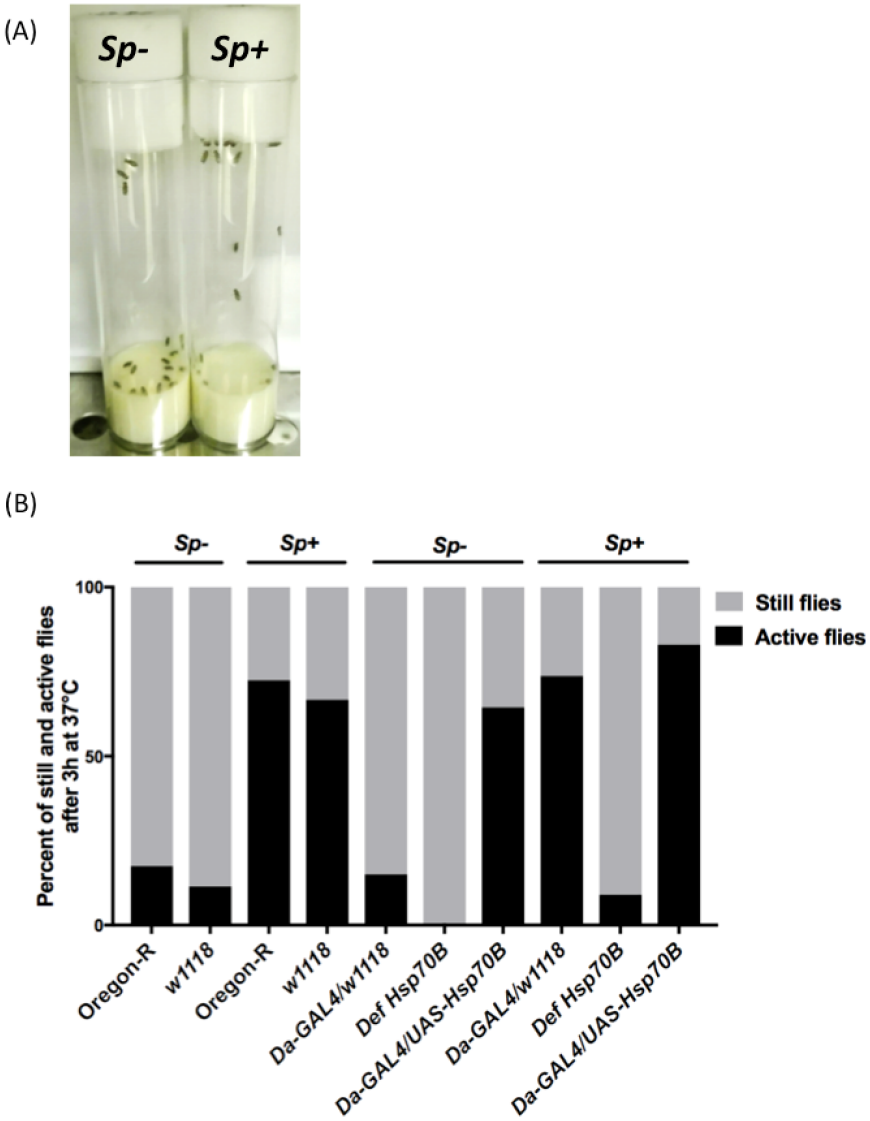


Figure 19. (A) Representative pictures of vials containing infected and uninfected flies incubated for 3h at 37°C. **(B)** Quantification of *Spiroplasma*-infected and uninfected flies behavior after 3h at 37°C in two different wild-type genetic background (Fisher's exact test p***<0.001). Comparison of the level of activity after 3h at 37°C upon *Spiroplasma* infection (Fisher's exact test between infected and uninfected wild-type flies p***<0.001; Infected wild-types flies and infected flies deficient for *Hsp70A* or *Hsp70B* p***<0.001; uninfected wild types flies and uninfected flies over-expressing *Hsp70B* p**<0.007). *Sp+* and *Sp-* refer to infected or uninfected condition respectively.

Importantly, *Spiroplasma Df(3R)Hsp70B* flies display a reduction of activity after heat shock similar to uninfected wild-type flies (Figure 19B). This indicates that the *Spiroplasma*-induced increase in activity is mediated by *Hsp70B*. Consistent with this hypothesis, over-expressing *Hsp70B* tends to further increase the activity of flies upon heat stress regardless of the infection status. (Figure 19B). However, no significant difference in life span was observed between infected and uninfected flies when raised at 29°C, 32°C or 37°C (supplementary figure 8). This suggests that the heat tolerance provided by *Spiroplasma* is transient. Collectively, our study indicate that *Spiroplasma* promote a behavioral resistance upon short exposure to a heat stress and that this effect is mediated *Hsp70B*.

Spiroplasma* RIPs can induce the expression of *Hsp70B* in *Drosophila

Spiroplasma secretes two toxins belonging to the family of Ribosomes Inactivating Proteins (RIPs) that were initially implicated in endosymbiont-mediated protection of its host against parasites (Ballinger and Perlman, 2017; Hamilton et al., 2016). RIP toxins are produced by *Spiroplasma* during the whole life cycle and contribute to the premature death of adult infected flies (Garcia-Arraez et al., 2018). Interestingly, rats fed with *Ricinus*, a poisonous plant containing a high quantity of the RIP toxin Ricin, show increased expression of *Hsp70* in their intestine (Stojadinovic et al., 1997). We thus hypothesized that *Hsp70B* induction *in Spiroplasma*-infected flies could be a secondary consequence of *Sp*RIP production that mitigate the deleterious effects of these toxins, with an increased thermo-tolerance as a side effect. To further investigate this hypothesis, we analyzed the expression of *Hsp70B* in flies over-expressing *SpRIP1* or *SpRIP2* in embryos and only *SpRIP2* in adults (flies expressing *SpRIP1* die before adult stage) by the inducible *GAL4/UAS* system (Duffy, 2002; Garcia-Arraez et al., 2018). Ubiquitous expression of *SpRIP2* with *Da-GAL4* in adult flies lead to increased expression of *Hsp70B*, similarly to what is observed in infected flies (Figure 20A). Interestingly, uninfected

flies expressing *SpRIP2* were also more active upon a short-term heat exposure, as *Spiroplasma*-infected flies (Figure 20C).

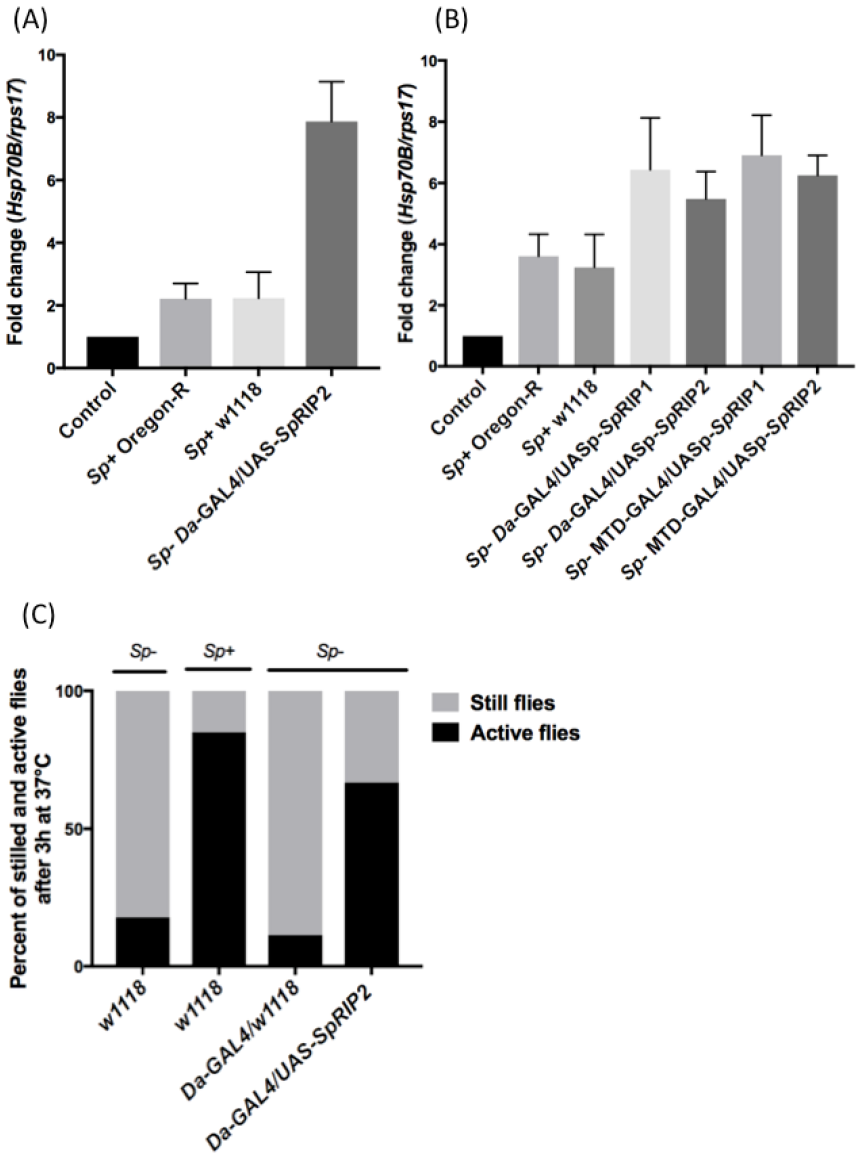


Figure 20. (A) Expression level of *Hsp70B* in embryos expressing *Sp*RIPs (Two way ANOVA for *Hsp70B* was between p***=0.0006 and p****<0.0001). **(B)** Expression level of *Hsp70B* in adult flies expressing *SpRIPs* (Two way ANOVA for *Hsp70B* expression p****<0.0001). Expression is indicated as a fold-change infected versus uninfected, uninfected being represented as 1. **(C)** Fly activity after 3h heat shock at 37°C upon *SpRIP* expression (Fisher's exact test between uninfected wild-type flies and uninfected flies expressing *SpRIP* p*<0.0321). *Sp-* and *Sp+* refer to uninfected or infected condition respectively.

Similarly, ectopic expression of *SpRIP1* or *SpRIP2* in embryos using either the ubiquitous zygotic *Da*-GAL4 driver or the maternal driver *MTD*-Gal4 resulted in the up-regulation of *Hsp70B* (Figure 20B). In contrast, *Hsp68* and *Hsp23* expression levels were not affected by the over-expression of *SpRIPs* (Supplementary figure 9). This suggests that the aforementioned up-regulation of *Hsp68* is not due to *SpRIPs* but rather to another yet unidentified factor. We conclude that the sole of expression of *SpRIP* toxin is sufficient to induce the specific expression of *Hsp70B*, suggesting that *Hps70B* induction by *Spiroplasma* is a secondary consequence of RIP production.

Discussion

In this work, we showed that the endosymbiotic bacteria *Spiroplasma poulsonii* trigger a specific stress response in its host, consisting in the upregulation of *Hsp70B*. Using *in vivo RNAi* silencing or deficiency lines for *Hsp70B,* we demonstrated that *Hsp70B* promotes *Drosophila* host survival upon *Spiroplasma* infection. Ectopic expression of *Spiroplasma* RIPs in uninfected flies induces *Hsp70B* suggesting that these toxins are responsible for the stress response of the host. Interestingly, the induction of *Hps70B* by *Spiroplasma* provides a short-term tolerance to heat. Collectively, our study supports a model by which endosymbiont-mediated host protection against stressful condition is a consequence of RIPs hormetic effect: the low level of stress pathway activation induced by the symbiont appears protective to the host upon more severe stresses.

Spiroplasma **causes an early transcriptome changes in both female and male embryos**

Spiroplasma-induced male killing in *Drosophila* is associated with abnormal apoptosis and neural disorganization in male embryos that can be detected after

stage 10 (Harumoto et al., 2016; Martin et al., 2013). The earliest known symptom of male killing is DNA damage on the X chromosome and can be detected at stage 8 (4 hours AEL) (Harumoto et al., 2016). The identification of early symptoms was hampered by the difficulty to sex embryos at this stage. Our RNA-seq analysis of single male and female embryos allowed us to monitor the impact of *Spiroplasma* at 3 hours after egg laying (AEL) (Edgar et al., 1994; Edgar and Schubiger, 1986; Foe et al., 1993; Lee et al., 2014) at the onset of zygotic transcription. Our RNA-seq analysis reveals that male embryos are strongly affected by *Spiroplasma* infection with a total of 1207 genes that were differently expressed between infected and uninfected male embryos. Gene ontology reveals changes in two categories, metabolism and splicing. The implication of splicing genes is interesting as *Drosophila* sex determination pathway involves a differential splicing cascade of genes between male and female embryos (Bell et al., 1991; Keyes et al., 1992). The massive transcriptional change and the over-transcription of the male X chromosome in *Spiroplasma*-infected male embryos is consistent with the recent identification of the male-killer toxin Spaid, a *Spiroplasma* toxin that co-localizes with the dosage compensation machinery and likely affects the X-chromosome transcription (Harumoto and Lemaitre, 2018).

Spiroplasma **induces a specific stress response in females**

In contrast to the situation observed in males, only 20 genes are differently expressed between uninfected and infected females. This is consistent with the observation that female embryos are spared by *Spiroplasma*. Interestingly, our transcriptome analysis reveals that females undergo a transcriptional stress response against the bacterium, reflected by the upregulation of a specific subset of *HSP* coding genes. This stress response is not limited to early embryos but is also observed in adult females suggesting that it is a generic response to *Spiroplasma*.

Interestingly, our study is not the first to associate Heat-Shock-Proteins to *Spiroplasma* infection. A proteomics analysis revealed a higher level of HSP in the aphid species *Acyrthosiphon pisum* upon *Spiroplasma* infection (Guidolin et al., 2018).

In this study, we provide evidences that activation of *Hsp70B* is caused by RIP toxins that are constitutively produced by *S. poulsonii* and inhibit protein synthesis. First, ectopic expression of *Spiroplasma* RIPs in uninfected flies induces the same transcriptional signature as in *Spiroplasma*-infected flies, including the induction of *Hsp70B* but not *Hsp23*. Second, *Hsp70B* is upregulated in early embryos and 5-week-old females, when RIP activity was shown to be the highest (Garcia-Arraez et al., 2018). We hypothesize that *Drosophila Hsp70B* provides an adaptive response to mitigate the action of *Spiroplasma* toxins. Consistent with this, ectopic expression of *Hsp70B* tends to promote the survival of infected embryos and extends the lifespan of infected females. In contrast, reduction of *Hsp70B* expression increases embryonic lethality in *Spiroplasma*-infected females. Strikingly, the lifespan of infected flies gets dramatically reduced in absence of *Hsp70B.* This reinforces the hypothesis that *HSP* genes play a role in host protection against *Spiroplasma* deleterious effects. As RIP toxins block protein synthesis, we hypothesize that *Hsp70B* can partially rescue infected flies by extending the half life of proteins and by diminishing the amount of proteins sent to degradation, allowing cell functioning despite reduced protein translation (Hartl and Hayer-Hartl, 2002; Mayer and Bukau, 2005; Ramos et al., 2005; Walter and Buchner, 2002).

The increased expression of *Hsp70* in presence of *Spiroplasma* promotes thermotolerance

Several studies, notably in aphids and whiteflies, have shown that heritable bacteria can promote thermo-tolerance in their hosts (Brumin et al., 2011; Chen et

al., 2000; Corbin et al., 2017; Heyworth and Ferrari, 2015; Russell and Moran, 2006). The mechanism underlying symbiont conferred resistance is still poorly characterized. It has been proposed that *Serratia symbiotica,* a facultative endosymbiont of aphids, whose titers decrease at high temperature, improves thermo-tolerance of its host by delivering protective metabolites either directly to its host or to its obligate heat-sensitive endosymbiont *Buchnera* (Gaelen et al., 2010). In contrast, the facultative endosymbiont *Rickettsia* is thought to indirectly influence thermo-tolerance to its host, the whitefly *Bemisia tabaci*, by inducing the expression of host proteins that promote tolerance to heat (Brumin et al., 2011). In this case, *Rickettsia* was shown to induce at normal temperature a set of cytoskeleton proteins (such as Actin or Myosin) that have been shown to contribute to heat tolerance. The higher expression of these proteins upon *Rickettsia* infection would subsequently provide an advantage to its host when placed at high temperature (Brumin et al., 2011). Here we provide evidence that the up-regulation of *Hsp70B* by *Spiroplasma* is not without consequence for its host. It can induce a mild tolerance to heat: flies carrying the symbiont remain more active than uninfected flies after heat treatment. This time window of protection is however short, likely due to fact that uninfected flies can also rapidly deploy their heat shock response and catch up with infected ones within a couple of hours. Importantly, we could show that *Spiroplasma*-induced thermo-tolerance indeed requires *Hsp70B*. This mild heat tolerance could turn out to be an ecological advantage in the wild, allowing infected flies to resist better to heat waves for example, although the exact consequence on fly fitness remains to be explored. Of note, a similar HSP-mediated heat tolerance has been described in *Paramecium caudatum,* whose symbiont *Holospora obtusa* enhances the expression of heat shock genes (Fujishima et al., 2005; Manabu and Masahiro, 2003). Collectively, the examples of whiteflies, paramecia and now *Drosophila* point to a common mechanism of endosymbiont-mediated protection against heat, in which the symbiont triggers a low level of stress in the host that appear

protective upon certain circumstance. Such mechanism is reminiscent of hormesis, in which a low exposure to stress improve further the resistance to more severe stresses.

Material and methods:

Fly stocks and handling

Infected lines were obtained several years before this study by injection of *Spiroplasma*-infected hemolymph in Oregon-R females (Herren and Lemaitre, 2011). For all the experiments flies were maintained at 25°C on standard cornmeal medium. Lifespan experiments were done as described in (Herren and Lemaitre, 2011). UAS-RNAi and deficiency fly lines were obtained from Bloomington: *Hsp23* (#4409), *Hsp70B* (#28787), *Hsp70A* (#35663). Df *Hsp70B* (#8843) and *Hsp70A* (#35663). *UASp-RIP1, UASp-SpRIP2* and *UAS-SpRIP2* were obtained from (Garcia-Arraez et al., 2018).

RNA-seq

RNA-seq was performed with three single embryos for each of the four conditions (infected and uninfected embryos, males and females). To collect the embryos at the same developmental stage, we selected them based on the first zygotic mark (cephalic furrow). Total RNA and DNA were isolated from each embryo and DNA was used to determine the sex by PCR on the sex chromosomes. Protocols and primers are described in (Lott et al., 2011). Mapping was performed using STAR aligner on the BDGP6.79 genome (ensembl) (Dobin et al., 2013). HTseq was used to count the number of reads per gene (Anders et al., 2015). The dataset was filtered, and only genes that have at least one count in 50% of the samples were kept. All the count data was converted to count per million (CPM) values using voom and normalized using quantile normalization (Law et al., 2014). RPKM values were computed by normalizing CPM values by gene lengths.

RNA, DNA extractions and RT-qPCR

RNA, DNA extractions, and RT-qPCR were performed as described in (Herren et al., 2014; Herren and Lemaitre, 2011; Paredes et al., 2016). Reverse transcription was done using 500 ng of RNA per sample, which was isolated from 3 adult flies, 3 larvae, 3 pupae, or 100 to 300 embryos.

Gene expression levels were normalized to that of *Drosophila* ribosomal gene *rps17*. All qPCR calculations have been done following the ΔΔCT method and figures represent the fold change between the experimental condition samples and the controls which are valued as 1 (Livak and Schmittgen, 2001). Primers for *Hsp23* are: Forward: 5'-CCCGGTTATGAGGCTGATAAGG-3' Reverse: 5'-GTTGCCCTTATCCTCGATTGCC -3' (efficiency = 1.82). Primers for *Hsp68* are: Forward: 5'- CTCTCGTTGGGCATAGAAACCG-3' Reverse: 5'-GCATAGGTGGTGAAGGTCTTGG-3' (efficiency = 1.87). Primers for *Hsp70B* are: Forward: 5'- AGATCGGGGTGGAGTATAAGGG-3' Reverse: 5'-CCGTCTCCTTCATCTTGGTCAG-3' (efficiency = 1.94). Results have been analyzed using two-way ANOVA followed by post-hoc Tukey HSD tests. Figures and statistical results were obtained using GraphPad Prism 7.0b software. All experiments have been repeated three independent times.

Design and construction of UAS-*Hsp70B* and UASp-*Hsp70B* constructs

Transgenic fly lines for *Hsp70B* were created from a clone obtained from the *Drosophila* Genomics Resource Center (clone ID: LP05203). The construct was cloned into a UASp and a UASt vector by Gateway LR reaction and injected in *D. melanogaster* w^{1118} embryos in the laboratory (Gompel and Schröder, 2005).

Embryo mortality

Embryos were collected from 5 to 7 days old flies by using cages and yeasted grape juice plates. A total of 100 embryos were collected per genotype on grape juice plates 15-20 hours AEL. After 10 more hours, the number of remaining embryos that did not hatch (dead embryos) was counted. Experiments were done simultaneously with two different drivers, the ubiquitous *Da*-GAL4, and the maternal MTD-GAL4.

Heat tolerance

Four tubes with 25 two-weeks-old female flies were put in an incubator at 37°C. After 3 hours, still flies at the bottom of the tube were counted without moving the tube from the incubator. All experiments have been repeated three independent times.

Acknowledgments

We sincerely thank Jean-Philippe Boquete for the *UASt-Hsp70B* and *UASp-Hsp70* plasmid injections in *Drosophila* embryos and Emile Bourban for technical help.

Chapter V: Conclusions

The goal of my PhD project was to better characterize the interactions between insects and their endosymbionts. Understanding the mechanisms that underlie such relationships could contribute to finding new leads to solve global humanitarian problems, as endosymbionts improve the adaptive and invasive power of disease vectors and plant pests. To address this, I used as a model *Drosophila melanogaster* infected by its facultative endosymbiont *Spiroplasma poulsonii.* While powerful genetics tools are available in *D. melanogaster*, molecular techniques are not yet applicable in *S. poulsonii.* Consequently, bacterial factors that contribute to the host-symbiont relationship remain largely unknown.

To investigate the bacterial factors further, we developed a new medium to culture *Spiroplasma in vitro*. Cultivating *Spiroplasma poulsonii* is an old idea, as a culture medium had been developed already in 1986 (Hackett et al., 1986). This is, however, the first time a medium allows the growth of *Spiroplasma poulsonii* in other laboratories. Key factors to achieve growth were to add fly extract and lipids to the medium. Using our medium, we compared the transcriptomes of *Spiroplasma* grown *in vitro* versus in the host. Our analysis revealed a total of 466 genes that are differentially expressed between the two conditions, a subset of which could be involved in the *Spiroplasma-Drosophila* interaction.

A very important discovery from this study was the identification and sequencing of one plasmid in *S. poulsonii,* together with the detection of a comE operon, indicating that *S. poulsonii* might be naturally competent for DNA uptake, which opens possibilities for genome engineering. Since a vast majority of *Spiroplasma* genes have no homology in databases, making any prediction about their function difficult, genome engineering will be crucial to study gene function in *Spiroplasma*.

I focused on the virulence factors that were up-regulated in bacteria extracted from the host compared to bacteria grown in culture medium. *S. poulsonii* encodes 18 virulence factors, and 2 of them (ARP5 and Spaid) are located on a plasmid, which indicates that extrachromosomal DNA may play an important role in bacteria-host interactions. This hypothesis is reinforced by the recent findings that the plasmid-borne gene Spaid is the main *Spiroplasma* male-killing factor (Harumoto and Lemaitre, 2018).

Among the virulence factors upregulated in host-grown *S. poulsonii,* I focused on *Spiroplasma* RIPs (*Sp*RIPs). These toxins were recently described for their involvement in host protection against parasites (Ballinger and Perlman, 2017; Hamilton et al., 2016). However, our transcriptomics analysis suggested that *Drosophila* hemolymph activates RIP expression in *Spiroplasma*. In this study, we have provided evidence that *Sp*RIPs shorten *Drosophila* life span and increase embryonic mortality. Of note, female embryos survived exposure to the toxin better than males, leading to a female bias in the adult population exposed to these toxins. This phenotype is especially relevant as it points to a possible secondary role of *Sp*RIPs in male killing.

Male killing enhances the spread of *Spiroplasma* in natural fly populations. Recently discovered proteins involved in *Spiroplasma* male killing, *Wolbachia* male killing and *Wolbachia* cytoplasmic incompatibility point to a common mechanism based on DNA deubiquitinase activity (Harumoto and Lemaitre, 2018; Le Page et al., 2017). However, our results indicate that *Spiroplasma* could still induce partial male killing in the absence of Spaid, suggesting the existence of additional factors.

Further characterization of the *Sp*RIP-*Drosophila* interaction also revealed a mechanism that partially mitigates the deleterious effects of these toxins. *Hsp70B* is up-regulated in *Spiroplasma*-infected flies and uninfected flies overexpressing *SpRIPs.* Ectopic expression of *Hsp70B* in infected flies increases life span and

embryo survival, indicating that this gene may be involved in mitigating the action of the toxin. However, the mechanism by which *Hsp70B* increases life span and embryo survival upon *Spiroplasma* infection remains unknown. As *Sp*RIPs target and inactivate ribosomes, we hypothesized that *Hsp70B* in infected flies extends the half-life of proteins, thus partially correcting for the lack of *de novo* synthesis (Meimaridou et al., 2009; Young et al., 2017).

Further characterization of infected flies revealed that the presence of *Sp*RIPs conferred a mild thermo-tolerance. Heat induces over-expression of *Hsp70B,* as a natural response to any stress. *Hsp70B* over-expression in *Spiroplasma*-infected flies could thus be a consequence of a general stress response. However, the fact that uninfected flies expressing *Sp*RIPs displayed the same thermo-tolerance suggests that this effect is directly linked to RIPs, although we cannot exclude the implication of other factors. This thermo-tolerance is biologically relevant because tolerance to a specific stress can result in a broader resistance to different environmental stresses (Gotcha et al., 2018). *Sp*RIPs thus shorten life span in adult flies, while conferring protection against natural parasites and promoting cross-tolerance to adapt the host to new niches. More interestingly, this process seems to be conserved between endosymbiotic *Spiroplasmas.* Aphids harboring *Spiroplasma* also up-regulate the expression of several Heat-Shock Proteins (Guidolin et al., 2018). Whether *Spiroplasma* can also confer tolerance to other types of stress such as dehydration or starvation remains to be tested. *Spiroplasma* RIP toxins are a good illustration of the hormesis concept, where a dose response to an external low stress results in stimulation or beneficial effect but a high dose becomes toxic (Calabrese et al., 2007).

This entire context highlights the complexity of symbiosis and how each factor affects the whole system. *Spiroplasma* RIP toxins induce a hormetic dose response in the host which ultimately benefits from the toxins. Parasitoid wasps and

Spiroplasma require *Drosophila* as the niche to survive. Both parasites have evolved to efficiently spread their own species in nature, either by killing male embryos, or by using fly larvae as storage of nutrients. Wasps and *Spiroplasma* are in direct competition, but *Spiroplasma* succeeded in biasing *Drosophila* co-evolution in its favor: to avoid being too harmful to their host, *Spiroplasma* secretes *Sp*RIPs that protect against parasites, re-enforce male-killing, shorten life span and promote thermo-tolerance to adapt to new niches.

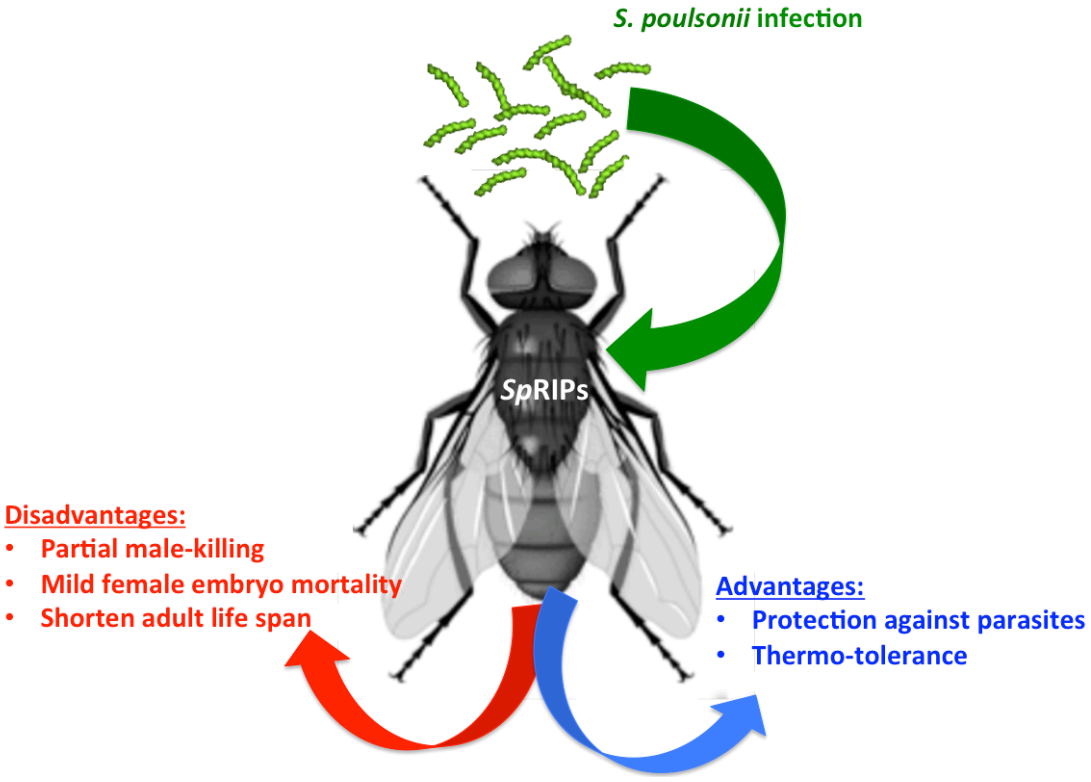
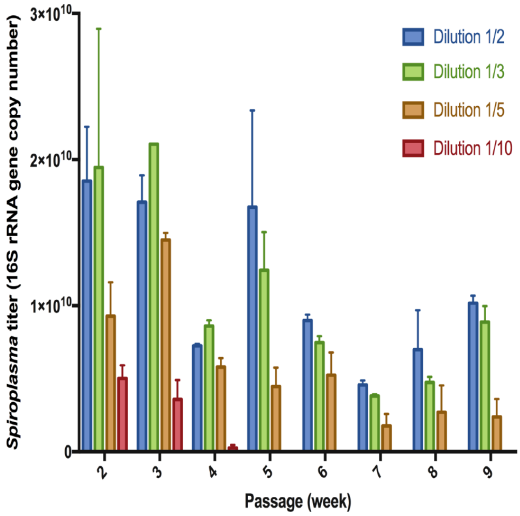
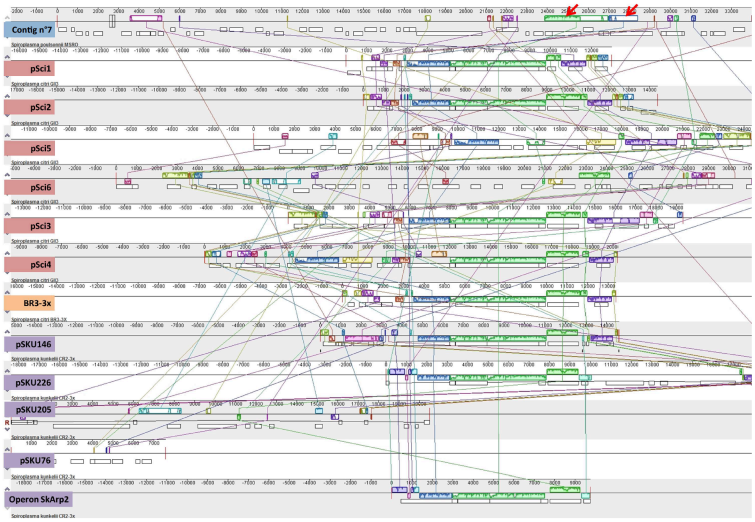


Figure 21. *Spiroplasma* infection trade-off regarding the effect of RIPs

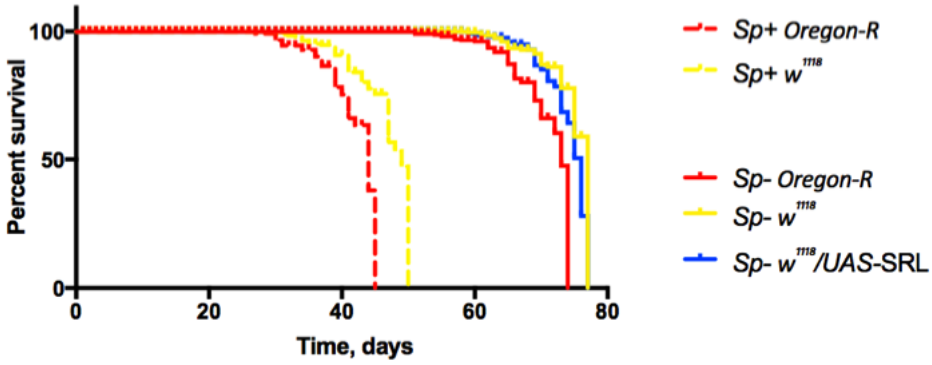
Appendices



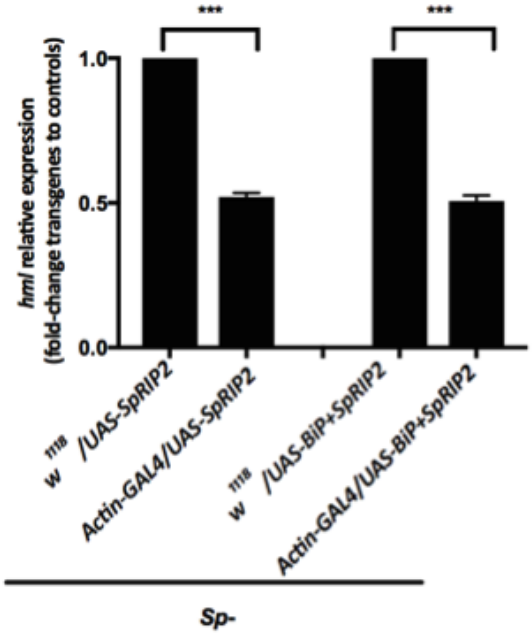
Supplementary figure 1. *S. poulsonii* titer in BSK-H-spiro diluted weekly with fresh medium. Each bar represents the average titer in three independent repetitions. Error bars represent standard deviations. *S. poulsonii* was not detectable by qPCR at the passage 5 onwards when the culture was diluted by 10.



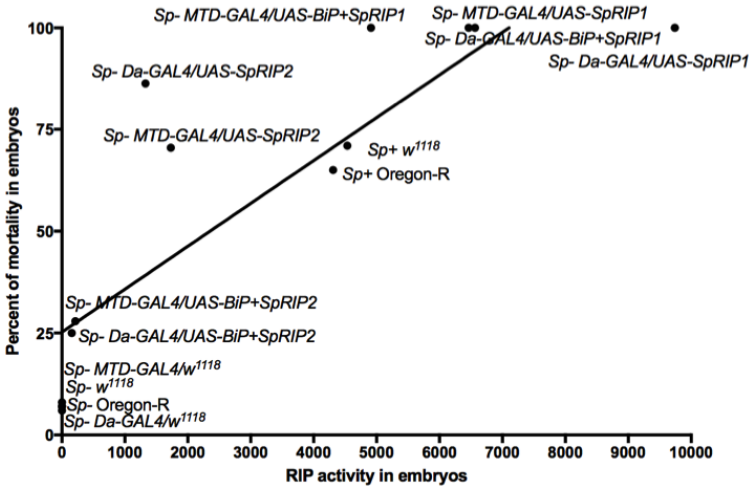
Supplementary figure 2. Multiple alignment of contig #7 with reference plasmid sequences of *Spiroplasma* citri and *Spiroplasma* kunkelii using the MAUVE progressive algorithm. Conserved synteny blocks (red arrows) contain an ARP (green block) and a traE operon (blue block).



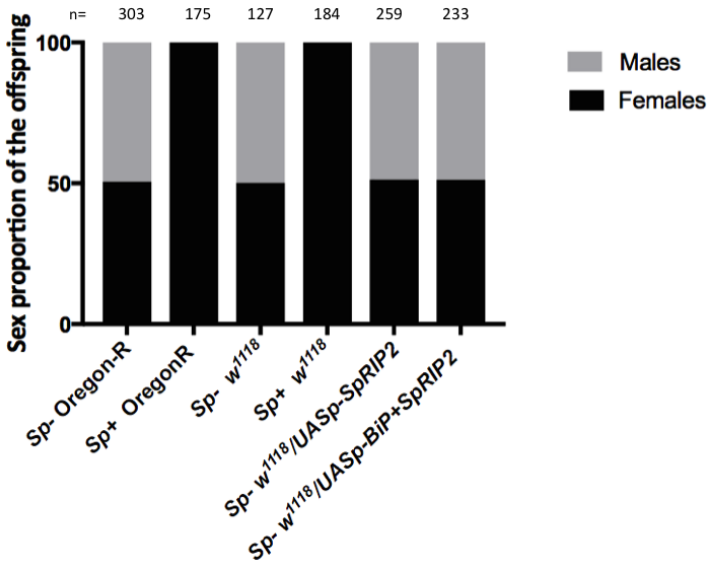
Supplementary figure 3. Lifespan of infected and uninfected wild types flies (controls for Figure 12). *Sp-* and *Sp+* refer to uninfected or *Spiroplasma*-infected condition respectively.



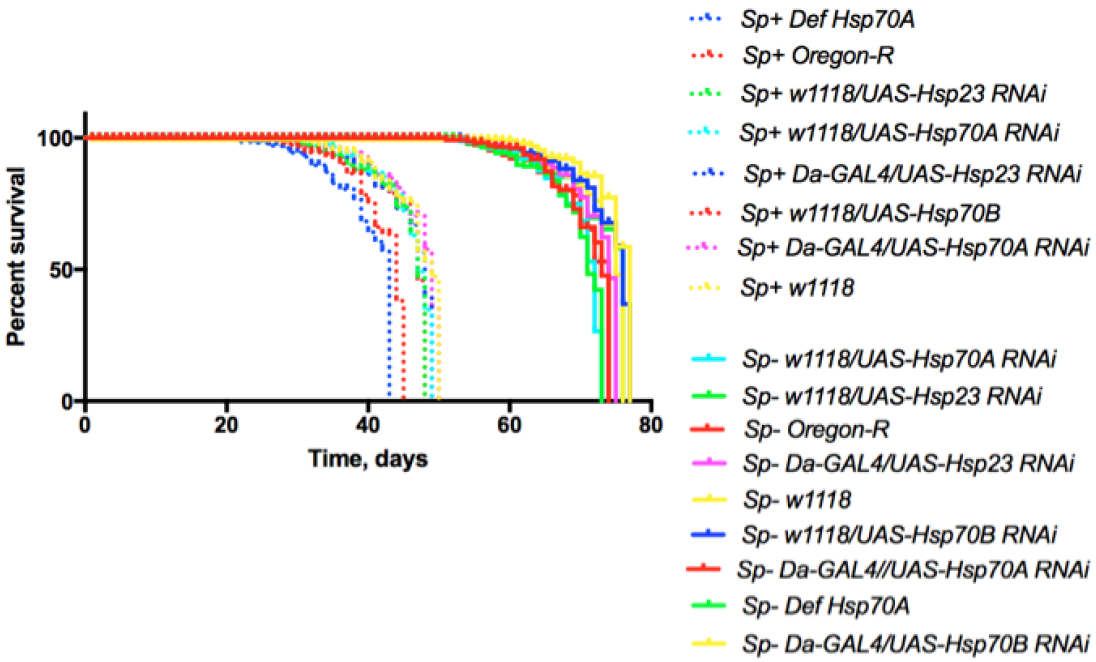
Supplementary figure 4. *hml* transcription level in lines expressing *UAS-SpRIP2* and *BiP+SpRIP2* under *actin-GAL4* control. Expression of both constructs also leads to a decrease in hemocyte number (Dunnett's multiple comparisons test p***< 0.0001). Controls are normalized as 1.



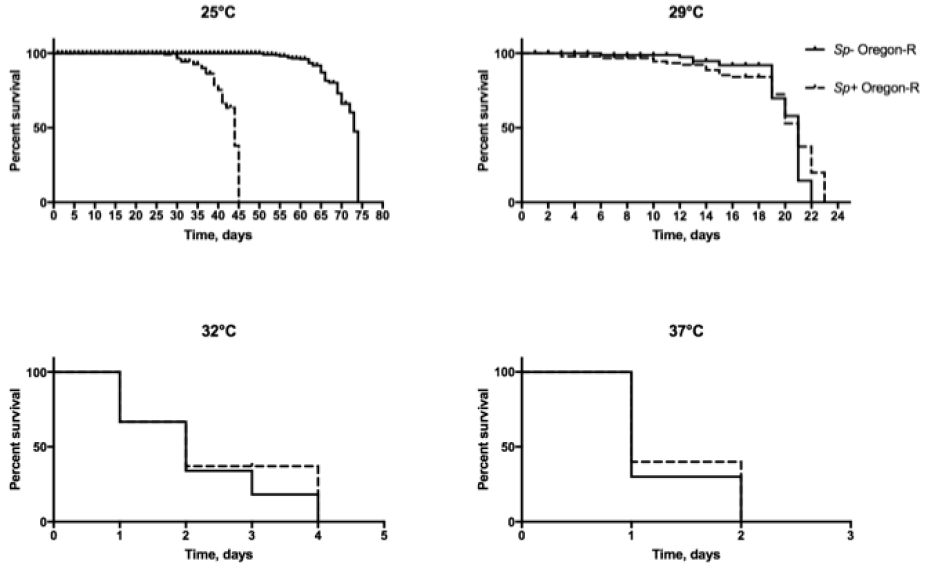
Supplementary figure 5. Correlation between RIP activity in embryos aged 0 to 24h after egg laying and embryo mortality (Pearson's test $p<0.0001$). RIP activity in infected wild types was normalized by uninfected samples. Transgenic fly lines were normalized by *Da*-GAL4/*w1118*. Controls are normalized as 1.



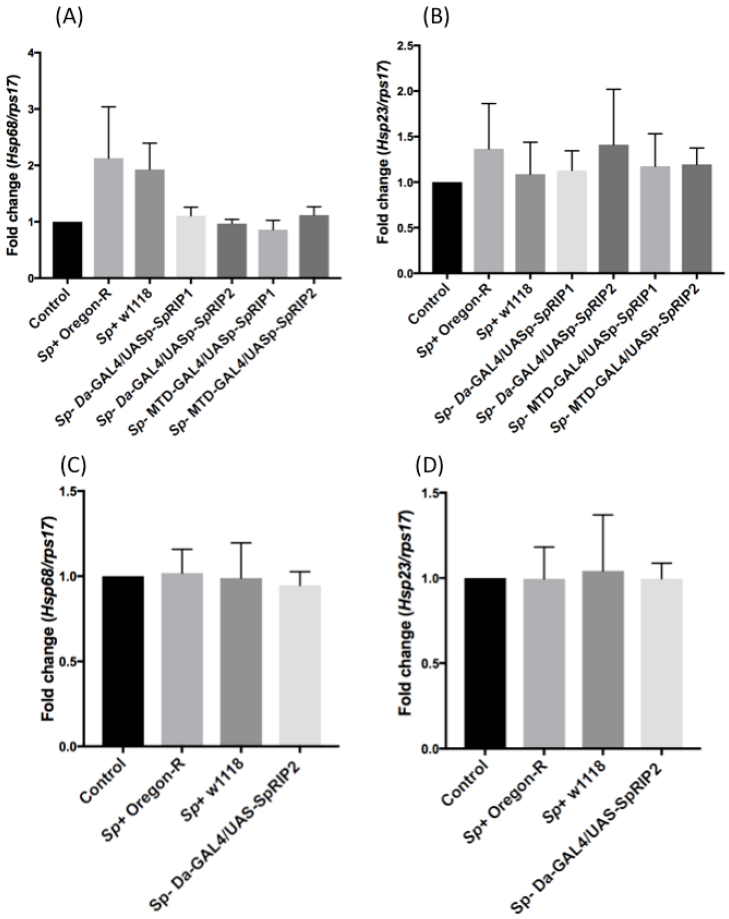
Supplementary figure 6. Sex ratio of the control fly lines for Figure 14. *Sp-* and *Sp+* refer to uninfected or *Spiroplasma*-infected condition, respectively.



Supplementary figure 7: Lifespan of infected and uninfected wild types flies and fly lines silenced for *Hsp70A* and *Hsp23* genes. *Sp-* and *Sp+* refer to uninfected or infected condition, respectively.



Supplementary figure 8: Lifespan of infected and uninfected wild types flies at 25°C, 29°C, 32°C and 37°c. *Sp+* and *Sp-* refer to infected or uninfected condition, respectively.



Supplementary figure 9: (A) Expression level of *Hsp68* in embryos expressing *SpRIPs* (Two way ANOVA for fly genotype p<0.5676). **(B)** Expression level of *Hsp23* in adult flies expressing *SpRIPs* (Two way ANOVA for fly genotype p<0.7231). **(C)** Expression level of *Hsp68* in adult flies expressing *SpRIPs* (Two way ANOVA for fly genotype p<0.9113). **(D)** Expression level of *Hsp23* in adult flies expressing *SpRIPs* (Two way ANOVA for fly genotype p<0.8143). Expression is indicated as a fold-change infected versus uninfected, uninfected being represented as 1. *Sp*- and *Sp*+ refer to uninfected or infected condition,v respectively.

Supplementary data 1:

Fly extract preparation

- Collect 1 to 7-days-old flies infected with *Spiroplasma*. Flies can be stored at -20°C for later use

- Add 30 mL of BSK-H medium without L-glutamin (BioSell) for 6 g of flies and crush thoroughly with a Dounce tissue grinder

- Incubate 20 minutes at 56°C

- Centrifuge 15 minutes at 3000 g and collect the supernatant

- Filter at 0.45 µm

- Filter at 0.22 µm

- Store at -20°C

Lipids mix preparation

- Dissolve 10 mg of cholesterol, 5 mg of palmitic acid and 10 mg of sphingomyelin in 1.8 mL of 100% ethanol preheated at 30°C.

- Add :

100 µL of 1-palmitoyl-2-oleoyl-sn-glycerol (stock at 10 mg/mL in ethanol)

b)100 µL of 1,2-dioleoyl-sn-glycerol (stock at 20 mg/mL in ethanol)

c) 50 µL of Tween 40

d) 50 µL of Tween 80

e)5.6 µL of oleic acid

- Vortex thoroughly

- Mix 400 µL of the lipids premix to 19.6 mL of fatty-acid-free BSA 6% to form the lipid mix usable in the BSK-H-spiro medium preparation. Store the premix and the mix at -20°C.

BSK-H-spiro preparation (400 mL of medium):

- 314 mL of BSK-H without L-glutamine (BioSell)

- 2 mL of penicillin G 130 mg/mL

- 10 mL of arginine 70 mg/mL

- 24 mL of rabbit serum heat-inactivated

- 30 mL of fly extract (7.5% final)

- 20 mL of lipids mix (5% final)

-Adjust the pH to 7.5 with HCl and filter at 0.22 µm.

Penicillin G and arginine stock solutions must be prepared in BSK-H rather than water. Do not autoclave the final medium or any of its components. Store at 4°C.

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Glossary

D

Drosophila: Genus of flies belonging to the family Drosophilidae. One specie of this genus in particular*, D. melanogaster*, has been heavily used in research in genetics and is a common model organism.

E

Ectosymbiosis: It refers to symbiotic associations where the symbiont resides on the surface of the host, including internal surfaces such as the epithelial tissues of the digestive tube and the ducts of glands.

Endosymbiosis: Symbiotic association where the symbiont lives inside the host.

F

Facultative endosymbiosis: Endosymbiotic associations whose prevalence is below 100% in wild populations.

H

Heat-Shock Proteins (HSPs): Cytosolic chaperones that play an essential role in protein homeostasis under normal and stressful conditions.

M

Male killing: Reproductive manipulation phenotype induced by endosymbionts. Male-killing results specifically in the death of infected male embryos, while infected females survive.

O

Obligate endosymbiosis: Endosymbiotic association that refers to an association whereby a host and an intracellular endosymbiont require each other to survive and reproduce.

R

Ribosome-inactivating proteins (RIPs): Toxins that prevents protein synthesis in eukaryotic cells.

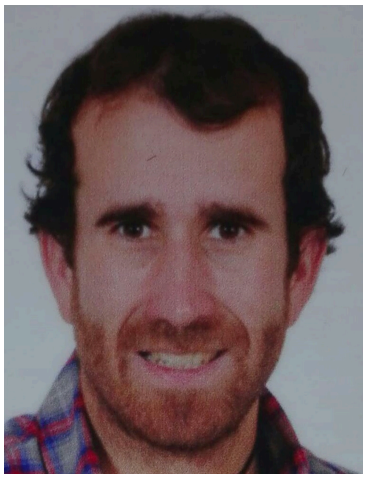
S

Spiroplasma: Long helical Gram-positive bacteria belonging to the Mollicute clade.

Symbiosis: association between organisms belonging to different species, regardless of the duration or the potential benefit or hindrance for the partners.

Index

Curriculum Vitae



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EDUCATION AND FORMATION

- **Degree**

  Biology
  Universidad de Valencia. Valencia, Spain
  Years: 2005-2010

- **Masters**

  Science teaching
  Universidad Jaume I. Castellon, Spain
  Years: 2011-2012

  Health and medical biotechnology
  Universidad Pablo de Olavide. Seville, Spain
  Kings's college London. England
  Years: 2012-2014

- **Online courses**

  Mini-MBA
  London Institute of management
  Year: 2017

  Business Consulting 101
  Universal class
  Year: 2017

Specialties during the degree

Cytogenetic (October, November & December 2006)
Molecular Genetic (November and December 2008)
Human genetic (year 2008-2009)
Developmental genetic (October, November and December 2009)

Animal (October, November and December 2007)
Microorganisms and diseases (October, November and December 2008)
Immunology (March, April and May 2009)
Protistology (October, November and December 2009)

Physical Anthropology (March, April and May 2007)
Animal Evolution (October, November and December 2008)
Developmental Biology (course 2007-2008)
Animal reproductive biology (March, April and May 2008)

Physiology of Plant Growth and Development (October, November and December 2009)
Plant Biotechnology (March, April and May 2010)

Oceanography (October, November and December 2006)
Ecology of fish stocks (March April / May 2010)
Marine Biology (course 2009-2010)

Complementary courses during the degree

Nutrition (March 2008)
Scientific English (October, November and December 2005)
The natural environment for sport (March 2006)
Botany for Educators (March, April and May 2008)
Participation in conferences of the Disability Sports Forum (November 2009)
Trail Design and Construction (April 2009)
Mountain biking (historical 2009nante November (March, April and May 2006)
Kayak Polo (March 2010)

Publications: (ORCID 0000-0002-6434-9127)

Masson, F., Copete Sandra, C., Schüpfer, F., Garcia-Arraez, G., Lemaitre, B., 2018. In Vitro Culture of the Insect Endosymbiont *Spiroplasma poulsonii* Highlights Bacterial Genes Involved in Host-Symbiont Interaction. mBio 9, 1–11.

Garcia-Arraez, G., Masson, F., Paredes, J.C., Lemaitre, B., 2018. Contribution of *Spiroplasma poulsonii* RIP toxins to *male-killing* and life span shortening phenotypes in Drosophila melanogaster. In revision (BMC Biology).

Garcia-Arraez, G., Masson, F., Rommelaere, S., Sleiman, M., Lemaitre, B., 2018.*The Drosophila heat-shock-protein 70B mitigates Spiroplasma poulsonii* deleterious effects and promotes endosymbiont-mediated heat tolerance. In progress.

Professional experience:

Science teacher

Institution: IES Francesc Ribalta Time: 6 months

Institution: EPFL Time: 300 hours

Kayak instructor

Institution: Club Nautico Castellon Time: 9 months

Life science researcher as PhD student

Institution: EPFL, GHI Time: 4 years

Awards and grants:

Best track record in kayaking for under 18 years old (Castellon -December 2000-)
https://www.castello.es/archivos/492/FIESTA-HOMENAJE%202000_web.pdf

Ls2 travel grant for PhD student
HTTPS://WWW.LS2.CH/FUNDING/TRAVEL-GRANT-REPORTS

LANGUAGES

NATIVE LANGUAGE **Spanish**

OTHER LANGUAGES	**Catalan** (Valenciano)	**English**	**French**
Reading	Fluent	Fluent	Basic
Writing	Good	Fluent	-----
Speaking	Good	Fluent	Basic

ARRANGE SKILLS Co-founder “Club regatas Castellon”

DRIVING LICENSE Category B

SOCIAL SKILLS One summer in Ireland through HelpX (2011)

Kayaking (since 1995)

- Real Club Nautico de Castellon (1995-2005)
- Kayak club Silla (2005-2007)
- Asociacion deportiva Pinatar (2008-2009)
- Real Club Nautico de Castellon (2009-2010)

Boy scout (1995-2004)