

# Testing cannibalism as a mechanism for horizontal transmission of *Wolbachia* in *Drosophila*

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**Abstract** *Wolbachia* are intracellular symbionts of many species of animals, mostly arthropods. Vertical transmission of *Wolbachia* is exclusively maternal and this endobacterium promotes reproductive manipulations of its hosts, increasing the fitness of infected females. Moreover, *Wolbachia* provides its hosts with a wide range of adaptive features ranging from protection against viral infections to dietary niche occupancy. Therefore, *Wolbachia* can potentially contribute to the evolutionary processes of sexual selection and speciation. The horizontal transmission of *Wolbachia* is strongly suggested by the non-concordant phylogeny of this endosymbiont and that of its hosts. However, the ecological mechanism(s) responsible for endosymbiont transmission between different hosts is still largely unknown. In the present study, we look at ingestion as a possible natural form of *Wolbachia* horizontal transmission. To this aim, we tested cannibalism between infected and uninfected *Drosophila* hosts, under different conditions of nutrition and gut integrity. Although ingestion represents a general and incontestable portal of entry for microorganisms, we did not find infection by *Wolbachia* in the progeny of cannibal individuals fed on infected flies. Our study suggests that if ingestion is a vehicle for horizontal transmission of *Wolbachia* in nature,

either it happens very rarely or it requires other factors or conditions to be effective. We discuss the likeliness of this mechanism with respect to the likelihood of each step necessary for horizontal transmission.

**Keywords** *Wolbachia* · Horizontal transmission · Cannibalism · *Drosophila* · Ingestion · Endosymbiosis

## 1 Introduction

The  $\alpha$ -proteobacteria of the Genus *Wolbachia* live intracellularly in a variety of animals, including arthropods and nematodes (Werren 1997; Harris et al. 2010). In arthropods, *Wolbachia* is typically transmitted vertically from mother to offspring. It causes a wide range of reproductive manipulations in different host species whereby increasing the fitness of infected females and, consequently, also increasing its own transmission rate (Charlat et al. 2003). These mechanisms include: (i) the induction of cytoplasmic incompatibility between individuals that do not share infection status, (ii) the induction of parthenogenesis in diploid females and (iii) the feminization or death of infected males (for revision see Werren et al. (2008)). Additionally, recent studies have shown that in *Drosophila melanogaster*, *Wolbachia* infection may also confer an advantage to its host through an increased resistance to RNA virus infection (Hedges et al. 2008; Teixeira et al. 2008).

It is estimated that *Wolbachia* infects 20–80 % of insect species (Jeyaprakash and Hoy 2000) possibly making it the most recurrent endosymbiont on the planet. The wide distribution of these bacteria is attributed to the high efficacy of vertical transmission. This efficacy may rely on *Wolbachia* using the host's cytoskeleton and intracellular transport system to migrate towards the germline precursors and ensure its

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presence inside future embryos (Ferree et al. 2005; Serbus and Sullivan 2007). In addition to the colonization of the germline during embryogenesis, *Wolbachia* remaining inside the embryo are internalized in progenitor cells of the somatic tissue (Frydman et al. 2006; Goto et al. 2006), with potential physiological and evolutionary consequences (Faria and Sucena 2013).

The widespread presence of *Wolbachia* must also rely on horizontal transmission, which can be attested by the presence of close strains of *Wolbachia* in phylogenetically distant hosts (Vavre et al. 1999; Baldo et al. 2008). Indeed, unlike mitochondria or obligatory bacterial endosymbionts, the molecular phylogeny of *Wolbachia* is not always concordant with that of its hosts (Werren and O'Neill 1997; Jiggins et al. 2002). These well-established patterns raise two important questions: i) which ecological conditions and mechanisms mediate horizontal transmission and ii) how does a transient horizontal transfer turn into a stable vertical transmission? Regarding this problem Frydman and colleagues reported that when haemolymph of an infected *D. melanogaster* fly is microinjected into adult uninfected females, *Wolbachia* could be transmitted vertically (Frydman et al. 2006). After 15 days upon haemolymph microinjection into uninfected female flies, *Wolbachia* could be detected in their offspring after preferentially establishing itself in the ovaries somatic stem cell niches. Also, it has been shown that *Wolbachia* is viable for several days outside the host's cell, thus allowing for a possible transfer across cells (Rasgon et al. 2006). Together these reports provide a link between horizontal and vertical transmission, indicating that any mechanism capable of introducing *Wolbachia* into the female's haemolymph may permit the establishment and perpetuation of *Wolbachia* in new hosts.

Despite their importance for understanding the epidemiological and evolutionary dynamics of *Wolbachia* infection, the ecological mechanisms responsible for the transfer of bacteria to new hosts in nature are still largely unknown (Haine et al. 2005). One strong candidate mechanism consists of parasitoid wasps acting as *Wolbachia* vectors. This is based on different evidence: i) the extensive similarities between *Wolbachia* strains found in parasitoids and their hosts (Vavre et al. 1999; Li et al. 2013); ii) *Wolbachia* can be transmitted to a parasitic wasp from its infected host (Heath et al. 1999; Morrow et al. 2014); iii) when infected and uninfected parasitoid wasp larvae share the same host egg, intra- and inter-specific horizontal transfer of parthenogenesis-inducing *Wolbachia* may occur (Schilthuisen and Stouthamer 1997; Huigens et al. 2000; Huigens et al. 2004). Another hypothetical vector for horizontal transmission of *Wolbachia* are ectoparasitic mites, known to transfer the *Drosophila* endosymbiont, *Spiroplasma poulsoni*, from infected *D. nebulosa* to *D. willistoni* (Jaenike et al. 2007). Based on our observations of *Drosophila* larval and adult behaviour in crowded

environments, we reasoned that cannibalism or scavenging, often witnessed not only in the laboratory but also in nature, could constitute a route for horizontal *Wolbachia* transfer. Moreover, occasional horizontal transmission via the oral route has been reported for the pea aphid *Bemisia*-like symbiont (Darby and Douglas 2003). Indeed, the digestive system is considered to be the major interface between the insect host and the microbial environment, constituting a privileged gateway for microorganism invasion (Douglas and Beard 1996). However, as most ingested bacteria are eliminated by the immune system or by peristalsis, few bacteria can persist in large numbers in the digestive tract of insects (Vallet-Gely et al. 2008). Nonetheless it is important to note that some bacterial species ensure their proliferation in recent hosts by passing through the digestive tract to other organs or cavities (Marsollier et al. 2005; Chiel et al. 2009).

Recent studies have demonstrated that, after predation of infected hosts, previously uninfected isopods, *Armadillidium vulgare* and *Porcellio dilatatus dilatatus*, would become infected with *Wolbachia* (Le Clec'h et al. 2013). Also, in the ant *Acromyrmex echinator*, it has been hypothesized that the faecal-oral route could constitute a means for horizontal transmission of *Wolbachia* (Frost et al. 2014).

In this work, we have tested if upon ingestion *Wolbachia* could be transmitted stably to the offspring of a *Drosophila* host. For this, several ingestion experiments were performed using infected and uninfected hosts of *D. melanogaster* and *D. simulans*, at different developmental stages. Nutritional variation, dehydration and intestinal injury were used in an attempt to mimic naturally-occurring potentiating factors for the passage of *Wolbachia* into the body cavity of the fly and the subsequent establishment of a symbiotic relationship with the new host. Through a PCR-based analysis of the offspring we were unable to find any infection by *Wolbachia*, both in early and late progeny. This result suggests that the ingestion of *Wolbachia* by a non-infected new host is not sufficient in itself to establish a stable infection horizontally or is too rare to be detected within the limits of our experiment.

## 2 Materials and methods

### 2.1 Foundation and maintenance of *drosophila* outbred populations

Outbred populations of *Drosophila melanogaster* and *Drosophila simulans* were established in the laboratory (Martins et al. 2013). *Wolbachia*-infected *D. melanogaster* and *D. simulans*, collected from the southwest of Portugal (Azeitão) were used to establish two laboratory populations (MeO<sup>+</sup> and SimO<sup>+</sup>, respectively). After over 50 generations in the laboratory, MeO<sup>+</sup> and SimO<sup>+</sup> were replicated for the

establishment of four new populations: two infected with *Wolbachia* as the founding populations ( $mel^+$  and  $sim^+$ ) and two treated with tetracycline during four generations for total *Wolbachia* elimination ( $mel^-$  and  $sim^-$ ). We confirmed the absence of *Spiroplasma* in all populations. For the *Serratia* assays, the *D. simulans* populations were established using two isofemale lines from the Drosophila Species Stock Centre (UC San Diego, California, US)  $sim^+$  (14,021–0251.138) and  $sim^-$  (14,021–0251.01). All populations were kept in cages with an effective size between 1500 and 2000 individuals with non-overlapping generations, in a day/night cycle of 12 h, constant temperature of 25 °C, standard level of relative humidity (70 %) and fed on standard cornmeal-agar medium. The infection status of populations was monitored regularly through PCR (see below).

## 2.2 *Wolbachia* extraction

*Wolbachia* was extracted by crushing 100 infected adults or approximately 500 embryos of *D. melanogaster* or *D. simulans*, previously washed in 70 % ethanol, and transferred to 1 mL of ice-cold PBS (adapted from (Frydman et al. 2006)). For adult co-infected ingestion assays and adapting a protocol described previously (Rasgon et al. 2006), *Wolbachia* was extracted by smashing approximately 500 infected flies in 10 mL of Schneider's medium. The confirmation of bacterial viability after extraction was also performed as described in Rasgon et al. (2006). In all cases, the homogenate was used entirely.

## 2.3 Adult ingestion assay

For ingestion experiments with adults, 4–7 day old females were used from the  $mel^-$  population. From the regular stock of flies (which were maintained in rich medium), 20 replicates of 20 adult females were used to exclusively ingest 250  $\mu$ L of a *Wolbachia*-containing suspension homogenized in PBS (from infected adults of  $mel^+$  populations) for a period of 48-h. These experiments were also undertaken with a previous 72-h treatment either with a poor medium (rich medium diluted 1:10 in water) or in a condition of starvation, where the females spent a 48-h period in total absence of nutritional resources until the beginning of the ingestion treatment.

## 2.4 Larval ingestion assay

For the ingestion experiments with larvae, we used  $mel^-$  larvae from the three larval stages. Larvae ingested a homogenate, containing adults (or embryos), from  $mel^+$  or  $sim^+$  populations infected with *Wolbachia* for a period of 24 h. In each of the experiments, 5 replicates of 50 larvae were fed on 500  $\mu$ L of homogenate from 40 flies.

## 2.5 Adult co-infected ingestion assays

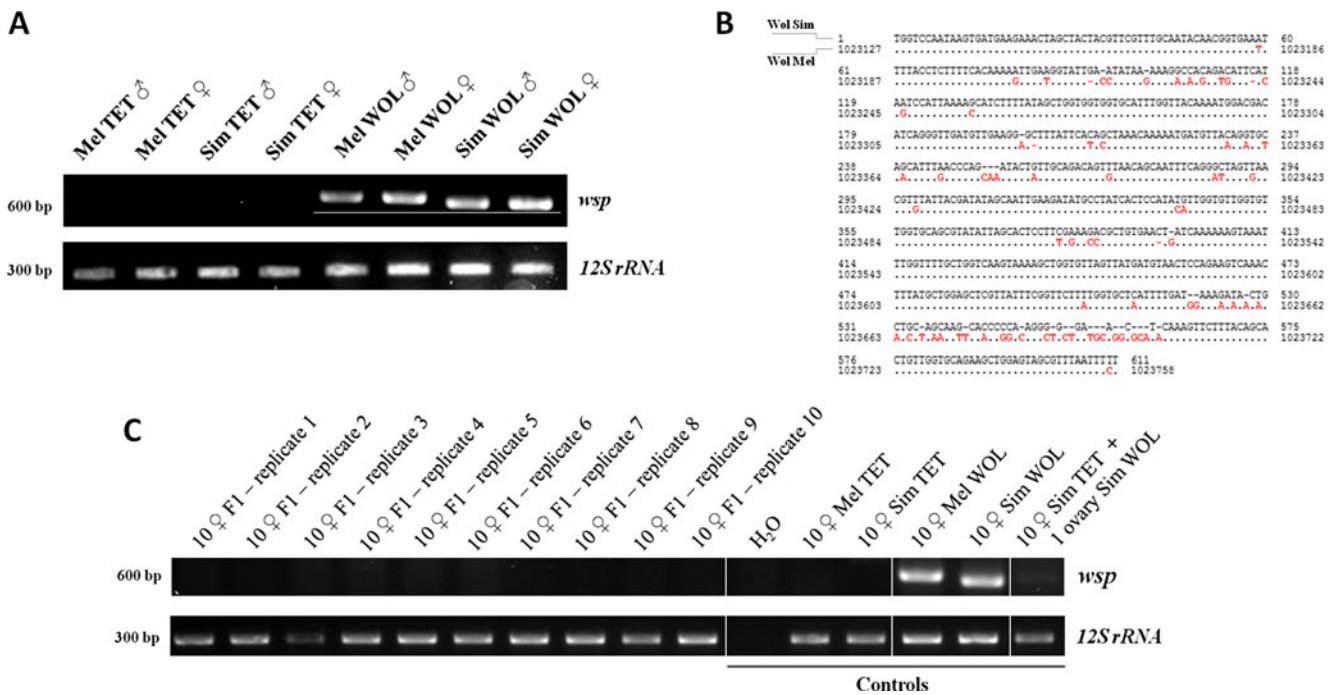
For ingestion experiments with adults, 4–7 day old females were used from the  $mel^-$  population. From the regular stock of flies, 10 adult females were used per replicate to exclusively ingest i) 250  $\mu$ L of *Serratia marcescens* (a kind gift from B. Lemaitre) for a period of 24 h, or ii) 250  $\mu$ L of a *Wolbachia*-containing suspension for a period of 24 h. The food solution containing *Serratia* was prepared from an overnight culture grown exponentially at 37 °C and was diluted with a sterile 50-mM sucrose solution to a final  $OD_{600} = 15$ . These experiments were also undertaken either with *Wolbachia* with a previous 24-h ingestion treatment with LB or with *Serratia* and posterior treatment with  $sim^-$  and  $mel^-$ .

## 2.6 Diagnostic PCR

In all procedures, tested females gave rise to the adult  $F_1$  from which genomic DNA was extracted (in pools of 10 adult females) and screened for *Wolbachia* infection by PCR through the amplification of a *wsp* gene fragment using primers  $wsp81F$  5'TGG TCC AAT AAG TGA TGA AGA AAC 3' and  $wsp691R$  5'AAA AAT TAA ACG CTA CTC CA 3' (Zhou et al. 1998). *Wolbachia* strains of *D. melanogaster* and *D. simulans* generate PCR amplicons of different sizes, 632 bp and 611 bp, respectively. This diagnostic PCR was further confirmed in 10 % of the samples chosen randomly by sequencing the respective PCR products.

## 3 Results and discussion

We fed *D. melanogaster* larvae and adults of the *Wolbachia* negative outbred population ( $mel^-$ ) with embryo or adult fly homogenates from *Wolbachia* infected populations of *D. melanogaster* ( $mel^+$ ) and *D. simulans* ( $sim^+$ ). As controls we applied the same procedures using homogenates from uninfected populations referred to as  $mel^-$  and  $sim^-$ . The status of *Wolbachia* infection of the populations used in these experiments is shown in Fig. 1A, also illustrating the size difference between *wsp* gene amplification products of *Wolbachia* strains from *D. melanogaster* and *D. simulans*. Confirmation of the different strains was obtained by sequencing the *wsp* gene fragment (Fig. 1B). These results validate our procedure for the simultaneous determination of the infection status and *Wolbachia* strain present in individual or pooled adult flies (as to ascertain instances of intra- or interspecific transmission). We tested the  $F_1$  of fed females at two time points: early  $F_1$  (8 to 10 days) and in late  $F_1$  (more than 15 days), determined by the description of *Wolbachia* dynamics upon entry into the haemolymph and subsequent stable establishment in the germline (Frydman et al. 2006). A representative gel of the



**Fig. 1** Screen for *Wolbachia* in the initial and tested populations. **a** Infection status in males and females of initial populations – F0; **b** Differentiation of *Wolbachia* strains of *D.melanogaster* and *D.simulans*

by *wsp* gene sequencing; **c** Representative PCR for *Wolbachia wsp* gene in tested females progeny, indicating *Wolbachia* absence in F1 (10 replicates + controls)

PCR-based screen for *Wolbachia* infection is presented in Fig. 1C.

Larval ingestion could lead to the stable transmission of *Wolbachia* by one of two ways: i) establishing itself in cells of somatic tissue, surviving the metamorphosis stage of the host and colonizing the ovaries of adult females, or ii) crossing the epithelium of the digestive system and colonizing the stem cells of the future ovary. We fed *D. melanogaster* larvae of different stages, previously maintained in normal medium, a homogenate of mel<sup>+</sup> and sim<sup>+</sup> infected embryos or adults for 24 h (Table 1). In a second set of experiments, we placed mel<sup>-</sup> adult flies on a diet composed of a mel<sup>+</sup> adult homogenate for 48 h (Table 2 – A). If ingestion of *Wolbachia* occurs in the adult stage, it should be enough for a successful transmission that the endosymbiont

crosses the midgut and passes to the haemolymph (Frydman et al. 2006). Yet, it should be stressed that it is unclear what is the necessary concentration of haemolymph *Wolbachia* for the establishment of these bacteria in the ovaries.

Both in the larvae and adult ingestion experiments, the early and late F1 flies tested did not show the presence of *Wolbachia* (Tables 1 and 2 – A, “Wol F1e and Wol F1l”). This negative result holds true even when varying the *Wolbachia* source, both *D. melanogaster* and *D. simulans* (intra- or interspecific), and the stage at which the *Wolbachia* homogenate was extracted, embryos or adults. Our findings indicate that if horizontal transmission by ingestion occurs in nature, within or between *Drosophila* species, it is a rare event.

Another aspect to consider is that our progeny analysis treats the whole putative process of infection as a binary

**Table 1** Summary of larval ingestion assays and respective female progeny analyses

Stage	Condition	N test	Wol ext	Analyzed	Wol F1e	Wol F1l
Larvae	Rich food	50 (5) <sup>2</sup>	Adults Sim <sup>+</sup>	10 ♀	–	–
Larvae	Rich food	50 (5) <sup>2</sup>	Adults Sim <sup>-</sup>	10 ♀	–	–
Larvae	Rich food	50 (5) <sup>4</sup>	Adults Mel <sup>+</sup>	10 ♀	–	–
Larvae	Rich food	50 (5) <sup>4</sup>	Adults Mel <sup>-</sup>	10 ♀	–	–
Larvae	Rich food	50 (10) <sup>2</sup>	Embryo Mel <sup>+</sup>	10 ♀	–	–
Larvae	Rich food	50 (10) <sup>2</sup>	Embryo Mel <sup>-</sup>	10 ♀	–	–

*Stage* Developmental stage of tested individuals, *Condition* Previous treatment, *N test* number of tested individuals, ( ) number of replicates, *Superscript* number of independent experimental sets, *Wol ext* *Wolbachia* extraction, *Analyzed* number of F1 females analyzed (per replicate), *Wol F1e* presence (+) or absence (–) of *Wolbachia* in early F1, *Wol F1l* presence (+) or absence (–) of *Wolbachia* in late F1



**Table 2** Summary of adult ingestion assays and respective female progeny analyses

Stage	Condition	N test	Wol ext	Analyzed	Wol F1e	Wol F1l
<b>A</b>						
Adults	Rich food	20 (20) <sup>2</sup>	Adults Mel <sup>+</sup>	10 ♀	–	–
Adults	Rich food	20 (20) <sup>2</sup>	Adults Mel <sup>–</sup>	10 ♀	–	–
<b>B</b>						
Adults	Poor food	20 (10)	Adults Mel <sup>+</sup>	10 ♀	–	–
Adults	Poor food	20 (10)	Adults Mel <sup>–</sup>	10 ♀	–	–
Adults	Starvation	20 (10)	Adults Mel <sup>+</sup>	10 ♀	–	–
Adults	Starvation	20 (10)	Adults Mel <sup>–</sup>	10 ♀	–	–

*Stage* Developmental stage of tested individuals, *Condition* Previous treatment, *N test* number of tested individuals, () number of replicates, *Superscript* number of independent experimental sets, *Wol ext* *Wolbachia* extraction, *Analyzed* number of F1 females analyzed (per replicate), *Wol F1e* presence (+) or absence (–) of *Wolbachia* in early F1, *Wol F1l* presence (+) or absence (–) of *Wolbachia* in late F1

outcome (F<sub>1</sub> infected or non-infected) and cannot pinpoint the critical step at which the infection fails to progress. We may consider the absence of *Wolbachia* in the *D. melanogaster* F<sub>1</sub> flies as the product of low probability events, each one necessary for the occurrence of horizontal transmission. We can formalize this idea through the equation:

$$P_{HT(W)} = P_{EI}(\alpha) \times P_{AH}(\beta) \times P_{BS}(\gamma) \times P_{OC}(\delta) \times P_{VT}(\epsilon)$$

where the probability of any horizontal transmission of *Wolbachia* ( $P_{HT(W)}$ ) is equal to multiplying the probabilities of all the independent steps required for its occurrence: the environmental interaction between *Wolbachia* infected and non-infected individuals ( $P_{EI}$ ), here tested as ingestion; the access of *Wolbachia* to the haemolymph ( $P_{AH}$ ); the bacterial survival in the new host ( $P_{BS}$ ); the colonization of ovaries ( $P_{OC}$ ); and the vertical transmission ( $P_{VT}$ ). Each of these steps can still be associated with a correction factor ( $\alpha$ ,  $\beta$ ,  $\gamma$ ,  $\delta$  and  $\epsilon$ ) linked to specific ecological conditions.

*Wolbachia* ingestion by a non-infected new host is not in itself sufficient to establish a stable infection in *Drosophila* but specific ecological conditions may favour this process (here, formalized as  $\alpha$ ,  $\beta$ ,  $\gamma$ ,  $\delta$  and  $\epsilon$ ). Indeed, there is ample evidence that several aspects of host life-history have a significant impact on the transmission of *Wolbachia* (McGraw and O’Neill 1999; Hurst et al. 2001; Mouton et al. 2007). Thus, we have manipulated some of these factors in order to favour horizontal transmission via ingestion, namely starvation and infection with a known natural bacterial pathogen. Interestingly, under nutritional restriction, the apoptotic region present in the ovaries (region 2a/2b of the germarium) (Drummond-Barbosa and Spradling 2001) overlaps with the region of *Wolbachia* entrance into the germinal tissue (Frydman et al. 2006), raising the hypothesis that the invasion of the germinal tissue by *Wolbachia* is opportunistic ( $\delta$ ). Additionally, the absence of nutritional resources in nature could also trigger an increase in cannibalism ( $\alpha$ ) and in bacterial infections due to the weakening of the host’s tissue barrier by cell death ( $\beta$ ). With this

aim, we placed mel<sup>–</sup> adult females, previously maintained in nutritionally poor medium or under starvation, on a diet composed of a mel<sup>+</sup> adult homogenate for 48 h (Table 2 – B). Under these conditions we observed a total absence of *Wolbachia* in F<sub>1</sub> tested females. Next, we used an oral infection model by previous infection with *Serratia marcescens* as an enhancer of secondary infection with ingested *Wolbachia* ( $\beta$ ). Indeed, it has been shown that severe intestinal injury produced by *S. marcescens* promotes its crossing from the gut to the fly’s body cavity (Nehme et al. 2007). The subsequent ingestion of *Wolbachia* could follow the same route, increasing the probability of *Wolbachia* entry into the *Drosophila* haemolymph. In this experiment, adult females ingested a suspension of the entomobacterium *S. marcescens* and, subsequently, ingested *Wolbachia* extracted from infected adults of *D. melanogaster*

**Table 3** Summary of adult co-infection ingestion assays and respective female progeny analyses

Stage	Condition
Adults	LB
Adults	LB
Adults	LB
Adults	LB
Adults	<i>Serratia</i>
Adults	<i>Serratia</i>
Adults	<i>Serratia</i>
Adults	<i>Serratia</i>

*Stage* Developmental stage of tested individuals, *Condition* Previous treatment, *N test* number of tested individuals, () number of replicates, *Superscript* number of independent experimental sets, *Wol ext* *Wolbachia* extraction, *Analyzed* number of F1 females analyzed (per replicate), *Wol F1e* presence (+) or absence (–) of *Wolbachia* in early F1, *Wol F1l* presence (+) or absence (–) of *Wolbachia* in late F1, *F0 Mortality %* of dead females 3 days after treatments

and *D. simulans* (mel<sup>+</sup> and sim<sup>+</sup>) (Table 3). Here, only the late progeny of female flies was analyzed and the percentage of female mortality three days after ingestion of *S. marcescens* is shown (Table 3 – “F0 Mortality”). Regardless of a previous exposure to injury stress, these females did not give rise to *Wolbachia* infected F<sub>1</sub>s, indicating the absence of *Wolbachia* transmission (Table 3 – “Wol F1”). Despite the absence of *Wolbachia* in late progeny of tested females, this co-infection scenario presents itself as an excellent model to study the horizontal transmission of several endosymbionts to different potential new hosts. Indeed, recently it has been proposed that the ingestion of mushrooms could constitute the gateway for *Wolbachia* transmission between species (Stahlhut et al. 2010).

After an ingestion episode and once inside a potential new host, bacteria must endure the local defence deployed by the digestive system, such as low pH, the production of Reactive Oxygen Species (ROS) and the action of Anti-Microbial Peptides (AMPs). Insect parasitoids, mites or wounding can avoid this immune local challenge by providing a more direct path for bacteria to penetrate the body cavity of the new host. This route is not without danger as invading *Wolbachia* must survive the host melanization reaction triggered by injury. Finally, for *Wolbachia* to establish a viable horizontal infection once in the haemolymph (Frydman et al. 2006), it must overcome the systemic action of AMPs and phagocytosis by haemocytes. As a result, it is still unclear if the individual frequencies or efficiencies of each one of these potential mechanisms would be enough to explain all the evidence for horizontal transmission. An additional important element consists on the effects that ecological co-factors (such as those studied here: resource limitation and co-infection) have on *Drosophila* immune response translating into changes in the success of bacteria to invade and establish ( $\gamma$ ) (Schneider 2009).

Thus, the mechanisms governing horizontal transmission of facultative endobacteria, particularly of *Wolbachia*, remain unknown. As mentioned above, insect parasitoids and parasitic mites may promote some of these symbiotic exchanges; however, other mechanisms that complete the puzzle of the pathways that facultative endobacterial species utilize to accomplish a new invasion, have yet to be explained. Although *Wolbachia* has been specializing throughout evolution in the vertical transmission strategy, we do not know the true horizontal transmission capacity of this endobacterium, a feature which is an ancestral characteristic of rickettsial bacteria and is still conserved in close related Genera (Anderson and Karr 2001). Therefore, it is essential to continue the study of the mechanisms responsible for horizontal transmission phenomena that, associated with several phenotypic and reproductive manipulations, may play an important role in the generation of the enormous diversity of arthropods (Faria and Sucena 2015).

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#### Compliance with ethical standards

**Conflict of interest** All authors have declared that they have no conflict of interest.

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