Title

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

Authors

Vitor G. Faria[1]✉, Tânia F. Paulo[1,2], Élio Sucena[1,2]✉

Affiliations

[1] Instituto Gulbenkian de Ciência, Apartado 14, Oeiras, 2780-901; Portugal

[2] Universidade de Lisboa, Faculdade de Ciências, Depto. Biologia Animal, Lisboa, Campo Grande, 1700; Portugal

Correspondence

✉ esucena@igc.gulbenkian.pt

✉ vfaria@igc.gulbenkian.pt

phone/fax: +351 21 446 4639/ +351 214 407 970

Running title:

No evidence for *Wolbachia* horizontal transfer via host cannibalism

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.

Key Words: *Wolbachia*, horizontal transmission, cannibalism, *Drosophila*, ingestion, endosymbiosis

47

48 # 1 Introduction

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

60 It is estimated that *Wolbachia* infects 20-80% of insect species (Jeyaprakash and Hoy
61 2000) possibly making it the most recurrent endosymbiont on the planet. The wide
62 distribution of these bacteria is attributed to the high efficacy of vertical transmission. This
63 efficacy may rely on *Wolbachia* using the host's cytoskeleton and intracellular transport
64 system to migrate towards the germline precursors and ensure its presence inside future
65 embryos (Ferree et al. 2005; Serbus and Sullivan 2007). In addition to the colonization of the
66 germline during embryogenesis, *Wolbachia* remaining inside the embryo are internalized in
67 progenitor cells of the somatic tissue (Frydman et al. 2006; Goto et al. 2006), with potential
68 physiological and evolutionary consequences (Faria and Sucena 2013).

69 The widespread presence of *Wolbachia* must also rely on horizontal transmission,
70 which can be attested by the presence of close strains of *Wolbachia* in phylogenetically
71 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 interspecific horizontal transfer of parthenogenesis-inducing *Wolbachia* may occur (Schilthuizen and Stouthamer 1997; Huigens et al. 2000; Huigens et al. 2004). Another hypothetical vector for horizontal transmission of *Wolbachia* are ectoparasitic mites, known

97 to transfer the *Drosophila* endosymbiont, *Spiroplasma poulsonni*, from infected *D. nebulosa*
98 to *D. willistoni* (Jaenike et al. 2007). Based on our observations of *Drosophila* larval and adult
99 behaviour in crowded environments, we reasoned that cannibalism or scavenging, often
100 witnessed not only in the laboratory but also in nature, could constitute a route for horizontal
101 *Wolbachia* transfer. Moreover, occasional horizontal transmission via the oral route has been
102 reported for the pea aphid *Bemisia*-like symbiont (Darby and Douglas 2003). Indeed, the
103 digestive system is considered to be the major interface between the insect host and the
104 microbial environment, constituting a privileged gateway for microorganism invasion
105 (Douglas and Beard 1996). However, as most ingested bacteria are eliminated by the immune
106 system or by peristalsis, few bacteria can persist in large numbers in the digestive tract of
107 insects (Vallet-Gely et al. 2008). Nonetheless it is important to note that some bacterial
108 species ensure their proliferation in recent hosts by passing through the digestive tract to other
109 organs or cavities (Marsollier et al. 2005; Chiel et al. 2009).
110 Recent studies have demonstrated that, after predation of infected hosts, previously
111 uninfected isopods, *Armadillidium vulgare* and *Porcellio dilatatus dilatatus*, would become
112 infected with *Wolbachia* (Le Clec'h et al. 2013). Also, in the ant *Acromyrmex echinatior*, it
113 has been hypothesized that the faecal-oral route could constitute a means for horizontal
114 transmission of *Wolbachia* (Frost et al. 2014).
115 In this work, we have tested if upon ingestion *Wolbachia* could be transmitted stably
116 to the offspring of a *Drosophila* host. For this, several ingestion experiments were performed
117 using infected and uninfected hosts of *D. melanogaster* and *D. simulans*, at different
118 developmental stages. Nutritional variation, dehydration and intestinal injury were used in an
119 attempt to mimic naturally-occurring potentiating factors for the passage of *Wolbachia* into
120 the body cavity of the fly and the subsequent establishment of a symbiotic relationship with
121 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 ($MelO^+$ and $SimO^+$, respectively). After over 50 generations in the laboratory, $MelO^+$ and $SimO^+$ were replicated for the establishment of four new populations: two infected with *Wolbachia* as the founding populations (mel^+ and sim^+) and two treated with tetracycline during four generations for total *Wolbachia* elimination (mel^- and sim^-). We confirmed the absence of *Spiroplasma* in all populations. For the *Serratia* assays, the *D. simulans* populations were established using two isofemale lines from the Drosophila Species Stock Centre (UC San Diego, California, US) sim^+ (14021-0251.138) and sim^- (14021-0251.01). All populations were kept in cages with an effective size between 1500 and 2000 individuals with non-overlapping generations, in a day/night cycle of 12 hours, constant temperature of 25º C, standard level of relative humidity (70%) and fed on standard cornmeal-agar medium. The infection status of populations was monitored regularly through PCR (see below).

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 1mL of ice-cold PBS (adapted from (Frydman et al. 2006)). For adult co-infected ingestion assays and

147 adapting a protocol described previously (Rasgon et al. 2006), *Wolbachia* were extracted by
148 smashing approximately 500 infected flies in 10mL of Schneider's medium. The confirmation
149 of bacterial viability after extraction was also performed as described in Rasgon et al. (2006).
150 In all cases, the homogenate was used entirely.
151
152 **2.3 Adult Ingestion assay**
153 For ingestion experiments with adults, 4-7 day old females were used from the mel^-
154 population. From the regular stock of flies (which were maintained in rich medium), 20
155 replicates of 20 adult females were used to exclusively ingest 250µL of a *Wolbachia*-
156 containing suspension homogenized in PBS (from infected adults of mel^+ populations) for a
157 period of 48-hours. These experiments were also undertaken with a previous 72-hour
158 treatment either with a poor medium (rich medium diluted 1:10 in water) or in a condition of
159 starvation, where the females spent a 48-hour period in total absence of nutritional resources
160 until the beginning of the ingestion treatment.
161
162 **2.4 Larval ingestion assay**
163 For the ingestion experiments with larvae, we used mel^- larvae from the three larval stages.
164 Larvae ingested a homogenate, containing adults (or embryos), from mel^+ or sim^+ populations
165 infected with *Wolbachia* for a period of 24 hours. In each of the experiments, 5 replicates of
166 50 larvae were fed on 500µL of homogenate from 40 flies.
167
168 **2.5 Adult co-infected ingestion assays**
169 For ingestion experiments with adults, 4-7 day old females were used from the mel^-
170 population. From the regular stock of flies, 10 adult females were used per replicate to
171 exclusively ingest i) 250µL of *Serratia marcescens* (a kind gift from B. Lemaitre) for a period

of 24 hours ii) 250µL of a *Wolbachia*-containing suspension for a period of 24 hours. The food solution containing *Serratia* was prepared from an overnight culture grown exponentially at 37 ºC and was diluted with a sterile 50-mM sucrose solution to a final OD_{600}= 15. These experiments were also undertaken either with *Wolbachia* with a previous 24-hour ingestion treatment with LB or with *Serratia* and posterior treatment with sim^- and mel^-.

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 *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, 632bp and 611bp, respectively. This diagnostic PCR was further confirmed in 10% of 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 Figure 1A, also illustrating the size difference between *wsp* gene amplification products of *Wolbachia* strains from *D. melanogaster* and *D. simulans*. Confirmation of the different strains was obtained by sequencing the *wsp* gene fragment (Figure 1B). These results validate

197 our procedure for the simultaneous determination of the infection status and *Wolbachia* strain
198 present in individual or pooled adult flies (as to ascertain instances of intra- or interspecific
199 transmission). We tested the F1 of fed females at two time points: early F1 (8 to 10 days) and
200 in late F1 (more than 15 days), determined by the description of *Wolbachia* dynamics upon
201 entry into the haemolymph and subsequent stable establishment in the germline (Frydman et
202 al. 2006). A representative gel of the PCR-based screen for *Wolbachia* infection is presented
203 in Figure 1C.

204 Larval ingestion could lead to the stable transmission of *Wolbachia* by one of two
205 ways: i) establishing itself in cells of somatic tissue, surviving the metamorphosis stage of the
206 host and colonizing the ovaries of adult females, or ii) crossing the epithelium of the digestive
207 system and colonizing the stem cells of the future ovary. To validate these findings, we fed *D.*
208 *melanogaster* larvae of different stages, previously maintained in normal medium, a
209 homogenate of mel^+ and sim^+ infected embryos or adults for 24 hours (Table 1). In a second
210 set of experiments, we placed mel^- adult flies on a diet composed of a mel^+ adult homogenate
211 for 48 hours (Table 2 – A). If ingestion of *Wolbachia* occurs in the adult stage, it should be
212 enough for a successful transmission that the endosymbiont crosses the midgut and passes to
213 the haemolymph (Frydman et al. 2006). Yet, it should be stressed that it is unclear what is the
214 necessary concentration of haemolymph *Wolbachia* for the establishment of these bacteria in
215 the ovaries.

216 Both in the larvae and adult ingestion experiments, the early and late F1 flies tested
217 did not show the presence of *Wolbachia* (Table 1 and Table 2 – A, "Wol F1e and Wol F1l").
218 This negative result holds true even when varying the *Wolbachia* source, both *D.*
219 *melanogaster* and *D. simulans* (intra- or interspecific), and the stage at which the *Wolbachia*
220 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 outcome (F_1 infected or non-infected) and cannot pinpoint the critical step at which the infection fails to progress. We may consider the absence of *Wolbachia* in the *D. melanogaster* F_1 flies as the product of low probability events, each one necessary for the occurrence of horizontal transmission. We can formalize this idea through the equation:

$$\mathbf{P_{HT(\mathit{w})}} = P_{EI}(\alpha) \times P_{AH}(\beta) \times P_{BS}(\gamma) \times P_{OC}(\delta) \times P_{VT}(\varepsilon)$$

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

Wolbachia ingestion by a non-infected new host is not in itself sufficient to establish a stable infection in *Drosophila* but specific ecological conditions may favour this process (here, formalized as α, β, γ, δ and ε). Indeed, there is ample evidence that several aspects of host life-history have a significant impact on the transmission of *Wolbachia* (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 (δ). Additionally, the absence of nutritional resources in nature could also trigger an increase in cannibalism (α) and in bacterial infections due to the weakening of the host's tissue barrier by cell death (β). With this aim, we placed mel^- adult females, previously maintained in nutritionally poor medium or under starvation, on a diet composed of a mel^+ adult homogenate for 48 hours (Table 2 – B). Under these conditions we observed a total absence of *Wolbachia* in F_1 tested females. Next, we used an oral infection model by previous infection with *Serratia marcescens* as an enhancer of secondary infection with ingested *Wolbachia* (β). Indeed, it has been shown that severe intestinal injury produced by *S. marcescens* promotes its crossing from the gut to the fly's body cavity (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* and *D. simulans* (mel^+ and sim^+) (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_1s, indicating the absence of *Wolbachia* transmission (Table 3 – "Wol F1l"). Despite the absence of *Wolbachia* in late progeny of tested females, this co-infection scenario presents itself as an excellent model to study the horizontal transmission of several endosymbionts to different potential new hosts. Indeed, recently it has been proposed that the ingestion of mushrooms could constitute the gateway for *Wolbachia* transmission between species (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 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 (γ) (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 and may play an important role in the enormous diversity of arthropods (Faria and Sucena 2015).

Acknowledgements

The authors wish to thank Alexandre Leitão, Nelson Martins, Inês Trancoso and Luis Teixeira for discussions and Patrícia Beldade for the critical reading of the manuscript. Fundação para a Ciência e a Tecnologia (FCT), Portugal, supported this work (PPCDT/BIA-BDE/60950/2004) and VGF (#SFRH/BD/ 82299/2011). Tânia F. Paulo is a student of the Masters Programme in Evolutionary and Developmental Biology of Faculdade de Ciências da Universidade de Lisboa.

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

References

Anderson CL, Karr TL (2001) *Wolbachia*: evolutionary novelty in a rickettsial bacteria. BMC Evol Biol 1:10.

Baldo L, Ayoub NA, Hayashi CY, et al (2008) Insight into the routes of *Wolbachia* invasion: high levels of horizontal transfer in the spider genus *Agelenopsis* revealed by *Wolbachia* strain and mitochondrial DNA diversity. Mol Ecol 17:557–69. doi: 10.1111/j.1365-294X.2007.03608.x

Charlat S, Hurst GD, Mercot H (2003) Evolutionary consequences of *Wolbachia* infections. Trends Genet 19:217–23. doi: S0168952503000246

Chiel E, Zchori-Fein E, Inbar M, et al (2009) Almost there: transmission routes of bacterial symbionts between trophic levels. PLoS One 4:e4767.

Darby AC, Douglas AE (2003) Elucidation of the transmission patterns of an insect-borne bacterium. Appl Environ Microbiol 69:4403–7.

Douglas AE, Beard CB (1996) Microbial symbiosis in the midgut of insects. Chapman and Hall, London

Drummond-Barbosa D, Spradling AC (2001) Stem cells and their progeny respond to nutritional changes during *Drosophila* oogenesis. Dev Biol 231:265–278. doi: 10.1006/dbio.2000.0135

Faria VG, Sucena É (2015) Novel Endosymbioses as a Catalyst of Fast Speciation. In: Gontier N (ed) Reticulate Evolution: Symbiogenesis and Lateral gene Transfer. Springer, Dordrecht, Germany,

Faria VG, Sucena É (2013) *Wolbachia* in the Malpighian Tubules: Evolutionary Dead-End or Adaptation? Journal of Experimental Zoology Part B: Molecular and Developmental Evolution 320:195–199. doi: 10.1002/jez.b.22498

Ferree PM, Frydman HM, Li JM, et al (2005) *Wolbachia* utilizes host microtubules and Dynein for anterior localization in the Drosophila oocyte. PLoS Pathog 1:e14. doi: 10.1371/journal.ppat.0010014

Frost CL, Pollock SW, Smith JE, Hughes WOH (2014) Wolbachia in the Flesh: Symbiont Intensities in Germ-Line and Somatic Tissues Challenge the Conventional View of *Wolbachia* Transmission Routes. PLoS ONE 9:e95122. doi: 10.1371/journal.pone.0095122

Frydman HM, Li JM, Robson DN, Wieschaus E (2006) Somatic stem cell niche tropism in *Wolbachia*. Nature 441:509–12. doi:10.1038/nature04756

Goto S, Anbutsu H, Fukatsu T (2006) Asymmetrical interactions between *Wolbachia* and *Spiroplasma* endosymbionts coexisting in the same insect host. Appl Environ Microbiol 72:4805–10. doi:10.1128/AEM.00416-06

Haine ER, Pickup NJ, Cook JM (2005) Horizontal transmission of *Wolbachia* in a *Drosophila* community. Ecological Entomology 30:464–472.

347 Harris HL, Brennan LJ, Keddie BA, Braig HR (2010) Bacterial symbionts in insects:
348 balancing life and death. Symbiosis 51:37–53. doi: 10.1007/s13199-010-0065-3

349 Heath BD, Butcher RD, Whitfield WG, Hubbard SF (1999) Horizontal transfer of *Wolbachia*
350 between phylogenetically distant insect species by a naturally occurring mechanism.
351 Curr Biol 9:313–6. doi: S0960-9822(99)80139-0

352 Hedges LM, Brownlie JC, O'Neill SL, Johnson KN (2008) *Wolbachia* and virus protection in
353 insects. Science 322:702. doi: 322/5902/702

354 Huigens ME, de Almeida RP, Boons PA, et al (2004) Natural interspecific and intraspecific
355 horizontal transfer of parthenogenesis-inducing *Wolbachia* in *Trichogramma* wasps.
356 Proc Biol Sci 271:509–15. doi: 10.1098/rspb.2003.2640

357 Huigens ME, Luck RF, Klaassen RH, et al (2000) Infectious parthenogenesis. Nature
358 405:178–179. doi: 10.1038/35012066

359 Hurst GD, Jiggins FM, Robinson SJ (2001) What causes inefficient transmission of male-
360 killing *Wolbachia* in *Drosophila*? Heredity (Edinb) 87:220–226.

361 Jaenike J, Polak M, Fiskin A, et al (2007) Interspecific transmission of endosymbiotic
362 *Spiroplasma* by mites. Biol Lett 3:23–5.

363 Jeyaprakash A, Hoy MA (2000) Long PCR improves *Wolbachia* DNA amplification: wsp
364 sequences found in 76% of sixty-three arthropod species. Insect Mol Biol 9:393–405.

365 Jiggins FM, Bentley JK, Majerus ME, Hurst GD (2002) Recent changes in phenotype and
366 patterns of host specialization in *Wolbachia* bacteria. Mol Ecol 11:1275–83.

367 Le Clec'h W, Chevalier FD, Genty L, et al (2013) Cannibalism and Predation as Paths for
368 Horizontal Passage of *Wolbachia* between Terrestrial Isopods. PLoS ONE 8:e60232.
369 doi: 10.1371/journal.pone.0060232

370 Li J, Wang Z, Bourguet D, He K (2013) *Wolbachia* Infection in Populations of *Ostrinia*
371 *furnacalis*: Diversity, Prevalence, Phylogeny and Evidence for Horizontal
372 Transmission. Journal of Integrative Agriculture 12:283–295. doi: 10.1016/S2095-
373 3119(13)60227-0

374 Marsollier L, Aubry J, Coutanceau E, et al (2005) Colonization of the salivary glands of
375 *Naucoris cimicoides* by *Mycobacterium ulcerans* requires host plasmatocytes and a
376 macrolide toxin, mycolactone. Cell Microbiol 7:935–43. doi: 10.1111/j.1462-
377 5822.2005.00521.x

378 Martins NE, Faria VG, Teixeira L, et al (2013) Host adaptation is contingent upon the
379 infection route taken by pathogens. PLoS Pathog 9:e1003601. doi:
380 10.1371/journal.ppat.1003601

381 McGraw EA, O'Neill SL (1999) Evolution of *Wolbachia pipientis* transmission dynamics in
382 insects. Trends in Microbiology 7:297–302. doi: 10.1016/S0966-842X(99)01531-0

383 Morrow JL, Frommer M, Shearman DCA, Riegler M (2014) Tropical tephritid fruit fly
384 community with high incidence of shared *W olbachia* strains as platform for

horizontal transmission of endosymbionts: Horizontal transmission of *Wolbachia*. Environmental Microbiology 16:3622–3637. doi: 10.1111/1462-2920.12382

Mouton L, Henri H, Charif D, et al (2007) Interaction between host genotype and environmental conditions affects bacterial density in *Wolbachia* symbiosis. Biol Lett 3:210–213. doi: 10.1098/rsbl.2006.0590

Nehme NT, Liegeois S, Kele B, et al (2007) A model of bacterial intestinal infections in *Drosophila melanogaster*. PLoS Pathog 3:e173.

Rasgon JL, Gamston CE, Ren X (2006) Survival of *Wolbachia pipientis* in cell-free medium. Appl Environ Microbiol 72:6934–7. doi: AEM.01673-06

Schilthuizen M, Stouthamer R (1997) Horizontal transmission of parthenogenesis-inducing microbes in *Trichogramma* wasps. Proc Biol Sci 264:361–6. doi: 10.1098/rspb.1997.0052

Schneider DS (2009) Physiological integration of innate immunity. In: Insect infection and immunity: evolution, ecology, and mechanisms. Oxford University Press, pp 106–118

Serbus LR, Sullivan W (2007) A cellular basis for *Wolbachia* recruitment to the host germline. PLoS Pathog 3:e190. doi: 10.1371/journal.ppat.0030190

Stahlhut JK, Desjardins CA, Clark ME, et al (2010) The mushroom habitat as an ecological arena for global exchange of *Wolbachia*. Mol Ecol 19:1940–52. doi: 10.1111/j.1365-294X.2010.04572.x

Teixeira L, Ferreira A, Ashburner M (2008) The bacterial symbiont *Wolbachia* induces resistance to RNA viral infections in *Drosophila melanogaster*. PLoS Biol 6:e2. doi: 10.1371/journal.pbio.1000002

Vallet-Gely I, Lemaitre B, Boccard F (2008) Bacterial strategies to overcome insect defences. Nat Rev Microbiol 6:302–13. doi: nrmicro1870

Vavre F, Fleury F, Lepetit D, et al (1999) Phylogenetic evidence for horizontal transmission of *Wolbachia* in host-parasitoid associations. Mol Biol Evol 16:1711–23.

Werren JH (1997) Biology of *Wolbachia*. Annu Rev Entomol 42:587–609. doi: 10.1146/annurev.ento.42.1.587

Werren JH, Baldo L, Clark ME (2008) *Wolbachia*: master manipulators of invertebrate biology. Nat Rev Microbiol 6:741–51. doi: nrmicro1969

Werren JH, O'Neill SL (1997) The evolution of heritable symbionts. Oxford University Press, Oxford

Zhou W, Rousset F, O'Neil S (1998) Phylogeny and PCR-based classification of *Wolbachia* strains using wsp gene sequences. Proc Biol Sci 265:509–15. doi: 10.1098/rspb.1998.0324

Figure legends

Figure 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).