YOAN CAMILO GUZMAN SARMIENTO

Wolbachia IN THE *Drosophila Saltans* SUBGROUP: EFFECTS ON FITNESS AND REPRODUCTIVE ISOLATION

Thesis submitted to the Entomology Graduate Program of the Universidade Federal de Viçosa in fulfillment of the requirements for the degree of *Doctor Scientiae*.

Advisor: Karla Suemy Clemente Yotoko

VIÇOSA - MINAS GERAIS
2021

Ficha catalográfica elaborada pela Biblioteca Central da Universidade Federal de Viçosa - Campus Viçosa

T
G993w
2021

Guzman Sarmiento, Yoan Camilo, 1988-
Wolbachia in the *Drosophila saltans* subgroup: *effects on fitness and reproductive isolation* / Yoan Camilo Guzman Sarmiento. – Viçosa, MG, 2021.
1 tese eletrônica (92 f.): il. (algumas color.).

Inclui anexo.
Inclui apêndice.
Orientador: Karla Suemy Clemente Yotoko.
Tese (doutorado) - Universidade Federal de Viçosa, Departamento de Biologia Geral, 2021.
Inclui bibliografia.
DOI: https://doi.org/10.47328/ufvbbt.2021.240
Modo de acesso: World Wide Web.

1. *Wolbachia*. 2. *Drosophila prosaltans*. 3. *Drosophila septentriosaltans*. 4. Insetos - Comportamento sexual. 5. Acasalamento de animais. 6. Infecundidade em animais. I. Yotoko, Karla Suemy Clemente, 1974-. II. Universidade Federal de Viçosa. Departamento de Biologia Geral. Programa de Pós-Graduação em Entomologia. III. Título.

CDD 22. ed. 595.774

Bibliotecário(a) responsável: Renata de Fátima Alves CRB6/2578

YOAN CAMILO GUZMAN SARMIENTO

Wolbachia IN THE *Drosophila Saltans* SUBGROUP: EFFECTS ON FITNESS AND REPRODUCTIVE ISOLATION

Thesis submitted to the Entomology Graduate Program of the Universidade Federal de Viçosa in fulfillment of the requirements for the degree of *Doctor Scientiae*.

APPROVED: September 17th, 2021.

Assent:

Yoan Camilo Guzman Sarmiento
Author

Karla Suemy Clemente Yotoko
Advisor

ACKNOWLEDGMENTS

I thank the Coordination for the Improvement of Higher Education Personnel – CAPES for granting the Doctoral Scholarship.

To the Graduate Program in Entomology-UFV and to the professors who contributed to my training.

To Professor Karla Yotoko for her guidance and friendship during my master's and doctoral degree.

To Marina Magalhães and Luísa Dias for their collaboration, dedication, and friendship.

To Nicole Ibagon for constructive discussion and friendship.

To Professor Wolfgang J. Miller (Centre of Anatomy and Cell Biology, Medical University of Vienna) for providing the two of the drosophila strains used in this work.

To colleagues at the Laboratory of Bioinformatics and Evolution-UFV for their friendship and companionship.

To my wife Jessenia Moncaleano and my daughter Isabel Sophia for their patience and moral support.

To the colleagues of the Entomology for the advice, tips, supports, smiles, and help.

To all the people who contributed to the realization of this work in one way or another.

ABSTRACT

GUZMAN, Sarmiento Yoan Camilo, D.Sc., Universidade Federal de Viçosa, September, 2021. ***Wolbachia* in the *Drosophila saltans* subgroup: effects on fitness and reproductive isolation.** Advisor: Karla Suemy Clemente Yotoko

Population genetics and speciation process studies owe tribute to a small group of Neotropical species that caught the attention of Theodosius Dobzhansky. During his visits to Brazil, Dobzhansky noticed that *Drosophila* species from the *saltans* and the *willistoni* groups, widely distributed throughout Central and South America, presented a wide variety of cryptic species, which can be recognized only by details of the male genitalia. Such characteristics suggested that these groups were under speciation process. Among the most studied models, he highlighted the seven species of the *saltans* subgroup species, which show reproductive isolation but generate hybrids in the laboratory. Later, two of these species, *D. prosaltans* and *D. septentriosaltans*, were revealed to be infected by *Wolbachia*. This infection may play a relevant role in speciation because it can alter reproductive behavior, besides causing death or sterility of hybrids, especially males. This work aimed to investigate the phenotypic consequences of *Wolbachia* infection in a Brazilian strain of *D. prosaltans* (Pro101) and a Panamanian strain of *D. septentriosaltans* (SepPLR2). We assembled intraspecific crosses between infected and antibiotic-treated strains, evaluated the pre and post-mating components of reproductive isolation, and simulated the spread of infection in an uninfected population. Our results suggested that *Wolbachia* enhances, through different processes, the fitness of Pro101 and SepPLR2, while ensuring their survival and reproduction in these hosts. We also investigated the role of *Wolbachia* in reproductive isolation between Pro101 and a Mexican strain of *D. saltans* (Sal02). To do this, we set up interspecific crosses and evaluated the pre and post-mating components of reproductive isolation. The results of this second study suggested that infection does not interfere with the isolation of these species. However, we confirmed that the cross involving females of *D. saltans* and males of *D. prosaltans* produces sterile males, which added to further evidence, led us to the hypothesis that another endosymbiont may be responsible for the reproductive isolation between them.

Keywords**:** *Wolbachia*. *Drosophila prosaltans*. *Drosophila septentriosaltans*. Pre and post-mating isolation mechanisms. Mate recognition. Asymmetric hybrid male sterilit

RESUMO

GUZMAN, Sarmiento Yoan Camilo, D.Sc., Universidade Federal de Viçosa, setembro de 2021. ***Wolbachia* no subgrupo *Drosophila saltans*: efeitos no valor adaptativo e isolamento reprodutivo.** Orientadora: Karla Suemy Clemente Yotoko

O estudo da genética de populações e do processo de especiação devem tributos a um pequeno grupo de espécies Neotropicais que chamou a atenção de Theodosius Dobzhansky. Nas coletas que fez em suas visitas ao Brasil, Dobzhansky percebeu que espécies de *Drosophila* dos grupos *saltans* e *willistoni*, com ampla distribuição ao longo de toda América Central e do Sul, apresentam uma grande variedade de espécies crípticas, que só podem ser reconhecidas por detalhes da genitália masculina. Estas características indicam que as espécies desses grupos estão em pleno processo de especiação. Dentre as espécies mais estudadas, destacam-se as sete espécies do subgrupo *saltans*, que apresentam isolamento reprodutivo mas hibridos em laboratório. Posteriormente foi constatado que duas dessas espécies, *D. prosaltans* e *D. septentriosaltans*, encontram-se infectadas por *Wolbachia.* A infecção por esta bactéria pode desempenhar um papel relevante na especiação por ser capaz de alterar o comportamento reprodutivo, além de causar a morte ou esterilidade dos híbridos, especialmente os machos. Neste trabalho, nos propusemos a investigar as consequências fenotípicas da infecção por *Wolbachia* em uma linhagem brasileira de *D. prosaltans* (Pro101) e uma linhagem panamenha de *D. septentriosaltans* (SepPLR2). Para isso, montamos cruzamentos intraespecíficos com linhagens de infectadas e tratadas com antibióticos, avaliamos os componentes de isolamento reprodutivo pré e pós-copula, e simulamos a disseminação da infecção numa população não infectada. Nossos resultados sugeriram que *Wolbachia* aumenta, por meio de diferentes processos, o valor adaptativo de Pro101 e SepPLR2, enquanto garante sua sobrevivência e reprodução nesses hospedeiros. Nós também investigamos o papel da bactéria no isolamento reprodutivo entre Pro101 e uma linhagem mexicana de *D. saltans* (Sal02). Para isso, montamos cruzamentos interespecíficos e avaliamos os componentes de isolamento pré e pós-copula. Os resultados desse segundo estudo revelaram que a infecção não parece interferir no isolamento dessas espécies. No entanto, confirmamos a informação pré-existente de

que o cruzamento de fêmeas de *D. saltans* com machos de *D. prosaltans* produz machos estéreis, o que, juntamente com outras evidências, nos levou a levantar a hipótese de que outro endossimbionte pode ser o responsável pelo isolamento reprodutivo entre elas.

Palavras-chave: *Drosophila prosaltans*. *Drosophila saltans*. *Drosophila septentriosaltans*. Mecanismos de isolamento pré e pós-copula. Reconhecimento de parceiros. Esterilidade assimétrica do macho híbrido

CONTENTS

GENERAL INTRODUCTION

The field of population genetics owes much to the repeated visits of Theodosios Dobzhansky to Brazil to study Neotropical groups of *Drosophila* since 1948, especially *Drosophila willistoni* Burla and Da Cunha, 1949 and *Drosophila saltans* Sturtevant, 1942 groups. In his repeated travels, Dobzhansky focused his research on the genetic variation of *Drosophila* species related to adaptability to the ecological conditions of the tropics. He thus contributed to such group genetics, ecology, and taxonomy (DE CARVALHO, 2020; PAVAN; DA CUNHA,2003).

In 1942, Sturtevant established the *Drosophila saltans* group and subdivided it into two subgroups. Later, Magalhães & Bjorkholm (1957) divided the group into five subgroups based on morphological characters, mainly of male genitalia. In 1962, Magalhães finally redefined the subgroups, and Mourão & Bicudo (1967) confirmed its 21 species. Among these subgroups, studies of the speciation processes in the subgroup *saltans* provided excellent models to understand the steps of population differentiation and the isolation mechanisms that originate new species. Therefore, Bicudo (1973) tested the reproductive isolation within the subgroup *saltans* and concluded that "there exist in the *saltans* subgroup, different factors that influence, in varying degrees, the isolation among the seven species." She set crosses including individuals of different species and found the isolation levels showed in Figure 1, pointing *D. saltans* Sturtevant, 1916 and *D. prosaltans* Duda, 1927 as the less isolated species (61 % of reproductive isolation). Indeed, the first studies regarding population genetics of *D. prosaltans* confounded the species and considered *D. saltans* as *D. prosaltans* (DOBZHANSKY; STREISINGER, 1944, HOENIGSBERG; SANTIBANEZ, 1960). The researchers believed that *D. prosaltans* occurred from North of Mexico to South of Brazil at that time. The work of Magalhães (1962) distinguished these species, showing that *D. saltans* occurs from Mexico to Costa Rica while *D. prosaltans* occurs from Costa Rica to southern Brazil. Bicudo (1973) showed that the sympatric lineages of *D. saltans* and *D. prosaltans* (in Costa Rica) were wholly isolated while lineages from allopatric populations of these species generated offspring in both directions. She further argued that populations collected at the extremes of the distribution (Northern Mexico for *D. saltans* and Southern Brazil for *D. prosaltans*) were less isolated than combinations involving geographically closer populations.

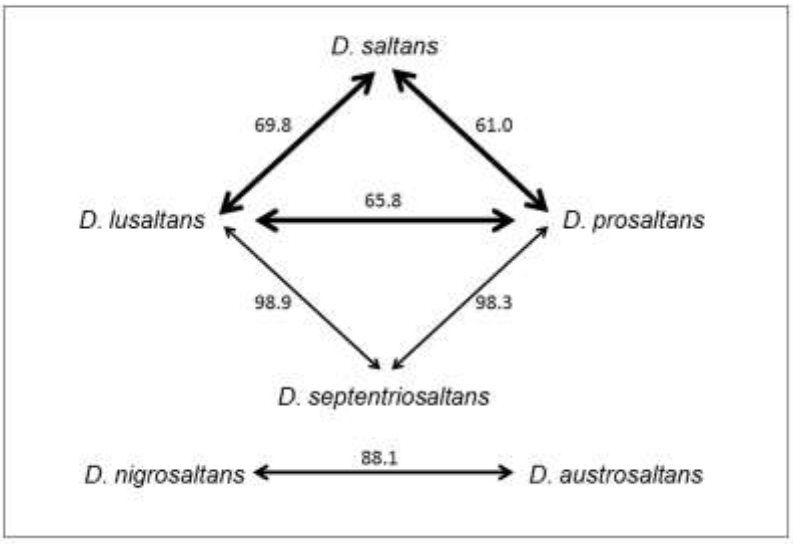

Figure 1: Diagram of the seven species of the saltans subgroup showing the average of the total isolation in both directions of the crosses (sensu Bicudo, 1973). Arrows connect no isolated species. The thicker the arrow, the less isolated they are. Unconnected species indicate that they present 100% isolation. Based on Bicudo's (1973) results**.**

More recently, Miller & Riegler (2006), studying *Wolbachia* infection at Neotropical Drosophila, found that *D. prosaltans* and *D. septentriosaltans* Magalhaes & Buck 1962 were both infected by similar strains of these bacteria, called *w*Sep and *w*Pro. Furthermore, they found a high similarity of such strains with *w*Au, infecting *D. simulans* Sturtevant, 1919 and other old-world *Drosophila*. They thus suggested that Neotropical *Wolbachia* recently infected these old-world species. Given the sequence similarity of *w*Sep and *w*Pro, they hypothesized that *D. prosaltans* and *D. septentriosaltans* inherited these strains (*w*Sep and *w*Pro) from their ancestral.

Wolbachia has been identified as a possible cause of reproductive isolation, especially pre-mating isolation, because it alters the reproductive behavior (BRUCKER; BORDENSTEIN, 2012; SHROPSHIRE; BORDENSTEIN, 2016), enhancing mate discrimination among closely related fly lineages (MILLER; EHRMAN; SCHNEIDER, 2010). In addition, this bacteria can also trigger post-mating isolation because it reduces the hybrid viability (throughout Cytoplasmic Incompatibility - CI);

kills the male offspring (WERREN; BALDO; CLARK, 2008); or causes hybrid male sterility in crosses involving individuals harboring different *Wolbachia* strains (MILLER; EHRMAN; SCHNEIDER, 2010).

Additionally, recent findings suggest that *Wolbachia* infection confers fitness benefits to arthropod hosts (review in ZUG; HAMMERSTEIN, 2015), such as protection against viruses (HEDGES *et al.*, 2008); increasing female fecundity (TURELLI, 1994; VAVRE et al., 1999), longevity (DOBSON; RATTANADECHAKUL; MARSLAND, 2004); or nutritional provisioning (MORIYAMA *et al.*, 2015).

Wolbachia of the *w*Au-like strains induce several different phenotypes in their old and new-world hosts, but the infection consequences at their putative (sensu Miller and Riegler, 2006) original hosts are unknown. Therefore, the first chapter of this work investigated the phenotypic consequences of *Wolbachia* infection in a Brazilian lineage of *D. prosaltans* (Pro101) and a Panamanian lineage of *D. septentriosaltans* (SepPLR2). We tested the hypothesis that old and stable relationships produce similar and positive impacts for the hosts.

In the second chapter, we wondered whether *Wolbachia* had a role in the reproductive isolation of *D. saltans* and *D. prosaltans*, the less isolated species pair of the subgroup *saltans* (Figure 1).

References

BICUDO, H. E. M. Reproductive isolation in the saltans group of *Drosophila*. I. The *saltans* subgroup. **Genetica**, vol. 44, no. 3, p. 313–329, Sep. 1973. DOI 10.1007/BF00161311. Available at: http://link.springer.com/10.1007/BF00161311.

BRUCKER, R. M.; BORDENSTEIN, S. R. Speciation by symbiosis. **Trends in Ecology & Evolution**, vol. 27, no. 8, p. 443–451, 1 Aug. 2012. DOI 10.1016/J.TREE.2012.03.011. Available at: https://www.sciencedirect.com/science/article/pii/S0169534712000766.

BURLA, H.; DA CUNHA, A. B. The *Willistoni* group of sibling species of *Drosophila*. **Evolution; international journal of organic evolution**, vol. 3, no. 4, p. 300–314, 1 Dec. 1949. DOI 10.1111/j.1558-5646.1949.tb00031.x. Available at: https://onlinelibrary.wiley.com/doi/full/10.1111/j.1558-5646.1949.tb00031.x.

DE CARVALHO, T. B. Modern evolutionary biology and Brazilian population genetics: Theodosius Dobzhansky at the University of Sao Paulo. **Perspectives on Science**, vol. 28, no. 2, p. 223–243, 1 Apr. 2020. DOI 10.1162/posc_a_00339. Available at: https://doi.org/10.1162/posc_a_00339.

DOBSON, S. L.; RATTANADECHAKUL, W.; MARSLAND, E. J. Fitness advantage and cytoplasmic incompatibility in *Wolbachia* single- and superinfected A*edes albopictus*. **Heredity**, vol. 93, no. 2, p. 135–42, 5 Aug. 2004. DOI 10.1038/sj.hdy.6800458. Available at: http://dx.doi.org/10.1038/sj.hdy.6800458.

DOBZHANSKY, T.; STREISINGER, G. Experiments on Sexual Isolation in Drosophila: II. Geographic Strains of *Drosophilap prosaltans*. **Proceedings of the National Academy of Sciences**, vol. 30, no. 11, p. 340–345, 15 Nov. 1944. https://doi.org/10.1073/PNAS.30.11.340.

HEDGES, L. M.; BROWNLIE, J. C.; O'NEILL, S. L.; JOHNSON, K. N. *Wolbachia* and Virus Protection in Insects. **Science**, vol. 322, no. 5902, 2008.

HOENIGSBERG, H. F.; SANTIBANEZ, S. K. Intraspecific sensory Discrimination in *Drosophila aequinoctialis* Dobzhansky and *Drosophila prosaltans* Duda. **Zeitschrift für Tierpsychologie**, vol. 17, no. 2, p. 133–140, 12 Jan. 1960. DOI 10.1111/J.1439-0310.1960.TB00197.X. Available at: https://onlinelibrary.wiley.com/doi/full/10.1111/j.1439-0310.1960.tb00197.x.

MAGALHAES, L. D. Notes on the taxonomy, morphology, and distribution of the *saltans* group of Drosophila, with descriptions of four new species. **University of Texas Publication**, vol. 6205, p. 135–154, 1962.

MAGALHÃES, L. E.; BJÖRNBERG, A. J. S. Estudo da genitália masculina de Drosophila do grupo *saltans* (Diptera). **Revista brasileira de Biologia**, vol. 17, no. 4, p. 435–450, 1957. .

MILLER, W. J.; EHRMAN, L.; SCHNEIDER, D. Infectious speciation revisited: Impact of symbiont-depletion on female fitness and mating behavior of *Drosophila paulistorum.* **PLoS Pathogens**, vol. 6, no. 12, p. e1001214, 2 Jan. 2010. DOI 10.1371/journal.ppat.1001214. Available at: http://journals.plos.org/plospathogens/article?id=10.1371/journal.ppat.1001214.

MILLER, W. J.; RIEGLER, M. Evolutionary dynamics of *w*Au-like *Wolbachia* variants

in neotropical *Drosophila* spp. **Applied and environmental microbiology**, vol. 72, no. 1, p. 826–35, 1 Jan. 2006. DOI 10.1128/AEM.72.1.826-835.2006. Available at: http://aem.asm.org/content/72/1/826.abstract.

MORIYAMA, M.; NIKOH, N.; HOSOKAWA, T.; FUKATSU, T. Riboflavin provisioning underlies *Wolbachia*'s fitness contribution to its insect host. **mBio**, vol. 6, no. 6, 10 Nov. 2015. https://doi.org/10.1128/mBio.01732-15.

MOURAO, C. A.; BICUDO, C. H. E. Duas novas espécies de *Drosophila* do grupo *saltans* (Drosophilidae, Diptera). **Papéis Avulsos de Zoologia**, vol. 20, p. 123–134, 1967. .

PAVAN, C.; DA CUNHA, A. B. Theodosius Dobzhansky and the development of Genetics in Brazil. **Genetics and Molecular Biology**, vol. 26, no. 3, p. 387–395, 2003. DOI 10.1590/S1415-47572003000300027. Available at: http://www.scielo.br/j/gmb/a/99X6vb8Lj8t49bBQvPyBTCj/?lang=en.

SHROPSHIRE, J. D.; BORDENSTEIN, S. R. Speciation by Symbiosis:Tthe Microbiome and Behavior. **mBio**, vol. 7, no. 2, p. e01785, 31 Mar. 2016. DOI 10.1128/mBio.01785-15. Available at: http://www.ncbi.nlm.nih.gov/pubmed/27034284.

STURTEVANT, A. H. I.The Classification of the Genus *Drosophila*, with Descriptions of Nine New Species. **The University of Texas Publication**, vol. 4213, no. 4213, p. 6–51, 1942. Available at: https://repositories.lib.utexas.edu/handle/2152/27735.

TURELLI, M. Evolution of incompatibility-inducing microbes and their hosts. **Evolution**, vol. 48, no. 5, p. 1500–1513, 1 Oct. 1994. https://doi.org/10.1111/j.1558-5646.1994.tb02192.x.

VAVRE, F.; FLEURY, F.; LEPETIT, D.; FOUILLET, P.; BOULÉTREAU, M. Phylogenetic evidence for horizontal transmission of *Wolbachia* in host-parasitoid associations. **Molecular biology and evolution**, vol. 16, no. 12, p. 1711–23, 1 Dec. 1999. Available at: http://mbe.oxfordjournals.org/content/16/12/1711.abstract.

WERREN, J. H.; BALDO, L.; CLARK, M. E. *Wolbachia*: master manipulators of invertebrate biology. **Nature Rev Microbiol**, vol. 6, no. 10, p. 741–751, Oct. 2008. DOI 10.1038/nrmicro1969. Available at: http://www.ncbi.nlm.nih.gov/pubmed/18794912. Accessed on: 14 Jul. 2014.

ZUG, R.; HAMMERSTEIN, P. Bad guys turned nice? A critical assessment of *Wolbachia* mutualisms in arthropod hosts. **Biological reviews of the Cambridge Philosophical Society**, vol. 90, no. 1, p. 89–111, 1 Feb. 2015. DOI 10.1111/brv.12098. Available at: http://doi.wiley.com/10.1111/brv.12098.

CHAPTER 1: FITNESS BENEFITS AND *WOLBACHIA* FIXATION STRATEGIES OF *w*AU-LIKE INFECTION IN NEOTROPICAL *DROSOPHILA* (*SALTANS* SUBGROUP).

Guzman Sarmiento, Yoan Camilo[1]; Moreira, Marina Magalhães[1]; Bouzada-Dias Luísa[2]; Yotoko, Karla[2]

[1]Programa de Pós-graduação em Entomologia, Universidade Federal de Viçosa, 36570-900, Viçosa-MG, Brasil [1]

[2]Departamento de Biologia Geral Universidade Federal de Viçosa, 36570-900, Viçosa-MG, Brasil

Abstract

Wolbachia is an α-proteobacterium widespread among arthropods whose main route of transmission is vertical. However, phylogenetic trees of Arthropod hosts rarely agree with their *Wolbachia* infections, suggesting that horizontal transmission, introgression, and symbiont extinction are ordinary events at *Wolbachia*-Arthropoda relationships. Commercial trade activities provided contact between species formerly isolated with the consequent interchange of their simbionts. Indeed, *Wolbachia* strains of the *w*Au-like clade would have originated in the tropical region, in hosts of the *Drosophila saltans* group, and recently spread worldwide. Members of the clade cause different phenotypes in their hosts, but there are no studies about effects on their putative original hosts. In this work, we investigated a Brazilian lineage of *D. prosaltans* (Pro101) and a Panamanian lineage of *D. septentriosaltans* (SepPLR2), both members of the *saltans* group. We found that similar but not identical *Wolbachia* strains of *w*Au-like clade infect the lineages. Furthermore, we set intraspecific crosses involving infected and antibiotics-treated individuals and found no cytoplasmatic incompatibility or biases at sex ratio, but found that depletion of *Wolbachia* impacted the fitness of both lineages. In Pro101, we got a clue that infected females prefer infected males, even though they produce more offspring with treated males, which we interpreted as host manipulation. In SepPLR2, the disadvantages of *Wolbachia* depletion were even more evident, both for males and females and at pre and post-mating fitness

components, which led us to question the ability of uninfected SepPLR2 to survive and compete with infected individuals in field conditions. Our simulations suggest that *Wolbachia* manages to increase in frequency until the fixation in both fly lineages. Overall, we found that the bacteria manipulate the behavior of infected females of Pro101 and induce dependence for the progeny success in SepPLR2.

Keywords: *Drosophila prosaltans*; *Drosophila septentriosaltans*; antibiotics treatment; host manipulation, infection spread.

1. Introduction

Wolbachia is an α-proteobacterium largely widespread among arthropods (JEYAPRAKASH; HOY, 2000; WERREN; JAENIKE, 1995). Its primary mode of transmission is vertical (from mothers to their progeny), but there are reports of horizontal transmission (HEATH *et al.*, 1999; VAVRE *et al.*, 1999), introgression (JIGGINS, 2003), strain replacements (KRIESNER *et al.*, 2013; RIEGLER *et al.*, 2005), and *Wolbachia* extinction (KOEHCKE *et al.*, 2009). Bailly-Bechet *et al.* (2017) suggested that most of such events occurred within the last million years, explaining why phylogenetic trees of Arthropod species rarely agree with trees of *Wolbachia* (but see (BLEIDORN; GERTH, 2018; RAYCHOUDHURY *et al.*, 2009).

There are indications that this kind of modification occurred even more recently: during the last 500 years, world trade and the transport of human-associated fauna promoted the contact between isolated species with the consequent interchange of their symbionts. Based on the phylogeny of the *Wolbachia* clade *w*Au-like and the geographical origin of their hosts, Miller and Riegler (2006) suggested a recent transfer of *Wolbachia* from the neotropical *Drosophila saltans* group Burla and Da Cunha, 1949 (*w*Spt and *w*Pro) to *D. willistoni* Sturtevant, 1916, (another neotropical species – *w*Will) and several old-world species, such as *D. simulans* Sturtevant, 1919 (wAu), *D. yakuba* Burla, 1954 (*w*Yak), *D. teissieri* Tsacas, 1971 (*w*Tei), and *Rhagoletis cerasi* Linnaeus, 1758 (Diptera: Tephritidae) (*w*Cer2). More recently, Müller *et al.* (2013) found *w*Au-like strains infecting other neotropical (*D. tropicalis* Burla and Da Cunha, 1949, *D. paulistorum* Dobzhansky and Pavan, 1949, and *D. arawakana* Heed, 1962) and old-

world *Drosophila* (*D. ananassae* Doleschall, 1858 and *D. santomea* Lachaise & Harry 2000).

Theoretically, vertically inherited symbionts should evolve towards mutualism since they depend on their host to survive (FINE, 1975; LIPSITCH *et al.*, 1995). Notwithstanding, *Wolbachia* is traditionally known as a parasite that owes its success to its ability to manipulate its host reproduction, causing cytoplasmic incompatibility (CI) or male-killing (WERREN; BALDO; CLARK, 2008). Recent findings, however, suggested that the infection confer fitness benefits to arthropod hosts (review in ZUG; HAMMERSTEIN, 2015), such as protection against viruses (HEDGES *et al.*, 2008); increasing female fecundity (TURELLI, 1994; VAVRE *et al.*, 1999), longevity (DOBSON; RATTANADECHAKUL; MARSLAND, 2004); or nutritional provisioning (MORIYAMA *et al.*, 2015).

Specifically, *w*Au-like strains cause unidirectional CI in the fruit fly *Ragoletis cerasi* infected by *w*Cer2 (RIEGLER; STAUFFER, 2008; ZABALOU *et al.*, 2004); show a neutral effect in *D. yakuba* infected by *w*Yak (CHARLAT; BALLARD; MERÇOT, 2004); and protect *D. simulans* infected by *w*Au against the effects of virus infection (OSBORNE *et al.*, 2009). James and Ballard (2000) found that *w*Au also causes a weak CI in *D. simulans* (but see CHARLAT; LE CHAT; MERÇOT, 2003; HOFFMANN; CLANCY; DUNCAN, 1996). In the neotropical *D. paulistorum* superspecies, depletion of *w*Pau *Wolbachia* reduced the egg production and assortative mating between subspecies (MILLER; EHRMAN; SCHNEIDER, 2010) and between infected females and antibiotics treated males (SCHNEIDER *et al.*, 2019). However, there are no studies about phenotypic consequences of *w*Au-like *Wolbachia* in their putative original hosts: the species of the *Drosophila saltans* subgroup.

Miller & Riegler (2006) found two of the six studied *Drosophila* species of the subgroup *saltans, D. prosaltans* Duda, 1927 and *D. septentriosaltans* Magalhaes & Buck 1962, harboring similar *w*Au-like clade strains (*w*Pro and *w*Spt). Because of the close relatedness of hosts and the sequence similarity of the bacteria strains, they inferred a long-term *Wolbachia*-host relationship. They also hypothesized that the ancestral of *w*Pro and *w*Spt infected the common ancestral of *D. prosaltans* and *D. septentriosaltans* and diverged after the hosts' speciation.

In this work, we investigated the *Wolbachia* infection in a Brazilian lineage of *D. prosaltans* (Pro101) and a Panamanian lineage of *D. septentriosaltans* (SepPLR2) (see Table 1). We questioned (*i)* whether Pro101 harbors *Wolbachia*; (*ii)* used molecular sequences to identify and differentiate the strains harbored by Pro101 and SepPLR2; (*iii)* set intraspecific crosses to measure the fitness consequences of the infection; and (*iv)* developed a computer simulation to infer the spread of *Wolbachia* in a host population considering the fitness consequences of the infection in different combinations of crosses.

2. Materials and methods

3.

2.1. Fly lineages

Table 1 shows all isofemale fly lineages used in the crosses and the positive and negative controls used in the procedures of *Wolbachia* detection. All strains were kept at 23 °C, with a 12L/12D photoperiod, in the banana-barley-agar medium [100 ml of medium contains 13.8 g banana, 3 g barley flour, 9.5 g corn syrup, 2.8 g yeast extract, 0.6 g agar, and 0.22 g Nipagin].

2.2. DNA extraction

The total DNA of each fly lineage (including the controls) was extracted from a pool of five females using the Wizard® Genomic DNA Purification Kit (Promega A1120, animal tissue protocol). To test the quality of the extracted DNA, we performed standard PCR using universal primers of the mitochondrial COI gene. Samples with negative results for this reaction were discarded.

2.3. *Wolbachia* detection

We tested the fly lineages for *Wolbachia* infection using a standard PCR (primer annealing at 59°C) of the gene *wsp* (*Wolbachia* Surface Protein) with the primers *wsp*-226F (5'-GTGGTGGTGCATTTGGTTATAAAATGG-3'), and *wsp*-588R (5'-CATAAGAACCGAAATAACGAGCTCCAG-3') (ARTHOFER *et al.*, 2009). The PCR product was visualized into the electrophoresis gel (agarose 1.0 %), and we interpreted

the band visualization as indicative of high titer infection. In contrast, we interpreted the band absence as low-titer or no-titer.

We included a positive control (JS 6.3 – Table 1) and three negative controls for each experiment: D.mel 1118 – (Table 1), sample of DNA extraction without tissues, and a PCR reaction without template.

Table 1. *Drosophila* lineages used in this work.

Lineage	Species	Stock	Sampling site
Pro101	*D. prosaltans*	Lab. Bioinformática e Evolução, Universidade Federal de Viçosa	Mata da Biologia, Universidade Federal de Viçosa, MG, Brazil, 2016.
SepPLR2[1]	*D. septentriosaltans*	Lab. Genome Dynamics, Medical University of Vienna, Vienna, Austria.	Gamboa, Panama, 2002
JS 6.3[1,2]	*D. willistoni*	Lab. Genome Dynamics, Medical University of Vienna, Vienna, Austria.	Reserva Biológica Jatun Sacha, Tena, Ecuador, 1997
Dmel w1118[3]	*D. melanogaster* Maigen, 1830	Bloomington Drosophila Stock Center, Indiana, USA white mutant	-

[1]High titer *Wolbachia*, [2]Positive control, [3]Negative control). The infection status was inferred by PCR using the *wsp* gene (see section 2.3).

2.4. *Wolbachia* strain identification

To identify the infected strains of the *Drosophila saltans* group species studied here, we amplified and sequenced the MLST (MultiLocus Sequence Typing) and the *wsp* (*Wolbachia* Surface Protein) gene. The genes were amplified using the standard protocols provided on the *Wolbachia* MLST website (JOLLEY; BRAY; MAIDEN, 2018). In addition, we amplified two variable tandem repeated regions (*VNTR-105* and *VNTR-141*) and one Ankyrin repeat domain gene (*RO766*) (RIEGLER *et al.*, 2012) (Table 2).

Table 2 List of primers used for molecular *Wolbachia* strain characterization: *wsp* (*Wolbachia* surface protein), MLST [Multilocus sequence typing: *gatB* (Glutamyl-tRNA Gln amidotransferase subunit B), *coxA* (Cytochrome c oxidase subunit 1), *hcpA* (conserved hypothetical protein), and *ftsZ* (cell division protein)], VNTR (Variable tandem repeat regions, 105 and 141), and ANK (Ankyrin repeat domain); their nucleotide sequences, PCR product expected size (bp) and annealing temperature (TA).

ID	Locus	Primers sequences (5'-3')	bp	TA
wsp[1]	*wsp*1	F1:GTCCAATARSTGATGARGAAAC	546	59
		R1:CYGCACCAAYAGYRCTRTAAA		
MLST[1]	*gatB*	F1:GAKTTAAAYCGYGCAGGBGTT	471	54
		R1:TGGYAAYTCRGGYAAAGATGA		
	coxA	F1:TTGGRGCRATYAACTTTATAG	487	54
		R1:CTAAAGACTTTKACRCCAGT		
	hcpA	F1:GAAATARCAGTTGCTGCAAA	515	54
		R1:GAAAGTYRAGCAAGYTCTG		
	ftsZ	F1:ATYATGGARCATATAAARGATAG	524	54
		R1:TCRAGYAATGGATTRGATAT		
	fbpA	F1:GCTGCTCCRCTTGGYWTGAT	509	59
		R1:CCRCCAGARAAAAYYACTATTC		
VNTR[2]	*VNTR*-105	F:GCAATTGAAAATGTGGTGCC	*	57
		R:ATGACACCTTACTTAACCGTC		
	VNTR-141	F:GGAGTATTATTGATATGCG	*	57
		R:GACTAAAGGTTAGTTGCAT		
ANK[2]	*RO 766*	F:GACCACCATGAAATATGACAAATTT	*	57
		R:TCAAGTAAGTGCTTTTTCTGTC		

[1] Baldo *et al.* (2006), [2] Riegler *et al.* (2012); *Polymorphic fragments.

2.5. Antibiotic treatment

Performing crosses between different combinations of infected and non-infected flies requires antibiotic treatment of the infected lineages. Therefore, we added tetracycline

(0.03 % W/V) to the culture medium (LI *et al.*, 2014) and let adult females lay eggs in tubes containing such medium. We maintained the lineages under antibiotic treatment for four generations and transferred the fifth generation to a medium without antibiotics. We waited for another five generations, which theoretically allows the recovery of gut microbiota and treats direct adverse effects (LI *et al.*, 2014). These generations in a recovery medium are essential to avoid reducing sperm viability of treated males (ZEH; ZEH, 1996) and significantly reduce the progeny drop that Poinsot and Merçot (1997) observed in the fifth generation of treated lineages. We identified the treated lineages as tet and the infected lineages as w+.

2.6. Gut microbiota restoration treatment

To ensure the restoration of gut microbiota depleted by tetracycline, we supplemented the food medium with feces of infected co-lineage males for one generation to re-infect the flies with a similar microbiota. According to Douglas (2018), the *Drosophila* gut is an open system, and microbiota cells are both ingested with food and shed in feces. Therefore, we tested the first generation of adults after treating *Wolbachia* infection (see item 2.3) to ensure that they were not re-infected.

2.7. Crosses

We controlled larvae density (CLANCY; HOFFMANN, 1998) of the vials used to obtain the adults to get homogeneous sets of adults. For that, we initially established, for each lineage, pool vials of 50 ml of culture medium with 20 sexually mature males and females (1:1) that mated and laid eggs for three days. After that, we discarded the adults and maintained larvae and pupae at the vial to collect virgins. We thus searched for newly emerged adults in the pool vials, transferred them to two different vials for males and females, and maintained them for six days to wait for sexual maturity (BICUDO, 1973).We kept the vials where the females were separated for additional ten days. If we detected no larvae in ten days, we considered that the females were virgins; otherwise, we discarded those crosses related to the vial with larvae.All the crosses performed in this work (Table 3) included one female and two males (FRY; PALMER; RAND, 2004). Initial (G_0) crosses were set up in vials with reduced volume (we left 1.0 cm between the surface of the fly food and the foam plugin in 2.5 cm

diameter glass tubes). This procedure enhances the chances of mating. We kept the tubes in this way for 48 h, discarded the males, and kept the females in the tube for another 24 h to lay eggs. After this first period, we dissected the female to check for sperm in her spermathecas. We monitored the tubes for 30 days to collect adults, counted, sexed, and checked for *Wolbachia* infection (see item 2.3). We applied Fisher exact tests to compare the number of inseminated and fertilized females between pairs of crosses to infer the effects of pre and post-mating fitness components.

Table 3. Intraspecific crosses involving infected (w+) and antibiotic-treated (tet) fly lineages of Pro101 and SepPLR2. Control crosses involved couples with the same infection status. Inter-line crosses involved w+ x tet couples.

Cross	♀	♂
Control	w+	w+
	tet	tet
Inter-lineage	w+	tet
	tet	w+

We compared the total progeny of each cross combination considering all females to estimate a pre-mating isolation component and only inseminated or fertilized females to estimate post-mating isolation components. We used generalized linear models (GLM) with the quasi-Poisson distribution to correct for overdispersion in count data. We used ANOVA F test to compare models and evaluate the variability explained by the factor crosses for each lineage through a complete model for all females, inseminated and fertilized females. The complete model included all the crosses. We thus amalgamated the levels of the factors using all possible combinations and evaluated the changes in deviance to get to the minimum adequate model (MAM). Finally, we ran an ANOVA of the MAM, determined the number of levels and *p*-values of the significant explanatory factors (CRAWLEY, 2007).

Another fitness component usually affected by *Wolbachia* infection is the proportion of females in the progeny. Thus, we compared sex ratio using a GLM with the x^2 test for binomial error distribution.

All statistical analyses used in this work were performed with the software R version 4.0.2 (R CORE TEAM, 2017). All figures were produced using the package ggplot2 (WICKHAM, 2009).

2.8. Simulating the spread of infection

Using the data obtained according to section 2.7, we simulated the spread of the infection considering the arrival of infected females. We developed a visualization in a Shiny App (https://yoan-sarmiento.shinyapps.io/simulation/) with Shiny 1.5.0 (CHANG *et al.*, 2017) using the R version 4.0.2 (R CORE TEAM, 2017). Figures were produced using the package ggplot2 (WICKHAM, 2009) and the functions of the plotly package (SIEVERT, 2020).

The input data for the simulation at the Shiny App includes crosses, population, and *Wolbachia* infection parameters. Among cross parameters, we considered the number of inseminated (ins) and non-inseminated ($nins$) females, the number of fertilized females (fe), and the median number (M) of adults at the F_1 of fertilized females (the user can input other measures of F_1 production). Among population parameters, we included the population size (N) and the number of arriving infected females (ni). The user also can choose the number of generations to simulate (g). *Wolbachia* infection parameters include vertical transmission rate (vt) and sex ratio (proportion of females) produced by infected females (fsr); we also considered that the non-infected females' progeny has 50 % of females (The appendix gives a complete description of how the simulation works, as well as the R and Shiny scripts.).

3. Results

3.1 Molecular identification of *Wolbachia* strains

The *wsp* PCR detected *Wolbachia* in Pro101 and confirmed the infection of SepPLR2. The molecular characterization showed that the strains infecting these lineages are similar (GenBank accession numbers XXXX to YYYY). Figure 1 shows that we found only two base substitutions when comparing the strain infecting Pro101 with the strain infecting SepPLR2: the first in the gene encoding *fbpA* (ferric binding protein) and the second in the gene encoding *wsp* (*Wolbachia* surface protein). The other sequenced

loci showed no substitutions. Figure 2 reveals that from the three polymorphic *loci*, only the VNTR-141 presented differences.

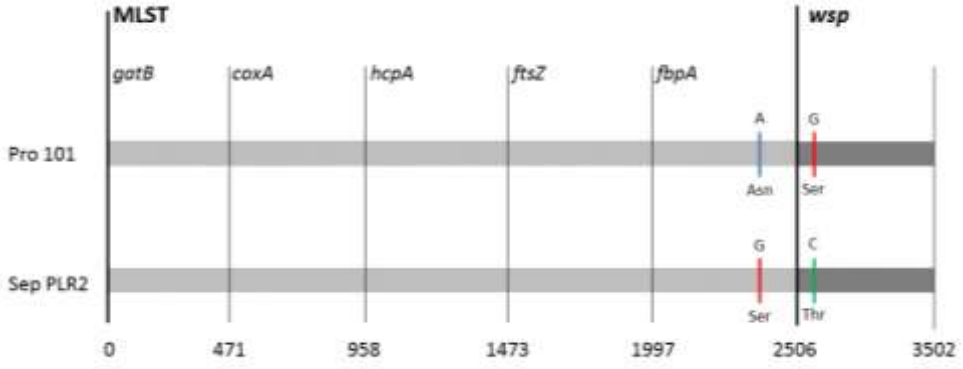

Figure 1. MLST (Multi Locus Sequence Typing System – in light grey) and *wsp* (Wolbachia Surface Protein – in dark grey) sequence representation of the strains infecting Pro101 (a Brazilian lineage of *D. prosaltans*) and SepPLR2 (a Panamanian lineage of *D. septentriosaltans*). The sequences of *gatB* (Glutamyl-tRNA Gln amidotransferase subunit B), *coxA* (Cytochrome c oxidase subunit 1), *hcpA* (conserved hypothetical protein), and *ftsZ* (cell division protein) were identical. The only differences were found at position 386 of the *fbpA* (ferric binding protein) and position 68 of the *wsp* (Wolbachia surface protein).

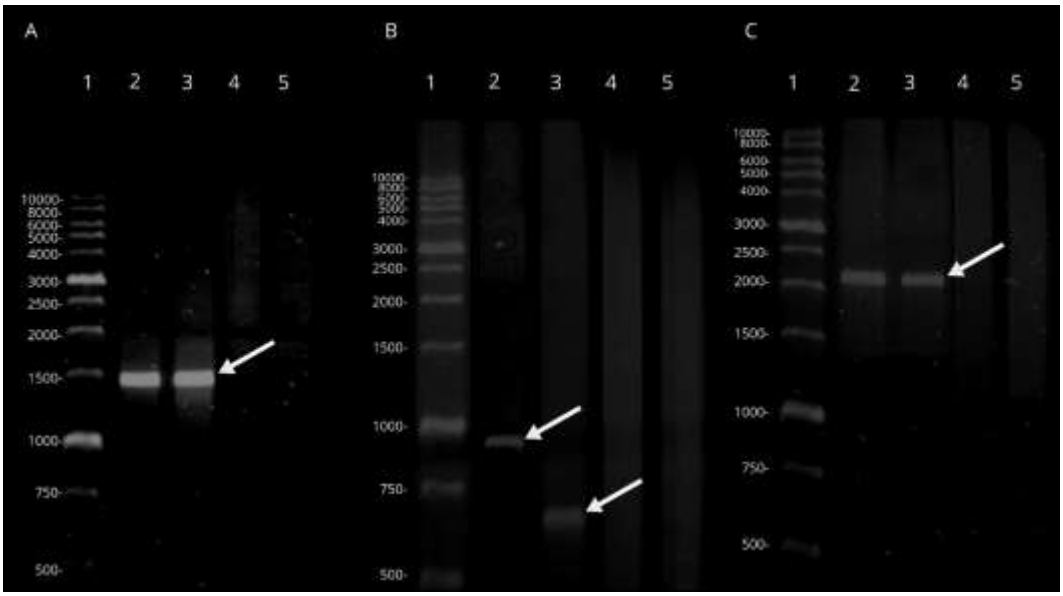

Figure 2. Electrophoresis visualization of the polymorphic loci **A.** Variable tandem repeat regions (*VNTR*-105)*,* **B.** VNTR-*141,* and **C**. Ankyrin repeat domain (*RO766*)*.* **1**-Molecular size marker (the fragment sizes are indicated beside each band). **2-**Pro101 (*D. prosaltans*). **3-**SepPLR2 (*D. septentriosaltans*). **4-**Negative control (Dmel W1118). **5-**No DNA PCR control.

Table 4 compares the *wsp* sequences of 10 alleles of *w*Au-like strains previously published (MILLER; RIEGLER, 2006) and the alleles found in Pro101 and SepPLR2. As we expected, the allele of SepPLR2 was identical to *w*Spt PLR2 (accession number AY620212), hosted by *D. septentriosaltans*. The allele of Pro101 was identical to *wPro* SG2 (accession number DQ118779) collected in Panama. According to the PubMLST database (https://pubmlst.org/organisms/ *Wolbachia*-spp), Pro101 and SepPLR2 host *Wolbachia* strains belonging to the supergroup A.

Table 4. Simplified DNA and amino-acid alignment (only variable sites) of the *wsp* alleles of Pro101 (*D. prosaltans*) and SepPLR2 (*D. septentriosaltans*) obtained in this work (in bold) and other ten *w*Au-like strains alleles (MILLER; RIEGLER, 2006) obtained from other species: *D. simulans* (*w*Au, accession number AF020067), *D. willistoni (w*Wil, accession number DQ118778), *D. prosaltans* (*w*Pro SG1, accession number AY620208; *w*Pro SG2 accession number DQ118779.1), and *D. septentriosaltans* (*w*Spt PLR1, accession number AY620211; *w*Spt PLR2, accession number AY620212; *w*Spt BCI1, accession number AY620209; *w*Spt BCI2, accession number AY620210; *w*Spt PNM1, accession number AY620213; *w*Spt PNM2, accession number AY620214). The first line of the alignment shows a consensus sequence. The dots indicate coincidences with the consensus sequence.

Strain	**DNA variable sites**								**aa variable sites**				
	68*	**70**	**258**	**263**	**333**	**340**	**363**	**377**	**23**	**24**	**88**	**114**	**126**
Consensus	C	C	T	G	T	G	A	A	T	H	G	A	D
*w*Au	.	T	.	.	.	A	.	.	.	Y	.	T	.
*w*Wil	.	T	.	.	.	A	.	.	.	Y	.	T	.
*w*Pro SG1	G	.	.	A	.	.	.	.	S	.	E	.	.
*w*Pro SG2	G	.	.	.	.	.	.	.	S	.	.	.	.
*w*Spt PLR1	.	.	.	.	.	.	.	G	.	.	.	.	G
*w*Spt PLR2	.	.	.	.	.	.	.	.	.	.	.	.	.
*w*Spt BCI1	.	T	.	.	.	.	.	.	.	Y	.	.	.
*w*Spt BCI2	.	.	.	.	.	.	.	.	.	.	.	.	.
*w*Spt PNM1	.	.	.	.	.	.	.	.	.	.	.	.	.
*w*Spt PNM2	.	.	C	.	C	.	C	.	.	.	.	.	.
Pro101	G	.	.	.	.	.	.	.	S	.	.	.	.
SepPLR2	.	.	.	.	.	.	.	.	.	.	.	.	.

*A position 1 of the consensus sequence corresponds to position 164 in the *wsp* sequence of *w*Au of *D. simulans* (accession number AF020067). Table based on Miller and Riegler's (2006) Table 4.

3.2. Crosses

3.2.1. *Drosophila prosaltans*

Table 5 shows the number of inseminated and fertilized females in each cross combination of Pro101 (Table 3). The table rows show the results for females, while the columns show the results for males, infected (w+) and treated (tet). The numbers withinparentheses correspond to the total number of each type involved in the crosses. We considered inseminated (ins) those females with sperm in their spermatheca after 72 h (48 h with males + 24 h alone) and fertilized (fe) those females that produced at least one larva. We applied Fisher exact tests to compare the number of inseminated and fertilized females between pairs of crosses. To visualize the pairs, consider, for example, only infected (w+) females: we compared the insemination of these females by infected (25 of 26 crosses) and treated males (20 of 35 crosses). The numbers were highlighted in green to show that these numbers were significantly different according to the Fisher test ($p < 0.05$). This difference impacted the total insemination rate of males (Sum), once more infected than treated males inseminated females (numbers in blue at Table 5). Finally, infected females were more fertilized than treated ones regardless of the infection status of males (numbers in red, Table 5). The remaining comparisons showed no significant differences (numbers in black).

Table 5. Number of females inseminated ($\boldsymbol{ins}$), not inseminated ($\boldsymbol{nins}$), fertilized ($\boldsymbol{fe}$), and not fertilized ($\boldsymbol{nfe}$) females in crosses between infected (w+) and treated (tet) lineages of Pro101 (a *D. prosaltans* lineage). Colored numbers highlight significant differences between pairs of crosses. In green: w+ males inseminated more w+ females than tet ones ($\boldsymbol{p = 0.0008018}$). In blue: w+ males inseminated more females than tet ones ($\boldsymbol{p = 0.0001492}$). In red: w+ females were more fertile than tet females ($\boldsymbol{p = 0.02017}$).

Sex		♂								**Sum**			
	status	**w+ (59)**				**tet (63)**							
		$\boldsymbol{ins}$	$\boldsymbol{nins}$	$\boldsymbol{fe}$	$\boldsymbol{nfe}$	$\boldsymbol{ins}$	$\boldsymbol{nins}$	$\boldsymbol{fe}$	$\boldsymbol{nfe}$	$\boldsymbol{ins}$	$\boldsymbol{nins}$	$\boldsymbol{fe}$	$\boldsymbol{nfe}$
♀	**w+(61)**	**25**	**1**	24	1	**20**	**15**	20	0	45	16	**44**	**1**
	tet(61)	31	2	24	7	23	5	21	2	54	7	**45**	**9**
Sum		**56**	**3**	48	8	**43**	**20**	41	2	99	23	89	10

Regardless of the advantages of crosses involving infected males or females, Figure 3A shows that the total progeny of the four cross combinations did not significantly differ. However, when we discarded the non-inseminated females (pre-mating effects, Figure 3B), we surprisingly found that the crosses involving infected females and treated males produced the highest F_1. Furthermore, we found this same pattern when discarded females whose eggs were not fertilized (Figure 3C).

3.2.2.*Drosophila septentriosaltans*

Table 6 shows that the infected (w+) males inseminated more females than treated (tet) ones (numbers in blue), both in crosses involving infected (numbers in green) and treated (numbers in red) females. Even though the fertilization rate did not differ among the crosses, Figure 4 reveals that the infected couple produced more F_1 than the others, suggesting that the antibiotic treatment negatively impacted both the insemination capacity of males and the viability of larvae.

Table 6 Number of females inseminated ($\boldsymbol{ins}$), not inseminated ($\boldsymbol{nins}$), fertilized ($\boldsymbol{fe}$), and not fertilized ($\boldsymbol{nfe}$) from crosses between infected (w+) and treated (tet) lineages of SepPLR2 (a *D. septentriosaltans* lineage). Colored numbers highlight significant differences between pairs of crosses. In green: w+ males inseminated more w+ females than tet males ($\boldsymbol{p = 0.009668}$). In red: w+ males inseminated more tet females than tet ones ($\boldsymbol{p = 0.01704}$). In blue: w+ males inseminated more females than treated ones ($\boldsymbol{p = 0.0005855}$).

Sex		♂								**Sum**			
	status	**w+ (39)**				**tet (38)**							
		$\boldsymbol{ins}$	$\boldsymbol{nins}$	$\boldsymbol{fe}$	$\boldsymbol{nfe}$	$\boldsymbol{ins}$	$\boldsymbol{nins}$	$\boldsymbol{fe}$	$\boldsymbol{nfe}$	$\boldsymbol{ins}$	$\boldsymbol{nins}$	$\boldsymbol{fe}$	$\boldsymbol{nfe}$
♀	**w+(40)**	**13**	**3**	12	1	**9**	**15**	8	1	22	18	20	2
	tet(37)	**15**	**8**	10	5	**3**	**11**	2	1	18	19	12	6
Sum		**28**	**11**	22	6	**12**	**26**	10	2	38	37	32	8

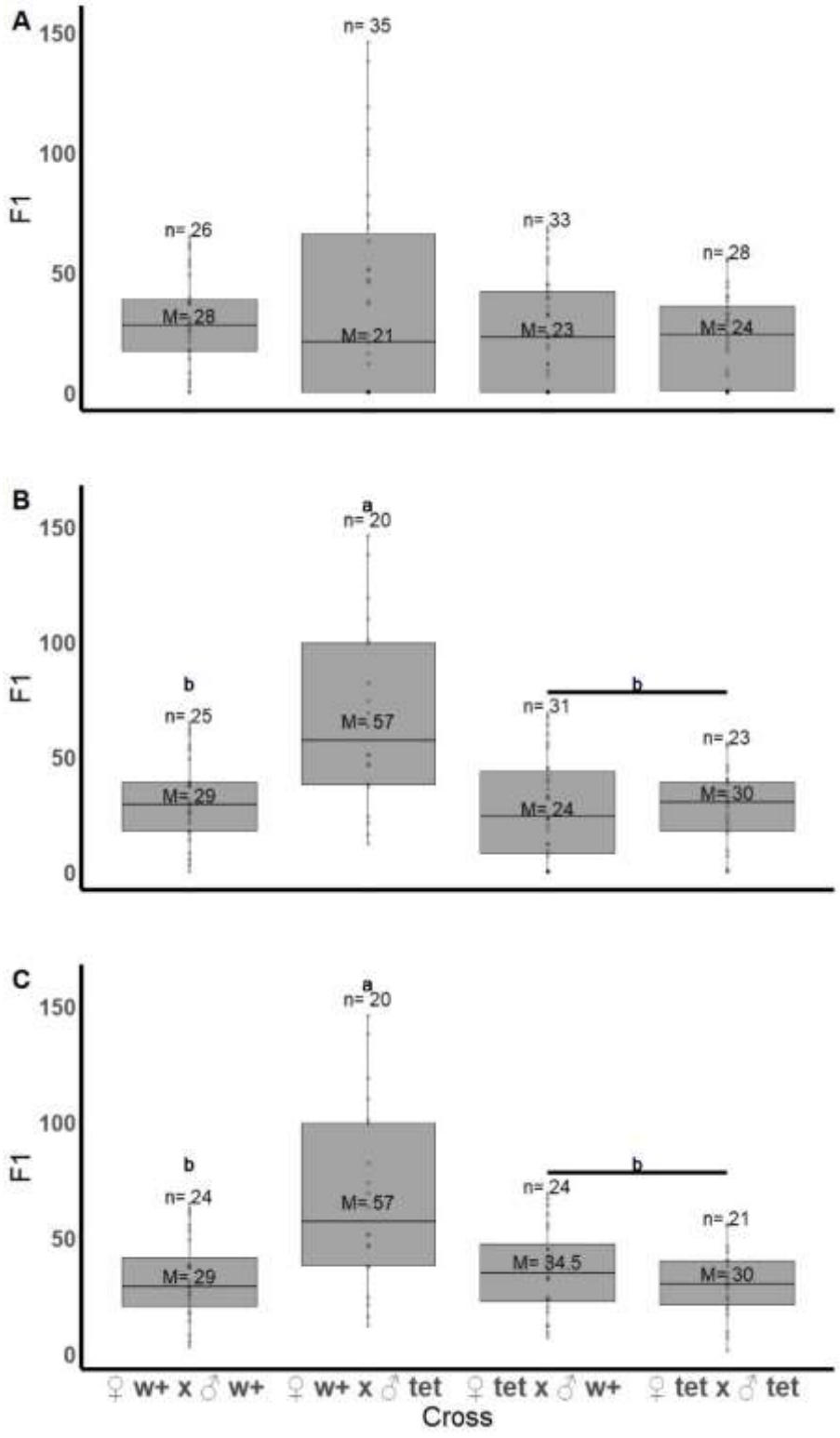

Figure 3. Total progeny $(\mathbf{F_1})$ of infected (w+) and antibiotic-treated (tet) combinations within Pro101 (a *D. prosaltans* lineage) individuals considering **A**. all females $(\mathbf{F_{3,118} = 1.8665\ p = 0.139})$; **B.** only inseminated females $(\mathbf{F_{1,97} = 36.094, p = 3.284\ e^{-8}})$; and **C.** only females whose eggs were fertilized $(\mathbf{F_{1,87} = 32.49, p = 1.697e^{-7}})$. M is the median size of $\boldsymbol{F_1}$ produced, and n is the number of crosses considered in each combination.

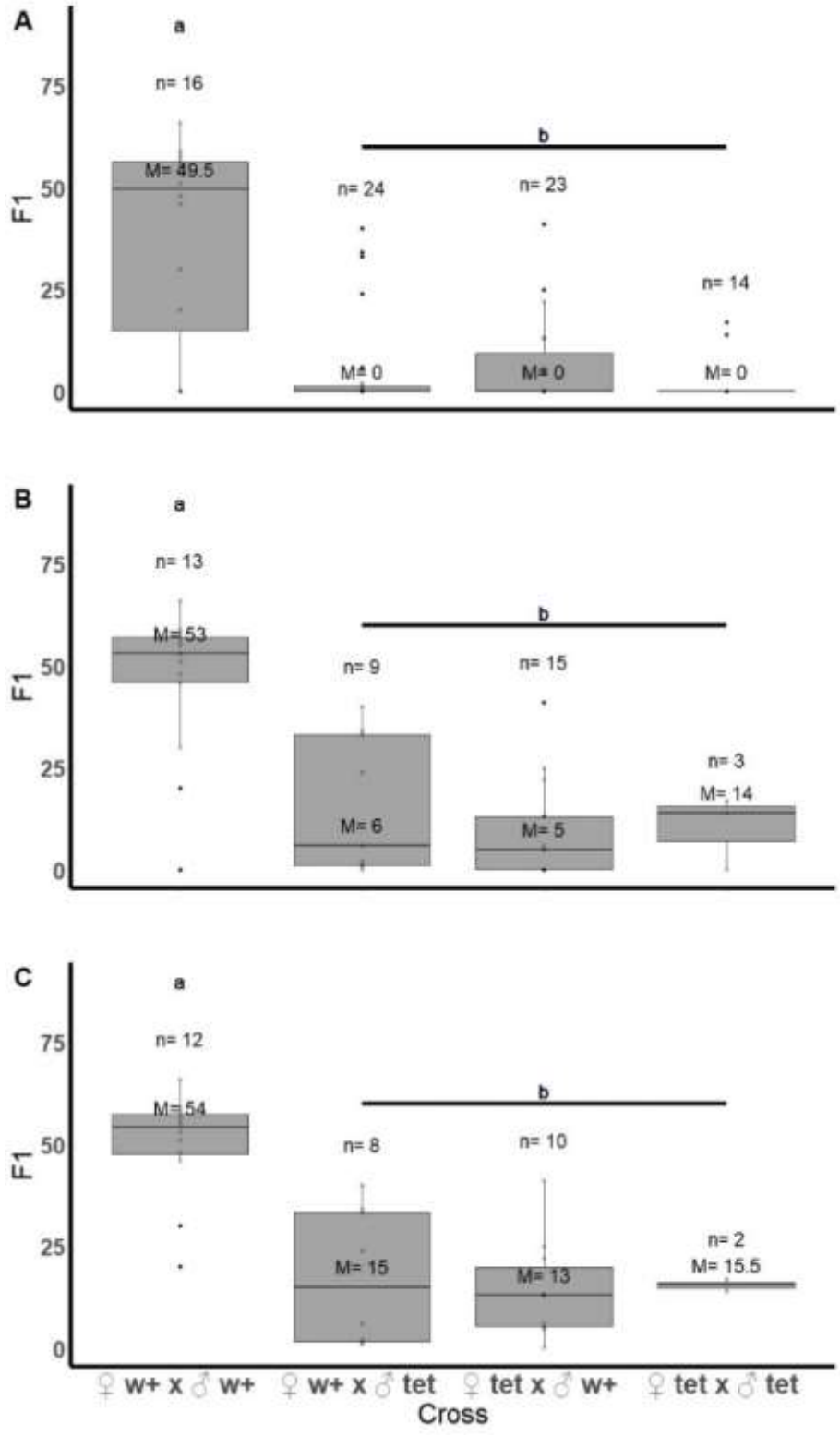

Figure 4. Total progeny (F_1) of non-treated (w+) and antibiotic-treated (tet) combinations within SepPLR2 (a *D. septentriosaltans* lineage) individuals considering **A**. all females($F_{1,75} = 414.288$, $p = 1.085e^{-8}$); **B**. only inseminated females($F_{1,38} = 32.385$, $p = 1.511\,e^{-6}$); and **C**. only females whose eggs were fertilized ($F_{1,30} = 35.137, p = 1.707\,e^{-6}$). M is the median size of F_1produced and n is the number of crosses considered in each combination.

3.2.3 Sex ratio and F_1 infection status

The four cross combinations produced the same sex ratio (50 % females and 50 % males) in both species ($LTR_{3,\ 85} = 5.7658,\ p = 0.1236$ for Pro101 a lineage of *D. prosaltans,* and $LTR_{3,27} = 2.95,\ p = 0.3982$ for SepPLR2 lineage of *D. septentriosaltans*). As expected, the offspring of infected females also host *Wolbachia*.

3.3. Simulating the infection spread

Figure 5 shows the spread of *Wolbachia* infection in Pro101 (a lineage of *D. prosaltans*) and SepPLR2 (a lineage of *D. septentriosaltans*) considering the data shown in Tables 5 and 6 (ins, $nins$ and fe), a sex ratio (fsr) of 50 % (item 3.2.3) and 100 % vertical transmission rate (vt, we did not measure this parameter in this work). Our simulation is available at https://yoan-sarmiento.shinyapps.io/simulation/. We simulated 100 generations (g) in a population of 100 individuals (N) with only one infected female in the first generation (in). For the numbers in F_1, we used the median (M) number of adults produced by fertilized females (indicated in Figure 3C for Pro101 and 4C for SepPLR2). Supplementary Figure 1A shows the parameters set for Pro101. In Pro101 simulation, the infection was not fixed in 100 generations. Instead, it reached 99 % of individuals in 64 generations and 99,9 % in 99 generations. Other simulations showed (data not shown) that the infection takes 289 generations to fix under such parameters. In SepPLR2, the infection was fixed in 15 generations. It is important to note that the fixation depends on 100 % of vertical transmission rate (vt) in both simulations: the lower the transmission rate, the lower the proportion of infected individuals in the population. Table 7 shows the number of generations required to reach 50 % and the maximum prevalence expected in each lineage under different values of vt. We did not consider the effects of drift but can predict that the loss of the bacteria in Pro101 is more likely than in SepPLR2 because, in Pro101, *Wolbachia* takes more generations to fix than in SepPLR2, increasing the chance of losses by drift.

In the second round of simulations (Supp. Fig. 1B), we inverted the median number of individuals found in F_1 of infected females (M) of the crosses involving infected females, *i.e.,* infected females (♀w+) x infected males (♂w+) and infected females (♀w+) x treated males (♂wtet), to investigate the impact of differences in F_1 production on the spread of the infection (the original data have $M = 29$ for the cross

♀w+ x ♂w+ and $M = 57$ for the cross ♀w+ x ♂tet, *i.e.*, as already mentioned, the Pro101 infected females produce more F_1 with treated than with infected males, regardless to be more inseminated by infected males). We found that whether the infected females produced more F_1 when crossed with infected instead of treated males, the infection prevalence could not rise in the simulated population considering the invasion of only one infected female ($ni = 1$). Other simulations (data not shown) revealed that at least 13 infected females must invade the population to ensure increased prevalence.

Finally, in the third round of simulations, we inverted the insemination rate of w+ females (ins and $nins$) when crossed with infected or treated males (Supp. Fig. 1C) to investigate the impact of the insemination rate on the spread of infection. We found that whether the treated males inseminated more infected females than the infected males, the infection reaches a maximum (Pmax) of 79 %. Therefore, to increase the chances of fixing infection in a population with the parameters found in Pro101, infected males need to be more efficient than treated males in inseminating infected females.

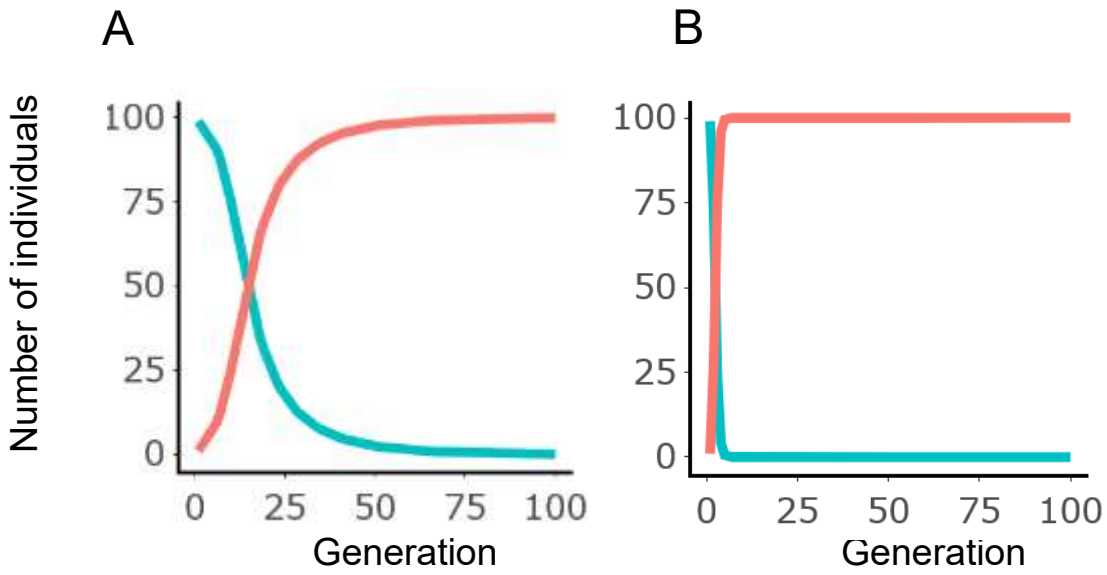

Figure 5. Simulation of *Wolbachia* infection spread in **A**. Pro101 and **B**. SepPLR2 inferred at https://yoan-sarmiento.shinyapps.io/simulation/ considering the parameters of Tables 5 and 6 ($\boldsymbol{ins}$, $\boldsymbol{nins}$ and $\boldsymbol{fe}$), the median ($\boldsymbol{M}$) of $\boldsymbol{F_1}$ showed in Figures 3C and 4C, 50 % of sex ratio ($\boldsymbol{fsr}$) and 100 % of vertical transmission ($\boldsymbol{vt}$). Abbreviations according to the simulator parameters Red line: infected individuals; blue line: non-infected individuals.

Table 7. Simulations of *Wolbachia* spread in Pro101 and SepPLR2 at https://yoan-sarmiento.shinyapps.io/simulation/ considering the parameters used in Figures 5A and B but varying the values of the vertical transmission rate ($\boldsymbol{vt}$). $\boldsymbol{g_{50}}$ corresponds to the number of generations required to infect 50 % of the host population, and $\boldsymbol{P_{max}}$ corresponds to the highest prevalence expected.

vt	Pro101		SepPLR2	
	$\boldsymbol{g_{50}}$	$\boldsymbol{P_{max}}$	$\boldsymbol{g_{50}}$	$\boldsymbol{P_{max}}$
100	14-15	99.9	4-5	100
0.99	15-16	88.9	4-5	99.8
0.95	19-20	65.6	4-5	94.17
0.9	x	46.6	5-6	88.3
0.8	x	18.4	6-7	76.45
0.7	x	0.09	6-7	64.34
0.6	x	0	9-10	21.79

4. Discussion

4.1. Molecular identification of *Wolbachia* strains

In this work, we tested whether an isofemale lineage of *D. prosaltans* (Pro101), sampled in Minas Gerais state (Brazil), harbors *Wolbachia,* and found evidence for high titer infection. In 2016, we collected three other females of *D. prosaltans* and verified that the *wsp* (Wolbachia surface protein), a highly variable sequence, were identical for the three isofemale lineages. Therefore, we chose to work with Pro101. (data not shown).

The molecular characterization revealed that the *D. prosaltans* lineage Pro101 and the *D. septentriosaltans* lineage SepPLR2 harbor similar but not identical *Wolbachia* strains, which probably explains the different effects on their fitness. It is relevant to highlight that different from the results of Miller and Riegler (2006), SepPLR2 did not present evidence of double or multiple strain infections. Even the high variable loci (*VNTR-105, VNTR-141*, and *RO766* - Figure 2) presented only one amplified fragment.

According to the *wsp* alleles obtained (Table 4), SepPLR2 harbors, as expected, the strain *w*Spt PLR2. The *Wolbachia* strain found in Pro101 presents the same *wsp* allele as the wPro SG2 allele found in *D. prosaltans* populations sampled in Panama. We should mention that regardless of the high polymorphism of the gene *wsp*, we have no data on other *loci* harbored by the Panamanian lineage, which led us to leave to further work to determine whether Pro101 carries the *w*Pro SG2 *Wolbachia* strain. This precaution makes even more sense if we consider that populations of *D. prosaltans* from Central and Southern South America may belong to different subspecies, presenting evidence for reproductive isolation (BICUDO; PRIOLI, 1978; DOBZHANSKY; STREISINGER, 1944). Moreover, while we found an allele of 1450 bp for *VNTR-105* and 930 bp for *VNTR-141* in Pro101 (Figure 2), Patarro and Bicudo (2019) found fragments of 1018 bp for both *loci*. In a *D. prosaltans* lineage from Cachoeira dos Monteiros (BA), Brazil. This fact points to an undercover diversity in *Wolbachia* strains in Brazilian *D. prosaltans* populations.

4.2. Crosses:

4.2.1. Drosophila prosaltans

We showed that the infection by *Wolbachia* provides fitness advantages for both females and males (Table 5). *Wolbachia* increases fertility for females (more infected than treated females produced at least one larva - numbers in red in Table 5). Pereira-Filho (2021) compared egg production of infected and treated Pro101 and found that infected females produced significantly more eggs than the treated ones over the first 14 days after adult emergence. He obtained these data by counting the eggs at aged female ovaries, suggesting that the infection enhances females' reproductive potential. Fast *et al.* (2011) showed an increased mitotic activity of germline stem cells, which decreases apoptosis in the germarium, resulting in a fourfold increase in *Drosophila mauritiana* egg production. Zug and Hammerstein (2015) stated that *Wolbachia* could use these mechanisms to enhance host fecundity, explaining the higher progeny of infected Pro101 females.

For males, *Wolbachia* increased the insemination of infected, but not treated females (numbers in green at Table 5), providing a clue that perhaps females prefer infected males or are less able to recognize treated males as mating pairs. However, we did not set up choice experiments and thus cannot confirm that infected females chose which should be tested in further studies. Koukou *et al.* (2006) reported that antibiotic removal of *Wolbachia* reduces levels of premating isolation between cage populations of *D. melanogaster*. They suggested that the premating isolation could be due to alterations on pheromone profiles of males or females, affecting mating preferences, or subtly altering mating behaviors. They inferred that such assortative mating is a side effect of infection rather than direct selection to induce mate discrimination. Later, studying Neotropical *D. paulistorum* infected by *w*Pau (a *w*Au-like strain), Schneider *et al.* (2019) showed that infected females prefer infected males because antibiotic-treated males express altered sexual pheromone profiles and lack endosymbiont pheromone producing cells. Therefore, modifications of pheromone production mediated by *Wolbachia* could cause the preference of infected females for infected males in Pro101.

Because of the fitness advantages of infected individuals, one could predict that the cross combination involving w+ females and w+ males would produce more F_1.

Figure 3A, however, contradicts such predictions and shows that the four combinations produce equivalent F_1. It is important to note that while Figure 3A shows the F_1 of all females, including the non-inseminated ones, Figure 3B reveals that when we discard such females, the combination ♀w+ x ♂tet (infected female x treated males) produces more F_1 then the other pairs. Figure 3C, which considers only fertilized females, shows the same pattern. This difference suggests that treated males can fertilize more eggs than infected ones. Unfortunately, it was impossible to notice the selective advantages of treated males in crosses with treated females, probably because of the low egg production of such females. Snook *et al.* (2000) compared infected and uninfected strains of *D. simulans* and found that infected males produced fewer sperm cysts and presented lower fertility than uninfected males. Also, Lewis *et al.* (2011) showed that infected males transferred fewer fertile sperm than uninfected males to females of *Ephestia kuehniella* Zeller 1879 (Lepidoptera: Pyralidae).

Fry, Palmer, and Rand (2004) also found that infected females produced more F_1 with uninfected males of *D. melanogaster* strains and suggested that such an unexpected pattern promotes the spread of the infection when *Wolbachia* is rare. To test this prediction in our simulator, https://yoan-sarmiento.shinyapps.io/simulation/, we tested what happens when the infected couple produces more F_1 than the pair including an infected female and a treated male. The supplemental material 1B shows the result and indicates that if only one or a few infected females immigrate to an uninfected population, the prevalence cannot increase. Thus, the infection tends to disappear from the population in the first generation. Therefore, in agreement with the Fry, Palmer, and Rand (2004) prediction, our simulator indicated that the increased fitness of the pair infected female x treated male not only promotes the spread but ensures the permanence of *Wolbachia* in a new host population given the other parameters found in Pro101.

Because they produced more F1 with treated than infected males, it would be better for infected females to be inseminated by treated than by infected males. In a second simulation (supplementary material 1C), we tested this hypothesis's effects on the infection's spread. We thus fixed all parameters and inverted the insemination rates of infected females by infected and treated males. For the sake of simplicity, we considered that all inseminated infected females produced at least one larvae. The

simulation showed that, under such circumstances, the spread of *Wolbachia* would be faster (in only 5-6 generations, 50 % of the population would be infected, against 15-16 generations using the original parameters(see Table 7). However, the prevalence could not reach 99.9 % over 100 generations but stuck at 79 %, probably because this change imposes a stronger selection against infected males. Further studies would answer whether the infected females prefer infected males over treated ones or whether this is a collateral effect of the antibiotic treatment. For now, taking such preference as an assumption, we can infer that it is more beneficial to the bacteria than to the female, suggesting that we are facing a case of host manipulation.

4.2.2.*Drosophila septentriosaltans*

Table 6 shows that *Wolbachia* also increases the insemination probability of SepPLR2 males, both with infected and treated females, indicating that, different from Pro101, *Wolbachia* infection seems to interfere with male instead of female behavior. De Crespigny, Pitt, and Wedell (2006) found that males of *D. melanogaster* and *D. simulans*, infected by *w*Mel and *w*Ri, mate in higher frequency than uninfected males. As these *Wolbachia* strains induce CI, they interpreted mating frequency as an adaptation to reduce CI consequences. However, we did not find CI in SepPLR2, and other studies would be necessary to explain the higher rate of insemination in this species.

Figure 4A shows that the crosses involving w+ couples produced more progeny than the other combinations. Therefore, we can infer that in this case, *Wolbachia* favors both males and females. Different from the pattern found in Pro101 (Fig. 3), , when we considered only the inseminated or fertilized females (Figure 4B and 4C), the pattern remained, indicating that infected males, in addition to inseminate and fertilize more females, produce more offspring than the treated ones. Such an effect is rare since *Wolbachia* is known to harm males, and its maternal inheritance, in theory, precludes selection in favor of infected males. (ZUG; HAMMERSTEIN, 2015). However, Wade and Chang (1995) and Hariri, Werren, and Wilkinson (1998) found *Wolbachia* increasing male fertility.

We should mention that a similar result could be obtained if antibiotics impair the reproductive potential of SepPLR for reasons other than *Wolbachia*. We tried to

minimize such impact by leaving the strain for at least five generations without antibiotics and feeding larvae and adults of the sixth generation with conspecific male feces to ensure the re-establishment of the intestinal microbiota. Given that, and considering that the PCR results ensured the drop of *Wolbachia* titer to undetectable levels, we considered the fitness effects as consequences of lack of *Wolbachia* rather than side effects of antibiotic treatment. Similar results led to the conclusion that in *D. paulistorum* superspecies, composed of at least seven subspecies, from which four harbor *w*Au-like strains, *Wolbachia* is a fixed obligate mutualism (EHRMAN, 1968; EHRMAN; WILLIAMSON, 1969; KERNAGHAN; EHRMAN, 1970; MILLER; EHRMAN; SCHNEIDER, 2010; SCHNEIDER *et al.*, 2019). In the case of *D. septentriosaltans*, we maintained the treated lineage for several generations (this lineage is still living in the lab – Nov/2021). However, we found no evidence of uninfected populations of this species in the literature, which could indicate that *D. septentriosaltans*, like *D. paulistorum*, depend on *Wolbachia* to survive or reproduce at field conditions as shown by Miller; Ehrman; and Schneider, (2010). Further investigations should address this subject.

4.3. Simulating the infection Spread

Using our simulator https://yoan-sarmiento.shinyapps.io/simulation/, Figure 5), we found that the strategy adopted by the strain harbored by SepPLR2 promotes the rapid spread of the infection at the host population more efficiently than the strain harbored by Pro101. In addition, in Table 7, we showed that when we change the vertical transmission rate (vt), the infecting Pro101 strain depends on values of vt above 95 %, to infect least half the population. On the contrary, SepPLR2 achieved 50 % of infection with a vt of only 70 %, vt which could help to explain why all *D. septentriosaltans* mentioned in the literature are infected (acho que precisa de referências). Unfortunately, we could not calculate the transmission rate (vt) in this work since it would be necessary to collect a sample of females of each species to calculate vt in F_1 and F_5, as described in Moreira (2020). However, over six years of collections, we only obtained three females of *D. prosaltans*, an admittedly rare species that occurs in small populations spread over a wide geographic distribution (DOBZHANSKY; PAVAN, 1950; DOBZHANSKY; PAVAN, 1943; DOBZHANSKY;

STREISINGER, 1944; MAGALHAES, 1962), and never collected any individual of *D. septentriosaltans*.

5. Conclusion

The present work showed that the Brasilian *D. prosaltans* lineage, Pro101, harbors a *Wolbachia* strain of the *w*Au-like clade, with the *wsp* identical to wPro SG2 found in Panama. Both Pro101 and SepPLR2 proved to survive and reproduce, at least under laboratory conditions, with no or low-titer *Wolbachia*. Furthermore, we found no evidence of cytoplasmatic incompatibility or biases at sex ratio, two common phenotypes induced by the α-proteobacterium, but noted that the depletion of *Wolbachia* impacted the fitness of both lineages.

In Pro101, we found a clue that infected females prefer infected males, even though they produce more offspring with treated males, which we interpreted as host manipulation. As we did not set behavior experiments, we still need to confirm whether infected Pro101 females prefer infected males. Notwithstanding, our simulations showed that the production of more offspring with treated males results in a rapid spread of the infection in uninfected populations. Our simulations also showed that the preference for infected males might slow down the spread but ensure the fixation of the infection at the settled populations, possibly because it reduces the natural selection against infected males that presumably produce less viable sperm.

In SepPLR2, the disadvantages of *Wolbachia* depletion were even more evident, both for males and females, at pre and post-mating fitness components, which led us to question the ability of uninfected SepPLR2 to survive and compete with infected individuals in field conditions. Indeed, to the best of our knowledge, there are no registers of uninfected *D. septentriosaltans*.

6. Acknowledgments

This study was financed in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES) - Finance Code 001. The authors thank Dr. Wolfgang Miller from the Center of Anatomy and Cell Biology, Medical University of Vienna, for kindly providing some of the drosophila lineages used in this work.

7. References

ARTHOFER, W.; RIEGLER, M.; SCHNEIDER, D.; KRAMMER, M.; MILLER, W. J.; STAUFFER, C. Hidden *Wolbachia* diversity in field populations of the European cherry fruit fly, *Rhagoletis cerasi* (Diptera, Tephritidae). **Molecular Ecology**, vol. 18, no. 18, p. 3816–3830, 1 Sep. 2009. DOI 10.1111/j.1365-294X.2009.04321.x.

BAILLY-BECHET, M.; MARTINS-SIMÕES, P.; SZÖLLOSI, G. J.; MIALDEA, G.; SAGOT, M. F.; CHARLAT, S. How long does *Wolbachia* remain on board? **Molecular Biology and Evolution**, vol. 34, no. 5, 2017. https://doi.org/10.1093/molbev/msx073.

BALDO, L.; HOTOPP, J. C. D.; JOLLEY, K. A.; BORDENSTEIN, S. R.; BIBER, S. A.; CHOUDHURY, R. R.; HAYASHI, C.; MAIDEN, M. C. J.; TETTELIN, H. H.; WERREN, J. H.; DUNNING HOTOPP, J. C.; JOLLEY, K. A.; BORDENSTEIN, S. R.; BIBER, S. A.; CHOUDHURY, R. R.; HAYASHI, C.; MAIDEN, M. C. J.; TETTELIN, H. H.; WERREN, J. H. Multilocus sequence typing system for the endosymbiont *Wolbachia pipientis.* **Applied and Environmental Microbiology**, vol. 72, no. 11, p. 7098–7110, Dec. 2006. DOI 10.1128/AEM.00731-06.

BICUDO, H. E. M. Reproductive isolation in the *saltans* group of *Drosophila*. I. The *saltans* subgroup. **Genetica**, vol. 44, no. 3, p. 313–329, Sep. 1973. DOI 10.1007/BF00161311.

BICUDO, H. E. M. de C.; PRIOLI, A. J. Reproductive isolation in the *Saltans* group of *Drosophila* II. The *saltan*s subgroup. **Genetica**, vol. 48, no. 1, p. 17–22, Jan. 1978. DOI 10.1007/BF00125282.

BLEIDORN, C.; GERTH, M. A critical re-evaluation of multilocus sequence typing (MLST) efforts in *Wolbachia*. **FEMS Microbiology Ecology**, vol. 94, no. 1, p. 133710, 2018. DOI 10.1093/femsec/fix163.

CHANG, W.; CHENG, J.; ALLAIRE, J.; XIE, Y.; MCPHERSON, J.; OTHERS. Shiny: web application framework for R. **R package version**, vol. 1, no. 5, p. 2017.

CHARLAT, S.; BALLARD, J. W. O.; MERÇOT, H. What maintains noncytoplasmic incompatibility inducing *Wolbachia* in their hosts: A case study from a natural *Drosophila yakuba* population. **Journal of Evolutionary Biology**, vol. 17, no. 2, p. 322–330, 1 Mar. 2004. DOI 10.1046/j.1420-9101.2003.00676.x.

CHARLAT, S.; LE CHAT, L.; MERÇOT, H. Characterization of non-cytoplasmic incompatibility inducing *Wolbachia* in two continental African populations of *Drosophila simulans*. **Heredity 2003 90:1**, vol. 90, no. 1, p. 49–55, 9 Jan. 2003. DOI 10.1038/sj.hdy.6800177.

CLANCY, D. J.; HOFFMANN, A. A. Environmental effects on cytoplasmic incompatibility and bacterial load in *Wolbachia*-infected *Drosophila simulans.* **Entomologia Experimentalis et Applicata**, 1998. https://doi.org/10.1046/j.1570-7458.1998.00261.x.

CRAWLEY, M. J. **The R Book**. [*S. l.*]: John Wiley & Sons, 2007. https://doi.org/10.1002/9780470515075.

DE CRESPIGNY, F. E. C.; PITT, T. D.; WEDELL, N. Increased male mating rate in

Drosophila is associated with *Wolbachia* infection. **Journal of Evolutionary Biology**, vol. 19, no. 6, p. 1964–1972, 1 Nov. 2006. DOI 10.1111/j.1420-9101.2006.01143.x.

DOBSON, S. L.; RATTANADECHAKUL, W.; MARSLAND, E. J. Fitness advantage and cytoplasmic incompatibility in *Wolbachia* single- and superinfected *Aedes albopictus*. **Heredity**, vol. 93, no. 2, p. 135–42, 5 Aug. 2004. DOI 10.1038/sj.hdy.6800458.

DOBZHANSKY, T.; PAVAN, C. Local and seasonal variations in relative frequencies of species of *Drosophila* in Brazil. **Journal of Animal Ecology,** vol. 19, no. 1, p. 1–14, 1950.

DOBZHANSKY, T.; STREISINGER, G. Experiments on Sexual Isolation in *Drosophila*: II. Geographic Strains of *Drosophila prosaltans*. **Proceedings of the National Academy of Sciences**, vol. 30, no. 11, p. 340–345, 15 Nov. 1944. https://doi.org/10.1073/PNAS.30.11.340.

DOBZHANSKY, T.; PAVAN, C. Studies on Brazilian Species of *Drosophila*. **Boletim da Faculdade de Filosofia e Ciencias e letras da Universidade de S. Paulo**, vol. 36, no. 4, p. 7–72, 1943.

DOUGLAS, A. E. The *Drosophila* model for microbiome research. **Lab Animal**, vol. 47, no. 6, p. 157–164, 1 Jun. 2018. DOI 10.1038/s41684-018-0065-0.

EHRMAN, L. Antibiotics and infectious hybrid sterility in *Drosophila paulistorum.* **MGG Molecular & General Genetics**, vol. 103, no. 3, p. 218–222, 1968. DOI 10.1007/BF00273689.

EHRMAN, L.; WILLIAMSON, D. L. On the etiology of the sterility of hybrids between certain strains of *Drosophila paulistorum*. **Genetics**, vol. 62, no. 1, p. 193, May 1969.

FAST, E. M.; TOOMEY, M. E.; PANARAM, K.; DESJARDINS, D.; KOLACZYK, E. D.; FRYDMAN, H. M. *Wolbachia* enhance *Drosophila* stem cell proliferation and target the germline stem cell niche. **Science**, vol. 334, no. 6058, p. 990–992, 18 Nov. 2011. DOI 10.1126/science.1209609.

FINE, P. E. M. Vectors and vertical transmission: an epidemiologic perspective. **Annals of the New York Academy of Sciences**, vol. 266, no. 1, p. 173–194, 1 Nov. 1975. DOI 10.1111/j.1749-6632.1975.tb35099.x.

FRY, A. J.; PALMER, M. R.; RAND, D. M. Variable fitness effects of *Wolbachia* infection in *Drosophila melanogaster*. **Heredity**, vol. 93, no. 4, p. 379–389, 11 Aug. 2004. DOI 10.1038/sj.hdy.6800514.

HARIRI, A. R.; WERREN, J. H.; WILKINSON, G. S. Distribution and reproductive effects of *Wolbachia* in stalk-eyed flies (Diptera: Diopsidae). **Heredity**, vol. 81, no. 3, p. 254–260, 1 Sep. 1998. DOI 10.1046/j.1365-2540.1998.00364.x.

HEATH, B. D.; BUTCHER, R. D. J.; WHITFIELD, W. G. F.; HUBBARD, S. F. Horizontal transfer of *Wolbachia* between phylogenetically distant insect species by a naturally occurring mechanism. **Current Biology**, vol. 9, p. 313–316, 1999.

HEDGES, L. M.; BROWNLIE, J. C.; O'NEILL, S. L.; JOHNSON, K. N. *Wolbachia* and Virus Protection in Insects. **Science**, vol. 322, no. 5902, 2008. .

HOFFMANN, A. A.; CLANCY, D.; DUNCAN, J. Naturally-occurring *Wolbachia* infection in *Drosophila simulans* that does not cause cytoplasmic incompatibility. **Heredity**, vol. 76, no. 1, p. 1–8, Jan. 1996. DOI 10.1038/hdy.1996.1.

JAMES, A. C.; BALLARD, J. W. O. Expression of cytoplasmic incompatibility in *Drosophila simulans* and its impact on infection frequencies and distribution of *Wolbachia pipientis*. **Evolution**, vol. 54, no. 5, p. 1661–1672, 2000. https://doi.org/10.1111/j.0014-3820.2000.tb00710.x.

JEYAPRAKASH, A.; HOY, M. A. Long PCR improves *Wolbachia* DNA amplification: *wsp* sequences found in 76% of sixty-three arthropod species. **Insect Molecular Biology**, vol. 9, no. 4, p. 393–405, Aug. 2000. DOI 10.1046/j.1365-2583.2000.00203.x.

JIGGINS, F. M. Male-killing *Wolbachia* and mitochondrial DNA: Selective sweeps, hybrid introgression and parasite population dynamics. **Genetics**, vol. 164, no. 1, p. 5–12, 2003.

JOLLEY, K. A.; BRAY, J. E.; MAIDEN, M. C. J. Open-access bacterial population genomics: BIGSdb software, the PubMLST.org website and their applications [version 1; referees: 2 approved]. **Wellcome Open Research**, vol. 3, 2018. DOI 10.12688/wellcomeopenres.14826.1.

KERNAGHAN, R. P.; EHRMAN, L. An electron microscopic study of the etiology of hybrid sterility in *Drosophila paulistorum*. **Chromosoma,** vol. 29, no. 3, p. 291–304, Sep. 1970. DOI 10.1007/BF00325944.

KOEHCKE, A.; TELSCHOW, A.; WERREN, J. H.; HAMMERSTEIN, P. Life and death of an influential passenger: *Wolbachia* and the evolution of CI-modifiers by their hosts. **PLoS ONE**, vol. 4, no. 2, p. e4425, 11 Feb. 2009. DOI 10.1371/journal.pone.0004425.

KOUKOU, K.; PAVLIKAKI, H.; KILIAS, G.; WERREN, J. H.; BOURTZIS, K.; ALAHIOTIS, S. N. Influence of antibiotic treatment and *Wolbachia* curing on sexual isolation among *Drosophila melanogaste*r cage populations. **Evolution**, vol. 60, no. 1, p. 87–96, 1 Jan. 2006. DOI 10.1111/j.0014-3820.2006.tb01084.x.

KRIESNER, P.; HOFFMANN, A. A.; LEE, S. F.; TURELLI, M.; WEEKS, A. R. Rapid sequential spread of two *Wolbachia* variants in *Drosophila simulans.* **PLoS Pathog**, vol. 9, no. 9, p. 1003607, 2013. DOI 10.1371/journal.ppat.1003607.

LEWIS, Z.; CHAMPION DE CRESPIGNY, F. E.; SAIT, S. M.; TREGENZA, T.; WEDELL, N. *Wolbachia* infection lowers fertile sperm transfer in a moth. **Biology Letters**, vol. 7, no. 2, p. 187–189, 23 Apr. 2011. DOI 10.1098/rsbl.2010.0605.

LI, Y.-Y. Y.; FLOATE, K. D.; FIELDS, P. G.; PANG, B.-P. P. Review of treatment methods to remove *Wolbachia* bacteria from arthropods. **Symbiosis**, n. 62, p. 1–15, 30 Jan. 2014.DOI 10.1007/s13199-014-0267-1.

LIPSITCH, M.; NOWAK, M. A.; EBERT, D.; MAY, R. M. The population dynamics of vertically and horizontally transmitted parasites. **Proceedings of the Royal Society B: Biological Sciences**, vol. 260, no. 1359, p. 321–327, 22 Jun. 1995. DOI 10.1098/rspb.1995.0099.

MAGALHAES, L. D. Notes on the taxonomy, morphology, and distribution of the *saltans* group of *Drosophila*, with descriptions of four new species. **University of Texas Publication**, vol. 6205, p. 135–154, 1962.

MILLER, W. J.; EHRMAN, L.; SCHNEIDER, D. Infectious speciation revisited: Impact of symbiont-depletion on female fitness and mating behavior of *Drosophila paulistorum.* **PLoS Pathogens**, vol. 6, no. 12, p. e1001214, 2 Jan. 2010. DOI 10.1371/journal.ppat.1001214.

MILLER, W. J.; RIEGLER, M. Evolutionary dynamics of *w*Au-like *Wolbachia* variants in neotropical *Drosophila* spp. **Applied and environmental microbiology**, vol. 72, no. 1, p. 826–35, 1 Jan. 2006. DOI 10.1128/AEM.72.1.826-835.2006.

MOREIRA, M. M. Negative fitness effects of *Wolbachia* in *Drosophila sturtevanti*. 2020. 29 f. Universidade Federal de Viçosa, 2020.

MORIYAMA, M.; NIKOH, N.; HOSOKAWA, T.; FUKATSU, T. Riboflavin provisioning underlies *Wolbachia*'s fitness contribution to its insect host. **mBio**, vol. 6, no. 6, 10 Nov. 2015. https://doi.org/10.1128/mBio.01732-15.

MÜLLER, M. J.; DÖRR, N. C. D.; DEPRÁ, M.; SCHMITZ, H. J.; VALIATI, V. H.; VALENTE, V. L. da S.; JOSIAS, M.; CAROLINA, N.; DÖRR, D.; DEPRÁ, M.; JOSÉ, H.; HUGO, V.; LÚCIA, V. Reevaluating the infection status by the *Wolbachia* endosymbiont in *Drosophila* Neotropical species from the *willistoni* subgroup. **Infection, genetics and evolution**, vol. 19, p. 232–239, Oct. 2013. DOI 10.1016/j.meegid.2013.07.022.

OSBORNE, S. E.; LEONG, Y. S.; O'NEILL, S. L.; JOHNSON, K. N. Variation in antiviral protection mediated by different *Wolbachia* strains in *Drosophila simulans.* **PLoS Pathogens**, vol. 5, no. 11, Nov. 2009. https://doi.org/10.1371/JOURNAL.PPAT.1000656.

PATARRO, T. de F.; BICUDO, H. E. M. de C. Intracellular Interactions in the reproduction control of introgressed strains involving species from *Drosophila saltans* group (*sophophora* subgenus) with emphasis to the effect of *Wolbachia* infection. **Open Journal of Animal Sciences**, vol. 09, no. 03, p. 324–340, 8 May 2019. DOI 10.4236/ojas.2019.93027.

PEREIRA-FILHO, H. P.; KALIMAN, K. C. G; GUZMÁN, Y. C.; YOTOKO, K.; LINO, J. (In prep.) The interaction *Wolbachia-Drosophila sturtevanti* as a model of infection of new hosts.

POINSOT, D.; MERÇOT, H. *Wolbachia* infection in *Drosophila simulans*: Does the female host bear a physiological cost? **Evolution**, vol. 51, no. 1, p. 180–186, 1 Feb. 1997. DOI 10.1111/j.1558-5646.1997.tb02399.x.

R CORE TEAM. R: A language and environment for statistical computing. R Foundation for Statistical Computing. **Vienna, Austria: URL https://www. R-project. org/.[Google Scholar]**, 2017.

RAYCHOUDHURY, R.; BALDO, L.; OLIVEIRA, D. C. S. G.; WERREN, J. H. Modes of acquisition of *Wolbachia*: horizontal transfer, hybrid introgression, and codivergence in the *Nasonia* species complex. **Evolution: International Journal of Organic Evolution**, vol. 63, no. 1, p. 165–183, 1 Jan. 2009. DOI 10.1111/j.1558-

5646.2008.00533.x.

RIEGLER, M.; ITURBE-ORMAETXE, I.; WOOLFIT, M.; MILLER, W. J.; O'NEILL, S. L.; O'NEILL, S. L. Tandem repeat markers as novel diagnostic tools for high resolution fingerprinting of *Wolbachia*. **BMC microbiology**, vol. 12 Suppl 1, no. Suppl 1, p. S12, 18 Jan. 2012. DOI 10.1186/1471-2180-12-S1-S12.

RIEGLER, M.; SIDHU, M.; MILLER, W. J.; O'NEILL, S. L.; O'NEILL, S. L.; O'NEILL, S. L. Evidence for a global *Wolbachia* replacement in *Drosophila melanogaster*. **Current biology : CB**, vol. 15, no. 15, p. 1428–33, 9 Aug. 2005. DOI 10.1016/j.cub.2005.06.069.

RIEGLER, M.; STAUFFER, C. *Wolbachia* infections and superinfections in cytoplasmically incompatible populations of the European cherry fruit fly *Rhagoletis cerasi* (Diptera, Tephritidae). **Molecular Ecology**, vol. 11, no. 11, p. 2425–2434, 9 Oct. 2008. DOI 10.1046/j.1365-294X.2002.01614.x.

SCHNEIDER, D. I.; EHRMAN, L.; ENGL, T.; KALTENPOTH, M.; HUA-VAN, A.; LE ROUZIC, A.; MILLER, W. J. Symbiont-driven male mating success in the Neotropical *Drosophila paulistorum* Superspecies. **Behavior Genetics**, vol. 49, no. 1, p. 83–98, 15 Jan. 2019. DOI 10.1007/s10519-018-9937-8.

SIEVERT, C. **Interactive Web-Based Data Visualization with R, plotly, and shiny**. [*S. l.*]: CRC Press, 2020. https://doi.org/10.1201/9780429447273.

SNOOK, R. R.; CLELAND, S. Y.; WOLFNER, M. F.; KARR, T. L. Offsetting effects of *Wolbachia* infection and heat shock on sperm production in *Drosophila simulans*: Analyses of fecundity, fertility and accessory gland proteins. **Genetics**, vol. 155, no. 1, p. 167–178, 2000. https://doi.org/10.1093/genetics/155.1.167.

TURELLI, M. Evolution of incompatibility-inducing microbes and their hosts. **Evolution**, vol. 48, no. 5, p. 1500–1513, 1 Oct. 1994. https://doi.org/10.1111/j.1558-5646.1994.tb02192.x.

VAVRE, F; FLEURY, F.; LEPETIT, D.; FOUILLET, P.; BOULÉTREAU, M. Phylogenetic evidence for horizontal transmission of *Wolbachia* in host-parasitoid associations. **Molecular biology and evolution**, vol. 16, no. 12, p. 1711–23, 1 Dec. 1999.

WADE, M. J.; CHANG, N. W. Increased male fertility in *Tribolium confusum* beetles after infection with the intracellular parasite *Wolbachi*a. **Nature**, vol. 373, no. 6509, p. 72–74, 5 Jan. 1995. DOI 10.1038/373072a0.

WERREN, J. H.; BALDO, L.; CLARK, M. E. *Wolbachia*: master manipulators of invertebrate biology. **Nature Rev Microbiol**, vol. 6, no. 10, p. 741–751, Oct. 2008. DOI 10.1038/nrmicro1969.

WERREN, John H.; JAENIKE, J. *Wolbachia* and cytoplasmic incompatibility in mycophagous *Drosophila* and their relatives. **Heredity**, vol. 75, no. 3, p. 320–326, 1 Sep. 1995. DOI 10.1038/hdy.1995.140.

WICKHAM, H. Elegant Graphics for Data Analysis. 2009. .

ZABALOU, S.; CHARLAT, S.; NIRGIANAKI, A.; LACHAISE, D.; MERÇOT, H.; BOURTZIS, K. Natural *Wolbachia* infections in the *Drosophila yakuba* species

complex do not induce cytoplasmic incompatibility but fully rescue the *w*Ri modification. **Genetics**, vol. 167, no. 2, p. 827–834, 1 Jun. 2004. DOI 10.1534/genetics.103.015990.

ZEH, J. A.; ZEH, D. W. The evolution of polyandry I: Intragenomic conflict and genetic incompatibility. **Proceedings of the Royal Society B: Biological Sciences**, vol. 263, no. 1377, p. 1711–1717, 1996. DOI 10.1098/rspb.1996.0250.

ZUG, R.; HAMMERSTEIN, P. Bad guys turned nice? A critical assessment of *Wolbachia* mutualisms in arthropod hosts. **Biological Reviews of the Cambridge Philosophical Society**, vol. 90, no. 1, p. 89–111, 1 Feb. 2015. DOI 10.1111/brv.12098.

Supplementary material 1

Simulation using the data obtained from Pro101 crosses (Table 5, in bold) and the median number of adults produced by fertilized females (Figure 3C, in bold). The table follows the logic of Table A1 of appendix. $\left(I_{c_i} = \frac{ins}{ins+nins}\right)$, $\left(Fe_{c_i} = \frac{fe}{ins}\right)$, and $\left(F_{1_{c_1}} = \frac{M_{c_i}}{M}\right)$.

Cross #	Couple	ins	$nins$	I_{c_i}	fe	Fe_{c_i}	M	$F_{1_{c_1}}$
c_1	♀w+ x ♂w-	**25**	**1**	0.96	**24**	0.96	**29**	0.19
c_2	♀w+ x ♂w-	**20**	**15**	0.57	**20**	1.0	**57**	0.38
c_3	♀w- x ♂w+	**31**	**2**	0.93	**24**	0.77	**34.5**	0.23
c_4	♀w- x ♂w-	**23**	**5**	0.82	**21**	0.91	**30**	0.2
Sum							150.5	

The simulation also needs the population parameters: the number of individuals of the uninfected population ($N = 100$), the number of infected females invading the population ($ni = 1$), the number of generations to be simulated ($g = 100$) the vertical transmission rate (vt=100) of the strain and the female sex ratio of the infected female´s offspring (fsr=0.5), according to Table A2.

Under these conditions, a D. prosaltans with the same parameters as Pro101 (inferred in this paper), when invaded by only one infected female, would reach 99 % of infected individuals in 64 generations, and 99.9 % in 99 generations.

Figure A. Simulation using the data obtained from Pro101 crosses (Table 5) and the median number of adults produced by fertilized females (Figure 3C).

Figure B. Simulation using the data obtained from Pro101 crosses (Table 5) and the median number of adults produced by fertilized females (Figure 3C), but inverting the number of adults produced by the couples for ♀w+ x ♂w+ (57) ♀w+ x ♂tet (29) (yellow squares show changed values compared with Supp. Fig. 1A).

Figure C. Simulation using the data obtained from Pro101 crosses (Table 5) and the median number of adults produced by fertilized females (Figure 3C), but inverting the number of w+ females inseminated by w+ and tet males, and considering that all inseminated females were fertilized (*). Yellow squares show changed values compared with Supp. Fig. 1A).

CHAPTER 2: THE ROLE OF ENDOSYMBIONTS IN THE REPRODUCTIVE ISOLATION OF *DROSOPHILA PROSALTANS* AND *D. SALTANS* (DIPTERA: DROSOPHILIDAE)

Guzman Sarmiento, Yoan Camilo[1]; Moreira, Marina Magalhães[1]; Bouzada-Dias Luísa[2]; Yotoko, Karla[2]

[1]Programa de Pós-graduação em Entomologia, Universidade Federal de Viçosa, 36570-900, Viçosa-MG, Brasil [1]

[2]Departamento de Biologia Geral Universidade Federal de Viçosa, 36570-900, Viçosa-MG, Brasil

Abstract

Species of the *Drosophila saltans* group, such as the cryptic species *D. saltans* and *D. prosaltans*, were essential in understanding the speciation process by Dobzhansky and his collaborators. *D. saltans* occurs from northern Mexico to Costa Rica, and *D. prosaltans* occurs from Costa Rica to southern Brazil. They do not generate offspring in sympatry, but lineages from distant populations placed together in the laboratory mate and generate offspring. More recent studies have revealed that populations of *D. prosaltans* harbor different strains of *Wolbachia*. Our previous work revealed that the strain infecting the lineage Pro101 (*D. prosaltans*), collected in southeastern Brazil, increases females' adaptive value but impairs the males, probably reducing their sperm production. In this work, we investigated the role of infection in the reproductive isolation of Pro101 and a strain of *D. saltans* collected in northern Mexico (Sal02), which, as far as we could verify, is free of *Wolbachia*. Our results showed that infection does not seem to affect pre-copulation components of isolation since infected Pro101 females were equally inseminated by Sal02 males or Pro101 males treated with antibiotics. The infection also does not cause cytoplasmic incompatibility or hybrid sterility, two post-copulation components of isolation. However, crossing Sal02 females with Pro101 males did cause hybrid male sterility, leading us to hypothesize that Sal02 produces some maternal effect capable of sterilizing the hybrid. Further studies should focus on the consequences of Sal02 treatment on interspecific crosses

and on finding the putative endosymbiont responsible for the unexpected phenotypes found here.

Keywords: speciation; pre and post-mating reproductive isolation; hybrid male sterility; *Wolbachia*.

1. Introduction

The field of population genetics owes much to the repeated visits of Theodosios Dobzhansky to Brazil to study Neotropical groups of *Drosophila* since 1948, especially *Drosophila willistoni* Burla and Da Cunha (1949) and *Drosophila saltans* Sturtevant, 1942. In his repeated travels, Dobzhansky focused his research on the genetic variation of *Drosophila* species related to adaptability to the ecological conditions of the tropics. Among the species of the *saltans* group, he was particularly interested in *Drosophila prosaltans* Duda, 1927, a species sampled in all his field samples, although in small numbers (SIÃO; MARTINS, 2020).

At that time, populations of *D. saltans* Sturtevant, 1916 were often misidentified as *D. prosaltans*, so that the species was believed to occur from northern Mexico to southern Brazil. several studies of sexual preference involving lineages from Mexico, Guatemala, and Brazil (DOBZHANSKY; STREISINGER, 1944), between Mexican and Brazilian populations (MAYR; DOBZHANSKY, 1945), or involving populations from Mexico, Trinidad, and Brazil (HOENIGSBERG; SANTIBANEZ, 1960) have their results compromise since there are two species and not populations of a single species.

Later, *D. saltans* and *D. prosaltans* were classified in the subgroup *saltans* Magalhaes ,1962, composed of seven sibling species (*sensu* MAYR, 1942), which are only adequately distinguishable by aspects of sperm morphology and male genitalia (MAGALHAES, 1962; MAGALHÃES; BJÖRNBERG, 1957). Since then, it was established that *D. saltans* occurs from Mexico to Costa Rica while *D. prosaltans* occurs from Costa Rica to southern Brazil (MAGALHAES, 1962). With this, it became clear that the sexual preference experiments focused on *D. prosaltans* previously mentioned mixed populations of *D. saltans* and *D. prosaltans*,

which presented isolation but generated different proportions of fertile offspring, depending on the combination studied (DOBZHANSKY; STREISINGER, 1944).

In 1973, Bicudo studied the reproductive isolation of the seven species of the *Drosophila saltans* subgroup and showed that sympatric lineages of *D. saltans* and *D. prosaltans* (in Costa Rica) were wholly isolated while lineages from allopatric populations generated offspring by crossing in both directions. She further argued that populations collected at the extremes of distribution (Northern Mexico for *D. saltans* and Southern Brazil for *D. prosaltans*) were less isolated than combinations involving geographically closer populations. Also, she discussed the origin of reproductive isolation and attributed it to two leading causes: (1) chance, as a side effect of genetic divergence (MULLER, 1942); and (2) natural selection that acts when two allopatric populations with incipient isolation become sympatric (DOBZHANSKY, 1940).

More recently, infections by endosymbionts that spread vertically through populations have been identified as another cause of reproductive isolation, especially pre-mating (BRUCKER; BORDENSTEIN, 2012; SHROPSHIRE; BORDENSTEIN, 2016), but also post-mating isolation (JAENIKE *et al.*, 2006). For example, the endosymbiont *Wolbachia*, an alfa-proteobacteria widely distributed in arthropods (JEYAPRAKASH; HOY, 2000; WERREN, WINDSOR; GUO, 1995), plays a relevant role in speciation (BOURTZIS; MILLER, 2003) by altering reproductive behavior; affecting mating discrimination (MILLER; EHRMAN; SCHNEIDER, 2010; SCHNEIDER *et al.*, 2019); impairing sperm-egg incompatibilities, known as Cytoplasmic incompatibility-CI (WERREN; BALDO; CLARK, 2008); or producing hybrid male sterility in crosses involving close related species (MILLER; EHRMAN; SCHNEIDER, 2010).

Our previous work found evidence that *Wolbachia* infection can disturb the recognition among individuals of the *D. prosaltans* strain, Pro101. Specifically, we saw that infected females avoid mating with treated males, as found in Schneider *et al.* (2019), even though this mating is the most prolific compared to other cross combinations involving infected and treated individuals (GUZMAN et al. *in prep* - Chapter 1). Therefore, in the present work, we set up interspecific crosses with individuals from the Brazilian Pro101 and a Mexican lineage of *D. saltans* (Sal 02 - see

methods) to investigate the role of *Wolbachia* in pre-and post-mating isolation between these lineages.

2. Materials and methods

2.1. Fly lineages

To investigate the *Wolbachia* role in the reproductive isolation between *D. prosaltans* and *D. saltans*, we used two lineages of these species: Pro101 and Sal02, respectively (Table 1). The infected and treated lineages of Pro101 were the same presented in Guzmán et al. (*in prep* - Chapter 1). We did a PCR reaction using a specific *Wolbachia* primer pair, *wsp* (226F and 588R), to ensure their infection status, as detailed in section 2.3 of the mentioned work. *D. saltans* is known as an uninfected species (BOURTZIS *et al.*, 1996; MILLER; RIEGLER, 2006). We applied the same protocol in Sal02 and found no evidence of high titer infection. All strains were kept at 23 °C, with a 12L/12D photoperiod, in the banana-barley-agar medium described in Guzman et al. (*in prep* - Chapter 1).

Table 1. *Drosophila* lineages used in this work.

Lineage	Species	Stock	Collection site
Pro 101	*D. prosaltans*	Lab. Bioinformática e Evolução, Universidade Federal de Viçosa	Mata da Biologia, Universidade Federal de Viçosa, MG, Brazil, 2016.
Sal 02	*D. saltans*	Lab. Genome Dynamics, Medical University of Vienna, Vienna, Austria.	Sinaloa, Mexico, 2004

2.2. Crosses

We controlled larvae density and female's virginity of the adult used in the experiment. Table 2 shows all the cross combinations set in this work with at least 15 replications, including one female and two males (FRY; PALMER; RAND, 2004) six days old (BICUDO, 1973). As described in section 2.7 of Guzmán et al. (*in prep* – Chapter 1), each cross was set up with virgin adults in vials of reduced volume. We kept the tubes for 48 h, discarded the males, and kept the females in the tube for another 24 h to lay

eggs. After this first period, we dissected the female to check for sperm in their spermatheca. We quantified and sexed F_1 until the end of the adult emergency.

Table 2. Crosse combination**.** Intraspecific crosses involving Sal02 linage (S). Inter-specific crosses involving Sal02 (S) and Pro101 treated (Ptet) and infected (Pw+)

Cross-type	♀	♂
Intra-specific	S	S
Inter-specific	Pw+	S
	Ptet	S
	S	Pw+
	S	Ptet

2.3. Cytoplasmic Incompatibility

To check whether *Wolbachia* causes Cytoplasmic Incompatibility in Pro101 or affects the egg-sperm compatibility in couples involving infected males and uninfected females, we set the four cross combinations involving infected treated Pro101 following the initial steps described in the 2.2 section. After the first 48 h (mating time), instead of placing each female to lay eggs in a new culture tube, we placed each female to lay eggs for 24 h in a Petri dish containing a culture medium composed of grape juice (30 %), agar (0.8 %), and a thin layer of yeast (CATTEL *et al.*, 2016). This procedure ensured the visualization of all eggs. We thus placed each female at a new dish and waited for another 24 h. Further, we dissected the female spermatheca to search for sperm, counted the number of eggs at the two dishes, and monitored the plates every day for seven days to search for and count larvae. We compared the larvae/egg ratio among the four combinations to test whether it is significantly lower in crosses involving treated females and infected males. We considered in our analyses only the inseminated females that laid at least ten eggs.

2.4 F_1 sterility

To determine whether the F_1 males and females produced in interspecific crosses were fertile or sterile, we transferred the F_1 of the pairs ♀Pw+ x ♂S and ♀S x ♂Pw+ and

waited for F_2. In case of the absence of F_2, we set up backcrosses (F_1 x G_0, two females and one male each) to determine whether both sexes or which sex was sterile.

2.5. Data analysis

Following the indexes proposed by Bicudo (1973), we calculated the Total Isolation (percentage of crosses not yielding progeny – Ti) and the proportion of Ti at insemination (Ii) or fertilization level (Fi) of interspecific crosses under study (Table 2). Then, we compared these indexes with those obtained from intraspecific crosses (within Sal02, and Pro101 – results from Guzmán *et al. in prep.* – Chapter 1). Considering that a is the number of uninseminated females, b the number of inseminated females without larvae and *c* the number of females that produced at least one larvae (fertilized females), we have that:

$$Ti = \frac{a+b}{a+b+c};\ Ii = \left(\frac{a}{a+b}\right)Ti\ ;\ \ Fi = \left(\frac{a}{a+b}\right)Ti$$

These indexes allow the discrimination between pre and post-mating isolation mechanisms. We also compared the total progeny produced by fertilized females to evaluate post-mating barriers between Sal02 and Pro101.

We applied generalized linear models (GLM) with a quasi-Poisson to correct for overdispersion in count (total F_1) data and a quasi-binomial distribution to correct for overdispersion in proportion data for cytoplasmatic incompatibility detection (item 2.3). We used R version 4.0.2 (R CORE TEAM, 2020) to perform the statistic analyses and produced the figures using the package ggplot2 (WICKHAM, 2009).

We used the $Ii$to estimate the pre-mating reproductive isolation components between Pro101 and Sal02, and the Fi, the number of adults in F_1, CI assays, and the sterility of hybrids to estimate the post-mating reproductive isolation components.

3. Results

Table 3 shows the isolation indexes calculated in crosses involving Sal02 (S) and infected and treated individuals of Pro101 (Pw+ and Ptet). We found that interspecific crosses involving S females presented higher Ii, Fi, and Ti than the intraspecific crosses, with few differences between crosses with Pw+ or Ptet males. With Pw+ females, the intralineage cross (♀Pw+ x ♂Pw+) resulted in less isolation than the interspecific cross (♀Pw+ x ♂S), which curiously had less isolation than the interlineage cross (♀Pw+ x ♂Ptet). In their turn, crosses involving ♀Ptet showed less Ii with ♂Pw+ than with ♂Ptet, and the couple ♀Ptet x ♂S showed the highest Ii index of all comparisons ($Ii = 0.7$), indicating that Ptet females were more effective in preventing interspecific mating than Pw+ or S females. Unexpectedly, in terms of Fi (isolation at the fertilization level), all interspecific crosses presented lower values than the pair ♀Ptet x ♂Pw+, while all interspecific crosses involving S males did not present Fi.

Table 3. Isolation index of intra and interspecific crosses involving Sal02 (S) and treated or infected Pro101 antibiotic treated (Ptet) or infected (Pw+). $\boldsymbol{Ti}$ is the total isolation, composed by $\boldsymbol{Ii}$ (isolation at the insemination level) and $\boldsymbol{Fi}$ (isolation at the fertilization level), $\boldsymbol{a}$ is the number of uninseminated females, $\boldsymbol{b}$ the number of inseminated and no fertilized females and $\boldsymbol{c}$ the number of females that produced at least one larva (according to item 2.5). Numbers in bold indicate the highest indexes in each category (intralineage, interlineage, and interspecific).

Female	Male	*a*	*b*	*c*	*Ii*	*Fi*	*Ti*
Intraspecific (intralineage) pairs							
S	S	1	0	15	0.06	0	0.06
Pw+*	Pw+*	1	1	24	0.04	0.04	0.08
Ptet*	Ptet*	5	2	21	**0.18**	**0.07**	**0.25**
Intraspecific (interlineage) pairs							
Ptet*	Pw+*	2	7	24	0.06	**0.21**	0.27
Pw+*	Ptet*	15	0	20	**0.43**	0	**0.43**
Interspecific pairs							
S	Pw+	8	2	18	0.29	0.07	0.36
Pw+	S	6	0	14	0.3	0	0.3
S	Ptet	4	2	12	0.22	**0.11**	0.33
Ptet	S	16	0	7	**0.7**	0	**0.7**

*Data presented in Guzmán et al. (*in prep* – chapter 1)

Figure 1 shows that all females produced more offspring with conspecific males; S and Ptet females produced similarly F_1 with Ptet and Pw+ males and, as mentioned in Guzman et al. (*in prep* – chapter 1), Pw+ females produce more offspring with Ptet than Pw+ males.

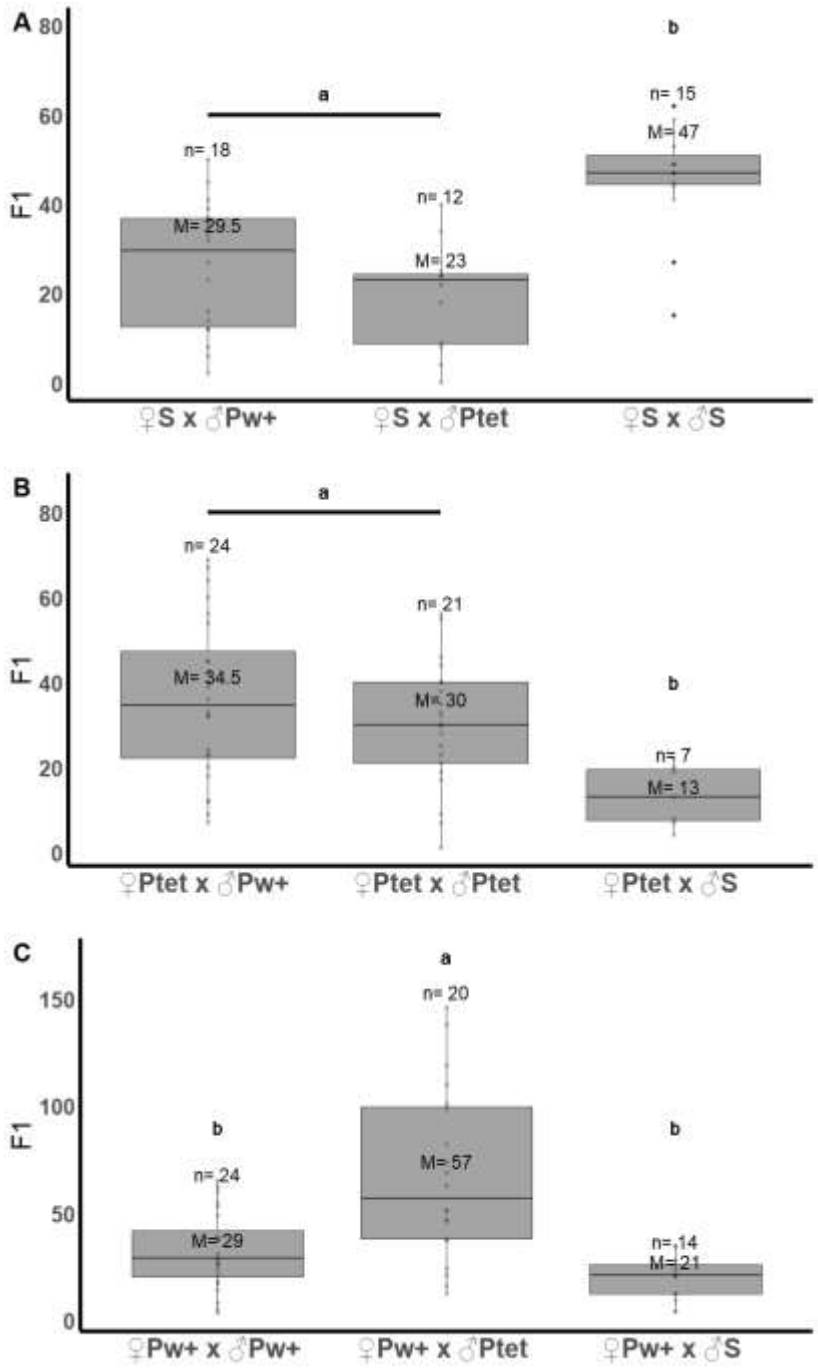

Figure 1. Progeny (F_1) in inter and intraspecific crosses involving fertilized females of **A.** *D. saltans* Sal 02 (S) ($F_{1,43} = 23.959, p = 1.428e^{-05}$); **B.** antibiotics treated *D. prosaltans* (Ptet) ($F_{1,50} = 11.686, p = 0.001261$); and **C.** infected *D. prosaltans* (Pw+) ($F_{1,56} = 31,866, p = 5.704e^{-07}$).

Figure 2 shows that the hatch proportion (larvae/eggs) of intraspecific crosses involving Pro101 were higher for the infected couple (♀Pw+ x ♂Pw+), with no

differences between the remaining combinations, indicating that the *Wolbachia* infecting Pro101 does not cause CI in this lineage.

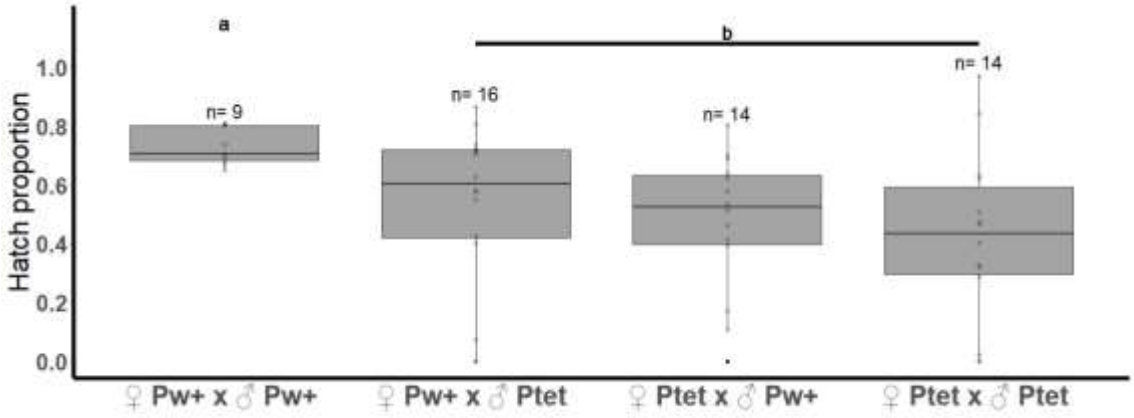

Figure 2. The hatch proportion (larvae/eggs) in four combinations of intraspecific crosses involving infected (Pw+) and antibiotic-treated (Ptet) *D. prosaltans*, Pro101 ($\boldsymbol{F_{1,51} = 7.1983, p = 0.009813}$).

Table 4 shows that among the 19 replications of the pair ♀Pw+ x ♂S, six produced no F_1, three produced no F_2 and seven produced at least one larva or pupa at F_2, showing that regardless of the decrease in progeny triggered by the interspecific combination, this pair can produce both fertile males and females. On the other hand, among the 18 replications of the pair ♀S x ♂Pw+, only three produced no F_1 while none produced larvae or pupae in F_2.

Table 5 shows the results of backcrossings involving hybrids (F_1) of the pair ♀S x ♂Pw+ in three different combinations: ♀F_1 x ♂S, ♀F_1 x ♂Pw+ and ♀S x ♂F_1. We also set the pair ♀Pw+ x ♂F_1 but realized that the females were not virgins at the end of the experiment and discarded the results. Nevertheless, our results allowed us to conclude that the sterility of F_1 was due to hybrid male sterility.

Table 4. F2 production of several replications (Rep) of ♀Pw+ x ♂S and ♀S x ♂Pw+ pairs. #F1 is the number of adults at the offspring of each Rep; Larvae = 1 means that we saw at least one larva or pupa into the tube, Larvae = 0 means that we did not see any larvae or pupae. NA means that the data is not available.

Rep	Pair	F_1	Larvae (F2)	Pair	F_1	Larvae (F2)
1	♀Pw+ x ♂S	25	1	♀S x ♂Pw+	39	0
2	♀Pw+ x ♂S	9	0	♀S x ♂Pw+	33	0
3	♀Pw+ x ♂S	35	0	♀S x ♂Pw+	27	0
4	♀Pw+ x ♂S	0	-	♀S x ♂Pw+	50	0
5	♀Pw+ x ♂S	21	1	♀S x ♂Pw+	6	NA
6	♀Pw+ x ♂S	26	1	♀S x ♂Pw+	2	NA
7	♀Pw+ x ♂S	28	1	♀S x ♂Pw+	12	0
8	♀Pw+ x ♂S	21	1	♀S x ♂Pw+	16	0
9	♀Pw+ x ♂S	0	-	♀S x ♂Pw+	41	0
10	♀Pw+ x ♂S	0	-	♀S x ♂Pw+	32	0
11	♀Pw+ x ♂S	12	0	♀S x ♂Pw+	0	-
12	♀Pw+ x ♂S	0	-	♀S x ♂Pw+	14	0
13	♀Pw+ x ♂S	23	1	♀S x ♂Pw+	37	0
14	♀Pw+ x ♂S	34	0	♀S x ♂Pw+	0	-
15	♀Pw+ x ♂S	0	-	♀S x ♂Pw+	8	NA
16	♀Pw+ x ♂S	4	NA	♀S x ♂Pw+	45	0
17	♀Pw+ x ♂S	0	-	♀S x ♂Pw+	0	-
18	♀Pw+ x ♂S	12	1	♀S x ♂Pw+	23	0
19	♀Pw+ x ♂S	4	NA			

Table 5. Backcrosses of ♀S x ♂Pw+ hybrids ($\boldsymbol{F_1}$) including one female and two males each. Larvae = 1 means that we saw at least one larva or pupa into the tube. Larvae = 0 means that we did not see any larvae or pupae. NA means that the data is not available.

Rep	♀	♂	Larvae	♀	♂	Larvae	♀	♂	Larvae
1	$\boldsymbol{F_1}$	S	1	$\boldsymbol{F_1}$	Pw+	1	S	$\boldsymbol{F_1}$	0
2	$\boldsymbol{F_1}$	S	1	$\boldsymbol{F_1}$	Pw+	NA	S	$\boldsymbol{F_1}$	0
3	$\boldsymbol{F_1}$	S	NA	$\boldsymbol{F_1}$	Pw+	0	S	$\boldsymbol{F_1}$	0
4	$\boldsymbol{F_1}$	S	0	$\boldsymbol{F_1}$	Pw+	NA	S	$\boldsymbol{F_1}$	0
5	$\boldsymbol{F_1}$	S	NA	$\boldsymbol{F_1}$	Pw+	1	S	$\boldsymbol{F_1}$	NA
6	$\boldsymbol{F_1}$	S	NA	$\boldsymbol{F_1}$	Pw+	1	S	$\boldsymbol{F_1}$	0
7	$\boldsymbol{F_1}$	S	1	$\boldsymbol{F_1}$	Pw+	0	S	$\boldsymbol{F_1}$	0
8	$\boldsymbol{F_1}$	S	0	$\boldsymbol{F_1}$	Pw+	NA	S	$\boldsymbol{F_1}$	0
9	$\boldsymbol{F_1}$	S	1	$\boldsymbol{F_1}$	Pw+	1	S	$\boldsymbol{F_1}$	NA
10	$\boldsymbol{F_1}$	S	1	$\boldsymbol{F_1}$	Pw+	0	S	$\boldsymbol{F_1}$	0

4. Discussion

In this work, we investigated the role of *Wolbachia* infection in the interspecific recognition between an uninfected Mexican linage of *D. saltans* (Sal02), and a *Wolbachia* infected Brazilian linage of *D. prosaltans* (Pro101). As expected for two close and well-defined species, we detected reproductive isolation between them. In crosses involving the Mexican uninfected linage Sal02 (♀S), the total isolation index (Ti) varied from 33 to 36 % for infected (♂Pw+) and uninfected (♂Ptet) males of Pro101, respectively. In both cases, the pre-mating isolation (denoted by the insemination index Ii) was at least twice as high as the post-mating isolation component (denoted by the fertilization index Fi) - Table 3. In her experiments, Bicudo (1973) found 72 % isolation (Ti) in crosses involving females of *D. saltans* from San Salvador, El Salvador, and males of *D. prosaltans* from Belém, PA, Brazil. Her results also indicated that the pre-mating component of isolation was only 2 %, *i.e.,* most of the isolation (70 %) occurred by fertilization failures, which do not depend on the behavior of the flies.

Interspecific crosses involving Pro101 females presented, on the one hand, 70 % pre-mating isolation between ♀Ptet x ♂S (Ii, Table 3). Considering that Ptet females

generate significantly more progeny in intraspecific crosses than in interspecific ones (Figure 1B), it is possible to state that such pre-mating isolation would be valuable in the unlikely event of Pro101 and Sal02 sympatry. On the other hand, the couple ♀Pw+ x ♂S presented only 30 % pre-mating isolation (Ii, Table 3). These results allow us to hypothesize that if the *Wolbachia* strain harbored by Pro101 acts in the same way in a population, it could harm *D. prosaltans* that contact *D. saltans* lineages by interfering with infected females' sexual discernment. It is crucial to highlight that Pw+ females, in intraspecific crosses, avoid copulation with Ptet males (Ii = 43 % - higher than with S males Ii = 30 %- Table 3), although they generate more offspring with Ptet males (Figure 1C) than with Pw+ males (GUZMÁN et al. in prep - Chapter 1). These difficulties in male recognition may be associated with modifications in cuticular hydrocarbons mediated by differential gene expression in infected hosts (BAIÃO *et al.*, 2019) which have critical roles in sexual partner recognition (FERVEUR, 2005). Figure 1C further shows that in terms of Pw+ females progeny, there are no differences between mates with ♂Pw+ or ♂S. It showed that *Wolbachia* infection impairs Pw+ males' fecundity by reducing their offspring with Pw+ females to the numbers obtained in interspecific crosses.

Given the differences between Pro101 infected and uninfected females, one should note that Bicudo's (1973) conclusion that the reproductive isolation between *D. saltans* and *D. prosaltans* reduces with the geographic distanceworks, but only to a certain extent. The prevailing theory at that time predicted that pre-mating isolation via behavioral elements tends to evolve by natural selection to avoid mating with members of other species in sympatry species through a mechanism known as reinforcement (DOBZHANSKY *et al.*, 1964). However, when Bicudo (1973) made crosses between interspecific strains collected at increasing distances from each other, she observed that some close strains did not show the relative isolation expected by theory. Therefore, we hypothesize that some populations of *D. prosaltans* included in her study would be infected and others free of *Wolbachia*, confounding effect and countering her expectations about correlations expected by the geographic distance between strains.

The higher isolation at the fertilization level (post-mating) was found in intraspecific pairs, ♀Ptet x ♂Pw+, leading us to question whether the *Wolbachia* strain

infecting Pro101 also causes cytoplasmic incompatibility (CI). While Figure 2 reveals, that the most fecund pair (highest ratio of larvae to eggs) was ♀Pw+ x ♂Pw+, it also shows that there were no differences between ♀Ptet x ♂Pw+ (IC couple) and ♀Ptet x ♂Ptet. The fact that crosses of ♀S x ♂Pw+ generated as much progeny as ♀S x ♂Ptet (Figure 1A) contributes to the conclusion that Pw+ males do not cause CI. Curiously, although the pair ♀Pw+ x ♂Pw+ generated more larvae (higher hatch rate), it generated fewer adults than ♀Pw+ x ♂Ptet (Figure 1C), suggesting that *Wolbachia* infection impairs the development of larvae from the ♀Pw+ x ♂Pw+ pair into adults. In our previous work (GUZMAN *et al. in prep*. - Chapter 1), we hypothesized that Pw+ males would produce fewer spermatozoa or transfer fewer fertile spermatozoa to females' spermatheca than Ptet males. However, the larvae/egg ratio results indicate that the problem may not be in the number or fecundity of the spermatozoa but the viability of the larvae or pupae. Islam and Dobson (2006) found evidence of an adverse effect of *Wolbachia* infection on the survival of immature males of *Aedes albopictus* (Diptera: Culicidae) under optimal rearing conditions. In models aimed to predict the likelihood of *Wolbachia* infection in mosquitoes, Crain et al. (2011) suggested that the infected larval viability is a good predictor for the colonization success of new hosts by *Wolbachia*.

In our F_2 experiments, we found that the pair ♀Pw+ x ♂S produced less F_1 ($\bar{x} = 13.4 \pm 0.66$) than the pair ♀S x ♂Pw+ ($\bar{x} = 21.4 \pm 0.95$), but produced fertile hybrids, while the pair ♀S x ♂Pw+ produce sterile males. Haldane's rule predicts that "when in the offspring of two different animal races one sex is absent, rare, or sterile, that sex is the heterozygous sex." Spassky (1957) and Bicudo (1973) already found this same reciprocal male sterility in the hybrids of *D. saltans* and *D. prosaltans*. More recently, Miller; Ehrman; and Schneider (2010) proposed that reciprocal asymmetry could be attributed to factors of female cytoplasms, such as *Wolbachia,* or other symbiont infections (reviewed in MATUTE; COOPER, 2021; SOMERSON *et al.*, 1984).

Sal02 is in thesis free of *Wolbachia* (our PCR showed no bands using the *wsp* primers - item 2.1). Thus, we did not test the effects of antibiotic treatment in this lineage. Nevertheless, if one returns to the reproductive isolation results and evaluates them from another point of view, one can interpret that Pw+ females show higher Ii

(Table 3) with antibiotic-treated (Ptet - 43 %) than with untreated males (Pw+ - 6 %, and S - 30 %). With this, we can hypothesize that the antibiotic treatment performed in Pro101 eliminated or knocked down, in addition to *Wolbachia*, some other bacterium, which is probably also present in Sal02 and confuses the Pw+ females mating recognition system.

Further work should address the antibiotic treatment of Sal02 to check whether it affects the isolation at insemination level between Sal02 and Pro101 and the hybrid sterility of the pair ♀S x ♂P. Our next step is thus to investigate if and which bacteria, presumably an endosymbiont, infects Sal02 and triggers the unexpected phenotypes found in this work.

5. Conclusion:

In a previous work (GUZMÁN *et al.*, in prep Chapter 1), we concluded that infection in *D. prosaltans* enhances females' fitness, despite the evident drawbacks caused in males, presumably in spermatozoa production. In this work, by investigating the effects of infection on sexual behavior in interspecific crosses with a closely related species, *D. saltans*, we found evidence that infection might be more detrimental to *D. prosaltans* than previously estimated. Our data suggested that *Wolbachia* reduces offspring viability of infected pairs in Pro101 (♀Pw+ x ♂Pw+), in comparison to other combinations, but does not reduce spermatozoa production in males. Also, the *Wolbachia* strain infecting Pro101 seems to impair the discernment of infected females between treated Pro101 and Sal02 males, reducing their fitness in the hypothesis of sympatry. Also, we found that the infection does not cause cytoplasmic incompatibility nor hybrid male sterility in crosses with Pro101 infected females (♀Pw+) and Sal02 males (♂S) since hybrids fertile, showing that *Wolbachia* does not trigger these post-mating isolation mechanisms between the studied lineages. Therefore, the infection seems to have unimmportant or no role in these lineages' isolation, which could be explained by the fact that they occur in both species' extreme distributions. However, male sterility of the hybrid of the pair ♀S x ♂Pw+ could change the interpretation of our results by introducing the hypothesis that Sal02 harbors a low titer *Wolbachia* or another bacterium that could trigger both male sterility of F1 and the

mating recognition failures detected in Pw+ females. Further work should address *D. saltans* linage Sal02 antibiotic treatment and the search for Sal02s putative infection (or infections) responsible for the phenotypes found in this work.

6. Acknowledgments

This study was financed in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES) - Finance Code 001. The authors thank Dr. Wolgang Miller, from the Center of Anatomy and Cell Biology, Medical University of Vienna, for kindly providing the *Drosophila saltans* lineage (Sal02) used in this work.

7. References

BAIÃO, G. C.; SCHNEIDER, D. I.; MILLER, W. J.; KLASSON, L. The effect of *Wolbachia* on gene expression in *Drosophila paulistorum* and its implications for symbiont-induced host speciation. **BMC Genomics**, vol. 20, no. 1, p. 465, 7 Jun. 2019. DOI 10.1186/s12864-019-5816-9.

BICUDO, H. E. M. Reproductive isolation in the *saltans* group of *Drosophila*. I. The *saltans* subgroup. **Genetica**, vol. 44, no. 3, p. 313–329, Sep. 1973. DOI 10.1007/BF00161311.

BOURTZIS, K.; MILLER, T. A. **Insect symbiosis**. [*S. l.*]: CRC press, 2003. vol. 3, . https://doi.org/10.1201/9781420064117.

BOURTZIS, K.; NIRGIANAKI, A.; MARKAKIS, G.; SAVAKIS, C. C. C. *Wolbachia* infection and cytoplasmic incompatibility in *Drosophila* species. **Genetics**, vol. 144, no. 3, p. 1063–73, Nov. 1996. DOI 10.1186/1471-2105-15-293.

BRUCKER, R. M.; BORDENSTEIN, S. R. Speciation by symbiosis. **Trends in Ecology & Evolution**, vol. 27, no. 8, p. 443–451, 1 Aug. 2012. DOI 10.1016/J.TREE.2012.03.011.

BURLA, H.; DA CUNHA, A. B. The *Willistoni* group of sibling species of *Drosophila*. **Evolution; international journal of organic evolution**, vol. 3, no. 4, p. 300–314, 1 Dec. 1949. DOI 10.1111/j.1558-5646.1949.tb00031.x.

CATTEL, J.; MARTINEZ, J.; JIGGINS, F.; MOUTON, L.; GIBERT, P. *Wolbachia* -mediated protection against viruses in the invasive pest *Drosophila suzukii*. **Insect Molecular Biology**, vol. 25, no. 5, p. 595–603, Oct. 2016. DOI 10.1111/imb.12245.

CRAIN, P. R.; MAINS, J. W.; SUH, E.; HUANG, Y.; CROWLEY, P. H.; DOBSON, S. L. *Wolbachia* infections that reduce immature insect survival: Predicted impacts on population replacement. **BMC Evolutionary Biology 2011 11:1**, vol. 11, no. 1, p. 1–10, 5 Oct. 2011. DOI 10.1186/1471-2148-11-290.

DE CARVALHO, T. B. Modern evolutionary biology and Brazilian population genetics: Theodosius Dobzhansky at the University of Sao Paulo. **Perspectives on Science**, vol. 28, no. 2, p. 223–243, 1 Apr. 2020. DOI 10.1162/posc_a_00339.

DOBZHANSKY, T.; EHRMAN, L.; PAVLOVSKY, O.; SPASSKY, B. The superspecies *Drosophila paulistorum*. **Proceedings of the National Academy of Sciences of the United States of**, vol. 51, no. 1, p. 3–9, 1964. DOI 10.1073/pnas.51.1.3.

DOBZHANSKY, T.; STREISINGER, G. Experiments on Sexual Isolation in *Drosophila*: II. Geographic Strains of *Drosophila prosaltans*. **Proceedings of the National Academy of Sciences**, vol. 30, no. 11, p. 340–345, 15 Nov. 1944. https://doi.org/10.1073/PNAS.30.11.340.

DOBZHANSKY, Th. Speciation as a Stage in Evolutionary Divergence. **The American Naturalist**, vol. 74, no. 753, p. 312–321, 29 Sep. 1940. DOI 10.1086/280899.

FERVEUR, J. F. Cuticular hydrocarbons: Their evolution and roles in *Drosophila* pheromonal communication. **Behavior Genetics**, vol. 35, no. 3, p. 279–295, May 2005. DOI 10.1007/s10519-005-3220-5.

FRY, A. J.; PALMER, M. R.; RAND, D. M. Variable fitness effects of *Wolbachia* infection in *Drosophila melanogaster*. **Heredity**, vol. 93, no. 4, p. 379–389, 11 Aug. 2004. DOI 10.1038/sj.hdy.6800514.

GUZMÁN, Y. C.; MOREIRA, M. M.; BOUZADA-DIAS, L.; YOTOKO, K. Fitness benefits and *Wolbachia* fixation strategies of *w*Au-like infection in Neotropical Drosophila (saltans subgroup). [*s. d.*].(In prep)

HOENIGSBERG, H. F.; SANTIBANEZ, S. K. Intraspecific sensory Discrimination in *Drosophila aequinoctialis* Dobzhansky and *Drosophila prosaltans* Duda. **Zeitschrift für Tierpsychologie**, vol. 17, no. 2, p. 133–140, 12 Jan. 1960. DOI 10.1111/J.1439-0310.1960.TB00197.X.

ISLAM, M. S.; DOBSON, S. L. *Wolbachia* Effects on *Aedes albopictus* (Diptera: Culicidae) Immature survivorship and development. **Journal of Medical Entomology**, vol. 43, no. 4, p. 689–695, 1 Jul. 2006. DOI 10.1093/jmedent/43.4.689.

JAENIKE, J.; DYER, K. A.; CORNISH, C.; MINHAS, M. S. Asymmetrical reinforcement and *Wolbachia* infection in *Drosophila.* **PLoS Biology**, vol. 4, no. 10, p. 1852–1862, 10 Oct. 2006. DOI 10.1371/journal.pbio.0040325.

JEYAPRAKASH, A.; HOY, M. A. Long PCR improves *Wolbachia* DNA amplification: *wsp* sequences found in 76% of sixty-three arthropod species. **Insect Molecular Biology**, vol. 9, no. 4, p. 393–405, Aug. 2000. DOI 10.1046/j.1365-2583.2000.00203.x.

MAGALHAES, L. D. Notes on the taxonomy, morphology, and distribution of the *saltans* group of *Drosophila,* with descriptions of four new species. **University of Texas Publication**, vol. 6205, p. 135–154, 1962. .

MAGALHÃES, L. E.; BJÖRNBERG, A. J. S. Estudo da genitália masculina de *Drosophila* do grupo *Saltans* (Diptera). **Revista brasileira de Biologia**, vol. 17, no. 4, p. 435–450, 1957. .

MATUTE, D. R.; COOPER, B. S. Comparative studies on speciation: 30 years since Coyne and Orr. **Evolution**, vol. 75, no. 4, p. 764–778, 1 Apr. 2021. DOI 10.1111/EVO.14181.

MAYR, E.; DOBZHANSKY, T. Experiments on sexual isolation in *Drosophila*: IV. Modification of the degree of isolation between *Drosophila pseudoobscura* and *Drosophila persimilis* and sexual preferences in *Drosophila prosoltans*. **Proceedings of the National Academy of Sciences**, vol. 31, no. 2, 1 Feb. 1945.

MILLER, W. J.; EHRMAN, L.; SCHNEIDER, D. Infectious speciation revisited: Impact of symbiont-depletion on female fitness and mating behavior of *Drosophila paulistorum*. **PLoS Pathogens**, vol. 6, no. 12, p. e1001214, 2 Jan. 2010. DOI 10.1371/journal.ppat.1001214.

MILLER, W. J.; RIEGLER, M. Evolutionary dynamics of *w*Au-like *Wolbachia* variants in neotropical *Drosophila* spp. **Applied and environmental microbiology**, vol. 72, no. 1, p. 826–35, 1 Jan. 2006. DOI 10.1128/AEM.72.1.826-835.2006.

MULLER, H. Isolating mechanisms, evolution, and temperature. 6., 1942. **Biol. Symp.**, 1942. vol. 6, p. 71–125.

PAVAN, C.; DA CUNHA, A. B. Theodosius Dobzhansky and the development of Genetics in Brazil. **Genetics and Molecular Biology**, vol. 26, no. 3, p. 387–395, 2003. DOI 10.1590/S1415-47572003000300027.

R CORE TEAM. R: A Language and Environment for Statistical Computing. Vienna, Austria, 2020.

SCHNEIDER, D. I.; EHRMAN, L.; ENGL, T.; KALTENPOTH, M.; HUA-VAN, A.; LE ROUZIC, A.; MILLER, W. J. Symbiont-driven male mating success in the Neotropical *Drosophila paulistorum* Superspecies. **Behavior Genetics**, vol. 49, no. 1, p. 83–98, 15 Jan. 2019. DOI 10.1007/s10519-018-9937-8.

SHROPSHIRE, J. D.; BORDENSTEIN, S. R. Speciation by symbiosis: the microbiome and behavior. **mBio**, vol. 7, no. 2, p. e01785, 31 Mar. 2016. DOI 10.1128/mBio.01785-15.

SIÃO, J. F. M.; MARTINS, L. A. C. P. Dobzhansky and *dreyfus's* group: The introduction of natural population genetics studies in Brazil (1943–1960). **Perspectives on Science**, vol. 28, no. 2, p. 244–244, 1 Apr. 2020. DOI 10.1162/posc_a_00340.

SOMERSON, N. L.; EHRMAN, L.; KOCKA, J. P.; GOTTLIEB, F. J. Streptococcal L-forms isolated from *Drosophila paulistorum* semispecies cause sterility in male progeny. **Proceedings of the National Academy of Sciences**, vol. 81, no. 1, p. 282–285, 1 Jan. 1984. DOI 10.1073/PNAS.81.1.282.

SPASSKY, B. Morphological differences between sibling species of *Drosophila*. **University of Texas Publication**, vol. 5721, p. 48–61, 1957. .

STURTEVANT, A. H. I.The classification of the genus *Drosophila*, with descriptions of nine new species. **The University of Texas Publication**, vol. 4213, no. 4213, p. 6–51, 1942.

STURTEVANT, A. H. Notes on North American Drosophilidae with descriptions of twenty-three new species. **Annals of the Entomological Society of America**, vol. 9, no. 4, p. 323–343, 1 Dec. 1916. DOI 10.1093/aesa/9.4.323.

WERREN, J. H.; BALDO, L.; CLARK, M. E. *Wolbachia:* master manipulators of invertebrate biology. **Nature Rev Microbiol**, vol. 6, no. 10, p. 741–751, Oct. 2008. DOI 10.1038/nrmicro1969.

WERREN, John H.; WINDSOR, D.; GUO, L. Distribution of *Wolbachia* among neotropical arthropods. **Proceedings of the Royal Society B: Biological Sciences**, vol. 262, no. 1364, p. 197–204, 22 Nov. 1995. DOI 10.1098/rspb.1995.0196.

WICKHAM, H. Elegant Graphics for Data Analysis. 2009.

GENERAL CONCLUSION

In this work, we investigated the relationships of *Wolbachia* and three closely related species of the subgroup *saltans*, a neotropical clade that includes seven sibling species recognizable only by male genitalia morphology. Specifically, we investigated the phenotypic consequences the of *Wolbachia* infection in a Brazilian lineage of *D. prosaltans* Duda, 1927 (Pro101), and a Panamanian lineage of *D. septentriosaltans* Magalhaes & Buck 1962 (SepPLR2); and the role of the Pro101 infection at the reproductive isolation with the lineage Sal02 (*D. saltans* Sturtevant, 1916), sampled in the North of Mexico.

In the first Chapter, We found that *Wolbachia* enhances, through different processes, the fitness of Pro101 and SepPLR2, while ensures its survival and reproduction in these hosts. Furthermore, in both lineages, we did not find sex ratio biases or cytoplasmic incompatibility in crosses involving treated and infected individuals.

In Pro101, we found that the infection affects the insemination rates of infected females (Pw+) by infected and treated males. This result points to the probability that Pw+ females prefer to mate with Pw+ males, but we should test this hypothesis with proper behavior essays. The F_1 counting revealed that infected females produce more offspring in pairs with treated than infected males, regardless of their preference. We interpreted this result as host manipulation because the females´ preference reduces selection against infected males.

In SepPLR2, we found that the *Wolbachia* depletion with antibiotics reduces both males' and females' fitness, which leads us to question whether uninfected *D. septentriosaltans* can live and compete with infected individuals in a natural environment. Indeed, we could not find registers of *D. septentriosaltans* free of *Wolbachia*, which supports the hypothesis that they depend on Wolbachia in natural environments, even though we successfully maintained a treated SepPLR2 in laboratory conditions.

Using our simulator (available in https://yoan-sarmiento.shinyapps.io/simulation/), we found that the immigration of only one infected female could lead to

the fixation of *Wolbachia* in both lineages. However, the strain harbored by SepPLR2 was more efficient in spreading the infection in the host population than the strain harbored by Pro101.

Based on our results, we can infer that both relationships are old enough to promote benefits. Miller and Riegler (2006), based on *wsp* sequence identity, have suggested that *D. prosaltans* and *D. septentriosaltans* inherited the infection from their common ancestral. However, increased fitness in a *Wolbachia*-host relationship can be achieved more quickly than predicted by evolutionary time (SARIDAKI; BOURTZIS, 2010; TURELLI, 1994). Weeks et al. (2007) showed that *w*Ri, a strain that for a long time decreased fecundity of *D. simulans*, changed to increase the host's fecundity over only 20 years. Therefore, our work did not refute or reinforce Miller and Riegler (2006) hypothesis, which is still open.

In the second chapter, we investigated *Wolbachia's* role in the reproductive isolation of Pro101 and a Mexican lineage of *D. saltans* (Sal02). We set up crosses involving such lineages and measured pre-and post-mating isolation between them. Our results showed that the *Wolbachia* infection might be more detrimental to Pro101 than previously estimated. In addition, we tested for cytoplasmic incompatibility within Pro101 using the eggs hatch rate instead of the number of adults, as in Chapter 1. We found that the couple Pw+ x Pw+ had the highest egg hatch rate but not the highest F_1, suggesting that the infection reduces the fitness of the infected pairs' offspring, not the number of sperm or the sperm fertility of infected males, as previously inferred.

Regarding the interspecific crosses, we found that the *Wolbachia* infection harbored by Pro101 reduces the pre-mating isolation since females Ptet (females treated with antibiotics) were more efficient to avoid crosses with Sal02 males than Pw+ (infected females). Also, we confirmed the results already found by Spassky (1957) and Bicudo (1973) of asymmetric male hybrid sterility: crosses involving Pw+ females and Sal02 males generated fertile F1, while crosses involving Sal02 females and Pw+ males generated fertile females and sterile males. One of the hypotheses to explain such asymmetry stated that endosymbionts, inherited from mothers to the offspring, could generate male sterility because of over replication at the testis of hybrid males. Indeed, Miller et al. (2010) found that the antibiotics treatment reduced the male

sterility of hybrids produced with different subspecies of *D. paulistorum* Dobzhansky and Pavan, 1949 harboring different strains of *Wolbachia*. Unfortunately, we did not treat Sal02 with antibiotics, and our further studies will address the possibility that Sal02 harbor another endosymbiont (or harbor *Wolbachia* at low titer – not detectable by our PCR) that triggers the phenotypes found in our results.

References

BICUDO, H. E. M. Reproductive isolation in the *Saltans* group of *Drosophila*. I. The *saltans* subgroup. **Genetica**, vol. 44, no. 3, p. 313–329, Sep. 1973. DOI 10.1007/BF00161311.

MILLER, W. J.; RIEGLER, M. Evolutionary dynamics of *w*Au-like *Wolbachia* variants in neotropical *Drosophila* spp. **Applied and environmental microbiology**, vol. 72, no. 1, p. 826–35, 1 Jan. 2006. DOI 10.1128/AEM.72.1.826-835.2006.

SARIDAKI, A.; BOURTZIS, K. *Wolbachia:* more than just a bug in insects genitals. **Current Opinion in Microbiology**, vol. 13, no. 1, p. 67–72, 1 Feb. 2010. https://doi.org/10.1016/j.mib.2009.11.005.

SPASSKY, B. Morphological differences between sibling species of *Drosophila*. **University of Texas Publication**, vol. 5721, p. 48–61, 1957. .

TURELLI, M. Evolution of incompatibility-inducing microbes and their hosts. **Evolution**, vol. 48, no. 5, p. 1500–1513, 1 Oct. 1994. https://doi.org/10.1111/j.1558-5646.1994.tb02192.x.

WEEKS, A. R.; TURELLI, M.; HARCOMBE, W. R.; REYNOLDS, K. T.; HOFFMANN, A. A. From parasite to mutualist: Rapid evolution of *Wolbachia* in natural populations of *Drosophila*. **PLoS biology**, vol. 5, no. 5, p. 0997–1005, 17 May 2007. DOI 10.1371/journal.pbio.0050114.

APPENDIX

Simulations

Cross Parameters

From each cross combination (c_1 to c_4 – Table A1), the simulation requires the number of inseminated (ins), non-inseminated ($nins$), Fertilized (fe) females (ex. Table 5, Chapter 1) and (in our case) the median number of F_1 generated by the fertilized females (M) (ex. Figure 3, Chapter 1). These numbers should be used to infer the insemination rate (I_{c_i}), the fertilization rate (Fe_{c_i}) and the $F_{1_{c_i}}$ of each cross combination.

Table A1: Cross parameters obtained from experimental crosses: ins – number of inseminated females, $nins$ – number of non-inseminated females, I_{c_i} – rate of insemination in each cross combination (index $i = 1$ to 4), fe – number of fertilized females (those that produced at least one larvae), Fe_{c_i} = rate of fertilization, M – median number of F_1 from fertilized females, and $F_{1_{c_i}}$ – proportion of the total F_1, considering all crosses (c_1 to c_4).

Cross	Couple	ins	$nins$	I_{c_i}	fe	Fe_{c_i}	M	$F_{1_{c_1}}$
c_1	♀w+ x ♂w-	ins_{c_1}	$nins_{c_1}$		fe_{c_1}		M_{c_1}	
c_2	♀w+ x ♂w-	ins_{c_2}	$nins_{c_2}$	$\frac{ins_{c_i}}{ins_{c_i} + nins_{c_i}}$	fe_{c_2}	$\frac{fe_{c_i}}{ins_{c_i}}$	M_{c_2}	$\frac{M_{c_i}}{M}$
c_3	♀w- x ♂w+	ins_{c_3}	$nins_{c_3}$		fe_{c_3}		M_{c_3}	
c_4	♀w- x ♂w-	ins_{c_4}	$nins_{c_4}$		fe_{c_4}		M_{c_4}	
Sum							M	

Population Parameters

One should input the number of individuals of the simulated population (N), the number of infected females invading the population (ni), the number of generations to be simulated (g), the vertical transmission rate (vt) and the female sex ratio of the infected female's offspring (fsr). The last two parameters can be inferred by the user or simulated using different values.

Table A2: Population parameters to be input by the user to simulate the infection spread over g generations in a non-infected population of size N invaded by ni infected females considering a vertical transmission rate (vt) and a female sex ratio (fsr) of infected female´s offspring.

Parameter	Explanation
N	Size of the simulated population (arbitrary)
N_f	$\frac{N}{2}$
N_m	$\frac{N}{2}$
ni	Number of infected females invading the population (arbitrary)
g	Number of generations to be simulated (arbitrary)
vt	Vertical transmission (can be inferred in the user´s population)
fsr	Female sex ratio of the infected female´s offspring (can be inferred in the user´s population)

For the first generation, one should estimate how many crosses pairs are available for each cross combination considering the number of infected females invading the population (ni) and the number of individuals of the simulated population (N) to infer the proportion of infected females $\left(\frac{ni}{Nf}\right)$, non-infected females $\left(\frac{Nf-ni}{Nf}\right)$, infected males $\left(\frac{wm}{Nm}\right)$, non-infected males $\left(\frac{nm}{Nm}\right)$. Specifically, the number of infected males in this first generation is zero because we considered only infected females as invaders. Therefore, c_1 and c_3 form no pairs in first generation . For the sake of simplicity, all generations have the same number of individuals. Table A3 shows the inference of the frequency of mating pairs in the generation that received the infected female(s) (g_1).

Table A3: Inference of the frequency of mating pairs in generation 1 (g_1) of the simulation (the generation that received the ni infected females). $c_1 - c_4$, I_{c_i} , $Fe_{c_i}.F_{1_{c_i}}$ were defined in Table A1. N, Nf, Nm and ni were defined in Table A2. $p_{c_{i(g_1)}}$ is the proportion of pairs c_i available at the first generation. wf and wm are the infected females and males, and nm is the number of non-infected males.

Number of available pairs	Frequency
$c_{1(g_1)} = \frac{ni}{Nf} . \frac{wm}{Nm} . I_{c_1} . Fe_{c_1} . F_{1_{c_1}} . N$	$p_{c_{1(g_1)}} = \frac{c_{1(g_1)}}{Sum_{(g_1)}}$
$c_{2(g_1)} = \frac{ni}{Nf} . \frac{nm}{Nm} . I_{c_2} . Fe_{c_2} . F_{1_{c_2}} . N$	$p_{c_{2(g_1)}} = \frac{c_{2(g_1)}}{Sum_{(g_1)}}$
$c_{3(g_1)} = \frac{(Nf - ni)}{Nf} . \frac{wm}{Nm} . I_{c_3} . Fe_{c_3} . F_{1_{c_3}} . N$	$p_{c_{3(g_1)}} = \frac{c_{3(g_1)}}{Sum_{(g_1)}}$
$c_{4(g_1)} = \frac{(Nf - ni)}{Nf} . \frac{nm}{Nm} . I_{c_4} . Fe_{c_4} . F_{1_{c_4}} . N$	$p_{c_{4(g_1)}} = \frac{c_{4(g_1)}}{Sum_{(g_1)}}$
$Sum_{(g_1)} = \sum_{i=0}^{4} c_{i(g_1)}$	1.0

To simulate the second generation, one should calculate the number of infected and non-infected females (wf and nf) and males (wm and nm) generated by each cross combination. It is important to note that only infected females can produce infected offspring, while both can produce uninfected offspring.

We defined that the non-infected females (from c_3 and c_4 combinations) produce 50% of uninfected females (nf) and 50% of uninfected males (nm). The infected females should produce fsr (female sex ratio of the infected female´s offspring) females and $(1 - fsr)$ males. The infected offspring of the infected females depends on the infection´s vertical transmission rate (vt). Therefore, the non-infected offspring of infected females should be calculated by $1 - vt$.

Table A4 shows the calculation of the number of infected and non-infected females and males generated in generation 1 and available for generation 2.

Table A4: Inference of the number of infected and non-infected females (wf and nf) and males (wm and nm) produced by generation 1 (g_1) and available for generation 2 (g_2). $p_{c_{i_{(g_1)}}}$ was defined in Table A3. fsr, vt and N were defined in Table A2.

Individual infection status	Number of expected individuals of each type in the second generation
wf_{g_2}	$p_{c_{1_{(g_1)}}}.fsr.vt.N + p_{c_{2_{(g_1)}}}.fsr.vt.N$
nf_{g_2}	$p_{c_{1_{(g_1)}}}.fsr(1-vt)N + p_{c_{2_{(g_1)}}}.fsr(1-vt)N + \frac{p_{c_{3(g_1)}}}{2}N + \frac{p_{c_{4(g_1)}}}{2}N$
wm_{g_2}	$p_{c_{1(g_1)}}.(1-fsr).vt.N + p_{c_{2(g_1)}}.(1-fsr).vt.N$
nm_{g_2}	$p_{c_{1_{(g_1)}}}(1-fsr)(1-vt)N + p_{c_{2(g_1)}}(1-fsr)(1-vt)N + \frac{p_{c_{3(g_1)}}}{2}N + \frac{p_{c_{4(g_1)}}}{2}N$

With these numbers, it is possible to calculate the available pairs of each cross for the second generation (Table A5).

Table A5 Inference of the frequency of mating pairs in generation 2 (g_2) of the simulation. I_{c_1}, Fe_{c_1} and $F_{1_{c_1}}$ were defined in Table A1. N, Nf, Nm and ni were defined in Table A2. $p_{c_{i_{(g_2)}}}$ is the proportion of pairs c_i available at the second generation. wf and wm are the infected females and males and wm and nm are the numbers of infected and non-infected males, inferred in Table A4.

Number of available pairs	Frequency
$c_{1(g_2)} = \frac{wf_{g_2}}{Nf}.\frac{wm_{g_2}}{Nm}.I_{c_1}.Fe_{c_1}.F_{1_{c_1}}.N$	$p_{c_{1(g_2)}} = \frac{c_{1(g_2)}}{Sum_{(g_2)}}$
$c_{2(g_2)} = \frac{wf_{g_2}}{Nf}.\frac{nm_{g_2}}{Nm}.I_{c_2}.Fe_{c_2}.F_{1_{c_2}}.N$	$p_{c_{2(g_2)}} = \frac{c_{2(g_2)}}{Sum_{(g_2)}}$
$c_{3(g_2)} = \frac{nf_{g_2}}{Nf}.\frac{wm_{g_2}}{Nm}.I_{c_3}.Fe_{c_3}.F_{1_{c_3}}.N$	$p_{c_{3(g_2)}} = \frac{c_{3(g_2)}}{Sum_{(g_2)}}$
$c_{4(g_2)} = \frac{nf_{g_2}}{Nf}.\frac{nm_{g_2}}{Nm}.I_{c_4}.Fe_{c_4}.F_{1_{c_4}}.N$	$p_{c_{4(g_2)}} = \frac{c_{4(g_2)}}{Sum_{(g_2)}}$
$Sum_{(g_2)} = \sum_{i=0}^{4} c_{i(g_2)}$	1.0

Again, to simulate the third generation (and all following generation, or g_t), one should calculate the number of infected and no infected females and males of the former generation (g_{t-1}) (Table A6 and A7).

Table A6: Inference of the number of infected and non-infected females (wf and nf) and males (wm and nm) produced by generation $t-1$ (g_{t-1}) and available for generation t (g_t). $p_{c_{i_{(g_i)}}}$ was defined in Tables A5. fsr, vt and N were defined in Table A2.

Individual infection status	Number of expected individuals of each type in the second generation
wf_{g_t}	$p_{c_{1_{(g_{t-1})}}} fsr.vt.N + p_{c_{2_{(g_{t-1})}}}\ fsr.vt.N$
nf_{g_t}	$p_{c_{1_{(g_{t-1})}}} fsr(1-vt)N + \ p_{c_{2_{(g_{t-1})}}}\ fsr(1-vt)N + \frac{p_{c_{3(g_{t-1})}}}{2}N + \frac{p_{c_{4(g_{t-1})}}}{2}N$
wm_{g_t}	$p_{c_{1_{(g_{t-1})}}}.(1-fsr)vt.N + p_{c_{2(g_{t-1})}}(1-fsr).vt.N$
nm_{g_t}	$p_{c_{1_{(g_{t-1})}}}(1-fsr)(1-vt)N + \ p_{c_{2_{(g_{t-1})}}}(1-fsr)(1-vt)N + \frac{p_{c_{3(g_{t-1})}}}{2}N + \frac{p_{c_{4(g_{t-1})}}}{2}N$

Table A7 Inference of the frequency of mating pairs in generation t of the simulation. $Fe_{c_i}.F_{1_{c_i}}$ were defined in Table A1. N, Nf, Nm and ni were defined in Table A2. $p_{c_{i_{(g_t)}}}$ is the proportion of pairs c_i available at the t generation (g_t). wf and wm are the infected females and males and wm and nm are the numbers of infected and non-infected males, inferred in Table A6.

Number of available pairs:	Frequency:
$c_{1(g_t)} = \frac{wf_{g_t}}{Nf}.\frac{wm_{g_t}}{Nm}.I_{c_i}.Fe_{c_i}.F_{1_{c_1}}.N$	$p_{c_{1(g_t)}} = \frac{c_{1(g_t)}}{Sum_{(g_t)}}$
$c_{2(g_t)} = \frac{wf_{g_t}}{Nf}.\frac{nm_{g_t}}{Nm}.I_{c_2}.Fe_{c_2}.F_{1_{c_2}}.N$	$p_{c_{2(g_t)}} = \frac{c_{2(g_t)}}{Sum_{(g_t)}}$
$c_{3(g_t)} = \frac{nf_{g_t}}{Nf}.\frac{wm_{g_t}}{Nm}.I_{c_3}.Fe_{c_3}.F_{1_{c_3}}.N$	$p_{c_{3(g_t)}} = \frac{c_{3(g_t)}}{Sum_{(g_t)}}$
$c_{4(g_t)} = \frac{nf_{g_t}}{Nf}.\frac{nm_{g_t}}{Nm}.I_{c_4}.Fe_{c_4}.F_{1_{c_4}}.N$	$p_{c_{4(g_t)}} = \frac{c_{4(g_t)}}{Sum_{(g_t)}}$
$Sum_{(g_t)} = \sum_{i=0}^{4} c_{i_{(g_t)}}$	1.0

The simulator would run until the last generation marked by the user (g) and will produce three types of graphs: the first considering males + females, de second considering only females and the third considering only males.

R function

```
##### Parameter K
kcross<-function(ins,fec,pf1){

  kcross<-ins*fec*pf1

}

#########################################################################
######################

#Include the vertical transmission rate

N.wol<-function(n,nw,g,tv,rs,k1,k2,k3,k4){

  ge<-c(1:g) #generation number

  fw<-numeric()#infected females

  mw<-numeric()#infected males

  fn<-numeric()#non-infected females

  mn<-numeric()#non-infected males

  ni<-numeric()#population size

  h1<-numeric()#progeny proportion of w+ x w+ cross

  l1<-numeric()#progeny proportion of w+ x treated cross

  n1<-numeric()#progeny proportion of treated x w+ cross

  q1<-numeric()#progeny proportion of treated x treated cross

  h2<-numeric()#female progeny of w+ x w+ cross

  l2<-numeric()#female progeny of w+ x treated cross
```

```
n2<-numeric()#female progeny of treated x w+ cross
q2<-numeric()#female progeny of treated x treated cross
t1<-numeric()# total progeny
h3<-numeric()#male progeny of w+ x w+ cross
l3<-numeric()#male progeny of w+ x treated cross
for(i in 2:g){
 #initial numbers
  fw[1]<-nw
  mw[1]<-0
  fn[1]<-as.integer((n/2)-nw)
  mn[1]<-as.integer(n/2)
  ni[1]<-fw[1]+mw[1]+fn[1]+mn[1]

  h1[1]<-(fw[1]/(fw[1]+fn[1]))*(mw[1]/(mw[1]+mn[1]))*k1*ni[1]
  l1[1]<-(fw[1]/(fw[1]+fn[1]))*(mn[1]/(mw[1]+mn[1]))*k2*ni[1]
  n1[1]<-(fn[1]/(fw[1]+fn[1]))*(mw[1]/(mw[1]+mn[1]))*k3*ni[1]
  q1[1]<-(fn[1]/(fw[1]+fn[1]))*(mn[1]/(mw[1]+mn[1]))*k4*ni[1]

  t1[1]<-h1[1]+l1[1]+n1[1]+q1[1]

  h2[1]<-(h1[1]/t1[1])*rs
  l2[1]<-(l1[1]/t1[1])*rs
  n2[1]<-(n1[1]/t1[1])*0.5
  q2[1]<-(q1[1]/t1[1])*0.5
```

```
h3[1]<-(h1[1]/t1[1])*(1-rs)

l3[1]<-(l1[1]/t1[1])*(1-rs)


#loop begining

fw[i]<-(h2[i-1]*ni[i-1]*tv)+(l2[i-1]*ni[i-1]*tv)

mw[i]<-(h3[i-1]*ni[i-1]*tv)+(l3[i-1]*ni[i-1]*tv)

fn[i]<-(n2[i-1]*ni[i-1])+(q2[i-1]*ni[i-1])+(h2[i-1]*ni[i-1]*(1-tv))+(l2[i-1]*ni[i-1]*(1-tv))

mn[i]<-(n2[i-1]*ni[i-1])+(q2[i-1]*ni[i-1])+(h3[i-1]*ni[i-1]*(1-tv))+(l3[i-1]*ni[i-1]*(1-tv))


ni[i]<- fw[i]+mw[i]+fn[i]+mn[i]


h1[i]<-(fw[i]/(fw[i]+fn[i]))*(mw[i]/(mw[i]+mn[i]))*k1*ni[i]

l1[i]<-(fw[i]/(fw[i]+fn[i]))*(mn[i]/(mw[i]+mn[i]))*k2*ni[i]

n1[i]<-(fn[i]/(fw[i]+fn[i]))*(mw[i]/(mw[i]+mn[i]))*k3*ni[i]

q1[i]<-(fn[i]/(fw[i]+fn[i]))*(mn[i]/(mw[i]+mn[i]))*k4*ni[i]


t1[i]<-h1[i]+l1[i]+n1[i]+q1[i]


h2[i]<-(h1[i]/t1[i])*rs

l2[i]<-(l1[i]/t1[i])*rs

n2[i]<-(n1[i]/t1[i])*0.5

q2[i]<-(q1[i]/t1[i])*0.5
```

```
    h3[i]<-(h1[i]/t1[i])*(1-rs)
    l3[i]<-(l1[i]/t1[i])*(1-rs)
    }
  {
    dat_pro<-data.frame(ge,fw,fn,mn,mw,ni)
    #print(fw)
  }
}
```

##

Shinny app script

```
#Call the packages
library(ggplot2)
library(shiny)
library(plotly)
library(shinythemes)
##########################################################################
#Call the R function
source("pro_fun4.R")
##########################################################################
#Define the workspace and the input for cross parameters

ui <- fluidPage(theme = shinytheme("superhero"),
  #titlePanel("Population simulation"),
```

```
  #img(src = "LBE_logo.png", height = 80, width = 70 ),
  titlePanel(title=div(img(src="logo_lbe.PNG",  height = 100, width = 150 ), "Population simulation"), windowTitle = "Population simulation"),
  fluidRow(column(12,
                  h3("Cross parameters"),
                  ),
           fluidRow(
             column(1
                    ),
             column(2,
                    h4("C1 (W+ x W+)"),
                    numericInput("ins1",label="Number of inseminated females (ins)",value= 25),
                    numericInput("nins1",label="Number of non-inseminated females (nins)",value= 1),
                    numericInput("fert1",label="Number of fertilizated females (fe)",value= 24),
                    numericInput("F11",label="F1",value= 30)
                    ),
             column(2,
                    h4("C2 (W+ x W-)"),
                    numericInput("ins2",label="Number of inseminated females (ins)",value= 20),
                    numericInput("nins2",label="Number of non-inseminated females (nins)",value= 15),
```

```
            numericInput("fert2",label="Number of fertilizated females (fe)",value=
20),
            numericInput("F12",label="F1",value= 60)
            ),
        column(2,
            h4("C3 (W- x W+)"),
            numericInput("ins3",label="Number of inseminated females (ins)",value=
31),
            numericInput("nins3",label="Number of non-inseminated females
(nins)",value= 2),
            numericInput("fert3",label="Number of fertilizated females (fe)",value=
24),
            numericInput("F13",label="F1",value= 20)
            ),
        column(2,
            h4(" C4 (W- x W-)"),
            numericInput("ins4",label="Number of inseminated females (ins)",value=
43),
            numericInput("nins4",label="Number of non-inseminated females
(nins)",value= 20),
            numericInput("fert4",label="Number of fertilizated females (fe)",value=
41),
            numericInput("F14",label="F1",value= 30)
            )
        ),
 fluidRow(column(12
```

```
    ),
  fluidRow(
    column(1),
    column(3,
      h3("Population parameters"),
      numericInput("ni",label="Population size (N)",value= 100),
      numericInput("nw",label="Initial number of wolbachia infected females (ni)",value=1),
      numericInput("gi",label="Generations (g)",value=25),
      br(),
      h3("Wolbachia infection parameters"),
      sliderInput("tv",label="Vertical transmission rate (vt)",min=0.1,max=1,step=0.01,value=0.9),
      sliderInput("rs",label="Female sex ratio (fsr)",min=0.1,max=1,step=0.01,value=0.5)
      ),
    column(6,
      tabsetPanel(type="tabs",
        tabPanel("Summary table",tableOutput("table")),
        tabPanel("Females + Males",plotlyOutput("plot1")),
        tabPanel("Females",plotlyOutput("plot2")),
        tabPanel("Males",plotlyOutput("plot3"))
        ),
      textOutput("text")
      ),
```

```
      )
    )
  )
)

#################################

server <- function(input, output){

  output$table <- renderTable({

    ins1<-as.numeric(input$ins1)
    nins1<-as.numeric(input$nins1)
    fert1<-as.numeric(input$fert1)
    f11<-as.numeric(input$F11)

    ins2<-as.numeric(input$ins2)
    nins2<-as.numeric(input$nins2)
    fert2<-as.numeric(input$fert2)
    f12<-as.numeric(input$F12)

    ins3<-as.numeric(input$ins3)
    nins3<-as.numeric(input$nins3)
    fert3<-as.numeric(input$fert3)
```

```
    f13<-as.numeric(input$F13)


    ins4<-as.numeric(input$ins4)
    nins4<-as.numeric(input$nins4)
    fert4<-as.numeric(input$fert4)
    f14<-as.numeric(input$F14)


    Crosses<-c("C1", "C2", "C3" , "C4")
    Insemination_rate_I<-
c((ins1/(ins1+nins1)),(ins2/(ins2+nins2)),(ins3/(ins3+nins3)),(ins4/(ins4+nins4)))
    Fertilization_rate_Fe<-c((fert1/ins1),(fert2/ins2),(fert3/ins3),(fert4/ins4))
    F1<-c(f11,f12,f13,f14)
    table_geral<-data.frame(Crosses,Insemination_rate_I,Fertilization_rate_Fe,F1)
    #head(table_geral)
    table_geral


  })


  output$plot1<-renderPlotly({


    ins1<-as.numeric(input$ins1)
    nins1<-as.numeric(input$nins1)
    fert1<-as.numeric(input$fert1)
    f11<-as.numeric(input$F11)
```

```
ins2<-as.numeric(input$ins2)

nins2<-as.numeric(input$nins2)

fert2<-as.numeric(input$fert2)

f12<-as.numeric(input$F12)


ins3<-as.numeric(input$ins3)

nins3<-as.numeric(input$nins3)

fert3<-as.numeric(input$fert3)

f13<-as.numeric(input$F13)


ins4<-as.numeric(input$ins4)

nins4<-as.numeric(input$nins4)

fert4<-as.numeric(input$fert4)

f14<-as.numeric(input$F14)


ins_rate1<-ins1/(ins1+nins1)

fert_rate1<-fert1/ins1

f1_rate1<-f11/(f11+f12+f13+f14)


ins_rate2<-ins2/(ins2+nins2)

fert_rate2<-fert1/ins1

f1_rate2<-f12/(f11+f12+f13+f14)
```

```
ins_rate3<-ins3/(ins3+nins3)

fert_rate3<-fert3/ins3

f1_rate3<-f13/(f11+f12+f13+f14)


ins_rate4<-ins4/(ins4+nins4)

fert_rate4<-fert4/ins4

f1_rate4<-f14/(f11+f12+f13+f14)


k1<-kcross(ins_rate1,fert_rate1,f1_rate1)

k2<-kcross(ins_rate2,fert_rate2,f1_rate2)

k3<-kcross(ins_rate3,fert_rate3,f1_rate3)

k4<-kcross(ins_rate4,fert_rate4,f1_rate4)


ni<-as.numeric(input$ni)

nw<-as.numeric(input$nw)

g<-as.numeric(input$gi)

tv<-as.numeric(input$tv)

rs<-as.numeric(input$rs)

#(n,nw,g,tv,rs,k1,k2,k3,k4)

dat1<-N.wol(ni,nw,g,tv,rs,k1,k2,k3,k4)

dat1$fwp<-dat1$fw/dat1$ni

dat1$fnp<-dat1$fn/dat1$ni

dat1$totalw<-dat1$fw+dat1$mw

dat1$totaln<-dat1$fn+dat1$mn
```

```
    ggplot(dat1, aes(x=ge))+geom_line(aes(y = totaln,colour="Non - Infected"), size=2)
+ geom_line(aes(y = totalw, colour="Infected"), size=2) +
labs(x="Generations",y="Females + Males", color="Wolbachia status") +
theme(axis.title.x=element_text(size=15,color="black"),axis.title.y=element_text(size=
15, color="black"),axis.text.y
=element_text(face="bold",size=10),axis.line=element_line(color="black", size=2),
panel.background=element_rect(fill="white"),axis.text.x = element_text(face="bold",
size=10), legend.title = element_text(face="bold"))


  })
   output$plot2<-renderPlotly({

     ins1<-as.numeric(input$ins1)
     nins1<-as.numeric(input$nins1)
     fert1<-as.numeric(input$fert1)
     f11<-as.numeric(input$F11)

     ins2<-as.numeric(input$ins2)
     nins2<-as.numeric(input$nins2)
     fert2<-as.numeric(input$fert2)
     f12<-as.numeric(input$F12)

     ins3<-as.numeric(input$ins3)
     nins3<-as.numeric(input$nins3)
     fert3<-as.numeric(input$fert3)
```

```
f13<-as.numeric(input$F13)

ins4<-as.numeric(input$ins4)
nins4<-as.numeric(input$nins4)
fert4<-as.numeric(input$fert4)
f14<-as.numeric(input$F14)

ins_rate1<-ins1/(ins1+nins1)
fert_rate1<-fert1/ins1
f1_rate1<-f11/(f11+f12+f13+f14)

ins_rate2<-ins2/(ins2+nins2)
fert_rate2<-fert1/ins1
f1_rate2<-f12/(f11+f12+f13+f14)

ins_rate3<-ins3/(ins3+nins3)
fert_rate3<-fert3/ins3
f1_rate3<-f13/(f11+f12+f13+f14)

ins_rate4<-ins4/(ins4+nins4)
fert_rate4<-fert4/ins4
f1_rate4<-f14/(f11+f12+f13+f14)

k1<-kcross(ins_rate1,fert_rate1,f1_rate1)
```

```
    k2<-kcross(ins_rate2,fert_rate2,f1_rate2)
    k3<-kcross(ins_rate3,fert_rate3,f1_rate3)
    k4<-kcross(ins_rate4,fert_rate4,f1_rate4)

    ni<-as.numeric(input$ni)
    nw<-as.numeric(input$nw)
    g<-as.numeric(input$gi)
    tv<-as.numeric(input$tv)
    rs<-as.numeric(input$rs)
    #(n,nw,g,tv,rs,k1,k2,k3,k4)
    dat1<-N.wol(ni,nw,g,tv,rs,k1,k2,k3,k4)
    dat1$fwp<-dat1$fw/dat1$ni
    dat1$fnp<-dat1$fn/dat1$ni

    ggplot(dat1, aes(x=ge))+geom_line(aes(y = fn,colour="Non - Infected"), size=2)+geom_line(aes(y = fw, colour="Infected"), size=2)+labs(x="Generations",y="Number of Females", color="Wolbachia status")+theme(axis.title.x=element_text(size=15,color="black"),axis.title.y=element_text(size=15, color="black"),axis.text.y=element_text(face="bold",size=10),axis.line=element_line(color="black", size=2), panel.background=element_rect(fill="white"),axis.text.x = element_text(face="bold", size=10), legend.title = element_text(face="bold"))

  })
  output$plot3<-renderPlotly({
```

```
ins1<-as.numeric(input$ins1)
nins1<-as.numeric(input$nins1)
fert1<-as.numeric(input$fert1)
f11<-as.numeric(input$F11)

ins2<-as.numeric(input$ins2)
nins2<-as.numeric(input$nins2)
fert2<-as.numeric(input$fert2)
f12<-as.numeric(input$F12)

ins3<-as.numeric(input$ins3)
nins3<-as.numeric(input$nins3)
fert3<-as.numeric(input$fert3)
f13<-as.numeric(input$F13)

ins4<-as.numeric(input$ins4)
nins4<-as.numeric(input$nins4)
fert4<-as.numeric(input$fert4)
f14<-as.numeric(input$F14)

ins_rate1<-ins1/(ins1+nins1)
fert_rate1<-fert1/ins1
f1_rate1<-f11/(f11+f12+f13+f14)
```

```
ins_rate2<-ins2/(ins2+nins2)

fert_rate2<-fert1/ins1

f1_rate2<-f12/(f11+f12+f13+f14)


ins_rate3<-ins3/(ins3+nins3)

fert_rate3<-fert3/ins3

f1_rate3<-f13/(f11+f12+f13+f14)


ins_rate4<-ins4/(ins4+nins4)

fert_rate4<-fert4/ins4

f1_rate4<-f14/(f11+f12+f13+f14)


k1<-kcross(ins_rate1,fert_rate1,f1_rate1)

k2<-kcross(ins_rate2,fert_rate2,f1_rate2)

k3<-kcross(ins_rate3,fert_rate3,f1_rate3)

k4<-kcross(ins_rate4,fert_rate4,f1_rate4)


ni<-as.numeric(input$ni)

nw<-as.numeric(input$nw)

g<-as.numeric(input$gi)

tv<-as.numeric(input$tv)

rs<-as.numeric(input$rs)

#(n,nw,g,tv,rs,k1,k2,k3,k4)

dat1<-N.wol(ni,nw,g,tv,rs,k1,k2,k3,k4)
```

```
    dat1$fwp<-dat1$fw/dat1$ni

    dat1$fnp<-dat1$fn/dat1$ni


    ggplot(dat1, aes(x=ge))+geom_line(aes(y = mn,colour="Non - Infected"), size=2)+geom_line(aes(y = mw, colour="Infected"), size=2)+labs(x="Generations",y="Number of Males", color="Wolbachia status")+theme(axis.title.x=element_text(size=15,color="black"),axis.title.y=element_text(size=15, color="black"),axis.text.y =element_text(face="bold",size=10),axis.line=element_line(color="black", size=2), panel.background=element_rect(fill="white"),axis.text.x = element_text(face="bold", size=10), legend.title = element_text(face="bold"))


  })


  output$text <- renderText({ "By Camilo Guzman" })


  }


shinyApp(ui = ui, server = server)
```