UNIVERSIDADE FEDERAL DE VIÇOSA

LETÍCIA CARLESSO DE PAULA SENA

SIMBIOSE NUTRICIONAL E FECUNDIDADE NA RELAÇÃO ENTRE *WOLBACHIA* E UM DROSOFILÍDEO NEOTROPICAL

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Dissertação apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Entomologia, para obtenção do título de *Magister Scientiae*.

Orientador: Gustavo Ferreira Martins
Coorientadora: Karla Suemy Clemente Yotoko

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Gustavo Ferreira Martins
Orientador

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RESUMO

Sena, L. C. P., M.Sc., Universidade Federal de Viçosa, julho de 2024. **Simbiose nutricional e fecundidade na relação entre *Wolbachia* e um drosofilídeo neotropical**. Orientador: Gustavo Ferreira Martins. Coorientador: Karla Suemy Clemente Yotoko.

Wolbachia é uma bactéria endossimbionte capaz de induzir fenótipos que em geral aumentam o valor adaptativo dos hospedeiros. A equipe do Laboratório de Bioinformática e Evolução monitorou a prevalência de *Wolbachia* em uma população de *Drosophila sturtevanti* Duda, 1927 (Diptera: Drosophilidae) em 2015, onde todos os indivíduos coletados estavam infectados. A comparação entre uma linhagem pré e pós-tratamento com antibiótico não revelou os fenótipos que seriam esperados com a alta prevalência da bactéria, como incompatibilidade citoplasmática. Ainda, estes resultados apontaram um aumento da fecundidade das fêmeas pós-tratamento, o que foi interpretado como evidência de um fenótipo que reduz o valor adaptativo dos hospedeiros. Logo, esperava-se redução da prevalência na população, de fato observada em 2019, onde a nova coleta indicou prevalência de 50 %. Em 2022, no entanto, a prevalência foi 75 % evidenciando oscilações temporais. O presente trabalho avaliou se a redução na fecundidade é um padrão da infecção na população de *D. sturtevanti* e se a disponibilidade de nutrientes modula a oscilação na prevalência através da relação de simbiose nutricional. A fecundidade foi investigada em três linhagens infectadas e três não infectadas, pré e pós-tratamento com antibiótico. Os resultados indicaram não haver qualquer relação entre fecundidade e status da infecção, de modo que ou a penetrância da redução da fecundidade é incompleta, ou outras bactérias, também tratáveis com antibióticos, afetam a fecundidade. Além disso, a viabilidade de larvas e pupas foi verificada em meios de cultura a 100 e 25 %, em uma linhagem infectada e uma naturalmente não infectada para testar a hipótese de que a cepa que infecta *D. sturtevanti* exerce papel como endossimbionte nutricional. Os resultados obtidos não permitiram concluir sobre tal papel, mas apontaram a estabilização por *Wolbachia* no desenvolvimento das larvas, reduzindo a mortalidade, por meio da mitigação da toxicidade do meio.

Palavras-chave: Disponibilidade nutricional; *Drosophila sturtevanti*; *w*Stv; toxinas; ovos maduros.

ABSTRACT

Sena, L. C. P., M.Sc., Universidade Federal de Viçosa, July, 2024. **Nutritional Symbiosis and Fecundity in the relationship between *Wolbachia* and a neotropical drosophilid**. Adviser: Gustavo Ferreira Martins. Co-adviser: Karla Suemy Clemente Yotoko.

Wolbachia is an endosymbiotic bacterium capable of inducing phenotypes that generally increase the adaptive value of hosts. The team from the Laboratório de Bioinformática e Evolução monitored the prevalence of *Wolbachia* in a population of *Drosophila sturtevanti* Duda, 1927 (Diptera: Drosophilidae) in 2015, where all collected individuals were infected. The comparison between a pre and post-antibiotic treatment lineage did not reveal the phenotypes that would be expected with the high prevalence of the bacterium, such as cytoplasmic incompatibility. Additionally, these results showed an increase in female fecundity post-treatment, which was interpreted as evidence of a phenotype that reduces the hosts' adaptive value. Consequently, a reduction in prevalence in the population was expected, which was indeed observed in 2019, where the new collection indicated a 50 % prevalence. In 2022, however, the prevalence was 75 %, highlighting temporal fluctuations. The present study evaluated whether the reduction in fecundity is a pattern of infection in the *D. sturtevanti* population and whether nutrient availability modulates the prevalence oscillation through the nutritional symbiosis relationship. In Chapter 1, fecundity was investigated in three infected and three uninfected lineages, pre and post-antibiotic treatment. The results indicated no relationship between fecundity and infection status, suggesting either incomplete penetrance of the induced phenotype or other bacteria, also treatable with antibiotics, affecting fecundity. In Chapter 2, the viability of larvae and pupae was tested in culture medium at 100 % and 25 %, in an infected lineage and a naturally uninfected one to test the hypothesis that the strain infecting *D. sturtevanti* acts as a nutritional endosymbiont. The results obtained did not allow a conclusion about such a role but pointed to the possibility that *Wolbachia* stabilizes larval development by mitigating medium toxicity.

Keywords: Nutritional availability; *Drosophila sturtevanti*; *w*Stv; toxins; mature eggs.

SUMÁRIO

INTRODUÇÃO GERAL

WOLBACHIA: FORMAS DE TRANSMISSÃO E FENÓTIPOS NOS HOSPEDEIROS

Wolbachia pipientis Hertig, 1924, é uma bactéria Gram-negativa, Alphaproteobacteria, da ordem Rickettsiales, conhecida apenas como *Wolbachia* por ser a única espécie do gênero (Kaur et al., 2021). Descrita há um século em gônadas do mosquito *Culex pipientis* (Hertig & Wolbach, 1924), é um dos endossimbiontes mais comuns do planeta, estimado em estar presente em 52 % dos insetos (Weinert et al., 2015). Outros estudos estimam uma incidência em cerca de 66 % dos artrópodes, visto que essa bactéria infecta outros táxons como nematóides filariais e aracnídeos, sendo esta incidência provavelmente subestimada devido ao maior foco em organismos modelo e a quantidade relativamente pequena de estudos em outros táxons (Hilgenboecker et al., 2008; Taylor et al., 2018; Werren et al., 2008). Apesar de não ter sido classificado em espécies, o gênero *Wolbachia* foi classificado em cepas, que compõe 17 supergrupos, nomeados com letras de A-F, H-Q e S, sendo que a maioria das cepas conhecidas está nos supergrupos A e B, que infectam apenas artrópodes (Kaur et al., 2021; Lefoulon et al., 2016).

Parte do sucesso de *Wolbachia* se deve às suas formas de transmissão, que pode ser classificada como horizontal (intraespecífica ou interespecífica) ou vertical (de mãe para prole). A transmissão horizontal é mais bem conhecida em artrópodes (Bailly-Bechet et al., 2017; Lefoulon et al., 2016; Taylor et al., 2018) e pode ocorrer por diversos mecanismos, como hibridização resultando em um fenômeno análogo à introgressão entre espécies próximas (Miyata et al., 2020; Raychoudhury et al., 2009), predação (Le Clec'h et al., 2013), parasitismo (Heath et al., 1999) e por meio de parasitóides (Zhao et al., 2021). Apesar da distribuição da bactéria ser sistêmica (por quase todo o organismo) no hospedeiro (Albertson et al., 2013; Diouf et al., 2018; Genty et al., 2014; Sacchi et al., 2010; Strunov & Kiseleva, 2016), a transmissão vertical ocorre apenas pela passagem de *Wolbachia* através dos ovócitos (Frydman et al., 2006). A transmissão vertical pode ser perfeita, resultando em toda a prole infectada, ou imperfeita, com apenas parte da prole de uma fêmea infectada (Hague et al., 2020).

A infecção por diferentes cepas de *Wolbachia* pode induzir fenótipos nos hospedeiros que por vezes podem afetar o valor adaptativo dos mesmos. Tais

fenótipos são características observáveis no hospedeiro infectado que estão ausentes nos não infectados (Ahmed et al., 2016). A maioria dos fenótipos que se tem registro são reprodutivos, e em geral beneficiam as fêmeas infectadas em relação às não infectadas, provavelmente em função da importância da transmissão vertical (Porter & Sullivan, 2023). Dentre estes fenótipos se destaca a incompatibilidade citoplasmática (IC), que resulta na redução da prole de fêmeas não infectadas que acasalaram com machos infectados (Shropshire & Borderstein, 2020; Beckmann et al., 2017; Hoffmann et al., 1998). Além da IC, também foram observados outros fenótipos que beneficiam as fêmeas infectadas como morte e feminização dos embriões machos, ao propiciar aumento de chance de sobrevivência das fêmeas (Bouchon et al., 2008; Hurst et al., 1999; Hurst & Jiggins, 2000), partenogênese (Ma & Schwander, 2017), aumento da fecundidade das fêmeas, pela indução de divisão celular na produção dos gametas femininos (Guo et al., 2018) ou alterações das preferências das fêmeas por machos também infectados (Miller et al., 2010). Existe também uma gama de fenótipos que beneficiam os hospedeiros e que não se restringem a modificações no processo reprodutivo, como auxílio na resposta imune (Zhang et al., 2020), redução da virulência viral (Dorigatti et al., 2018; Edenborough et al., 2021), alterações no comportamento de sono (Bi & Wang, 2020) ou simbiose nutricional (Newton & Rice, 2020; Lindsey et al., 2023).

Neste trabalho, nos concentramos em dois fenótipos induzidos por *Wolbachia*: modificações na fecundidade das fêmeas infectadas e simbiose nutricional. Ambos os casos elevam o valor adaptativo tanto das fêmeas infectadas quanto de *Wolbachia*, o que aumenta a chance de a infecção permanecer na população por transmissão vertical (Weeks et al., 2007). No primeiro caso, o aumento da fertilidade resulta no aumento da proporção de fêmeas infectadas na população, porque estas produzem mais filhas do que as fêmeas não infectadas. O segundo eleva o valor adaptativo dos hospedeiros pela produção, por *Wolbachia*, de nutrientes essenciais ou precursores metabólicos que os hospedeiros não sejam capazes de produzir ou captar do ambiente, temporária ou definitivamente (Hosokawa et al., 2010; Andersen et al., 2012; Newton & Rice, 2020; Lindsey et al., 2023).

ALÉM DE *DROSOPHILA MELANOGASTER*: *WOLBACHIA* EM *D. STURTEVANTI*

Com o intuito de entender a relação da interação *Wolbachia*-hospedeiro, diversos táxons já foram estudados (Gotoh et al., 2005; Noda et al., 2001; Porter & Sullivan, 2023), mas a relação *Wolbachia-Drosophila,* em especial *D. melanogaster* (Meigen, 1830), é a mais investigada (Burdina et al., 2021; Chen et al., 2024; Fry et al., 2004; Harcombe & Hoffmann, 2004; Strunov et al., 2022). A interação serve como modelo para outras interações com *Wolbachia*, devido à facilidade de manutenção em laboratório e a diversidade de fenótipos que são observados. Neste contexto, destaca-se o fato de que o genoma da cepa *w*Mel foi o primeiro genoma de *Wolbachia* a ser completamente sequenciado (Wu et al., 2004). A relação *Wolbachia–D. melanogaster* também forneceu a base dos estudos sobre incompatibilidade citoplasmática (IC) e morte dos embriões machos (Hoffmann et al., 1998; Hurst & Jiggins, 2000; Shropshire et al., 2020).

Pouco se sabe sobre a relação de *Wolbachia* com outros drosofilídeos, especialmente os neotropicais, mesmo os mais abundantes como *Drosophila sturtevanti* Duda, 1927, com distribuição do México ao sul do Brasil (Chaves & Tidon, 2008; Dobzhansky & Pavan, 1950; Magalhães, 1962). Em 2006, Miller e Riegler identificaram pela primeira vez a infecção por *Wolbachia* nesta espécie e a nomearam como *w*Stv. Provavelmente a infecção por *Wolbachia* em *D. sturtevanti* se deu por transferência horizontal, já que esta cepa apresenta alta identidade genética com *w*Whi descrita em *Lutzomyia shannoni* (Dyar 1929) (Diptera: Psychodidae) e *L. whitmani* (Antunes e Coutinho, 1939) (Ono et al., 2001) (Miller e Riegler, 2006) e é geneticamente muito diferente (Miller e Riegler, 2006) das cepas que infectam outras espécies do grupo *saltans*, ao qual *D. sturtevanti* pertence (Roman et al., 2022). Recentemente, uma nova espécie de drosofilídeo foi descrita no subgrupo *saltans*, *D. lehrmanae*, e uma cepa similar a *w*Stv foi encontrada, nominada como *w*Leh (Madi-Ravazzi et al., 2021), indicando uma possível transferência horizontal da *w*Stv para este hospedeiro, ou que a transferência ocorreu de uma destas espécies para *Lutzomyia* spp., algo que ainda precisa ser melhor investigado.

WOLBACHIA E *D. STURTEVANTI*: COMO EXPLICAR A OSCILAÇÃO NA PREVALÊNCIA?

Com o objetivo de estudar a dinâmica da infecção por *Wolbachia* em *D. sturtevanti* e em outras espécies do grupo *saltans*, sucessivas coletas de drosofilídeos

foram realizadas pelo nosso grupo de trabalho. Realizamos o monitoramento da infecção em espécies presentes na Mata da Biologia, localizada na Universidade Federal de Viçosa (UFV), em Minas Gerais (20° 45' 36.2" S, 42° 52' 14.6" W), com coletas realizadas em 2015/2016, 2019 e 2022. Na primeira coleta (2015/2016), todos os indivíduos de *D. sturtevanti* estavam infectados por *Wolbachia*, o que foi interpretado como evidência de 100 % de prevalência na população (Moreira et al., 2024). Para justificar esta elevada prevalência, presumimos que *Wolbachia* estaria beneficiando as fêmeas infectadas e, neste contexto, em 2016, investigamos os fenótipos causados nos indivíduos infectados, com ênfase na detecção de IC.

Normalmente, os experimentos envolvendo a análise dos fenótipos ocasionados por *Wolbachia* são realizados a partir da criação de linhagens isofêmea, cujo processo se dá pela coleta de uma fêmea que, em laboratório, oviposita em tubo com meio de cultura e tem sua prole mantida isolada de outros indivíduos por várias gerações (todos tendo o mesmo *background* genético) (Markow & O'Grady, 2006). Assim, a partir da coleta de 2016, uma linhagem isofêmea infectada por *Wolbachia* foi criada e nominada como st8 (Moreira et al., 2024). A fim de avaliar os fenótipos gerados por *Wolbachia* nesta linhagem, foi preciso reduzir a titulação da infecção com utilização de meio de cultura com Rifampicina a 0,01 % como substrato para desenvolvimento de larvas e comparar os fenótipos apresentados pelos indivíduos tratados e não tratados.

Ao contrário de nossas expectativas, a cepa *w*Stv Vi, identificada em st8, não conferiu incompatibilidade citoplasmática (a prole de fêmeas tratadas com machos infectados não foi menor que dos outros pares possíveis de indivíduos infectados e não infectados) nem provocou a morte dos embriões machos (a prole de fêmeas infectadas apresentou 50 % de machos e 50 % de fêmeas), mas aparentemente aumentou a fertilidade dos machos (machos tratados apresentaram menor fertilidade que os infectados) (Moreira et al. 2024) e reduziu a fecundidade das fêmeas (Moreira et al. 2024; Pereira-Filho, 2021). Diante destes resultados, nos perguntamos como *Wolbachia* se mantém em alta prevalência na população. Uma das alternativas seria que a taxa de transmissão vertical fosse muito alta e que as desvantagens encontradas na fecundidade das fêmeas, bem como a ausência dos fenótipos reprodutivos clássicos não fossem suficientes para eliminar a infecção. Para medir a transmissão vertical, foi necessário fazer outra coleta em campo, para avaliar a

frequência de prole infectada em fêmeas recém coletadas. Para nossa surpresa, apenas 50 % das linhagens coletadas estavam infectadas em 2019, sugerindo que talvez os efeitos negativos observados em st8 estivessem provocando a extinção gradual da infecção na população. Porém, em 2022, realizamos uma terceira coleta e constatamos que 75 % das linhagens coletadas estavam infectadas, o que coloca em foco um aumento na prevalência em relação a 2019 e evidencia uma oscilação na prevalência de *Wolbachia* nesta população. De fato, em um estudo envolvendo diversas populações de *D. sturtevanti* coletadas ao longo do Brasil e na América Central revelou que a prevalência da infecção variou entre 0 % e 70 % (Roman, 2022).

Considerando a capacidade de *Wolbachia* em causar mais de um fenótipo em indivíduos da mesma população (Sasaki et al., 2005), os fenótipos observados na linhagem st8 podem ser uma particularidade da linhagem, que respondeu de uma forma singular ao tratamento com antibiótico (Chaplinska et al., 2016). Cabe ressaltar que o tratamento altera toda a microbiota associada ao hospedeiro, reduzindo a titulação de algumas bactérias, eliminando outras e modificando as frequências relativas de outras tantas (Lesperance & Broderick, 2020; Li et al., 2014; Zug & Hammerstein, 2015).

A partir de tais considerações, surgiram as questões referentes ao primeiro capítulo deste trabalho: *Wolbachia* de fato reduz a fecundidade em *D. sturtevanti* ou esta é uma particularidade observada na linhagem st8? Por que a fecundidade aumentou após o tratamento com antibiótico? Será que esse aumento foi realmente resultado da retirada de *Wolbachia* ou foi um efeito do tratamento com antibiótico que resultou na retirada de outra bactéria? Para abordar estas questões, observamos o número de ovos produzidos nos ovários (estimador de fecundidade) em seis linhagens, sendo três infectadas e três naturalmente não infectadas por *Wolbachia*. Além disso, nos propusemos a avaliar a resposta de cada linhagem ao tratamento com antibiótico, comparando a fecundidade antes e depois do tratamento. Caso *Wolbachia* fosse a responsável pela redução na fecundidade das fêmeas na população de *D. sturtevanti*, conforme visto na linhagem st8 (Moreira et al. 2024), esperávamos que i) as linhagens infectadas apresentassem menos ovos maduros no ovário que as linhagens naturalmente não infectadas, ii) o tratamento com antibiótico aumentasse o número de ovos maduros nos ovários das linhagens naturalmente

infectadas e iii) não alterasse o número de ovos maduros nos ovários das linhagens naturalmente não infectadas.

Outro aspecto que foi considerado foi a possibilidade de que outros fatores estejam influenciando a prevalência da infecção por *Wolbachia*, incluindo a variação na disponibilidade de nutrientes ao longo do tempo (McPherson et al., 2023; Rion & Kawecki, 2007). Isto porque, drosofilídeos neotropicais geralmente se alimentam de frutas em decomposição (Markow & O'Grady, 2006), que nem sempre estão disponíveis em função de sazonalidade (Brownlie et al., 2009; Vijendravarma et al., 2012). Considerando a oscilação encontrada na prevalência de *Wolbachia* na população em estudo, é possível que a infecção não confira vantagens ou desvantagens reprodutivas detectáveis, fazendo com que seja plausível que a oscilação na prevalência de *Wolbachia* esteja relacionada à disponibilidade nutricional e que, portanto, *w*Stv Vi seja um simbionte nutricional. Se isto for verdade, *Wolbachia* pode fornecer nutrientes aos hospedeiros infectados nesta população, conferindo maior valor adaptativo, especialmente durante períodos de escassez nutricional, o que pode aumentar a prevalência da infecção (Correa & Ballard, 2016; Newton & Rice, 2020; Sudakaran et al., 2017; Zug & Hammerstein, 2015). Em contrapartida, em épocas de alta disponibilidade nutricional, o valor adaptativo de infectados e não infectados fica equivalente, fazendo com que a prevalência possa ser reduzida. Para abordar esta questão, avaliamos os componentes do valor adaptativo ao longo do ciclo de vida de *D. sturtevanti* em níveis de disponibilidade nutricional, utilizando, para tanto, diferentes concentrações de meio de cultura (25 % e 100 %) (adaptado de Lindsey et al., 2023). Se for este o caso, esperamos que i) a viabilidade de larvas e pupas seja menos afetada na linhagem infectada quando exposta a diferentes concentrações de nutrientes do que a linhagem não infectada; e que ii) o tempo de desenvolvimento seja mais lento no meio a 25 % do que a 100 % na linhagem não infectada, enquanto não haja diferença na linhagem infectada.

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CAPÍTULO I

Lack of pattern: Fecundity is not driven by *Wolbachia* infection in a Neotropical drosophilid

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

ABSTRACT

Maintaining facultative endosymbionts in hosts depends on the relationship between the parts. It often involves changes induced by the endosymbionts that improve female fitness and ensure the transmission of endosymbionts to the next generation. Experiments with *Wolbachia* and a specific lineage (st8) of the neotropical *Drosophila sturtevanti* revealed that antibiotic treatment increased fecundity (egg production) in infected females, suggesting that *Wolbachia* harms females. Paradoxically, the prevalence of *Wolbachia* fluctuated over time in the population that st8 belonged to, prompting us to question the penetrance of the phenotype induced by *Wolbachia* in that population. We therefore assessed the fecundity of females from six lineages from this population, three naturally infected and three naturally uninfected by *Wolbachia*. We treated three infected and one uninfected lineage with antibiotics and found no correlation between female fecundity and *Wolbachia* infection status. Also, the antibiotic treatment had no significant effects on one of the infected lineages while reducing fecundity in the remaining ones, including the uninfected. The lack of pattern could be due to infections by different *Wolbachia* strains. However, we found the same allele of *wsp* (*Wolbachia* Surface Protein) across all lineages, suggesting that the entire population hosts the same *Wolbachia* strain (*w*Stv). Therefore, the observed fecundity phenotype may result from the interaction

between *Wolbachia* and the specific genotype of each host, indicating a incomplete penetrance of the phenotype found in st8. Our results also point to the possibility that another symbiont increased fertility in three of the four lineages and was eliminated by the treatment. Similarly, it is possible that the previously studied lineage was being parasitized by another bacterium that reduced female fecundity. Our work highlighted the risk of considering *Wolbachia* as the sole factor in comparisons between infected and treated individuals of a single lineage and revealed the fragility of generalizations based on the study of a single host lineage.

KEYWORDS: *Drosophila sturtevanti*; mature eggs; Rifampicin; uninfected lineage; *w*Stv.

1 INTRODUCTION

Symbiosis is the association between two organisms, ranging from parasitism to mutualism (de Bary, 1879; Drew et al., 2021). These relationships evolve (Charlat et al., 2003; Zug & Hammerstein, 2015) based on the costs and benefits to the organisms involved (Ewald, 1987; Bronstein, 1994; Margulis, 1971). Consequently, the same symbiont can induce distinct phenotypes based on the extent and nature of the interaction, mainly when one of the symbionts is a microorganism (Zug & Hammerstein, 2015). The genomic plasticity, high mutation rates, large population sizes, and short life cycles provide microorganisms with a remarkable capacity for adaptation and alteration of host phenotypes (Dean et al., 2006; Schu & Schrallhammer, 2018; Drew et al., 2021; Weeks et al., 2007).

Endosymbiotic bacteria potentially influence host phenotype, altering it either beneficially or detrimentally, thereby impacting behaviors, nutrient biosynthesis, and even fecundity (Drew et al., 2021; Engelstädter & Hurst, 2009; Newton & Rice, 2020). In endosymbionts primarily transmitted vertically (from mother to offspring), enhancing host fecundity is an efficient strategy (Nouhaud et al., 2018; Strunov et al., 2022), which increases the adaptive value not only of the endosymbiont but also of the host (Turelli, 1994). *Wolbachia* (Rickettsiales: Alphaproteobacteria), a widely recognized endosymbiont, is known for inducing phenotypic changes in host fecundity. It infects approximately 66 % of arthropod species and 52 % of insect species (Charlat et al., 2003; Hilgenboecker et al., 2008; Weinert et al., 2015). Its broad distribution is attributed to both vertical transmission and horizontal transmission, which can occur

between unrelated individuals of the same species or different species (Werren et al., 2008; Taylor et al., 2018). In addition to enhancing female fecundity (Cao et al., 2019; Guo et al., 2020; Turelli, 1994), *Wolbachia* employs various strategies that alter host phenotypes, including the induction of reproductive phenotypes that typically increase the competitive advantage of infected females over uninfected ones (Kaur et al., 2021; Porter & Sullivan, 2023). Infected females benefit, for example, involves cytoplasmic incompatibility (CI) (Hoffmann et al., 1998; Beckmann et al., 2017; Shropshire & Bordenstein, 2020), male-killing (Hurst et al., 1999), induction of parthenogenesis (Ma & Schwander, 2017), and feminization of male embryos (Bouchon et al., 2008).

In recent years, the Laboratório de Bioinformática e Evolução team from Universidade Federal de Viçosa (UFV) has been investigating the relationship between *Wolbachia* and *Drosophila sturtevanti* Duda, 1927 (Diptera: Drosophilidae), one of the most abundant drosophilid species in the Neotropical region (Dobzhansky & Pavan, 1950; Da Mata et al., 2008; Chaves & Tidon, 2008). Our research has focused on a population inhabiting an urban fragment of the Atlantic Forest. Moreira et al. (2024) focused on comparing various components of adaptive value in infected individuals treated with antibiotics from the st8 lineage collected in 2016. Noteworthy was the discovery that, despite the high prevalence (100 %) observed that year, treated females exhibited higher fecundity than infected ones, as confirmed by Pereira-Filho (2021), suggesting that *Wolbachia* may reduce the adaptive value of females. Indeed, in a subsequent field sample in 2019, infection prevalence dropped to 50 %, indicating a potential extinction of the infection in the population. However, a new field sample in 2022 showed a rise in prevalence to 75 %, suggesting that prevalence fluctuates over time.

Studies involving individuals infected by *Wolbachia* and treated with antibiotics, such as those presented by Moreira et al. (2024) and Pereira-Filho (2021), benefit from the advantage that individuals with similar genetic backgrounds are compared, thereby reducing the number of factors to be investigated in the data analysis (Hickin et al., 2022; Guzmán, 2021; Miller et al., 2010; O'Shea & Singh, 2015; Serga et al., 2021; Weiland et al., 2022). However, this type of research does not consider variability within the host species (or studied population) nor the fact that antibiotic treatment may lead to the elimination of other endosymbionts, whose absence can affect immune

response, reproduction, nutrition, and metabolism of the hosts (Lesperance & Broderick, 2020; Zug & Hammerstein, 2015).

In this study, we aimed to understand how *Wolbachia* infection affects the fecundity of other lineages collected from the same population, testing the hypothesis that the bacterium reduces female fecundity in this *D. sturtevanti* population, as reported by Moreira et al. (2024) and Pereira-Filho (2021). To accomplish this, we assessed egg production in three naturally infected and three naturally uninfected lineages collected in 2022 and maintained in the laboratory. Additionally, to distinguish between the effects of antibiotic treatment and *Wolbachia* titer reduction, we treated part of these lineages with antibiotics and compared female fecundity before and after treatment.

We expected that if reduced female fecundity was a widespread effect of *Wolbachia* infection in this population of *D. sturtevanti*, i) females from infected lineages would exhibit lower fecundity (number of mature eggs) compared to naturally uninfected ones, ii) antibiotic treatment would increase the fecundity of females from infected lineages, and iii) it would not alter the fecundity of naturally uninfected lineages.

2 MATERIALS AND METHODS

2.1 FIELD SAMPLES AND MAINTENANCE OF LINEAGES

In 2022, six years after the field samples that initiated experiments with the st8 lineage (Moreira et al., 2024), we set up traps (Medeiros & Klaczko, 1999) to capture drosophilids in an urban fragment of the Atlantic Forest called Mata da Biologia, located at the Universidade Federal de Viçosa (UFV), Viçosa, Minas Gerais, Brazil (20° 45' 36.2" S, 42° 52' 14.6" W). Five traps baited with banana and yeast were placed in the field for 48 hours. Captured drosophilids were sorted at the Laboratório de Bioinformática e Evolução (LBE - UFV). The *Drosophila saltans* group, to which *D. sturtevanti* belongs, are dark-colored flies characterized by continuous black stripes at the abdomen, but species identification depends on evaluating diagnostic characters present in the male aedeagus (Markow & O'Grady, 2006). For this reason, each collected female was placed in a vial containing banana culture medium (Moreira et al., 2024) to oviposit and produce F1 offspring. Males of F1 were used for species

identification. The remaining individuals were kept in the vial to generate the second generation (F2) and establish isofemale lines. From this field sample, we obtained 12 isofemale lines of *D. sturtevanti* and maintained them in BOD incubators (Biochemical Oxygen Demand), under a 12 h light/ 12 h dark photoperiod, at 23 °C temperature and controlled humidity (Moreira et al., 2024). We monitored these lines for at least 15 generations until the start of the experiments.

2.2 LINEAGES USED

Of the 12 isofemale lineages established in the laboratory, nine were infected, and three showed no evidence of *Wolbachia* infection (Moreira et al., 2024). For this study, we utilized the three lineages that were either uninfected or had low *Wolbachia* titers (identified as L06, L18, and L22), along with three infected lineages (L01, L13, and L14).

2.3 *Wolbachia'* STRAIN IDENTIFICATION IN INFECTED LINEAGES

Similar strains of *Wolbachia* can induce distinct phenotypes in different host species (Cao et al., 2019; Miller et al., 2010; Schneider et al., 2019; Wolfe et al., 2021), and the identification of the infected strains is essential to evaluate their effects on hosts. Such identification typically involves the gene encoding *wsp* (*Wolbachia* Surface Protein), a locus known to vary among similar strains (Baldo et al., 2006). A sample of the lineages obtained in each field sample of *D. sturtevanti* (2015, 2016, 2019, and 2022) had their DNA extracted, and the *wsp* identified by Moreira et al. (2024). All tested samples so far exhibited *wsp* sequences identical to those detected by Miller and Riegler (2006) in a *D. sturtevanti* populations collected in Panama. The infecting strain was designated as wStv Vi in Moreira et al. (2024).

Since the *Wolbachia* strains infecting the three lineages used in our study (L01, L13, and L14) were not among the strains previously identified by Moreira et al. (2014), we proceeded with their identification. For this purpose, DNA was extracted from five females per lineage using a modified protocol of the Wizard® Genomic DNA Purification Kit (Promega A1120) (Moreira et al., 2024). Subsequently, we performed standard PCR (annealing temperature at 69 °C) with primers WSP-F1 5'-GTCCAATARSTGATGARGAAAC-3' and WSP-R1 5'-CYGCACCAAYAGYRCTRTAAA-3' (Baldo et al., 2006), which amplify a 546 base pair

of the *wsp* gene. PCR products were sequenced using the Sanger method. We used the software CodonCode Aligner Sequence (v 9.0.1 CodonCode Corporation) to evaluate the sequence quality, and the BLAST tool (Altschul et al., 1990), available at NCBI (National Center for Biotechnology Information, 1988), to compare our sequences with previously published ones.

2.4 ANTIBIOTIC TREATMENT

We subdivided each lineage under study (infected and uninfected) into two sub lineages, and treated one of them with antibiotics. We thus placed part of the individuals in vials with banana culture medium prepared with Rifampicin at 0.01 % dilluted in alcohol (Moreira et al., 2024). We maintained the treated sublineages in the antibiotic-containing culture medium for four generations, has already been proven to be effective in reducing *Wolbachia* title (Li et al., 2014) (each generation lasted approximately one month). Rifampicin is the second most used antibiotic for *Wolbachia* infection treatment, after Tetracycline (Li et al., 2014), and has been proven effective in eliminating infection in *D. sturtevanti* (Moreira et al., 2024).

To minimize the adverse effects of the treatment on individual fitness, we kept the treated sublineages in the antibiotic-free culture medium for two additional generations to allow the recovery of the intestinal biota (Ballard et al., 2007; Li et al., 2014; Zeh et al., 2012) before using them in our experiments.

2.5 EFFECTIVENESS OF THE ANTIBIOTIC TREATMENT

To assess the effectiveness of antibiotic treatment in each lineage, we applied a protocol for detecting *Wolbachia* infection, which consists of a DNA extraction using five females (item 2.3) followed by two PCRs, the first using the primers COI 106F 5’-ATTCAGAATATGTTCAG-3’ and COI 154R 5’- TTTAATTTTACCTGGATTTGG-3’ (Madi-Ravazzi et al., 2021), which generates a 650-base pair fragment of the mitochondrial COI gene. Samples that did not produce bands on 1.0 % agarose gel were discarded and the others had their DNA used in the second PCR, with an annealing temperature of 67 °C, using the primers WSP-81F 5’-TGGTCCAATAAGTGATGAAGAAACTAGCTA-3' and WSP-691R 5’-AAAAATTAAAC GCTACTCCAGCTTCTGCAC-3' (Arthofer et al., 2009). This reaction produces a 365 base-pair fragment of the *wsp* gene. We interpreted the presence of a band on 1.0 %

agarose gel as high-titer *Wolbachia* infection and its absence as no or low-titer infection. As a positive control, we used the DNA of five females of *D. prosaltans* Duda, 1927 from the Pro101 lineage, infected with *Wolbachia* and maintained in the laboratory. As negative controls, we used the DNA of five females from a Pro101 sub-lineage treated with 0.03 % Tetracycline (Guzmán, 2021) and samples without biological material to control DNA extraction and PCR conditions.

2.6 OVARY DISSECTION

Females of *Drosophila* spp. start producing eggs on the second or third day after emergence (Wyman, 1979). Therefore, we dissected ovaries from virgin females aged three to 15 days (every two days) across the six studied lineages. We used virgin females because insemination can interfere with egg production (Barnes et al., 2008; Qazi et al., 2003; Chapman, 2001). We dissected ovaries from 20 females per age per lineage, totaling 140 females per treatment and 840 overall. Additionally, we treated some of these lineages with antibiotics and dissected ovaries from 140 females in each treated lineage. To ensure the accurate age of the females, we monitored their emergence daily from tubes containing culture medium set to produce adults. These adults were monitored daily for removal, sexing, and separation of females. Females detected on a given day were transferred to a new tube containing culture medium and labeled with the transfer date, where they were kept until the day of ovary dissection (3-15 days). We counted the eggs in the dissected ovaries to measure fecundity. To confirm female virginity, all vials containing females transferred on a given date were kept for an additional ten days to detect any larvae. All data obtained from those vials were excluded from analyses whenever larvae were found.

For ovary dissection, we first anesthetized females with CO2, sank them in 1.0 % PBS (phosphate-buffered saline), and used fine-tipped forceps. The ovaries were then placed on histological slides, covered with a coverslip, viewed under a microscope at 40 x magnification, and photographed using a cell phone camera. We counted only mature eggs, identified by the presence of the chorionic membrane (thin layer around the egg) and two dorsal appendages (Cummings & King, 1970). We counted mature eggs with the aid of the DotDotGoose software (Ersts, 2022).

2.7 STATISTICAL ANALYSIS

In all tests, we used the number of mature eggs produced in the ovaries of each dissected female as the response variable and the females' age, lineage, infection status, or treatment status as independent variables. In some analyses, we used the total number of eggs produced by each lineage or by the three lineages with the same infection status. In other analyses, we used the number of eggs produced per female age in each lineage or treatment.

All datasets were tested for normality using the Shapiro-Wilk test (Shapiro & Wilk, 1965), which revealed that the majority were not normally distributed, precluding the use of parametric methods. Therefore, we employed the Kruskal-Wallis test (Kruskal & Wallis, 1952). Whenever the test indicated significant differences ($p < 0.05$) and more than two measurements were being compared, we applied the Dunn's post-hoc test with Bonferroni correction for multiple comparisons (dunn.test package, version 1.3.5; Dinno & Dinno, 2017), considering as significative, comparisons which $p < 0.025$. All analyses were conducted using R 4.2.2 environment (R Core Team 2022), and the ggplot2 package (Wickham, 2016) was used to plot the graphs. All scripts and experimental data are available at the following link: https://encurtador.com.br/0X8ZH

3 RESULTS

3.1 STRAIN OF *Wolbachia* IN INFECTED LINEAGES

The *wsp* sequencing of the infected lineages of *D. sturtevanti*, L01, L03, and L14, revealed sequences identical to the sequence identified as *w*Stv Vi, found in all *D. sturtevanti* lineages previously identified at LBE (including st8, studied by Moreira et al., 2024, and Pereira-Filho, 2021) and in a population collected in Panama (Miller & Riegler, 2006). We have deposited the sequences in NCBI (accession number PP952407 - PP952409 – not available until publication).

3.2 EFFECTIVENESS OF THE ANTIBIOTIC TREATMENT

Based on the infection detection protocols (section 2.5), we did not detect any signs of infection in individuals after the fourth generation of antibiotic treatment. We

treated the three infected lineages and the uninfected lineage L18. The other uninfected lineages (L06 and L22) could not be treated in time for the writing of this document. The four treated lineages continue to be maintained in the laboratory and are periodically checked to confirm the absence of *Wolbachia* infection.

3.3 FERTILITY PRE-ANTIBIOTIC TREATMENT

Female *D. sturtevanti* produced from 0 to 114 mature eggs in their ovaries (0 to 106 in infected lineages and 0 to 114 in uninfected ones). The Kruskal-Wallis test did not detect significant differences based on infection status (Kruskal-Wallis $\chi^2 = 0.0054$, df = 1, $p = 0.9414$). When data were subdivided by female age, we observed higher egg production in infected females compared to uninfected ones at age 5, and higher in uninfected compared to infected at age 15 (age 5: Kruskal-Wallis $\chi^2 = 10.967$, df = 1, $p < 0.001$, age 15: Kruskal-Wallis $\chi^2 = 5.1793$, df = 1, $p = 0.02286$) (Fig. 1). No differences were found in egg production among females of other ages (Tab. S1 – Supplementary Material).

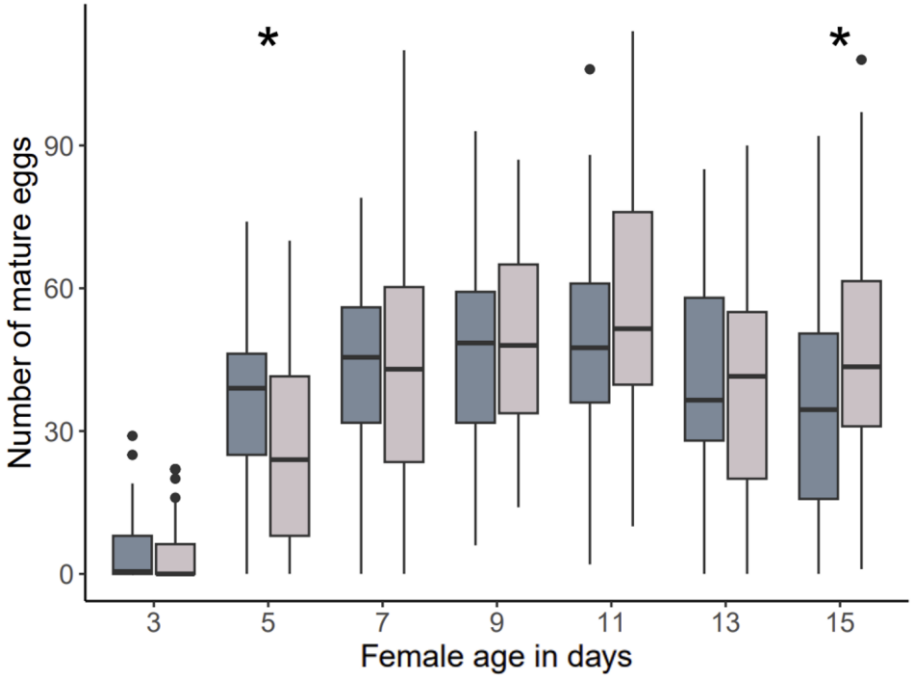

Figure 1. Number of mature eggs produced per day of female maturity in infected lineages (dark gray boxes) and uninfected lineages (light gray boxes) of *Drosophila sturtevanti*. Asterisks indicate differences between infected and uninfected females at each age.

The females of the lineages produce different numbers of eggs (Kruskal-Wallis $\chi^2 = 167.86$; df = 5; $p < 0.001$), but these differences could not be related to infection status: L18, an uninfected lineage, produced the highest number of eggs, while L14

(infected) and L22 (uninfected) produced the lowest numbers (Fig. 2). Among the infected lineages, L01 and L13 showed similar egg production, whereas L14 had reduced egg production (Tab. S2 – Supplementary Material). Each uninfected lineage showed a different fecundity.

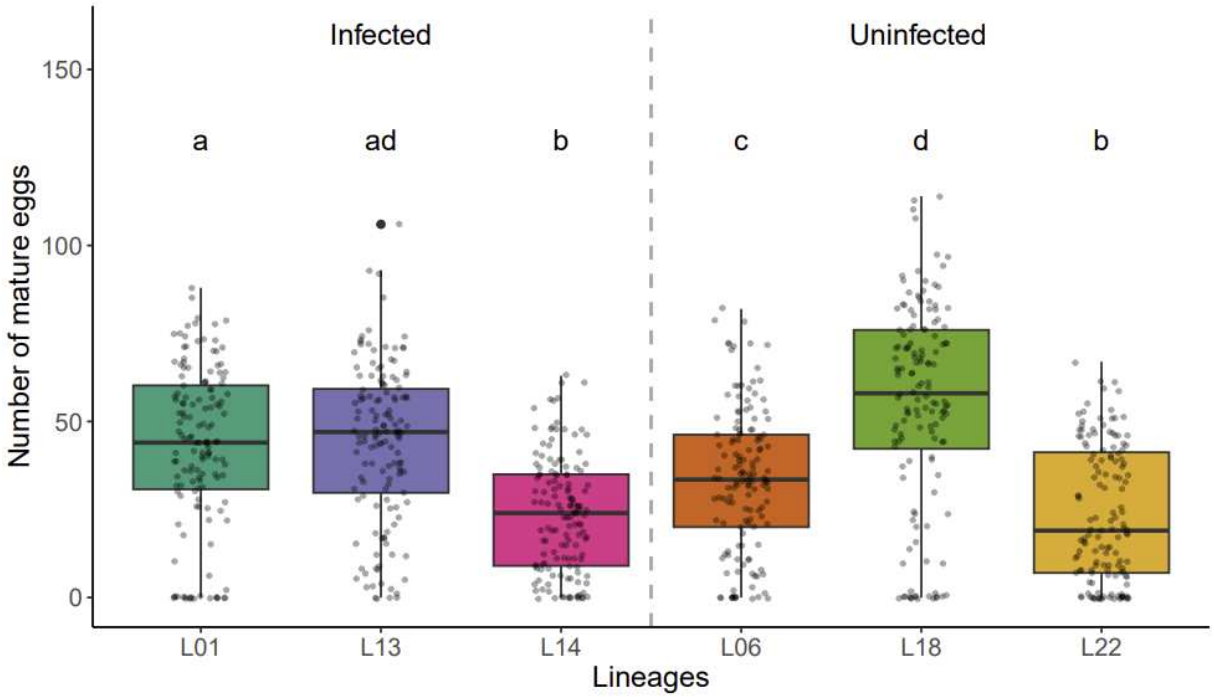

Figure 2. Number of mature eggs produced in the ovaries of females of nfected (L01, L13, and L14) and uninfected lineages (L06, L18, and L22) of *Drosophila sturtevanti*. Each point on the graph represents the total eggs counted in each dissected female, at different ages. Matching letters indicate similar groups.

Figure 3 details egg production by lineage, considering the age of females. In Figure 3A (infected lineages), starting from five days of age, lineages L01 (represented in green) and L13 (blue) exhibited similar and higher egg production compared to lineage L14 (pink) (Tab. S3 – Supplementary Material). In Figure 3B (uninfected lineages), we observed that egg production in L18 (light green) was significantly higher than the other two lineages in females aged 9 and 15 days, while L22 (yellow) showed significantly lower production than the others in females aged 5, 7, 9 and 15 days (Fig. 3B; Tab. S4 – Supplementary Material).

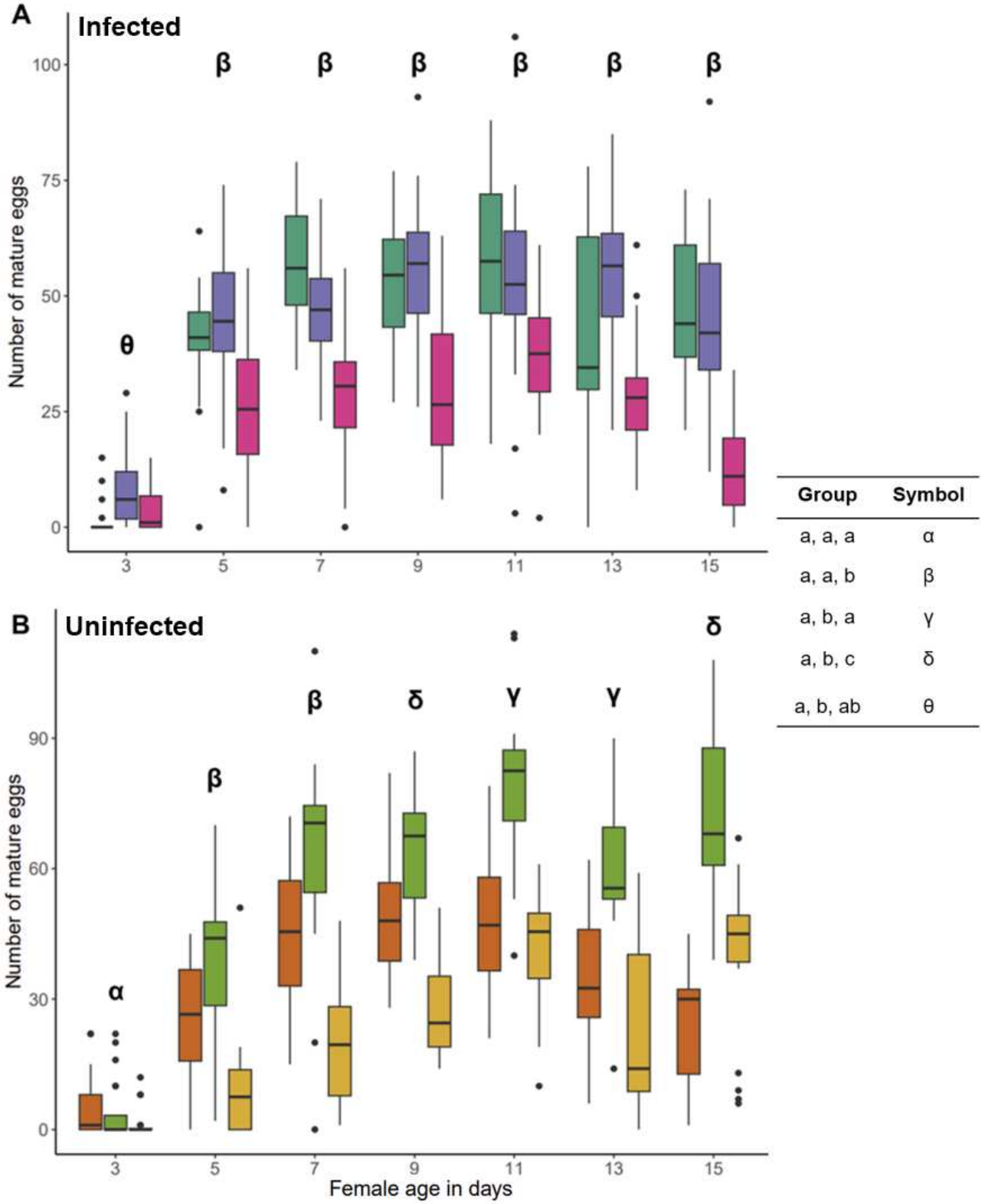

Figure 3. Number of mature eggs produced in the ovary across females ages (in days) of *Drosophila sturtevanti* lineages. A) Infected lineages, L01 (dark green), L13 (purple), and L14 (pink). B) Uninfected lineages, L06 (orange), L18 (light green), and L22 (yellow). In each graph (A or B), all comparisons were made daily among the three lineages. Greek letters denote similar patterns of significant differences among the three lineages, as encoded in the table next to the graphs.

3.4 FERTILITY POST-ANTIBIOTIC TREATMENT

The antibiotic treatment had distinct effects on the fecundity of the three *Wolbachia*-infected lineages (L01, L13, and L14). In lineage L01, we found no significant differences (Kruskal-Wallis $\chi^2 = 1.1921$, df = 1, $p = 0.2749$) while in lineages L13 and L14, the treatment reduced fecundity (in L13: Kruskal-Wallis $\chi^2 = 95.858$, df = 1, $p < 0.001$; in L14: Kruskal-Wallis $\chi^2 = 5.3888$, df = 1, $p = 0.0203$). The antibiotic-treated uninfected lineage, L18, showed a reduction in the total number of eggs produced after antibiotic treatment (Kruskal-Wallis $\chi^2 = 8.3333$, df = 1, $p = 0.003892$) (Fig. 4).

The egg production over females ages in the three infected lineages did not show a consistent pattern of increased or decreased fecundity in response to the treatment (Fig. S1 – Supplementary Material). Tables S5, S6, and S7 in the supplementary material detail the significance between treated and untreated individuals across days in lineages L01, L13, and L14, respectively. Conversely, in the uninfected lineage, antibiotic treatment resulted in three distinct outcomes (Fig. S2 – Supplementary Material). On day 5, treatment led to an increase in egg production; on days 7, 11, and 13, it significantly reduced egg production, with no differences found on other days. Table S8 in the supplementary material provides details of significance between pre- and post-treatment across days in lineage L18.

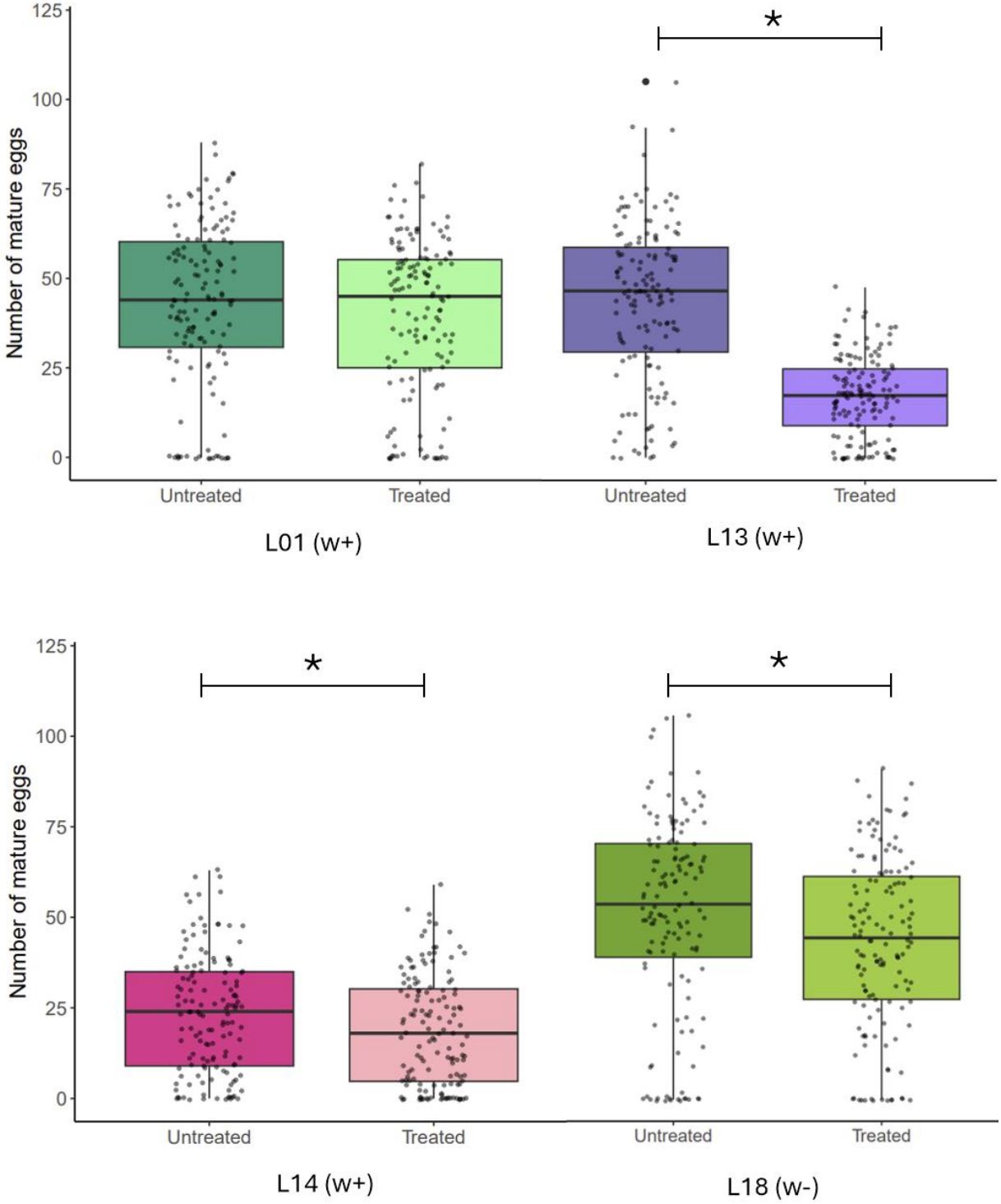

Figure 4. Number of mature eggs produced in the ovaries across pre- (dark shades) and post-antibiotic treatment (light shades) of infected lines (L01, L13, and L14) and naturally uninfected line (L18) of *Drosophila sturtevanti*. Each point on the graph represents the total eggs counted in each dissected female of different ages. Asterisks indicate a significant reduction in mature eggs between pre- and post-treatment in the line.

4 DISCUSSION

In this study, we tested the hypothesis that *Wolbachia* infection reduces the fecundity of *Drosophila sturtevanti* females, as observed by Moreira et al. (2024) and Pereira-Filho (2021), who found that the st8 strain showed increased egg production after antibiotic treatment. In our study, we compared lineages naturally infected and uninfected by *Wolbachia*, some of which we treated with antibiotics. Our results revealed no clear difference in fecundity between naturally infected and uninfected lineages and that antibiotic treatment did not systematically increase the fecundity of infected strains as we predicted.

The infected lineages investigated here exhibited different fecundities (Fig. 2 and 3A), and one of the simplest hypotheses to explain this difference is the infection by different strains of *Wolbachia* (Arai et al., 2019). Indeed, in two species of the *saltans* group (*D. prosaltans* Duda, 1927 and *D. septentriosaltans* Magalhães & Buck, 1962), multiple *wsp* (*Wolbachia* Surface Protein) sequences have been found, indicating the existence of multiple infecting strains within the same host population or even within the same lineage (Miller & Riegler, 2006; Guzmán, 2021). The *wsp* gene exhibits a high substitution rate, and nucleotide substitutions are evidence of different strains (Zhou et al., 1998). However, when sequencing the *wsp* in our lineages, we did not find any difference. In fact, the sequences obtained here are identical to those previously obtained in the st8 lineage (Moreira et al., 2024) and other studies with *D. sturtevanti* (Miller & Riegler, 2006; Roman 2022). There is still a chance that these strains are distinct, but this needs to be verified through whole-genome sequencing of the *Wolbachia* infecting the lineages studied here.

In the second phase of the study, we assessed the difference in egg production caused by antibiotic treatment. We expected that if the reduction in *Wolbachia* titers was responsible for increased fecundity, infected lineages should show enhanced fecundity after antibiotic treatment (Moreira et al., 2024), but this was not observed. Also, the treatment generated an unexpected decrease in female fecundity in three lineages (two infected and one uninfected), while no differences were observed in the remaining infected lineage. It is important to note that different genotypes may interact differently with *Wolbachia* and react differently to antibiotic treatment (Miller et al., 2010).

Overall, our results suggest that if the phenotype observed in st8 by Moreira et al. (2024) and Pereira-Filho (2021) was induced by the strain of *Wolbachia* infecting this population (the *wsp* sequences were identical over time in the population), its penetrance is incomplete or the phenotype no longer induced due to environmental changes and population shifts.

Our results also point to the possibility that another bacterium increased fertility in three of the four treated lineages and was eliminated by the treatment. A good candidate to do it is the genus *Spiroplasma*, an endossymiont capable of enhancing female fecundity (Haselkorn et al., 2009). Similarly, it is possible that the previously studied lineage was being parasitized by another bacterium that reduced female fecundity. In fact, several bacteria can live in the bodies of drosophilids, some of which cause harm to organisms, such as reducing fecundity (Adair et al., 2018; Chandler et al., 2011; Staubach et al., 2013). However, testing all these possibilities is impossible, as it would require mapping the bacterial community associated with the st8 lineage (Henry et al., 2020; Sharon et al., 2010; Staubach et al., 2013), which was extinct in the laboratory, likely due to low fecundity caused by the bacteria associated with it.

This study highlighted the risk of considering *Wolbachia* as the sole factor in comparisons between *Wolbachia*-infected and treated individuals of a single lineage. Although this approach has the clear advantage of comparing individuals with the same genetic background, no matter how careful the treatment and post-treatment recovery were, it is impossible to ensure the reestablishment of the entire microbiota and that the different groups of bacteria will be exactly in the same proportions after antibiotic treatment (Chaplinska et al., 2016; Weiland et al., 2022). The fecundity reduction after the treatment of the uninfected lineage illustrates the concern. Furthermore, the treatment can be quite detrimental to many larvae, producing a bottleneck effect that can further reduce genetic variability in an isofemale lineage, such that the pre- and post-treatment comparison may involve genetically distinct lineages.

Our study also challenges the traditional use of a single lineage to study the consequences of *Wolbachia* infection since other bacteria can be involved besides the putative incomplete penetrance of some phenotypes. Therefore, understanding ecological patterns and evolutionary processes involved in the *Wolbachia*-hosts

relationships requires experiments and observations involving multiple host lineages, making interpretation more complex and complete.

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SUPLEMENTARY MATERIAL

Table S1. Kruskal-Wallis test results for the total number of mature eggs produced in the ovary among infected lineages (L01, L13, and L14 grouped) and among uninfected lineages (L06, L18, and L22 grouped) per female age (in days) (Fig. 1). Asterisks indicate significant differences ($p < 0.05$) between infected and uninfected lineages. X^2 = Chi-square; df = degrees of freedom.

Female age	X^2	df	*p*
3	1.6158	1	0.2037
5	10.967	1	**0.0009276***
7	0.016544	1	0.8977
9	0.021606	1	0.8831
11	2.9022	1	0.08846
13	0.30096	1	0.5833
15	5.1793	1	**0.02286***

Table S2. *P* values of Dunn tests for the total number of mature eggs produced in the ovary between infected (L01, L13, and L14) and uninfected lineages (L06, L18, and L22) (Fig. 2). Asterisks indicate significant differences ($p < 0.025$) between the lineages.

	L01	L13	L14	L18	L22
L13	1.0000				
L14	**0.0000***	**0.0000***			
L18	**0.0054***	0.0261	**0.0000***		
L22	**0.0000***	**0.0000***	1.0000	**0.0000***	
L06	**0.0083***	**0.0015***	**0.0035***	**0.0000***	**0.0076***

Table S3. *P* values of Dunn tests for the total number of mature eggs produced in the ovary between infected lineages (L01, L13, and L14) per female age (in days) (Fig. 3A). Asterisks indicate significant differences ($p < 0.05$) between infected and uninfected lineages,

Female age	L01-L13	L01-L14	L13-L14
3	**0.0005***	0.1511	0.0774
5	0.8987	**0.0020***	**0.0003***
7	0.0883	**0.0000***	**0.0052***
9	1.0000	**0.0001***	**0.0000***
11	0.8115	**0.0010***	**0.0077***
13	0.1093	**0.0201***	**0.0000***
15	0.9085	**0.0000***	**0.0000***

Table S4. *P* values of Dunn tests for the total number of mature eggs produced in the ovary between uninfected lineages (L06, L18 and L22) per female age (in days) (Fig. 3B). Asterisks indicate significant differences ($p < 0.025$) between the lineages.

Female age	L06-L18	L06-L22	L18-L22
3	0.5100	0.1009	0.5719
5	0.0448	**0.0081***	**0.0000***
7	0.0407	**0.0014***	**0.0000***
9	**0.0228***	**0.0009***	**0.0000***
11	**0.0000***	0.6148	**0.0000***
13	**0.0005***	0.1974	**0.0000***
15	**0.0000***	**0.0166***	**0.0006***

CAPÍTULO II

Could *Wolbachia* be a nutritional symbiont in a Neotropical drosophilid? Accessing traits on viability and development

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

ABSTRACT

Over several years, our laboratory monitored the prevalence of *Wolbachia* infection in a population of *Drosophila sturtevanti* and found that it fluctuates despite previous experiments demonstrating that this infection reduces female fecundity. In this work, we tested whether this *Wolbachia* acts as a nutritional symbiont using a naturally infected and an naturally uninfected lineage in two nutrient concentrations medium: 25 % and 100 %. We observed that in the infected lineage, nutrient concentration did not affect larval viability, development time to pupation, or adult production. However, in the uninfected lineage, we found high larval mortality, especially in the tubes with more concentrated medium, precluding conclusions about nutricional symbiosis. We also observed that in the uninfected lineage, larvae at 25 % developed faster than those at 100 %, but we did not find differences in pupal viability. Thus, our findings were not conclusive about the nutritional role of *Wolbachia*, but they pointed to an unexpected role: mitigating environmental toxicity. This finding could be crucial for future work aimed at insect preservation in a world undergoing constant changes, including increased use of pesticides and more issues related to pollutants that certainly increase toxicity.

KEYWORDS: *Drosophila sturtevanti*; nutritional availability; toxins; *w*Stv; larval density.

1 INTRODUCTION

Adequate nutrient availability is a fundamental requirement for the survival of organisms, playing a crucial role in developmental processes and reproduction. However, such availability is not always guaranteed, as nutrients can be limited in quantity or quality, either across different sites or over time within the same site. This nutritional limitation exerts strong selection pressure (Rion & Kawecki, 2007), which can affect development time, viability, and reproductive potential (Kolss et al., 2009; Rion & Kawecki, 2007; McPherson et al., 2023; Wayne et al., 2006). Nutritional deficiencies may also facilitate the establishment and maintenance of interspecific interactions, such as those with endosymbionts, which otherwise might be eliminated from the host (Alarcón et al., 2022; Broderick & Lemaitre, 2012; Newton & Rice, 2020).

In such relationships, endosymbionts play a crucial role. They can offset the cost of occupying space and consuming nutrients by supplying essential nutrients or metabolic precursors that the hosts cannot produce or acquire from the environment, either temporarily or permanently (Newton & Rice, 2020). This type of relationship is classified as nutritional symbiosis, which facilitates host adaptations to previously inaccessible ecological niches, such as environments with limited resources or modified environments that provide fewer nutrients (Correa & Ballard, 2016; Sudakaran et al., 2017). In this scenario, the endosymbiont ensures its survival and reproduction within a host that, rather than attempting to eliminate it, develops strategies to maintain it due to the relationship that arises from nutritional scarcity (Shigenobu et al., 2000; Akman et al., 2002). However, demonstrating the existence of a nutritional symbiosis between species is challenging since it is necessary to present evidence of direct advantages in the host's fitness.

Establishing a nutritional symbiotic relationship is particularly challenging in cases where the same endosymbiont induces different phenotypes in the host, as in the case of *Wolbachia* (Rickettsiales: Alphaproteobacteria), one of the most successful of endosymbionts, present in approximately 52 % of arthropod species and 66 % of insect species (Hilgenboecker et al., 2008; Weinert et al., 2015). *Wolbachia* can cause different phenotypes depending on the host and the time of infection (Weeks et al., 2007). This bacterium can modify host reproduction by inducing reproductive phenotypes such as cytoplasmic incompatibility (CI) (Hoffmann et al., 1998; Beckmann et al., 2017) and male-killing (Dyer & Jaenike, 2004), which can sometimes increase

its fitness at the expense of the hosts (Werren et al., 2008; Newton & Rice, 2020). In addition, some strains manipulate host's behavior (Bi & Wang, 2020; Hoffmann et al., 2011; Miller et al., 2010) or protect against viral infections (Dorigatti et al., 2018; Teixeira et al., 2008). It should be noted, however, that not all strains induce any of these phenotypes and yet continue to be highly prevalent and successfully invade new populations (McPherson et al., 2023; Kriesner & Hoffmann, 2018; Zug & Hammerstein, 2015), potentially due to the nutritional advantages they provide to their hosts (Gill et al., 2014; Zug & Hammerstein, 2015). Some *Wolbachia* strains can synthesize nutrients or contribute to the control of metabolic pathways related to nutrition in scarce or unbalanced environments (Zug & Hammerstein, 2015). The sharing of metabolic pathway precursors between endosymbionts and hosts has been demonstrated, as most of the *Wolbachia* strains already sequenced show genes responsible for producing precursors of essential metabolic pathways (De Clerck et al., 2015; Newton & Rice, 2020; Moriyama et al., 2015). More directly, some strains can contribute to the synthesis of biotin (B7) (Ju et al., 2020; Nikoh et al., 2014), riboflavin (B2) (Ju et al., 2020; De Clerck et al., 2015; Moriyama et al., 2015), lysine (De Clerck et al., 2015), purines and pyrimidines (Lindsey et al., 2023; Darby et al., 2012), and ATP (Gill et al., 2014), as well as to collaborate in iron metabolism, specifically in the production of heme groups and bacterioferritin (Kremer et al., 2009; Gill et al., 2014). However, few studies correlate these contributions of infection with the fitness of infected hosts, neglecting factors that promote an increase in the prevalence of the endosymbiont due to nutritional advantages (Zug & Hammerstein, 2015; Newton & Rice, 2020).

Wolbachia infection occurs at variable prevalence in numerous populations of *D. sturtevanti* Duda 1927 (Roman, 2022), one of the most abundant Neotropical drosophilids (Chaves & Tidon, 2008; Dobzhansky & Pavan, 1950). Since 2015, our Laboratory of Bioinformatics and Evolution (LBE) team has monitored *D. sturtevanti* populations inhabiting an urban renmant of the Atlantic Forest. In our first samples, collected in 2015 and 2016, we found that all individuals of *D. sturtevanti* were infected with *Wolbachia*. To understand the reason for the great success of the bacterium in this population, we began to study this interaction (Moreira et al., 2024).

However, the results suggested that the strain of *Wolbachia* found in the population, named *w*Stv Vi, and studied in the st8 strain, contrary to all expectations, did not show the traditional phenotypes that increase the adaptive value of females

(such as CI and male-killing). Specifically, treatment with antibiotics that reduced the titer of *Wolbachia* to undetectable levels increased the fecundity of females (Pereira-Filho, 2021), induced protogyny and reduced the fertility of males. We interpreted the results as evidence that *Wolbachia*, in this lineage, reduced female fecundity, eliminated protogyny, and increased the fertility of males.

In the face of this paradox (high prevalence vs. absence of beneficial phenotypes for females), new samples were collected at the same site in 2019 and 2022 to understand the infection dynamics. In 2019, only 50 % of the individuals were infected, consistent with the data obtained in the experiments, suggesting a decrease in prevalence and possible extinction of the strain in the population. However, in 2022, the prevalence of the bacteria increased to 75 %, revealing a fluctuation in prevalence rather than a tendency to fix or lose the infection.

In light of these findings, we hypothesized that an environmental component may modulate the prevalence of the bacteria over time (Moreira et al., 2024). Because drosophilids live in environments with varying nutrient availability, and most neotropical drosophilids feed on decomposing organic matter (Markow & O'Grady, 2006), competition for this ephemeral resource is expected to be high (Broderick & Lemaire, 2012; Brownlie et al., 2009; Vijendravarma et al., 2012). As mentioned above, Moreira et al. (2024) based their conclusions on individuals from a single isofemale lineage (st8) that was previously adapted to the laboratory environment, which includes maintenance in a nutrient-rich culture medium and stability over generations (Moreira et al., 2024). The oscillations in *Wolbachia* prevalence in the *D. sturtevanti* studied population are likely driven by the variable resource availability. Unlike the controlled environment of the laboratory, where resources are always available and abundant, natural resources are limited (Berryman, 2020; Malthus, 1798). As additional evidence, electron microscopy images revealed that st8 females of *D. sturtevanti* had *Wolbachia*-filled bacteriocytes (Pereira-Filho, 2021), structure typically found in insects dependent on nutritional symbionts, especially those living in nutritionally poor or unbalanced environments (Hosokawa et al., 2009; Luan, 2024; Porter & Sullivan, 2023).

In this work, we aimed to investigate whether the availability of nutrients in the environment explains the observed *Wolbachia* prevalence fluctuation in the studied population of *D. sturtevanti*. Based on the nutritional symbiosis hypothesis, the infected lineage would be favored over the uninfected during periods of reduced nutrient

availability. We set experiments to quantify the viability and development time of infected and uninfected larvae under different nutrient availability.

2 MATERIALS AND METHODS

2.1 LINEAGES STUDIED

We studied two lineages of *D. sturtevanti*: one infected with *Wolbachia* (lineage L13 w+) and one naturally uninfected (lineage L18 w-). Both lineages were collected in 2022 from the population of Mata da Biologia, in Viçosa, Minas Gerais, Brazil (20° 45' 36.2" S, 42° 52' 14.6" W), an urban remnant of the Atlantic Forest. The lineages were maintained in the laboratory for at least 15 generations before the beginning of the experiments, in standard banana and yeast culture medium, kept on BOD (Biochemical Oxygen Demand incubator) with a temperature of 23 ºC, 12 h light/12 h dark photoperiod and controlled humidity (Moreira et al. 2024).

Before setting up the experiments, we confirmed the *Wolbachia* infection status of the two lineages using PCR reactions. We extracted total DNA from five females per lineage using the Wizard® Genomic DNA Purification Kit (Promega A1120) (Moreira et al., 2024). Thus, we checked the quality of the extracted DNA with a PCR reaction (annealing temperature 55 ºC) based on the *Drosophila* COI 106F 5'-ATTCAGAATATGTTCAG-3' and COI 154R 5'- TTTAATTTTACCTGGATTTGG-3' primers (Madi-Ravazzi et al., 2021), which amplify a 650 base pair fragment of the mitochondrial COI gene (cytochrome C oxidase I). We discarded the samples that did not generate a band on the 1.0 % agarose gel and used the remaining samples to test the status of *Wolbachia* infection with another PCR (annealing temperature 67 ºC) using primers WSP-81F 5'-TGGTCCAATAAGTGATGAAGAAACTAGCTA-3' and WSP-691R 5'-AAAAATTAAAC GCTACTCCAGCTTCTGCAC-3' (Arthofer et al., 2009), which amplify a 365 base pair fragment of the *wsp* (*Wolbachia* surface protein). exclusive to *Wolbachia*. We interpreted the 365 bp band in a 1 % agarose gel as evidence of *Wolbachia* infection at high titer, and the absence of the band as evidence of no or low titer. As a positive control, we used the DNA of five females of *D. prosaltans* Duda, 1927 from the Pro101 lineage, infected with *Wolbachia* and maintained in the laboratory. As negative controls, we used the DNA of five females from a Pro101 sub-lineage treated with 0.03 % Tetracycline (Guzmán, 2021) and samples without

biological material to control DNA extraction and PCR conditions. The *wsp* sequence of L13 was deposited in NCBI, (accession number PP952408 – not available until publication; Sena et al., *in prep.* - Chap. 1 of this document).

2.2 EXPERIMENTAL PILOT

Before investigating whether *Wolbachia* infection can benefit *D. sturtevanti* in conditions of low nutrient availability, we needed to ensure that the infected lineage has some chance of survival in a diluted culture medium. Therefore, we conducted a pilot study with the infected lineage to define the dilution of the culture medium for carrying out the experiments. We thus diluted the standard laboratory culture medium (100 % concentration) to 50 % and 25 %, with the concentrations of agar, methyl paraben (antifungal), and volume of distilled water held constant (adapted from Lindsey et al., 2023; see Tab. 1). The culture media were prepared in three concentrations on the same day using the same ingredients and the same cooking process. It should be noted that the tubes with different concentrations can be visually distinguished, allowing their visual identification.

Larval density can influence the amount of food consumed per individual. To avoid competition for nutrients and the accumulation of excretions, we controlled the larval density (Chen & Sokolowski, 2022; Dombrovski et al., 2017; Dombrovski et al., 2020; Gregg et al., 1990; Liao et al., 2024). Furthermore, to ensure consistency in the experiment, all the larvae used in the experiments were derived from a single generation. This caution is crucial because natural selection (or genetic drift) can alter the characteristics of a lineage over time in laboratory conditions (Dias, 2024). To control larvae density, we introduced groups of five sexually mature females (at least eight days old) that had been previously inseminated (kept together with the males during this period) into new tubes containing standard culture medium (Tab. 1) (Fig. 1). All the tubes mentioned above were assembled on the same day.

After 48 hours, we removed the females and waited for six days to detect the larvae. Larvae from all the tubes were simultaneously transferred to Petri dishes containing PBS (Phosphate-Salt Buffer). After visual inspection, some larvae were selected for the experiment, based on size and mobility to ensure the most homogeneous set possible for distribution among the treatments. A total of six tubes were prepared for each concentration, with 20 larvae transferred to each. Each tube

contained 10 ml of culture medium with 100, 50, or 25 % nutrients. Daily observations were conducted to record the number of pupae produced in each tube. All tubes were maintained until the emergence of the first adult. We did this procedure to identify the minimum dilution that would ensure the survival until the adult stage in the infected lineage. This experiment was adapted from the one described by Lindsey et al. (2023), who studied nutritional symbiosis in *Drosophila melanogaster*.

A similar number of pupae was observed in the tubes with different concentration of culture medium during the pilot phase (18.83 ± 0.52; mean ± standard error) and the same occurred with the first adults' emergence date (12.33 ± 0.66 days). As the infected lineage survived on 25 % diluted medium, subsequent experiments were conducted using 100 % medium as a control and 25 % diluted medium to investigate the hypothesis that *Wolbachia* acts as a nutritional endosymbiont in this population.

Table 1. Quantity of ingredients required to prepare a standard culture medium, comprising banana and yeast, at three different concentrations: 100 %, 50 %, and 25 %. The values were used to assemble 10 tubes with 10 ml of medium each.

Ingredients	[100 %]	[50 %]	[25 %]
Distilled water (ml)	100,0	100,0	100,0
Honey (g)	9,50	4,75	2,38
Banana (g)	13,80	6,90	3,45
Agar-agar (g)	0,90	0,90	0,90
Barley flour (g)	3,00	1,50	0,75
Beer yeast (g)	2,80	1,40	0,70
Methylparaben (g)	0,22	0,22	0,22

2.3 NUTRITIONAL SYMBIOSIS AND COMPONENTS OF FITNESS

The same procedure described in section 2.2 was employed with individuals from the infected (L13 w+) and uninfected (L18 w-) lineages to set up the tubes for the four treatments (infected at 100 % and at 25 % and uninfected at 100 % and at 25 %). Each treatment was tested using 30 tubes containing 20 larvae each. The tubes were inspected daily to search for pupae and adults. The pupae were allowed to emerge from the tubes while the adults were frozen in 98 % alcohol everyday of emergency for subsequent analysis. The tubes were monitored until no adults emerged for five consecutive days.

If *Wolbachia* is a nutritional endosymbiont in *D. sturtevanti*, in terms of viability of larvae and pupae (how many larvae empupates and how many pupae emerge in adults, respectively), the infected lineage (L13 w+) should be less affected by the reduction in nutrient availability than the uninfected lineage (L18 w-). Regarding development time, it would be reasonable to assume that development is slower in nutritionally poor environments (Kolss et al., 2009; Lindsey et al., 2023). Therefore, development in 25 % medium should be slower than in 100 % medium, while the development time difference should be less pronounced in the infected lineage compared to the uninfected lineage.

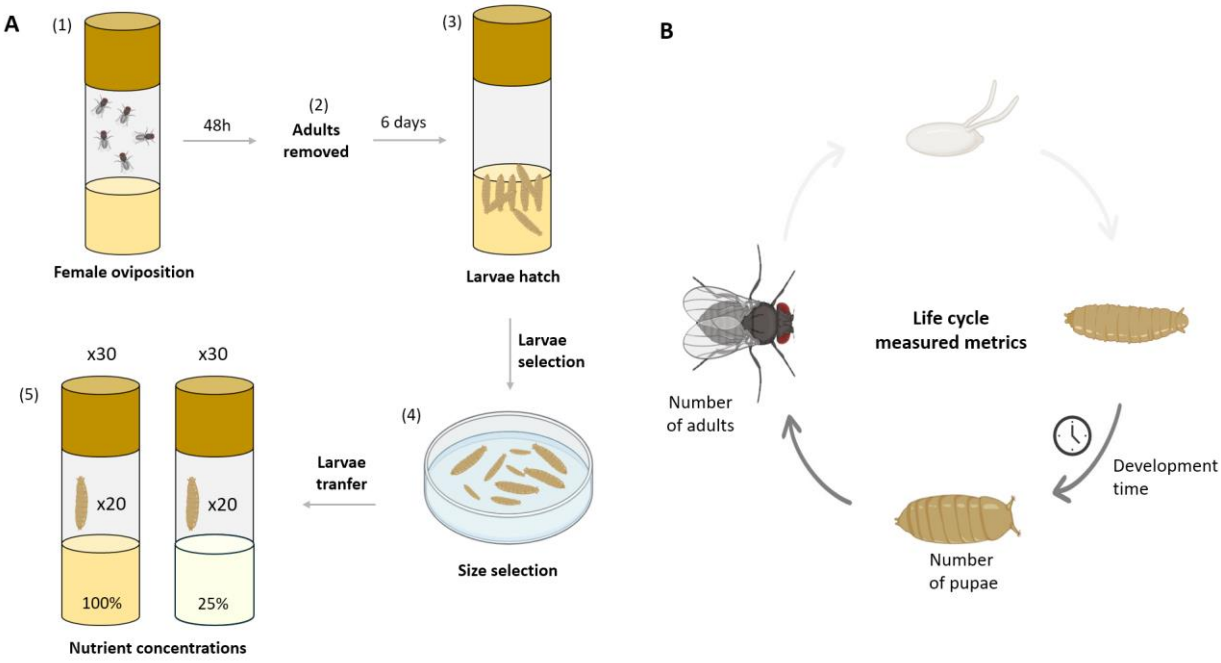

Figure 1. Schematic representation depicts the nutritional symbiosis experiment conducted on both the infected and not infected lineage with *Wolbachia*. A) The procedure started with (1) oviposition by five previously inseminated females for 48 hours in the standard culture medium, followed by (2) their removal. (3) The larvae were allowed to develop for six days, after which they were transferred to Petri dishes containing phosphate-buffered saline (PBS) buffer (1 %) and (4) larvae of a similar size were selected for (5) subsequent transfer to tubes containing culture medium at two different nutrient concentrations: 100 % and 25 %. Twenty larvae were transferred to each tube, with 30 replicates carried out in each treatment. (B) Life cycle metrics: development time from larvae to pupae, number of pupae and number of adults were observed daily from larvae transfer until the end of the experiment (adapted from Lindsey et al., 2023).

2.4 STATISTICAL ANALYSIS

We plotted a survival curve to evaluate differences in the developmental time from larvae to pupa in the four treatments (infected at 25 % and 100 %, uninfected at 25 % and 100 %). We performed a survival analysis using the Kaplan-Meier method (Kaplan & Meier, 1958) with Weibull distribution with on censored data (Therneau, 2015), whereby instead of counting the number of individuals that died over a period (standard data of survival curve), we counted the number of larvae that pupated per day throughout the experiment. Consequently, at the initial stage of the experiment, all the individuals were in the larval stage, and by the experiment's conclusion, a proportion of them had reached the pupal stage. Those who did not reach the pupal stage by the end of the experiment were classified as deceased. We tested the survival model considering infection status, nutrient concentration, and the interaction between these two factors. We employed the survival package (Therneau, 2015) survminer package (Kassambara et al., 2021) to generate and analyze the survival graphs.

The results obtained were analyzed to estimate the viability of the larvae and pupae in each tube. The viability of the larvae was calculated as the number of pupae produced per 20 larvae, while the viability of the pupae was calculated as the number of adults produced per number of pupae. The normality of these data was tested in each treatment (infected at 25 % and 100 %, uninfected at 25 % and 100 %) using the Shapiro-Wilk test (Shapiro & Wilk, 1965), which indicated that most of the data did not exhibit normal distribution, making parametric methods unsuitable for comparison. Given these circumstances, we employed the Kruskal-Wallis test (Kruskal & Wallis, 1952). When significant differences were indicated by the test ($p < 0.05$), and more than two measurements were involved, Dunn's post-hoc test (Dunn, 1964) with Bonferroni correction for multiple comparisons was employed (Haynes, 2013; dunn.test package, version 1.3.5; Dinno & Dinno, 2017) which comparisons with $p < 0.025$ were considered significant. Additionally, a Levene's test (Levene, 1960), adjusted to the median (car package - Fox & Weisberg, 2019), was employed to ascertain the variability of the data across treatments. When significant differences were identified across all treatments, the number of treatments was reduced (*e.g.*, from 3 to 3 and from 2 to 2), to identify the specific groups exhibiting differential dispersion in their data.

All analyses were conducted using the R 4.2.2 environment (R Core Team 2023) and the ggplot2 package (Wickham, 2016) to facilitate graph editing. The full complement of scripts and data from the experiment is available for consultation at https://encurtador.com.br/U4rdy.

3 RESULTS

3.1 PERIOD OF EXPERIMENT

The number of days from the transfer of the larvae to the emergence of the last adult was 28 days in the tubes of both lineages that developed in the 100 % medium. In the 25 % medium, the period was 31 days for the infected lineage and 32 days for the uninfected lineage.

3.2 PUPAE DEVELOPMENT TIME

The larvae began to pupate simultaneously. The first pupa was observed in the tubes of uninfected lineage with 25 % culture medium on the first day after larval transfer. In the remaining treatments (25 % infected, 100 % infected, and 100 % uninfected), the first pupa was observed on the second day after transfer. In light of the survival curve model based on the developmental time from larvae to pupae, no effect of the interaction between infection status and nutrient availability was observed ($p = 0.1493$). Consequently, the interaction was not accounted for in the subsequent analysis.

The definitive model demonstrated that the development time of infected lineage did not significantly vary as a function of the culture medium concentration (25 and 100 %) (Fig. 2A; $p = 0.100$). In contrast, the uninfected lineage exhibited a faster developmental rate in larvae cultured in a 25 % medium compared to 100 % medium (Fig. 2B; $p = 0.001$). Furthermore, comparing the survival curves of infected and uninfected larvae at different nutritional concentrations revealed that the infected lineage developed faster than the uninfected one ($p < 0.001$; Fig. 2C-D).

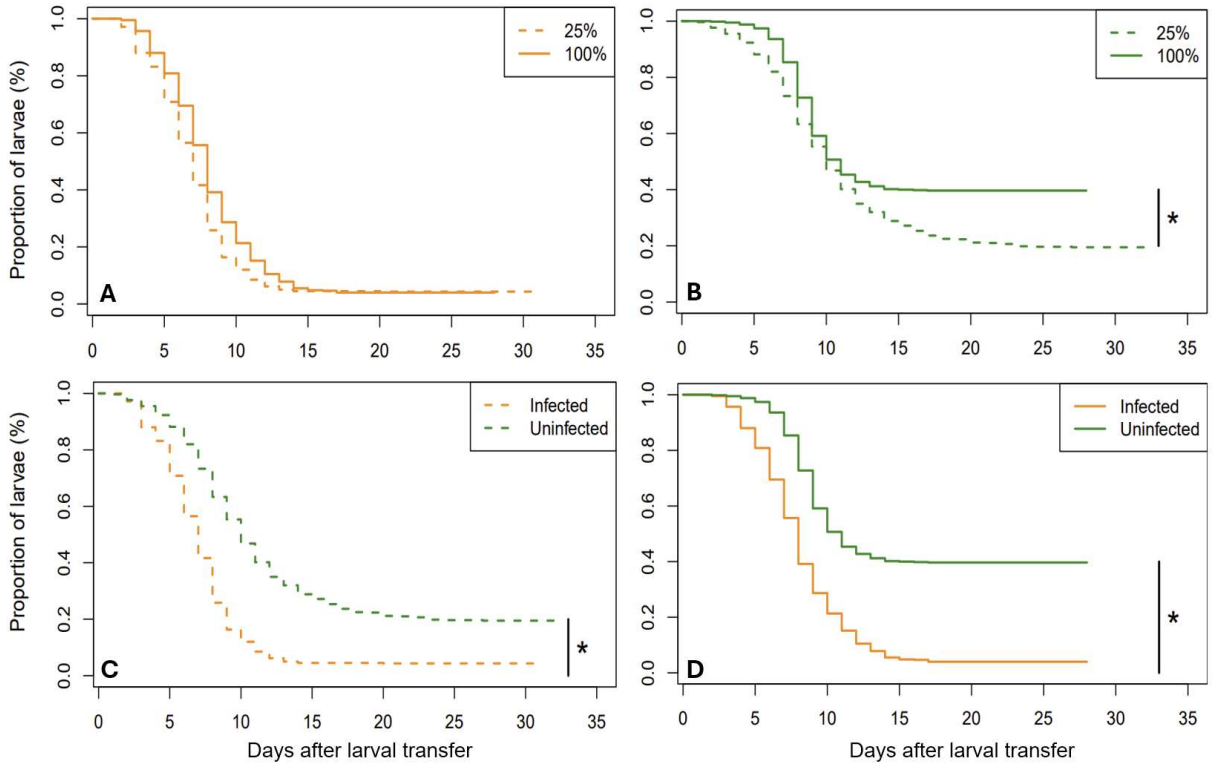

Figure 2. The survival curve depicts the development time of larvae to pupae for a *Wolbachia*-infected lineage (orange line) and an uninfected lineage (green line). Both lineages were reared in standard culture medium with 100 % nutrients (solid line) and in a diluted culture medium with 25 % nutrients (hatched line). A) Infected lineage in 25 % and 100 % nutrient medium; B) Uninfected lineage in 25 % and 100 % nutrient medium; C) Infected and uninfected lineages in 25 % nutrient medium; D) Infected and uninfected lineages in 100 % nutrient medium. All tubes began with 20 larvae (y = 1.0). Asterisks indicate significant differences.

3.3 VIABILITY OF LARVAE

The four treatments (infected at 25 % and 100 %, uninfected at 25 % and 100 %) exhibited differences in larval viability, as evidenced by the pupae to larvae ratio (Kruskal-Wallis $\chi^2 = 20.09$; df = 3; $p < 0.001$). However, we did not find significant differences in the function of nutrient concentrations in either the infected ($p = 1.0$) or uninfected ($p = 0.2057$) lineages (Fig. 3; see Tab. 1 in the Supplementary Material). The viability of infected and uninfected larvae in the 25 % medium was also the same ($p = 0.0969$). However, uninfected larvae in tubes containing 100 % medium were less viable than infected larvae, regardless of the medium concentration ($p < 0.001$ in both comparisons).

The viability variance, defined as the variance of the total number of larvae that pupated over the tubes, was found to be small and not significantly different between infected larvae that developed in 25 % or 100 % medium ($p = 1.0000$) (Fig. 3, Greek

letters; Tab. 2, Supplementary Material). However, the tubes containing uninfected larvae exhibited significantly more variance than those containing infected larvae in the same nutrient concentrations (p< 0.001 for both comparisons). The tubes containing uninfected larvae that developed in 100 % medium exhibited more variance than those containing uninfected larvae that developed at 25 % (p < 0.001).

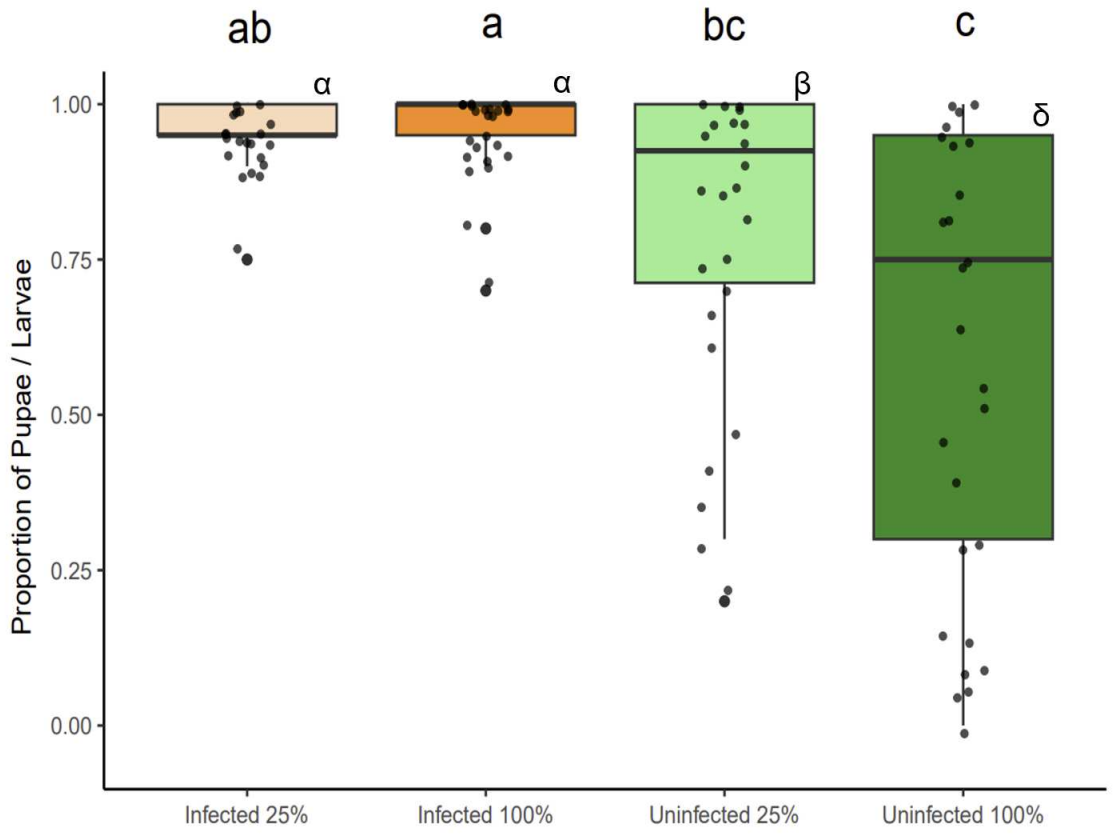

Figure 3. Viability of larvae with 25 % and 100 % nutrient concentration in infected and uninfected *D. sturtevanti* lineages. Larval viability was calculated as the number of pupae divided by the number of larvae in each of the 30 tubes set up per treatment. All tubes began with 20 larvae, and each point on the graph represents a tube (repetition). Roman letters indicate the treatments with similar outcomes, inferred by the Dunn test ($p \geq 0.025$) while Greek letters indicate the treatments with similar variance, inferred by the Levene test ($p \geq 0.05$).

3.4 VIABILITY OF PUPAE

No statistically significant difference was identified in the pupal viability, as evidenced by the number of adults to pupae ratio number, among the four treatments studied (infected at 25 % and 100 %, uninfected at 25 % and 100 %) (Fig. 4 – Kruskal-Wallis $\chi^2 = 1.89$; df = 3; $p = 0.5939$). The median viability of pupae across all treatments was approximately 50 %. An analysis of variance indicated that the infected individuals

that developed in 25 % medium had more variance than those developed in 100 % medium ($p < 0.001$) (Fig. 4; Tab. 3 - Supplementary material). In contrast, no statistically significant difference was observed in function of differences in nutrient concentrations in the uninfected lineage ($p = 0.0836$). Among the tubes containing 100 % medium, the infected lineage exhibited a significantly reduced variance compared to the uninfected lineage ($p < 0.001$), whereas no discernible differences in variance were observed between the tubes containing 25 % medium ($p = 0.2503$).

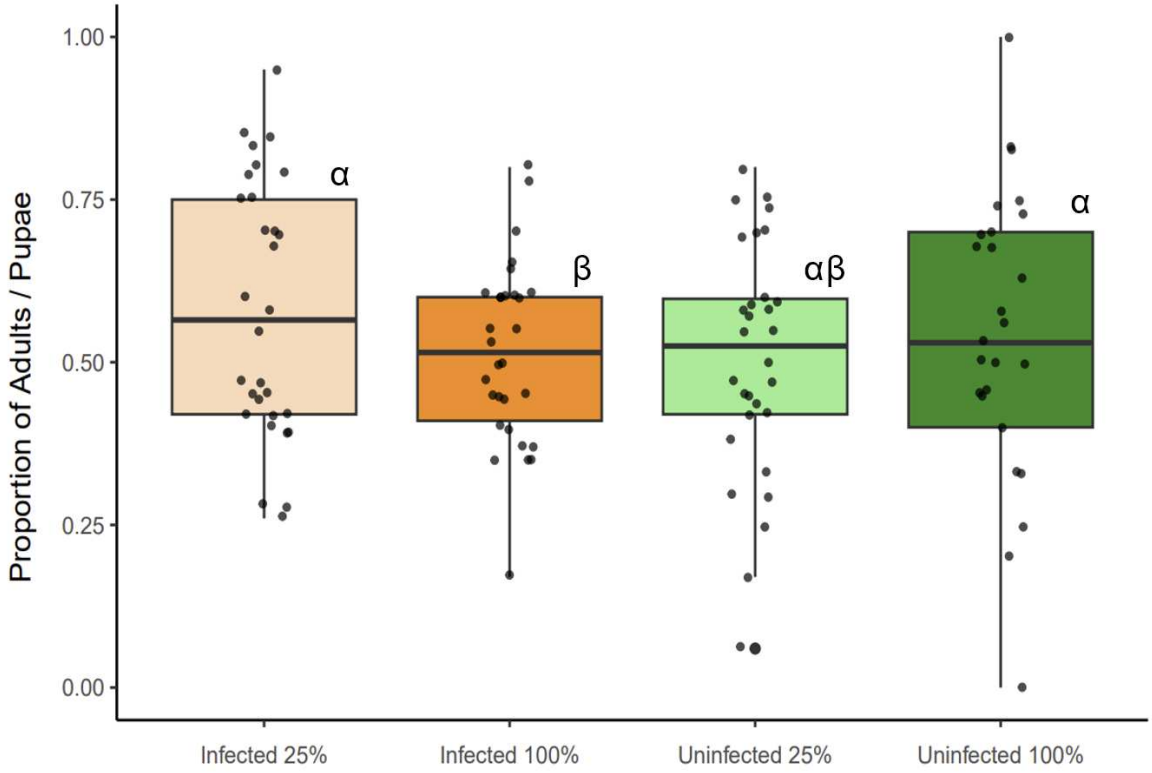

Figure 4. Viability of pupal with 25 % and 100 % nutrient concentration in infected and uninfected *D. sturtevanti* lineages. Pupal viability was calculated as the number of adults divided by the number of pupae in each of the 30 tubes set up per treatment. Each point on the graph represents a tube (repetition). The Kruskal-Wallis test indicated that no significant differences were observed between the groups. Greek letters indicate treatments with similar variance that were inferred by Levene's test ($p \geq 0.05$).

3.5 ADULT EMERGENCE

The number of adults varied significantly between lineages and nutrient concentrations (Fig. 5, Kruskal-Wallis $\chi^2 = 10.87$, df = 3, $p = 0.0124$). In particular, the treatment involving the lineage infected at 25 % resulted in a more significant number of adults than that involving the uninfected lineage at 100 % ($p = 0.0047$) (Tab. 4 - Supplementary Material - Dunn post hoc). In terms of variance, the infected lineage at

25 % exhibited more variance than at 100 % ($p < 0.001$), while the uninfected lineage demonstrated similar variance at both concentrations ($p = 0.1467$) (Tab. 5 - Supplementary material). The infected and uninfected lineages presented the same variance in tubes with 25 % of nutrients ($p = 0.6627$). In comparison, among the tubes with a 100 % nutrient concentration, the uninfected group displayed more variance ($p < 0.001$).

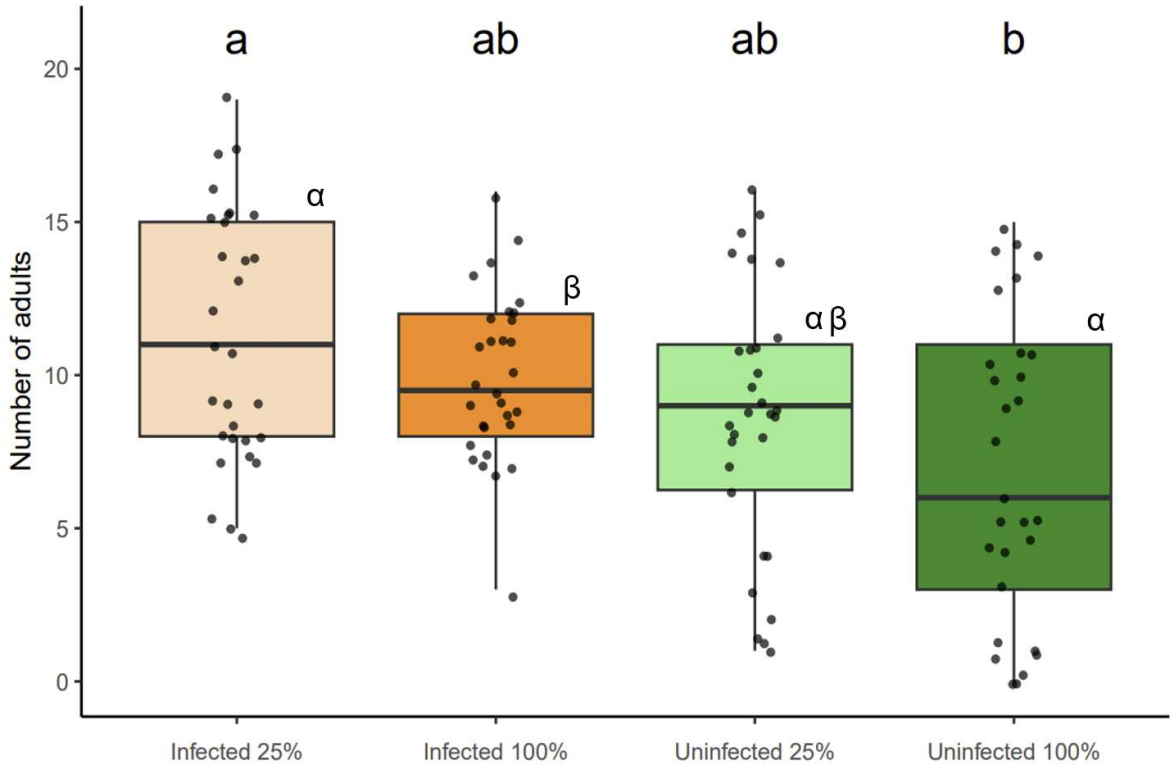

Figure 5. Number of adults obtained per tube, with 25 % and 100 % nutrient concentration, in infected and uninfected lineages. All tubes began with 20 larvae, and each point on the graph represents a tube (repetition). Roman letters indicate similar treatments inferred by the Dunn test ($p \geq 0.025$), while greek letters indicate treatments with similar variance, inferred by the Levene test ($p \geq 0.05$).

4 DISCUSSION

The objective of this study was to ascertain whether the fluctuations in the prevalence of *Wolbachia* infection observed in the population over time are due to fluctuations in nutrient availability and, consequently, whether *w*Stv Vi acts as a nutritional endosymbiont. Our results indicated that the infected lineage exhibited superior performance at both nutrient concentrations, suggesting that the different nutrient concentrations are equivalent for the survival of this lineage. This result could be interpreted as evidence that this strain of *Wolbachia* functions as a nutritional symbiont, providing nutrients to the host when they are scarce in the medium.

However, this conclusion would require corroboration from the uninfected lineage in 100 % medium, which should be equivalent to those found in the infected lineage and superior to the uninfected lineage in 25 % medium, which was not the case.

Our results align with the hypothesis that nutritional symbiosis influences the rate of larval development. As shown in Figure 2, the discrepancy in the development time of larvae grown in different nutrient concentrations was smaller in the infected lineage. The larvae of the infected lineage developed at a markedly faster rate than those of the uninfected lineage, regardless of the nutrient concentration. Contrary to our expectations, the larvae of the uninfected lineage that developed in the 25 % medium pupated significantly faster than those that developed in the 100 % medium. Our hypothesis predicted that, in the 100 % medium, the larvae would reach a minimum size first and then pupate first. Contrary, Rodrigues et al. (2015) proposed that low nutrient availability can lead to faster development, even if the larva has not reached a minimum size. In light of the potential for resource exhaustion, these authors posited that a more advantageous strategy would enable the entire life cycle to occur in a shorter time frame, thereby accelerating the generation of adults and conferring competitive advantages in the acquisition of resources (Krijger et al., 2001). Nevertheless, it is noteworthy that approximately 40 % of the uninfected larvae in the 100 % medium died before pupation. Therefore, these differences are likely more due to the higher mortality of larvae in the 100 % medium rather than a faster development of larvae in the 25 % medium.

The study of viability differences in culture medium with different nutrient concentrations was inconclusive. We expected that the difference in viability due to nutrient availability would be greater in the uninfected than in the infected lineage. However, we did not find differences in viability related to nutrient concentration in the infected or non-infected lineages. Nonetheless, the variance in viability (number of larvae that pupated per tube) was significantly higher in the uninfected lineage than in the infected one, especially when fed with 100 % medium. Such enhanced variance is likely due to the high mortality previously mentioned in this treatment, which was not homogeneous among the tubes.

It can be concluded from the results of this study that the experimental design developed was not appropriate to test the hypothesis of nutritional symbiosis. Indeed, to elucidate the results obtained from 100 % uninfected larvae, it is necessary to

consider the digestive process of drosophilids. In addition to internal digestion, drosophilid larvae engage in external digestion by secreting salivary enzymes that digest and break down macronutrients in the culture medium (Gregg et al., 1990). This process is dependent on group behavior (cluster or "social digestion" - Chen & Sokolowski, 2022; Dombrovski et al., 2017; Dombrovski et al., 2020; Gregg et al., 1990), which has an optimal larval density where cooperation is maximized (Allee Effect - Allee 1927; Liao et al., 2024) and liquefies the medium, which is shared between the larvae (Gregg et al., 1990). For instance, Liao et al. (2024) observed that 40 larvae in a single tube is the optimal density for utilizing the medium in *Drosophila melanogaster*.

Considering that both lineages were maintained for at least 15 generations before the experiments in a culture medium identical to the medium of 100 % of the experiment, we concluded that it was possible because of the high larval density within the tubes in each generation sufficient to digest the food and maintain the larvae. However, when planning the experimental procedure, we set up the tubes with only 20 larvae (adapted from Lindsey et al., 2023) to avoid the potential confounding effects of overcrowding, which has been demonstrated to interfere with the development of the larvae to the adult stage (Henry et al., 2020; Horvath & Halinka, 2016; Sokolowski, 1997). Consequently, to reduce the impact of potential biases associated with high larval density, we employed a reduced number of larvae. However, this approach may have introduced an opposite bias, as the lower density might have been insufficient for effective digestive processing of the medium, leading to increased larval mortality in the more nutritious medium.

But what could have caused the increased mortality in the richer medium? In the absence of appropriate modifications by the larvae, culture mediums can facilitate the proliferation of opportunistic bacteria (Henry et al., 2020; Ramírez-Camejo et al., 2017), which can result in the production of toxic metabolites that may alter the composition and function of the intestinal microbiota of larvae (Broderick & Lemaitre, 2012; Miguel-Aliaga et al., 2018; Wong et al., 2015), and increase larval mortality (Olcott et al., 2010). Indeed, it was observed that among the tubes containing uninfected larvae and 100 % medium, eight had at least three dead larvae at the outset of the observations, which may indicate that there was a major issue in some tubes, but not in all, thereby corroborating the hypothesis of infection by opportunistic

microorganisms in certain cases. It is important to note that our first run of analyses excluded the tubes in which we found more than three dead larvae (uninfected at 100 %) as we believed it was an isolated issue. In a second round of analyses, we included all the data without any exclusions and obtained the same result, leading us to conclude that this mortality was part of the overall results (results available in the script). In fact, the survival curves show that only 60 % of the uninfected larvae placed in 100 % culture medium pupated, meaning that 40 % of them still died as larvae.

Since this pattern of high mortality and decreased larval survival was not seen in infected larvae, *Wolbachia* infection could provide benefits in terms of more efficient digestion or mechanisms to mitigate toxicity. Indeed, *Wolbachia* infection has been demonstrated to mitigate the effects of nutrient-overloaded (Brownlie et al., 2009) and reduce the toxic effects of insecticides (Kline & Jhosi, 2024; Liu & Guo, 2019; Tang et al., 2021). However, infection with certain *Wolbachia* strains can make hosts more susceptible to insecticides (Evans et al., 2018; Liu & Guo 2019), so it is essential to evaluate the efficacy of inserting *Wolbachia* strains into insects to increase this resistance to pesticides.

Our pupal viability and number of adults results (Fig. 4 and 5) show similarity across treatments, including similarity in variance, reinforcing that the larval stage of holometabolous insects is most affected by variations in nutrient availability and possibly by food toxicity. Indeed, the larval stage is expected to be critical for nutrition, given that most of the feeding of holometabolous insects is necessary for survival, especially from the end of the larval stage to the beginning of the adult stage, when there is a feeding pause for developmental reorganization (Aguila et al., 2013; Chiang, 1963; Edgecomb et al., 1994; Klepsatel et al., 2020; Rehman & Varghese, 2021; Riddiford, 1993). Our results also show that the role of *Wolbachia* in viability, as measured in our experiment, is limited to promoting larval development, as *Wolbachia* infection did not increase pupal viability. Remarkably, pupal viability in our experiment was only 50 % in all treatments. However, we expected a higher percentage given that the lineages survived in the medium for 15 generations and we supposed that were fully adapted to the laboratory environment (*e.g.*, Lindsey et al., 2023). It is possible that the supposed toxicity had impacted the viability of pupa, but further experiments with different larval densities will be required to investigate pupal viability in these lineages.

Our results also raised questions about other metrics that were not evaluated here, leading us to investigate the adults generated from the experiments, which were preserved in alcohol. We are using geometric morphometrics tools applied to adult wings to test if the uninfected larvae that developed in 100 % medium produced larger adults compared to those that developed in 25 % medium, and whether this difference is greater than in infected larvae under the same conditions. This would prove that, although we could not support a nutritional symbiosis in this study, it could exist and impact adult size. In another approach, we aim to test whether the high mortality of larvae in the 100 % medium was due to toxins, which likely affect embryonic development. In this case, we expect the wing size asymmetry of adults from these tubes to be greater than that of adults whose larvae developed in the 25 % medium. If *Wolbachia* is responsible for mitigating the toxicity, we expect that the difference in asymmetry will be smaller in the infected lineage. Therefore, this paper reveals an important and understudied aspect of the *Wolbachia*-host relationship related to host adaptation to an environment of increasing toxicity.

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SUPPLEMENTARY MATERIAL

Table S1. Dunn's post-hoc analysis of larvae viability using 25 % and 100 % nutrient concentration with the infected and uninfected lineages (Fig. 3, Roman letters). Larvae viability was calculated as the ratio of pupae to larvae in each tube, with a range from 0 (indicating no pupation of larvae) to 1 (indicating 100 % pupation). All tubes were initiated with 20 larvae. Asterisks indicate that a statistically significant difference exists between the groups under comparison, with *p*-values less than 0.025 considered to be statistically significant.

	Infected 100 %	**Infected 25 %**	**Uninfected 100 %**
Infected 25 %	1,0000		
Uninfected 100 %	**< 0,001***	**< 0,001***	
Uninfected 25 %	**0,0167***	0,0969	0,2057

Table S2. Levene analysis of larvae viability using 25 % and 100 % nutrient concentration with the infected and uninfected lineages (Fig. 3, Greek letters). Larvae viability was calculated as the ratio of pupae to larvae in each tube, with a range from 0 (indicating no pupation of larvae) to 1 (indicating 100 % pupation). All tubes were initiated with 20 larvae. Asterisks indicate that a statistically significant difference exists between the groups under comparison, with *p*-values less than 0.05 considered to be statistically significant.

	Infected 100 %	**Infected 25 %**	**Uninfected 100 %**
Infected 25 %	1,0000		
Uninfected 100 %	**< 0,001***	**< 0,001***	
Uninfected 25 %	**< 0,001***	**< 0,001***	**0,014***

Table S3. Levene analysis of pupae viability using 25 % and 100 % nutrient concentration with the infected and uninfected lineages (Fig. 4, Greek letters). Pupae viability was calculated as the ratio of adults to pupae in each tube, with a range from 0 (indicating no emergence of adults) to 1 (indicating 100 % emergence). Asterisks indicate that a statistically significant difference exists between the groups under comparison, with *p*-values less than 0.05 considered to be statistically significant.

	Infected 100 %	**Infected 25 %**	**Uninfected 100 %**
Infected 25 %	**< 0,001***		
Uninfected 100 %	**< 0,001***	0,3270	
Uninfected 25 %	0,2043	0,2503	0,0836

Table S4. Dunn's post-hoc analysis of number of adults using 25 % and 100 % nutrient concentration with the infected and uninfected lineages (Fig. 5, Roman letters). Asterisks indicate that a statistically significant difference exists between the groups under comparison, with *p*-values less than 0.025 considered to be statistically significant.

	Infected 100 %	**Infected 25 %**	**Uninfected 100 %**
Infected 25 %	0,9157		
Uninfected 100 %	0,0957	**0,0047***	
Uninfected 25 %	1,0000	0,1518	0,6627

Table S5. Levene analysis of number of adults using 25 % and 100 % nutrient concentration with the infected and uninfected lineages (Fig. 5, Greek letters). Asterisks indicate that a statistically significant difference exists between the groups under comparison, with *p*-values less than 0.05 considered to be statistically significant.

	Infected 100 %	**Infected 25 %**	**Uninfected 100 %**
Infected 25 %	**< 0,001***		
Uninfected 100 %	**< 0,001***	0,1939	
Uninfected 25 %	0,0581	0,6627	0,1467

CONCLUSÃO

1) Os resultados do Capítulo 1 desta dissertação apontam para os perigos de assumir, em estudos que envolvem indivíduos de uma mesma linhagem, tradados e não tratados com antibióticos, que a titulação de *Wolbachia* seja o único fator a ser considerado ao comparar os fenótipos em estudo. Embora seja verdade que neste tipo de trabalho a titulação de *Wolbachia* seja reduzida a níveis não detectáveis por PCR, e que todos os cuidados sejam tomados no sentido de recompor a biota intestinal, o tratamento ainda pode eliminar outras bactérias e alterar as frequências relativas dos micro-organismos do intestino;

2) Ainda que a diferença observada entre indivíduos infectados e não infectados se deva à infecção por *Wolbachia*, a penetrância dos fenótipos pode não ser completa, o que significa que a mesma cepa pode induzir fenótipos diferentes em indivíduos diferentes, seja por questões genéticas (indivíduos com genótipos diferentes) ou ambientais (indivíduos que habitam diferentes ambientes);

3) No Capítulo 2, não foi possível testar o papel de *Wolbachia* como simbionte nutricional na população estudada de *Drosophila sturtevanti.* Em nossos experimentos, ao tentar evitar vieses causados por altas densidades larvais, controlamos a densidade larval, mas, conforme descobrimos depois de concluídos os experimentos, a densidade larval ficou aquém da necessária para o processamento (digestão externa) apropriado do meio. Com isto, acabamos encontrando evidências de que *Wolbachia* estabiliza o ambiente das larvas, possivelmente por meio de uma ação de mitigação de toxicidade, um fenótipo até então pouco conhecido, mas que pode ajudar a compreender o sucesso da bactéria entre os insetos;

4) Conforme recomendado no final do Capítulo 1, a possibilidade de que *Wolbachia* desempenhe um papel de mitigar a toxicidade do meio, levantada no Capítulo 2, precisa ser estudada envolvendo mais linhagens infectadas e não infectadas, para evitar conclusões sobre a indução de fenótipos a partir de apenas duas linhagens.