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Dinâmica do endossimbionte *Wolbachia* (Anaplasmataceae) nos níveis populacional e ontogenético de hospedeiros do subgrupo *willistoni* de *Drosophila* (Drosophilidae)

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Science is not only a disciple of reason but, also, one of romance and passion.

- Stephen Hawking

Sumário

Abreviaturas, símbolos e unidades.

bp	pares de base
COI	*Citocromo Oxidase Subunidade I*
D	teste de neutralidade de Tajima
DNA	ácido desoxirribonucleico
F	teste de neutralidade de Fu
Hd	diversidade haplotípica
kb	Quilobases
Kl-3	*Male fertility factor*
min	minuto (s)
mM	Milimolar
ng	nanograma
p	probabilidade
pb	pares de base
PCR	reação de polimerização em cadeia
s	Segundos
U	unidade
wsp	*Wolbachia surface protein*
µg	micrograma
µL	microlitro
π	diversidade nucleotídica

Resumo

Endossimbionte muito famosa e bem sucedida, a bactéria *Wolbachia pipentis* vive dentro de células de artrópodes e nematoides, e é conhecida por causar manipulações reprodutivas sobre seus hospedeiros, com o objetivo de aumentar sua própria frequência nas populações. Entretanto, apesar de ser conhecida classicamente como uma parasita reprodutiva, muitas evidências recentes demonstram que ela pode desenvolver relacionamentos mais amigáveis e estáveis com seus hospedeiros, mediante alguns acordos genéticos realizados com eles. Uma das características mais importantes de qualquer relação simbiótica é a densidade de infecção, já que a replicação do simbionte dentro do hospedeiro tem consequências diretas sobre, por exemplo, o nível de manipulação reprodutiva, o grau de virulência e as principais características da relação endossimbiótica. Dentre sua vasta gama de hospedeiros, *Wolbachia* desenvolve relações bastante interessantes com moscas do subgrupo *willistoni* de *Drosophila*. Foram registradas infecções em populações de *D. willistoni* com diferenças bem acentuadas no nível de infecção, com algumas linhagens apresentando titulação muito alta, enquanto outras possuem níveis apenas detectáveis por técnicas de hibridização. Tendo em vista a importância da densidade intracelular para o desfecho da relação com o hospedeiro, a manutenção de níveis tão desiguais de infecção em populações simpátricas se torna um problema biológico bastante interessante para ser investigado. Desta forma, o objetivo desta Dissertação de Mestrado foi averiguar mais aprofundadamente algumas características deste sistema simbiótico, para tentar entender como ele está sendo mantido durante o tempo evolutivo. Os principais achados deste trabalho são que, primeiramente, as infecções em alta titulação por *Wolbachia* são todas causadas pela linhagem *w*Wil, enquanto que as infecções em baixa titulação são mantidas também por esta linhagem em alguns casos, porém em sua maioria por uma outra linhagem próxima, *w*Au-like. Como baixas e altas infecções são mantidas segregando aproximadamente em uma proporção 1:1 em populações de moscas de *D. willistoni* desde a Amazônia até os Pampas, nós investigamos se existiria uma associação entre os padrões de densidade de infecção e haplótipos mitocondriais das moscas hospedeiras, o que poderia explicar o padrão observado pelo efeito de uma varredura seletiva conduzida por *Wolbachia*. Nossos dados contradisseram tal hipótese, porém demonstraram que o padrão de variação mitocondrial das isolinhagens avaliadas poderia ser explicada por uma expansão demográfica. Ainda, nós desenvolvemos uma série de ensaios de avaliação de caracteres adaptativos das moscas, objetivando verificar se algum dos padrões de infecção observados poderia causar algum aumento de valor adaptativo em seus hospedeiros, assim explicando sua manutenção na população. Nós verificamos que a baixa infecção pela linhagem *w*Au-like está associada a uma maior fecundidade das fêmeas hospedeiras, enquanto que moscas infectadas com altas e baixas infecções pela linhagem *w*Wil mostraram uma fecundidade menor. Unindo este dado com uma série de quantificações deste endossimbionte realizadas em estágios ontogenéticos de isolinhagens infectadas com diferentes modelos de infecção, nós propusemos que os dois tipos principais de infecção (*w*Wil em alta densidade e *w*Au-like em baixa densidade) estão sendo mantidos em populações através de um equilíbrio entre o a maior fecundidade das fêmeas infectadas por *w*Au-like a maior eficiência de transmissão materna da infecção por *w*Wil.

Abstract

A quite famous and successful endossimbiont, the bacterium *Wolbachia pipientis* lives inside the cells from its arthropod and nematode hosts, and it is known to cause reproductive manipulations upon them in order to increase its own frequency in host populations. However, even though it is classically known as a reproductive parasite, recent evidences have demonstrated that it may develop more friendly relationships with its hosts by genetic agreements established between them. One of the most important characteristics of any symbiotic relationship is infection density, since the within-host symbiont replication has direct consequences upon the level of reproductive manipulation, the virulence degree and the endosymbiotic relationship's main characteristics. Among its wide range of hosts, *Wolbachia* establishes quite interesting relationships with flies from the *willistoni* subgroup of *Drosophila*. It was recorded outstanding differences concerning the infection density among *D. willistoni* populations, with some isofemale lines presenting a very high density, while others are infected with *Wolbachia* in such low densities that are only rescued by hybridization techniques. Considering the importance of intracelular density to the symbiosis outcome, the maintenance of such unequal levels of infection in sympatric populations establishes an interesting biological issue that should be investigated. Therefore, this Masters Thesis aimed to more deeply ascertain some characteristics of such symbiotic system, in order to understand how it has been maintained throughout the evolutionary time. The main findings from this work are, first, that high-titer infections are all held by the *w*Wil strain, whereas low-titer infectins are, in a few cases, also maintained by this same strain, although the majority of them are maintained by a closely-related strain, *w*Au-like. Since low and high-titer infections are segregating in an approximately 1:1 ratio in *D. willistoni* populations from the Amazonian Rainforest until the Pampas, we investigated if there is an association between the infection densities and mitochondrial haplotypes from the host flies, which would explain the observed pattern by a *Wolbachia*-conducted selective sweep. Our data contradicted such hypothesis, although it demonstrated that the mitochondrial variation could be explained by a demographic expansion of the species. Furthermore, we developed a series of fitness experiments with the flies, aiming to verify if any of the *Wolbachia* infection patterns could be associated to an increased adaptative value from their hosts, which would in turn explain their maintenance in host populations. We verified that the *w*Au-like low-titer infection is associated with an increased fecundity in its female hosts, while flies infected with either low or high-titer *w*Wil showed a lower fecundity. Gathering this data with a series of qPCR *Wolbachia* quantifications among ontogenetic estages from flies of all infection types, we proposed that the two main infection types (high-titer *w*Wil and low-titer *w*Au-like) are being maintained in the host populations by an equilbrium between the higher fecundity by females infected with *w*Au-like and a higher efficiency of transmission by *w*Wil-infected flies.

CAPÍTULO 1: Introdução

A Simbiose e uma endossimbionte bem sucedida, *Wolbachia pipientis*

A simbiose é considerada um importante maquinista de novidades evolutivas e diversidade ecológica, e os simbiontes microbianos, em particular, tem sido grandes catalizadores evolutivos ao longo dos 4 bilhões de anos de vida na Terra, tendo auxiliado na conformação da evolução de organismos complexos. A endossimbiose é um tipo específico de simbiose em que um parceiro, geralmente microbiano, vive dentro de seu hospedeiro, representando assim uma forma muito íntima de interação entre dois organismos (Wernegreen, 2004). Os diversos tipos de simbioses entre microorganismos e eucariotos tem recebido atenção crescente no que se refere às características de suas interações, tais como a diversidade e a abundância de simbiontes, o tipo de vantagem ou prejuízo que os parceiros experenciam, como a interação começa e, mais recentemente, também quanto a quais genes são responsáveis pelo estabelecimento e manutenção da simbiose (Hentschel *et al*., 2000; Zilber-Rosenberg e Rosenberg, 2008).

Atualmente já é claro que a maioria das espécies de artrópodes carregam endossibiontes herdáveis, e que tais microorganismos são extremamente diversos (Duron e Hurst, 2013). Estes processos são particularmente frequentes nos insetos, os quais desenvolvem associações com diferentes linhagens procarióticas, assim como com microsporídios (protistas eucarióticos) (Werren e O'Neill apud Bandi *et al.*, 2001). Dentre estes microrganismos, há de se chamar atenção para bactérias que vivem exclusivamente dentro das células de seus hospedeiros, fazendo uso do citoplasma do ovócito materno para garantir sua transmissão para prole. Estes organismos tem chamado atenção de evolucionistas devido ao amplo espectro de estratégias evolutivas por eles empregadas, que variam desde o mutualismo obrigatório até o parasitismo reprodutivo (Ishikawa apud Wernegreen, 2004; Buchner apud Wernegreen, 2004).

De um lado do "continuum" de relações simbióticas, os endossimbiontes mutualistas auxiliam no desenvolvimento e sobrevivência de seus hospedeiros, geralmente a partir de parcerias estabelecidas há muito tempo na história evolutiva, e com características bastante especializadas. Permitindo que seus hospedeiros explorem fontes de alimento e habitats anteriormente inadequados, aumentando o seu valor adaptativo e protegendo-os contra inimigos naturais, a aquisição destes mutualistas pode ser vista como

uma inovação chave na evolução do hospedeiro (Moran e Telang, 1998; Herre *et al*., 1999; Dheilly, 2014). No outro extremo deste espectro se encontram os chamados parasitas reprodutivos, que se propagam através das linhagens de seus hospedeiros manipulando sua reprodução. Estes microrganismos herdados maternalmente infligem um arsenal impressionante de alterações em seus hospedeiros, permitindo o aumento da frequência de fêmeas infectadas na prole e, por consequência, garantindo sua própria propagação para a geração seguinte (Wernegreen, 2004).

O modo de transmissão dos endossimbiontes é considerado um determinante-chave na evolução da virulência (Ebert e Herre, 1996). Por exemplo, uma maior virulência é favorecida quando o modo de transmissão é horizontal, quando existe uma alta possibilidade de o hospedeiro estar infectado por múltiplas linhagens de parasitas, ou quando a virulência do parasita é associada com a fecundidade e chance de transmissão do mesmo. Por outro lado, no caso de simbiontes transmitidos verticalmente (da mãe para a prole), os sucessos reprodutivos do hospedeiro e parasita são mais intrincados, e a seleção favoreceria linhagens parasitárias mais benignas. De fato, a seleção poderia também promover uma contribuição positiva do agente herdado para o hospedeiro. Assim, em adição à possibilidade de se tornarem benignos para os hospedeiros responsáveis por sua transmissão (fêmeas infectadas), a seleção sobre microrganismos herdados pode favorecer a disseminação de fenótipos que são prejudiciais aos hospedeiros não envolvidos em sua transmissão (machos ou fêmeas não infectadas) (Werren, 1997; Stouthamer *et al.*, 1999; Werren e O'Neill apud Bandi *et al.*, 2001). Tais manipulações frequentemente envolvem a determinação de uma razão sexual distorcida em favor das fêmeas, já que, para microrganismos herdados maternalmente, os machos significam becos sem saída.

Ainda, apesar de a herança materna ser o modo padrão de transmissão dos parasitas reprodutivos, eles também podem se utilizar da transmissão horizontal, permitindo a infecção de novas populações (Noda *et al.*, 2001), como no caso de microsporídios indutores de androcídio em mosquitos e da linhagem de *Wolbachia* indutora de partenogênese em vespas parasitoides (Huigens *et al.*, 2000). A inserção destes parasitas nos processos populacionais podem ter efeitos muito fortes sobre a evolução dos hospedeiros através de alterações diretas dos padrões reprodutivos que, por sua vez, podem ser agentes causadores de eventos de especiação nos hospedeiros (Bordenstein *et al.*, 2001).

Nesse contexto, um importante e reconhecido parasita reprodutivo é a bactéria do gênero *Wolbachia*, que determina diferentes tipos de manipulações reprodutivas em artrópodes (indução de partenogênese, feminização, morte de embriões machos, incompatibilidade citoplasmática), mas que também é considerada benéfica para os nematoides filariais (Werren, 1997; Stouthamer *et al*., 1999).

Wolbachia é gram-negativa e vive dentro de vacúolos nas células de espécies hospedeiras, principalmente em tecidos reprodutivos, porém também encontrada nos somáticos (Dobson *et al.*, 1999). Ela foi descoberta primeiramente nos ovários de mosquitos da espécie *Culex pipiens*, sendo então denominada *Wolbachia pipientis* (Hertig, 1936). Mais tarde, entretanto, foi verificado que ela é na verdade uma infecção extremamente disseminada entre os artrópodes e nematoides (Werren, 1997). Uma análise recente estimou, usando inferências estatísticas, que cerca de 40% de todos os artrópodes terrestres devem estar infectados por *Wolbachia* (Zug e Hammerstein, 2012).

Até o momento, *Wolbachia pipentis* é a única espécie reconhecida do gênero *Wolbachia*, posicionada taxonomicamente junto à família Anaplasmatacea, ordem Rickettsiales, da classe das alfa-proteobactérias (Lo *et al.*, 2007). Apesar de ser considerado um gênero monoespecífico, o mesmo apresenta uma alta diversidade molecular entre as estirpes e tem sido classificado em, até o momento, 14 supergrupos ou clados (chamados de "A" a "O"). A atribuição dessas linhagens a estes supergrupos tem sido alvo de extensos debates (Werren *et al*., 1995a; Bandi *et al*., 1998; Gorham *et al.*, 2003; Casiraghi *et al.*, 2004; Czarnetzki *et al.*, 2004; Rowley *et al*., 2004; Bordenstein *et al.*, 2005; Casiraghi *et al*., 2005; Dunn e Stabb, 2005; Sakamoto *et al.*, 2006; Baldo *et al.*, 2007; Covacin e Barker, 2007; Panaram e Marshall, 2007; Vaishampayan *et al.*, 2007; Haegeman *et al.*, 2009; Ros *et al.*, 2009; Augustinos *et al.*, 2011; Glowska *et al.*, 2015).

Da mesma forma que para outros parasitas reprodutivos, *Wolbachia* é normalmente herdada maternalmente, todavia, a disseminação tão marcada desta bactéria na biodiversidade de artrópodes e nematoides é também explicada pela ocorrência de eventos de transmissão horizontal entre hospedeiros. Este movimento lateral da bactéria tem sido bem documentado em laboratório e inferido nas reconstruções filogenéticas de diferentes genes de *Wolbachia* (Werren *et al*, 1995a). Por exemplo, Panaram e Marshall (2007), encontraram a linhagem do supergrupo F de *Wolbachia* (característica de nematoides filarias) em insetos ortópteros. Ainda, a similaridade entre as sequências do gene *ftsZ* de

Wolbachia encontrada em diferentes hospedeiros das ordens Coleoptera, Diptera, Hymenoptera e Lepidoptera, demonstra ter havido transmissão horizontal de *Wolbachia*, já que as sequências deste gene teriam divergido há aproximadamente 1,6 milhões de anos (Werren *et al.*, 1995a), enquanto as espécies hospedeiras há aproximadamente 200 milhões de anos. O mecanismo de transmissão entre táxons até o momento é desconhecido, embora haja evidências indiretas da transferência entre parasitoides e seus hospedeiros insetos (Werren *et al.*, 1995b; Huigens *et al.*, 2004), e de eventos de transmissão através de canibalismo e predação, por exemplo (Le Clec'h *et al*., 2012).

Os fenótipos induzíveis por *Wolbachia*

Em seus hospedeiros nematoides filiariais, *Wolbachia* não manipula a reprodução de forma óbvia, porém, experimentos baseados em tratamento com antibióticos demonstram a importância da bactéria na embriogênese e outros estágios do desenvolvimento destes nematoides (Bandi *et al.*, 1999). Desta forma, neste caso *Wolbachia* seria mais bem caracterizada como um "parceiro essencial" do hospedeiro (Stevens *et al.*, 2001). Este papel é enfatizado pela congruência das filogenias de *Wolbachia* e dos nematoides por mais de 100 milhões de anos, o que é típico de interações em forma de parceria (Charlat *et al.*, 2003).

Além deste papel mutualista com os nematoides, a infecção por *Wolbachia* pode induzir em seus hospedeiros diversos efeitos clássicos de parasitismo reprodutivo que geram resultados diretos sobre a razão sexual das populações, como a indução de partenogênese, a feminização e o androcídio (*male killing*). Além disso, *Wolbachia* também pode causar a incompatibilidade citoplasmática que, apesar de não causar distorção na razão sexual, tem efeitos profundos na evolução dos hospedeiros (Werren *et al.*, 2008).

Na indução à partenogênese, que ocorre em espécies haplodiplóides, *Wolbachia* torna machos em fêmeas. Nestes organismos, os machos normalmente se desenvolvem a partir de ovos haploides não fertilizados (partenogênese arrenótoca), enquanto as fêmeas se desenvolvem de ovos diploides fertilizados. A bactéria desencadeia a duplicação do número cromossômico dos ovos haploides não fertilizados, tornando-os diploides. Isto então leva à formação de fêmeas produzidas assexuadamente (Stouthamer apud Charlat *et al*., 2003). Apesar de o mecanismo de atuação não estar claro, sabe-se da interrupção do ciclo celular durante o desenvolvimento embrionário precoce, resultando em ovos

diploides não fertilizados. Linhagens de *Wolbachia* que induzem partenogênese frequentemente estão fixadas dentro da população, convertendo espécies hospedeiras sexuais em assexuais. Em algumas destas espécies, a capacidade de reproduzir-se sexualmente se perdeu completamente ao longo do tempo. Apesar do tratamento com antibióticos poder restaurar a produção de machos na progênie, estes machos se mostram não funcionais. Em outras espécies, a infecção permanece em um equilíbrio polimórfico, com indivíduos infectados e não infectados. Tal polimorfismo se acredita ser promovido por fatores do hospedeiro que resistem à ação ou transmissão da bactéria (Stouthamer apud Bandi *et al.*, 2001).

Na feminização em diplóides, machos genéticos são transformados em fêmeas fenotípicas e funcionais por ação hormonal (Kageyama *et al.*, 1998). Algumas linhagens de *Wolbachia* podem causar a feminização através de ação sobre a glândula androgênica ou sobre a recepção do hormônio androgênico, enquanto que outras apenas rompem o desenvolvimento da glândula, resultando em eficiência imperfeita de feminização, com alguns machos com *Wolbachia* (Marcadé *et al.*, 1999; Rigaud *et al.*, 1999). Em termos populacionais este fenótipo tem grande importância, visto que fêmeas infectadas passam a produzir o dobro de fêmeas em relação às não infectadas, aumentando então tanto a frequência de fêmeas infectadas quando a do próprio endossimbionte (Charlat *et al.*, 2003). No exemplo clássico de feminização em isópodos terrestres, *Wolbachia* é responsável por reversão de sexo. Cerca de metade das espécies de isópodos de diferentes famílias é infectada, cada espécie carregando uma linhagem única de *Wolbachia* (Bouchon *et al*., 1998).

No fenótipo de androcídio, a bactéria consegue detectar o sexo do hospedeiro e assim matar os machos, ou então interfere diretamente com a determinação do sexo, de forma a causar a morte específica de machos. Como a morte ocorre durante a embriogênese, o endossimbionte provavelmente interage com componentes acima das vias de determinação de sexo (Charlat *et al.*, 2003). Assim, observa-se uma interferência direta na determinação do sexo, que por fim ocasiona desvios nas proporções sexuais da prole, em favor das fêmeas. As espécies hospedeiras geralmente colocam muitos ovos de uma única vez, e a prevalência é maior em espécies onde os ovos não eclodidos são consumidos pelos irmãos logo após a eclosão destes, ou onde existe competição entre os irmãos eclodidos por recurso limitado de alimento (Hurst apud Bandi *et al*., 2001). Assim, a morte

dos machos acaba por aumentar a sobrevivência das fêmeas irmãs, que possuem a bactéria manipuladora por descendência.

Na incompatibilidade citoplasmática (IC), certos cruzamentos entre machos e fêmeas apresentando diferentes estados de infecção por *Wolbachia* geram prole inviável. *Wolbachia* foi documentada como causadora de IC pela primeira vez por Yen e Barr (1976), e desde então este fenótipo foi descrito em diversas ordens de insetos, porém o fenômeno foi e é mais amplamente estudado em *Drosophila simulans* (Hoffmann *et al*., 1986; Merçot *et al*., 1995; Hoffmann *et al*., 1996; Clark *et al*, 2002). Especificamente, na chamada IC unidirecional a prole é inviável em cruzamentos em que apenas o macho esteja infectado, já que tal cruzamento não seria ótimo para a transmissão de *Wolbachia* a sua prole, que se dá apenas pela via materna. Entretanto, o cruzamento no sentido contrário, ou seja, de uma fêmea infectada com um macho não infectado, gera prole viável (Serbus *et al.*, 2008; Werren *et al.*, 2008). Já na IC bidirecional, cruzamentos em que macho e fêmea estejam infectados com linhagens incompatíveis de *Wolbachia* podem levar à prole inviável (Werren, 1997).

A morte determinada por IC ocorre no início do desenvolvimento, e parece estar associada com alterações na condensação e descondensação apropriada da cromatina paterna (Werren, 1997; Stouthamer *et al.*, 1999; Merçot e Poinsot, 2009). Assim, quando o espermatozoide adentra o óvulo, a IC é expressa pela incapacidade dos cromossomos paternos de se condensarem, enquanto os cromossosmos maternos adentram a mitose normalmente, levando então a condições haploides ou aneuploides. Ainda, seguindo o modelo Modificação/Recuperação (*mod/resc*) proposto por Poinsot *et al*. (2003) para linhagens compatíveis do simbionte, acredita-se que *Wolbachia* provoque tais modificações nos espermatozoides, porém é permitido o desenvolvimento normal do embrião se a fêmea, também infectada, resgatar as modificações provocadas nesses cromossomos paternos para completar a cariogamia (Lassy e Karr, 1996; Callaini *et al.*, 1997). Fêmeas livres da bactéria não recuperam as modificações provocadas nos espermatozoides e, por consequência, sua descendência é bloqueada (McGraw *et al.*, 2002).

Este fenótipo é induzido em diversos artrópodes, particularmente insetos, porém também em ácaros e crustáceos isópodes (Werren, 1997; Stouthamer *et al.*, 1999). Os custos impostos pela IC não são fáceis de predizer. Para as fêmeas, ter *Wolbachia* se torna

vantajoso, visto que a infecção protege os ovos da mortalidade causada por IC. Para os machos, por outro lado, a infecção é deletéria, visto que diminui a fertilidade em cruzamentos com fêmeas não infectadas. Assim, a direção da seleção depende da prevalência da infecção: quando a prevalência de *Wolbachia* é baixa, o custo sofrido por machos infectados é muito maior que o benefício obtido por fêmeas infectadas; entretanto, quando a prevalência é alta, os custos sofridos pelos machos infectados serão muito menores que os benefícios obtidos pelas fêmeas infectadas. De uma forma geral, os custos e benefícios de possuir *Wolbachia* entram em equilíbrio quando a frequência de indivíduos infectados e não infectados é aproximadamente a mesma, porém este é um estágio apenas transitório (Charlat *et al.*, 2003). De qualquer forma, esta estratégia de manipulação confere uma vantagem reprodutiva à infecção, consequentemente possibilitando a disseminação de *Wolbachia* em populações naturais até uma alta prevalência (Duron, 2008). Desta maneira, este fenótipo está envolvido em implicações evolutivas importantes, principalmente no processo de especiação (Telschow *et al.*, 2005; Werren, 2008), devido à redução do fluxo gênico entre as populações, acentuando assim o isolamento reprodutivo.

De acordo com Miller *et al.* (2010), *Wolbachia* é considerada o agente de especiação entre as seis semiespécies (Orinocana, Andino-Brasileira, Interior, Amazônica, Centro Americana e Transicional) de *Drosophila paulistorum*, na qual cada uma seria portadora de uma linhagem específica de *Wolbachia*. As semiespécies são consideradas espécies *in statu nascendi*, em conjunto formando a superespécie *D. paulistorum* (Dobzhansky e Pavan) (Dobzhansky e Spassky, 1959). Nos híbridos formados pelo cruzamento entre as semiespécies, a bactéria apresenta comportamento patogênico, provocando a esterilidade do macho (100%) ou morte do embrião (> 90%), sendo as fêmeas todas férteis. Além de barreira pós-zigótica, observa-se um forte isolamento pré-zigótico em relação à preferência de parceiros. No entanto, após as semiespécies serem tratadas com antibiótico para diminuição da densidade de *Wolbachia*, o cruzamento entre elas é possível e os híbridos são viáveis, indicando o envolvimento de *Wolbachia* no reconhecimento e preferência de parceiros nestas espécies (Miller *et al.*, 2010).

É interessante também salientar que a indução de IC por *Wolbachia* tem sido utilizada em esforços na proposição de estratégias para diminuir a competência de vetores de doenças (Bourtzis, 2008; Hoffmann *et al.*, 2011), devido à impressionante capacidade

deste fenótipo de efetivamente dirigir *Wolbachia* pela população hospedeira (Turelli e Hoffmann, 1991).

Acreditava-se que a IC era um fenótipo induzido exclusivamente por *Wolbachia*. Entretanto, verificou-se que este não é o caso, com a identificação da bactéria *Cardinium* (Bacteroidetes) como outro agente causador, estabelecendo um modelo interessante para estudos comparativos (Hunter *et al*., 2003).

Especificamente em *Drosophila*, nosso organismo modelo neste trabalho, a interação entre *Wolbachia* e seu hospedeiro pode provocar tanto alterações na proporção sexual devido à morte dos machos, como o efeito da incompatibilidade citoplasmática (Werren, 1997).

Wolbachia, seus hospedeiros e uma imensidão de interações

Hospedeiros e seus parasitas estão continuamente envolvidos em uma corrida armamentista de adaptação e contra adaptação (Dawkins e Krebs, 1979), e esta corrida recíproca requer variação genética para traços envolvidos no desfecho final da interação (Thompson apud Rouchet e Vorburger, 2012). As relações estabelecidas por *Wolbachia* com seus hospedeiros podem gerar diferentes "acordos" entre as partes, que são refletidos em interações mais amigáveis ou não. Dentro disso, custos de *fitness* induzidos por *Wolbachia* em *Drosophila* têm sido revelados por medidas de traços clássicos de história de vida, como redução da fecundidade (Hoffmann *et al.*, 1990), sobrevivência do ovo ao adulto (Clancy e Hoffmann, 1997), tempo de desenvolvimento (Reynolds *et al.*, 2003) e expectativa de vida (Min e Benzer, 1997; McGraw *et al.*, 2002). A magnitude de tais efeitos parece depender do genótipo do hospedeiro e da bactéria, e se as medidas são realizadas a campo ou no laboratório (Olsen *et al.*, 2001; Reynolds *et al.*, 2003).

Entretanto, o efeito de uma infecção endossimbiótica em um hospedeiro é mais complexo do que previamente se considerava. Apesar de muitas vezes as relações terem características de parasitismo, mais comumente do que se imaginava os simbiontes podem aumentar o fitness do hospedeiro ou ter impactos múltiplos sobre eles. *Wolbachia* não é uma exceção e há diversos casos reportados de relações com artrópodes em que ela se comporta como um mutualista condicional, podendo conferir vantagens sob certas condições ambientais. Alguns estudos demonstraram situações em que a bactéria auxilia no fornecimento de ATP ao hospedeiro, aumenta a longevidade e fecundidade (Fry *et al.*, 2004; Weeks *et al.*, 2007; Darby *et al.*, 2012) e protege contra patógenos e parasitas

(Teixeira *et al.*, 2008; Brownlie e Johnson, 2009; Osborne *et al.*, 2009; Duron e Hurst, 2013), por exemplo.

Assim, é possível verificar uma grande variedade de fenótipos induzíveis e diferentes desfechos das interações estabelecidas por *Wolbachia* e seus hospedeiros, o que implica a existência de muitos processos subjacentes responsáveis pela manutenção destes padrões. Tais modelos fornecem um campo rico de estudo para explorar conflitos genéticos (Frank, 1996a, 1996b), os quais podem envolver, por exemplo, o modo de transmissão do simbionte, a densidade intracelular e o sexo da prole do hospedeiro. Desta forma, as características das associações inseto-bactéria podem ser entendidas como diferentes desfechos na negociação destes conflitos genéticos, principalmente se levarmos em conta o fato de que hospedeiros e simbiontes frequentemente possuem diferentes interesses evolutivos (Wernegreen, 2004).

Dentro do vasto campo de estudo acerca das interações entre *Wolbachia* e seus hospedeiros, tem havido um interesse substancial em buscar esclarecer os detalhes que determinam os processos verificados na natureza, incluindo, por exemplo, a dinâmica populacional da infecção, os mecanismos de manutenção intracelular de *Wolbachia* nas células e como ela manipula a reprodução dos seus hospedeiros (Masui *et al.*, 2000; Mouton *et al.*, 2004; Clark *et al.*, 2006).

Alguns pesquisadores buscam entender os mecanismos empregados por *Wolbachia* para assegurar sua transmissão vertical à prole, visto que este é seu modo padrão de disseminação e, portanto, essencial a quaisquer discussões adicionais (revisado por Serbus *et al.*, 2008). Dentro disso, uma estratégia de transmissão eficiente seria a interação com fatores-chave do desenvolvimento do hospedeiro, e *Wolbachia* usa uma estratégia conceitual semelhante, associando-se a determinantes da linhagem germinativa do hospedeiro que promovem a sua inclusão na linhagem materna de células germinativas (Serbus e Sullivan, 2007). Nesse contexto, apesar de os mecanismos moleculares ainda serem pouco compreendidos, há um consenso acerca da concentração de *Wolbachia* na região posterior dos oócitos, o que promove a supracitada incorporação nas células germinativas posteriores durante a oogênese. Kose e Karr (1995) propuseram a interação entre *Wolbachia* e os microtúbulos das células hospedeiras, a qual explicaria a sua distribuição equitativa em direção aos polos do fuso durante a mitose e a localização desses

endossimbiontes no córtex do embrião, na região organizada pelos microtúbulos durante o desenvolvimento, o que foi também verificado por análises citológicas (Tram *et al.*, 2003).

Já no caso do comportamento de *Wolbachia* na espermatogênese dos machos, estudos com *Drosophila* tem tido bastante foco ultimamente. *Wolbachia* é encontrada nos testículos de *Drosophila*, porém se encontra praticamente ausente no esperma maduro, devido à sua remoção dos cistos em desenvolvimento, juntamente com o citoplasma e a maioria das outras organelas. A variação na densidade de diferentes linhagens de *Wolbachia* dentro dos cistos é correlacionada com a variação nos níveis de incompatibilidade citoplasmática (IC), sugerindo que a abundância de *Wolbachia* nos testículos é necessária (apesar de não suficiente) para induzir IC (Clark *et al*., 2003; Veneti *et al.*, 2003). Além disso, a densidade de *Wolbachia* nos testículos diminui com o envelhecimento em *Drosophila*, o que se correlaciona com a força diminuída da IC em machos mais velhos (Clark *et al.*, 2003). A partir destas observações, se formulou a hipótese do espermatócito/ espermátide infectados por *Wolbachia* (WISSH, do inglês "*Wolbachia*-infected spermatocyte/ spermatid hypothesis"), que defende que cistos infectados representam a base celular para a IC (Clark *et al.*, 2003; Serbus *et al.*, 2008). Entretanto, o escopo desta hipótese parece se restringir ao modelo *Drosophila*, não podendo ser generalizado para todas as interações entre *Wolbachia* e seus diferentes hospedeiros (Duron, 2008).

Quanto à convivência de *Wolbachia* com seus hospedeiros, experimentos de manipulação demonstraram que a severidade dos efeitos induzidos por *Wolbachia* é determinada por uma combinação do genótipo do hospedeiro, a linhagem de *Wolbachia*, a sua localização tecidual e a interação com o ambiente (Sakaguchi e Poulson, 1963; Boyle *et al.*, 1993; Poinsot *et al.*, 1998; McGraw *et al.*, 2001; Riegler *et al.*, 2004; Tinsley e Majerus, 2007). Um caso bem documentado acerca da influência do genótipo do hospedeiro é o da linhagem de *Wolbachia* que infecta *D. melanogaster*, *w*Mel, que induz IC muito fraca em seu hospedeiro. Entretanto, quando esta linhagem é transfectada para a espécie irmã, *D. simulans*, *w*Mel induz forte incompatibilidade citoplasmática (Poinsot *et al.*, 1998; Merçot e Charlat, 2004). Por outro lado, a linhagem que ocorre naturalmente em *D. simulans*, *w*Ri, que induz forte IC em *D. simulans*, quando transfectada em *D. melanogaster* causa baixa IC (Boyle *et al.*, 1993).

A garantia de transmissão do endossimbionte para a prole depende da manutenção de um nível apropriado do simbionte no hospedeiro. Baixas densidades podem resultar em falha da transmissão, porém uma densidade excessiva pode causar a morte do hospedeiro (Serbus *et al.*, 2011). A densidade intracelular é também muito importante na determinação do desfecho da interação endossimbiótica (Mouton *et al.*, 2003). Por exemplo, Kondo *et al.* (2005) verificaram os efeitos de regulação da densidade de *Wolbachia* em besouros da espécie *Callosobruchus chinensis*, e verificaram que os genótipos dos hospedeiros influenciavam a densidade de *Wolbachia* de maneiras diversas, e que a coinfecção por diferentes linhagens de *Wolbachia* causava a supressão da densidade de infecção como um todo. Por outro lado, Mouton *et al.* (2004), investigando o sistema de simbiose entre a vespa *Asobara tabida* e suas três linhagens de *Wolbachia* naturalmente infectantes (duas facultativas e que induzem IC, e uma que é necessária à oogênese do hospedeiro), verificaram justamente o contrário: os custos fisiológicos do hospedeiro aumentaram com o número de linhagens coinfectadas, correspondendo ao aumento na densidade bacteriana total.

Assim, quanto ao controle da densidade de *Wolbachia* nos tecidos de seus hospedeiros, Mouton *et al.* (2003) defenderam que deve haver um mecanismo no qual as células liberem fatores que possam regular a densidade da bactéria, minimizando os efeitos das alterações reprodutivas observadas. Isso significa que o sucesso reprodutivo de hospedeiros infectados resultaria de uma forte seleção natural controlando a multiplicação de *Wolbachia* nos tecidos (McGraw *et al.*, 2002). Este conceito também pode ser entendido a partir de alguns recentes estudos que verificaram uma densidade muito aumentada (superreplicação) de *Wolbachia* nos tecidos germinativos de híbridos formados a partir de cruzamentos entre as semiespécies do complexo da superespécie *Drosophila paulistorum* (Miller *et al.*, 2010) e entre espécies do gênero *Glossina* (Schneider *et al.*, 2013). Com base nisso, foi especulado que o equilíbrio entre hospedeiro e simbionte é perdido nos híbridos, onde os *backgrounds* genéticos misturados dos hospedeiros podem transformar *Wolbachia* em um verdadeiro patógeno devido à falta de controle na replicação.

Apesar de ser claro que fatores do hospedeiro tem uma forte influência sobre o título de *Wolbachia* (Boyle *et al.*, 1993; Poinsot *et al.*, 1998; McGraw *et al.*, 2002; Veneti *et al.*, 2004; Kondo *et al.*, 2005; Serbus *et al.*, 2008), pouco se sabe acerca da identidade e função destes fatores. Serbus *et al.* (2011) indicaram que *gurken* (*grk*), um gene de

Drosophila que codifica um determinante de eixo crucial, tem um impacto controlador, cumulativo e dosagem-sensitivo no crescimento e proliferação de *Wolbachia* durante a oogênese de *Drosophila*, através de seu mRNP (complexo do mRNA de *grk* com as proteínas Squid e Hrp48/Hrb27C). Os achados do grupo sugerem um "feedback loop" no qual a interação de *Wolbachia* com o mRNP de *grk* afeta tanto o título de *Wolbachia* quanto a função do mRNP *grk*. Os resultados dos autores sugerem que *Wolbachia* alcança um balanço no qual seu título é maximizado, porém sem romper o desenvolvimento do oócito, já que situações de titulação anormalmente alta produzem defeitos na formação de apêndices dorsais. Talvez a interação entre *Wolbachia* e *grk* represente um passo em direção à evolução da simbiose em que *Wolbachia* se torne integral à regulação da morfogênese do hospedeiro, aproximando-se de um modelo mutualista, semelhante ao observado nos nematoides filariais.

Nosso modelo de trabalho: moscas do subgrupo *willistoni* de *Drosophila*

A família Drosophilidae tem sua origem datada há cerca de 50 milhões de anos nas regiões tropicais e, atualmente, possui representantes em praticamente todas as partes do mundo (Throckmorton, 1975). É composta por moscas de pequeno porte, sendo que muitas espécies vivem associadas ao homem e definidas como espécies domésticas (Dobzhansky, 1965). Estas moscas são conhecidas ecologicamente como consumidoras primárias de microrganismos, em especial de leveduras associadas a frutos em estágio inicial de decomposição (Carson, 1971).

A família Drosophilidae está dividida em duas subfamílias: Drosophilinae e Steganinae, as quais são representadas por 73 gêneros e 3.938 espécies (Bächli *et al.*, 2000). A subfamília Drosophilinae atualmente é composta por 35 gêneros, dentre os quais se destaca o gênero *Drosophila*. Este é composto por 15 subgêneros e mais de 1.400 espécies, o que corresponde aproximadamente à metade das espécies da família, e o mais amplo espectro de distribuição (Wheeler, 1986). Dentre estes 15 subgêneros, destacam-se os subgêneros *Sophophora* e *Drosophila*. O subgênero *Sophophora* compreende 233 espécies subdivididas em 7 grupos, dentre os quais os grupos *melanogaster, obscura, saltans* e *willistoni*, estes dois últimos representando a radiação do subgênero ocorrida no Novo Mundo, e o último sendo um dos principais grupos alvo de trabalho de nosso grupo de pesquisa nos últimos anos.

O grupo *willistoni* de *Drosophila* é constituído por seis espécies crípticas e por dezenove não crípticas, sendo o grupo de espécies mais bem representado nas comunidades neotropicais do gênero (Val *et al.*, 1981). As espécies crípticas, que compõe o subgrupo *willistoni*, são: *Drosophila willistoni* Sturtevant, *Drosophila equinoxialis* Dobzhansky, *Drosophila paulistorum* Dobzhansky e Pavan, *Drosophila tropicalis* Burla e Da Cunha, *Drosophila insularis* Dobzhansky *e Drosophila pavlovskiana* Kastritsis e Dobzhansky (esta última não mais registrada há muitos anos, possivelmente tendo sido primeiramente identificada erroneamente). A este grupo de espécies é atribuída origem no Brasil central (Da Cunha *et al.*, 1950), em associação a florestas quentes e úmidas.

Das espécies crípticas, *D. willistoni* figura como a mais estudada e conhecida, inclusive por sua característica peculiar, dentro do grupo, de possuir uma grande versatilidade ecológica, haja vista sua capacidade de explorar diversos tipos de ambientes, como matas, formações abertas (Da Cunha *et al*, 1950, 1959; Da Cunha e Dobzhansky, 1954), cidades (Santos e Valente, 1990; Valiati e Valente, 1997; Goñi *et al.*, 1997, 1998), assim como diferentes tipos de substratos (Valente e Araújo, 1986). Sua distribuição geográfica é também a mais extensa do grupo, e se estende desde o sul dos Estados Unidos (Flórida) e México na América do Norte, até o norte da Argentina (Spassky *et al.*, 1971; Ehrman e Powell, 1981).

Drosophila tropicalis apresenta distribuição desde o centro do México, passando pela América Central e alcançando muito da América do Sul, até o estado de São Paulo. *D. equinoxialis* tem distribuição coextensiva ao norte com a distribuição de *D. tropicalis*, porém não se estendendo tão ao sul (as amostras mais ao sul de *D. equinoxialis* são do Peru central). *D. paulistorum* é considerada uma superespécie, a qual consiste de pelo menos seis semiespécies (espécies incipientes, conhecidas como: Amazônica, Orinocana, Andino-Brasileira, Centro-americana, Interior e Transicional). A distribuição geográfica da superespécie se estende da Guatemala, pela América Central, até a América do Sul, mais especificamente o sul do Brasil. O isolamento reprodutivo entre as semiespécies de *D. paulistorum* é quase completa, sendo os híbridos obtidos em laboratório estéreis quando machos, porém férteis quando fêmeas. O isolamento etológico entre elas é suficiente para permitir que duas ou até mesmo três delas coexistam em simpatria em muitas localidades (Ayala *et al.*, 1974).

As espécies crípticas do subgrupo da *D. willistoni* são praticamente indistinguíveis morfologicamente, porém isoladas reprodutivamente. Spassky (1957) verificou diferenças leves, porém confiáveis, na genitália masculina dos machos das espécies (forma do hipândrio), possibilitando assim sua identificação morfológica. Além dos caracteres morfológicos, as espécies deste subgrupo exibem também diferenças no padrão de bandeamento de seus cromossomos politênicos (Dobzhansky *et al.*, 1950; Rohde *et al.*, 2006), no som de corte produzido pelos machos durante a corte sexual (Ritchie e Gleason, 1995) e ao nível molecular, com variação em aloenzimas (Ayala *et al.*, 1970; Ayala e Powell, 1972; Garcia *et al.*, 2006) e em sequências de DNA (Gleason *et al.*, 1998; Robe *et al.*, 2010).

O problema biológico em tela e a justificativa deste trabalho

A simbiose entre *Wolbachia* e *Drosophila* provê um sistema onde a filogenia das espécies hospedeiras é bem definida, porém os hospedeiros exibem variações no grau de fenótipos parasíticos e mutualistas. Como as variações nestes fenótipos também está presente dentro das espécies, este sistema oferece um excelente material para o estudo dos processos estabelecidos para manter as interações, e para investigar possíveis transições entre modos de vida parasíticos e mutualistas de simbiontes (Fry *et al.*, 2004).

Especificamente no sistema de interação de *Wolbachia* com moscas do subgrupo *willistoni* de *Drosophila*, que representam uma biodiversidade nativa do Neotrópico, Miller e Riegler (2006) realizaram um *screening* em estoques de laboratório de espécies deste subgrupo e do subgrupo *saltans*, não encontrando *Wolbachia* em linhagens estabelecidas antes da década de 70. Já Mateos *et al.* (2006) registraram novas linhagens de *Wolbachia* e *Spiroplasma* em espécies de *Drosophila*, e reafirmam serem estes os únicos endossimbiontes amplamente distribuídos nas moscas deste gênero.

Entretanto, recentemente nosso grupo de pesquisa demonstrou a descontinuidade da densidade de infecção por *Wolbachia* em populações de *Drosophila* do subgrupo *willistoni* coletadas a partir de diferentes regiões do Brasil, desde os Pampas, passando pela Mata Atlântica, até a Floresta Amazônica. Verificou-se que existem diferenças marcadas quanto à densidade de infecção, com populações apresentando alta densidade do endossimbionte (por uma mesma linhagem de *Wolbachia*, *w*Wil), enquanto outras, muitoas vezes simpátricas às anteriores, apresentam níveis de *Wolbachia* apenas detectáveis via qPCR,

escapando ao limite de detecção da PCR convencional, e não sendo passíveis de identificação (Müller *et al.*, 2013).

Considerando a importância da densidade sobre as características de uma interação simbiótica, o estabelecimento deste cenário complexo de infecções surge como um problema biológico extremamente interessante e que carece de explicações. Por que este sistema está estabelecido desta forma? Qual é, ou quais são, as linhagens de *Wolbachia* que infectam as isolinhagens de *Drosophila* em baixa densidade? Estas linhagens conferem alguma vantagem adaptativa? Por que as linhagens de alta infecção não conseguem se fixar dentro de uma população e alastrar-se para todas as populações avaliadas?

Esta dissertação de mestrado surge como um esforço para buscar responder ao menos em parte estas muitas perguntas, usando a estratégia de abordar o problema por todos os seus lados, ou seja, buscando primeiramente compreender a fundo o sistema estabelecido, para permitir assim a formulação de hipóteses que possam explicar os padrões observados.

Objetivo Geral

Tendo em vista que foi verificado, em estudos prévios do nosso grupo de pesquisa (Müller *et al.*, 2013), que *Wolbachia* mantém diferentes padrões de infecção em populações naturais brasileiras de espécies do subgrupo *willistoni* de *Drosophila*, inclusive com marcadas variações intraespecíficas em regiões geográficas compartilhadas, o objetivo geral deste trabalho é aprofundar o conhecimento acerca deste problema biológico, e buscar entender as possíveis estratégias e respostas empregadas pela bactéria e seus hospedeiros na manutenção destas densidades diferenciais de infecção.

Objetivos específicos

1. Diagnosticar e estimar a taxa de infecção por *Wolbachia* em isolinhagens de espécies do subgrupo *willistoni* de *Drosophila*, especificamente *D. willistoni* e *D. paulistorum*, a partir de coletas realizadas em populações naturais.

2. Caracterizar molecularmente as linhagens de *Wolbachia* que infectam tais espécies de *Drosophila.*

3. Verificar e quantificar a densidade de infecção por *Wolbachia* ao longo do desenvolvimento das moscas, caracterizando assim a cinética da interação deste sistema.

4. Investigar possíveis interações entre infecção por *Wolbachia* e marcadores de *fitness* das moscas.

5. Verificar a ocorrência de fenótipos de parasitismo reprodutivo causados por *Wolbachia* sobre o hospedeiro, especificamente androcídio.

CAPÍTULO 2

Living together in the same fly: population and evolutionary dynamics between *w*Au and *w*Wil *Wolbachia* strains and *Drosophila willistoni* mitochondria

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Living together in the same fly: populational and evolutionary dynamics between *w*Au and *w*Wil *Wolbachia* strains and *Drosophila willistoni* mitochondria

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Wolbachia relationships with Neotropical *Drosophila* flies

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Abstract

Wolbachia is one of the most widely distributed microorganisms that can establish endosymbiotic relationships with invertebrates. It is known to classically induce reproductive manipulations on its hosts, thereby improving its own transmission through populations. The symbiont load within host cells is one of the most basic features of any symbiotic relationship, which can therefore partially explain the symbiotic outcome. Neotropical flies from the *willistoni* subgroup of *Drosophila* are infected with variable *Wolbachia* titre levels in populations from geographically distant locations in Brazil. Considering the importance of the infection titre to the symbiosis outcome, we aimed to investigate how this biological system has been evolutionary maintained. Hence, the infection scenario, as well as the *Wolbachia* strains that infect *D. willistoni* and *D. paulistorum* lines, were evaluated. We demonstrated that all high-titre infections are maintained by *w*Wil *Wolbachia*, whereas low-titre infections are possessed by either *w*Wil or *w*Au-like. Moreover, we performed a large-scale association study between *Wolbachia* infection status (high- or low-titre) and *D. willistoni* mitochondrial haplotypes, in order to verify whether the infection pattern seen nowadays could have been established due to a *Wolbachia*-conducted selective sweep. Our data disagreed with such an hypothesis, although the mtDNA variation scenario was better explained by a *D. willistoni* demographic expansion. Furthermore, we propose an evolutionary hypothesis that might account for all our findings.

Keywords: *Wolbachia*-conducted selective-sweep; *VNTR-141*; *COI*; Neotropical *Drosophila* flies; Endosymbiosis

1. Introduction

Living together presents challenges, especially when it occurs so intimately. In the course of evolution of life on Earth, microorganisms have evolved countless ways to

associate with each other and with other organisms. The most intimate way is via intracellular symbiosis.

In this context, the most widely studied endosymbiont is *Wolbachia pipientis*, a gram-negative alphaproteobacterium that infects a variety of arthropods and nematodes (Werren *et al.*, 2008). It inhabits vacuoles within host cells primarly in reproductive tissues, although it can also be present in somatic tissues (Dobson *et al*, 1999). *Wolbachia* is mainly considered a reproductive parasite, even though accumulating evidence has shown that it might increase host fitness and develop mutualistic relationships (Fry *et al.*, 2004). Since *Wolbachia* lives in the cytoplasm, it is vertically transmitted from the mother to the offspring. As a classic reproductive parasite, it can induce parthenogenesis, feminisation, male killing and cytoplasmic incompatibility (reviewed in Werren *et al*., 2008). All these phenotypes ultimately favour the females, since they are responsible for the transmission of *Wolbachia*, in a sense subverting the host's reproductive system for its own benefit (Ballard, 2004).

Considering its ability to propagate throughout host populations due to its inducible phenotypes, *Wolbachia* may also influence mitochondrial DNA (mtDNA) evolution, since they both share maternal inheritance. Furthermore, because the mitochondrial genome lacks recombination, it is particularly susceptible to genetic hitchhiking (Ballard *et al.*, 1996). Therefore, if for instance, *Wolbachia* induces cytoplasmic incompatibility between the hosts's mtDNA lines where some harbour *Wolbachia* infection and others do not, its own frequency will increase in the population, bringing together associated mitochondrial haplotypes in a phenomenon known as selective sweep. From a genetic point of view, the spread of a specific *Wolbachia* strain through a host population can have a parallel effect on the variation of its cytoplasmic partner, the mtDNA (Rokas *et al.*, 2001; Dean *et al.*, 2003; Ballard, 2004).

Considering a selective sweep scenario, the mtDNA will be forced through a bottleneck of one host female, from which all mtDNA haplotypes in the population will descend. Consequently, infected populations might have a lower mtDNA diversity (Fine, 1978; Turelli, 1994). For instance, evidence from *Drosophila* suggests that a single mtDNA haplotype might have become widespread in the host population through hitchhiking with a successful *Wolbachia* strain (Turelli *et al.*, 1992; Ballard *et al.*, 1996). Furthermore, selective sweeps not only reduce mitotype diversity, but also cause the

remaining set of host haplotypes to deviate from predictions based on neutrality (Johnstone and Hurst, 1996).

Although the *Drosophila–Wolbachia* model has been extensively studied elsewhere, particularly with the Old World species *D. melanogaster* and *D. simulans* (Merçot and Charlat, 2004; Riegler *et al.*, 2005), less is known about the dynamics that *Wolbachia* establishes with Neotropical *Drosophila* species. Among these, the *willistoni* group of *Drosophila* (constituted by six cryptic and 19 non-cryptic species), is the best-represented group of species of the genus in Neotropical communities (Val *et al.*, 1981). The cryptic species that compose the so called *willistoni* subgroup are named as follows: *D. willistoni*, *D. equinoxialis*, *D. paulistorum*, *D. tropicalis*, *D. insularis* and *D. pavlovskiana*. The origin of this group of species is attributed to hot and humid forests from Central Brazil (Cunha *et al.*, 1950).

Since approximately 2006, the *willistoni* subgroup has been investigated for *Wolbachia*, and some interesting assumptions have been made. Miller and Riegler (2006) screened laboratory stock lines of species from *D. willistoni* and *saltans* subgroups and found no evidence of *Wolbachia* infection in stocks collected before the 1970s. Furthermore, they were able to establish that all iso- and oligofemale lines collected after this decade were infected by a high-titre *Wolbachia* strain then called *w*Wil.

Nevertheless, our research group recently demonstrated that *Wolbachia* does not evenly infect Brazilian populations of species from the *willistoni* subgroup, when considering flies from the Pampas biome, throughout the Atlantic Forest to the Amazonian Rainforest. Specifically, standard PCR assays were unable to identify an infection signal for all isofemale lines tested, although results from dot-blot hybridisations and quantitative PCR assays confirmed that all species were infected but often at a very low density. For *D. willistoni*, where differences in infection levels were more clear, high-titre infections were all assigned to the same *w*Wil strain, but all low-titre infections from this species and the others remain to be identified (Müller *et al.*, 2013).

This complex infection scenario represents an extremely interesting biological problem that needs to be clarified. This is particularly true for *D. willistoni*, where such strong differences in infection densities by *Wolbachia* are maintained in fly lines collected in sympatry, and whose pattern is seen in all the evaluated populations. Infection density is an important factor in the determination of the symbiosis outcome (Mouton *et al.*, 2003;

2004). An appropriate symbiont density is important to ensure its own transmission to offspring, since very low densities might result in the failure of transmission, but too strong titres might result in the host's death (Serbus *et al.*, 2011). Considering this, it is quite surprising that low- and high-titre infections are maintained side by side in the same host species. In this context, some key questions arise, such as: (i) which *Wolbachia* strain or strains are responsible for low-titre infections? (ii) how can this system be evolutionarily mantained? (iii) is it due to a selective sweep conducted separately by both infection types? (iv) why do these high-titre infections not spread throughout all host populations? In this study, we aimed to investigate such issues with flies from a new field survey, but also using information from our previous analyses, and to make some evolutionary assumptions based on our new findings. For this purpose, the infection scenario, as well as the *Wolbachia* strains that infect *D. willistoni* and *D. paulistorum* lines, were evaluated. Furthermore, we performed a large-scale association study between *Wolbachia* infection status (high- or low-titre) and *D. willistoni* mitochondrial haplotypes. We attempted to investigate whether the infection pattern observed nowadays could have been established due to a selective sweep conducted by *Wolbachia* in the evaluated populations, which would carry the flies' mitochondria as a hitchhiker.

2. Materials and Methods

2.1 Fly samples and isofemale line establishment

Drosophila flies were captured from a location in the Atlantic Forest (Osório, Rio Grande do Sul, Brazil – 29º53′08.20"S, 50º16'39.81"W) in May 2013, using an insect entomological net and plastic bottles containing banana and yeast bait. Flies were subsequently taken to the laboratory and isofemale lines that were roughly identified as belonging to the *willistoni* subgroup were established in cornflour and yeast medium, and were further maintained in Biochemical Oxygen Demand (B.O.D.) chambers at 19°C with a 12h/12h photoperiod, with the medium being changed weekly (as in Müller *et al.*, 2013).

Drosophila willistoni and *D. paulistorum*, which are the only species from this subgroup that occur in that location (Spassky *et al*., 1971; Valiati and Valente, 1996), were identified through hypandrium shape examination following Bächli *et al.* (2004) and the hypandrium was compared with that described by Malogolowkin (1952). This

identification was subsequently confirmed by *Cytochrome Oxidase I* (*COI*) gene sequence analysis (see below).

2.2 Determination of *Wolbachia* infection titre

The determination of *Wolbachia* infection titre in all *Drosophila* isofemale lines was performed using two methodologies: PCR and PCR-blot hybridisation. In both techniques, DNA was extracted using the NucleoSpin® Tissue Kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's instructions.

The initial survey for *Wolbachia* infection was performed with single-female fly DNA through standard PCR of a 0.6-kb fragment from the *Wolbachia Surface Protein* (*wsp*) gene using the primers Wsp-F and Wsp-R (Jeyaprakash and Hoy, 2000). A blank (ultrapure water) and a positive control (*D. willistoni* Gd-H4, Guadeloupe – Lesser Antilles, 12 Genomes Consortium) were included in all reactions. A complete description of the primer sequences, concentrations and specific parameters used in all performed PCR reactions is presented in Table S1 (Supplementary Material).

Drosophila isofemale lines whose DNA amplified the *wsp* fragment were assumed to be infected with high-titre *Wolbachia*, in agreement with our previous experience in this matter (Müller *et al*., 2013). Alternatively, samples that did not amplify in the PCR were assumed to be infected with low-titre *Wolbachia*, and this status was subsequently confirmed by PCR-blot hybidrisation, a more sensitive technique that is widely used to screen for *Wolbachia* low-titre infections (Arthofer *et al*., 2009; Miller *et al*., 2010).

For this assay, we performed a new DNA extraction from each isofemale line that did not amplify *wsp*, using 15 ovaries instead of a single female fly. Since *Wolbachia* preferentially infects reproductive tissues due to its vertical mode of transmission (Dobson *et al.*, 1999), this strategy increases the chance of identifying any sign of low-intensity infections.

A PCR-blot assay was performed according to Arthofer *et al.* (2009) with some modifications. Standard PCR was performed with DNA from ovaries using the same set of primers and cycling conditions as for the *wsp* gene. Furthermore, a 387-bp fragment from the *Wolbachia* housekeeping gene *Glutamyl-tRNA amidotransferase - subunit B* (*gatB*) was amplified using the primers gatBF and gatBR (Baldo *et al.*, 2006) (Table S1). Since this technique aimed to verify low-titre infections, the use of two different *Wolbachia*

probes rules out the chances of false positive hybridisation signals by comparing the patterns obtained by those different genes. Moreover, two single-fly DNA-positive controls (*D. willistoni* and *D. paulistorum* lines infected with high-titre *Wolbachia*) were included in PCR and hybridisation reactions.

The PCR products were run on a 0.8% agarose gel with GelRed (Biotium, Hayward, CA, USA) at low voltage for approximately three hours. After specific buffer incubations, the DNA was transfered from the gel to a Hybond N+® (GE Healthcare, Amersham, UK) nylon membrane by capilarity, following the manufacturer's instructions. The PCR-blot hybridisation was performed using the Amersham Gene Images AlkPhos Direct Labelling and Detection System (GE Healthcare, Amersham, UK), according to the manufacturer's instructions. Membranes were hybridised overnight at 55ºC. The probes consisted of the same fragments (*wsp* and *gatB*) amplified by PCR from the standard *D. willistoni* isofemale line Gd-H4, which were cloned into pGEM-T® vectors (Promega, Madison, USA). Following amplification, these fragments were gel-purified using the Wizard® SV Gel and PCR Clean-Up System (Promega, Madison, USA) kit and quantified using a NanoDrop (Thermo Scientific, Delaware, USA). After hybridisation, detection was performed using a chemiluminescence substrate CDP-*Star* Detection Module (GE Healthcare, Amersham, UK), according to the manufacturer's instructions, and the exposure time varied from 6 to 16 h.

2.3 Identification of the *Wolbachia* strains in fly infection

The same DNA that was used to determine the *Wolbachia* infection status (single-fly DNA for high-titre infections and 15-ovary DNA for low-titre infections) was used to verify which *Wolbachia* strains infected the isofemale lines. We amplified a minisatellite marker initially isolated from the anotated genome of *w*Mel *Wolbachia* from *D. melanogaster* (Riegler *et al.*, 2005). This marker can successfully distinguish closely related *Wolbachia* strains that might be impossible to differentiate through *wsp* screening alone (Miller and Riegler, 2006). The use of specific primers flanking this tandem repeat region (VNTR-141, since the basic repeat consists of 141-bp) for PCR results in fragments with different sizes according to each *Wolbachia* strain, making the identification possible even via gel examination only (Riegler *et al*., 2005).

The PCR of this minisatellite region was performed using the primers Wob-F and Wob-R (Riegler *et al.*, 2005) (Table S1). All reactions included DNA from fly lines infected with *Wolbachia* strains with standard fragment sizes, in order to compare sizes on the gel (Miller and Riegler, 2006) as follows: *w*Wil *Wolbachia* strain (387-bp fragment) from *D. willistoni* GdH4 line and *w*Mel strain (1,320-bp) from *D. melanogaster* Oregon-R line. All amplicons were run on a 1% agarose gel with Gel Red (Biotium, Hayward, CA, USA) and were visualised under UV-light.

All fragments generated were subsequently cloned into pGEM®-T Vectors (Promega, Madison, USA), and successful cloning was tested by PCR using the vector primers T7 and SP6 (Promega, Madison, USA) (Table S1). The PCR products were visualised on a 1% agarose gel and were subsequently enzymatically purified using FastAP (Thermosensitive Alkaline Phosphatase, Thermo Scientific, Delaware, USA) and EXO I (Exonuclease I, Thermo Scientific, Delaware, USA). Reactions were incubated for 15 min at 37ºC and 15 min at 85ºC. Purified products were sent for direct and bidirectional sequencing at Macrogen (Macrogen Inc., Seoul, Korea) using the same vector primers.

Chromatogram quality was evaluated using Chromas Pro 1.5 (http://www.technelysium.com.au). Segments of the sequence that were of vector origin were removed using the VecScreen tool (http://www.ncbi.nlm.nih.gov/tools/vecscreen/). Sequence alignment was performed with ClustalW implemented into Mega 5, with a later edition in BioEdit 5.0.9, if necessary. A consensus sequence was generated, based on the alignment of two independent sequences, and sequence similarity was obtained by comparison with sequences deposited in GenBank using BLASTN (NCBI, available online), taking into account values of maximum identity.

2.4 Large-scale association study between *Drosophila willistoni* mitochondrial haplotypes and *Wolbachia* infection titre

Single-female fly DNA from *D. willistoni* samples collected in Osório was used to amplify an approximately 0.95-kb fragment of the mitochondrial *COI* gene using the set of primers TY-J-1460 and C1-N-2329 (Simon *et al.*, 1994) (Table S1). The PCR products were enzymatically purified (as mentioned in the previous section) and were subjected to direct and bidirectional sequencing, using the same primers, at Macrogen (Macrogen Inc., Seoul, Korea).

The data were combined with sequences obtained in our previous studies with flies collected in the Atlantic and Amazonian Forests (Müller *et al.*, 2012; 2013), resulting in a total of 100 sequences. Sequence quality analyses, aligment and consensus sequence generation were executed according to the methodology stated previously.

Furthermore, DNAsp software was used to analyse frequencies, distribution and haplotype coalescence, as well as genetic diversity measures (haplotype and nucleotide diversity). The Arlequin program was employed to perform neutrality tests based on the sequence polymorphisms Fu and Li and Tajima *D*. In order to infer the relationships between mitochondrial haplotypes and their respective *Wolbachia* infection status, we constructed a haplotype network by the Median-Joining method incorporated into the Network software.

3. Results

3.1 Fly samples and *Wolbachia* infection titre determination

Our fly collection in Osório resulted in the establishment of 27 *D. willistoni* isofemale lines and eight *D. paulistorum* isofemale lines in the laboratory, according to the expected occurence of flies from the *D. willistoni* subgroup in Brazil. All isofemale lines were submitted to *Wolbachia* screening, first by standard PCR via amplification of the *Wolbachia*-specific *wsp* marker from single-fly DNA, which would account for high-titre infections. Of all 27 *D. willistoni* and eight *D. paulistorum* isofemale lines established, 11 (40.7%) and three (37.5%) were confirmed to be infected with high-titre *Wolbachia*, respectively.

The remaining samples were submitted to a new DNA extraction from 15 ovaries and this DNA was used in PCR-blot hybridisations with *Wolbachia*-specific *wsp* and *gatB* probes. In addition to the screening performed with flies recently collected in the field, five *D. equinoxialis* isofemale lines previously collected in the Amazonian Rainforest (Müller *et al.*, 2013) were included. Since these lines were already shown to be *wsp*-PCR negative and to display a rather low amount of *Wolbachia* in qPCR assays (Müller *et al.*, 2013), they were also submitted to PCR-blot assay with a 15-ovary DNA extraction. All samples generated a hybridisation signal in both PCR-blot reactions (*wsp* and *gatB*) (Fig. 1), confirming them to be infected by low-titre *Wolbachia*.

3.2 Identification of *Wolbachia* strains in fly infection

The VNTR-141 cloned sequences from high- (single-fly DNA) and low-titre- (15-ovary DNA – including *D. equinoxialis* isofemale lines) infected flies were compared to those in the GenBank database through BLASTn. As already stated, all *D. equinoxialis* isofemale lines analysed were infected with low-titre *Wolbachia*; VNTR-141 sequencing of two of these lines confirmed them to be infected with a *w*Au-related *Wolbachia* strain (see below).

For *D. willistoni*, all high-titre infections were identified as *w*Wil, a *Wolbachia* strain that was first described in this particular species (Miller and Riegler, 2006). However, among the 16 *D. willistoni* lines infected with low-titre *Wolbachia*, three (18.7%) were also assigned as being infected by *w*Wil, three (18.7%) could not be identified, and nine lines (56.3 %) were shown to be infected with *w*Au-like *Wolbachia*, the same found in *D. equinoxialis*. This strain has a 502-bp VNTR-141 fragment, thus showing a 26-bp deletion when compared to the 528-bp canonical *w*Au strain. The latter was first recognised in Australian *D. simulans*, but today has already been found in Neotropical *Drosophila* species (Miller and Riegler, 2006; Müller *et al.*, 2013). Moreover, we found one case of coinfection by both *w*Wil and *w*Au-like low-titre *Wolbachia* strains in *D. willistoni* (we obtained two fragments in the 15-ovary PCR reaction; both were excised from the gel, purified and cloned).

Furthermore, for *D. paulistorum*, all high- and low-titre infections were shown to be maintained by the same *w*Au-like strain found in *D. equinoxialis* and *D. willistoni*, thus, this was the only *Wolbachia* strain shown to infect this species in this study.

Notably, when comparing VNTR-141 sequences of *w*Wil (387-bp; Accession Number DQ118778), canonical *w*Au (528-bp; Accession Number KF311111) and *w*Au-like (502-bp; please check Data Archiving section), the nucleotide block that is absent in the latter strain is located within the larger block that is absent in *w*Wil (Fig. 2 and 3).

3.3 Large-scale association study between *D. willistoni* mitochondrial haplotypes and *Wolbachia* infection titre

We obtained a more robust dataset for *D. willistoni* that consisted of *COI* sequences from flies collected in Osório (analysed here) and from studies carried out with flies from the Atlantic and Amazonian Forests (Müller *et al*., 2012; 2013). Therefore, we performed

and analysed an alignment of 100, 645-bp *COI* sequences, to verify whether the maintenance of high- and low-titre *Wolbachia* infections could be due to a *Wolbachia*-conducted selective sweep.

Firstly, we verified a roughly balanced segregation between low- (n = 46) (46%) and high-titre (n = 54) (54%) *Wolbachia* infections in *D. willistoni* (no deviation from a theoretical 1:1 segregation between infection types; $\chi^2 = 0.64$; $p = 0.001$), as can be verified in the haplotypic network (Fig. 4). The high- and low-titre infections are represented by grey and black colours on each haplotype circle, respectively, demonstrating no prevalence of either *Wolbachia* infection type in these populations.

We confirmed a total of 24 mitochondrial haplotypes that were distributed in a star-like network. All 28 mutations detected in the 24 haplotypes were shown to be synonymous, suggesting that this gene is evolving under purifying selection. The central haplotype (H1) was the most frequent (n = 49) (49%), from which all the others derived. Fly lines with this mitochondrial haplotype were infected with either low- or high-titre *Wolbachia*, although low-titre infections were more frequent (low-titre: n = 31, 63.2%; high-titre: n = 18, 36.7%). Besides H1, haplotypes H4 (n = 11; 11%) and H9 (n = 12; 12%) were slightly more frequent than the others, and were also shown to have both infection types recorded. Haplotype H2 (n = 5; 5%) was an exception, since only high-titre *Wolbachia* infections were recorded, although this haplotype was also slightly more frequent. All remaining haplotypes (n = 20, 83.3%) were quite rare (one or two isofemale lines included) and were infected by only one type of *Wolbachia* infection.

We also recorded a considerable haplotype diversity (Hd: 0.735), a low nucleotide diversity ($\pi = 0.00192$), negative and significant Tajima's *D* and Fu and Li's *F* values ($D = -2.32792$, $p < 0.0000$; $F = -24.36101$, $p < 0.0000$), and a non-significant Mismatch Sum-of-Squares value (0.00191; $p = 0.40600$). Taken together, these data suggest that the verified pattern is not explained by a *Wolbachia*-conducted selective sweep, but is rather the result of a demographic expansion in *D. willistoni*.

4. Discussion

4.1 Evolutionary dynamics of *Wolbachia* strains in *Drosophila* species

We recently re-evaluated *Wolbachia* infections among populations from the *willistoni* subgroup of *Drosophila* and confirmed a remarkable difference in the infection

densities among them. Although it was not possible at this point to identify low-titre strains, our results suggested *w*Wil to be the *Wolbachia* strain responsible for all high-titre infections (Müller *et al.*, 2013).

In this study, we were able to refine our analyses and to confirm the presence of a variety of *Wolbachia* infection densities among *D. willistoni* and *D. paulistorum* isofemale lines. The results from PCR-blotting using two *Wolbachia* genes, *wsp* and *gatB*, confirmed the disseminated presence of low-titre *Wolbachia* infections in those fly species by using 15-ovary DNA in the reactions. The use of two different gene fragments as probes allowed us to confirm the presence of those infections with confidence, and enabled us to identify the *Wolbachia* strains responsible for those infections.

The *VNTR-141* PCR and cloning results confirmed *w*Wil as the *Wolbachia* strain responsible for high-titre infections in *D. willistoni*, as previously stated (Müller *et al.*, 2013), and the same strain was also recognised to infect some of the the low-titre isofemale lines. However, the majority of those infections were shown to be maintained by a closely related *Wolbachia* strain (Miller and Riegler, 2006), *w*Au-like, whose canonical form was first recorded in *D. simulans* from Australia (Hoffmann *et al.*, 1996).

With regard to *Wolbachia* infections in *D. paulistorum*, although we only evaluated eight isofemale lines, we imediately observed a difference in infection density in comparison to that in the 2013 survey. At that time, we only confirmed low-titre *Wolbachia* infections, which could not be identified at the VNTR-141 level (Müller *et al*, 2013). Our new survey confirmed that three out of eight *D. paulistorum* isofemale lines were infected with high-titre *w*Au-like *Wolbachia*, and the same strain was confirmed in low-density infections. Importantly, the VNTR-141 sequence of *w*Au-like *Wolbachia* in those isofemale lines was identical to that amplified from *D. willistoni* lines.

We could also identify low-titre *w*Au-like *Wolbachia* infections in two *D. equinoxialis* isofemale lines that we have maintained in the laboratory since their 2011 collection in the Amazonian Rainforest. This finding demonstrates that *w*Au-related *Wolbachia* strains are highly disseminated in Neotropical *Drosophila* species, since related strains were also identified in flies from the *saltans* group (Miller and Riegler, 2006).

The presence of *w*Au-like in all species evaluated here, combined with our previous results showing that high-titre canonical *w*Au *Wolbachia* infected *D. tropicalis* (Müller *et al.*, 2013), another species from the *D. willistoni* subgroup, increases evidence in favour of

a Neotropical origin for *w*Au and its horizontal transmission from a Neotropical *Drosophila* species to the Old World and the worldwide dissemination of *D. simulans*.

Miller and Riegler (2006) previously performed an extensive survey for *Wolbachia* in Neotropical *Drosophila* species (*saltans* and *willistoni* groups), with the aim of identifying potential sources of *Wolbachia* infection to *D. simulans*. They proposed a scenario for the evolution of *w*Au-related *Wolbachia* strains in the Neotropics from the *saltans* group, which could be the potential donor to *D. willistoni* (the only infected species of the subgroup recognised at that time). Furthermore, *Wolbachia* would have evolved within *D. willistoni* lines during years of maternal transmission until it became what we know today as the *w*Wil strain. This infection in *D. willistoni* or other *w*Au-related infections have been suggested as the source of transmission to *D. simulans*, which has then spread the *w*Au infection worldwide (Ballard, 2004; Miller and Riegler, 2006).

However, we proposed a slightly different hypothesis to explain the established infection patterns (Müller *et al*., 2013). In this model, *D. tropicalis*, rather than *D. willistoni*, is the more probable donor of *w*Au *Wolbachia* to *D. simulans*. This is supported by the evidence that this species harbours the identical *Wolbachia* strain (canonical *w*Au) as *D. simulans* and that these species also occur sympatrically. As *D. willistoni* infections were considered quite recent at that time, because Miller and Riegler (2006) only encountered *wsp*-PCR-positive fly lines collected since the 1970s, either *D. tropicalis* or *D. simulans* were considered as possible *Wolbachia* donors to *D. willistoni*.

Our new results, however, allow other alternatives to be proposed. The previously considered recent *Wolbachia* infection in *D. willistoni* might have been misleading due to methodological barriers (Miller and Riegler, 2006), since the screening used only standard PCR to establish *Wolbachia* infection, which is not sufficient to unravel the complete low density infection diversity. The discovery that *w*Au-like *Wolbachia* maintains low-titre infections in *D. willistoni* raises the possibility that the infection in this species is in fact older than we had previously assumed and might have been maintained at low density levels, possibly by *w*Au-like *Wolbachia*.

Moreover, the discovery of *w*Au-related strains among all the *willistoni* subgroup flies evaluated here and in our previous study (Müller *et al.*, 2013), reinforces the idea of a horizontal transmission event having occurred from a Neotropical *Drosophila* species to the Old World *D. simulans*, more likely from *D. tropicalis*, as it harbours the identical

Wolbachia strain as *D. simulans*. Furthermore, Ballard (2004) made some assumptions regarding the worldwide *Wolbachia* infection pattern in *D. simulans* and formulated hypotheses about the dynamics of those infections and the host mitochondrial DNA evolution. The author suggested that a certain uninfected *D. simulans* mitochondrial line arrived in Ecuador, where it became infected with *w*Au *Wolbachia* from an unknown source. Since *D. tropicalis* can occur in Ecuador (Spassky *et al.*, 1971), the possibility of horizontal transmission of *Wolbachia* from this species to *D. simulans* becomes even more plausible.

Considering the evolution of *Wolbachia* infections within the *willistoni* subgroup, together with the verification of *w*Au-related strains in the species evaluated, and the almost complete absence of nucleotide polymorphisms in such a rapidly-evolving genome region as VNTR-141 (Riegler *et al*., 2005), it is plausible to infer that this infection was established in this group of species by horizontal transmission events rather than by cladogenesis.

The presence of low-titre *w*Au-like in *D. willistoni* supports the hypothesis that *w*Wil *Wolbachia* evolved within *D. willistoni* from a *w*Au-like ancestral infection. Furthermore, the discovery of a *D. willistoni* line coinfected with both *w*Wil and *w*Au-like at low titre is particularly relevant. Moreover, this might be a more frequent event than we can assess using our methodologies, since low-titre infections are often difficult to rescue, even with 15-ovary DNA. We also cannot rule out the possibility that the so-called high-titre-infected flies might be coinfected with a low-titre *Wolbachia* partner.

Moreover, as the *w*Au-like strain is confirmed to be present either at a low or high density in *D. paulistorum*, it is more plausible to assume that this species was the donor of the infection to *D. willistoni*, either by horizontal transmission or hybrid introgression (Robe *et al*., 2010), which then evolved until it became *w*Wil. This reasoning is strengthened by the VNTR-141 sequence alignment of all these strains (*w*Wil – 387 bp; *w*Au-like – 502 bp; canonical *w*Au – 528 bp). As can be observed in Figure 3, the small 26-bp deletion that has been lost in *w*Au-like compared with the canonical *w*Au, is located within the large block of nucleotides that is deleted from the *w*Wil fragment. Furthermore, *w*Wil infections have been observed in only one other *Drosophila* species besides *D. willistoni*, namely, *D. arawakana* (Hamm *et al*., 2014), a Neotropical species from the *cardini* group. As *w*Wil only represents a small percentage of *Wolbachia* infections in *D.*

arawakana, which are mostly caused by *w*Spt *Wolbachia* (Hamm *et al.*, 2014), the hypothesis of the evolution of *w*Wil in *D. willistoni* with further horizontal transmission to *D. arawakana* is more plausible.

Horizontal transmission events of *Wolbachia* between closely or distantly related species have already been documented either phylogenetically (Vavre *et al.*, 1999) or experimentally (Le Clec'h *et al.*, 2013). Such events clearly require an intimate ecological relationship or the existence of potential vectors, such as parasitoids or parasite mites. For instance, Huigens *et al.* (2004) reported the natural inter- and intraspecific horizontal transfer of a parthenogenesis-inducing *Wolbachia* strain between parasitoid wasps, which was observed when infected and uninfected larvae shared the same host egg. In the specific case of drosophilids, the flies live in association with mites, which might potentially be vectors for infection, as has been demonstrated in the laboratory (Rozhok *et al.*, 2011), thus confirming the proposed routes of transmission and evolutionary models considered here.

4.2 Large-scale association study between *D. willistoni* mitochondrial haplotypes and the *Wolbachia* infection titre

Examples of *Wolbachia*-induced selective sweeps abound in the literature. Perhaps the most widely studied case is the spread of a specific *Wolbachia* strain among *D. simulans* populations in California over time. For instance, Turelli *et al.* (1992) recorded incompatible crosses in this species due to *Wolbachia* infection, which rapidly spread northwards within California. Analyses of mtDNA restriction fragment length polymorphisms demonstrated that all infected flies contained the same mtDNA allele, whereas uninfected flies remained polymorphic, representing a classic example of *Wolbachia*-conducted selective sweep. Similar results were obtained by Ballard *et al.* (1996), who analysed the same host–symbiont complex, but associated instead, the lack of mitochondrial variation in infected lines with a normal polymorphism for the autosomal locus Adh^r, and showed that the marker was not affected in uninfected lines.

In a complex of *Drosophila* sister species, Shoemaker *et al.* (2004) examined patterns of mtDNA diversity in *D. recens*, which was infected with *Wolbachia*, and its uninfected sister species, *D. subquinaria*. They observed that the level of diversity of mitochondrial but not of nuclear DNA was much lower in *D. recens* than in *D.*

subquinaria, consistent with the diversity-purging effects of an evolutionarily recent *Wolbachia* sweep.

The specific case of *Wolbachia* infections in *D. willistoni* is interesting, since it demonstrates that two infection density types (low- and high-titre), which are apparently maintained by two closely related *Wolbachia* strains, can be transmitted in host populations. Infection density is an important characteristic of any symbiotic relationship, since it corresponds to the amount of symbiont replication within host cells, which is directly connected to the long-term conservation of the system (Mouton *et al.*, 2003; 2004). Considering the repetitive incidence of *Wolbachia*-induced selective sweep events reported in the literature, we hypothesised that the maintenance of differential infection densities and *Wolbachia* strains in sympatric populations of *D. willistoni* from distantly related localities might be due to selective sweeps conducted by either infection type. Therefore, if each infection status/*Wolbachia* strain is segregating independently within the populations, we might expect them to be associated with certain mitochondrial haplotypes, as a result of a *Wolbachia*-induced selective sweep.

We verified a pattern where the high- and low-titre *Wolbachia* infections showed a 1:1 segregation. Therefore, the estimate of mtDNA variation from *D. willistoni* isofemale lines might indicate the occurrence of selective sweep conducted separately by either *Wolbachia* infection type, with each one being associated with a certain set of host haplotypes (Rokas *et al.*, 2001; Dean *et al.*, 2003; Ballard, 2004).

The *COI*-sequence analysis from *D. willistoni* collected in Brazilian localities remarkably far from each other recovered 24 haplotypes that were shown to be the product of 28 synonymous mutations. However, high- and low-titre *Wolbachia* infections were randomly spread throughout the network, with no specific association of any haplotype with a particular infection type (similar to the findings of Avtzis *et al.*, 2014, for a different purpose). Moroever, since we would expect a rather low haplotype diversity in mtDNA from host populations that had undergone a selective sweep (Turelli *et al.*, 1994), the considerably higher haplotype diversity (Hd: 0.735) reported here, also argues against such an hypothesis.

However, the star-shaped network obtained from the distribution of haplotypes, including several rare haplotypes, is a rough indicator of population expansion (Mirol *et al.*, 2008). Moreover, the aforementioned considerable haplotype diversity, which is

associated with a low nucleotide diversity and an excess of rare variants, highlight a possible population expansion for this species, as has already been mentioned elsewhere (Müller *et al.*, 2012). Furthermore, the unimodal pattern of the Mismatch distribution (data not shown) that considers pairwise differences, showed a sum of squares that did not reject the demographic expansion hypothesis for this species (as obtained by Rogers and Harpending, 1992; Avtzis *et al.*, 2014).

Theory suggests that the long-term coexistence of incompatible *Wolbachia* strains is not possible unless they coinfect the same individuals (Rousset *et al.*, 1991; Dean *et al.*, 2003), and we did not retrieve clear signs of a *Wolbachia*-induced selective sweep. Therefore, two hypotheses exist to explain the maintenance of the polymorphism of *Wolbachia* strains and infection densities in *D. willistoni* populations: (i) *w*Wil and *w*Au-like are not incompatible strains and their infection distribution among *D. willistoni* populations is random and the fixation of either strain might have evolved by drift; or (ii) assuming a scenario where *w*Wil evolved from *w*Au-like within *D. willistoni*, we might be visualising an intermediate point in the evolution of a *w*Au-like replacement by *w*Wil, and would therefore expect that after some time, *w*Wil would evolve towards fixation. A replacement scenario would be facilitated if *w*Wil could improve its host fitness, even slightly.

The first hypothesis, although possible, is unlikely, since we would have to rely on random factors to explain the maintenance of a roughly 1:1 segregation pattern of *Wolbachia* infection types that is evident in *D. willistoni* populations from at least three geographically distant locations in Brazil. Therefore, the second hypothesis is more plausible, although future work to evaluate and compare classical fitness markers such as fecundity, viability and survival (Fry *et al.*, 2004) between flies from different infection models should be performed. Furthermore, experimental crosses with flies infected with different *Wolbachia* strains would be useful to infer the extent to which these strains are compatible.

These approaches would enable the intimacies regarding the relationship that has been established between such closely-related *Wolbachia* strains and their fly hosts to be unravelled. Living together presents challenges, especially when it occurs so intimately, even for us who study these amazing relationships.

Supplementary information is available at Heredity's website.

5. Acknowledgements

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6. Conflict of Interest

The authors declare that they have no competing interests.

7. Data archiving

*w*Au-like *VNTR*-141sequences from *D. willistoni* and *D. paulistorum* were deposited in GenBank (in process, accession numbers to be provided).

8. References

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9. Figures

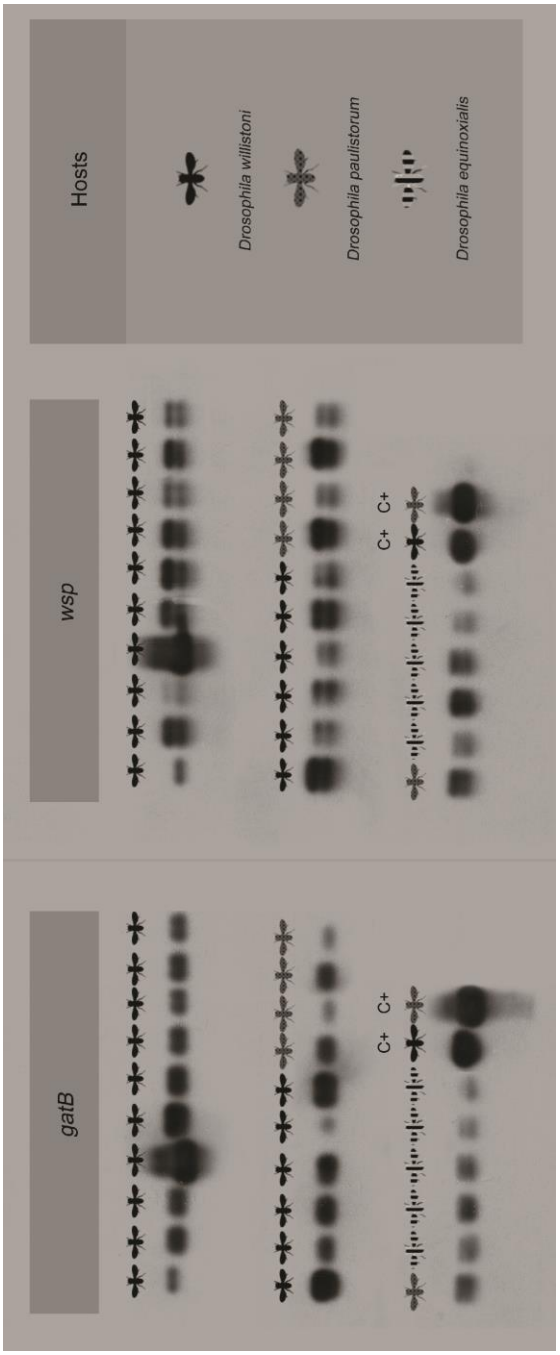

Figure 1. PCR-blot of 15-ovary DNA from low-titre-infected *D. willistoni*, *D. paulistorum* and *D. equinoxialis* isofemale lines, probed with *gatB* and *wsp* plasmids of *D. willistoni* GdH4. Icons above each hybridisation signal represent one isofemale line, whose species is identified by colour. Single-female fly DNA of high-titre *Wolbachia*-infected *D. willistoni* and *D. paulistorum* isofemale lines were used as positive controls (C+) in each reaction.

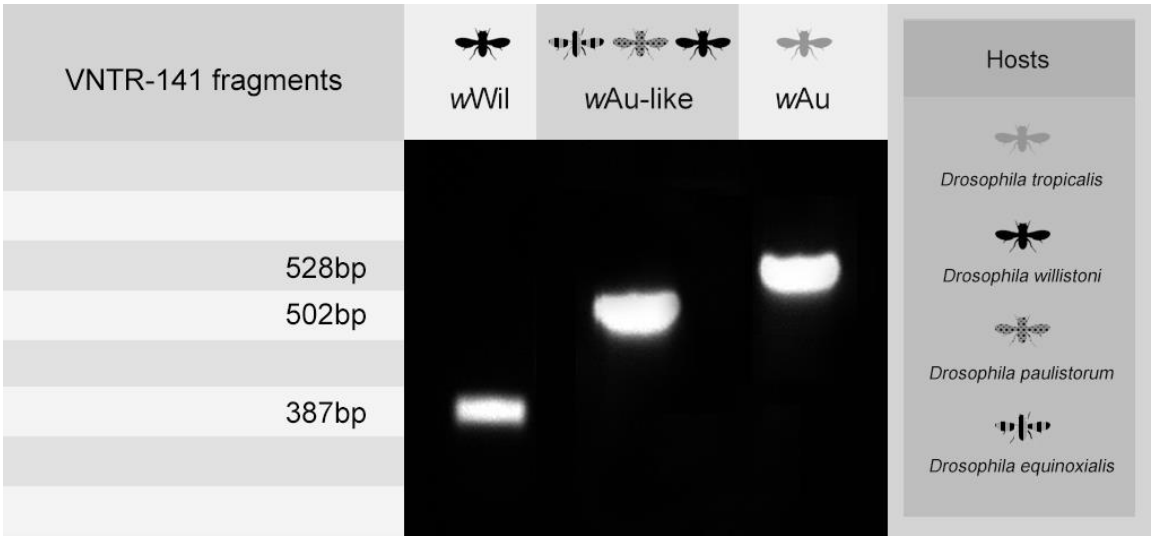

Figure 2. *VNTR-141* fragments representing *Wolbachia* strains found in Neotropical flies from the *willistoni* subgroup. PCR-amplification shows *w*Wil (387 bp), *w*Au-like (502 bp) and *w*Au (528 bp) *VNTR-141* fragments. Icons above PCR fragments represent *Drosophila* hosts where such strains were found, either in this study or in Müller *et al*. (2013).

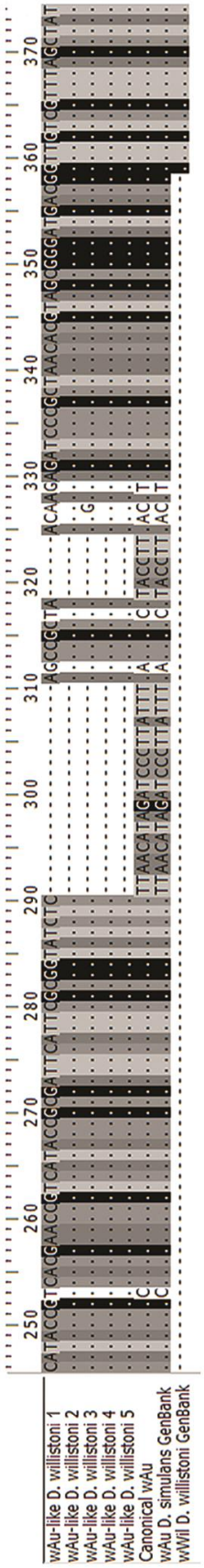

Figure 3. Partial alignment of *VNTR-141* fragments representing *Wolbachia* strains found in Neotropical flies from the *willistoni* subgroup. The alignment specifically depicts a portion of the fragment where a large block of nucleotides has been lost in *w*Wil compared to canonical *w*Au. Furthermore, the 26-bp deletion in *w*Au-like is located within the same fragment region

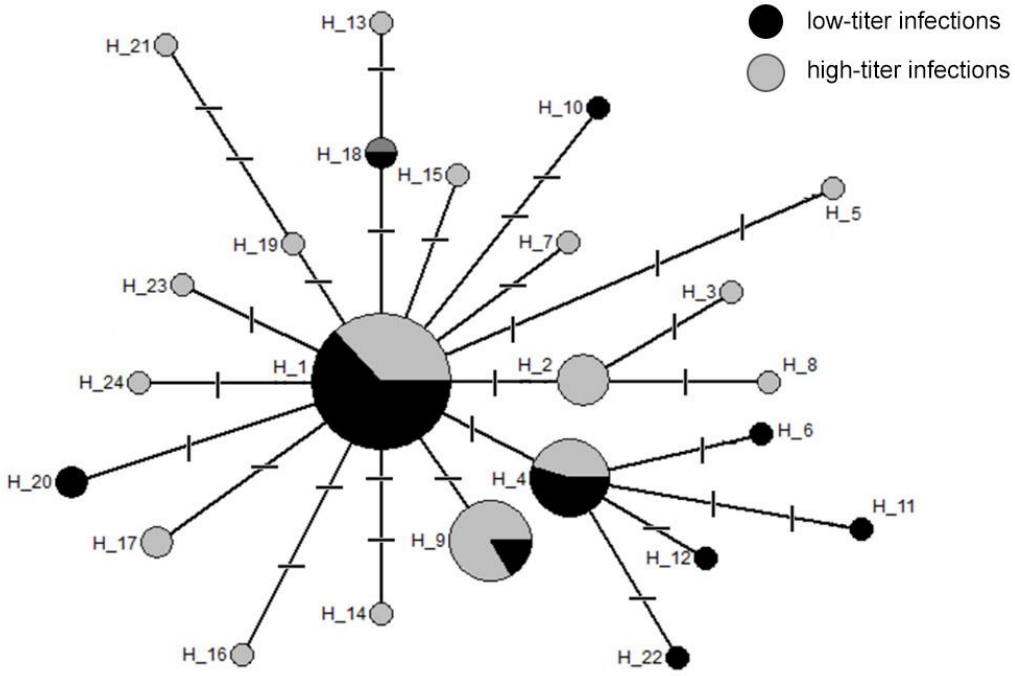

Figure 4. Haplotype network based on *Cytochrome Oxidase I* gene from *Drosophila willistoni* isofemale lines. The network was constructed by the Median-Joining method, combining data from fly lines collected in Osório (this study) with sequences from fly lines collected in the Atlantic and Amazonian Rainforest. Low and high-titre *Wolbachia* infections are represented by black and grey colours, respectively. Size of the circles represent the number of individuals, and crossing lines in branches represent the mutational steps. Low and high-titre infections do not depart from a 1:1 segregation (χ^2 = 0.64; p = 0.001), and are not associated to specific *COI* haplotypes.

CAPÍTULO 3

Wolbachia polymorphic infections in *Drosophila willistoni* populations: an unexpected journey.

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Artigo em preparação para submissão à revista **BMC Biology.**

Wolbachia polymorphic infections in *Drosophila willistoni* populations: an unexpected journey.

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Abstract

Background

The bacterium *Wolbachia* is known to cause reproductive manipulations upon its arthropod hosts increasing its own frequency. However, recent evidence has demonstrated that it may develop more friendly relationships by genetic agreements established between the symbiotic partners. One of the most important characteristics of any symbiotic relationship is infection density, since the within-host symbiont replication has direct consequences upon the level of reproductive manipulation, the virulence degree and the endosymbiotic relationship's main characteristics. *Wolbachia* establishes interesting relationships with flies from the *willistoni* subgroup of *Drosophila*. It was recorded outstanding differences concerning the infection density among *D. willistoni* populations, with some isofemale lines presenting a high density infection by *w*Wil *Wolbachia*, while others are infected with the *w*Au-like strain in such low densities that are only rescued by hybridization techniques. Considering the importance of intracelular density to the symbiosis outcome, the maintenance of such polymorphic infections in sympatric populations establishes an interesting biological issue that should be investigated.

Results

We studied *Wolbachia* quantification dynamics during host ontogeny, considering each infection model that can be found in *D. willistoni*. We verified that its behaviour is density, rather than strain-specific. Besides, we observed a significant increase in *Wolbachia* titer during embryos and 20-day old female ontogenetic stages in high-titer infected flies, which is consistent with a transmission fidelity strategy. Furthermore, we developed a series of fitness experiments with the flies, aiming to verify if any of the infection models could be associated to a higher adaptative value from their hosts, which would in turn explain their maintenance in host populations. We verified no sign of sex-ratio distortions, proving there is no male-killing effect by these *Wolbachia* infections, and that these infections apparetly have no effect on host survival. On the other hand, we verified that the *w*Au-like low-titer infection is associated with a higher female fecundity.

Conclusions

We proposed that the two main infection types (high-titer *w*Wil and low-titer *w*Au-like) are being maintained in the host populations by an equilbrium between the higher fecundity by females infected with *w*Au-like and a higher efficiency of transmission by *w*Wil-infected flies.

Keywords

Endosymbiosis; Fecundity; Viability; Survival; Symbiont density; Ontogeny

Background

We can not deny our innate effort to categorize natural phenomena into conceptual boxes, as this makes considerably easier our understanding of all things that surround us. Nevertheless, associations between organisms are quite often blurry to categorize as they might not always fit assumptions that would include them in one box or another. Particularly blurry are symbiotic relationships, specially when one partner lives inside the other, in a system called endosymbiosis [1,2,3].

Endosymbionts are considered great evolutionary catalysts since they helped to adapt the evolution of a variety of complex organisms as we know today. Among them, special attention should be given to bacteria that live exclusively inside host cells, using the maternal cytoplasm to insure their own transmission to the offspring. These organisms are quite impressive due to the wide range of evolutionary strategies they developed to relate with their hosts, from cozy and quiet partnerships until continuous arms races [2,4]. One side of the continuum of possible symbiotic strategies comprises mutualist endosymbionts which may help during the development and survival of their hosts, in relationships that often go a long way back in evolutionary history [5]. On the other side are the so called reproductive parasites which propagate through host lineages manipulating their reproduction. These microorganisms often inflict an astonishing set of phenotypes in the host that ultimately benefit their own transmission to the offspring [2].

Among the classic reproductive parasites stands *Wolbachia pipientis* [6], an alphaproteobacterium from the Rickettsiales order that is famous due to its respectable armory of possible parasitic phenotypes it may induce upon a wide range of arthropod and nematode hosts [7]. *Wolbachia* may cause parthenogenesis, feminization, male killing or

cytoplasmic incompatibility (CI), all of which ultimately favor the females and therefore *Wolbachia*'s own propagation since it is mainly maternally transmitted [8,9,10,11].

However, even though *Wolbachia* is considered a reproductive parasite and thus quite harmful to their hosts, accumulating examples have shown that it may not always be the Smaug of endosymbionts. *Wolbachia* is known to develop mutualistic relationships with nematodes, in fact being indispensable for proper development and fertility [12,13]. It may also have only negligible effects [14] or behave as a neutral variant in host populations [15]. Furthermore, *Wolbachia* may increase host fitness for instance by providing ATP, increasing longevity [16] and fecundity [17,18], protecting against virus [19,20], or even being indispensable for host oogenesis, as demonstrated by Dedeine *et al.* [**21]** in *Wolbachia* associations with the parasitic wasp *Asobara tabida.*

Since *Wolbachia* may establish such discordant relationships with its hosts, it is natural to imagine the intrincate net of interactions that may be regulated in order to result and maintain those symbiotic outcomes. In fact, the characteristics of an host-bacteria association may be understood as different outcomes in the negotiation of the genetic conflicts established between them, particularly if we consider that hosts and symbionts quite often have different evolutionary interests [22,23,2].

Moreover, an important feature of any symbiotic negotiation is the regulation of the symbiont density inside host cells. The density and tissue distribution of *Wolbachia* within its hosts is thought to determine a number of characteristics of the host-endosymbiont association [24] such as the expression of cytoplasmic incompatibility and the virulence of *Wolbachia* to its host [25,26,27,28,29]. Furthermore, in order to maintain *Wolbachia* infections inside host populations, it should be ensured the proper transmission fidelity along hosts generations. The assurance of an appropriate symbiont transmission to the offspring is dependent upon the maintenance of a proper replication rate and density level inside host cells, since low densities might result in fail of transmission whereas excessive densities might lead to the host pathology or death [26,30]. Moreover, the replication control can be considered a defining feature that separates vertically transmitted symbionts from horizontally transmitted pathogens. While the former tend to develop a more quiet relationship with their hosts, since their own transmission is dependent upon host survival, horizontally transmitted pathogens perceive their hosts only as bridges to achieve new hosts, so the replication rate is not so regulated [26].

The regulation of *Wolbachia* density is hypothesized to be the result of highly complex interactions involving the host and symbiont genotypes, as well as the environment [31,32,33]. The specific mechanisms that underlie such replication control and the extent to which it is influenced by each variable considered are not well understood, although it has been proven that the host genotype in fact has a strong involvement [34,35,26,36,37]. Such involvement may be verified in the case of *Wolbachia* overreplication in the germinative tissues of hybrids, which makes them ultimately quite problematic. Since hybrids are the product of mixed genetic backgrounds from parental species, it is hypothesized that the equilibrium maintained between host and symbiont in the parents might get lost in the hybrids, leading to overreplication [38,39]. Furthermore, a well documented case is that *w*Mel, the *Wolbachia* strains that infects *D. melanogaster* and induces negligible CI effects, when transinfected to *D. simulans* induces high CI [34,40]. On the other hand, the naturally-occuring *D. simulans Wolbachia*, *w*Ri, that induces high levels of CI in its natural host, causes low CI when transinfected to *D. melanogaster* [41].

In populational terms, the rate of spread and prevalence equilibrium of *Wolbachia* infections depend mainly on the maternal transmission fidelity and the relative fitness of infected lineages, and both of these variables often depend on the endosymbiont density within hosts [42,43]. Therefore, the population dynamics of endosymbiont infections is likely to be governed by environmental and genetic variables that affect within-host density of these infections [42].

The regulation of within-host *Wolbachia* density might usually be investigated by either the exchange of *Wolbachia* strains between different hosts, thus conjecturing mostly on the host genotype influence, or by comparing distincts *Wolbachia* strains within the same host species [33]. Having this in mind, we have recently investigated the intimacies of a quite complex infection scenario developed by *Wolbachia* and Neotropical flies from the *willistoni* subgroup of *Drosophila*, which is comprised by six cryptic species that may occur in sympatry, particularly in some parts of northern South America [44,45]. In the specific case of *D. willistoni*, the most widely studied and known species from the subgroup, populations collected from geographically distant localities in Brazil all share the same pattern of infection, which is a polymorphism of infection densities maintained by different *Wolbachia* strains. While high-titer infections are held only by the *w*Wil strain of *Wolbachia*, low-titer infections are either established by the same aforementioned strain,

though mainly by a closely-related strain, called *w*Au-like. This same strain was also found to be infecting other subgroup species, such as *D. tropicalis* in high titer [46], *D. paulistorum* in either low or high-titer, and *D. equinoxialis* only in low densities (data being prepared for publication).

Furthermore, our characterization of the infections established in those flies also showed that in the particular case of *D. willistoni*, for which we had a more robust dataset, infection density status (low and high-titer) did not display any sort of correlation with mitochondrial haplotypes from the flies, thus suggesting that the scenario was not established due to a *Wolbachia*-conducted selective sweep (data being prepared for publication).

We have proposed an explanation in which the low-titer *w*Au-like infection would be the oldest in *D. willistoni*, based on the evolution of a minisatellite marker and since this strain is quite widespread among Neotropical *Drosophila* species, and that *w*Wil would have evolved from *w*Au-like inside *D. willistoni* lines [47,46, data being prepared for publication]. Since we verified a roughly 1:1 segregation between low and high-titer infections in all populations evaluated, we proposed that *w*Wil might be replacing *w*Au-like, and that the observable scenario nowadays would be an intermediate point towards *w*Wil fixation. Although *Wolbachia* is classically considered as a reproductive parasite, accumulating evidence have demonstrated that *Wolbachia* may influence host fitness in many possible positive ways. On that grounds, we conjectured if the *w*Wil infection would be providing higher fitness profits to the flies when comparing to *w*Au-like effects, thus facilitating its way towards fixation, in a similar scenario as proposed by Fry *et al.* [17] to explain the worldwide *Wolbachia* distribution among *D. melanogaster* populations without perceivable cytoplasmic incompatibility induction.

Therefore, considering the major importance of *Wolbachia* density in the symbiosis mantainance and outcome, we performed qPCR assays to unravel *Wolbachia*'s behavior during host ontogenesis for each symbiotic system, in order to deepen our knowledge concerning the dynamics of this association during host development. Furthermore, in order to access the phenotypic effects of all the *D. willistoni* - *Wolbachia* associations considered, we evaluated classical fitness in flies from all these symbiotic systems, aiming to evaluate if the apparently youngest infection *w*Wil improves host fitness, which would greatly facilitates its propagation throughout host populations.

Our results show that *Wolbachia* behavior during *Drosophila* ontogeny has suggested to be density rather than strain-specific, and that high-titer infections apparently increase their density during ontogenetic stages that are critical to insure transmission. Moreover, our fitness results disproved our first evolutionary scenario, although we were able to propose a quite plausible alternative, that explains the maintenance of different infection types by a counterbalance mechanism between fitness profits by one infection type and more effective transmission by the other.

Results and Discussion

Wolbachia ontogenetic behaviour is density, rather than strain-specific

Measuring the within-host *Wolbachia* density is perhaps the most direct way to access the host-symbiont evolutionary dynamics, since this information can shed light on the virulence degree and maternal transmission efficiency of the infection, for example [33]. Moreover, considering the complex infection scenario maintained by *Wolbachia* strains and within-host densities among *D. willistoni* populations throughout Brazil [46], we assume that deepen our knowledge in what concerns *Wolbachia* titer oscillation during host's ontogeny would render meaningful statements towards understanding the maintenance of such symbiotic models. Specifically, we expect that the quantification of *Wolbachia* during ontogenetic stages can shed light on the possible mechanisms that might be responsible for the discrepancies in infection titer we verify during the female adult stage in the field and in the laboratory [46].

As mentioned before, *Wolbachia* mantains at least three models of symbiotic interactions with *D. willistoni* flies: (i) High-titer infection by the *w*Wil strain; (ii) Low-titer infection by *w*Wil; (iii) Low-titer infection by *w*Au-like strain. These categories were established after evaluation through PCR and blot-PCR hybridization of either old-female fly DNA or 15-ovaries DNA [data being prepared for publication], so we conjectured on how these types of infection would behave during their hosts' ontogeny. Although proximately related, the *Wolbachia* strains reported in theses systems were shown to behave differently during their hosts' embryonic development. While *w*Au infects both germ line and somatic tissues of *D. simulans*, *w*Wil is found exclusively in the primordial

germ line cells of *D. willistoni* embryos [47], so there is no reason not to expect some sort of different ontogenetic behaviors due to relatadness.

We used two independent "biological replicates" for each symbiotic model considered, that is, two *D. willistoni* isofemale lines that have different mitochondrial haplotypes but harbour the same *Wolbachia* infection type (strain and density). Although these biological replicates did not behave perfectly equal when considering *Wolbachia* quantification among ontogenetic stages, specifically in what concerns the absolute quantification itself, we were able to estimate an average number of *wsp* (*Wolbachia Surface Protein* – gene used in qPCR assays) copies for each stage between both replicates and to state some interesting comparisons among the infection types. Furthermore, since we had *D. paulistorum* isofemale lines harbouring *w*Au-like *Wolbachia* in high-titer, that is, an infection type not found among *D. willistoni* isofemale lines, we also submit them to *Wolbachia* ontogenetic quantification for comparison purposes. We depict below the main findings for each infection model.

Drosophila willistoni* lines infected with low-titer *w*Wil *Wolbachia

Both *D. willistoni* isofemale lines infected with low-titer *w*Wil *Wolbachia* presented a remarkably low copy number among the ontogenetic stages evaluated, ranging from around 2 to 100 *wsp* copies. When considering the average number of *wsp* copies from both replicates in each stage (Fig. 1), ANOVA results showed that *Wolbachia* quantification significantly oscilated during the ontogeny of these flies ($F_{7,32}$= 2.782; p = 0.02228). It is possible to verify that the lowest *Wolbachia* quantification was verified in 20-day old females, although this quantification did not differ significantly from embryos, female larvae, pupae, virgin male and 20-day old male quantification by Tukey's range test. It is particulary interesting to verify that while 20-day old females presents the lowest *Wolbachia* titer, virgin females showed the highest *Wolbachia* infection density (which did not statiscally differed from male larvae), which shows that the density level inside female host cells decreases with age. On the other hand, virgin and 20-day old males did not consistently differed regarding *Wolbachia* quantification.

Drosophila willistoni* lines infected with low-titer *w*Au-like *Wolbachia

Even though we used two isofemale lines both considered as infected by low-titer *w*Au-like, one displayed a considerable lower *Wolbachia* density than the other. The isofemale line infected with a subtly higher density sometimes showed more abrupt differences between ontogenetic stages but they both maintained roughly the same behaviour pattern along the entire ontogeny. Furthermore, as the within-groups variance was considerably high, neither ANOVA ($F_{7,36}$= 1.878; p = 0.1051) nor Kruskal-Wallis (H= 7.713; p= 0.3586) were able to rescue significant differences among groups, although we may verify some differences by visual verification (Fig. 2).

In agreement with low-titer infection by the *w*Wil strain, embryos showed a rather low infection density, ranging from 3 to 15 *wsp* copies. In addition, *Wolbachia* quantification increased when entering the larvae stages, with higher density in male larvae according to what was seen in the aforementioned low-titer infection by *w*Wil. Pupae had a somewhat average infection between female and male larvae, but this density was followed by a dramatic decrease when entering the virgin female adult fly stage, which had an indistinguishable infection density when comparing to 20-old female flies. As regards to male fly samples, virgin male had the highest *Wolbachia* infection recorded, but it apparently decreases with age (Fig. 2).

Drosophila willistoni* infected with high-titer *w*Wil *Wolbachia

Both biological replicates showed common features and comparable *Wolbachia* oscillations during host ontogeny, and ANOVA confirmed the symbiont titer significantly oscillated during the ontogenetic stages considered ($F_{7,40}$= 9.382; p = $7.921e^{-7}$).

Wolbachia quantification on embryos was remarkably high, in fact being the highest quantification recorded for both lines evaluated (Fig. 3). Interestingly, the infection density dropped tremendously when we look at the larvae stages (especially in the case of female larvae, which was considered as lowest average value, although it did not statiscally differed from male larvae sample). Pupae sample showed a subtle increase in *Wolbachia* copies, although it might have been biased since we can not distinguish pupae sex. Furthermore, pupae showed *Wolbachia* quantification that is not distinguishable in statistic grounds from the quantification in embryos, virgin female and 20-day old females. Moreover, when looking at the adult fly samples, we can see the classic *Wolbachia*

behaviour in *Drosophila*: a substantial increase in *Wolbachia* titer when considering female samples, and a contrasting decrease when looking at male samples, though these differences were not shown to be statistically relevant. It is known that *Wolbachia* density tends do decrease in male testes during aging in *Drosophila*, which correlates with a diminished cytoplasmic incompatibility induction in old males, when this phenotype occurs [48]. On the other hand, it is hypothesized that *Wolbachia* titer in females should increase with age and thus reproductive season, therefore improving the chance of *Wolbachia* proper transmission to the offspring [49].

Drosophila paulistorum* infected with high-titer *w*Au-like *Wolbachia

Here we evaluated the the ontogenetic behaviour of a high-titer *Wolbachia* strain that is found in low-titer in *D. willistoni*, so the following results have general grounds comparison purposes, since we are evaluating this strain behaviour against a different host genetic background. High-titer *w*Au-like *Wolbachia* was shown to significantly oscilate during ontogenetic stages of *D. paulistorum* (ANOVA $F_{7,46}$= 15.04; p = 4.994e-^{10}) (Fig. 4). First of all, *Wolbachia* quantification on the embryos is much more similar to the *w*Wil high-titer infection rather than the low-titer infection by the same strain both found in *D. willistoni*, namely the highest density among all ontogenetic stages, being also indistinguishable from 20-day old female fly quantification at Tukey's range test. Furthermore, *Wolbachia* density in larvae stages is almost indistinguishable between female and male samples, and the same holds until the pupae stage. Male and female virgin flies displayed a quite similar *Wolbachia* density, but their destiny towards 20-day old stage where quite the opposite, with an increased density in female samples and decreased in male samples (and this was the lowest density found in this system), the same pattern found in the high-titer infection performed by *w*Wil in *D. willistoni*.

Probably the most remarkable feature concerning the comparison of all ontogenetic *Wolbachia*-quantifications here reported is the fact that both low-titer infections by either *w*Wil or *w*Au-like *Wolbachia* behave quite similarly during *D. willistoni* ontogeny, and the same holds for high-titer infections, even though the *w*Au-like infection is maintained against a different host genetic background. This finding leads us to assume that the

control of *Wolbachia* replication, at least considering the systems here evaluated, is more density-specific rather than strain-specific.

Although it is possible to verify a fluctuation in the symbiont's titer during the ontogeny of isofemale lines infected with low-titer *Wolbachia*, such variation is much more pronounced in high-titer infections (Y-axis of all graphs). In fact, this feature is so clear that some ontogenetic stages from the latter systems displayed *wsp*-quantifications in amounts that are similar to those observed throughout the development of isofemale lines infected with low-titer *Wolbachia*. In order to gain deeper knowledge on this matter, we performed statystical analyses that compared the number of *wsp* copies among symbiotic models considering each ontogenetic stage separetely, as follows.

Increase in *Wolbachia*-titer in embryos and 20-day old females in high-titer infected *Drosophila* lines is consistent with a transmission fidelity strategy

The comparison of *wsp* copies among symbiotic system when considering each ontogenetic stage presented some interesting insights. The clearer feature when comparing low and high-titer *Wolbachia* infections is seen in the evaluation of embryos (Kruskal-Wallis: H= 18.47; p= 0.00035) and 20-day old female samples (Kruskal-Wallis: H= 14.71; p= 0.002086) (Fig. 5), when the infection density factor clearly separates the symbiotic models in two groups.

This finding is consistent with field populational surveys where old females are used in the screening for *Wolbachia*, where high-titer infections are easily rescued by standard *wsp*-PCR using single-female fly DNA, while low-titer infections are only confirmed after *wsp* blot PCR hybridization using 15-ovaries DNA [46, data being prepared for publication).

Moreover, it is quite meaningful to observe that the highest *Wolbachia* quantifications in high-titer infected flies were recorded precisely in embryos and 20-day old females, which are the most relevant ontogenetic stages when considering the need of a high fidelity of transmission to the offspring. 20-day old females represent flies in their reproductive age and, as *Wolbachia* is maternally-transmitted, an increase in *Wolbachia* titer would be evolutionary advantageous to insure transmission to the offspring. The same holds for embryos, that ultimately came directly from 20-day old females. They have the

load of *Wolbachia* that will be further distributed among cells during the fly development, thus a high density would insure the maintenance of infection.

Furthermore, comparison between *Wolbachia* quantification in virgin females also displays a statistically significant difference among infection models, altough with a rather less clear categorization among density types (Kruskal-Wallis: H= 15.44; p= 0.00147) (Fig. S1). High-titer infections are indistinguishable from each other even when considering two different *Wolbachia* strains in two different host species, the same we observed for embryos and 20-day old females. Moreover, low-titer infection by *w*Au-like in *D. willistoni* was the lowest quantification recorded, whereas low-titer *w*Wil was not able to be differentiated from either of the other categories. Virgin females were obtained by DNA extraction from newborn flies, far from reproductive age. As it is known that young virgin flies might display quite pronounced oscilation in *Wolbachia* titer [43,33], it is possible that this quantifications here recorded are not representative of a whole-population sample.

As regards to *Wolbachia* quantification in male flies, virgin males from each infection system did not display so clear categorization by infection density, although showing significant differences among types (Kruskal-Wallis: H= 9.048; p= 0.02866). While *w*Au-like high-titer infection in *D. paulistorum* and *w*Wil low-titer infection in *D. willistoni* are clearly different, high-titer *w*Wil and low-titer *w*Au-like are indistinguishable from each other and from each of the others (Fig. 6). In 20-day old males, as mentioned previously, even highly infected isofemale lines displayed rather low *Wolbachia* quantifications (Kruskal-Wallis: H= 9.358; p= 0.02489), such that although high-titer infected *D. paulistorum* presented the highest quantification recorded after Mann-Whitney pairwaise comparison, the likewise highly infected by *w*Wil *Wolbachia* males showed the lowest. Both low-titer infections remained indistinguishable from each other and from other categories (Fig. 6). The remarkable decline in *Wolbachia* titer with age in *Drosophila* males infected with high-titer *Wolbachia* (*w*Wil for *D. willistoni* and *w*Au-like for *D. paulistorum*) is in accordance with several cases reported to a variety of hosts in the literature [50,51,52,47]. In the specific cases of CI-inducing *Wolbachia,* the maintenance of *Wolbachia* infection during reproductive age in males might have lethal effects since crosses with incompatible females would ultimately render lack of transmission of its own nuclear genes to the offspring. Therefore, theory suggests that selection would act on host

nuclear genes in a way that would prevent such events, for example by decreasing *Wolbachia* titer in males before they are sexually mature [53,52]. Although neither *w*Wil nor *w*Au (-like) *Wolbachia* are known to express CI in their hosts [47], the occurrence of such phenotype has not yet been tested in our models of study, and worth being investigated in the future.

Considering larvae stages, while only females from *w*Au-like high-titer infected *D. paulistorum* were distinguishable from the remaining samples (ANOVA: Kruskal-Wallis: H= 17.19; p= 0.00064), male larvae from all infection models were indistinguishable under statistic grounds (Kruskal-Wallis: H= 3.701; p= 0.2957) (Fig. S2).

Finally, considering pupae, although this might be a biased quantification since it is not possible to distinguish pupae sex and therefore the sample might have more individuals of one sex or the other, high-titer infected samples regardless the strain were considered to be more infected than low-titer infections (Kruskal-Wallis: H= 9.499; p= 0.0233). Low-titer infection by *w*Au-like was not able to be clearly differentiated from either other categories, whereas *w*Wil low-titer *D. willistoni*-infected displayed the lowest quantification of all (Fig. S3).

In the case of *w*Au-like infections, it is quite interesting to verify that we could only retrieve this strain infecting *D. willistoni* flies with extremely low-titer. In fact, our whole line of hypothesis assumes that this infection is older inside this species, and that the *w*Wil might have evolved from it inside this host lineage. On the other hand, we can verify *w*Au-like infecting *D. paulistorum*, a closely-related species [review in 54] in either low or high-titer [data being prepared for publication]. It is possible that the *w*Au-like infection sometime had higher densities in *D. willistoni*, but we might not see it anymore. Unckless *et al.* [43] verified a substantial (20,000-fold) variation in male-killing *Wolbachia* in *D. innubila* flies from Arizona, and a few flies were shown to carry extremely low *Wolbachia* densities, which they suggested to be relicts from previously robust infections, since low-titer infections were often characterized by low fidelity of *Wolbachia* transmission and low efficiency of male-killing. Nevertheless, we can not rule out the possibility that in fact the host genotype plays an important role regarding the replication control inside host cells, since the exactly same strain remains in extremely low density in *D. willistoni*.

Although considering some pronounced differences in some specific cases, the general picture obtained from our qPCR results suggests that *Wolbachia* has a density rather than strain-specific behaviour during the ontogeny of *Drosophila* hosts. However, this finding does not imply that the specific cellular dynamics performed by either strain (*w*Wil or *w*Au-like) within each density are equal, since we performed whole-body measures rather than tissue specifications, and we also did not perform immunostaining localization in order to verify the specific localization of *Wolbachia* inside tissues, as has been done in embryos, for example [47].

Wolbachia does not induce male-killing in the evaluated fly lines

We verified no signs of female-biased sex-ratios in offsprings of *D. willistoni* isofemale lines with different *Wolbachia* infections, as follows: low-titer *w*Wil (Iso1: p= 0.142 and Iso 2: p= 0.676), high-titer *w*Wil (Iso 1: p= 0.549; Iso 2: p= 0.54); low-titer *w*Au-like (Iso 1: p= 0.482; Iso 2: p= 0.365). Furthermore, we also did not verified biased sex-ratios in the biological replicates of *D. paulistorum* infected with high-titer *w*Au-like (Iso 1: p= 0.2; Iso 2: 0.382). Therefore, we assume *Wolbachia* is not inducing male-killing in the evaluated fly lines.

Wolbachia infection models do not influence host survival

We constructed Kaplan-Meier survival curves and plotted the probability of survival of *D. willistoni* flies infected with different *Wolbachia* infection models against time, considering data from two independet biological replicates (comprised of eight experimental replicates with 20 flies each) for all infection models, so we evaluated around 320 individual flies per infection model. Survival curves from *D. willistoni* isofemale lines infected with low and high-titer *w*Wil and low-titer *w*Au-like *Wolbachia* are presented in Figure 7, and these results combined with data from *D. paulistorum* infected with high-titer *w*Au-like are presented in Figure S4 (Supplementary Material). Survival data from each infection model were pairwaise compared with each other by log-rank and Wilcoxon tests. None of the comparisons rendered significant results (Table S1).

Even though statistical analysis did not accuse a higher or lower survival probability of flies from any of the *Wolbachia* infection models considered, *D. willistoni* flies infected with low-titer *w*Wil presented a survival curve (Fig. 7) that visually

negatively departs from the others. In fact, the average hazard of death from this category is slightly higher (0.00315) than either high-titer *w*Wil (0.00268) or low-titer *w*Au-like (0.00159) infected flies, and the average time to death is less (50.21 days) than either high-titer *w*Wil (74.55 days) or low-titer *w*Au-like (82.8 days) infected flies.

Low-titer *w*Au-like *Wolbachia* infection is associated with a higher female fecundity

The number of eggs laid by flies during a 12-days period was recorded for each symbiotic model (comprising two independent biological replicates with 8 experimental replicates each), and they were further compared by Kruskal-Wallis followed by Mann-Whitney test. Outlier values (less than 5 eggs/ evaluation) were excluded from analysis. Kruskal-Wallis results prove there was a statistical difference regarding the fecundity (average number of eggs laid) among symbiotic models (H= 9.04; p= 0.01086). As can be seen in Figure 8, *D. willistoni* isofemale lines infected with both low or high-titer *w*Wil *Wolbachia* presented statistically indistinguishable fecundity measures after Mann-Whitney pairwaise comparisons (p= 0.977). Low-titer *w*Au-like infections, on the other hand, renderend almost the double of eggs considering the same time frame, significantly departing from fecundity measures for either low-titer *w*Wil (p= 0.01233) or high-titer *w*Wil (p= 0.009776) infected *D. willistoni* flies. Furthermore, when including high-titer *w*Au-like infected *D. paulistorum* isofemale lines (H: 22.75; p= $4.553e^{-5}$), their fecundity measures grouped with low-titer *w*Au-like infected *D. willistoni* lines (p= 0.085750) (data not shown in graphs), but in this case the higher fecundity measures might be due to the host's features rather than due to *Wolbachia* infection differences.

Although it is known that high *Wolbachia* densities might have negative effects upon host fitness [26], we can disregard that the higher fecundity in *w*Au-like infections is explained by its low density level alone, since fecundity of isofemale lines infected with *w*Wil in equally low densities are indistinguishable from high-titer infections by the same strain.

Moreover, we analysed the fraction of eggs laid by flies from all symbiotic systems that rendered adult flies, thus representing the viability. However, we recorded a tremendously low viability for all models evaluated, with repeating zero-viability records, thus any statistical inferences were arrested. Nevertheless, we could verify that low-titer *w*Au-like infected *D. willistoni* lines displayed subtly higher average viability measures,

around 12%, while low and high-titer *w*Wil infected lines showed 4,5% and 0% viability, respectively. We can not discard the possibility that both fecundity and viability measures are dependent on each other, since coincidentally low-titer *w*Au-like infections, which showed the highest fecundity, also showed the highest viability. However, we prefer not to state any assumptions on this matter, and to further rely on additional experiments to confidently declare any substantial differences regarding viability measures among symbiotic systems.

Wolbachia polymorphism of strains and densities is probably maintained by a balance between fecundity and transmission fidelity

We have recently proposed a model to explain the evolutionary scenario maintained by *Wolbachia* infections among *D. willistoni* populations. We claimed that the rough equilibrium between low and high-titer infections is in fact probably an intermediate point towards *w*Wil fixation, since this strain would have evolved from *w*Au-like inside *D. willistoni* host. Furthermore, in this paper we aimed to verify if high-titer *w*Wil could cause any slight fitness benefit to its hosts, which would facilitate its population dissemination towards fixation. However, our experimental results disproved our hypothesis since, unlinke what we expected, the forementioned *w*Au-like strain that was hypothetically being replaced in fact was associated to a higher female fecundity when compared to both high and low-titer infections by *w*Wil, which in turn are indistinguishable by those measures.

Based on theoretical and empirical assumptions, *Wolbachia* dynamics through host populations depends mostly on (i) the level of cytoplasmic incompatibility (CI) induction, (ii) the maternal transmission fidelity and (iii) fitness benefits. Furthermore, infection dynamics models also predict that *Wolbachia* strains that do not cause cytoplasmic incompatibility or male-killing-derived sex-ratio distortions should be lost from their host populations unless the transmission efficiency from mothers to offspring is perfect or the infection causes any fitness advantage [55,56,53,57,58,37].

Given that neither *w*Wil nor *w*Au-like are known to induce CI in Neotropical *Drosophila* flies [47], and we found no signs of male-killing effects on the offsprings of all evaluated isofemale lines, we shall further consider only transmission efficiency and fitness effects as possible mechanisms to explain *Wolbachia* maintenance in *D. willistoni*

populations. That said, if we previously considered that high-titer *w*Wil should increase host fitness and this would facilitate its dissemination, our new findings may otherwise agree with a new scenario where the higher fecundity by *D. willistoni* females infected with low-titer *w*Au-like *Wolbachia* is in fact preventing *w*Wil dissemination throughout the host populations, probably maintaining the roughly 1:1 segregation pattern between high and low-titer infections that is seen in populations from geographically distant locations from Brazil [46, data being prepared for publication].

This scenario does not consider low-titer infections by *w*Wil as they are populationally rare, although our screening may not be geographically relevant [data being prepared for publication]. Nevertheless, this lower prevalence of low-titer infections by *w*Wil at the populational level might in fact be true and could be explained by the considerably lower fecundity from its infected females and lower survival probability of infected flies, even though such results were not statistically relevant (Fig. 7, 8; Table S1). This matter could be more deeply evaluated in the future; if these findings prove to be correct, this infection combination is expected to be excluded from host populations.

Furthermore, our *Wolbachia*-quantification throughout host ontogeny results have shown that the infection by high-titer *w*Wil reaches its higher density precisely during embryos and 20-day old females stages, which represent host stages that are critically involved in *Wolbachia* transmission to offspring. On the other hand, this is not seen in the *wsp*-quantification in neither *w*Wil nor *w*Au-like low-titer infections, where *Wolbachia* quantification at first seems to oscilate during host ontogeny with no specific transmission strategies. Therefore, it is plausible to assume that, although we did not perform specific experiments in the lab, the transmission fidelity in low-titer infected flies is less effective than high-titer, as has been proposed elsewhere [43].

Since *w*Wil is most frequently found in a high within-host density, and is thus expected to have a higher transmission fidelity, it is plausible to assume that both *Wolbachia* strains (*w*Wil and *w*Au-like) and densities (high and low) are being maintained at the *D. willistoni* populational level by a balance between a higher fecundity by *w*Au-like-infected females and a higher transmission fidelity by *w*Wil-infected females. Charlat *et al.* [58] investigated *D. yakuba* populations from Africa infected by a non-CI-inducing *w*Au-like *Wolbachia* and explained its maintenance in host populations as a neutral variant due to its high maternal transmission efficiency, whereas fecundity effects were negligible.

Their findings match with what we found in high-titer infections by *w*Wil in *D. willistoni*, although our scenario also includes the population counterbalance with higher female fecundity associated to low-titer infections by *w*Au-like.

Moreover, *Wolbachia* theoretically must cross three filters in order to be established in a new host species, (i) ecological, which corresponds to the interaction between an existing and a potential new host species; (ii) physiological, which considers the ability of *Wolbachia* to colonize the germ line of an individual, and (iii) populational, which conditions the ability of *Wolbachia* to invade and maintain itself in the host population [Combes apud 37]. Therefore, considering that we assume the *w*Wil infection arised inside *D. willistoni* itself, specifically from the *w*Au-like *Wolbachia*, it is plausible to assume that *w*Wil in fact used an evolutionary shortcut on its way to populational dissemination, since both the ecological and physiological filters were subdued by its mode of appearance, if our presuppositions are correct.

Furthermore, even though *w*Wil does not cause fitness advantages on its hosts (at least not for the markers we evaluated), we could assume that it increased in frequency at the populational level using its higher power of transmission fidelity and is now being maintained by counterbalancing the higher fecundity by low-titer *w*Au-like-infected females. Moreover, as quickly mentioned before, it is worth to note that we did not performe exhaustive experimental assessment of fitness markers that could prove high-titer *w*Wil infection to be causing some fitness improvement that we thus are not considering here. For example, we could have also tested egg-to-adult developmental time, larval viability, body size, resistance to environmental stresses, and so on [15,59,33]. Besides, we can not confidently state that *w*Au-like infection increases host fecundity or if *w*Wil infection diminishes it, principally if we consider that, under our scenerio, *w*Au is the oldest *Wolbachia* infection in the species, and might thus be more familiarized with *D. willistoni* hosts. Either way, the evolutionary hypothesis to explain the polymorphic *Wolbachia* infection for this species remains valuable regardless the specific infection effect upon fecundity.

Also, we also did not assess if flies infected with different *Wolbachia* strains and densities might experience any level of incompatibility when crossed, which would also help to explain their maintenance at the populational level. Considering that it has been previously postulated that there is an association between levels of incompatibility and

density of *Wolbachia* in cells [60], though not in all cases [15], and although neither *w*Wil nor *w*Au are considered as CI-inducing strains, it is possible that crosses between flies infected with either strain might render less viable offsprings when compared to parental crosses, thus producing a populational subdvision.

Conclusions

We end this paper by confirming the primary assumption we had in mind when started working on the *D. willistoni – Wolbachia* interaction system: it shall not be easy! Even though this was an unexpected journey, our results were able to state some new grounds that improved our knowledge on this system. (i) We verified that *Wolbachia* in fact behaves differently during *Drosophila* species ontogeny in a density rather than strain-specific manner; (ii) High-titer *Wolbachia* apparently increases its density especially during ontogenetic stages that are critical to insure vertical transmission, while low-titer *Wolbachia* oscilates quite randomly during the host ontogeny; (iii) The observed *Wolbachia* infection models do not cause female-biased sex-ratios in their hosts; (iv) *Wolbachia* infection does not seem to affect host survival in a significant way; (v) Low-titer infection by the *w*Au-like strain is associated to higher female fecundity, when compared to *w*Wil infections either in low or high-titer; (vi) We propose a new model for the maintenance of different *Wolbachia* infection types in *D. willistoni* populations, which is the spread of the presumably highly transmissible high-titer infection by *w*Wil being controlled by the higher female fecundity by low-titer *w*Au-like infection, thus permiting both infection types to occur side-by-side at the populational level.

It is already difficult to frame *Wolbachia* in understandable boxes after a first look in all the interactions it may establish with different host species. Who would say that such framing would prove even harder after knowing the intimacies of an infection system a little bit deeper?

Methods

Flies collection, isofemale lines establishment and identification of *Wolbachia* infection

Flies were captured from the field in an Atlantic Forest site located in Rio Grande do Sul, Southern Brazil (Osório city, 29º53’08.20’’ S, 50º16’39.81’’ W) in May 2013,

using appropriate methodological approaches [61]. *Drosophila willistoni* flies were identified through hypandrium shape examination [62,63] and sequence analysis from the mitochondrial gene *Cytochrome Oxidase I* (*COI*). Isofemale lines were further established in the lab in standard cornflour and yeast medium [64], maintained in Biochemical Oxygen Demand (B.O.D.) chambers at 19 °C with a 12/12-h photoperiod.

Wolbachia infection was confirmed by standard PCR or PCR-blot hybridization in the case of high or low-titer infections, respectively, and strain identification was carried out by the amplification and cloning of a minisatellite marker [65] that is able to distinguish between closely related *Wolbachia* strains [data being prepared for publication].

We were able to identify two *Wolbachia* strains maintaing different infection levels (high or low density), in combinations that we will further refer to as "infection models", as follows: (i) *w*Wil high-titer infection; (ii) *w*Wil low-titer infection; (iii) *w*Au-like low-titer infection. Furthermore, two isofemale lines infected with each of those infection models (biological replicates) were separated from the others and maintained in bigger cornflour medium tubes, which were changed and supplemented with yeast twice a week, in order to increase fly population. It is worth to mention that each isofemale line replicate from each system considered had different mitochondrial haplotypes.

Furthermore, for comparison purposes, we also included in all experiments two biological replicates of *D. paulistorum* Andean-Brazilian (AB) isofemale lines infected with high-titer *w*Au-like *Wolbachia*, thus representing a symbiotic model not found in *D. willistoni*, but that contains a *Wolbachia* strain and an infection density that are found separetely in the latter.

Wolbachia quantification during *Drosophila* ontogeny

We aimed to verify *Wolbachia* dynamics during *Drosophila* ontogenetic stages, and to compare such dynamics among the symbiotic models here considered (two biological replicates of each system: low and high-titer *w*Wil; low-titer *w*Au-like for *D. willistoni*; high-titer *w*Au-like for *D. paulistorum*), so we performed a *Wolbachia*-absolute quantification by real-time PCR using DNA extracted from each developmental stage, as follows.

DNA extraction

DNA was extracted using NucleoSpin® Tissue Kit (Macherey-Nagel, Düren, Germany) or UltraClean® Tissue & Cells DNA Isolation Kit (MO BIO Laboratories, Inc., Carlsbad, CA, USA) according to the manufacturer's instructions. We collected samples from the ontogenetic stages of each isofemale line according to appropriate methodologies, as follows.

Embryos

We placed adult flies in "embryos collection chambers" comprising clock glasses cointaing oviposition medium (1.5% agar; 15% w/v honey; 0,3% w/v propionic acid; *Ponceau* dye) and yeast, which was covered by an upsidedown glass vial to hold the flies in. After 16 hours, we collected around 80 embryos using needles and tweezers under stereomicroscope, which were transferred to a 1.5-mL tube containing 400 µL of 70% ethanol. Embryos were rinsed twice with 70% ethanol prior to DNA extraction [66,38].

Third-instar larvae

We collected third-instar larvae directly from fly tubes, placing them in 70% ethanol in appropriate handling glass plates, and sexed them under stereomicroscope using tweezers by analyzing male gonads (testes), which are much larger than female gonads (ovaries) [67]. Fifteen male and female larvae were selected and separated into 1.5-mL tubes containing 400 µL 70% ethanol. Larvae were rinsed twice with 70% ethanol prior to DNA extraction. To confirm larvae sex, we amplified the Y-linked gene *kl3* (male fertility factor – codes to a heavy chain dynein) using the primers Kl-3Y_F 5'-AGC CAG GCG TTC AGT AAC AC-3' and Kl-3Y_R 5'-GAT GCC ATT GCT ACA GTT GAC TT-3'. PCRs were performed in 25 µL-reactions, using 2 µL DNA with 12.5 µL PCR Master Mix 2X (Fermentas, Lithuania) (0.05U/ µl Taq DNA Polymerase, 10X buffer, 4mM $MgCl_2$, 0.4mM of each dNTP), 1 µL of each primer (10 µM) and ultrapure water up to the final volume. A 624-pb fragment was amplified by the following cycling conditions: 95 ºC during 3 min, followed by 40 cycles of 95 ºC 1min, 60 ºC 1 min and 72 ºC 1 min 30 s, and final extension at 72 ºC during 5 min.

Pupae

We collected five pupae from fly tubes and directly transferred them to a 1.5-mL tube containing 400 µL of 70% ethanol. Pupae were rinsed twice with 70% ethanol prior to DNA extraction.

Adult flies

Virgin flies were collected from fly tubes and sexed under stereomicroscope after etherization; one female and one male were separated and directly transferred to a 1.5-mL tube and used to DNA extraction, and further considered "virgin" flies (male and female). Furthermore, a few virgin female and male flies were kept separetely in tubes containing medium during 20 days, after which we performed DNA extraction and further considered them as "20-day old" flies (male and female). We considered it important to include adults with different ages since previous work have shown *Wolbachia* titer oscilation with aging; furthermore, *Drosophila* usually mate in the field when they are two to three weeks old, so a quantification measure of reproductive age would be meaninguful [57,33]. Adult flies were not rinsed with 70% ethanol prior to DNA extraction since they are usually not contaminated with medium.

***Wolbachia*-quantification by real-time PCR (qPCR)**

DNA samples were quantified in Nanodrop (Thermo Scientific, Waltham, MA, USA) and diluted to the same 10 ng⁄ µL concentration. Absolute quantification of *Wolbachia* genome numbers was performed by construction of a standard curve using serial dilutions of a fragment of the *wsp* (*Wolbachia Surface Protein*) gene from the standard laboratory line *D. willistoni* GdH4, which was cloned into the pGEM-T® vector (Promega, Madison, WI, USA). In order to specifically amplify a portion of the *wsp* gene, we used the following primers: WSPq-F325 (5'-GGTGCARCGTATATTAGCACTCC-3') and WSPq-R470 (5'-GAACCGAAATAACGAGCTCCAG-3') [46].

The total reaction volume was 20 µL, consisting of 2X Platinum® SYBR® Green qPCR SuperMix-UDG 2X (Invitrogen, Carlsbad, CA, USA), a final concentration of 200 nM for each primer, 60 ng of sample DNA and water to fill up the volume. We used a StepOne Plus® (Life Technologies, Carlsbad, CA, USA) Real-Time PCR System, where the qPCR conditions were: 50 ºC for 2 min to UDG incubation, initial denaturation at 95

°C for 5 min followed by 40 cycles at 95 °C for 15 s and 60 °C for 60 s to measure fluorescence. Furthermore, samples were heated from 60 to 95 °C at a 0.3 °C/ s temperature gradient to construct the denaturing curve of the amplified products.

Wsp standard curves from all plates presented between 95% and 99% efficiency. This fact and the positive controls included in each reaction enabled between-plates calibration. All samples were analyzed in quadruplicates, and *wsp* copies in each sample were estimated by the software comparing Ct values to the standard curve.

Statistical analysis

After separately analyzing the ontogenetic *wsp*-quantification in each biological replicate to confirm common *Wolbachia* behavior features for a given symbiotic model, we combined the data from both biological replicates for statistical analysis. We log-transformed absolute quantification values and performed ANOVA followed by Tukey's range test or Kruskal-Wallis followed by Mann-Whitney test, according to within-groups variance. We performed two sorts of statistical comparison regarding *Wolbachia* quantification: (i) among ontogenetic stages of each symbiotic model, thus showing *Wolbachia* dynamics along the ontogenetic development; (ii) among symbiotic models for each ontogenetic stage. All statistical analyses were carried out in PAST 2.17 c software. Average *wsp* copy values and standard error deviations were used to construct graphs.

Phenotypic measures

In order to assess phenotypic features specific for each symbiotic model evaluated in this paper, thus possibly allowing us to make assumptions regarding the adaptative value of each system, we performed experiments to evaluate classic fitness measures (survival, fecundity, viability) and offspring sex-ratio (since *Wolbachia* may induce male-killing in dipterans). In the same way that was performed for qPCR assays, we considered two biological replicates (isofemale lines) for each symbiotic model, and we also included two biological replicates of *D. paulistorum* isofemale lines infected with high-titer *w*Au-like *Wolbachia* for comparison purposes. Each biological replicate was submitted to a certain number of technical replicates in each experiment, as follows.

Sex-ratio experiment

We set up this experiment in order to verify if flies infected with different *Wolbachia* strains and tissue densities experienced any male-killing effect due to *Wolbachia* infection, which would be represented by a female-biased sex-ratio. We placed 10 individuals each of 2-day old virgin males and females flies into three replicate experimental tubes containing cornflour medium, that were maintained in a Biochemical Oxygen Demand (B.O.D.) chamber at 19 °C with a 12/12-h photoperiod. The flies were replaced to a new tube every week until all flies had died. Remaining tubes were supplemented with yeast suspension and maintained in B.O.D. chambers for approximatelly 14 days, during which period all offspring were counted and sexed. Total number of counted male and female offspring were submitted to a qui-square test in order to verify if observed frequencies significantly departed from a 1:1 sex-ratio.

Survival experiment

In order to verify if *Drosophila* flies infected with a given *Wolbachia*-symbiotic model (strain and tissue density combination) experienced any difference regarding their age-specific survival, 10 individuals each of 2-day old virgin males and females flies were placed into eight replicate experimental tubes containing cornflour medium, that were maintained in a Biochemical Oxygen Demand (B.O.D.) chamber at 19 °C with a 12/12-h photoperiod. The flies were replaced to a new tube every other day, when all dead flies that remained in the old tube were sexed and counted, until all the flies had died.

Survival analysis was carried out by constructing nonparametric maximum-likelihood Kaplan-Meier survival curves for each symbiotic model (*Wolbachia* density and strain), which were further pairwaise compared by log-rank (Mantel-Cox) and Wilcoxon tests, all in PAST 2.17 c software.

Fecundity experiment

This experiment aimed to verify if *Drosophila* flies infected with a given *Wolbachia*-symbiotic model (strain and tissue density combination) experienced any difference regarding the female fecundity, here represented by the number of laid eggs. For this, 1 and 2 individuals of 2-day old virgin female and males, respectively, were placed into eight replicate experimental glass vials containing oviposition medium (1.5% agar;

15% w/v honey; 0,3% w/v propionic acid; *Ponceau* dye), supplemented with dried yeast, that were maintained in a Biochemical Oxygen Demand (B.O.D.) chamber at 19 °C with a 12/12-h photoperiod. The flies were replaced to a new tube every other day, when all eggs laid during that 48-hour period were counted and recorded, and old tubes were supplemented with yeast suspension. The procedure was repeated until both males or the female had died.

Statistical analyses were performed by comparing the average number of eggs laid during the first 12 days of experiment (6 evaluations) among symbiotic models by ANOVA followed by Tukey's range test, all in PAST 2.17 c software.

Viability experiment

This experiment was the continuation of the fecundity experiment, and aimed to verify if *Drosophila* flies infected with a given *Wolbachia*-symbiotic model (strain and tissue density combination) experienced any difference regarding the viability of eggs, here represented by the percentage of adults that emerged from the eggs already recorded. Fecundity tubes with laid eggs and yeast suspension were maintained in a Biochemical Oxygen Demand (B.O.D.) chamber at 19 °C with a 12/12-h photoperiod during approximately 14-days, during which period all adults that emerged from the eggs were counted and sexed.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

NCDD conceived the design of the study, performed DNA work, carried out fitness and qPCR experiments, analyzed the data and drafted the manuscript. LC performed DNA work, carried out fitness and qPCR experiments, analyzed the data and helped to draft the manuscript. MJM helped in the design and execution of qPCR experiments, data analysis and helped to draft the manuscript. VHH and VLSV helped to design the study, to analyze the data and to draft the manuscript. All authors read and approved the final manuscript.

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Figures

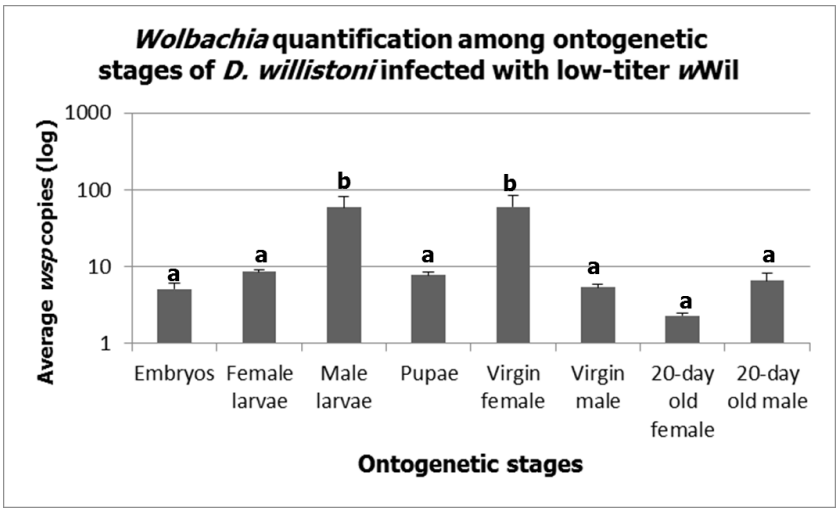

Figure 1. Ontogenetic stage-specific density of low-titer *w*Wil *Wolbachia* in *D. willistoni* isofemale lines.

Graph shows the absolute density of *w*Wil throughout the fly life cycle. Density was estimated using qPCR of the *Wolbachia*-specific *wsp* gene. Each bar represents the average number of *wsp* copies, estimated by quantification of DNA samples from two independent biological replicates of isofemale lines infected with the same *Wolbachia* strain and within-host density. Comparisons marked with the same letter ("a" or "b") were not statistically different using a Tukey's range test, while different letters prove statistical significance of difference concerning *Wolbachia* quantification.

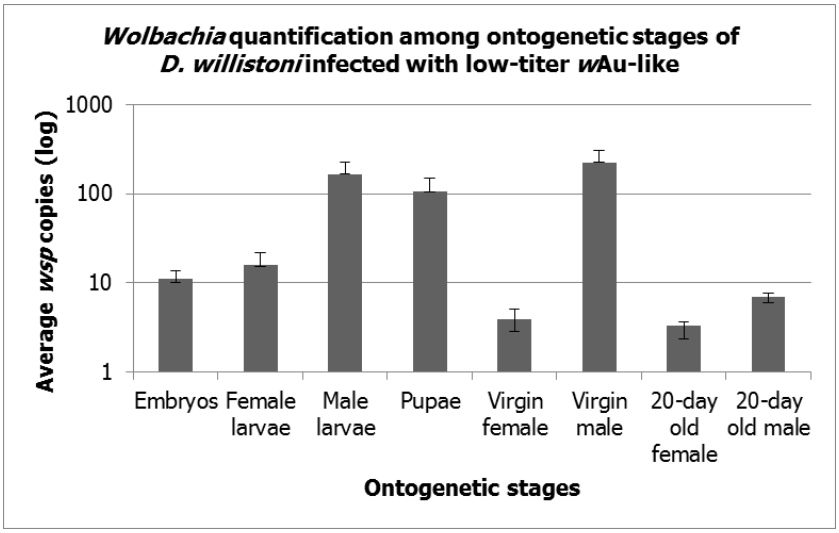

Figure 2. Ontogenetic stage-specific density of low-titer *w*Au-like *Wolbachia* in *D. willistoni* isofemale lines.

Graph shows the absolute density of *w*Au-like throughout the fly life cycle. Density was estimated using qPCR of the *Wolbachia*-specific *wsp* gene. Each bar represents the average number of *wsp* copies, estimated by quantification of DNA samples from two independent biological replicates of isofemale lines infected with the same *Wolbachia* strain and within-host density. Comparisons between ontogenetic stages were not statistically significant (Kruskal-Wallis H= 7.713; p= 0.3586).

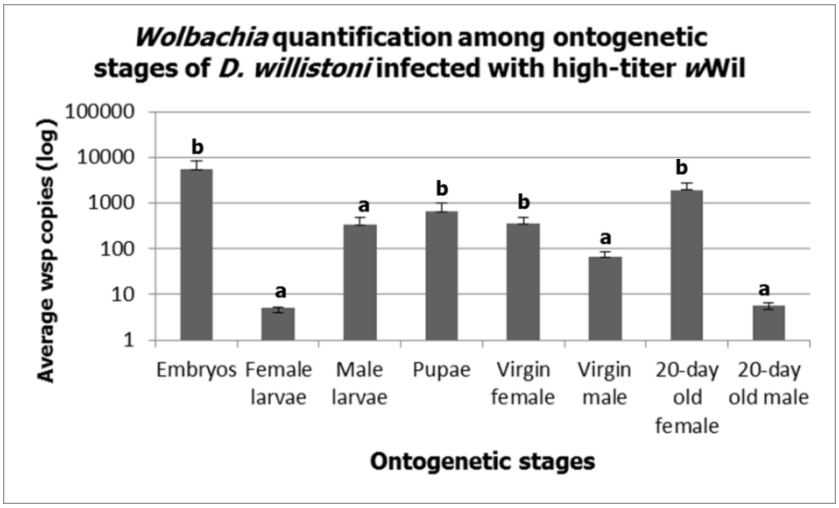

Figure 3. Ontogenetic stage-specific density of high-titer *w*Wil *Wolbachia* in *D. willistoni* isofemale lines.

Graph shows the absolute density of *w*Wil throughout the fly life cycle. Density was estimated using qPCR of the *Wolbachia*-specific *wsp* gene. Each bar represents the average number of *wsp* copies, estimated by quantification of DNA samples from two independent biological replicates of isofemale lines infected with the same *Wolbachia* strain and within-host density. Comparisons marked with the same letter ("a" or "b") were not statistically different using a Tukey's range test, while different letters prove statistical significance of difference concerning *Wolbachia* quantification.

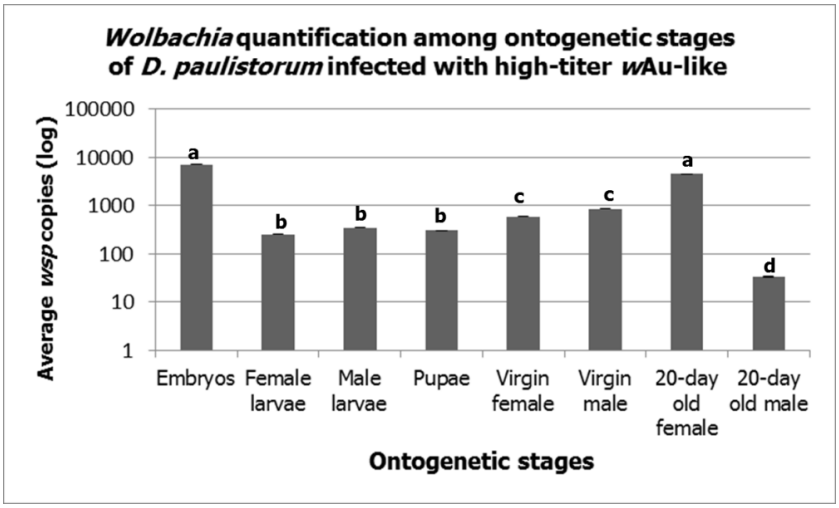

Figure 4. Ontogenetic stage-specific density of high-titer *w*Au-like *Wolbachia* in *D. paulistorum* isofemale lines.

Graph shows the absolute density of *w*Au-like throughout the fly life cycle. Density was estimated using qPCR of the *Wolbachia*-specific *wsp* gene. Each bar represents the average number of *wsp* copies, estimated by quantification of DNA samples from two independent biological replicates of isofemale lines infected with the same *Wolbachia* strain and within-host density. Comparisons marked with the same letter ("a"; "b"; "c" or "d') were not statistically different using a Tukey's range test, while different letters prove statistical significance of difference concerning *Wolbachia* quantification.

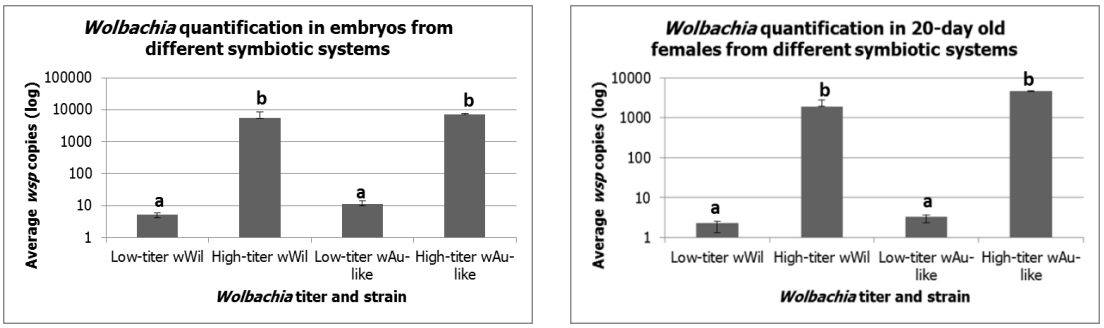

Figure 5. Symbiotic model-specific density of *Wolbachia* in embryos (left) and 20-day old females (right) from *D. willistoni* and *D. paulistorum* isofemale lines.

Wolbachia density was estimated using qPCR of the *Wolbachia*-specific *wsp* gene. Each bar represents the average number of *wsp* copies, estimated by quantification of DNA samples from two independent biological replicates of isofemale lines infected with the same *Wolbachia* strain and within-host density (as depicted in the X-axis). For each graph, comparisons marked with the same letter ("a" or "b") were not statistically different using a Mann-Whitney test, while different letters prove statistical significance of difference concerning *Wolbachia* quantification.

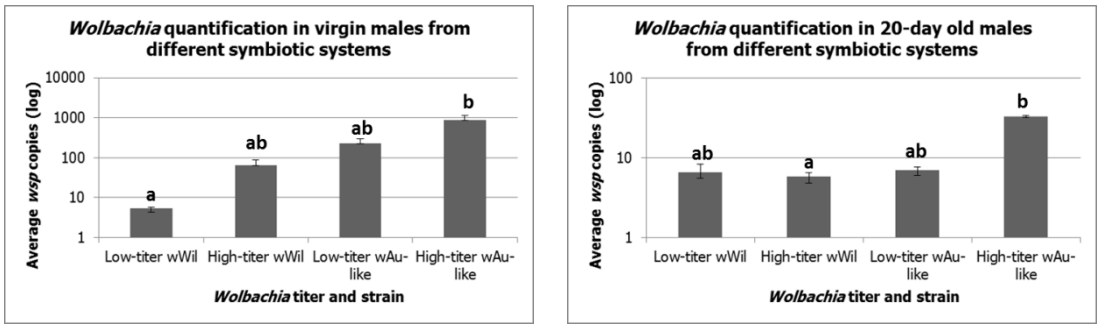

Figure 6. Symbiotic model-specific density of *Wolbachia* in virgin (left) and 20-day old males (right) from *D. willistoni* and *D. paulistorum* isofemale lines.

Wolbachia density was estimated using qPCR of the *Wolbachia*-specific *wsp* gene. Each bar represents the average number of *wsp* copies, estimated by quantification of DNA samples from two independent biological replicates of isofemale lines infected with the same *Wolbachia* strain and within-host density (as depicted in the X-axis). For each graph, comparisons marked with the same letter ("a" or "b") were not statistically different using a Mann-Whitney range test, while different letters prove statistical significance of difference concerning *Wolbachia* quantification.

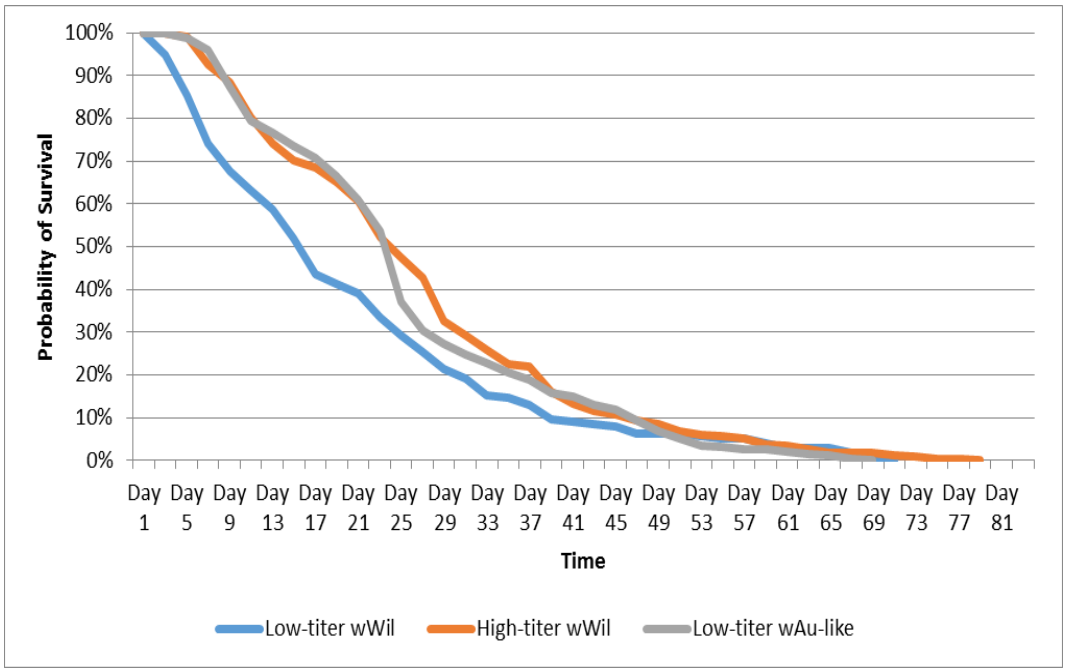

Figure 7. *Wolbachia* infection types have no effect on the longevity of their respective adult *D. willistoni* fly hosts.

Graph shows Kaplan-Meier survival curves for female and male adults of *D. willistoni* flies infected with low-titer *w*Wil (blue), high-titer *w*Wil (orange) and low-titer *w*Au-like (grey) *Wolbachia*. The data shown for each category were pooled from two independent biological replicates, with eight experimental replicates each, and analysed together. The survival curves for each infection model did not differed significantly (see Table S1).

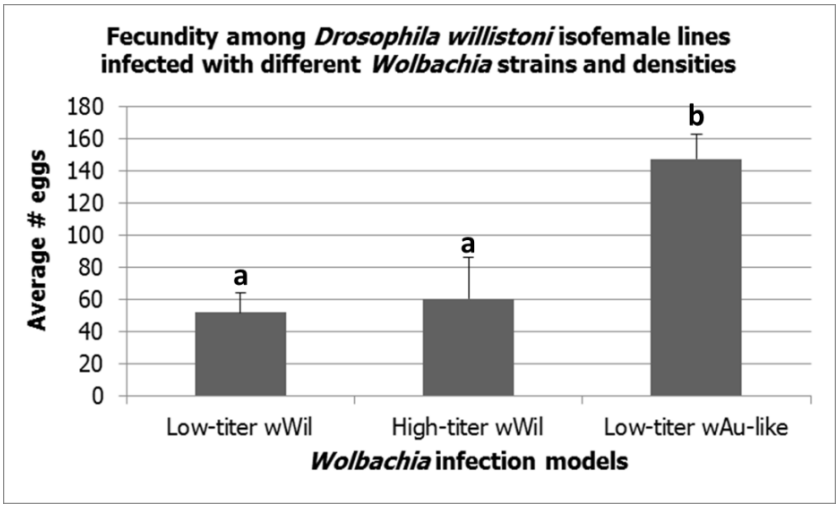

Figure 8. *D. willistoni* flies infected with low-titer *w*Au-like *Wolbachia* laid more eggs than females infected with *w*Wil *Wolbachia*.

Graph shows the number of eggs laid by females infected with different *Wolbachia* strains and within-host densities. The data shown for each category were pooled from two independent biological replicates, with eight experimental replicates each, and analysed together. Comparisons marked with the same letter ("a" or "b") were not statistically different using a Mann-Whitney test, while different letters prove statistical significance of difference concerning *Wolbachia* quantification.

Supplementary Material

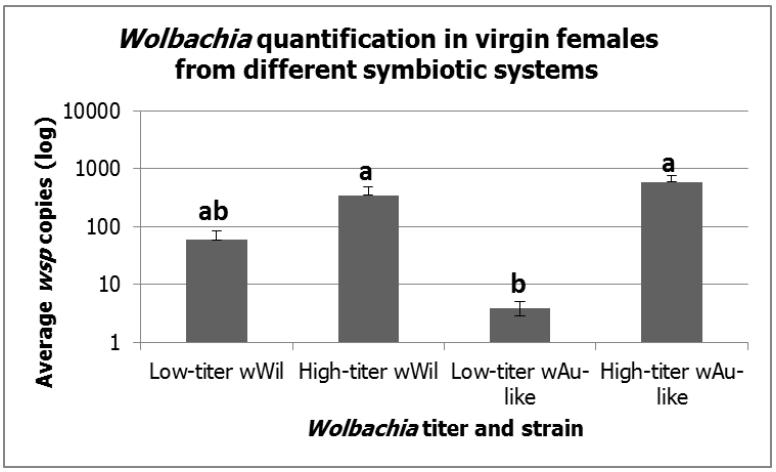

Figure S1. Symbiotic model-specific density of *Wolbachia* in virgin female flies from *D. willistoni* and *D. paulistorum* isofemale lines.

Wolbachia density was estimated using qPCR of the *Wolbachia*-specific *wsp* gene. Each bar represents the average number of *wsp* copies, estimated by quantification of DNA samples from two independent biological replicates of isofemale lines infected with the same *Wolbachia* strain and within-host density (as depicted in the X-axis). Comparisons marked with the same letter ("a" or "b") were not statistically different using a Mann-Whitney test, while different letters prove statistical significance of difference concerning *Wolbachia* quantification.

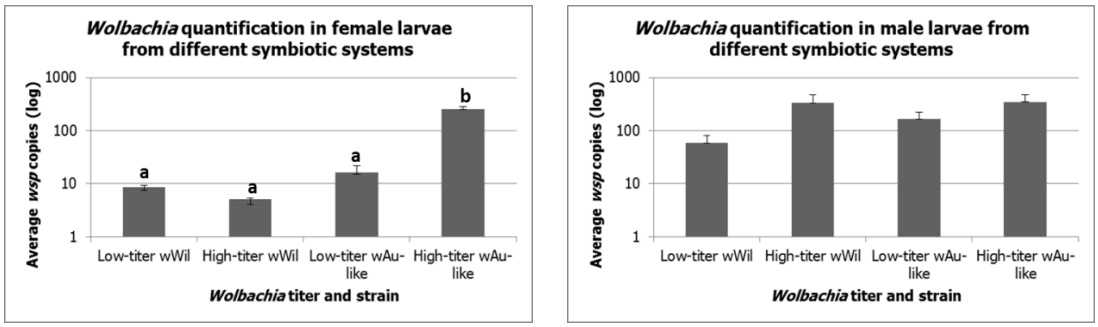

Figure S2. Symbiotic model-specific density of *Wolbachia* in female (left) and male larvae (right) from *D. willistoni* and *D. paulistorum* isofemale lines.

Wolbachia density was estimated using qPCR of the *Wolbachia*-specific *wsp* gene. Each bar represents the average number of *wsp* copies, estimated by quantification of DNA samples from two independent biological replicates of isofemale lines infected with the same *Wolbachia* strain and within-host density (as depicted in the X-axis). Comparisons marked with the same letter ("a" or "b") were not statistically different using a Mann-Whitney test, while different letters prove statistical significance of difference concerning *Wolbachia* quantification. Male larvae quantification among different infection models was not statiscally different (p= 0,2957).

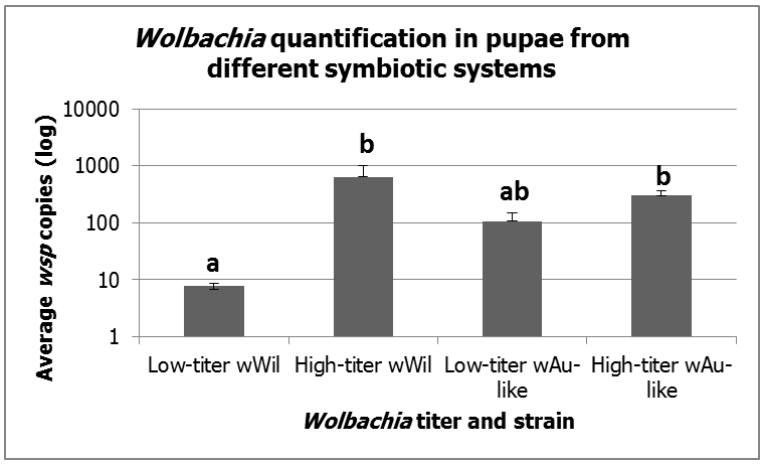

Figure S3. Symbiotic model-specific density of *Wolbachia* in pupae from *D. willistoni* and *D. paulistorum* isofemale lines.

Wolbachia density was estimated using qPCR of the *Wolbachia*-specific *wsp* gene. Each bar represents the average number of *wsp* copies, estimated by quantification of DNA samples from two independent biological replicates of isofemale lines infected with the same *Wolbachia* strain and within-host density (as depicted in the X-axis). Comparisons marked with the same letter ("a" or "b") were not statistically different using a Mann-Whitney test, while different letters prove statistical significance of difference concerning *Wolbachia* quantification.

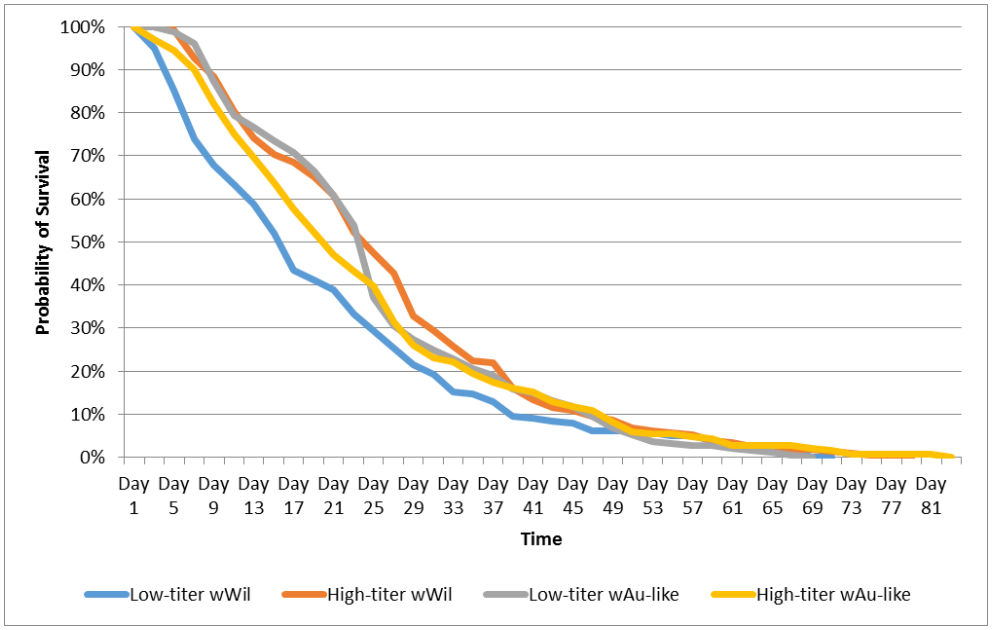

Figure S4. *Wolbachia* infection types have no effect on the longevity of their respective adult *D. willistoni* and *D. paulistorum* fly hosts.

Graph shows Kaplan-Meier survival curves for female and male adults of *D. willistoni* flies infected with low-titer *w*Wil (blue), high-titer *w*Wil (orange) and low-titer *w*Au-like (grey) *Wolbachia*, and curve for *D. paulistorum* flies infected with high-titer wAu-like (yellow) *Wolbachia*. The data shown for each category were pooled from two independent biological replicates, with eight experimental replicates each, and analysed together. The survival curves for each infection model did not differed significantly (see Table S1).

Table S1. Pairwise comparison among Kaplan-Meier survival curves from *D. willistoni* and *D. paulistorum* isofemale lines from four *Wolbachia* models of infection. Grey areas represent log-rank test results, whereas blue areas represent Wilcoxon test results (chi-square and p value).

		D. willistoni			***D. paulistorum***
		Low-titer *w*Wil	**High-titer *w*Wil**	**Low-titer *w*Au-like**	**High-titer *w*Au-like**
D. willistoni	**Low-titer *w*Wil**		X^2=0,427 **p**= 0,513	X^2= 0,086 **p**= 0,768	X^2= 1,100 **p**= 0,294
	High-titer *w*Wil	X^2= 0,726 **p**= 0,394		X^2= 0,912 **p**= 0,339	X^2= 2,279 **p**= 0,131
	Low-titer *w*Au-like	X^2= 0,001 **p**= 0,969	X^2= 0,846 **p**= 0,357		X^2= 0,377 **p**= 0,539
D. paul	**High-titer *w*Au-like**	X^2= 0,923 **p**= 0,336	X^2= 2,527 **p**= 0,112	X^2= 0,487 **p**= 0,485	

CAPÍTULO 4: Discussão e Conclusões

O trabalho desenvolvido durante estes dois anos de Mestrado possibilitou respondermos diversos questionamentos iniciais, e suscitou novos questionamentos sobre a evolução de estirpes de *Wolbachia* em espécies neotropicais de *Drosophila*. O primeiro artigo, resultado da primeira etapa de experimentos, visou destrinchar e caracterizar a diversidade da infecção por *Wolbachia* em populações de *Drosophila willistoni*, tendo em vista a conhecida descontinuidade dos níveis de densidade de infecção para esta espécie. Como previamente confirmado em trabalhos de outros e de nosso grupo (Miller e Riegler, 2006; Müller *et al.*, 2013), as infecções em alta titulação são mantidas pela linhagem *w*Wil de *Wolbachia*, que foi caracterizada pela primeira vez justamente nesta espécie. O diagnóstico foi possível utilizando-se de DNA extraído de uma fêmea adulta (*single-fly*). Por outro lado, as infecções em baixa titulação foram um verdadeiro desafio metodológico. Após uma série de tentativas, ajustes e melhoramentos técnicos, a extração de DNA a partir de 15 ovários permitiu a confirmação da infecção (já que estas eram linhagens novas no laboratório, provenientes de uma coleta recente) por hibridização por PCR-blot, assim como a amplificação de fragmentos de *Wolbachia* (*wsp* e *VNTR-141*) que foram usados para a identificação das estirpes dessa bactéria. Assim, conseguimos verificar que as baixas infecções são mantidas, em alguns poucos casos, pela mesma linhagem encontrada em alta infecção, *w*Wil, porém a maioria das isolinhagens de *D. willistoni* estavam infectadas por uma estirpe de *Wolbachia* chamada *w*Au-like, cuja forma canônica já foi descrita em diversas espécies neotropicais de *Drosophila* (Miller e Riegler, 2006). Além de *D. willistoni*, também caracterizamos molecularmente infecções por *Wolbachia* em *D. paulistorum*, onde tanto infecções com alta quanto com baixa densidade parecem ser mantidas pela mesma linhagem encontrada em *D. willistoni*, *w*Au-like.

O melhor entendimento da diversidade de infecção por *Wolbachia* nestas populações de *Drosophila* nos permitiu refinar algumas inferências quanto à dinâmica populacional destas infecções ao longo do tempo evolutivo. Tendo em vista a alta disseminação das infecções por *w*Au-like em espécies neotropicais de *Drosophila* (Miller e Riegler, 2006), algumas inferências quanto à similaridade entre marcadores VNTR-141 das estirpes e, em termos ecológicos, o grau de simpatria entre estas espécies (Spassky *et al*, 1977), sugerimos que a infecção por *Wolbachia* em *D. willistoni* provavelmente originou-

se de uma transmissão horizontal a partir de *D. paulistorum.* Em *D. willistoni*, a infecção por *w*Au-like seria a mais antiga e, a partir desta, teria surgido a linhagem que hoje chamamos de *w*Wil.

Ainda neste primeiro artigo, analisamos dados de variação mitocondrial do marcador *COI* (*Citocromo Oxidase I*) de moscas de *D. willistoni* e os padrões de infecção por *Wolbachia* (que foram categorizados em "infecção alta" x "infecção baixa"), buscando verificar se haveria alguma associação entre estes tipos de infecção e haplótipos mitocondriais específicos, o que explicaria a segregação 1:1 destes tipos em populações distantes da Amazônia ao Pampa gaúcho por um processo de varredura seletiva. Entretanto, nossos dados não corroboraram esta hipótese e apontaram para uma provável expansão demográfica nesta espécie e nenhuma relação entre as categorias e haplótipos mitocondriais. Como a varredura não explicou o cenário que observamos, propusemos o seguinte para o padrão de segregação 1:1 entre tipos de infecção: (i) poderia ter sido estabelecido por fatores randômicos que, entretanto, dificilmente explicariam a repetição deste padrão em locais tão afastados; (ii) seria um ponto intermediário na evolução da substituição das infecções *w*Au-like por *w*Wil, que culminaria na fixação de *w*Wil. Ainda, propusemos que a existência de vantagens adaptativas conferidas por esta última infecção poderia facilitar em muito este processo de fixação populacional.

Já no segundo artigo, procuramos investigar com mais afinco questões acerca das interações individuais entre *Wolbachia* e seus hospedeiros aqui trabalhados. Para tanto, primeiramente avaliamos a dinâmica do endossimbionte ao longo do desenvolvimento ontogenético de isolinhagens de *D. willistoni* infectadas com os diferentes modelos de infecção verificados: (i) *w*Wil em baixa titulação; (ii) *w*Wil em alta titulação; (iii) *w*Au-like em baixa titulação. Além do mais, a existências de isolinhagens de *D. paulistorum* infectas por *w*Au-like em alta titulação possibilitou a utilização das mesmas para estabelecermos algumas medidas de comparação. Avaliamos a quantificação absoluta de cópias de *Wolbachia* em embriões, larvas machos e fêmeas, pupas, além de fêmeas e machos adultos virgens e com 20 dias de idade. Dentre outros achados, conseguimos verificar que a dinâmica de *Wolbachia* ao longo do desenvolvimento parece ter um caráter mais ligado à densidade de infecção (se alta ou baixa) do que com a linhagem infectante (se *w*Wil ou *w*Au-like). Além disso, a infecção em alta titulação parece sofrer mais eventos de controle

por parte do hospedeiro, já que existe uma forte oscilação da quantificação entre os diferentes estágios, o que não é verificado nas infecções de baixa titulação. Ainda, ao compararmos o número de cópias de *Wolbachia* por estágio ontogenético entre os diferentes tipos de infecção, verificamos que as infecções fortes apresentam a maior densidade de *Wolbachia* nos estágios de embrião e mosca fêmea de 20 dias de idade (idade reprodutiva), que são justamente os estágios mais cruciais para a garantia de transmissão de infecção para a prole.

Além da avaliação ontogenética, investigamos se as moscas infectadas por diferentes padrões de infecção teriam alguma alteração quanto a medidas de fitness. Tal hipótese baseou-se no fato de que parte de nossa proposta de explicação para o cenário evolutivo das linhagens em populações de *D. willistoni* levaria em consideração um possível aumento de valor adaptativo de moscas infectadas pela linhagem *w*Wil. Primeiramente, verificamos que nenhum dos sistemas simbióticos avaliados possui razão sexual distorcida em favor das fêmeas, o que evidenciaria a existência do fenótipo de androcídio por *Wolbachia* (ou *Spiroplasma*, outro endossimbionte que pode causar este fenótipo em dípteros). Da mesma forma, não verificamos diferenças estatisticamente significantes quanto à probabilidade de sobrevivência de moscas infectadas por diferentes linhages e titulações de *Wolbachia*. Por fim, contrariamente ao que esperávamos, verificamos que as moscas infectadas em baixa titulação por *w*Au-like demonstraram ter uma fecundidade maior do que moscas infectadas por *w*Wil, tanto em alta quanto em baixa titulação.

Considerando que a dinâmica populacional por *Wolbachia* na ausência de indução de incompatibilidade citoplasmática é explicada pelo fitness das fêmeas e pela fidelidade de transmissão da infecção para a prole (Fine, 1978; Hoffmann *et al*., 1990; Turelli, 1994; Turelli and Hoffmann, 1995; Charlat *et al*., 2004; Riegler *et al*., 2004), nossas novas descobertas motivaram a revisão da hipótese da manutenção do cenário de infecções por *Wolbachia* em *D. willistoni*. Considerando que a infecção de alta titulação por *w*Wil confere uma alta fidelidade da sua transmissão à prole (vide os resultados de qPCR), enquanto a infecção de baixa densidade por *w*Au-like está associada a uma maior fecundidade de suas fêmeas, nós propusemos que estes tipos de infecção se mantem em níveis populacionais por um mecanismo de contrabalanço, em que a maior fecundidade de

moscas infectadas por *w*Au-like impede o alastramento da alta infecção por *w*Wil, que tem uma alta fidelidade de transmissão.

Perspectivas

Os resultados aqui apresentados são parte de uma ramificação de um projeto maior, que engloba o estudo das interações entre *Wolbachia* e espécies de *Drosophila*. Dentro deste, algumas perspectivas de trabalhos em andamento e futuros podem ser delineadas:

- Nos ensaios de quantificação de *Wolbachia* ao longo do desenvolvimento de isolinhagens de *Drosophila*, foi utilizado DNA extraído de apenas uma mosca, no caso de adultos (virgens e com 20 dias de idade). Tendo em vista que para os outros estágios utilizou-se DNA de mais indivíduos, e também considerando que uma quantificação individual pode levar a considerações enviesadas, serão realizadas novas quantificações dos estágios adultos, utilizando extrações de DNA feitas com cerca de 5 indivíduos.

- Cura, através de tratamento com antibióticos, de isolinhagens de *Drosophila* infectadas por *Wolbachia* em alta titulação. Tais linhagens curadas serão utilizadas em experimentos de fitness, para posterior comparação dos resultados com os obtidos para as linhagens infectadas.

- Cruzamentos inter- e intraespecíficos com isolinhagens de *D. willistoni* e *D. paulistorum* infectadas com diferentes linhagens e titulações de *Wolbachia*.

- Cruzamentos inter- e intraespecíficos entre isolinhagens de *D. willistoni* e *D. paulistorum* curadas e ainda infectadas com diferentes linhagens e titulações de *Wolbachia*.

- Avaliação de aspectos epigenéticos possivelmente envolvidos na interação entre *Wolbachia* e *D. willistoni* e *D. paulistorum*.

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