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Dissertação apresentada ao Instituto de Biociências da Universidade de São Paulo, para a obtenção de Título de Mestre em Ciências, na área de Ecologia de Ecossistemas Terrestres e Aquáticos.

Orientador(a): Prof. Dr. Rodrigo Cogni.

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Martins Oliveira da Silva, Marcos Aurélio.

Prevalence of the bacterial endosymbiont *Wolbachia* in neotropical drosophilid communities. Versão em português: Prevalência da bactéria endossimbionte *Wolbachia* em drosofilídeos neotropicais / Marcos Aurélio M. O. da Silva; orientador Rodrigo Cogni — São Paulo, 2021.

49p.

Dissertação (Mestrado) - Instituto de Biociências da Universidade de São Paulo. Departamento de Ecologia.

Palavras-chave: 1. *Wolbachia*. 2. levels of biological organization. 3. levels of selection. 4. neotropical drosophilids. 5. Atlantic Forest.

Universidade de São Paulo. Instituto de Biociências. Departamento de Ecologia.

Comissão Julgadora:

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Prof(a). Dr(a).	Prof. Dr. Rodrigo Cogni
	Orientador(a)

To suffer woes which Hope thinks infinite;

To forgive wrongs darker than death or night;

To defy power which seems omnipotent;

To love, and bear; to hope till Hope creates

From its own wreck the thing it contemplates.

Neither to change, nor falter, nor repent;

This, like thy glory, Titan, is to be

Good, great and joyous, beautiful and free;

This is alone Life; Joy, Empire, and Victory!

— Percy Bysshe Shelley, *Prometheus Unbound*

AGRADECIMENTOS

A todos que me apoiaram nessa jornada de conhecimento, obrigado. Um agradecimento especial a minha família pelo suporte e estímulo sempre presente desde tenra idade, e ao meu orientador Rodrigo Cogni por ter me apoiado e me orientado em um trabalho fascinante compreendendo muitas facetas da biologia. Agradeço também a todos os membros do Laboratório de Ecologia Evolutiva presentes durante esses anos e que me auxiliaram na minha pesquisa: André Coppe Pimentel e Eduardo Gomes Ragnoni que me apoiaram nos trabalhos de campo e Raquel Dietsche Monfardini e Dione Oliveira Jordan que me auxiliaram nos experimentos de biologia molecular. Agradeço também aos membros do comitê, Paulo Inácio de Knegt López de Prado e Paulo Roberto Guimarães Junior que propiciaram o debate de ideias fundamentais para o fechamento do presente trabalho.

Agradeço à Fundação de Amparo e Pesquisa (FAPESP) pela bolsa de estudo, viabilizando o projeto no país (Processo N.º 2018/01295-8) e a oportunidade de estágio nos Estados Unidos (Processo N.º 2019/07021-0), uma oportunidade que sem sombra de dúvidas acrescentou demais a minha formação e aos rumos que tomarei na pesquisa daqui em diante. A isso agradeço ao professor Michael Turelli (Universidade de Califórnia – Davis) e ao professor Brandon S. Copper (Universidade de Montana) que me instigaram e me auxiliaram nessa etapa.

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RESUMO

Interesse egoístas colocam endossimbiontes transmitidos por via maternal e o genoma do hospedeiro em conflito. *Wolbachia* é um exemplo de endossimbionte notavelmente bem-sucedido, presente em 50% de todos os artrópodes. Um processo que explica sua alta prevalência é sua capacidade de manipular a reprodução do hospedeiro a seu próprio favor por meio da incompatibilidade citoplasmática. A rápida propagação de *Wolbachia* em populações de *Drosophila* foi observada em tempo real; no entanto, foi demonstrado que algumas linhagens de *Wolbachia* mantêm altas frequências independentemente da manipulação, sugerindo que um efeito mutualístico pode manter sua alta prevalência em populações naturais. Curiosamente, a variação da frequência de *Wolbachia* em populações de hospedeiros naturais correlaciona com gradientes ambientais. Este estudo descreve a prevalência de *Wolbachia* em oito diferentes comunidades de drosofilídeos neotropicais da Mata Atlântica e questiona se fatores ecológicos da comunidade, além de fatores filogenéticos, podem aumentar a probabilidade de um indivíduo estar infectado por *Wolbachia*. Usando modelo logístico generalizado, nosso modelo proposto incluindo os fatores ecológicos abundâncias de espécies, estação e altitude não aprimora a explicação das chances de ocorrência de *Wolbachia*. Alternativamente, nosso modelo nulo contendo apenas as variáveis aleatórias espécie e grupo de espécie mostrou um coeficiente de correlação intraclasse que explica cerca de 40% de chance de componente de clado de predizer a ocorrência de *Wolbachia*. Nossos resultados sugerem que o impacto da ecologia na interação *Wolbachia*-hospedeiro é ofuscado pela força da seleção atuando no nível de genes que intercedem manipulação e mudança de hospedeiro. Esses resultados podem classificar *Wolbachia* como um “passageiro de passageiros”.

ABSTRACT

Selfish interests put maternally transmitted endosymbionts and host-nuclear genome into conflict. *Wolbachia* is an example of a remarkably successful endosymbiont present in 50% of all arthropods. One process that explains its high prevalence is its ability to manipulate host reproduction to its own favor through cytoplasmic incompatibility. The rapid spread of *Wolbachia* in *Drosophila* species populations was observed in real-time; however, it was shown that some successful *Wolbachia* strains maintain high frequencies regardless of manipulation, suggesting that a mutualistic effect may maintain its high prevalence in natural populations. Intriguingly, *Wolbachia* frequency variation in natural host populations correlates with environmental gradients. The present study describes the prevalence of *Wolbachia* in eight different neotropical drosophilid communities from the Atlantic Forest and queries whether community ecological factors, beyond phylogenetic factors, can improve the probability of explaining if an individual carry *Wolbachia*. By using generalized mixed logistic regressions, our proposed model that includes ecological factors species abundance, season, and altitude does not improve the explanation of the odds of *Wolbachia* occurrence. Alternatively, our null model containing only the random effects species and species group shows an intraclass correlation coefficient that explains about 40% of chance of clade components predicting *Wolbachia* occurrence. Our results suggest that the impact of ecology in *Wolbachi*a-host interaction is overshadowed by the strength of selection operating at the level of genes that mediate manipulation phenotype and host shift, these results might classify *Wolbachia* as “a passenger of passengers”.

INTRODUCTION

Selfish interests put maternally transmitted endosymbionts and host nuclear genome into conflict (HURST, 1992; WERREN, 2008; ZUG & HAMMERSTEIN, 2015; CORREA & BALLARD, 2016). The outcome of the interaction depends on the effects on host fitness and the ability of the endosymbiont to spread via reproductive manipulation (ZUG & HAMMERSTEIN, 2015; CORREA & BALLARD, 2016). Whereas some interactions show context and facultative associations, intimacy between host and endosymbionts essential traits evolves toward dependence (FISHER *et al*., 2017). Which outcome will be displayed depends on symbiont and host genomes interactions, the resulted induced phenotype, and the consistency of the environmental interplay (ZUG & HAMMERSTEIN, 2015; FISHER *et al*., 2017).

Wolbachia is a maternally transmitted α-proteobacteria endosymbiont that is widespread among invertebrates, having association with nematodes, more than 50% of all arthropods (WERREN, 2008, WEINERT *et al*., 2015) and present in more than 65% of all insect species (HILGENBOECKER *et al*., 2008). This remarkably widespread success has made *Wolbachia* an essential model to study host-symbiont interactions in gene, population, and species levels. One proposed reason of its success is its ability to attain high frequencies by acting as a reproductive manipulator, taking control of host mechanism of reproduction, and favoring its own matriline cytoplasmic transmission (e.g., inducing male killing, feminization, parthenogenesis, and cytoplasmic incompatibility) (WERREN, 2008; ZUG & HAMMERSTEIN, 2015). In addition, shifts among different lineages are often common over evolutionary timescales (CORREA & BALLARD, 2016), evidenced by discordant phylogenies between *Wolbachia* and their hosts (O'NEILL *et al*., 1992; HEATH *et al*., 1999; VAVRE *et al*., 1999; WERREN & WINDSOR, 2000; TURELLI *et al*., 2018; COOPER *et al*., 2019). Most intriguing, there is growing support that highlights *Wolbachia* as a powerful source of evolutionary innovation for many invertebrates (DURON & HURST, 2013), with *Wolbachia* inducing phenotypes beyond reproductive manipulation (ZUG & HAMMERSTEIN, 2015; CORREA & BALLARD, 2016).

The genus *Drosophila* has revolutionized molecular biology, including *Wolbachia*-related research. In *Drosophila*, cytoplasmic incompatibility (CI) and male killing seems to be the only phenotypes induced by *Wolbachia* (MILLER & RIEGLER, 2006; WERREN, 2008). However, CI has

long been considered the most important mechanism responsible for *Wolbachia* increase in frequency in natural host populations (ENGELSTÄDTER & TELSCHOW, 2009). Cytoplasmic incompatibility consists in a sperm modification of males bearing *Wolbachia*. Males are unable to transmit *Wolbachia*, but an infected male mating with a female not bearing *Wolbachia* results in non-developed embryos (WERREN, 1997; POINSOT *et al*., 2003). Females bearing *Wolbachia* can undo sperm modification, therefore rescuing embryo development and providing a relative fitness advantage to infected females. CI, therefore, is a mechanism that results in differential viability of offspring of *Wolbachia* infected females, driving *Wolbachia* infections to high frequencies within host populations (WERREN, 1997; POINSOT *et al*., 2003, ENGELSTÄDTER & TELSCHOW, 2009).

The rapid spread of CI-causing *Wolbachia* has been observed in real time over several decades in *Drosophila* field populations. The *Wolbachia* strain *w*Ri, for example, first spread through California, United States, and later spread through global populations of *D. simulans* (TURELLI & HOFFMAN, 1992; TURELLI & HOFFMAN, 1995; KRIESNER *et al*., 2013). It was also demonstrated that *w*Ri has spread among host diverged up to 50 million years in only the last few thousand years (TURELLI *et al*., 2018), showing its ability to successfully shift hosts. As CI is a positive frequency dependent trait, to attain its success, these *Wolbachia* and all others must first spread to intermediate frequencies before CI causes spread to higher frequencies (TURELLI & HOFFMAN, 1995). Additional studies have reported persistence of several *Wolbachia* that do not cause CI: *w*Au in *D. simulans* (HOFFMAN *et al*., 1996; KRIESNER *et al*., 2013), *w*Mau in *D. mauritiana* (MEANY *et al*., 2019), and *w*Suz in *D. suzukii* (HAMM *et al*., 2014).

All that suggests that *Wolbachia* increases components of host fitness, which is not surprising given that one predicted outcome of vertically transmitted symbionts is to coevolve towards mutualism with their hosts (WEEKS *et al*., 2007, ZUG & HAMMERSTEIN, 2015; FISHER *et al*., 2017). Mutualistic effects have already been described as increasing in female host fecundity in *w*Ri-infected *D. simulans* (WEEKS *et al*., 2007), nutritional complementation (BROWNLIE et al. 2009), and protection against different types of RNA viruses under laboratory conditions (TEIXEIRA *et al*., 2008, PIMENTEL *et al*., 2020). In transinfections performed in laboratory, different *Wolbachia* strains show antiviral protection in *D. simulans* when in high densities, this high density is also associate with costs as lower egg hatch rate, lower male fertility, and lower overall fecundity (MARTINEZ *et al*., 2014). Therefore, we may speculate that a context environment dependence

could modulate *Wolbachia* frequency offered by a balance of the beneficial induced phenotypes and the costs of *Wolbachia* infection.

Environmental factors may strongly influence the pattern of *Drosophila-Wolbachia* interaction. This was observed in *D. melanogaster* populations in different parts around the world, which show variation in *Wolbachia* frequency in a clinal latitude pattern (KRIESNER *et al*., 2016); higher frequencies were observed in tropical regions with higher temperatures and lower frequencies were observed in temperate regions with lower temperatures. COOPER *et al*., (2017) also showed for the African *D. yakuba* clade ((*Drosophila yakuba*, *D. santomea*), *D. teissieri*) that despite developing CI in the interaction with *Wolbachia*, the endosymbiont frequency consistently varies spatially and temporally (*D. yakuba*, *D. santomea*), potentially due to interactions with host genomes and/or environmental effects on the efficiency of maternal transmission. Interestingly, temperature affects immune function in insects, as example, flies infected with pathogens survive for longer when held at lower temperatures, a result expected both by the influence of temperature on both pathogen growth and the physiological responses of the host (LINDER *et al*., 2008). There is also evidence that temperature alter the potential of host shift by viruses, as an increase in temperature made susceptible species more susceptible, and the least susceptible less so (ROBERTS *et al*., 2018). Finally, temperature alters *Wolbachia* ability to protect against DCV virus in *D. melanogaster*, with lower protection when flies develop at 18°C and higher protection when develop at 25°C (CHROSTEK *et al*., 2020 - preprint). As *Wolbachia* interact directly with hosts and indirect with host's pathogens, understanding the effects of environmental variables is important as it might have fundamental impact in the three-way interaction and will shape the traits that natural selection will work upon.

The pervasive occurrence of horizontal transfer and induction of host phenotype are essential mechanisms for effective *Wolbachia* host shifts in arthropods and the attainment of high frequencies (ZUG & HAMMERSTEIN, 2015, SANAEI *et al*., 2021). There is evidence that *Wolbachia* can be transferred by host-parasitoid/parasitic interactions (VAVRE *et al*., 1999, BROWN & LLOYD, 2015) and the sharing of food resources (DOBSON *et al*., 2002, BROWN & LLOYD, 2015). Once *Wolbachia* had overcome host's immunity and successfully reached the germline, the induction of a phenotype, either reproductive manipulation or/and a host fitness enhancement phenotype, is crucial for its establishment in the newly infected host (ZUG & HAMMERSTEIN, 2015, SANAEI *et al*.,

2021). Although host shifts may happen across distant related lineages (SIOZIOS *et al*., 2018), genetic resemblance, evidenced by 'phylogenetic distance effect' (LONGDON *et al*., 2015) seems important as closely related host species are expected to be more alike, including in their ability to suppress or being pervious to *Wolbachia* (SANAEI *et al*., 2021). Supporting this, a positive correlation between success rates and relatedness of donor and recipient hosts to *Wolbachia* was observed in a review of 25 transinfection studies (RUSSEL *et al*., 2009). All those processes suggests that species sharing similar ecology (i.e., visiting the same type of food resources and being exposed to the same range of natural enemies) and near phylogeny (i.e., groups of sibling species) may share *Wolbachia* presence.

Little is known about *Drosophila* ecology in the neotropics, with few large surveys of the *Drosophila* species occurring in the Atlantic Forest with many species remaining to be described (MEDEIROS & KLACZO, 2004). Despite this, since Dobzhansky foundation of the Brazilian school of drosophilists, many following trained taxonomists and geneticists thoroughly described the known fauna and had made a consistent repertory of species groups (PAVAN & DA CUNHA, 2003; O'GRADY & DESALLE, 2018). Species groups, first employed by STURTEVANT (1939), are closely related assemblages of species erected based on a series of shared morphological traits (e.g., sex combs) and other characteristics (e. g chromosome evolution and partial reproductive isolation occurring during diversification) that homology statement can be made with certain confidence (O'GRADY & DESALLE, 2018).

The Neotropics presents the largest group of drosophilids belonging to the subgenus *Drosophila* (ROBE *et al*., 2005) where it includes at least 25 species groups. The classic THROCKMORTON (1975) Drosophilidae radiation, shows that those species groups are subdivided into two main lineages: the virilis-repleta radiation (15 species groups); and the quinaria-tripunctata radiation (9 species groups). The less diverse subgenus Sophophora, present the species groups *willistoni* and *saltans* as the main neotropical groups (O'GRADY & DESALLE, 2018). The new-world clade *saltans-willistoni* is sister group of the old-world well known clade *obscura-melanogaster* (MILLER & RIEGLER, 2006; O'GRADY & DESALLE, 2018). Important to note, both clades *saltans-willistoni* and *obscura-melanogaster* contain species associated with *Wolbachia* (MILLER & RIEGLER, 2006).

In the present study we describe eight different drosophilid (*Drosophilinae*) communities from the preserved region of the Atlantic Forest displaying seasonal and altitudinal differentiation. We investigate drosophilid community's patterns and link its features to *Wolbachia* presence. Specifically, we test the hypothesis that *Wolbachia* occurrence in individuals depends on environment and that ecological components may shape *Wolbachia* presence/absence in drosophilid communities, beyond what is only expected by the relationship of species and species group (clade component). *Wolbachia* literature has long being investigating processes and patterns at gene, individual and population levels, but few attempts have assessed many species across different environments. This study investigates patterns associate with environmental differences in collections of many species (i.e., community level) and inquire, in the field, the theoretical expectation that a description of a pattern in a different biological scale such as species communities may help to identify processes emerging and laying signatures from genes to communities (LEVIN, 1992).

MATERIAL AND METHODS

2.1 Study area

This study was conducted in the tropical rainforest of Serra do Mar State Park, São Paulo, Brazil, a major reminiscent of the original Atlantic Forest. Rainfall distribution is the main factor differentiating between types of forest within this biome (OLIVEIRA FILHO & FONTES, 2000; JOLY *et al.,* 2014). Flies' samples were collected in permanent plots (JOLY *et al.,* 2012) in the municipalities of Ubatuba, São Luís do Paraitinga and Cunha (Figure 1). Two permanent plots were chosen at lowland Ubatuba with altitudes ranging from 0m to 100m: Picinguaba – Lowland 1 (LL1); and Fazenda Capricórnio – Lowland 2 (LL2). And two, at highland sites with altitudes ranging from 800m to 1000m: São Luís do Paraitinga, Núcleo Santa Virgínia – Highland 1 (HL1); and, Cunha, Núcleo Cunha – Highland 2 (HL2).

The lowland regional climate is tropical humid, with no dry season, and with a mean annual temperature of 22°C (SETZER, 1966, REIS *et al.,* 2015). The average annual rainfall exceeds 2500 mm, and even in the driest months, from June to August, the average monthly precipitation is above 60 mm (REIS *et al.,* 2015). The highland climate is subtropical humid (SETZER, 1966, REIS *et al.,* 2015), the average annual temperature is 20°C and the average annual rainfall exceeds 1100 mm (REIS *et al.,* 2015). In the driest months, from April to September, the average monthly precipitation is above 30 mm (REIS *et al.,* 2015). As *Drosophila* communities are affected by pluviosity and their densities change with altitudinal gradient (GURUPRASAD *et al.,* 2010), altitude and seasonality were used as factors to discriminate communities. The field collections were accomplished on winter (August 2018) and on summer (January 2019) resulting in eight different *Drosophila* communities.

2.2 Sampling

To collect flies, plastic bottle traps were built as in MEDEIROS & KLACZKO (1999) with minor adaptations. Three plastics bottles were cut and assembled forming three compartments for capturing flies (Figure 2). Flies present negative geotropism and tend to walk upwards, then a series of holes were done in the downside of the trap just above the compartment where one and a half-spoiled banana plus dried yeast was used as bait. A thin fabric was used to separate the bait

Figure 1. Location of study sites at Serra do Mar State Park, São Paulo, Brazil. Two sites were selected at highland tropical rainforest (Santa Virginia and Cunha - dark green) and lowland sites (Fazenda Capricórnio and Picinguaba - light green). Figures obtained with Google Maps ™.

compartment from the fly's entrance. Ten traps were distributed in each site by at least 100m distance from each other. Traps were set at 75-100 cm from the ground. Flies were collected from traps after 24 hours of setup, and then again after 48 hours.

2.3 Morphospecies and species group classification

Flies were collected and stored in ethanol (92,4%) and then at -20ºC in the lab. Flies were sorted by sex, species group and morphospecies. An identification species key for neotropical drosophilids was used (PAVAN, 1949) to separate flies into species group. Additional external morphological characters from a general *Drosophila* species key (MARKOW AND O'GRADY, 2006) was used to double check the previous identification and to characterize morphospecies and choose species group and species candidates, as is impossible to characterize most species without dissection of male terminalia. Males were then used to identify at species level by desiccation of terminalia and characterization of aedeagus. Labels were then created to match each aedeagus with each remaining body of an individual.

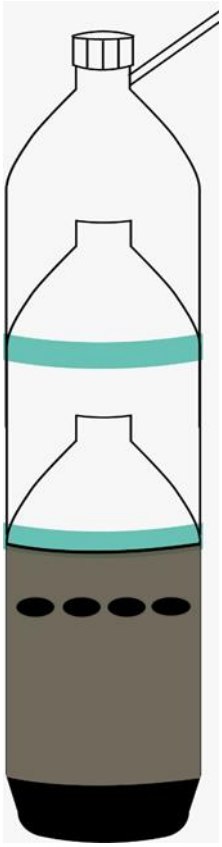

Figure 2. Plastic bottle traps built as described in Medeiros & Klaczko (1999) with minor adaptations. Three compartments are present to capture flies as they exhibit negative geotropism. The black holes are flies' entrance, and a half-spoiled banana plus dried yeast at the compartment in the bottom.

The extraction of aedeagus and other key structures from male terminalia was made using a dissection needle and a combination of techniques that lose the terminalia parts to facilitate the detachment and dye. The terminalia was transferred from each labelled storage microtube to a microtube containing 100 ul of KOH, then those tubes were left in a dry heater for 65°C for 10 min. The sample was cleaned from KOH in natural water and then transferred to a microtube containing 10 ul of GAGI dye (storage: 300ml of H_20, 0.5g of acid fuchsin and 10ml of HCl). The terminalia were then left dyeing for 2 minutes and then washed in natural water. The terminalia was observed and dissected thoroughly in a slide containing glycerin. After dissection, aedeagus and attached structures were transferred to a new slide containing glycerin and coverslip and then stored in a slide box for future observation at a stereo microscope.

2.4 DNA extraction and PCR amplification

DNA extractions were performed individually for each male (the terminalia was removed for species identification) using Gentra® Puregene® Cell Kit (Qiagen, Chatsworth, CA, USA) with minor adjustments from manufacturer's protocol. This protocol yields on average 20-70 ng of DNA per fly per extraction, with final DNA solution of 20 ul.

Each fly DNA sample was amplified using the same protocol for both the mitochondrial Cytochrome Oxidase Subunit I gene COI, and *Wolbachia*'s surface protein gene *wsp* (Table 1) to test *Wolbachia* presence. A 12.5 PCR reaction was prepared for each sample: 1 µl from sample DNA was mixed with 6.25 µl of reaction mix (MyTaq™ Mix - Bioline) and 3.75 µl of primer mix (0.125 of *forward* primer, 0.125 of *reverse* primer, 4.5 µl of Milli-Q water). The PCR program set was: 1. 95°C for 5 minutes; 2. 95°C for 30 seconds; 3. 55°C for 30 seconds; 4. 72°C for 1 minute; 5. Steps 2 to 4 for 32 cycles; 6. 72°C for 1 minute; 7. hold at 12°C. The amplified genes were visualized using electrophoresis in agarose gel (0.01g agarose/ml of MilliQ water, 20 µl TAE (50x)/ml, 0.05 µl ethidium bromide/ml).

2.5 Species identity through DNA barcode

For cryptic species and for species in which the determination by morphology was uncertain, the species identity was confirmed by DNA barcoding and then by comparing the acquired sequences among then and with available public sequences (NCBI Genbank®, UCSC In-Silico PCR tool). The DNA of twelve individuals from each of the three dominant species from the group *willistoni* and one from the remaining morphospecies were extracted, amplified (following the steps from the previous session) and prepared for DNA sequencing of a fragment of the mitochondrial region of cytochrome oxidase subunit I (COI), with 858 bp. A PCR product cleanup was made using 10 µl of each post-PCR sample product combined with 4 µl of the reagent ExoSap-IT™ following standard protocol. Sample's reactions were made with BigDye® Terminator v3.1 Cycle Sequencing Kit (4337456) and Sanger sequencing was then performed in the ABI 3730 DNA Analyser (Applied Biosystems™). To get the best estimation of the sequences, for each sample two labelled microtubes with 5 µl of the cleaned PCR product were run in the sequencing machine. As the Sanger sequencing recreate each strand independently the first labelled tube contained the cleaned DNA solution added with 2.5 µl with *forward* COI_2183 primer (5 µM) and the second

Table 1. Primers used in this study. *Wolbachia* surface protein (*wsp*) was used to identify *Wolbachia* presence and cytochrome oxidase subunit I (COI) was used to access amplification quality and to identify *Drosophila* species (Barcode). When amplification of COI result in a good quality amplification, but not the one from *wsp.* RT-PCR of the *Wolbachia* atpD synthase was used to confirm *Wolbachia* presence and to detect the possible presence at low titer.

Gene	Primer Sequence	Fragment Size	Published Name	Reference
wsp	5'-TGGTCCAATAAGTGATGAAGAAAC-3'	610 bp	WSP_81FW	Braig *et al.* (1998)
	5'-AAAAATTAAACGCTACTCCA-3'		WSP_691RV	Braig *et al.*, (1998)
COI	5'-CAACATTTATTTTGATTTTTTGG-3'	858 bp	C1-J-2183 ("Jerry")	Simon *et al.* (1994)
	5'-TYCATTGCACTAATCTGCCATATTAG-3'		R3037	Oliveira *et al.* (2005)
atpD	5'-CCTTATCTTAAAGGAGGAAA-3'	107 bp	atpDQALL_FW	Martinez *et al.* (2014)
	5'-AATCCTTTATGAGCTTTTGC-3'		atpDQALL_RV	Martinez *et al.* (2014)

tube the cleaned DNA solution added with 2.5 µl *reverse* COI_3041 primer (5 µM). Chromatograms were checked and sample sequences were trimmed in their best quality regions also aiming for same sequences size. All analyses were performed using Bioconductor and its incorporated packages in R Software. When a low-quality sequence was obtained, both forward and reverse strands were removed from the subsequent analyses. Consensus sequences were attained from pairwise alignment of the forward and the reverse complement of the reverse sequence, increasing the overall quality of sequences that were used to perform multiple alignment. Multiple sequence alignment was then executed on the best acquired sequences and reference sequences obtained from NCBI GenBank® and UCSC In-Silico PCR tool with CLUSTAL W (THOMPSON *et al.*, 1994). The converted distance matrix was obtained from the multiple sequence alignment and a phylogenetic tree was made using unweighted pair group method with arithmetic mean – UPGMA (SNEATH & SOKAL, 1973) and plotted with "ape'' package within Bioconductor.

2.6 *Wolbachia* status

The presence of *Wolbachia* was checked individually by PCR amplification of the *wsp* gene. For DNA samples in which the *wsp* amplification result was negative, the amplification of the mitochondrial gene COI was done as a control for DNA amplification.

For DNA samples that do not show amplification of the *wsp* gene, but show a good quality COI amplification, it is possible that PCR screening was not sensitive enough to detected *Wolbachia* present at low titer. For those, a high-sensitive real time PCR (RT-PCR) of the *Wolbachia* ATP synthase gene *atpD* (Table 1) was used to detected *Wolbachia* infections present at low titer. To do so, 1 ul of sample DNA was mixed with 5 ul of SensiFAST™ SYBR® HI-ROX and 3ul of the *atpD* primers mix in 96 well plate for RT-PCR and run-on Applied Biosystems StepOneplus™ Real-Time PCR, following manufacturer's protocol. Quantification cycle values (C_T) and melting temperature (T_M) were checked using StepOne™ Software v2.3. Samples were considered infected when C_T values were below 30 and T_M values matched positive control samples.

2.7 Communities' data analysis and comparison

Each sampled community was described referring to its *Drosophila* richness, abundance, and diversity. As sites are different regarding to space (geolocalization and altitude) and time (season) a non-metric multidimensional scaling (NMDS) was used to collapse information from the eight communities and, therefore, to quantitatively access the dissimilarity between communities. Ranking abundance distributions (RADs) were used to describe communities' diversity by comparing patterns of evenness and dominance (Figure 3). Model selection of five different RADs models were executed using the package "vegan" implemented in the R Software. As described in WILSON (1991), diversity models can be divided into the ecological resource-apportioning models and statistical models. Two of the five models used in model selection are resource-apportioning models, they are: the null model ("Broken-Stick"); and species geometric decay ("Preemption"). The remaining three are statistical models, they are: General Lognormal, Zipf and Zipf-Mandelbrot. Generally, "Broken-Stick" describes individuals from different species randomly distributed along a unique niche axis, the result is a distribution with a low degree of dominance and subsequent shallow decay of the distribution, constituting evenness for the

following species in the rank. The geometric decay ("Preemption") characterizes a species

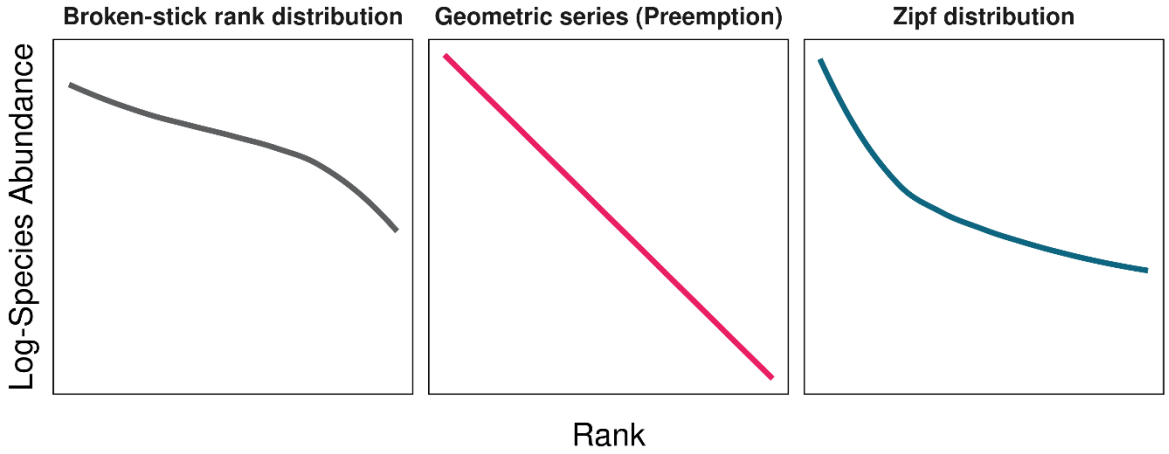

Figure 3. Rank-abundance distribution (RADs) models. Model selection (AIC) assign each community distribution to one known RAD. Grey line shows the Broken-Stick null model, representing a community with shallow decrease in abundance. Pink solid line shows community Preemption, with a steep decrease in abundance. This steep decrease is followed by depletion of the total richness (reach first zero abundance). Dashed black line shows the statistical model - Zipf. This model is characterized by steep decrease in abundance of the first species in the rank, followed by shallowness in the tail end, this represents a cumulative richness, even in the presence of dominant species.

distribution in which the sequence of species shows a fast decrease in abundance, with species domination prevailing and with subsequent steep decay of the distribution, resulting in unevenness of species abundances in the rank and low richness of the overall community. Lognormal and Zipf models are generalized linear models (GLMs). Zipf–Mandelbrot differs from the pure Zipf by adding one nonlinear parameter.

2.8 Estimate *Wolbachia* occurrence in species populations

Some *Drosophila* species and species group do not present *Wolbachia* infections in their natural populations (MATEOS *et al.*, 2006). Therefore, species that are known to not harbor *Wolbachia* were discriminated from those that are found naturally infected. As neotropical drosophilids show many species and species group not well known by *Wolbachia* literature, the *Wolbachia* occurrence in its species populations is currently unknown. For those, an estimation of *Wolbachia* frequency was performed based in the minimum number of individuals collected to find

at least one infected.

To estimate and characterize *Wolbachia* status, three categories of infection in species populations were used: *Wolbachia* infected, *Wolbachia* non-infected and non-determined. To characterize the category as non-infected, a simulation was conducted to estimate the minimum number of individuals required to assort different frequencies of *Wolbachia*. For example, if *Wolbachia* frequency is intermediate in the estimated population, the minimum number of individuals sampled to found at least one infected is given by the reduction in the probability of false negatives (Figure 4). Therefore, to reduce to less than 5% chance to estimate false negatives there is a need of five flies. When the frequency of *Wolbachia* is low in the estimated population, about 10%, the number of individuals that show a percentage of 5% or less of false negatives is higher, about 30 individuals. Therefore, all species for which we collected less than 30 individuals in the overall communities and which none were found infected were classified as non-determined.

2.9 *Wolbachia* frequency at different levels of biological organization

To answer the questions that *Wolbachia* is present at significant levels in the communities and with high frequencies in their natural populations, the prevalence was accessed in two ways in this study: first as a community feature and second as a population feature. At the community level, *Wolbachia* prevalence was accessed as the frequency of species infected in each community (richness infected) and as the frequency of all individuals infected regardless of species (overall abundance infected). At population level, prevalence was evaluated as the frequency of *Wolbachia* infected individuals in each species population within communities. This approach allowed to investigate the relationship between drosophilid diversity and *Wolbachia* success in the community in the field. Significance (< 0.05) was attained using Pairwise Fisher exact tests with Bonferroni correction, to test the pairwise *Wolbachia* frequency difference between the eight communities over the different scales.

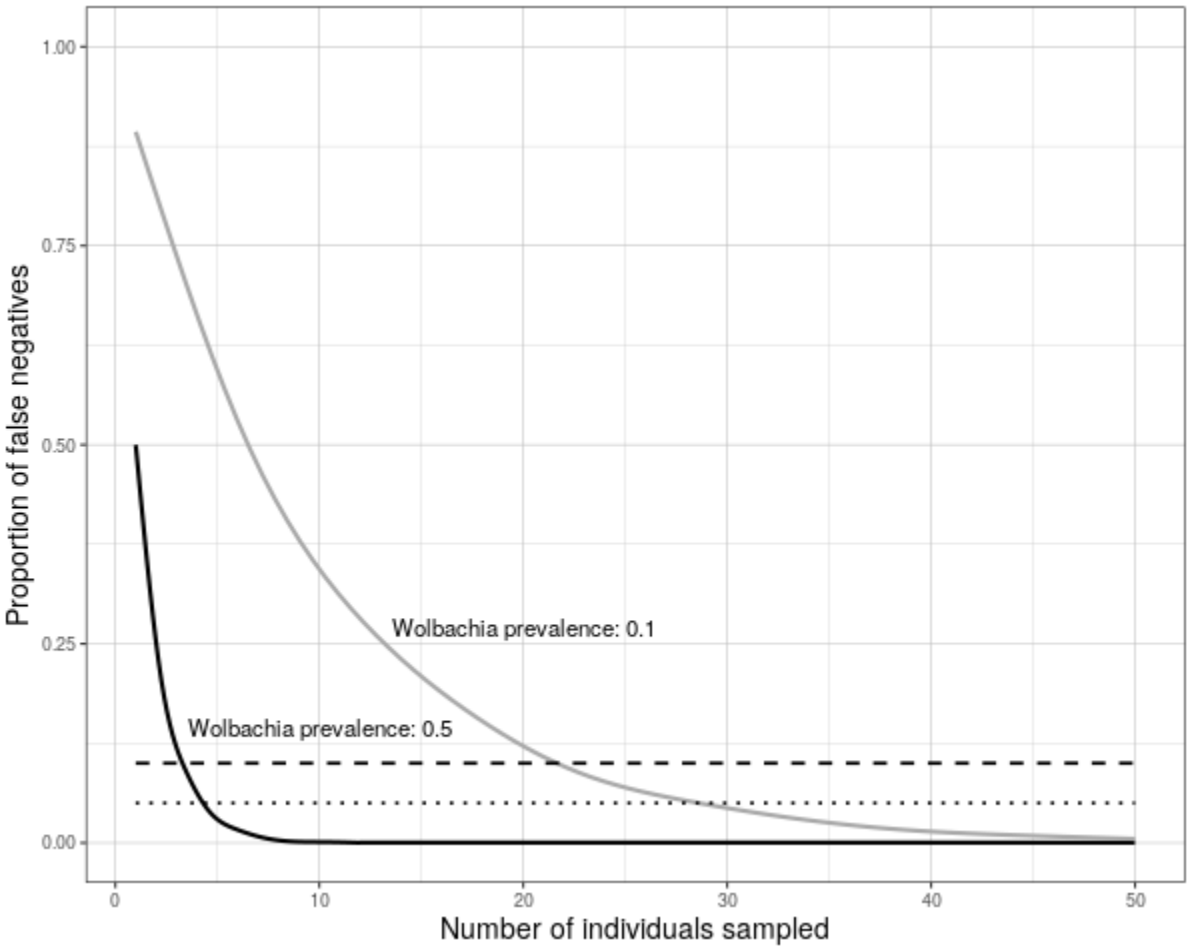

Figure 4. Simulation test to estimate the minimal number of individuals found non-infected to characterize a species population as not infected with *Wolbachia* given the reduce in the probability of false negatives as the number of individuals characterized increased. Black line shows this feature when the frequency of *Wolbachia* is intermediate (p = 0.5) and grey line when *Wolbachia* frequency is low (p = 0.1). Horizontal lines represent the 10% margin (dashed) and 5% margin (dotted) of false negatives.

2.10 Predicting *Wolbachia* presence under clade and ecological components

Furthermore, to predict the importance of clade and ecology on *Wolbachia* prevalence a generalized mixed logistic regression was carried out. This was done to express how much of the probability of an individual being infected by *Wolbachia* is explained by species and species group

(clade component) and by environmental factors and community structure considered in this study (ecological component). If *Wolbachia* presence is important in ecology, ecology and community structure could improve the explanation of the odds of individuals being infected. Alternatively, if clade component overshadows the importance of ecology, species and species group will explain the probability of an individual being infected by *Wolbachia* and the inclusion of ecological component will not improve this explanation. To expresses this in the models, factors on clade component were chosen as random effects, whereas factors on ecological component were chosen as fixed effects.

RESULTS

3.1 Drosophilids communities vary in species richness, abundance, and composition.

Abundance differs between lowland and highland sites. The amount of drosophilids caught in each community are about one order of magnitude higher in lowland sites than highland sites as represented by the total number of individuals collected and the number of individuals collected per trap in each community (Figure 5).

Drosophilid communities are diverse. Lowland sites had both more individuals and species count in total. By comparing sites in different seasons, LL1 and HL2 sites differ in the number of species sampled. LL1 had 34 species described in winter, being the richest site in that season, however, in summer a considerable loss of species was observed, showing about half species (18 sp.) (Table 2). HL2, in the other hand, had the opposite and more prominent effect. Being the poorest site in species richness in winter and of all collections, only 7 was described, it became the richest site in the summer with 43 species becoming the richest site of that season and of all collections (Table 2).

Diversity is higher in winter communities, excepting the HL2 in which the summer community is the most diverse as showed by the Simpson's Diversity Index (D) (Table 2). LL1 has the highest D value in winter. In summer, the prevalence of dominant species is higher which should had contributed to a decrease in D value. HL2 became the richest site in summer and reach the highest D value, which show that all its species has evenness in abundance.

Species composition is different among communities. The predicted ordination distance (Bray-Curtis metric) is fitted with R^2 0.999 and R^2 0.992 for, respectively, non-linear, and linear methods against the original distance matrix in the non-metric multidimensional scaling (NMDS) analysis. Excluding one community from the highland being more similar with the ones at lowland (HL2S), altitude factor presents communities with different species composition (Figure 6), as showing by the dissimilarity along the first axis of variation, and the appearance of two altitudinal clusters, with high values for highland sites and low values for lowland sites, again excluding one community. A less prominent differentiation along the second axis of variation is also observed. Interestingly, it shows a trend in season differentiation, as lower values are associated with the

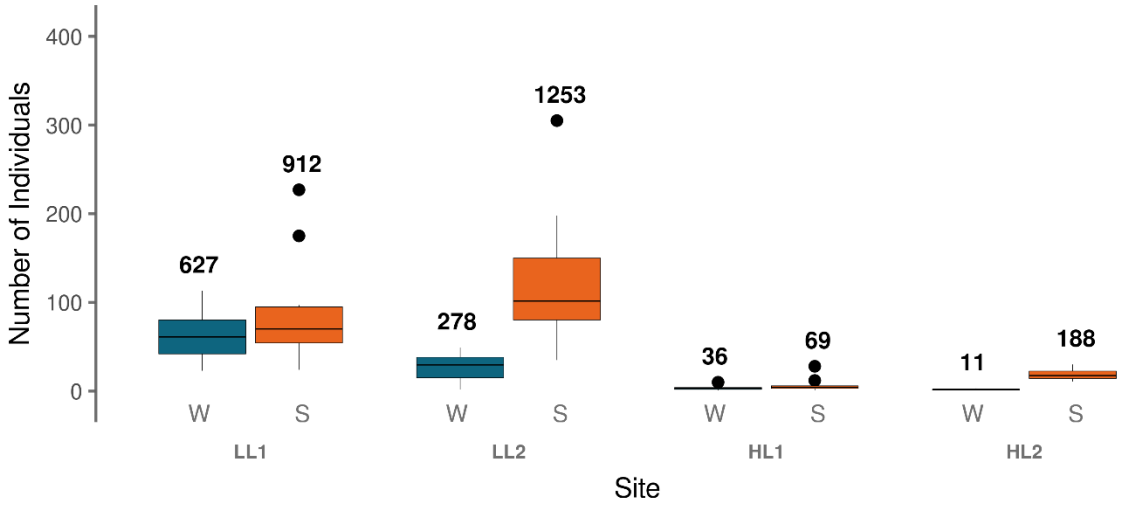

Figure 5. Boxplot of number of individuals collected in ten traps distributed in four sites in winter (blue) and in summer (orange) totalizing eight communities. Bold horizontal line represents median. Upper and lower box limits represent 95% confidence interval and dots are outliers. The number of individuals collected in each community is shown above each boxplot.

winter communities and higher values with the summer communities, but this trend seems to be site specific, or showing a great site dependence (as showing by the size of the transition lines between different seasons of each site) and is not present in HL2 site (this line is almost orthogonal with the second axis).

Communities' species rank-abundance distributions (RADs) show different levels of evenness. The eight communities show different levels of evenness in its species rank-abundance distribution (Table 3, Figure 7), being LL1W and LL1S the two most uneven. In those, RAD is characterized by a dominance preemption, showing a fast (geometric) decrease in abundance as species rank order goes from the most abundant to the less abundant. It is important to note that despite AIC is lower for the model selected in these two communities, a clear sub estimation of rarer species is observed (Figure 7a, 7b). All the other communities show a flatter RAD than LL1W and LL1S and even flatter than that from the statistical lognormal distribution. In fact, LL2W, LL2S, HL1W and HL1S all show the same RAD (Zipf), defined by a fast decrease in abundance

Table 2. Species abundance, richness, and the associate Simpson's Diversity Index (D) for all the eight communities.

	Abundance		Richness		Simpson's Diversity Index (D)	
	Winter	**Summer**	**Winter**	**Summer**	**Winter**	**Summer**
LL1	627	912	31	14	0.84	0.61
LL2	278	1253	26	25	0.73	0.42
HL1	36	69	9	12	0.70	0.60
HL2	11	188	7	38	0.74	0.92

for the first species in the rank order, followed by a shallow decrease for the subsequent species in the rank, resulting in higher richness than the model that show niche preemption. HL2 site, in the other hand, was the only site exhibiting difference in model selection when season is considered. HL2W did not show difference from the null model ("broken-stick"), which show a less expressive decrease in abundance of the initial dominant species, and more modest decrease in the tail end of the distribution. However, this inference is uncertain due to sample size limitation. HL2S, best model is selected to be a Mandelbrot distribution. That differs from Zipf, by the addition of one nonlinear parameter. Overall, the null model distribution shows the most even distribution over all models, and all the different models selected show deviation from this expectation. Important to say, however, is that both highland communities sampled in winter (HL1W and HL2W) had low abundances, assuredly affecting the outcome of this result. HL2W show high evenness and richness, which contribute to it having the highest diversity (D) estimate (Table 2).

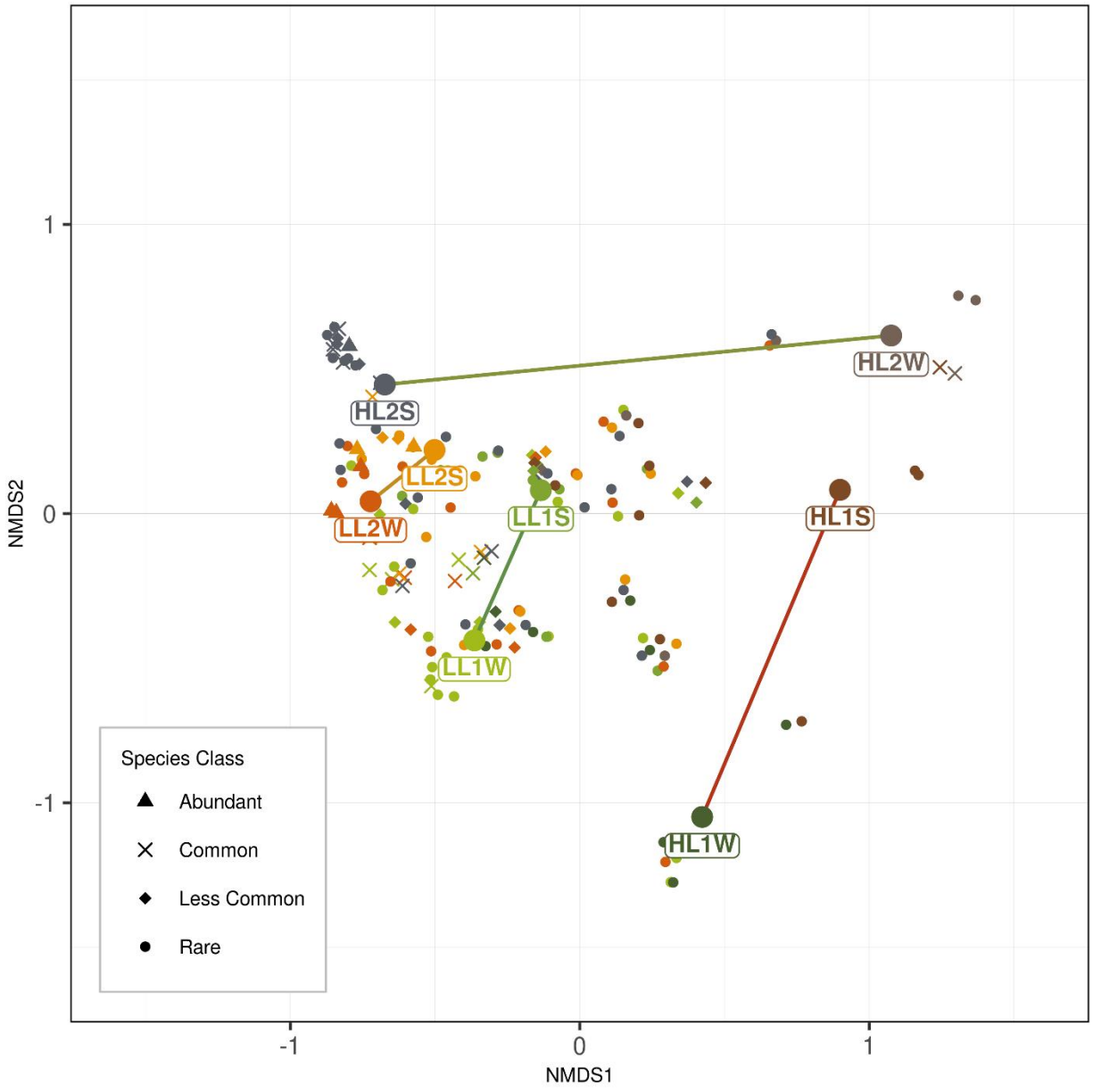

Figure 6. Non-metric multidimensional scaling (NMDS) collapsing information from the eight communities. Each community is represented by a colored circle next to its highlighted name. The drawn lines represent the transition of the same site from winter to summer. Small colored shapes represent species from the respective community and different shapes represent four levels of species abundance.

Table 3. AIC scores for the eight communities assessed in this study. The lowest AIC score represent the best model selected (in bold) for that specific community among five known rank-abundance distribution (RADs).

Species Rank-Abundance Distributions (RADs) AIC values					
	Broken-Stick	**Preemption**	**Lognormal**	**Zipf**	**Mandelbrot**
LL1W	554.66	**167.70**	233.36	288.79	171.70
LL2W	322.67	183.60	107.55	**89.1**	90.0
HL1W	32.61	31.25	31.69	**29.61**	31.61
HL2W	**19.13**	20.41	21.82	20.82	22.82
LL1S	891.32	**102.86**	145.72	179.63	106.86
LL2S	2502.21	748.61	192.91	**131.97**	133.97
HL1S	82.01	58.58	49.18	**40.78**	42.78
HL2S	145.82	132.78	125.74	129.09	**120.16**

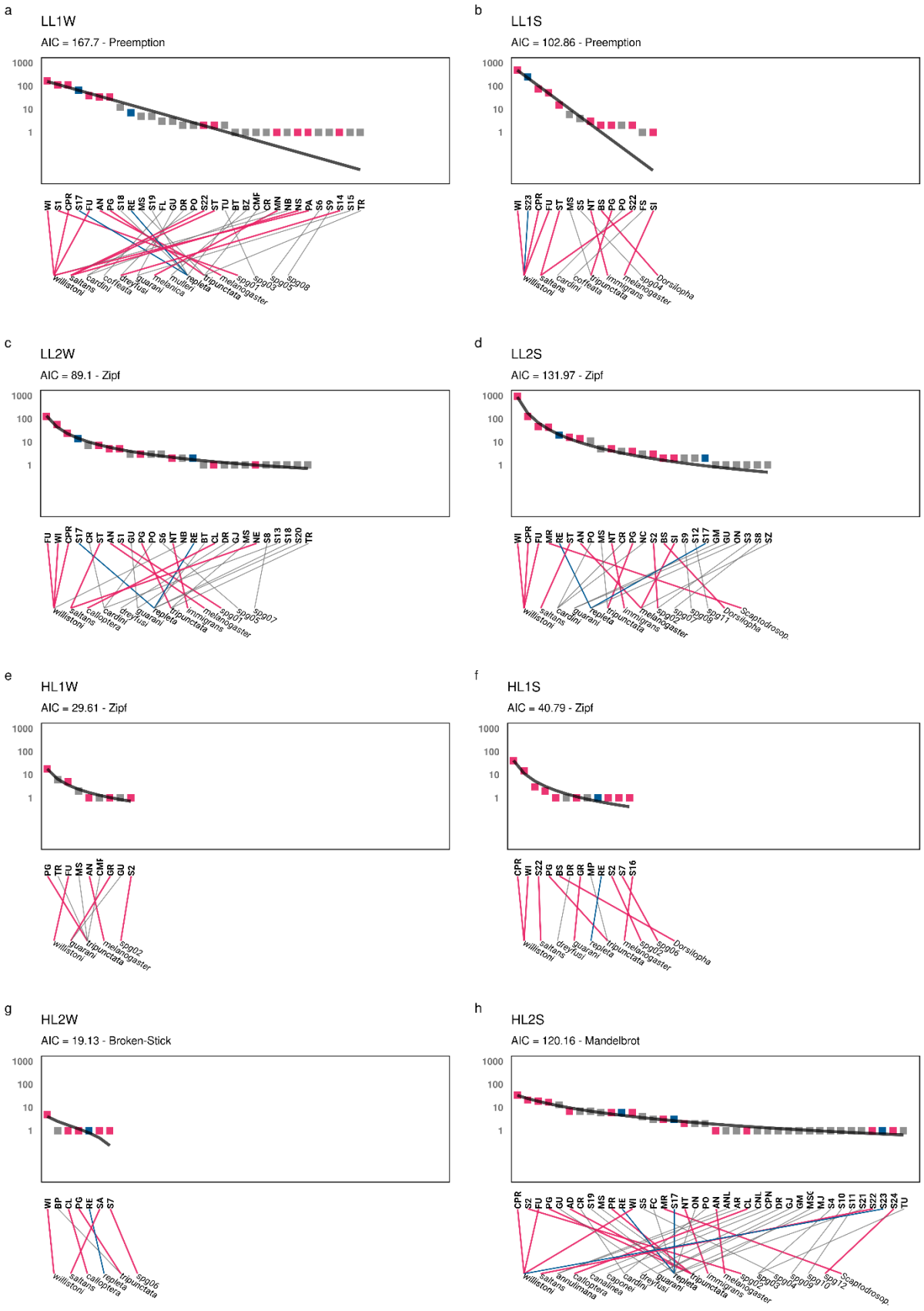
a
LL1W
AIC = 167.7 - Preemption
b
LL1S
AIC = 102.86 - Preemption
c
LL2W
AIC = 89.1 - Zipf
d
LL2S
AIC = 131.97 - Zipf
e
HL1W
AIC = 29.61 - Zipf
f
HL1S
AIC = 40.79 - Zipf
g
HL2W
AIC = 19.13 - Broken-Stick
h
HL2S
AIC = 120.16 - Mandelbrot

Figure 7. Rank-abundance distributions and *Wolbachia-Drosophila* bipartite graphs interactions of the eight communities. Squares represent the total abundance of each species in each community. The scale of abundance was log transformed for better view of low abundances. Each species was related with its associate species group showing relationship between clade and *Wolbachia* presence. Pink squares and lines represent species that was found with *Wolbachia*. Green squares and lines represent species that *Wolbachia* was not found and estimated to be uninfected. Grey squares and lines show species that *Wolbachia* infection were not determined. Species IDs are coded in Table 4.

3.2 *Wolbachia* is present in less than half of species in each community.

Wolbachia frequency was estimated in two ways in this study. First, as an attribute of each community species richness (community scale), that means the percentage of *Wolbachia* presence in the total species of the community. Second, as an attribute of species population within communities (population scale), that means the percentage of *Wolbachia* presence in each species population. The total of species screened in this study is provided in Table 4.

At the community scale the frequency of *Wolbachia* infected species was similar between communities in the winter (< 30%), except for the site HL2 with ~45% of infected species (Figure 8a), however the estimation for this community has a high standard error because of low number of flies collected. In the summer, the values are slightly higher (less or about 30%). HL2, which is the richest and most diverse community, has only 14% of its species infected. Pairwise Fisher exact test with Bonferroni correction did not detect significant difference on frequency estimations of *Wolbachia* presence in different species of each community.

Wolbachia presence was also accessed by looking to the proportion over the total abundance of individuals in each community at the community scale. This gives a rough estimation of *Wolbachia* success in the community, as its frequency is expected to be high if advantageous to the host or if it is a successful reproductive manipulator in many species. The proportion of individuals infected in each community, regardless of species, is also below the 50% mark (Figure 8b), in fact most of them are below 30%. HL1 has the lowest proportion of infection (11%) in winter and of all communities and became the site with highest infection (44%) in summer and of all communities. The use of Pairwise Fisher exact test with Bonferroni correction detects significant difference on frequency between summer and winter collections from respectively, LL1

Table 4. Number of samples screened for *Wolbachia* in this study over the total of samples collected in each assemblage.

***Wolbachia* Screening**			
	Winter	**Summer**	**Total**
LL1	338/627	92/912	768/3070
LL2	196/278	142/1253	
HL1	27/36	9/69	134/304
HL2	11/11	87/188	
Total	572/ 952	330/ 2422	902/3374

and LL2 sites. The pairwise test also detect significant difference between the winter community, LL1W and the communities LL2W and LL2S. Difference was also attained between the summer community LL1S and HL2S. Interestingly, the pattern of *Wolbachia* infection is inversed when season is considered in different lowland sites: LL1W show a frequency of 12,4% and LL1S a frequency of 39.1%, whereas LL2W show a frequency of 38.2% and LL2S a frequency of 27.5%.

Dominant species are infected. In 7 out of the 8 communities, the first species in the rank, therefore the dominant species, is from the *Drosophila willistoni* group. The cryptic nature found in species of this group was investigated in our phylogenetic analysis of COI sequences showing that our methodology was able to differentiate some species, but not *D. willistoni* from *D. paulistorum* which is all grouped in *D. willistoni* species name (Figure 9). At lowlands sites *Drosophila willistoni* dominate in three communities (LL1S, LL2S, LL1W), being present in LL2S by a fair amount (one order of magnitude more individuals than the second species in the rank). *Drosophila willistoni* also dominate in HL2S, however the overall sample size is so small that it is difficult to attribute a pattern. *Drosophila willistoni* was known to harbour *Wolbachia* (Miller & Riegler, 2006) and were found infected with intermediate frequency in this study (Figure 10a). Interestingly, generally the second, third and fourth species in the rank at lowland sites are also from the *willistoni* group (Figure 7, Table 4). In LL2W the dominant species is *Drosophila fumipennis*, followed by *Drosophila willistoni* and *Drosophila capricorni*. Those three species are related, with *D. fumipennis* and *D. capricorni* being more closely related (subgroup *bocainensis*). However, only *D. fumipennis* was found infected with *Wolbachia*, also with intermediate frequency

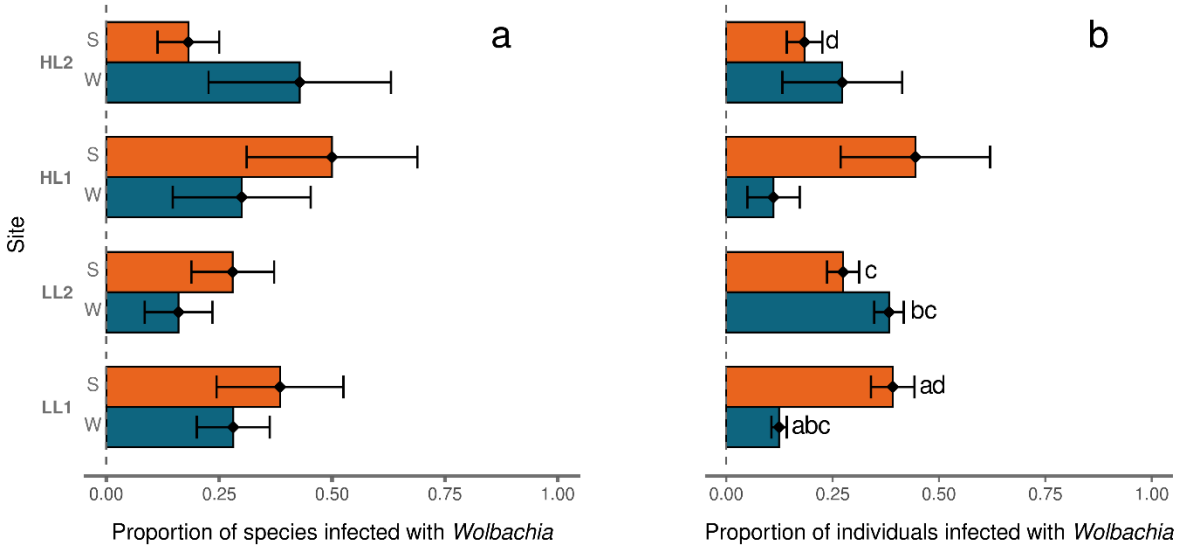

Figure 8. Proportion of species infected with *Wolbachia* (a). Proportion of individuals infected with *Wolbachia* (b). Orange colors represent summer assemblages whereas blue color represent winter assemblages. Error bars show the error associated with frequency estimation given the number of individuals sampled. Letters show the significance of the pairwise Fisher exact test with Bonferroni correction (< 0.05).

(Figure 10b). In only one community (HL1W) the first species in the rank was not from *Drosophila willistoni* group, being from the group tripunctata, *Drosophila paraguayensis*, this species shows a low level of *Wolbachia* infection.

Many species are uninfected. Many species were not found infected with *Wolbachia*. None of the species from the species group cardini, guarani, repleta and many species from species group tripunctata (except *Drosophila paraguayensis*, and *Drosophila prosimilis*) were found infected (Figure 7, Table 4). Those groups contain species found sometimes at an intermediate rank position in the RADs, especially at lowland sites. Eleven groups were classified as unknown as their unique representative species were not able to be determined. From those, only spg.2 (sp. 2) and spg.6 (sp. 7) were found infected. Excepting HL2S, those species groups being not infected with *Wolbachia* contribute to more than half of the diversity being uninfected in the communities (Figure 7, Table 4). Species from the species group annulimana, canalinea, caponei, coffeata, mulleri were all present with low abundance in all communities (n < 3). None of those were found infected with *Wolbachia*.

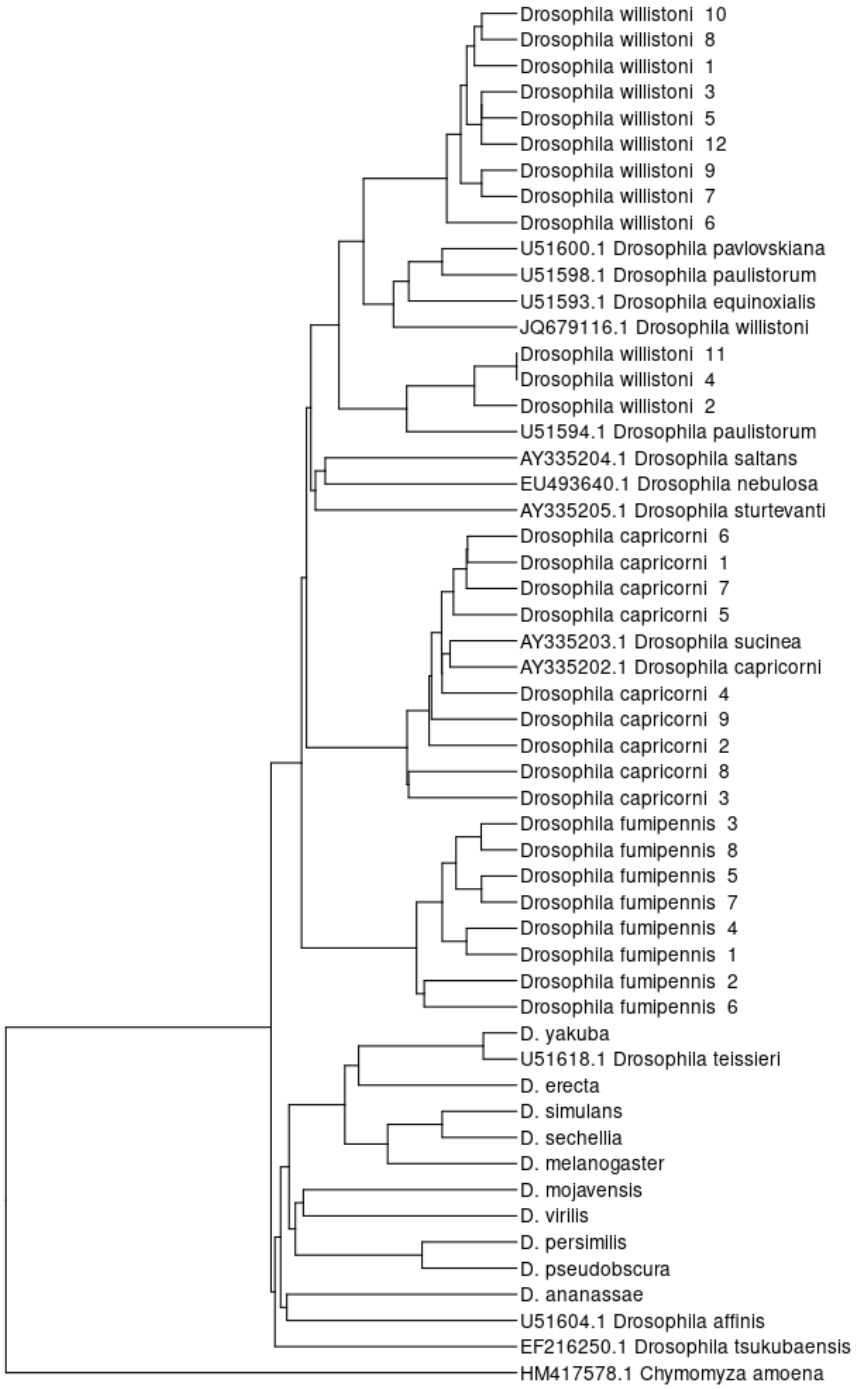

Figure 9. Phylogenetic tree containing twelve replicates on 858 bp barcode COI sequences of each of the three dominant species of willistoni group (*D. willistoni*, *D. fumipennis* and *D. capricorni*) among with sequences obtained from NCBI GenBank® and UCSC In-Silico PCR tool. Tree was made using UPGMA method. *D. willistoni* is cryptic with *D. paulistorum.* Samples identified as *D. willistoni* 2, 4 and 11 probably are *D. paulistorum*.

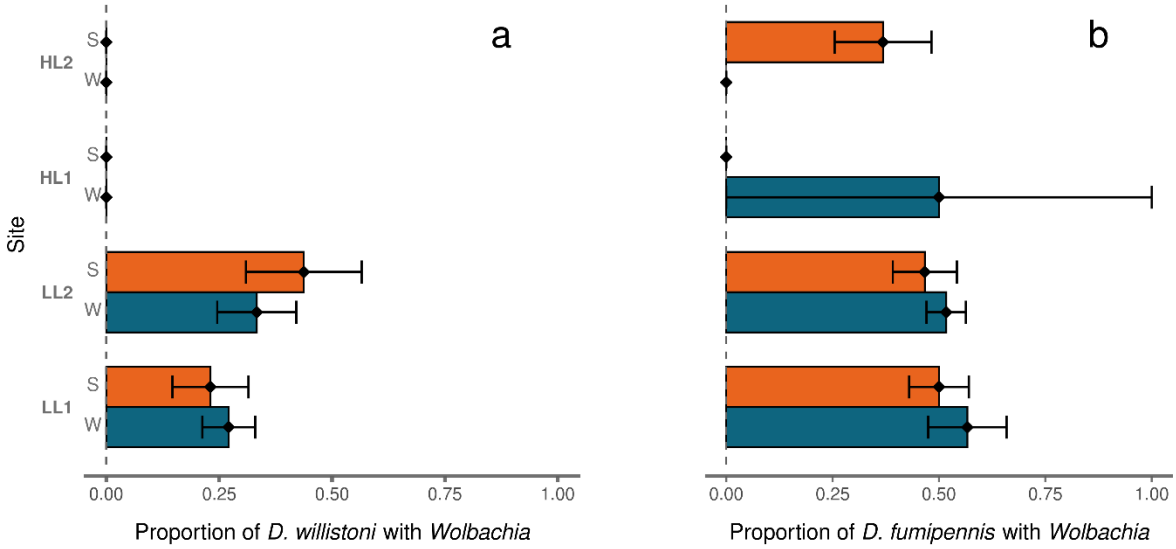

Figure 10. *Wolbachia* frequency in *D. willistoni* (a) and *D. fumipennis* (b) in each of the eight communities. Orange colors represent summer assemblages whereas blue color represent winter assemblages. Error bars show the error associated with frequency estimation given the number of individuals sampled. Letters show the significance of the pairwise Fisher exact test with Bonferroni correction (< 0.05).

Nonnative and cosmopolitan species were collected and carries *Wolbachia*. One species that was not present in the winter communities appeared in the summer assemblages. This is from the *Drosophilinae* nonnative subgenus *Scaptodrosophila* (group *latifasciaeformis*), with *Drosophila mirim* as the only representant. This species was found infected in LL2S and is at the intermediate positions in the RAD and is also present and found infected in HL2S but with low abundance. Another nonnative subgenus present in the communities is *Dorsilopha*. The representant species *Drosophila busckii*, was found on summer with only two individuals in the lowland communities LL1S and LL2S, all uninfected, and with one individual in the highland HL1S infected. Moreover, the cosmopolitan group *melanogaster* was also found in the neotropical communities, with the species *D. ananassae*, *D. simulans* and *D. suzukii*. All of those were found infected with *Wolbachia*, but only *D. ananassae* was present at intermediate RADs, the other two were rare species in the communities. The recent described as cosmopolitan species *Drosophila nasuta* (Vilela and Goñi, 2015), was found in the communities LL1S, LL2W, LL2S and HL2S with low abundances (< 5 individuals), in LL1S and LL2S individuals were found infected.

Some of the rarest species were found infected. Including the unidentified species sp.2 and sp.7 from the unidentified groups that are both infected and at low rate in the communities and the cosmopolitan and nonnative species. Many species from the group saltans, were found infected and present with low abundance, excepting *Drosophila sturtevanti*, that was found at intermediate rank positions in LL1S, LL2S and LL2W. Species from the neotropical groups calloptera, dreyfusi and melanica were present in all communities with low abundance ($n < 6$). But species within those groups were found infected with *Wolbachia* (Figure 7, Table 4).

3.3 Ecology does not improve the odds of explaining *Wolbachia* occurrence.

The inclusion of ecological component in our logistic multilevel regression model does not improve the explanation of *Wolbachia* occurrence when compared with our null model only containing the clade components species and species groups. Clade component shows an intraclass correlation coefficient that explains about 6.5 % of chance that species alone predict *Wolbachia* occurrence, whereas species and species group combined increase this explanation to about 40%. The inclusion of ecological factors abundance, seasonal shift, and altitudinal differentiation in our proposed model does not change these odds (Figure 11). Our obtained AIC from the proposed model was 229.2 versus 224.0 from the null model. Competing models containing different combinations of our ecological variables did not perform better than our null model. In fact, the probability of *Wolbachia* infection shows a clear independence of ecological components, namely, individuals do not have more chances of carrying *Wolbachia*, when present with high densities with conspecifics and neither when present in any of the communities from different seasons and different altitudes.

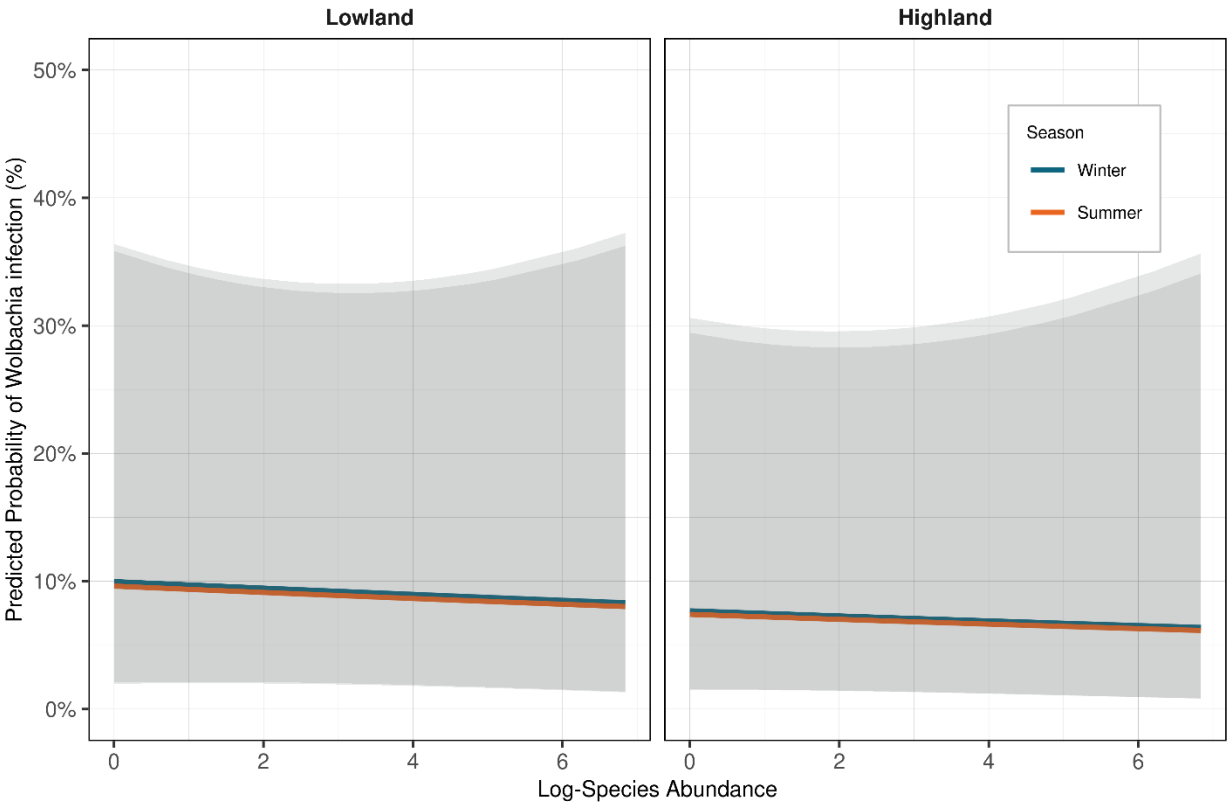

Figure 11. Logistic multilevel modelling shows the independence of the prediction of *Wolbachia* infection from ecological factors abundance, season, and altitude. The large confidence intervals show that most of the variation remains unexplained and that it did not improve the explanation obtained by our null model.

Table 5. Description of species and species groups identified in this study communities.

	Winter				Summer			
Site	**Species Group**	**Species**	**N**	**ID**	**Species Group**	**Species**	**N**	**ID**
LL1	willistoni				willistoni			
		D. willistoni	167	WI		*D. willistoni*	500	WI
		D. capricorni	113	CPR		*D.* sp23	248	S23
		D. fumipennis	38	FU		*D. capricorni*	74	CPR
		D. nebulosa	1	NB		*D. fumipennis*	52	FU
		D. paulistorum	1	PA				
					saltans			
	spg1					*D. sturtevanti*	15	ST
		D. sp1	115	S1		*D.* sp22	2	S22
	repleta				tripunctata			
		D. sp17	68	S17		*D. mediostriata*	6	MS
		D. sp18	12	S18		*D. paraguayensis*	2	PG
		D. repleta	7	RE	spg4			
		D. sp19	5	S19		*D.* sp5	4	S5
		D. betari	1	BT	immigrans			
	melanogaster					*D. nasuta*	3	NT
		D. ananassae	34	AN	cardini			
	tripunctata					*D. polymorpha*	2	PO
		D. paraguayensis	34	PG	dorsilopha/busckii			
		D. mediostriata	5	MS		*D. busckii*	2	BS
		D. tripunctata	1	TR	coffeata			
						D. fumosa	1	FS
	coffeata				melanogaster			
		D. flavolineata	3	FL		*D. simulans*	1	SI
	guarani							
		D. guaru	3	GU				
		D. sp15	1	S15				
	cardini							
		D. polymorpha	2	PO				
		D. cardini	1	CR				
	dreyfusi							
		D. dreyfusi	2	DR				
		D. sp14	1	S14				
	saltans							
		D. sp22	2	S22				
		D. sturtevanti	2	ST				
		D. neosaltans	1	NS				
	spg3							
		D. tuchaua	2	TU				
	melanica							
		D. melanica	1	MN				
	mulleri							
		D. buzzatii	1	BZ				
	spg5							
		D. sp6	1	S6				
	spg8							
		D. sp9	1	S9				
		D. campestris	1	CMP				
LL2	willistoni				willistoni			
		D. fumipennis	129	FU		*D. willistoni*	936	WI
		D. willistoni	58	WI		*D. capricorni*	126	CPR
		D. capricorni	24	CPR		*D. fumipennis*	46	FU
		D. nebulosa	2	NB				
					Scaptodrosophila/latifasciaeformis			
						D. mirim	44	MI

(continued)

	Winter				Summer			
Site	**Species Group**	**Species**	**N**	**ID**	**Species Group**	**Species**	**N**	**ID**
LL2	repleta				repleta			
		D. sp17	14	S17		*D. repleta*	20	RE
		D. repleta	2	RE		*D.* sp17	2	S17
		D. betari	1	BT		*D. onca*	1	ON
		D. sp18	1	S18	saltans			
		D. sp20	1	S20		*D. sturtevanti*	16	ST
	cardini				melanogaster			
		D. cardini	7	CR		*D. ananassae*	14	AN
		D. polymorpha	3	PO		*D. simulans*	2	SI
		D. sp13	1	S13		*D. suzukii*	1	SZ
	saltans				cardini			
		D. sturtevanti	7	ST		*D. polymorpha*	11	PO
		D. neoelliptica	1	NE		*D. cardini*	4	CR
	melanogaster					*D. neocardini*	3	NC
		D. ananassae	5	AN	immigrans			
	spg1					*D. nasuta*	5	NT
		D. sp1	5	S1	tripunctata			
	guarani					*D. mediostriata*	5	MS
		D. guaru	3	GU		*D. paraguayensis*	4	PG
		D. guaraja	1	GJ	spg2			
	spg5					*D.* sp2	3	S2
		D. sp6	3	S6		*D.* sp3	1	S3
	tripunctata				dorsilopha/busckii			
		D. paraguayensis	3	PG		*D. busckii*	2	BS
		D. mediostriata	1	MS	spg11			
		D. tripunctata	1	TR		*D.* sp12	2	S12
	immigrans				spg8			
		D. nasuta	2	NT		*D.* sp9	2	S9
	calloptera				guarani			
		D. calloptera	1	CL		*D. guaramunu*	1	GR
	dreyfusi					*D. guaru*	1	GU
		D. dreyfusi	1	DR	spg7			
	spg7					*D.* sp8	1	S8
		D. sp8	1	S8				
HL1	tripunctata				willistoni			
		D. paraguayensis	18	PG		*D. capricorni*	41	CPR
		D. tripunctata	6	TR		*D. willistoni*	15	WI
		D. mediostriata	2	MS	saltans			
	willistoni					*D.* sp22	3	S22
		D. fumipennis	5	FU	tripunctata			
	guarani					*D. paraguayensis*	2	PG
		D. guarani	1	GR		*D. mediopuctata*	1	MS
		D. guaru	1	GU	dorsilopha/busckii			
	melanogaster					*D. busckii*	1	BS
		D. ananassae	1	AN	dreyfusi			
	tripunctata iii					*D. dreyfusi*	1	DR
		D. campestris	1	CMP	guarani			
	spg2					*D. guarani*	1	GR
		D. sp2	1	S22	melanogaster			
						D. sp16	1	S16
					repleta			
						D. repleta	1	RE
					spg2			
						D. sp2	1	S22
					spg6			
						D. sp7	1	S7

(continued)

	Winter				Summer			
Site	**Species Group**	**Species**	**N**	**ID**	**Species Group**	**Species**	**N**	**ID**
HL2	willistoni				willistoni			
		D. willistoni	5	WI		*D. capricorni*	35	CPR
	calloptera					*D. fumipennis*	19	FU
		D. calloptera	1	CL		*D. willistoni*	6	WI
	repleta					*D.* sp23	1	S23
		D. repleta	1	RE	spg2			
	saltans					*D.* sp2	21	S2
		D. saltans	1	SA	tripunctata			
	spg6					*D. paraguayensis*	17	PG
		D. sp7	1	S7		*D. addisoni*	7	AD
	tripunctata					*D. mediostriata*	6	MS
		D. paraguayensis	1	PG		*D. prosimilis*	6	PR
		D. bipunctata	1	BP		*D. mediosignata*	1	MSG
					guarani			
						D. guaru	13	GU
						D. guaraja	1	GJ
						D. guaramunu	1	GM
					cardini			
						D. cardini	7	CR
						D. polymorpha	2	PO
					repleta			
						D. sp19	7	S19
						D. repleta	6	RE
						D. fascioloides	3	FC
						D. sp17	3	S17
						D. onca	2	ON
						D. moju	1	MJ
						D. sp21	1	S21
					spg4			
						D. sp5	4	S5
					Scaptodrosophila/latifasciaeformis			
						D. mirim	3	MR
					immigrans			
						D. nasuta	2	NT
					annulimana			
						D. annulimana	1	ANL
						D. ararama	1	AR
					calloptera			
						D. calloptera	1	CL
					canalinea			
						D. canalinea	1	CN
					caponei			
						D. caponei	1	CPN
					dreyfusi			
						D. dreyfusi	1	DR
					melanogaster			
						D. ananassae	1	AN
					saltans			
						D. sp22	1	S22
					spg10			
						D. sp11	1	S11
					spg3			
						D. sp4	1	S4
						D. tuchaua	1	TU
					spg9			
						D. sp10	1	S10
					spg12			
						D. sp24	1	S24

DISCUSSION

The neotropical drosophilid communities described in this study show a total of 71 species divided into 30 species groups within a total sample size of 3374 male individuals. This richness and abundance are unevenly distributed among communities contributing to our finding that the communities are different regarding to our selected ecological factors (Figure 6, Figure 7). Specifically, communities show overall high richness and great difference in the diversity metrics due to altitude. Our *Wolbachia* screening on communities shows less than 50% of species and individuals being infected, however, this does not show any pattern of ecological differentiation, adding to our finding that the inclusion of ecological factors did not improve the explanation of *Wolbachia* occurrence.

Communities show a notable difference in the diversity metrics employed, that is, diversity and composition differ among communities that are present at the same forest continuum and in the vicinity of each other. This emphasizes that the ecological factors investigated, namely, altitudinal differentiation and seasonal shift, impact drosophilid community's structure following that species populations are not isolated by distance, an effect displayed in many insects' communities (HODKINSON, 2005). Generally, at the finest scale, insect's composition and richness may peak at high, low, or mid altitude depending on the community studied and location (HODKINSON, 2005). At a broader scale of many insect's species, however, there is evidence of decrease in abundance and species richness due to elevation in tropical forests (WOLDA, 1987). Sometimes peaking at intermediate altitudes but with subsequent decrease in abundance in higher altitudes (JANZEN, 1973). A study with diversity of wasps in Atlantic Forest also show a consistent pattern of decrease in abundance and diversity following increase in altitude (RIBEIRO *et al.,* 2019). Our study show abundance drop of one order of magnitude in highland sites in comparison with lowland sites. Season variation in tropical forests, in the other hand, shows variation concerning rainfall volumes, when temperature does not vary considerably (PEEL *et al.,* 2007; JOLY *et al.,* 2014). In accordance with an assessment on drosophilid fauna in the Atlantic Forest of northeast Brazil (Coutinho-Silva *et al.,* 2017), our data show that drosophilid abundance in summer (wet) season is higher than winter (dry) season, the same result was obtained in their paper when they only consider neotropical species, but not when considering nonnative species. Overall, even with our low sample size, expectations found in community description reproduce what is expected for

insects in the tropical forest (Wolda, 1978. Coutinho-Silva *et al.*, 2017) with only the caveat that our diversity estimation was higher for winter assemblages than the summer ones, when the opposed was found for drosophilids of the Atlantic forest (DE TONI *et al.*, 2007).

Moreover, a singular dominance of species from the group *willistoni* was observed in almost all communities, suggesting its independence of ecological factors. Interestingly, however, is that *D. willistoni* shows ten times more individuals in summer assemblages than winter ones, suggesting higher densities due to season, a result also expressed elsewhere (Coutinho-Silva *et al.*, 2017). Despite this, the group *willistoni* ordinary presence does not seem to be associate with any of the diversity models that imply resource-apportioning in our attempt to describe rank-abundance distributions. Indeed, our best adjustments in model selection shows Zipf distribution, a model that can be described as presenting high degree of dominance but also high richness, as displayed by the presence of many rare species in the communities. Finally, our dimension reduction approach shows a trend of altitudinal differentiation along the first axis of variation and season differentiation along the second axis of variation, a pattern only disrupted by one highland community that show species that are more similar to lowland sites, but processes behind this could be site specific.

Following that, sample effort might offer a caveat in our analyses. Previous assessment on the diversity of drosophilids in the Atlantic Forest shows that even after large sample efforts (more than 5000 individuals) species rarefaction curves does not seem to reach a plateau (DE TONI *et al.*, 2007, DÖGE *et al.*, 2008). Our study features collections with abundances below than that, assuredly sub estimating the diversity. Our data also features an expressive number of unidentified species (23 out of 71). MEDEIROS & KLACZO (2004) inferred that the proportion of unidentified species increase with sample size and did not reach a plateau even with an extensive sample effort and predicted that half of the drosophilid species in the Atlantic Forest remain to be described. Moreover, drosophila taxonomy is challenging. We plan to combine traditional morphological taxonomy with identification through DNA barcode to improve the description and relationship of those unidentified species.

The endosymbiont literature has long demonstrated the successful and widespread nature of *Wolbachia* infections, with *Wolbachia* present in more than 50% of all arthropods (WERREN 2008, WEINERT *et al.*, 2015) and in 65% of all insect species (HILGENBOECKER *et al.*, 2008). Beyond that, *Wolbachia* commonly reaches high infection frequency within species populations,

expressing its success in matriline transmission in each generation (KRIESNER *et al*., 2016). In the present study, *Wolbachia* shows moderate to low occurrence on species from the same community (Figure 8), with less than half of species infected in all the eight communities. The predominant species from group *willistoni, D. willistoni* and *D. fumipennis* also display low to intermediate *Wolbachia* frequency in their lowland populations (Figure 10).

Many of the intermediate rank species, in the other hand, does not show *Wolbachia* infection, the conservative explanation is that although processes of horizontal transfer of *Wolbachia* is common (ZHOU *et al.,* 1998; VAVRE *et al.,* 1999; BALDO *et al.*, 2008), some clades show restrained association with *Wolbachia,* or its species genomes does not interact in such ways that infection is possible (SANAEI *et al.*, 2021). The *Drosophila* group repleta, for example, has never show any species that had *Wolbachia* (MATEOS *et al.,* 2006). The same could happen with groups cardini and guarani, for which there are no records of *Wolbachia* association. Despite this, due to small samples size, we were not able to classify those species as non-infected following the fixed number obtained in our *Wolbachia* discovery estimation. This fixed number was based on the proportion of false negatives under an arbitrary *Wolbachia* frequency, therefore, to reduce the proportion of false negatives to less than 0.05 in a hypothetical population with 10% of *Wolbachia* frequency, a screening of 30 flies were necessary, but only 5 flies if the frequency is set to be 50% (Figure 4). Moreover, just a few species were classified as non-infected due to sample size limitations, but a corollary of this is that if *Wolbachia* is present in small sample sizes with just five non-positive flies or more, we may say that their frequency is below the 50% mark. This can be attributed to *D. cardini* and *D. polymorpha* from group *cardini;* to *D. tripunctata* and *D. mediostriata* from group *tripunctata;* and, to the unidentified species *D. sp5.*

Given all those considerations and that our analysis deal with presence/absence data under the scope of community ecology (PODANI *et al.,* 2018), that is, mitigating the effects that are possible problematic in population ecology, our main aim in this study was to characterize and explore *Wolbachia* prevalence in a high scale level of biological organization, namely the one of communities' interactions. Methods to achieve this were integrated in our logistic regression analysis contributing to our finding that the probability of explaining host's individuals *Wolbachia* infections does not depend on ecology. Our null model shows that species explain at most 6.5% of *Wolbachia* odds of infection, whereas the combination of species and species group explains about

39%. Thus, the partition of variation not explained remain over 60%, and the processes behind this can be complex with our analysis barely scratch its surface. Even though, the combination of species and species group multiplies by six the explanation of *Wolbachia* occurrence when compared to species partition alone. This result might show that *Wolbachia* infection and its potential in ecology is constrained by deeper phylogenetic relationships, mainly due to clade association. Alternatively, if *Wolbachia* induced phenotypes is pervasive in many species, a possible scenario is that ecological differentiation displayed in our collection sites may not be high enough to affect *Wolbachia* frequencies.

Our data is pioneer in the assessment of *Wolbachia* on a community scale and it is among the few that performs its description in natural neotropical drosophilids. Much remains to be understood about neotropical drosophilids, especially their ecology and genomic response to environmental variation. *Wolbachia* doubtless have an important role in that regard, and this can be illustrated by the merely *Wolbachia* presence offering costs that hosts are constantly evolving against (McGraw & O'Neill, 2004). Important to say is that our analysis does not reduces the influence of ecology on *Wolbachia* presence. Instead, our results show that the influence of *Wolbachia* on host's ecology is not widespread and strong enough to leave a signature in the communities, a result that might be expected due to *Wolbachia* status as the one of the most abundant endosymbionts on Earth (Werren *et al.*, 1995). *Wolbachia* and *Drosophila* might have complex interactions with species populations in the field, and if there is interaction of ecological features, an outcome is populations experiencing variability in *Wolbachia* frequency due to balance of costs (including host reproductive manipulation) and the associated benefits. This is a plausible scenario given the spatial and temporal variation already described in *Wolbachia*'s host populations (Kriesner *et al*., 2016; Cooper *et al*., 2019). To fully understand how *Wolbachia-Drosophila* interaction can be mediated by those process and specially to understand why this does not leave a signature in the community level, three considerations must be done, namely, the nature and context of innovative endosymbionts induced traits; selection to maintain the unit of mutualisms; and finally; selfish genetic elements and the concept of clade selection. This will be described in the following paragraphs.

The strength of *Wolbachia* adaptations and hosts counteradaptations leading to an innovative evolutionary outcome depends on stability of some evolutionary forces and relaxation

in others over time, affecting, among other things, endosymbiont genome sizes (FISHER *et al.*, 2017). Thus, environmental constancy and the category of phenotype induced by endosymbionts (i.e., an essential or context-based phenotype), can either result in an ultimate outcome as evolutionary dependence or in facultative associations which might result in shifts between conflict and cooperation (ZUG & HAMMERSTEIN, 2015; FISHER *et al.*, 2017). To illustrate those outcomes, *Wolbachia* strains of filarial nematodes are all associated with nutrition provisioning phenotype (MERÇOT & POINSOT, 2009). This mutualism in which the induced phenotype is an essential requirement of hosts shows direction towards evolutionary dependence (obligatory mutualism), as evidenced by the death or fitness drop of nematodes when their *Wolbachia* are removed (MERÇOT & POINSOT, 2009; NEWTON & RICE, 2020). Therefore, those patterns show the strong association of genotypes that evolves as a single unit. In addition, *Wolbachia* shows phylogenetic congruency in filarial nematodes (SANAEI *et al.*, 2021), showing the maintenance of coevolution with no other forces such as host shifts disrupting the long-term association. Alternatively, phylogenetic congruency does not occur between *Wolbachia* and arthropods, mainly by pervasive host shifts (SANAEI *et al.*, 2021, TURELLI *et al.*, 2018). Interestingly, following the endosymbiont evolutionary theory, *Wolbachia* hijacking of host's reproductive machinery in arthropods also shows potential to lead to host dependence once redundance in this machinery may lead to relaxation of important genes associate with host's ability of reproduction. However, this does not seem to be the case in the plethora of *Wolbachia* strains and its phenotypes already described in arthropods (HAMM *et al.*, 2014, ZUG & HAMMERSTEIN, 2015), possible due to costs associated with *Wolbachia* infection, or in other words, the disruption of a unit of selection due to endosymbiont and host conflict. Instead, the recent literature showing that *Wolbachia* interaction might also mediate fitness benefits in arthropods such as, increased fecundity (WEEKS *et al.*, 2007), nutrition provisioning (NEWTON & RICE, 2020), and protection against RNA virus (TEIXEIRA *et al.*, 2008, PIMENTEL *et al.*, 2020), might show context environmental dependence. Protection against viral pathogens, specially, is a context-based trait, and might show only facultative associations due to biotic challenges.

Indeed, mutualisms operating in host-endosymbiont interactions might work under multilevel selection (HEATH & STINCHCOMBE, 2014), with evolutionary forces expected to be attached in the long-term maintenance of genes, endosymbiont, and hosts genomes interactions once there are mutually benefits in doing so. The evolution of mutualism expects punishment and sanctions to maintain cooperation between partners (WERNER *et al.*, 2014). If the mutualistic

induced trait is not an essential trait independent of environment change, environmental stability and predictability certainly will have an important role in the high-level selective pressures maintaining mutualisms (FISHER *et al.,* 2017), whereas environmental uncertainty may lead to partners shifting its display towards parasitism (ZUG AND HAMMERSTEIN, 2015). Among the beneficial traits already described to be induced by *Wolbachia* in arthropods, protection against RNA virus might show quite different contextual dependencies due to different environments presenting contrasting pathogens abundances and costs associated with its display. In accordance with dependence on environmental factors CHROSTEK *et al.* (2020 – preprint) show that temperature alters *Wolbachia* ability to protect against DCV virus in *D. melanogaster*, with low protection when flies develop at 18°C and higher protection when develop at 25°C. The environmental factors described in our sampled communities does not exhibit such contrast, contributing to the alternative scenario that an ecological signal may be present in communities with more contrasting environments. In addition, antiviral protection in *D. simulans* only happens when *Wolbachia* is present in high densities, which is in turn associate with many costs to hosts (MARTINEZ *et al.* 2014). *Wolbachia* costs in hosts includes classical life history traits, namely reductions in fecundity, egg to adult survival, development time and lifespan (MCGRAW & O'NEILL, 2004).

Reproductive manipulation is the rule in arthropods, contrasting with *Wolbachia* strains in filarial nematodes this phenotype is not essential for survival, but have shown few evidence to became obligatory due to induction of parthenogenesis in parasitic wasps (DEDEINE *et al.,* 2001; KREMER *et al.,* 2009). In drosophilids, host's reproductive manipulation is mainly induced by cytoplasmic incompatibility (TURELLI & HOFFMAN, 1997, MILLER & RIEGLER, 2006, WERREN, 2008). In *Drosophila* clades, the pervasive host shifts contribute to mismatch phylogeny and coevolutionary forces (TURELLI *et al.,* 2018, MARTINEZ *et al.,* 2020), characterizing CI-inducing *Wolbachia* as selfish genetic elements. CI evolves under positive frequency dependence, and it must have to overcome a threshold first to start to be selected and reach fixation (BARTON & TURELLI, 2011). In fact, theory predicts that a mutualistic benefit is expected to occur to increase *Wolbachia* frequency above this threshold, and then CI would increase frequencies towards fixation (BARTON & TURELLI, 2011). However, high prevalence is also observed on some non-causing CI strains (HOFFMAN, 1988, HOFFMAN *et al.,* 1996; KRIESNER *et al.,* 2013, HAMM *et al.,* 2014, MEANY *et al.,* 2019). Recent studies have found the genetic architecture behind CI.

Wolbachia-modified sperm produces incompatibility proteins, namely CI-causing factors (*cifs*) in a *Wolbachia* genomic region associated with a WO prophage insertion (BECKMANN & FALLON, 2013). These include CI-inducing deubiquitylase (*cid*) and CI-inducing nuclease (*cin*) pairs predicted to generate toxicity of infected male sperm (*B*) and rescue in infected female egg cells (*A*) (BECKMANN & FALLON, 2013; BECKMANN *et al.,* 2017, LEPAGE *et al.,* 2017; LINDSEY *et al.,* 2018; SHROPSHIRE *et al.,* 2018, BECKMANN *et al.,* 2019). It happens that in some systems, like *Wolbachi*a that infect the *D. yakuba*-clade flies, WO prophage regions contain *cif* loci that diverged up to 50 million years ago from *D. yakuba*-clade *Wolbachia*, with evidence that flanking *Wolbachia*-specific transposons mediate horizontal transfer of these loci independent of phage (COOPER et al. 2019). Furthermore, this pattern of transposons mediating horizontal transfer is observed in many *Wolbachia* strains, with *Wolbachia* specific *cifA*-*cifB* pairs appearing more present in related hosts clades than distant clades, with support of frequently horizontal transmission happening along the phylogeny but with partial congruency, probably due to host's phylogenetic restriction (MARTINEZ *et al.,* 2020). The outcome of this might have some crucial evolutionary consequences. Indeed, selection under the scope of gene transferences among different lineages (i.e., at lower levels of biological organization), detaches evolutionary forces operating in the long-term maintenance of genes, endosymbiont, and hosts genomes interaction. This is supported in the same study of MARTINEZ *et al.,* (2020) that showed that the modification pair (*cifB*) accumulates loss of function mutations, consisting in a dead end for the incompatibility factors within *Wolbachia* and hosts genomes. In the perspective of the evolution of the pair itself, however, being selected to be horizontally transmitted consists in its ability to make copies that survive a transient functional life elsewhere.

Clade selection have been proposed as one mechanism behind the solid and pervasive horizontal transfer of *Wolbachia* to new species, with evidence of CI-inducing *Wolbachia* strains being more likely to be horizontally transferred between host species (HURST & MCVEAN, 1996). Clade selection consists in the ability of selfish genetic elements to invade new populations and species but doing so by creating new conditions for its spread and evade inevitable extinction within hosts (HURST & MCVEAN, 1996, MARTINEZ *et al.,* 2020). Indeed, this process appears to be what is acting at the level of the *cif* genes, beyond *Wolbachia* entire genomes, a process analogous to the evolution of transposable elements, which frequently go extinct within host species, but persist long enough to jump into new species (SCHAACK *et al.,* 2010; MARTINEZ *et al.,* 2020). This

finding characterizes *Wolbachia* as "a passenger of passengers" with the possibility that the outcome of *Wolbachia-Drosophila* interactions overshadows the importance of ecology on evolution of parasitic or mutualistic interactions. Our result of independence of ecology on the high level of community's biological interactions support this. In this level, important processes can be contrasted at the different nodes and ends of the phylogeny (species nested in species groups), and no signal of ecology suggests a pervasive selection at a lower level, showing a theoretical expectation that patterns at the community scale can emerge from complex interactions revealing many signatures left from genes all the way up to population and community level (LEVIN, 1992).

In conclusion, neotropical drosophilid communities are diverse and dissimilar in respect to the ecological features investigated here. *Wolbachia* show less than 50% of species and individuals being infected, a result that is below previous estimations of its success in arthropods and insects in general (WERREN 2008, WEINERT *et al.*, 2015; HILGENBOECKER *et al.*, 2008). And, finally, *Wolbachia* occurrence does not show any concrete pattern of ecological differentiation, adding to the alternative that clade components should be more important, with processes occurring in the scale of gene transfers overshadowing the importance of ecology of *Wolbachia-Drosophila* interaction. Our community approach to investigate *Wolbachia* success was key here as it expose the potentials and limitations of some proposed important biological phenomena leaving signal at the community level, namely, our results may suggest that cooperation between *Wolbachia* and their hosts seems to be more limited to singular cases than a ubiquitous phenomenon of *Wolbachia* and host interaction facilitated by certain environments, whereas conflict may be pervasive and might result in a dead end of manipulation phenotype and *Wolbachia* interaction (MARTINEZ *et al.*, 2020). Despite this, new insights need to be done to understand if the prevalence of *Wolbachia* occurs as a byproduct of the action of selfish genes, or if it finds new routes to success under the umbrella of ecological interactions in more contrasting environments. The recent find of the genetic basis of incompatibility shows an exciting opportunity to utilize genomic approaches to describe the occurrence and diversity of CI-inducing factors in drosophilid fauna (BECKMANN *et al.*, 2019, MARTINEZ *et al.*, 2020). Currently, *Wolbachia* ability to induce manipulation of reproduction and other phenotypes in neotropical fauna is unknown. Understand this, together with the mode and tempo of *Wolbachia* and CI-inducing phenotype acquisitions may help to describe the dynamics behind the pattern found here. Moreover, biotic interactions such as those mediated by pathogens prevalence in the field is also unknown and gradients showing pathogen prevalence differentiation

should be investigated and established to determine challenge in *Wolbachia*-host interaction. One important step to achieve this, is to describe the prevalence of viral pathogens present on neotropical communities, first looking for its diversity and then the potentials in causing fitness drop in hosts, finally linking correlations with environmental variables and *Wolbachia* prevalence. Although *Wolbachia* association was not found to depend on ecology here, thoroughly describing those ecological challenges is important to accurately evaluate the strength of competing evolutionary forces. The outcome of this ecological and evolutionary approaches will readily retain the classic *Wolbachia* cognomen "masters of manipulation" (WERREN, 2008) or will reclassify it to the new passive nickname "passenger of passengers".

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