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Wolbachia-density variation in weevils of the tribe Naupactini (Coleoptera, Curculionidae)

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Abstract

The intracellular bacteria *Wolbachia pipientis* infects arthropods and filarial nematodes and is able to manipulate host reproduction. It has been reported an association between parthenogenesis and *Wolbachia* infection in weevils from the tribe Naupactini. A curing experiment suggested that a threshold density of *Wolbachia* is required for parthenogenetic reproduction to occur. The aim of this study was to analyze *Wolbachia* infection status and density in two sexually reproducing species from the tribe Naupactini, *Naupactus xanthographus* and *Naupactus dissimulator*. *Wolbachia* infection was detected in individuals from both species in several geographic locations, not being fixed. Quantification through real time PCR confirmed that *Wolbachia* loads in sexual species were significantly lower than in parthenogenetic ones; these results support the hypothesis of a threshold level for parthenogenetic reproduction to occur in Naupactini weevils. Strain typing showed that both sexual species carry *w*Nau1, the most frequent strain in parthenogenetic Naupactini weevils. In addition, the presence of the WO phage, which might be an important factor regulating infection density in some hosts, was detected in this strain. Finally, *Wolbachia w*Nau1 was located throughout the whole insect body, which is in agreement with the idea of a recent acquisition by horizontal transfer of *w*Nau1 across the tribe Naupactini.

Keywords *Wolbachia* · Naupactini · Weevils · *Wolbachia* density · Parthenogenesis

1 Introduction

The obligate intracellular Gram-negative bacteria *Wolbachia pipientis* (Hertig 1936) (Rickettsiales: Anaplasmataceae) are the most widespread endosymbionts in nature, infecting arthropods and filarial nematodes (López-Madrigal and Duarte 2019). Although they are mainly transmitted vertically by females, horizontal transfer is also extensive (Duron and Hurst 2013). *Wolbachia* are able to manipulate host reproduction by inducing several disorders including cytoplasmic incompatibility, feminization of genetic males, embryonic and larval male killing and thelytokous parthenogenesis (Werren et al. 2008). These reproductive alterations give a selective advantage to the bacteria, enhancing infection spread (López-Madrigal and Duarte 2019). Nevertheless, *Wolbachia* can play plenty of roles in symbiotic associations, such as production of nutrients or resistance against pathogens (Zug and Hammerstein 2015). It has been suggested that *Wolbachia* tissue location may be important in relation to vital functions and transmission routes (Frost et al. 2014; Osborne et al. 2012; Pietri et al. 2016). Conversely to what was first thought, most *Wolbachia* infections are not restricted to reproductive organs. Different studies have detected that *Wolbachia* can colonize diverse somatic tissues such as muscle, digestive tract, brain, fat body and the hemolymph (Albertson et al. 2009; Cheng et al. 2000;

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Frydman et al. 2006; Osborne et al. 2012; Schneider et al. 2011; Strunov et al. 2017), implicating somatic tissue tropism as a key aspect in the evolution of these microorganisms (Pietri et al. 2016). At the same time, *Wolbachia* somatic location might also explain many phenotypic alterations (e.g. Hosokawa et al. 2010) as well as aiding horizontal transmission within and between species, thus serving as a mechanism to increase the genetic diversity of these bacteria. Furthermore, a recent study reported that the differential distribution of *Wolbachia* within *Drosophila* oocytes may affect strain's transmission route to the next generation, including not only germline-to-germline but also soma-to-germline vertical transmission pathways (Radousky et al. 2023).

Expression of some *Wolbachia*-induced host phenotypes are deeply influenced by bacterial titers (Baião et al. 2019; López Madrigal and Duarte 2019; Negri et al. 2009; Osborne et al. 2012; Unckless et al. 2009; Veneti et al. 2004). For instance, a two-step mechanism of parthenogenesis reported for parasitoid wasps (i.e. diploidization of the unfertilized egg followed by feminization) occurs only if *Wolbachia* exceeds a density threshold within eggs (Ma et al. 2015). Something similar has been seen for feminization (Negri et al. 2009), male killing (Hurst and Jiggins 2000) and cytoplasmic incompatibility (Breeuwer and Werren 1993). Recently, Hidayanti et al. (2022, 2023) reinforced this idea by artificial manipulation of bacterial communication, which would ensure a quorum of cells to reach a threshold density, thus activating gene regulatory systems that result in phenotypic changes. Thus, those factors affecting *Wolbachia* density may influence the induction of reproductive distortions, like it has been hypothesized for the temperate phage WO (Bordenstein et al. 2006; Kent and Bordenstein 2010). Complete understanding of the association between phenotype and *Wolbachia* density remains unclear and requires further investigation. Thus, quantitative investigation of tissue tropism and density could provide valuable information regarding to host phenotype (Pietri et al. 2016).

The tribe Naupactini comprises more than 500 species of weevils distributed mostly in Central and South America. Many of them reproduce by parthenogenesis like *Naupactus cervinus* Boheman 1840 (Buchanan 1939), many others reproduce strictly sexually like *Naupactus dissimulator* Boheman 1840 (Marvaldi 1999), and some others exhibit a mixed mode of reproduction (parthenogenetic and sexual) like *Pantomorus postfasciatus* (Hustache 1947) (Elias-Costa et al. 2019). An association has been reported between the parthenogenetic reproductive mode and *Wolbachia* infection in these weevils (Elias-Costa et al. 2019; Rodriguero et al. 2010a). Nine bacterial strains were found in 20 parthenogenetic Naupactini species or lineages. Although closely related, they compose a polyphyletic group, what is a hint of horizontal transmission (Rodriguero et al. 2010a). Conversely, sexually reproducing species or lineages were uninfected (Elias-Costa et al. 2019; Rodriguero et al. 2010a).

A recent curing experiment performed on parthenogenetic lineages of *P. postfasciatus* suggested that a threshold density of *Wolbachia* is required for reproductive manipulation to occur (Rodriguero et al. 2021). This inference points to bacterial density as a relevant variable in the interaction between *Wolbachia* and Naupactini weevils. Considering that *Wolbachia* density would be important in triggering parthenogenesis in Naupactini species (Rodriguero et al. 2021), we decided to undertake a quantitative analysis of *Wolbachia* strains in both sexually reproducing and parthenogenetic Naupactini species, especially because small loads of *Wolbachia* infection might have not been detected by Rodriguero et al. (2010a), similar to what happened in bark beetles studies (Arthofer et al. 2009). In fact, Arthofer et al. (2009) had to use extremely sensitive molecular techniques to prove evident *Wolbachia* infection in light of incompatible crosses.

In the present contribution two sexually reproducing species from the tribe Naupactini, *N. dissimulator* and *Naupactus xanthographus* (Germar 1824), were selected as models to analyze the *Wolbachia* infection status and density. Both species are native to South America and harmful for several crops, mainly alfalfa and fruit trees (Lanteri et al. 2002). *Naupactus xanthographus*, known as "the fruit weevil", causes damage on grapes, peach, nectarine, apple, berries, cherries, and other deciduous fruit trees, as well as alfalfa, potatoes, soybean and other plants of commercial relevance in Argentina and Chile (Lanteri and del Río 2017). It is a quarantine pest for Japan and the USA (Pinto and Zaviezo 2003) and several measures have been established to intercept this weevil from grape exports from Chile to Peru (SENASA-Perú and SAG-Chile 2005–2006). *Naupactus dissimulator* also causes damage to important commercial crops like citrus species and "yerba mate" tea (*Ilex paraguariensis* Saint Hill), among others (Lanteri and del Río 2017). In addition, we further characterized bacterial strains by investigating their tissue tropism and the presence of the *Wolbachia* infecting temperate phage known as WO phage. We compared these patterns with the parthenogenetic species *P. postfasciatus*, which is infected with the same strain (Rodriguero et al. 2010a; Elias-Costa et al. 2019). The results from this work may be useful as a first step in increasing the knowledge about the dynamics of *Wolbachia* titers in weevil pests, considering these bacteria as a potential tool for Integrated Pest Management.

2 Materials and methods

2.1 Sampling of biological material

Adult weevils were collected using a beating sheet (0.55 × 0.55 m) during the summer seasons of 2004–2022. Specimens of *N. xanthographus* and *N. dissimulator* were sampled in 14 locations from Argentina and in 8 locations from Argentina and Brazil, respectively (see Online Resources 1). Parthenogenetic weevils sampled in two locations from Argentina (see Online Resources 2) were also included for comparative purposes: *N. cervinus*, *Naupactus dissimilis* Hustache 1947, and *P. posfasciatus*. Specimens were stored at -20 °C until DNA extraction.

2.2 Experimental procedures

2.2.1 DNA extraction

For *Wolbachia* prevalence survey and quantification in different tissues, total genomic DNA was extracted from weevils following the protocol devised by Sunnucks and Hales (1996), based on alcohol precipitation. For *Wolbachia* density quantification in whole weevils, total genomic DNA was extracted using the DNeasy Blood & Tissue Kit (Qiagen, Germany), following manufacturer instructions.

2.2.2 *Wolbachia* detection and strain typing

Wolbachia infection status of *N. dissimulator* and *N. xanthographus* was determined in 1–10 individuals per location (N = 105) (Online Resources 1). DNA from *N. cervinus* and *N. dissimilis* were used as positive controls. These are the closest species to *N. dissimulator* and *N. xantographus*, respectively, and are parthenogenetic and naturally infected with *Wolbachia* (Rodriguero et al. 2010a). Besides, parthenogenetic (infected) populations of *P. postfasciatus* were included in this study. Distilled water was used as negative control.

Wolbachia infection was diagnosed through multilocus PCR amplification of the cytochrome C oxidase subunit I (*coxA*), fructose-bisphosphate aldolase (*fbpA*), aspartyl/glutamyl-tRNA amidotransferase subunit B (*gatB*), and *Wolbachia* surface protein (*wsp*) genes (Baldo et al. 2006; Braig et al. 1998). Primers S1718 and A2442 specific for the insect mitochondrial cytochrome C oxidase subunit I (*COI*) gene (Normark 1994) and *16S rRNA* primers specific to eubacteria (O'Neill et al. 1992) were used to amplify these genes and check the quality of the DNA extraction. Amplifications were carried out in a 15 µL final volume reaction containing 100 ng of genomic DNA used as template, 0.5 µM of each primer (Thermo Fisher Scientific, USA), 0.1 mM of each dNTP (GenBiotech, Argentina), 25 mM $MgCl_2$ (Thermo Fisher Scientific, USA), 1 unit of Taq polymerase (Thermo Fisher Scientific, USA) and 1X buffer (Thermo Fisher Scientific, USA). The reactions were performed on an Applied Biosystems Veriti thermal cycler under the conditions described by Scataglini et al. (2005) for the *COI* gene, Baldo et al. (2006) for the *coxA*, *fbpA* and *gatB* genes, and Braig et al. (1998) for the *wsp* gene. In the case of the *16S rRNA* gene, the thermal conditions were those described in O'Neill et al. (1992) but using 50° C as annealing temperature. PCR products were run on a 1% agarose gel with TAE buffer and visualized using GelRed® staining (GenBiotech S.R.L.). All experiments were repeated at least twice.

Wolbachia strain typing for *N. xanthographus* and *N. dissimulator* was accomplished through sequencing of the *fbpA* gene, which is considered a barcode for *Wolbachia* strain identification (Bailly-Bechet et al. 2017), and comparing it with the *Wolbachia* PubMLST database (https://pubmlst.org/organisms/wolbachia-spp/) in samples from Colón (Co) and Ciudad de Buenos Aires (BA), respectively. This gene is the most rapidly evolving of the five *Wolbachia* MLST genes, it is highly variable among Naupactini's strains (Rodriguero et al. 2010a) and the most sensitive to detect the maximum diversity of these bacteria (Bailly-Bechet et al. 2017; Baldo et al. 2006; Simoes et al. 2011). Further sequencing of the *coxA* gene confirmed the previous strain typing because it allows distinction between those ones which have the same *fbpA* allele (i.e. *w*Nau2 and *w*Nau3, see Rodriguero et al. 2010a for details). Because of the presumed low density of *Wolbachia* infecting sexual host species, several PCR products of both *fbpA* and *coxA* genes were pooled, purified with a QIAquick Gel Extraction Kit (Qiagen, Germany) and cloned into the pGEM-T easy-cloning vector (Promega, USA) in order to increase the amount of DNA for sequencing. Four clones per amplification product were isolated and sequenced using the vector primers T7 and SP6. DNA was sequenced using a 3130-XL Automatic Sequencer (Applied Biosystems, USA) at the Sequencing and Genotyping Unit of the Facultad de Ciencias Exactas y Naturales – Universidad de Buenos Aires (FCEyN, UBA, Buenos Aires, Argentina).

In addition, the WO phage was surveyed by sequencing its *orf7* locus. Primers and conditions described by Masui et al. (2000) were used and reactions were performed in both sexual species and in a parthenogenetic population of *P. postfasciatus* sampled in BA. PCR amplifications were carried out as formerly described, but T-Holmes Taq polymerase kit (Inbio Highway, Argentina) was used because of its higher sensitivity. PCR products were enzymatically purified using Exonuclease I (ExoI) and Thermosensitive Alkaline Phosphatase (FastAP) (Thermo Fisher Scientific, USA). Sequencing in both directions was performed in a

3130-XL Automatic Sequencer (Applied Biosystems). BlastN sequence analyses (Altschul et al. 1997) were conducted to identify the *orf7* sequences. The MLST sequences obtained in this work were deposited in Figshare database (https://doi.org/10.6084/m9.figshare.24179076).

2.2.3 Real time quantitative PCR

Four specimens from two locations were selected for each species (two males and two females) during the summer seasons 2018–2019: *N. dissimulator* from Ciudad de Buenos Aires (BA) and Paulino Island (PI); and *N. xanthographus* from BA and Parque Provincial Pereyra Iraola (PIP) (references to geographic locations are included in the Online Resources 1). Parthenogenetic populations of *P. postfasciatus* were also sampled for comparative purposes in BA and Colonia (Uruguay). Specimens were processed as previously described.

For *Wolbachia* quantification and localization in weevil's tissues, five females of *N. dissimulator, N. xanthographus* and *P. postfasciatus* were selected. They were sampled in BA during the summer season 2022, were kept alive after collection and then sacrificed and aseptically dissected in PBS with a scalpel using a stereo-microscope (100X). Each individual was separated in four parts: head (H), reproductive tissue (R), digestive tissue (D) and rest of the body (B). Each sample was conserved in absolute ethanol at -20° C.

For relative quantification, *gatB* was used as target gene, while weevil's *ITS1* was selected as endogenous control gene. A nested PCR was optimized in order to increase sensitivity and specificity of the method. For both genes, end-point PCR was performed as a first step using 100 ng of total genomic DNA as template, followed by qPCR of the product. 50 fold dilutions of end-point PCR products were used for each gene. The primers and thermal cycling conditions for *gatB* gene were previously described herein (Sect. 2.2.2), and for *ITS1* gene were published by Rodriguero et al. (2013).

Primers for *gatB* and *ITS1* genes suitable for qPCR analyses were designed using Primer3Plus software (Rozen and Skaletsky 2000). Primer sequences for *gatB* were based on the sequence of *w*Nau1 strain described in Rodriguero et al. (2010a), whereas primer sequences for *ITS1* were designed using conserved regions of *ITS1* sequences from the three weevil species retrieved from GenBank (NCBI, NIH) (Table 1).

The qPCR reactions were conducted in a Step One Plus Real-Time PCR System (Thermo Fisher Scientific, USA) using the SYBR Green methodology. Reactions were prepared in 20 µL total volume mixtures, consisting of 10 µL SYBR™ Select Master Mix (Thermo Fisher Scientific, USA), 200 nM of each primer (Macrogen, Korea) and DNA template (1 µL of ITS1 or *gatB* PCR product 500-fold diluted). All qPCRs were run in triplicate and each run also included three replicates of a negative control with no added DNA template. The thermal cycling conditions for both genes were 95° C for 2 min followed by 40 cycles of 95° C for 3 s and 60° C for 30 s. After that, melting curve analyses of the PCR products were performed. Standard curves were constructed using a qPCR amplicon obtained for each species serially 10-fold diluted. The qPCR amplification efficiency was calculated from the formula $E = (10^{-1/S})$, being S the slope of the linear fit in the standard curve (Rodríguez et al. 2015). The three species showed similar and adequately high efficiencies for both amplicons.

Relative *Wolbachia* levels were analyzed by the comparative Cq method (Pfaffl 2001), which standardizes target genes against an endogenous host gene and adjusts for differences in PCR efficiency between the amplicons, using the formula:

$$Ratio = \frac{E_{gatB}^{\Delta Cq(Pp-sex)}}{E_{ITS1}^{\Delta Cq(Pp-sex)}} \quad (1)$$

E = mean efficiency for each gene

Pp = mean Cq obtained for all *P. postfasciatus* individuals

sex = mean Cq obtained from the three replicates for each individual with sexual reproduction

Table 1 Oligonucleotide sequences of primers designed in this study for qPCR

Primer pairs	Sequence	Position
gatBqPCR-F	5'-CTGTGATGCAAA TGTTTCT-3'	87[a]
gatBqPCR-R	5'-CTTATTTCTCCTC CGCTTT-3'	224[a]
ITS1qPCR-F	5'-CGCTTATCCGGC CTAGTCG-3'	848[b]
ITS1qPCR-R	5'-AGCGCTACTGTC CGTTTTGA-3'	939[b]

[a]Positions are in accordance with the published sequence of the *gatB* gene of *w*Nau1 from *Pantomorus postfasciatus* (GenBank accession no. GU573910).

[b]Positions are in accordance with the published sequence of the *ITS1* gene of *Naupactus dissimulator* (GenBank accession no. JX440505).

PCR product size amplified with gatBqPCR-F/R is 157 bp

PCR product size amplified with ITS1qPCR-F/R is 92 bp

2.3 Data analyses

Differences in *Wolbachia* relative quantification were analyzed through general linear mixed models with the library *nlme* (Pinheiro et al. 2023), using R software environment v. 3.0.1 (R Core Team 2019) and RStudio v. 1.2.5033 (RStudio Team 2019). Normality was evaluated using the

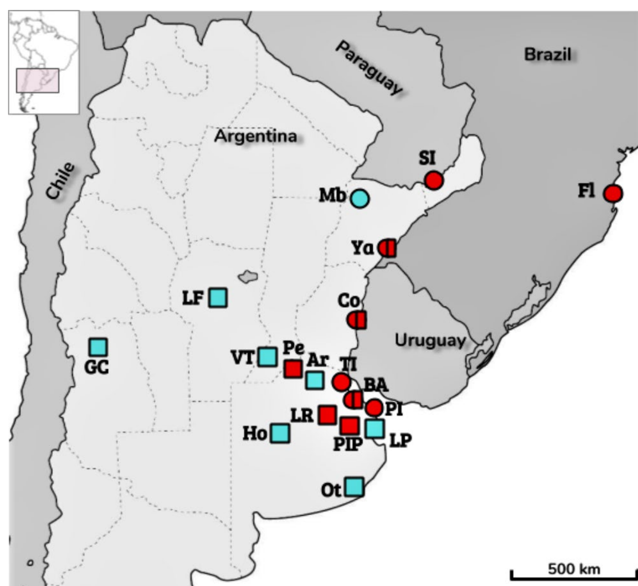

Fig. 1 Distribution of sampling sites of *Naupactus dissimulator* (circles) and *Naupactus xanthographus* (squares). Colors indicate *Wolbachia* infection status at each sampling point (red, infected; blue, uninfected). For interpretation of the references to geographic locations in this figure, the reader is referred to the Online Resources 1

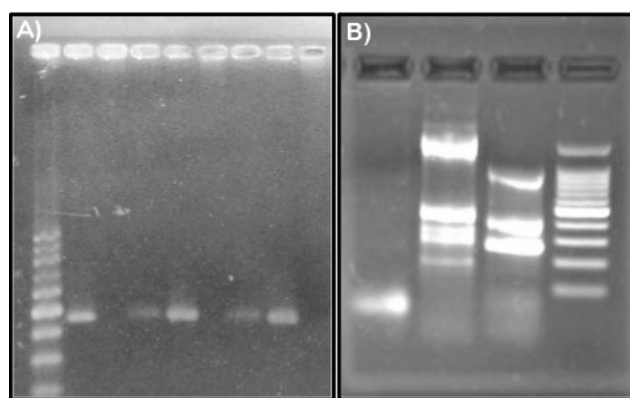

Fig. 2 Electrophoretic profiles of the: (**A**) *Wolbachia gatB* gene; (**B**) *orf7* locus from the *Wolbachia* WO phage obtained from end-point PCR. White line indicates a splicing in the gel. Lanes in A: 1) DNA size marker; 2) *Naupactus cervinus* (parthenogenetic); 3) *Naupactus dissimulator* from Mburucuyá (sexual, uninfected); 4) *Naupactus dissimulator* from Buenos Aires City (sexual, infected); 5) *Naupactus dissimilis* (parthenogenetic); 6) *Naupactus xanthographus* from La Falda (sexual, uninfected); 7) *Naupactus xanthographus* from Colón (sexual, infected); 8) *Pantomorus posfasciatus* (parthenogenetic); 9) Negative control (distilled water). Lanes in B: 1) *Pantomorus posfasciatus* (parthenogenetic); 2) *Naupactus dissimulator* (sexual, infected); 3) *Naupactus xanthographus* (sexual, infected); 4) DNA size marker

Shapiro-Wilk test, while homoscedasticity was evaluated graphically. In case of heteroscedasticity, variance was modeled using varIdent. Analyses were carried out in two separate groups, including: (i) the whole dataset to evaluate the effect of the reproductive mode (n = 24); (ii) *N. xanthographus* + *N. dissimulator* data to test the effect of the species, sex and location (n = 16).

For (i), a model with log (10) of the ratio obtained from Eq. 1 as response variable, and reproductive mode as explanatory variable with fixed effects was applied. Geographic location was included as a random effect variable and was used to model variance.

For (ii), significance of explanatory variables (species, sex and location) was tested by dropping explanatory variables and their interactions from the models. Models considering the interactions among the explanatory variables did not fulfill the assumptions of normality and homoscedasticity, even after variance modeling. Sex was not considered in the final model because both the Akaike Information Criterion (AIC) and Bayesian Information Criterion (BIC) values were higher in models including this variable ($AIC_{sp+loc+sex} = -79.607 > AIC_{sp+loc} = -100.133$; $BIC_{sp+loc+sex} = -76.884 > BIC_{sp+loc} = -96.950$). An additive model with the ratio obtained from Eq. 1 as response variable, and species and geographic location as explanatory variables with fixed effects was selected. Variance was modeled by geographic location. PCR plate was used as explanatory variable with random effects. All charts were performed with the R package *ggplot2* (Wickham 2016).

To compare *Wolbachia* density per tissue among Naupactini species we fitted a linear mixed effect model through the R library *lme* (Pinheiro et al. 2023). Normality and homocedasticity were tested as described above. A model with the log (10) of the ratio obtained from Eq. 1 as response variable, and tissue, species and the interaction between the two as the fixed effects was considered (n = 44), as it fulfilled all assumptions. Sample was introduced in the model as a random factor.

3 Results

3.1 *Wolbachia* survey in sexually reproducing species

Wolbachia infection was detected in individuals from 8 out of 14 (57%), and 7 out of 8 (87.5%) geographic locations investigated for *N. xanthographus* and *N. dissimulator*, respectively (Fig. 1).

The bands observed in the agarose gel of the four genes (*coxA, gatB, wsp* and *fbpA*) were faint for both sexual species, while parthenogenetic species used as positive controls showed intense bands for all *Wolbachia* genes (Fig. 2a). On the other hand, the analysis of *COI* and *16S rRNA* genes revealed the presence of intense bands for all weevil species, which was a sign of good quality DNA extraction.

Sequencing of *fbpA* and *coxA* genes showed that both sexual species have the "181" (GenBank accession

number GU573907) and "14" (GenBank accession number GU079631) alleles, respectively, which belong to the Sequence Type (ST) "190" of the *Wolbachia* PubMLST database, unequivocally diagnosing infection with the *w*Nau1 strain (see Table 2 in Rodriguero et al. 2010a). The same strain was previously detected in most parthenogenetic populations of *P. postfasciatus* (Elias-Costa et al. 2019). Alleles for *N. dissimulator* and *N. xanthographus* are deposited in Figshare (https://doi.org/10.6084/m9.figshare.24179076).

The presence of WO phage was detected in both sexually reproducing species and in parthenogenetic *P. postfasciatus*. Although the three of them share the same *Wolbachia* strain, the electrophoretic profile revealed a differential band pattern between parthenogenetic and sexual hosts. While in *P. postfasciatus* a single bright band was observed, *N. dissimulator* and *N. xanthographus* showed multiple bands (Fig. 2b). Sequencing of the *orf7* locus from *P. postfasciatus* confirmed identity with the WO phage (99.71% nucleotide identity with the WO capsid protein gene encoded by a *Wolbachia* strain from a butterfly native to India, GenBank accession no. FJ392499.1, E-value = 6e-174). The sequence obtained in the present study is available at GenBank (accession number MT526906).

3.2 Comparison of *Wolbachia* density in sexually reproducing vs. parthenogenetic weevil species

The relative quantification obtained for *Wolbachia* strain *w*Nau1 in sexual species *N. xanthographus* and *N. dissimulator* was significantly lower than that in parthenogenetic lineages of *P. postfasciatus* (Fig. 3a). The fold change related to reproductive mode was at least twice. Additionally, an elevated inter-individual variation in bacterial densities within sexual species was detected. Thus, reproductive mode seems to have a significant effect on *w*Nau1 load ($p = 0.0331$; estimated mean ratio for a sexual population $= 1.73 \times 10^{-4}$; $DF = 3$).

When comparing only sexual species, no significant effects were detected in *Wolbachia* loads in relation to species, sex or geographic location ($p > 0.05$) (Fig. 3b-c).

3.3 Distribution and density of *Wolbachia* in tissues of sexually reproducing vs. parthenogenetic weevils

Relative quantification by qPCR of *Wolbachia w*Nau1 in the different weevil's tissues is summarized in Fig. 4. The infection was detected throughout all the tissues analyzed in the three surveyed species.

Wolbachia density was significantly higher in all the tissues from the parthenogenetic *P. postfasciatus* than in the sexual species ($p < 0.002$), while there was no difference between *N. dissimulator* and *N. xanthographus* levels. The interaction between the variables species and tissue was not statistically significant ($p > 0.05$).

4 Discussion

In a previous contribution we proposed that "*Wolbachia* infected species or lineages of Naupactini reproduce by parthenogenesis while uninfected do not" (Rodriguero et al. 2010a). However, the quantitative molecular techniques applied herein revealed that some sexually reproducing species are also infected, but at very low densities. Accordingly, we could say that "species or lineages of Naupactini infected with high levels of *Wolbachia* reproduce by parthenogenesis and species or lineages infected with low density or uninfected do not". Low *Wolbachia* titers were also described for populations of the bark beetle *Pityogenes chalcographus* (Linnaeus, 1761) (Curculionidae, Scolytinae) (Arthofer et al. 2009) and for the fly *Drosophila melanogaster* Meigen 1830 (Drosophilidae, Drosophilinae) (Hoffmann et al. 1998; Merçot and Charlat 2004).

Both sexually reproducing weevils herein studied, *N. dissimulator* and *N. xanthographus*, are infected with the *w*Nau1 *Wolbachia* strain, the most widespread within parthenogenetic Naupactini, including *P. postfasciatus* and *Naupactus minor* (Buchanan), among other species (Elias-Costa et al. 2019; Rodriguero et al. 2010a). This suggests a high incidence and an efficient transmission rate of the strain within this tribe. No difference was found in bacterial titers between *N. dissimulator* and *N. xanthographus*, regardless of sex or geographic location, although they show higher intraspecific variability in *Wolbachia* loads than the parthenogenetic *P. postfasciatus*.

There is a complex host-symbiont relationship within Naupactini. In some species like *N. cervinus*, which has long-standing *Wolbachia* infections, the *w*Nau5 strain is fixed (Rodriguero et al. 2010b). Conversely, in other species like *P. postfasciatus* and *N. xanthographus*, both infected and uninfected populations coexist, pointing to still-evolving processes. Since *Wolbachia* strain *w*Nau1 is spatially scattered in parthenogenetic *P. postfasciatus* and sexual *N. xanthographus* (see Fig. 1 in Elias-Costa et al. (2019) and Fig. 1 of the present work), interesting questions arise: is low *Wolbachia* density of infection in *N. xanthographus* indicative of evolution towards endosymbiont loss? Or is it an example of persistent *Wolbachia* infection at low levels and frequencies, as in the bark beetle *P. chalcographus* (Arthofer et al. 2009)? Several studies have stated that microbial symbionts in eukaryotes are not transient passengers randomly acquired from the environment (Brucker and Bordenstein 2012; Fraune and Bosch 2010;

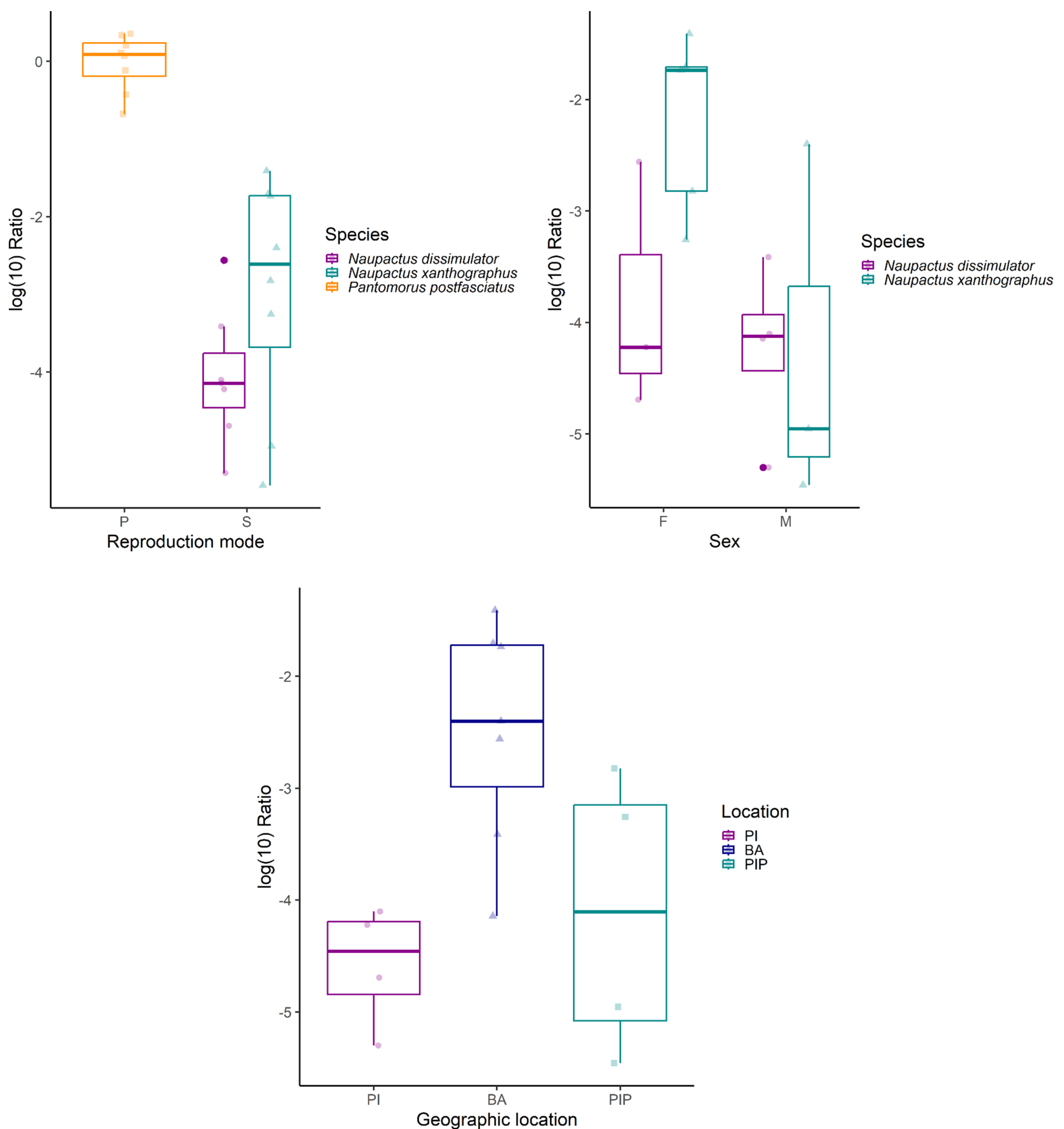

Fig. 3 Relative *Wolbachia* levels (log(10) Ratio) for weevils with different reproductive modes: *Naupactus dissimulator* and *Naupactus xanthographus* (sexual); and *Pantomorus postfasciatus* (parthenogenetic). *Wolbachia* loads were compared by: (**A**) reproductive mode for the three species (P: parthenogenetic reproduction; S: sexual reproduction); (**B**) sex split by sexual species (F: female; M: male); and (**C**) geographic location for sexual species (PI: Paulino Island; BA: Buenos Aires City; PIP: Pereyra Iraola Park). Thick horizontal lines indicate median values, markers indicate individual data points, boxes the interquartile range (IQR), whiskers 1.5 times the IQR, and dots show outlier values

Lee and Mazmanian 2010; McCutcheon et al. 2009; Werren et al. 2008). Then, under the latter hypothesis we wonder which role *Wolbachia* plays in sexual species. Is there any beneficial fitness effect like protection against pathogens even at low densities (Arthofer et al. 2009)? Or maybe is it

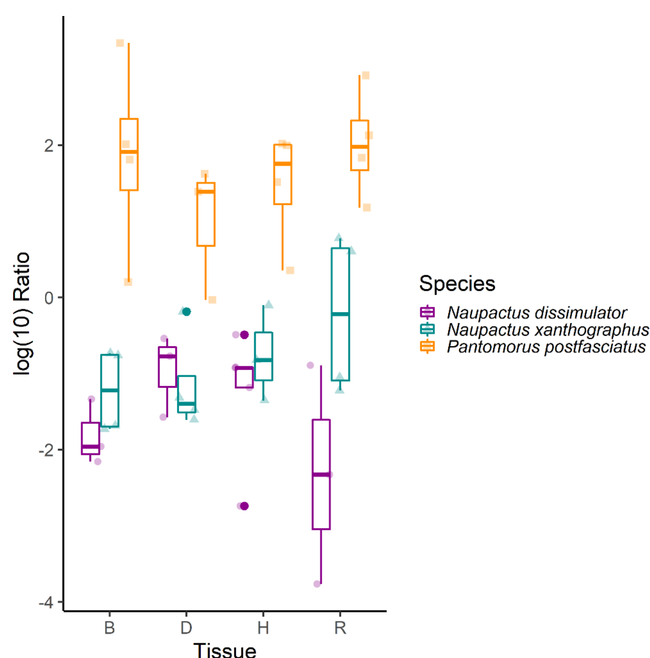

Fig. 4 Relative *Wolbachia* levels (log(10) Ratio) for different tissues from weevils with different reproductive modes: *Naupactus dissimulator* and *Naupactus xanthographus* (sexual); and *Pantomorus postfasciatus* (parthenogenetic). Tissues considered are digestive tissue (D), head (H), reproductive tissue (R) and rest of the body (B). Thick horizontal lines indicate median values, markers indicate individual data points, boxes the interquartile range (IQR), whiskers 1.5 times the IQR, and dots show outlier values

inducing a reproductive disorder other than parthenogenesis like cytoplasmic incompatibility?

In *N. dissimulator*, *Wolbachia* infection appears to be uniformly widespread throughout its geographic range, encompassing the gallery forests of Paraná and Uruguay rivers, down to the banks of La Plata River (Lanteri and del Río 2017). In this area, *N. dissimulator* coexists with its closest species, *N. cervinus* (Scataglini et al. 2005). However, they do not share the same endosymbiont strain; *N. dissimulator* is infected with *w*Nau1 strain, while *N. cervinus* carries *w*Nau5. Something similar occurs with the sister species *N. xanthographus* - *N. dissimilis* (carrying *w*Nau1 and *w*Nau7, respectively, Rodriguero et al. 2010a). This fact suggests that both *w*Nau1 and *w*Nau5 (or *w*Nau1 and *w*Nau7) were independently acquired, and that horizontal transmission is an important force driving *Wolbachia* evolution and diversity in this group of insects, as proposed by Rodriguero et al. (2010a). In such a study, bacterial strains appeared distantly related in the phylogenetic tree, not comprising a monophyletic clade and supporting the occurrence of horizontal transfer. Multiple mechanisms of *Wolbachia* horizontal transmission have been proposed, including predators, parasitoids, hemolymph transfer, cohabitation, and foraging on the same host plants (Ahmed et al. 2015; Huigens et al. 2004; Kolasa et al. 2017; Le Clec'h et al. 2013). So far, in the case of weevils, indirect evidence of such transmissions by parasitoids has been provided (Fernandez Goya et al. 2022; Kajtoch et al. 2018; Kotásková et al. 2018; Mengoni Goñalons et al. 2014; Rodriguero et al. 2014).

Additionally, we report the temperate phage WO for the first time in the *Wolbachia* strain infecting *N. xanthographus*, *N. dissimulator* and *P. postfasciatus*. The differential electrophoretic profile in WO phage region suggests potential divergence in the WO phage region infecting the three weevil species. This information could be relevant since some authors have proposed that low *Wolbachia* densities might be a consequence of high densities of its associated bacteriophage (Bordenstein et al. 2006. This subject deserves additional studies.

Another aspect analyzed in this work is the localization of *Wolbachia* in the tissues of its hosts. It has been proven that not only it is restricted to the reproductive tissues, but that this parasite can colonize other organs beyond the gonads (Pietri et al. 2016). Thus, somatic tissue tropism seems to be an important aspect of this endosymbiont's life history. According to our results, *w*Nau1 infection was not circumscribed to a specific tissue; instead, it has ubiquitous distribution in weevil tissues and its density is homogeneous throughout the whole body both in the sexual and the parthenogenetic species studied. From an evolutionary point of view, somatic localization might be maintained because it confers the arena for advantageous bacterial phenotypes to evolve in the host that enhance its germ-line transmission and/or because it facilitates the horizontal transmission within and between species, thus increasing the genetic diversity of *Wolbachia* and accounting for its pandemic distribution (Pietri et al. 2016). Indeed, the somatic localization of the *w*Nau1 strain is consistent with its super-spreader ability (Rodriguero et al. 2010a).

The study of Rodriguero et al. (2021) suggested that a threshold of bacterial density might be required for egg hatching and/or changes in oogenesis and other mechanisms related to parthenogenesis. Our present results are in agreement with the hypothesis that a threshold level of *w*Nau1 strain is needed to trigger reproduction in the parthenogenetic lineages of Naupactini weevils, and if this level is not sufficiently high, parthenogenesis does not occur despite the genetic background and physiological capacities of the hosts (Braig et al. 2002). Some of the reasons why the *w*Nau1load might not be sufficient to trigger parthenogenesis include the following: *Wolbachia* levels might be modulated by specific proteins produced by the host, as it was demonstrated with hybrid hosts of the tsetse fly (Schneider et al. 2013); some host and endosymbiont metabolic and signaling pathways involved in nutrient sensing might be affecting *Wolbachia* levels (Serbus et al. 2015); and competitive interactions with other members of the microbiota

could be detrimental for *w*Nau1, as incompatibility among microorganisms could be a potential barrier to transmission of heritable symbionts (Hughes et al. 2014). Future studies of high-throughput sequencing of the 16 S rRNA gene comparing microbiotas of parthenogenetic vs. sexual weevil species will be conducted to further investigate this hypothesis and give insight into whether *Wolbachia* loads are regulated by partners of the microbiota. Additionally, *Wolbachia* titers could be modulated by the host. Experiments aimed to discover alterations in host expression targeting *Wolbachia* proliferation, trafficking or maintenance may reveal the host genetic basis of *Wolbachia* density.

Several studies have pointed out the importance of a quantitative measure of *Wolbachia* to correlate the effects of reproductive manipulation on host (Baião et al. 2019; Bordenstein and Bordenstein 2011; Hurst et al. 2000; Ikeda et al. 2003; Ma et al. 2015; Osborne et al. 2012). However, there are few reports specifically evaluating the relationship between *Wolbachia* titers and the parthenogenetic phenotype, and all of them are referred to Hymenoptera (Lindsey and Stouthamer 2017; Pascal et al. 2004; Tulgetske and Stouthamer 2012; Zchori-Fein et al. 2000). To the best of our knowledge, this contribution would be the first report for Coleoptera.

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Declarations

Conflict of interest The authors declare no conflict of interest.

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