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Dependence of egg hatching on *Wolbachia* density in a parthenogenetic weevil revealed by antibiotic treatment

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Abstract

Naupactini is a tribe of Neotropical broad-nosed weevils highly diverse in South America. This group includes several parthenogenetic species, some of them harmful for agriculture and invasive around the world. Although some hypotheses based on polyploidy and hybridization have been proposed to explain the origin of parthenogenesis in weevils, the infection with the bacterium *Wolbachia pipientis* may be involved in the origin of parthenogenetic reproduction of some species. In this contribution, we studied the role of *Wolbachia* in the reproductive biology of *Pantomorus postfasciatus* Hustache (Coleoptera: Curculionidae) through a curing experiment using tetracycline. This weevil has a mixed mode of reproduction including sexual and parthenogenetic populations. Exposure to an antibiotic did not affect fecundity, but did reduce egg hatching in comparison with untreated individuals. Consequently, we inferred that *Wolbachia* most probably takes part in the reproduction of *P. postfasciatus*, either by exerting nutritive functions in oogenesis necessary for egg hatching, or by induction of thelytokous parthenogenesis. Although infection was not totally cured, *Wolbachia* load was significantly lower in treated than in control females. Thereby, we hypothesize that a minimum threshold density of *Wolbachia* is required for weevil reproduction. We conclude that all analyses support a role of *Wolbachia* in *P. postfasciatus* reproduction.

Introduction

Wolbachia are obligate intracellular, maternally transmitted bacteria infecting 40% of terrestrial arthropods (Zug & Hammerstein, 2012). They manipulate host reproduction through several strategies which enhance vertical transmission. These bacteria can spread within a host population by cytoplasmic incompatibility, male killing, feminization, and thelytokous parthenogenesis, reaching high frequencies, and even fixation (Engelstädter & Hurst, 2009). For this reason they were considered reproductive parasites (Werren et al., 1995). They represent the most frequent infection among the reproductive parasites so far reported (e.g., *Arsenophonus*, *Cardinium*, *Rickettsia*, or *Spiroplasma*) (Duron et al., 2008), mainly because of their markedly higher prevalence and diverse range of effects on the host (Duron & Hurst, 2013). Although maternal transmission is the primary mode within host species, interspecific horizontal transfer is pervasive (Duron & Hurst, 2013). The capacity of *Wolbachia* to overcome the species barriers may account for its pandemic distribution (Zug et al., 2012).

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Thelytokous parthenogenesis induction by *Wolbachia* is fairly common in haplo-diploid insects such as the parasitic wasps of the genera *Trichogramma* (Stouthamer & Kazmer, 1994), *Leptopilina* (Pannebakker et al., 2004), *Muscidifurax* (Gottlieb et al., 2002), *Asobara* (Kremer et al., 2009), *Telenomus* (Arakaki et al., 2000), and *Diplolepis* (Stille & Dävring, 1980), and the thrips genera *Franklinothrips* (Arakaki et al., 2001) and *Hercinothrips* (Kumm & Moritz, 2008). In insects with this sex determination system, thelytokous parthenogenesis can be easily explained by the diploidization of the unfertilized oocyte through bacterial induction. However, there are also many parthenogenetic insects with a sex determination system involving sex chromosomes. Then, the question arises: could *Wolbachia* take part in the origin of parthenogenesis in such insects? And if so, what is the underlying mechanism?

Within Coleoptera, the family Curculionidae (weevils) comprises a large number of parthenogenetic species belonging to bark beetles of the subfamily Scolytinae and to broad-nose weevils of the Listroderini (Cyclominae) and Entiminae (Suomalainen, 1969; Smith & Virkki, 1978; Lanteri & Normark, 1995). Some hypotheses have been proposed to explain the origin of parthenogenetic weevils, mainly hybridization and polyploidy (Suomalainen, 1969; Saura et al., 1993). Because experimental evidence supports plausibility for both scenarios, Tomiuk et al. (1994) have invoked a variety of factors that may account for the unisexuality in this group. We proposed another one: *Wolbachia* may also play an important role in the origin of unisexual reproduction, at least in some weevil species. Rodriguero et al. (2010) showed an association between parthenogenetic reproduction and *Wolbachia* infection status in some South American Naupactini, on the basis of 30 bisexual and parthenogenetic species. Although no correlation was observed for weevils from central Europe (Stenberg & Lundmark, 2004; Lachowska et al., 2010), North America (Chen et al., 2012), Great Britain, and Azores Isles (Piper et al., 2001), the pattern seen in the tribe Naupactini is striking and prompted us to investigate the role of *Wolbachia* in the reproductive biology of this group. The best way to determine the effect of *Wolbachia* infection on a host species is to compare an infected host strain with either a naturally uninfected or artificially cured host strain (Stouthamer & Mack, 2002). *Pantomorus postfasciatus* Hustache (frequently misidentified as *Naupactus ambiguus* Boheman) is a stimulating model to investigate this issue. Elias-Costa et al. (2019) found that parthenogenetic populations of this weevil were infected with *Wolbachia*, whereas bisexual ones were free of the infection, mirroring the macroevolutionary trend reported by Rodriguero et al. (2010). Moreover, infected populations were genetically divergent from the bisexual pool probably due to a long-term disruption of gene flow between them. This population structure provided hints of an on-going speciation mediated by the *Wolbachia* infection and pointed to these bacteria as the agent behind thelytokous parthenogenesis induction.

Since Werren et al. (1995) reported *Wolbachia* infection in the parthenogenetic Naupactini *Aramigus tessellatus* (Say), several authors have pointed out the importance of accomplishing curing experiments in parthenogenetic diplo-diploid species (Werren et al., 1995; Braig et al., 2002; Koivisto & Braig, 2003). The aim of the present work was to test the hypothesis of infectious parthenogenesis in the weevil *P. postfasciatus*. To reach this goal, we studied the influence of *Wolbachia* on the reproductive biology of this species (fecundity and egg viability) using an antibiotic treatment.

Materials and methods

Sampling and specimens assayed

During summer 2013, we collected overwintering adult parthenogenetic females of *P. postfasciatus* (n = 32) on blue passion flower, *Passiflora caerulea* L. (Passifloraceae), from the neighborhoods Belgrano (n = 10), Ciudad Universitaria (n = 4), and Villa Lugano (n = 18) in Buenos Aires City, Argentina.

Experimental design

Antibiotic concentration and administration time should be carefully considered when evaluating the effects of infection on offspring production (Dobson & Rattanadechakul, 2001; Stouthamer & Mack, 2002). Consequently, we chose a concentration low enough to avoid toxicity but high enough to cure the infection, following Lawson et al. (2001), Son et al. (2008), Rodriguero (2009), and Chen et al. (2012), who used a 2.5 mg ml^{-1} dose, with effects in reproduction and harmless to weevil biology (see Son et al., 2008; Chen et al., 2012). Son et al. (2008) and Chen et al. (2012) also tested 0.25 mg ml^{-1}, although this dose was not enough to maintain sustained effects on reproduction during the whole experiment.

On the day after sample collection, 16 weevils (nine from Lugano, five from Belgrano, and two from Ciudad Universitaria) were randomly assigned to one of the following treatments: (1) distilled water (control), or (2) tetracycline hydrochloride (2.5 mg ml^{-1}). Fresh *P. caerulea* leaves were dipped in water or the tetracycline solution

for 30 min, and then offered to the females (Son et al., 2008). They were kept individually in clear plastic jars (90 mm diameter, 55 mm high) containing moist cotton and maintained at room temperature under L16:D8 photoperiod.

The antibiotic was administered for 24 days. At intervals of 2 days, we checked for the presence of eggs in every container and recorded clutch size. Prior to the first date of egg collection, the control and antibiotic-treated groups were allowed to feed on *P. caerulea* leaves during 5 days. Then, an accordion-shaped oviposition device constructed according to Guedes & Parra (2004) was placed in each jar and replaced after egg collection.

On the 6th day of the antibiotic supply, eggs were thoroughly disinfected against fungi and mites with 2% copper sulphate for 4 min and rinsed 3× with distilled water (Rodriguero, 2009). Subsequently, each egg clutch identified by female ID and collection date was placed in a sterile Petri dish (50 mm diameter) containing sterilized moist paper. Petri dishes were transferred to a rearing chamber and kept under constant conditions until egg hatching (27 °C, 75% r.h., and L16:D8 photoperiod).

We regularly checked Petri dishes for larval emergence. After larvae stopped emerging, we estimated the proportion of hatched eggs per clutch, thus obtaining the egg hatching rate. On these occasions we monitored the presence of fungi and mites, which might decrease larval emergence and hence may have a misleading effect. The adult females that survived at the end of the experiment were stored in 100% alcohol to check for elimination of *Wolbachia* through tetracycline curing by means of standard and quantitative real-time PCR of the *Wolbachia* gatB gene.

Statistical analysis

Response variables considered in our analysis were clutch size per time interval (as a proxy of fecundity) and egg hatching rate per time interval (as a proxy of egg viability). Every weevil was considered as a replicate and each treatment had 16 replicates. Mean (± SE) and median (Q1-Q3) of clutch size (i.e., egg counts) and egg hatching rate (i.e., percentage of hatched eggs per clutch) were estimated for each group of females at each time interval. Statistical analyses and charts were performed with the R software package v.3.0.1 (R Development Core Team, 2010), using RStudio 2013 (RStudio Team, Boston, MA, USA).

We applied generalized linear mixed models (GLMM) because they accommodate incomplete, unbalanced data sets, non-normal response distributions, and random effects (Bolker et al., 2009). Specifically, for egg count data we tested several probability distributions because of the excess of zeros (Sileshi, 2006) using the glmmadmb function (library: glmmADMB) (Fournier et al., 2012) and applying the Akaike information criterion (AIC) to select the best-fitting model. For egg hatching rate we applied the binomial distribution using the glmer function (library: lme4) (Bates et al., 2009). We also accounted for the excess of zeros and selected the best-fitting model as before. To solve the pseudoreplication problem, female ID was included as a random effect in all models.

To test for differences in fecundity between control and tetracycline-treated females, we modeled clutch size of *P. postfasciatus* (response variable), using treatment and time (i.e., days of tetracycline treatment) as explanatory variables and sampling location (Lugano, Belgrano, or Ciudad Universitaria) as a covariate. P-values for coefficient parameters were calculated with Laplace's approximation (Raudenbush et al., 2000). To test for differences in egg viability between both groups of females, we modeled the egg hatching rate (response variable) using treatment and time intervals as explanatory variables. Sampling location and presence of fungi and/or mites were considered as covariates. Again, P-values for coefficient parameters were calculated with Laplace's approximation (Raudenbush et al., 2000).

Significance of explanatory variables and covariates was tested by dropping explanatory variables and their interactions and covariates from the models and comparing the resulting change in deviance with a Z-test for clutch size and performing a likelihood-ratio test (χ^2 test) for egg hatching rate. We began with a maximal model containing all the above variables, and this was then simplified by first removing non-significant interactions and then main effects, until no further reduction in residual deviance was observed (Bolker et al., 2009). Parameter estimates for fixed effects were tested for significance with a *Z*-test, as this provides a more robust test than the alternative likelihood-ratio test when sample sizes are small (Bolker et al., 2009).

PCR assay to test for antibiotic curing of *Wolbachia* infection

At the end of the experiment, antibiotic curing of the *Wolbachia* infection was assessed by standard and quantitative real-time PCR (qPCR) specific to the *Wolbachia* gatB gene, with the nuclear ITS1 sequence used as control. Total genomic DNA was extracted following the protocol of Sunnucks & Hales (1996).

First, we checked *Wolbachia* curing by amplification of the *Wolbachia* surface protein (wsp) and the aspartyl/glutamyl-tRNA amidotransferase subunit B (gatB) genomic regions using the primers designed by Braig et al. (1998) and Baldo et al. (2006), respectively. DNA from *Naupactus*

cervinus Boheman was used as a positive control and distilled water as negative control. Primers S1718 and A2442 specific for the insect mitochondrial cytochrome C oxidase subunit I (COI) gene (Normark, 1994) were used to 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, Waltham, MA, USA), 0.1 mM of each dNTP (GenBiotech, Buenos Aires, Argentina), 25 mM $MgCl_2$, 1 unit of Taq polymerase, and 1× buffer (all Thermo Fisher Scientific). The reactions were performed on an Applied Biosystems Veriti thermal cycler under the conditions described in Scataglini et al. (2005) for the COI gene, Braig et al. (1998) for the wsp gene, and Baldo et al. (2006) for the gatB gene. PCR products were run on a 1% agarose gel with TAE buffer and visualized using GelRed staining (GenBiotech). All experiments were repeated at least twice.

We designed new primers for every sequence to optimize qPCRs. Primer sequences for gatB were based on the sequence of the strain *w*Nau1 described by Rodriguero et al. (2010), whereas primer sequences for ITS1 were based on the ITS1 sequences of a single individual from each sampled neighborhood in Buenos Aires (see primers and conditions for PCR amplification of ITS1 in Rodriguero et al., 2013). Two alleles were obtained: one from Belgrano and Ciudad Universitaria, and the other from Villa Lugano (GenBank accession numbers KY499667 and KY499668). Primers were designed from invariant regions using Primer3 (Koressaar & Remm, 2007; Untergasser et al., 2012). The following primers were used: qgatBF: 5'-GTAGCTATGCTACGGTGCTC-3' and qgatBR: 5'-ATTCGTCGTCTCTTATGACC-3', producing a 156-bp gatB amplicon, and qITS1F 5'-CTGTGATGCAAATGTTTCTG-3' and qITS1R5' -ACTTATTTCTCCTCCGCTTT-3', producing a 156-bp ITS1 amplicon.

Wolbachia intensities were quantified in the two groups of mothers: control (C) and treated (T). Each group (C, T) consisted of six individuals. DNA from these samples was subjected to qPCR analysis using a LightCycler 96 (Roche Diagnostics Deutschland, Mannheim, Germany). Each qPCR was conducted in 20-µl reaction volume, containing 50–100 ng of DNA, Fast Start Essential DNA Green Master (Roche), and 5 µM of each primer (Invitrogen, Carlsbad, CA, USA). Reaction conditions were as follows: an initial step of 5 min at 95 °C, followed by 40 cycles of 10 s at 95 °C, 45 s at 60 °C, and 15 s at 72 °C. A melting curve analysis was performed after each PCR to check for primer-dimers and non-specific amplification. Each run also included a negative control with no added template. All qPCR assays were performed in triplicate and replicates with high standard deviation (0.5 C_q) or outside of the calibration range were removed from analysis. Data analyses were performed on the average of the three replicates. PCR efficiencies were calculated from calibration curves with a purified-PCR product in serial dilutions (1 000×, 100×, 10×, and 1×). The efficiency values were adequately high for both amplicons. Relative *Wolbachia* intensities were analyzed by the comparative C_q method (Pfaffl, 2001), which standardizes target genes against an endogenous host gene and adjusts for differences in PCR efficiency between the amplicons. In particular, ITS1 was used as host control gene and gatB as bacterial target gene. Differences in relative *Wolbachia* intensity between control and treated groups were examined using the unpaired t-test with Welch's correction that assumes unequal variances. Transformation of *Wolbachia* density ($\log_2$) was implemented to account for the non-normality and lack of homogeneity of residuals.

Results

Almost all females survived at the end of the experiment, as only one treated and three control females died before days 8 and 24, respectively. After the first egg-collection day (day 6), larval emergence occurred within 2–3 weeks and ended after 2 months.

For the analysis of fecundity (inferred from clutch size), we tested the Poisson (P), zero-inflated Poisson (ZIP), and negative-binomial (NB) distribution models. The negative binomial was the best fitting model, given that it showed the lowest AIC value ($AIC_{NB} = 1617.822 < AIC_{ZIP} = 1675.256 < AIC_P = 3465.622$).

The number of eggs per clutch ranged from 0–40 and 0–37 for control and treated females, respectively (Figure 1A). Overall, tetracycline treatment had no effect on fecundity ($\Lambda = 0.662$, d.f. = 1, $P = 0.42$), although the elapsed time since the first egg collection seemed to affect this parameter ($\Lambda = 47.436$, d.f. = 9, $P<0.001$). Both, control and treated females showed a decrease in clutch size with time, with values being significantly lower at the end of the experiment (Figure 1A,B). Sampling location had no influence in clutch size ($\Lambda = 1.6$, d.f. = 2, $P = 0.45$).

For the analysis of egg viability (inferred from egg hatching rate), we tested models of binomial (B) and zero-inflated binomial (ZIB) distributions. Both models had the same AIC value ($AIC_B = AIC_{ZIB} = 55.511$), so we preferred the simplest one (i.e., binomial distribution model). Larval emergence ranged from 0 to 50% in control females and from 0 to 41% in treated females, who showed a remarkable reduction after the first date of egg collection (Figure 1C). Time had no effect on egg hatching rate

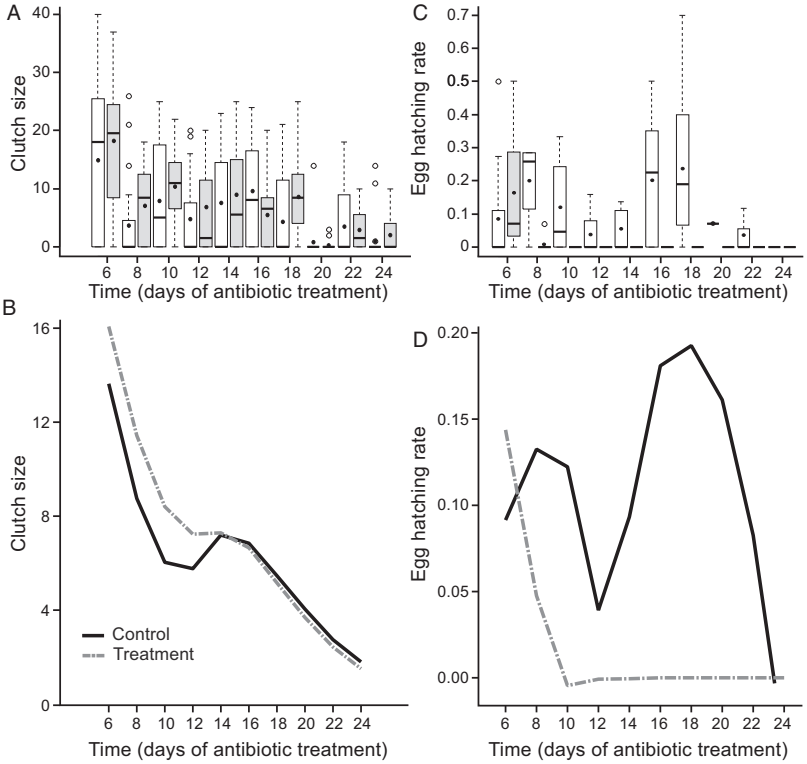

Figure 1 Clutch size at each collection day from the beginning of the experiment and hatching rate of the eggs laid by control and treated females of *Pantomorus postfasciatus*. (A, C) Box-plot for (A) clutch size and (C) hatching rate for control (white) and treated (grey) females. Black dots indicate the mean values, the thick horizontal lines the medians, the bottom and top of the boxes indicate the 25th and 75th percentiles, respectively, the whiskers represent 1.5× the interquartile range, and the open dots the outliers. (B, D) Relationship between (B) clutch size and (D) egg hatching rate for females and time from the beginning of the experiment fitted to a negative binomial generalized linear mixture model (GLMM). The clutch size decreased over time for both control and treatment, egg hatching rate decreased over time only in treated females.

(χ^2 = 4.746, d.f. = 9, P = 0.86), whereas egg hatching rate was significantly decreased by tetracycline (χ^2 = 6.17, d.f. = 1, P = 0.013; Figure 1D). The effect of sampling location and presence of fungi and mites on egg hatching rate was not significant (sampling location: χ^2 = 0.136, d.f. = 2, P = 0.93; fungi: χ^2 = 0.305, d.f. = 1, P = 0.58; mites: χ^2 = 0.043, d.f. = 1, P = 0.84).

Figure 1C shows that the emergence of larvae from eggs found on the first collection date (day 6) was not significantly different between control and treated females. Thus, it is reasonable to assume that the embryos derived from the first eggs escaped the effect of tetracycline, as they showed no evidence of decreased viability. The fact that almost no larvae emerged from eggs of treated females that were found on the second collection date (day 8; Figure 1C,D) may indicate that more antibiotic was required to effectively decrease *Wolbachia* levels.

Wolbachia infection was diagnosed by standard PCR amplification of the gatB fragment in the entire sample. Results suggested that the tetracycline treatment failed to completely remove the *Wolbachia* infection (Figure 2). Thus, we proceeded to carry out an assay of quantitative

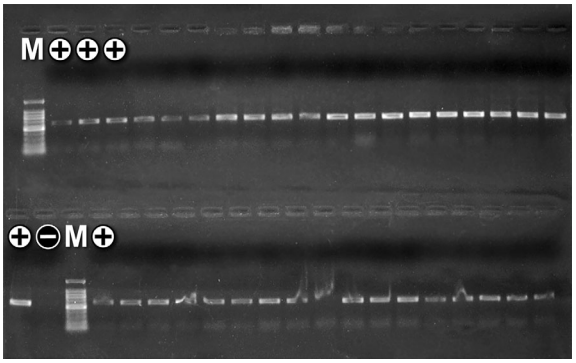

Figure 2 End-point PCR results of the *Wolbachia wsp* gene in an agarose gel. +: Positive control: *Naupactus cervinus*; −: negative control: distilled water; M: DNA size marker.

real-time PCR. Results evidenced that the treatment significantly reduced the intensity of *Wolbachia* infection (Welch-corrected t = 7.442, d.f. = 9, P<0.001), with mean (± SE) values of relative quantity of 10.42 (± 3.18) and 0.21 (± 0.1) for control and treated individuals, respectively, and a fold change due to treatment of 49.1.

Discussion

In the present study we tested the effect of *Wolbachia* infection on a parthenogenetic population of *P. postfasciatus* through a curing treatment. As a result, a decrease in the egg viability of treated females, as compared with control ones, was observed throughout the experiment, but we have not seen any effect of the treatment on fecundity. Accordingly, we realize that there is an involvement of *Wolbachia* in the viability of the eggs, probably because it is implicated in either oogenesis or parthenogenesis. Son et al. (2008) reported the effects and implications of an antibiotic treatment in broad-nosed weevils of the tribe Otiorhynchini, which belongs to the same subfamily (Entiminae) as Naupactini and also contains several parthenogenetic species, some of them polyploid. They concluded that *Wolbachia* affects egg hatching, in agreement with previous results for the Naupactini weevil *N. cervinus* (Rodriguero, 2009), and those obtained herein. Similar results were obtained for other assays performed in *Wolbachia*-infected parthenogenetic diplo-diploid species not closely related to Naupactini (Pike & Kingcombe, 2009; Timmermans & Ellers, 2009; Chen et al., 2012). Other factors leading to gradual decline of larval emergence as a consequence of antibiotic treatment should not be ruled out completely, such as toxic effects of tetracycline on weevil biology, or removal of other species of the microbiota than *Wolbachia* affecting egg hatching. However, partial clearance of this endosymbiont revealed by quantitative real-time PCR is highly suggestive of a link between *Wolbachia* and reproduction of *P. postfasciatus*.

Consumption of treated leaves for several days led to sterility of females. Occurrence of *Wolbachia* in treated females at a lower density than in untreated females suggests that a threshold of bacterial density in eggs may be required for parthenogenetic reproduction. This is not unexpected at all, as other *Wolbachia* thresholds were hypothesized for several interactions (e.g., Breeuwer & Werren, 1993; Hurst et al., 2000; Timmermans & Ellers, 2009; Negri et al., 2009; Ma et al., 2015).

It has been proven that *Wolbachia* plays a role in oogenesis in haplo-diploid wasps independently from any parthenogenesis-inducing mechanism (Dedeine et al., 2001), and also probably in some diplo-diploid weevils (Chen et al., 2012). But in these cases, there are differences in fecundity between control and antibiotic-treated females, contrary to what was seen in our experiment (but see Coelho et al., 2016). On the other hand, the association between *Wolbachia* infection status and parthenogenesis in Naupactini, both at the macro- and microevolutionary scales (Rodriguero et al., 2010; Elias-Costa et al., 2019), is in agreement with the outcome of this curing experiment.

Although all species with confirmed *Wolbachia*-induced thelytokous parthenogenesis are haplo-diploid, in some diplo-diploid species (weevils, booklice, scale insects, springtails, and ladybirds) (see table 3 in Ma & Schwander, 2017, and Magro et al., 2020) it is suspected, but not formally demonstrated (Ma & Schwander, 2017). In haplo-diploid species, curing parthenogenetic females of *Wolbachia* leads to the production of haploid sons instead of diploid daughters (Stouthamer et al., 1990), providing direct evidence for the role of endosymbionts in causing parthenogenesis in their hosts. Instead, removing endosymbionts from females in diplo-diploid species can result in the production of non-viable eggs or no eggs at all. Such a phenotype alone cannot be interpreted as *Wolbachia*-induced thelytokous parthenogenesis because it is equally likely that endosymbionts are required for successful oogenesis (Dedeine et al., 2001).

In diplo-diploid species, some obstacles must be overcome for parthenogenesis to occur: (1) inhibition of meiotic reproduction (if apomictic parthenogenesis) or ploidy restoration (if automictic parthenogenesis), and (2) initiation of the embryonic development, as the active centriole necessary to the assembly of the first zygotic centrosome is provided by the sperm in most animals (Manandhar et al., 2005). If *Wolbachia* were involved in the onset of parthenogenesis in these weevils, then it should be implicated, at least, in one of these steps of the reproductive process.

Unfortunately, disruption of any of these stages by partial *Wolbachia* clearance would yield unviable eggs. Thus, the outcome of our curing experiment did not allow us to decide on one hypothesis over the others. However, hints of meiosis in both *N. cervinus* and *P. postfasciatus* (MS Rodriguero, unpubl.) – which is unexpected for parthenogenetic weevils as all reported cases until now were apomictic (Smith & Virkki, 1978; Suomalainen et al., 1987; but see Rożek et al., 2009) – pose genome duplication as a golden candidate for a *Wolbachia* action in weevil parthenogenesis. Further research focusing on the cytogenetics and molecular biology of parthenogenetic Naupactini is needed to clarify this issue.

Wolbachia being the greatest ever panzootic reported (Werren et al., 2008), it is astonishing that only a few cases of parthenogenesis induction, the summum of reproductive manipulation, have been reported (see estimations in

Ma & Schwander, 2017). We suggest that a set of previous conditions in host species may facilitate invasion by strains capable of inducing this reproductive phenotype. In Curculionidae, for example, parthenogenesis is not a random trait. Most cases occur in the subfamily Entiminae, and most species with this reproductive mode lack metathoracic wings and live in xeric environments and grasslands (Lanteri & Normark, 1995). Surely, other aspects related to physiology and ecology still unexplored must be a common denominator for these species. In spite of the striking correlation between *Wolbachia* infection and parthenogenesis in Naupactini (Rodriguero et al., 2010; Elias-Costa et al., 2019), such correlation does not exist in the tribe Otiorhynchini, the Palearctic ecological counterpart of Naupactini. Whereas some parthenogenetic species are *Wolbachia*-infected [*O. sulcatus*, *Otiorhynchus singularis* (L.); Son et al., 2008; Lachowska et al., 2010], some others are not [e.g., parthenogentic lineages of *Otiorhynchus scaber* (L.); Stenberg & Lundmark, 2004]. Besides, bisexual lineages (*O. scaber*) (Stenberg & Lundmark, 2004) and species (*Otiorhynchus coecus* Germar) (Lachowska et al., 2010) are *Wolbachia*-infected. Thus, a clear pattern is precluded for this weevil group closely related to Naupactini and *Wolbachia* may be only one of the many pieces of this evolutionary puzzle. Because of the many obstacles that must be overcome for parthenogenesis to occur in a parthenogenetic weevil, even if *Wolbachia* solved one of these problems (e.g., egg activation, ploidy restoration, or early embryo cleavage), unidentified agents should be behind the other steps; thus, parthenogenesis should have a complex basis in these insects.

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