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Wolbachia Infections Responsible for Thelytoky in Dryinid Wasps. The Case of *Gonatopus bonaerensis* Virla (Hymenoptera: Dryinidae)

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Abstract
We studied the occurrence of *Wolbachia* in the parasitoid *Gonatopus bonaerensis* Virla (Hymenoptera: Dryinidae). In order to verify the existence of natural infections in the parasitoid, a field survey was conducted. Identification of *Wolbachia* was performed on the basis of 16S rDNA, wsp_F1, and wsp_R1-sequences. After the detection of the bacteria, infected specimens of *G. bonaerensis* were treated with a solution of tetracycline. In Tucumán, parasitoids hold *Wolbachia* endosymbiont, which seems to control the wasp's reproduction in the nature turning it into thelytokous. The symbiont was identified as the *Wolbachia* sp. wRi strain. The cure of infected unfertilized females determined the normal arrhenotokous parthenogenesis and the production of male offspring. As a consequence of this procedure, the male of *G. bonaerensis* is described for the first time.

Introduction

Dryinidae (Hymenoptera: Chrysidoidea) is a widespread family with approximately 1700 species (Olmi & Virla 2014). They are exclusive parasitoids of Hemiptera Cicadomorpha and Fulgoromorpha (Virla & Olmi 2007, Guglielmino *et al* 2013), with certain relevance as biocontrol agents (Olmi 1999, 2000). The general biology of the family is poorly known, and the lack of information on diverse aspects of the reproduction is notable. *Dryinids* are known mostly for its females, the males being infrequent; as an example, in the Neotropical Region, more than 80% of the species are known only for specimens of one sex (Virla & Olmi 2008); 83 of 123 species (67.5%) of the genus *Gonatopus* occurring in this region are known only by their females (Olmi & Virla 2014).

According to Normark (2003) most of the Hymenoptera species exhibit arrhenotokous parthenogenesis, where every male develops from an unfertilized egg and has only a haploid genome inherited from its mother. But in several families the existence of regular or irregular alternation between different genetic systems is commonly found, typically between amphimixis and thelytoky, or strict uniparental reproduction by thelytoky where females produce diploid female offspring from unfertilized eggs by apomixis or automixis, no mating occurs and there are no males. According to Arakaki *et al* (2000) two forms of thelytoky are recognized in Hymenoptera, reversible or microbe-associated thelytoky and non-reversible thelytoky.

Bacteria belonging to the genus *Wolbachia* are associated with a variety of reproductive anomalies in arthropods (Mazur *et al* 2016). These endosymbiotic bacteria are transmitted through the cytoplasm of eggs and have evolved various mechanisms for manipulating reproduction of their hosts, including induction of reproductive incompatibility, pathenogenesis, and feminization (reviewed by O'Neill *et al* 1997). They have been implicated as a possible mechanism for rapid speciation in arthropods (Werren & Windsor 2000). *Wolbachia* has been found associated with thelytoky in several hymenopteran parasitoids like Trichogrammatidae, Cynipidae, Aphelinidae, Pteromalidae, Scelionidae,

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Eucoilidae, etc. (Plantard & Solignac 1998, Plantard *et al* 1999, Arakaki *et al* 2000, Giorgini 2001). When this symbiotic relationship exists, the removal of these microbes by antibiotic or high-temperature induces the production of males (Stouthamer *et al* 1993, Arakaki *et al* 2000, Giorgini 2001). The existence of *Wolbachia* affecting Dryinidae was mentioned by Noda *et al* (2001) and Gan *et al* (2002). Noda *et al* (2001) analyzed samples from dryinid larvae (non identified species) affecting the small brown planthopper *Laodelphax striatellus* Fallén (Hemiptera: Delphacidae), and found four different strains; Gan *et al* discovered that both females of *Haplogonatopus apicalis* R. Perkins and specimens of its hosts (Delphacidae) were infected by the bacteria.

Within Dryinidae, most of the Gonatopodinae species exhibit biparental reproduction and "facultative" thelytokous parthenogenesis (see Table 4 in Guglielmino & Virla 1998). It is interesting to note that some species exhibit both amphimictic (biparental) and thelytokous populations, like *Gonatopus lunatus* Klug (Guglielmino & Virla 1998), *Gonatopus chilensis* Olmi (Olmi & Virla 2014), *G. caraibicus* (Olmi) (Olmi & Virla 2014), *G. clavipes* (Thungberg) (Waloff 1974), among others.

Gonatopus bonaerensis Virla is known for attacking Delphacidae, *Delphacodes sitarea* Remes Lenicov & Tesón (Hemiptera: Delphacidae) being its best-known host in Argentina (Olmi & Virla 2014). In Northern Argentina, their populations are active from late winter to the beginning of autumn; it is a solitary species with thelytokous parthenogenesis thus only female specimens were previously known (Virla 2004). These facts led us to think that *Wolbachia* might infect their populations.

In this contribution, we investigated the existence of natural *Wolbachia* infections in *G. bonaerensis* and test the possibility of obtaining males after antibiotic treatment.

Material and Methods

Insects

Obtaining dryinid specimens from the field. A survey was conducted fortnightly from August 2009 to September 2010 in a grassland composed mostly by St. Augustine grass, *Stenotaphrum secundatum* (Walt.) Kuntze (Poaceae), at Las Talitas (26°46′57.2″S; 65°12′17.5″W; elevation 496 m.asl) in Tucumán province (Argentina).

Specimens of *G. bonaerensis* were obtained from larvae affecting nymphs and adults of *D. sitarea*. The parasitized delphacids were brought to the laboratory and individually isolated in glass tubes containing a piece of stem of St. Augustine grass; the grass was changed daily and served to feed the delphacids and as pupation substrate for dryinids.

Adults of the dryinids were conserved in absolute ethanol immediately after they were found in the tubes. They were identified at species level by E.G. Virla using the keys provided by Virla (1997) and Olmi & Virla (2014).

Lab breeding. In order to maintain an experimental population, a colony was founded with four *G. bonaerensis* females obtained from attacked delphacids collected from field in Las Talitas. The colony was maintained using *D. sitarea* as host and St. Augustine grass as host-plants, according to the methodology exposed in Virla (2004).

Obtainment and characterization of Wolbachia

DNA extraction. DNA template was prepared by homogenizing a single female adult in a 25 µl mixture of STE buffer (100 mM NaCl, 10 mM Tris-HCl, 1 mM EDTA, pH 8.0) and proteinase K (10 mg/ml, 2 µl) in a 1.5 µl Eppendorf tube. The mixture was incubated at 37°C for 30 min and later at 95°C for 5 min. All PCR reactions were performed in 25 µ l of final volume. The primer pairs used in this study were wsp, 16S (Werren & Windsor 2000).

Wolbachia PCR assay. The presence of the endosymbiotic bacterium *Wolbachia* has been assessed by PCR using specific primers wsp_F1 GTCCAATARSTGATGARGAAAC, wsp_R1 CYGCACCAAYAGYRCTRTAAA. We have used primers amplifying the 16S ribosomal DNA of *Wolbachia* designed by O'Neill *et al* (1992) and Rousset & De Stordeur (1994) (positions 76–99 and 1012–994 of *E. coli* 16S rDNA) to check for the presence of *Wolbachia* in all the individuals of *G. bonaerensis* obtained in the field.

PCR was performed in a total volume of 25 µl, containing 5 µl of 10× buffers, 2.5 µl of dNTP mix, 0.3 µl of each primer (100 mM), 0.2 µl of Taq DNA polymerase, and 1 µl of DNA solution. DNA amplification was done in a Perkin Elmer thermal cycler; reactions were cycled 40 times at 95°C for 30 s, 52°C for 30 s, and 72°C for 2 min. After PCR, 5 µl of amplification product were separated electrophoretically on 2% agarose gel and visualized by ethidium bromide staining and UV fluorescence.

Antibiotic treatment and verifying Wolbachia removal

After the detection of *Wolbachia* affecting the field specimens of *G. bonaerensis*, 50 lab breeding newly emerged females were fed with a solution of 20 mg/ml of the antibiotic tetracycline dissolved in pure honey (Arakaki *et al* 2000, Giorgini 2001). This treatment was provided to females since the emergence.

Hosts were exposed 3 days after treated female emergence; 25 hosts (both nymphs of IV and V instars of

D. sitarea) were daily exposed during 24 h to female parasitism. Each parasitoid female was isolated in a glass pot (2 cm diameter × 25 cm long), with a St. Augustine grass stem fixed by a cotton plug to prevent the escape of the insects. After that, exposed planthoppers were located in breeding cages containing *S. secundatum* plants to permit the development of both hosts and parasitoids.

Ten unmated females of *G. bonaerensis* from lab breeding colony were used as control and were provided with a similar number of hosts as the treated females but fed only with honey.

Description of the male

The descriptions follow the terminology used by Olmi (1994, 1999) and partly revised after Gauld & Bolton (1988). The measurements reported are relative, except for the total length (head to abdominal tip, without the antennae), which is expressed in millimeters.

In the descriptions, POL is the distance between the inner edges of the two lateral ocelli, OL is the distance between the inner edges of a lateral ocellus and the median ocellu, OOL is the distance from the outer edge of a lateral ocellus to the compound eye, and OPL is the distance from the posterior edge of a lateral ocellus to the occipital carina.

Voucher specimens are deposited in the Instituto de Entomología, Fundación Miguel Lillo (Tucumán, Argentina) entomological collection (IMLA).

Results

Presence and characterization of Wolbachia affecting field populations of Gonatopus bonaerensis

From the 24 field samples held in Las Talitas, a total of 117 parasitized planthoppers were obtained, all belonging to *D. sitarea*. Only 46 of the parasitoids reached the adult state, resulting all females. The subsequent specific identification confirmed they belonged to *G. bonaerensis*.

The presence of *Wolbachia* sp. was detected for the first time in specimens of *G. bonaerensis* (25 females), by pcr amplification of the fragment of 438 bp that confirmed by 100% the identity of *Wolbachia* sp. wRi strain.

Antibiotic treatment

After being fed with the antibiotic solution, seven females (14%) survived 3 or more days, and only four of them reproduced successfully. Antibiotic treatment had effect on newly emerged females, so they produced only male offspring. All the untreated virgin females (control) produced only female progeny as in the origin lab colony.

Subsequently, a PCR study was performed on females that had been treated with tetracycline (cured) and on five male individuals (sons of them). The amplification did not show the presence of the endosymbiont in those dryinid specimens.

As a result of the experiment, the male of *G. bonaerensis* is described as follows:

Description of the male (based on five specimens): fully winged; length 1.88–2.22 mm (mean: 2.05 ± 0.12). Head black, except mandibles,brown; clypeus brown-dark; antennae brown; total length of head: 0.26–0.304 mm (mean: 0.28 ± 0.02). Mesosoma black, length 0.76–0.89 mm (0.83 ± 0.05); legs fully brown. Gaster brown-dark, in two specimens fully brown; total length of gaster: 0.85–1.05 mm (mean 0.94 ± 0.07). Antennae hairy, filiform; antennal segments in the following proportions: 6.3:4.5:10:9.5:10:10:9:9.5:8.5:10; antennal segment 3 more than three times as long as broad (10:2.2). Head shiny, slightly punctuate in the areas near the edge of the eyes; in some specimens, these areas reach the area between the ocelli; frontal line—absent; occipital carina absent; occiput concave; temples distinct; POL = 8; OL = 3; OOL = 3.5; OPL = 1.5; greatest breadth of anterior ocellus longer than OL (4:3). Palpal formula 2/1. Scutum slighty punctate, without sculpture among punctures hairy. Notauli complete, posteriorly not separated, joining approximately 1/5 before reaching posterior edge of the scutum (Fig 1A). Scutum with prominent parapsidal areas. Scutellum and metanotum shiny, finely hairy, very finely punctate, without sculpture among punctures. Propodeum dull, completely reticulate rugose, dorsal surface without a median longitudinal furrow, and posterior surface without keels. Forewing hyaline, without dark transverse bands; marginal cell open; distal part of stigmal vein much longer than proximal part (26:10). Dorsal process of paramere with distal apex broadened and margin deeply serrate; slightly longer than paramere but shorter than penis, with a lot of short hairs, mostly located in external area (Fig 1B). Tibial spurs 1, 1, 2.

Material examined: Argentina, San Miguel de Tucumán, five males obtained from antibiotic treated females, from October 25th to November 10th 2010, reared on *Delphacodes sitarea* (IMLA).

After the description of the male of *G. bonaerensis*, the key to the males of the neotropical species of *Gonatopus* presented by Olmi & Virla (2014) can be modified by replacing couplet 25 as follows:

25. Notauli almost joined at posterior margin of scutum 26
- Notauli joint at 1/5 before reach posterior edge of scutum (Fig 1 in this contribution) ..... *bonaerensis* Virla.
26. Minimum distance between notauli as long as greatest breadth of posterior ocelli ..... *lacualis* Olmi

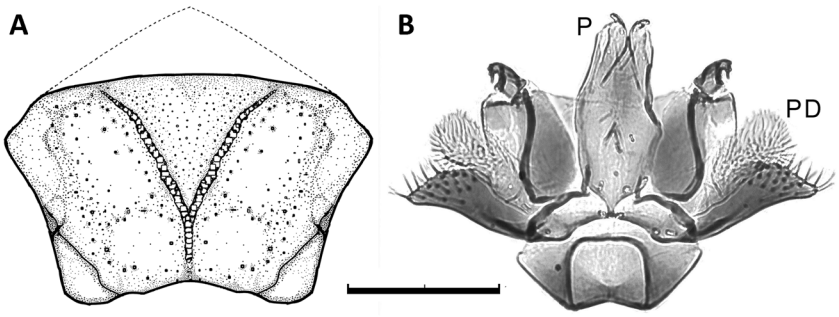

Fig 1 **A** Scutum in dorsal view in the male of *Gonatopus bonaerensis* Virla showing the notauli. **B** Stereoscopic microscope photograph of the male genitalia of *Gonatopus bonaerensis* (*P* penis; *PD* distal paramere process). *Scale bar* 0.39 mm for A, and 0.27 mm for B.

- Minimum distance between notauli much shorter than greatest breadth of posterior ocelli..... *autumnalis* Olmi.

Discussion

Wolbachia strains different from the wRi strain were recorded in the past from Asian dryinids (Noda *et al* 2001, Gan *et al* 2002). The wRi strain was previously mentioned as endosymbiont of *Drosophila simulans* Sturtevant and *Drosophila auraria* Peng (*Diptera*: *Drosophilidae*) (Zhou *et al* 1998, Jamnongluk *et al* 2002, Klasson *et al* 2009), *Aedes albopictus* Skuse (Diptera: Culicidae) (Zouache *et al* 2009), and experimentally in *Anopheles gambiae* Giles (Diptera: Culicidae) (Rasgon *et al* 2006). However, there were no records of this strain for dryinids or other Hymenoptera.

The discovery of *Wolbachia* wRi strain in *G. bonaerensis* in Argentina, represents the first record of this *Wolbachia* strain in dryinids worldwide.

After the antibiotic treatment, females produced only male offspring. These results strongly suggest that *Wolbachia* is responsible for the exclusive thelytoky found in natural populations of *G. bonaerensis* from Las Talitas.

In the Dryinidae, sexual dimorphism is so strong that corresponding sexes are not recognizable and, consequently, the classification is based mostly on females (Olmi *et al* 2000, Olmi & Virla 2014). Until now, males were assigned to a concrete species only after obtaining them in copula, from the offspring of a wild female compound by the two sexes, or via unfertilized eggs. The possibility that several species of Dryinidae that reproduce usually by thelytoky are infected by *Wolbachia* is high.

In addition, the fact that uniparental and biparental populations with a different system of reproduction (arrhenotoky or thelytoky), and showing similar morphology, belong to the same biological species is questionable. The antibiotic treatment is a tool that could provide "induced males" from thelytocous populations allowing the beginning of crossing test with females from biparental ones in order to confirm the validity of the species. This methodology opens a big perspective to solve taxonomical problems in the family.

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