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SHORT COMMUNICATION

Is the parthenogenesis of the yellow scorpion (*Tityus serrulatus*) promoted by endosymbiont bacteria (*Wolbachia* sp.)?

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Abstract. The reproduction of the yellow scorpion *Tityus serrulatus* Lutz & Mello, 1922 (Scorpiones: Buthidae) mainly occurs by parthenogenesis, and sexual reproduction is known for only a few populations. Recently, bacteria of the genus *Wolbachia*, a group of intracellular symbionts known to induce asexual reproduction in many groups of arthropods, were reported in a population of *T. serrulatus*. This finding suggests that the parthenogenesis in this scorpion could be caused by these bacteria. We tested the correlation between *Wolbachia* presence and parthenogenesis in *T. serrulatus* through PCR amplification tests of three bacterial genes (WSP, ftsZ and 16S) in parthenogenetic and sexual individuals. The results for *Wolbachia* were negative both in individuals from a sexual population and parthenogenetic individuals. This suggests that the *Wolbachia* infection previously reported for this species would be restricted to just the single population analyzed, or that the results previously obtained could be related to sample contamination.

Keywords: Intracellular bacteria, PCR, Sexual reproduction

Parthenogenesis is the reproduction form in which embryos develop without fertilization of the female gamete. Among the arachnids, it has been reported in many mite species (Oliver 1971), in a few species of harvestmen (Tsurusaki 1986), spiders (Edwards et al. 2003) and scorpions (Francke 2008). In the latter, the parthenogenesis is usually thelytokous, in which the offspring consists only of females (Lourenço 2008). Of approximately 1750 species of scorpions described in the world (Kovařik 2011), parthenogenesis has been demonstrated or suggested for only 15 (Francke 2008; Ayrey 2017). The first case of parthenogenesis reported for the order, and probably the better known one, is the Brazilian yellow scorpion *Tityus serrulatus* Lutz & Mello, 1922 (Matthiensen 1962). The species has been considered exclusively asexual for many years, until a sexual population was described from the Brazilian state of Minas Gerais (Souza et al. 2009). The exact mechanisms involved in the parthenogenesis in the yellow scorpion are still unknown, though evidence suggests that, at least in one southern Brazilian population, asexual reproduction may have been induced by bacteria of the genus *Wolbachia* (Suesdek-Rocha et al. 2007).

Wolbachia are intracellular bacteria, belonging to the group of α-proteobacteria, which induce host cytoplasmic incompatibility, sex ratio distortion, feminization of genetic males, killing of male embryos, and parthenogenesis (O'Neill et al. 1997). The main form of *Wolbachia* proliferation is vertical transmission (Hoffman et al. 1990) and, because it is transmitted by maternal inheritance, the bacteria induce an increase in female frequency in parasitized populations (Werren 1997; Koivisto & Braig 2003). *Wolbachia* is present in a wide range of hosts and has been reported in nematodes (Sironi et al. 1995) and in several arthropods, including crustaceans (Juchault et al. 1994; Cordaux et al. 2001; Maniatsi et al. 2010), insects (Werren & Windsor 2000; Werren et al. 1995) and chelicerates (Johanowicz & Hoy 1995; Rowley et al. 2004). Among scorpions in particular, besides *T. serrulatus,* it has been found in gonads of *Opistophthalmus* Koch, 1837 (Scorpionidae, Baldo et al. 2007) and venom glands of *Hemiscorpius lepturus* Peters, 1861 (Hemiscorpiidae, Baradaran et al. 2011; Ashtian et al. 2017). However, there is no report of parthenogenesis in the latter two species.

In species in which parthenogenesis is induced by *Wolbachia*, individuals may reproduce sexually when treated with antibiotics (Zchori-Fein et al. 2004). This indicates that the presence of the bacteria is necessary for asexual reproduction to occur. Although *T. serrulatus* had been considered exclusively asexual for many years, three sexual populations are currently known from Brazil (Souza et al. 2009; Santos et al. 2014; Lima et al. personal communication). If *Wolbachia* is responsible for parthenogenesis in the yellow scorpion, it is expected that only individuals from asexual populations will be infected, whereas individuals from sexual populations will not present the bacteria. In this study, we tested the correlation between *Wolbachia* and parthenogenesis in *T. serrulatus* by seeking for evidence of infection in parthenogenetic and sexual individuals.

This study is based on specimens collected specifically for DNA extraction, or relatively fresh museum material (Table 1). To discard the possibility of negative results due to DNA degradation, we used only individuals that had been previously used successfully for amplification and sequencing of mitochondrial genes, 16S rDNA (Gantenbein et al. 1999) and Cytochrome Oxidade I – COI (Simon et al. 1994; Tanaka et al. 2001, Fig. 1b), except the only individual used in the third experiment (see below). We collected specimens within the species distribution range in 2010 and 2012, storing the material in 95–100% ethanol at -20 °C. After DNA extraction, we deposited the material in the Centro de Coleções Taxonômicas of the Universidade Federal de Minas Gerais. In addition, we borrowed specimens of taxonomic collections from the Instituto Butantan, São Paulo and the Museu de Zoologia da Universidade de São Paulo, São Paulo (Table 1). We extracted DNA from 34 specimens of *T. serrulatus* collected from 16 urban locations (one sexual population and 15 parthenogenetic populations) in the Brazilian states of Minas Gerais, São Paulo, Rio de Janeiro and Santa Catarina (Fig. 1a). We characterized as parthenogenetic the populations composed only of females and/or populations in which the collected females reproduced asexually in the laboratory. In turn, we define as sexual populations those composed by both males and females. The control group for DNA extraction and amplification experiments consisted of fresh *Drosophila* Fallén, 1823 specimens, known to be contaminated with

Table 1.—*Tityus serrulatus* specimens used for *Wolbachia* detection. Asterisks indicate specimens whose reproductive tissue was used for DNA extraction.

UF	Localidade	Latitude	Longitude	Lot Number
MG	Belo Horizonte	-19.9167	-43.9333	UFMG 4071*, UFMG 4072, UFMG 4073, UFMG 12039*, Total extraction
MG	Frutal	-20.0264	-48.9359	UFMG 5360
MG	Morro do Pilar	-19.1630	-43.3645	UFMG 12539*, UFMG 12543*, UFMG 12550*
MG	Itacarambi	-15.1730	-44.1787	UFMG 7207*, UFMG 11106, UFMG 12038*
MG	Ituiutaba	-18.9746	-49.4601	MZSP 32443
MG	Santa Bárbara	-19.9731	-43.4992	UFMG 4705
MG	Varginha	-21.5561	-45.4369	IBSP 6857, IBSP 6858, IBSP 6859
RJ	Valença	-22.2459	-43.7069	MZSP 15505
SC	Balneário Camboriú	-26.9911	-48.6353	UFMG 13447, UFMG 13448
SC	Biguaçu	-27.4947	-48.6609	UFMG 13444, UFMG 13445
SC	Joinville	-26.3050	-48.8461	UFMG 13446
SP	Americana	-22.7378	-47.3336	IBSP 6845, MZSP 29049
SP	Espírito Santo do Pinhal	-22.1914	-46.7481	IBSP 6861, IBSP 6868
SP	Olímpia	-20.7375	-48.9147	UFMG 12035
SP	Piracicaba	-22.7343	-47.6481	IBSP 5900*, IBSP 6839, IBSP 6840
SP	Sumaré	-22.8209	-47.2732	IBSP 6853, IBSP 6864, IBSP 6867

Wolbachia, which were collected in a residence in Belo Horizonte, Minas Gerais, and fixed in 95–100% ethanol.

We extracted genomic DNA from all the specimens using the Wizard™ Genomic DNA Purification Kit (Promega), following the manufacturer's recommended protocol. To account for the possibility of unequal distribution of *Wolbachia* in differing scorpion tissues, we performed three experimental protocols. In the first, we extracted DNA from muscles of two legs of 33 individuals (30 parthenogenetic females, two sexual females, and one male). In the second, we extracted DNA from ovaries and embryos of six individuals from three parthenogenetic populations and one female ovary and one male hemispermatophore from the sexual population. The specimens used in this step were also tested in the first experiment. The third experiment was performed separately from the legs, pedipalps,

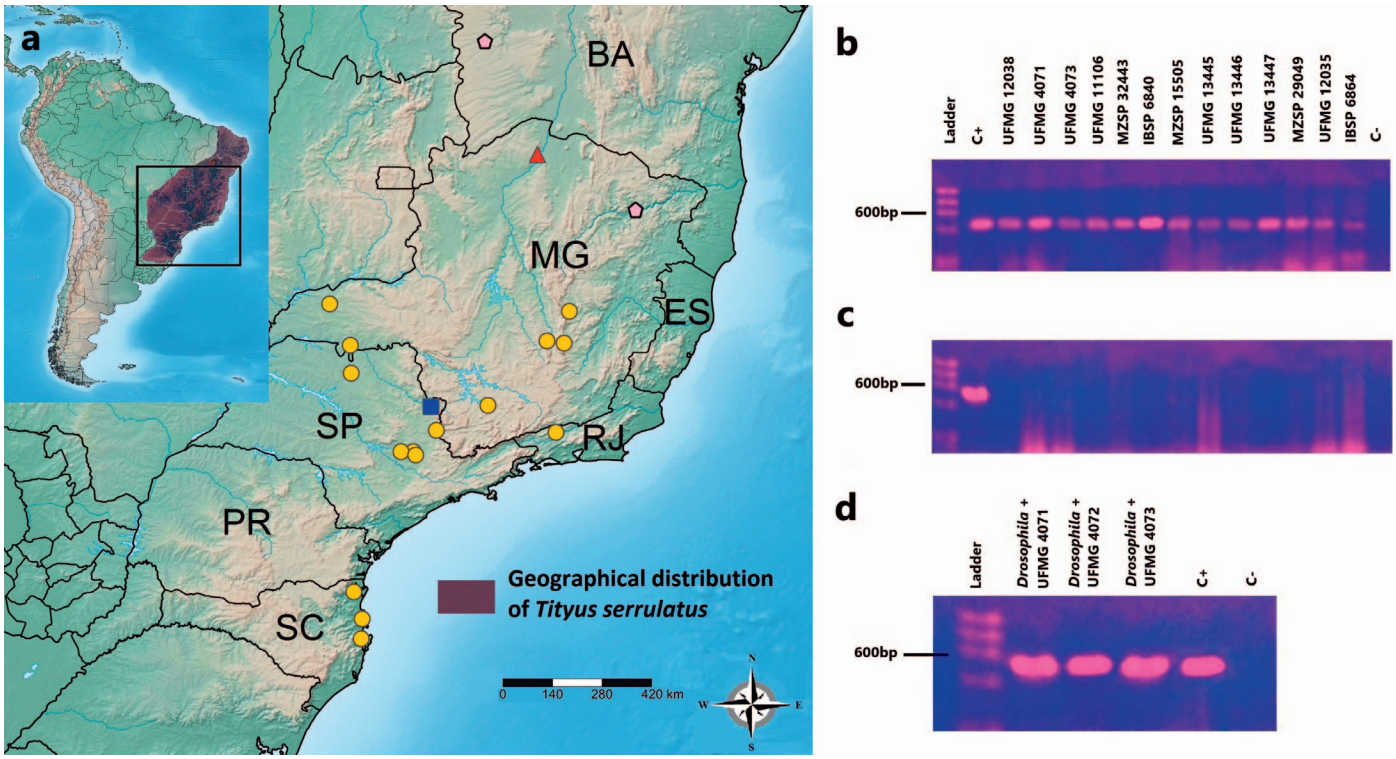

Figure 1.—(a) Geographic distribution of *Tityus serrulatus* specimens sampled in this study. Circles represent parthenogenetic populations, triangle sexual population, and pentagons sexual population not analyzed in the present study. The blue square indicates the population analyzed by Suesdek-Rocha *et al.* (2007), which was not sampled in this study. PCR amplification of (b) COI gene from *Tityus serrulatus*, (c) WSP gene of *Wolbachia* from leg muscles of *Tityus serrulatus*, (d) WSP gene from leg muscles of parthenogenetic *Tityus serrulatus* mixed with crushed fresh *Drosophila* flies.

metasoma, telson, reproductive tissues and embryos of a parthenogenetic individual euthanized by freezing at -20 °C and immediately subjected to extraction. Finally, to test whether any compound from the scorpion tissue could interfere on the *Wolbachia* DNA amplification, we extracted DNA from leg muscles of three parthenogenetic specimens mixed with crushed fresh *Drosophila* flies. The three individuals were also used in the first experiment.

We assessed the *Wolbachia* infection on scorpion and *Drosophila* tissues through PCR-amplification tests of three bacteria genes commonly used in phylogenetic studies on *Wolbachia*: cell cycle controller gene – ftsZ (Jeyaprakash & Hoy 2000), encoding surface protein gene – WSP (Braig *et al*. 1998) and 16S rDNA gene (O'Neill et al. 1992). The 16S and WSP primers have greater sensitivity for detecting *Wolbachia* (Werren & Windsor 2000; Xiao-Yue et al. 2002). Although the *ftsZ* gene has been extensively used in *Wolbachia* studies, it has shown low sensitivity, leading to false negatives in *Wolbachia* detection when used alone (Jeyaprakash & Hoy 2000; Marcon et al. 2011). Our tests were based on the primers described in the references above and the following PCR conditions: initial denaturation at 94 °C for three minutes, followed by 35 cycles with a denaturation step at 94 °C for one minute, annealing at 55 °C for one minute, extension at 72 °C for two minutes, and final extension at 72 °C for 10 minutes. To evaluate amplification results, we subjected the resulting material to 1% agarose gel electrophoresis, stained with GelRed™. We used the material extracted from *Drosophila* as a positive control, and material resulting from PCR reactions with all reagents, except extracted DNA, as a negative control. The DNA successfully amplified for ftsZ and WSP was sequenced in order to verify its identity. We purified the amplified material fragments using a cleaning protocol with Polyethylene Glycol (Polyethylene Glycol 20% and NaCl 2.5 M), performed the sequencing reaction using the BigDye Terminator Cycle Sequencing Kit, and purified the sequenced material with 7.5 M ammonium acetate. We sequenced genes in both directions in an ABI 3130x Genetic Analyzer (Applied Biosystems) automatic sequencer, with the same primers used for amplification. We compared the sequences obtained with sequences deposited in GenBank through the Blast™ tool (Basic Local Alignment Search Tool). We deposited the sequences in GenBank (ftsZ accession: MN097777, WSP accession: MN097776).

In all our experiments, no sample of pure *Tityus serrulatus* tissue resulted in successful DNA amplification of *Wolbachia* genes (Fig. 1c). The test using scorpion leg muscles, from either parthenogenetic or sexual populations, showed no positive results, though the *Drosophila* positive control was successfully amplified for all three genes. Although maternally inherited endosymbiont bacteria are sometimes reported only for reproductive tissues (Dobson et al. 1999), *Wolbachia* infection has been detected in non-reproductive tissues such as scorpion venom glands (Baradaran et al. 2011) and butterfly (Kodandaramaiah et al. 2011) and spider (Rowley et al. 2004) leg muscles. On the other hand, vertical transmission (Hoffman et al. 1990) makes it particularly easy to detect *Wolbachia* in reproductive tissues (Werren 1997) or on embryos (Zhao et al. 2013). However, we also obtained negative results for scorpion reproductive tissues, eliminating the possibility of the bacteria being concentrated only on the specific tissues, mostly in the reproductive system. This conclusion was further supported by the negative results of the amplification tests on all body parts of a specimen and on embryos. The amplification tests with ultra-fresh material were also negative, indicating that the results of our experiments cannot be attributed to bacterial DNA degradation due to scorpion preservation mode. Finally, amplification tests on scorpion muscle tissue macerated together with *Drosophila* flies were all positive for WSP, ftsZ and 16S, indicating that no substance in scorpion tissue could inhibit the bacterial DNA extraction or amplification (Fig. 1d). These results were further confirmed by the Blast tests on the successfully amplified DNA from the *Drosophila* samples, which showed 99% (ftsZ) and 100% (WSP) match with the *Wolbachia* sequences deposited on GenBank.

Our results are in conflict with those from Suesdek-Rocha et al. (2007), who detected *Wolbachia* in *T. serrulatus* parthenogenetic individuals using amplification of 16S rDNA bacterial genes. Despite the obvious possibility of accidental sample contamination during laboratory procedures, we suggest two possible explanations for Suesdek-Rocha et al. (2007) results. First, the scorpion specimens studied by them could contain remnants of an early infection that occurred in the past, but reversed over time. In the laboratory, *Wolbachia* can be de-activated or eliminated when the host is subjected to high and constant temperatures (28–30°C, Claney & Hoffmann 1998). This may have occurred naturally with *T. serrulatus*. However, this raises the question of why the parthenogenesis persisted on non-infected populations, since experiments based on antibiotic treatment demonstrated the importance of *Wolbachia* infection for maintenance of parthenogenesis in arthropods (Legner 1985; Stouthamer & Luck 1991; Stouthamer & Werren 1993). Another possible explanation would be a recent, local infection on the population sampled by Suesdek-Rocha et al. (2007). This is an interesting possibility, especially because the mechanisms of *Wolbachia* transmission between species are still poorly known (Cordaux et al. 2001). Whatever is the best explanation, our results clearly show that *Wolbachia* infection is not a general explanation for the parthenogenesis in *T. serrulatus*.

Our conclusion that the parthenogenesis in *T. serrulatus* is not caused by *Wolbachia* is indirectly supported by further evidence. Males and females of *T. serrulatus*, including specimens from parthenogenetic populations, have been shown to be diploids ($2n = 12$, Schneider & Cella 2010; Lima et al. personal communication). Results obtained in studies on wasps (Stouthamer & Kazmer 1994) and mites (Weeks & Breeuwer 2001) lead Weeks et al. (2002) to suggest that *Wolbachia*-induced parthenogenesis may occur only in haplodiploid hosts. In those cases, unfertilized eggs normally develop as haploid males, but generate diploid females when infected. However, *Wolbachia* also induces parthenogenesis in *Folsomia candida* Willem, 1902, a diplodiploid collembolan (Pike & Kingcombe 2009). Although *Wolbachia* induces parthenogenesis in both diploid and haplodiploid hosts, studies indicate that the first are infected by *Wolbachia* from the supergroup E (Lo et al. 2002; Pike & Kingcombe 2009), while the second are infected by *Wolbachia* from supergroup B. (Arakaki et al. 2001; Lindsey et al. 2016; Almeida & Stouthamer 2018). *Wolbachia* found in *T. serrulatus* is similar to the strain present in *Drosophila innubila* Spencer, 1943 (Suesdek-Rocha et al. 2007), which induces killing of male embryos and belong to the supergroup A (Dyer & Jaenike 2005), which probably does not have the capacity to induce parthenogenesis in diplodiploids. In addition, it has been shown in a wasp species that *Wolbachia*-infected females do not attract males, making *Wolbachia* an obligate partner for daughter production in thelytokous populations (Kremer et al. 2009). On the other hand, parthenogenetic *T. serrulatus* females accept copulation with males from sexual populations (Braga-Pereira, unpublished data).

Asexual reproduction in arthropods can be induced by other microorganisms, such as bacteria belonging to the CFB group (*Cytophaga*, *Flexibacter* and *Bacteroides*), which are associated with parthenogenesis in wasps and feminization in mites (Koivisto & Braig 2003). *Cardinium* bacteria, which belong to the CFB, are currently known to induce parthenogenesis in host species they infect, including mites (Nakamura et al. 2009; Ma & Schwander 2017). Whether any of these microorganisms occur in *T. serrulatus* is open to investigation. A characterization of the presence of the endosymbionts, using general bacterial primers, could help identifying other parthenogenesis-inducing microorganisms in the yellow scorpion. However, evidence gathered in ongoing studies lead us to discard other

symbiont bacteria as the promoters of the yellow scorpion parthenogenesis.

A first evidence against symbiont-induced parthenogenesis is the species' geographic distribution. Sexual populations of *T. serrulatus* occur within the distribution range of parthenogenetic populations (Fig. 1a). Additionally, during our field work, we observed no clear habitat differences between either sexual or asexual specimens, which are usually found in urban or rural, degraded habitats. Endosymbiont-induced parthenogenesis seems to facilitate the maintenance of reproductive polymorphism, with sexual (uninfected) and parthenogenetic (infected) strains presenting distribution differences, such as parthenogenetic individuals occurring at higher latitudes than their sexual relatives (Ma & Schwander 2017). If a symbiont bacterium is the cause of parthenogenesis in the yellow scorpion, what could have prevented the infection (and thus, exclusive parthenogenesis) in the sexual populations?

Besides induction by microorganisms, obligatory parthenogenesis can result from different processes, such as spontaneous, contagious or hybrid origin (Simon et al. 2003). Hybridization, in particular, is a major route to parthenogenesis in animals (Avise et al. 1992). Hybridization between species can disrupt meiotic processes and create opportunities for the selection of cytological processes that rescue egg production (Vrijenhoek 1998). This form of parthenogenesis results from crosses between two bisexual species and generally leads to the production of diploid, asexual lineages (Simon et al. 2003). Lourenço & Cloudsley-Thompson (1996) suggest the existence of a hybrid zone between *T. serrulatus* (although the *T. serrulatus* male was still unknown in 1996) and *T. stigmurus* Thorell 1876, another species in which parthenogenesis has been reported (Ross 2010). Thus, if lineages of these species suffered introgression during their evolutionary history, this factor may explain the emergence of parthenogenesis in *T. serrulatus* and *T. stigmurus*. If this hypothesis is true, specimens from parthenogenetic populations should have all mitochondrial alleles from only one parent species, but half nuclear alleles from each of two separate parent species (Welch & Meselson 2000).

Lastly, facultative parthenogenesis (or tychoparthenogenesis), in which eggs develop spontaneously, without fertilization (Simon et al. 2003), can give rise to obligatory parthenogenesis (Kramer & Templeton 2001). Since other scorpion species have been shown to reproduce by facultative parthenogenesis (Francke 2008), it is possible that the obligatory parthenogenesis of *T. serrulatus* evolved from facultative parthenogenesis.

This hypothesis can be evaluated in laboratory, analyzing whether specimens from sexual populations are able to perform parthenogenesis (Borges da Silva et al. 2010). In fact, we have already seen 19 females from a sexual population generating offspring without previous mating. These females were either collected in the second instar (nine individuals) or were born in the laboratory (10 individuals), and all of them were kept in separate containers, without contact with other individuals (unpublished results). This may be an indication of facultative parthenogenesis in *T. serrulatus*, and is not consistent with parthenogenesis induced by any endosymbiont.

Although the mechanism that gave rise to the parthenogenesis in *Tityus serrulatus* is unknown, there is evidence on its geographic origins. Parthenogenetic individuals may have emerged from areas of savanna with isolated palm trees (Lourenço 2008). Despite the widespread distribution of the yellow scorpion in Brazil (Fig. 1a), sexual populations have been recorded only in three, relatively near localities in northern Minas Gerais (Souza et al. 2009; Lima et al. personal communication) and western Bahia (Santos et al. 2014). Considering the geographic proximity of these sexual populations, it seems plausible to suppose the parthenogenesis in the yellow scorpion may have arisen between these regions. By discarding *Wolbachia* infection as the explanation for *T. serrulatus* parthenogenesis, our results suggest the asexual reproduction may actually be adaptive for this scorpion species, which is not being simply manipulated by an endosymbiont organism. This raises new and interesting possibilities on the investigation of the reproductive biology of the yellow scorpion.

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