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FACIAL MARKINGS AND *WOLBACHIA PIPIENTIS* INFECTION RATE IN *POLISTES CAROLINA* PAPER WASPS IN THE NORTHERN CHIHUAHUA DESERT

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ABSTRACT: During the summer of 2023, 115 *Polistes carolina* (Linnaeus, 1767) were captured in the Northern Chihuahua Desert. Ninety-two were females and 23 were males giving a sex-ratio distortion of exactly 4:1. Purposely, the queens were not captured. *Polistes carolina*, a common paper wasp, is important for two main reasons. First, they are native pollinators that have not been inbred like the domesticated honeybee, *Apis mellifera*. Therefore, they are more resistant to pathogens. Secondly, they are insect predators and help control insect populations. Insect larva in the Orders Lepidoptera and Diptera are usually targeted. Sixty-nine *Polistes carolina* were tested for *Wolbachia pipientis*, an endosymbiont that is changing the evolution of insects. The 16S rDNA gene of the small unit of the bacterial ribosome was targeted to identify *Wolbachia pipientis* infection. For insect DNA, the cytochrome C oxidase gene was targeted. Fifty-six-point five percent tested positive for *Wolbachia.* One-hundred and eight *Polistes carolina* wasps' facial markings were analyzed. Three facial marking categories were detected. Facial marking in the genus *Polistes* have been linked to communication. Facial markings in several other species in this genus have been analyzed, but not *Polistes carolina*. The wasps were identified using the Vespidae key from Buck, Marshall, and Cheung (2020).

KEYWORDS: *Wolbachia pipientis*, *Polistes carolina*, Facial Marking in Paper Wasps

BACKGROUND:

There is a dearth of information on the *Polistes carolina* populations living in the Northern Chihuahua Desert. *Polistes carolina*, a common ferrous colored paper wasp inhabits six continents and many oceanic islands (Reeve, 1991). Over the last several decades, these wasps have been studied to understand several behaviors including dominance hierarchy of reproduction (Jandt, Tibbetts, & Toth, 2014), the mechanisms of social communication (Cervo, Cini, & Turillazzi, 2015), and plasticity of social behavior (Patalano, Vlasova, Wyatt, Ewels, & Camara, 2015). *Polistes carolina* are non-specific native pollinators (Sumner & Cini, 2021), and are important predators in terrestrial communities (Stahlhut, Liebert, Starks, Dapporto, & Jaenike, 2006). They are an essential part of ecosystems. *Polistes carolina* mainly prey on the larval stages of Orders Diptera and Lepidoptera, which in large numbers can be agricultural pests (Southon, Fernandes, Nascimento, & Sumner, 2019). With the decline of *Apis melifera*, the domesticated European honeybee, the preservation of native pollinators is imperative (Hoehn, Tscharntke, Tylianakis, & Steffan-Dewenter, 2008).

Facial patterns have been shown to be a means of communications for some species of *Polistes* wasps. Differences in facial patterns have been linked to individual wasp identity (Jernigan, Stafstrom, Zava, Vogt, & Sheehan, 2023; Miller, 2023). Wasps are limited in their visual acuity due to small, compound eyes (Land, 1997). Facial markings are used to identify nest mates, enemies, and social hierarchy. Facial recognition among nest mates is used to reduce conflicts and stabilize social interactions (Tibbetts & Sheenan, 2011). Facial color patterns among *Polistes* wasps include yellow, red-brown and black markings (Miller, 2023). Facial pattern communications have been identified in three species of *Polistes* wasps, but not *Polistes carolina.*

Wolbachia pipientis is a rickettsia, alpha-proteobacteria endosymbiont that causes reproduction alterations that impact speciation (Knight, 2001; Werren, 1997). It infects insects, mites, crustaceans, and filarial nematodes (Serbus, Casper-Lindley, Landmann, & Sullivan, 2008). Although, it is now known that *Wolbachia* can go through an extracellular phase that can last up to seven days (Rasgon, Gamston, & Ren, 2006). *Wolbachia* causes cytoplasmic incompatibility, (LePage, et al., 2017; Werren, 1997) parthenogenesis, feminization, and male-killing (Hurst, et al., 1999: Werren, 1997). It can be transmitted horizontally

from prey to predator, through extracellular surfaces, and vertically from mother to off-spring (Rasgon, Gamston, & Ren, 2006; Werren, 1997). Besides causing reproductive alterations, *Wolbachia pipientis* can block numerous diseases that are commonly transmitted by mosquitoes such as Zika virus, Dengue virus, Drosophila C virus, West Nile virus and many more (Frentiu, Robinson, Young, McGraw, & O'Neill, 2010; Iturbe-Ormactxe, Walker, & O'Neill, 2011). Also, *Wolbachia* has made some populations of insects resistant to insecticides and RNA viruses (Li, Lui, & Guo, 2018; Pimentel, Cesar, Martins, & Cogni, 2021; Eleftherianos, Atri, Accetta, & Castillo, 2013). Females from several populations *Polistes dominulus* in Italy and the U.S. were tested for the *Wolbachia* infections rates in 2006. The infection rates varied immensely. Italian wasps' infection rates ranged from 33% to 71%. The U.S. populations ranged from 16% to 87% (Stahlhut, Liebert, Starks, Dapporto, & Jaenike, 2006).

METHODS: DNA Extraction and PCR Protocols

Two millimeters (mm) were removed from the specimen's abdomen. The abdominal segment was then placed in a 1.5 milliliters (mL) microfuge tube with 200 microliters (µL) of lysis buffer. The abdominal segment was macerated for 1 minute. Eight-hundred µL of lysis buffer was added to the microfuge tube then vortexed. The tube was placed in a 95°C water bath for 5 minutes. After heating, the tube was opened briefly to release pressure then centrifuged for 5 minutes at 10,000 rpm. Another microfuge tube was obtained and 400 µL of the supernatant and put into the new tube. Forty µL of 5.0 M NaCl was added and placed on ice for 5 minutes. Tubes were placed in the centrifuge at the same rpm and time as previously stated. Another clean microfuge tube was obtained and 300µL of supernatant was transferred. Four-hundred microliters of isopropanol was added and then centrifuged at 10,000 rpm for 5 minutes. The supernatant was carefully poured out and the mouth of tube was tapped lightly to remove most of the liquid. The tube was centrifuged for 1 minute and the rest of the liquid was pipetted out. The pellet was air dried for 10 minutes. Two-hundred µL of TE/RNase was added. The pellet was disturbed by pipetting then tube was centrifuged at 10,000 rpm for 1 minute. The DNA was frozen until PCR amplification. PCR amplification was done with a Bio-rad thermocycler t100. Five µL each of forward and reverse primer, 5 µL of insect DNA, and 10 µL of master mix were added to a 0.2 mL microfuge tube. PCR cycles included 95 degrees for 2 minutes, 30 cycles of: 94 degrees for 30 seconds, 55 degrees for 45 seconds, 72 degrees for 1 minute, then 72 degrees for 10 minutes, and finally left at 4 degrees for the rest of the allotted time. GelGreen Nucleic Acid Stain was added to the two percent agarose lithium bromide gels. The electrophoresis chamber was filled with lithium bromide buffer. The gels were run at 150 V for 30 minutes. A pearl biotech DNA illuminator was used to view the DNA.

Capture of *Polistes carolina* Paper Wasps

Five *Polistes carolina* wasp nests were located in a 5-hectare area of the Northern Chihuahuan Desert. A vacuum pump was used to extract a few wasps from each nest every few weeks so as not to destroy the nest. The queen wasps were not removed. The wasps were frozen to prevent DNA decay, then placed in 50 mL centrifuge tubes until be tested for *Wolbachia pipientis* and examined for facial markings. Safety clothing was worn at all times.

Collection Site: The specimens were collected from a 5-hectare area (~32.17°N, 107.63°W) at an elevation of 1400 m from the bajada on the north side of the Florida Mountains. The mountains are an inactive fault-block range comprised of Paleozoic limestone and dolomite rocks. The area receives ~23.37 cm of precipitation. The high temperature is ~35.5°C, and the low is ~18.3°C. The florae include creosote bushes, *Larrea tridentate*, Joshua trees, *Yucca brevifolia*, mesquite trees, *Prosopis glandulosa*, and several species of the genus *Opuntia,* prickly pear cacti.

Facial Markings

One-hundred eight wasps were decapitated and the heads viewed under a dissecting microscope. Classification of the facial markings were based on the difference locations of black lines on the edge of the clypeus, descending from the antenna, and also ascending from the base of the clypeus to the base of the eyes. Individual markings were noted, but discounted because of the limited numbers.

RESULTS

The present study was done for two purposes. First, the evaluate the facial markings of *Polistes carolina.* Facial marking of several other *Polistes* species had been evaluated, but not *P. carolina*. Secondly, the study assesses the *Wolbachia pipientis* infection rate of *Polistes carolina* in the Northern Chihuahua desert.

Facial Markings: One-hundred eight *Polistes carolina* paper wasps were decapitated, viewed under a dissecting microscope and analyzed.

Table 1. Number of male and female wasps with Alpha, Beta, and Gamma markings

Facial Type	Female	Male	Total	Percentage of Total Wasps
Alpha	1	1	2	1.8%
Beta	18	3	21	19.4%
Gamma	64	21	85	78.7%

Figure 1. (L to R) Specimen # 7 Alpha markings; Specimen # 18 Beta markings; Specimen # 50 Gamma markings

Alpha identification includes black lines on the margin of the clypeus that start at the base of the clypeus and extend upward to the base of the eyes.

Beta identification includes black line on the margin of the clypeus that start at the base of the clypeus and extend upwards to the antenna.

Gamma identification includes a black line starting at the antenna and descending down to the eye margin.

***Wolbachia pipientis* Infection Rate:** Sixty-nine *Polistes carolina* wasps were tested for *Wolbachia pipientis* infection. For *Wolbachia* DNA identification, the 16S rDNA gene of the small ribosome unit was targeted for identification. For insect DNA identification the cytochrome C oxidase gene was targeted. A second bacterium infection was identified by electrophoresis in a few of the specimens.

Table 2. Number of *Polistes carolina* wasps infected with *Wolbachia pipientis*.

Specimens	Number Tested	Number Infected	Percentage Infected
Total	69	39	56.5%
Male	16	10	62.6%
Female	53	29	54.7%

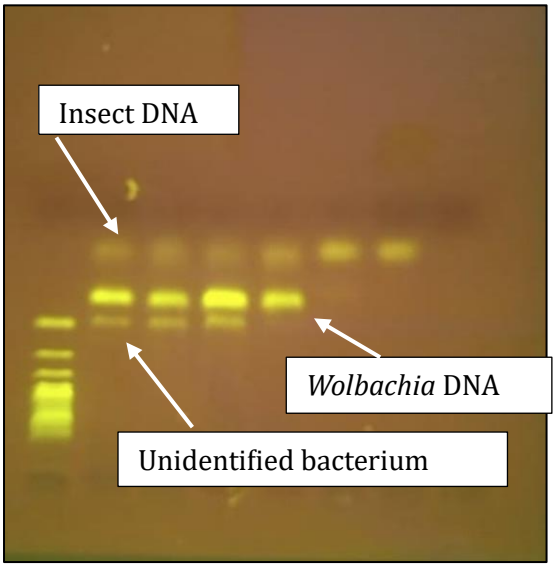

Figure 2 (Left) Is the electrophoresis get for specimens 59 through 64. The electrophoresis gel, on the left, shows the 100 bp ladder then DNA from specimens 59 through 64, in order. The insect DNA is located furthest from the wells at 709 bp, and the *Wolbachia* DNA is located below the insect DNA at 438 bp. Specimens 59 through 62 are infected with *Wolbachia.* Another bacterium's DNA is visible just below the *Wolbachia* DNA in specimens 59 through 61. Beings we targeted the 16S rDNA gene in bacterium and there are variations in the sequence with different bacterium, the primer we used picked an unidentified bacterium in specimens 59, 60 and 61. The university laboratory did not have the equipment or reagents necessary to identify the bacterium.

DISCUSSION

During the summer of 2023, 108 *Polistes carolina* paper wasps were captured from various nests in the Northern Chihuahua Desert. Facial markings are one method of communication used in *Polistes* wasps. Several species of facial markings in *Polistes* wasps have been analyzed, but not *Polistes carolina*. As mentioned earlier, the facial markings of *Polistes carolina* have not been analyzed or published. Also, purposely the queens were not captured, and only a few specimens were captured at one time from each nest to prevent harming the nests.

Three distinct facial marking were observed, 2 Alpha, 21 Beta, and 85 Gamma (Table 1, Figure 1). It seems obvious that Gamma facial marking denotes "workers". They made-up almost 79% of the wasps captured. The female with Alpha markings must have been a "foundress", a potential queen for a nest. The male is a quandary, but with such a small sample it is impossible to make any realistic assessment. More research is needed to ascertain the status of males with Alpha markings. Nineteen percent of the wasps have Beta markings. These wasps must have been captured while the nest was in its reproductive stage. All of the wasps had yellow faces with the exception of the black lines.

Sixty-nine *Polistes carolina* wasps were tested for *Wolbachia pipientis,* 16 male and 53 female (Table 2). To analyze the infection rate, the DNA was run through an agarose gel at 150 V for 30 minutes. Insect DNA was visible in bands at 709 base-pairs (bp) and *Wolbachia* was visible at 438 bp (Figure 2). Specimens 59 through 64 electrophoresis gel (Figure 2) shows that specimens 59 through 62 were infected with *Wolbachia pipientis*. Specimens 59, 60 and 61 were infected with a second bacteria, which showed up at about 400 bp. However, we did not have the reagents necessary to identify it. Fifty-four-point seven percent of the wasps were infected with *Wolbachia,* which aligns with the average infection rate across all insect species globally. Interestingly, *Aedes aegypti* collected in the same area had the exact same rate of *Wolbachia* infection (Kulkarni, et al., 2018). However, the infection rate of several *Polistes* species tested in the U.S. varied from 16% to 87% (Stahlhut, Liebert, Starks, Dapporto, & Jaenike, 2006).

When the gonads were removed, most of the *Polistes carolina* secreted a clear purple liquid. It was anticipated that the liquid was a reducing sugar. To verify that, the liquid was collected and analyzed with Benedict's reagent. The solution turned a brown/ red color which determined that the solution was indeed reducing sugar.

CONCLUSION

More research is needed on the *Polistes carolina* wasps in the Northern Chihuahua Desert, especially with the facial markings and *Wolbachia pipientis* infection rate. Presently, there is very little research being done on the insects inhabiting this desert region. *Wolbachia pipientis,* the fastest spreading endosymbiont on the planet, has an infection rate of approximately 50% in all insect species tested which includes Africanized Honey Bees (Rech, et al. 2020), *Brachystola magna* (Rech, et al., 2022), a

lubber grasshopper and *Hyles lineata* (Rech, et al., 2023), a sphinx moth, and *Ades aegypti* (Kulkarni, et al., 2018) a mosquito. Other insect populations must be tested to ascertain the spread of *Wolbachia* in this region.

Research into facial markings on *Polistes* wasps is an emerging area, which needs to be expanded. Few researchers have delved into this topic. The wasps are ecologically important as both non-specific pollinators and insect control. Our research shows three major groupings, workers with Gamma facial markings, individuals captured in the nests' reproductive phase with Beta markings, and the two wasps with Alpha markings. The female with Alpha marking was probably a foundress or potential queen. More studies have to be done to unravel the mystery of the male with Alpha markings. However, only two wasps captured had Alpha markings which is too small of a sample to make conclusions.

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