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Implications of the Infestation Rates of *Wolbachia pipientis* and *Oxyspirura petrowi* in *Brachystola magna* as Related to the Percentage of Eyeworm Disease in Northern Bobwhite Quail (*Colinus virginianus*)

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Abstract: Southern New Mexico encompasses a large portion of the Chihuahuan Desert that is located in the United States. The desert hosts a variety of flora and fauna. Orthopterans are an integral part of the ecosystem, and can also be vectors for diseases. *Brachystola magna* Girard (Orthoptera: Romaleidae), commonly known as Plains Lubber Grasshopper are large, polyphagous insects that emerge after a two-year diapause. Besides being a food source for many species, they can also be infected with parasites such as *Wolbachia pipientis* and *Oxyspirura petrowi*. *Wolbachia pipientis* is commonly known as the "male-killing bacteria" and is changing the sex ratio of insect populations. *Oxyspirura petrowi* is a nematode that causes "eyeworm" disease in Northern Bobwhite Quails (*Colinus virginianus*) and is decimating the quail population in New Mexico, Oklahoma and Texas. Sixty-nine *B. magna*, 14 males and 55 females, were randomly captured between July and November of 2021 in the northern section of the Chihuahua Desert at the base of the Florida Mountains. Thirty-seven specimens were tested for *W. pipientis* Hertig (Rickettsiates: Ehrlichiaceae). Thirty percent tested positive for *W. pipientis*. Fifty-five were tested for *O. petrowi* Skrjabin (Spirurida: Thelaziidae). Forty-nine percent tested positive most of them being females.

Keywords: *Wolbachia pipientis, Oxyspirura petrowi, Brachystola magna, Colinus virginianus* Plains Lubbers Grasshoppers, Eyeworm Disease, Northern Bobwhite Quail

INTRODUCTION:

Brachystola magna, commonly known as the Plains Lubber Grasshopper, inhabits the plains region of the United States and Mexico. They populate several types of prairies, including desert prairies (Capinera, Scott & Walker, 2004). Being omnivores, they feed on herbaceous plants, grasses, feces, detritus, and insects including those of their own species (Wyoming Agricultural Experiment Station, 1982). *Oxyspirura petrowi*, a heteroxenous parasitic nematode, is decimating the populations of birds in the Order Galliformes especially *C. viginianus*. The quails' populations, which have an important economic impact on the central plains of Oklahoma, Texas, and New Mexico, have been declining at a rate of 4% annually for decades (Houston & Easton, 2021; Sauer, et al., 2013). The United States Department of Agriculture (USDA) identified *B. magna* as an intermediate host, and the Northern Bobwhite Quails (*C. virginianus*) as a definitive host of the *O. petrowi* parasite (Brust, et al., 2014; Dunham, et al., 2016). The Northern Bobwhite quails have been especially vulnerable to the eyeworm disease though other species in the Order Galliformes can be infected. The life cycle of the nematode is poorly understood; however, it is known that two hosts are needed, an intermediate host and a definitive host (Kistler, et al., 2016). Additionally, there is paucity of information on the grasshopper as well (Bright, Bernays, & Moran, 1994). In 2016, 174 *B. magna* were collected from the plains in Mitchell County, Texas. Thirty-seven percent of the specimens were infected with third stage *O. petrowi* larvae. *Oxyspirura petrowi* eggs were found in the feces of both the *B. magna* and *C. virginianus* (Blanchard, et al., 2019).

Brachystola magna, a large flightless grasshopper with a reduced pink tagmen spotted in black (Richman, Lightfoot, Sutherland, & Ferguson, 1993), emerges after a two-year diapause. The nymphal period lasts for approximately 45 days in the wild. The maturation period before oviposition is around 23 days (Wyoming Experiment Station, 1982). Being a hemi-metabolic insect, it goes through five instars before reaching adulthood. *Brachystola magna* emergences tend to have a higher population level then grasshoppers with a one-year diapause (Burleson, 1974). The grasshopper is bivariant, flightless (Joern, 1981) and cryptic in rocky, gravelly soil with wild grasses, which it prefers (Jacques, 1953). Female *B. magna* have been

documented jumping 35.6 centimeters in one hop, and males up to 2.7 meters. The females are strikingly larger than the males, some with a mass of 4.5 grams (Wyoming Agricultural Experiment Station, 1982).

Populations of these grasshoppers can be problematic. They have been observed in Oklahoma defoliating cotton and sunflower crops creating a negative economic impact in the region (Coppock, 1962). Internal temperature regulation is important for insects. *Brachystola magna* regulates its internal temperatures within a tolerable parameter by varying behaviors between posturing and selection of microhabitats. Selection of microhabitat was shown to be more important than posturing (Joern, 1981). The feeding habits of *B. magna* have been of particular interest, due to their population numbers upon emergence. Once thought to be oligophagous (Burleson, 1974), it is now known that *B. magna* feeds on a variety of plants, soil, detritus, feces, plus live and dead insects including those of their own species (Wyoming Agricultural Experiment Station Bulletin, 1982). Being the largest grasshopper on the plains, *B. magna* readily subjugates smaller species. Females feed more often than males because of their size difference (Bright, Bernays, & Moran, 1994). *Brachystola magna* can also have a positive economic impact. They are known for their palatability, probably due to its to its feeding habits. They contain 64.7% unsaturated fats, plus they are high in potassium, magnesium, calcium and are low in sodium (Monter-Miranda, et al., 2018). Also of positive economic importance, the chitosan films from *B. magna* have proven to be excellent sausage casing that resist deterioration over time (Tirado-Gallegos, et al., 2021).

Oxyspirura petrowi, a heteroxenous parasitic nematode, infects all organs of the visual and the olfactory systems of avian species (Dunham, et al., 2016). The nematode has been found in many wild avian species, but has particularly decimated the Northern Bobwhite populations of Texas, New Mexico and Oklahoma. The occurrence of *Oxyspirura petrowi* infection has been increasing in quail populations in recent years (Dunham, et al., 2016; Kalyanasundaram, et al., 2019).

Wolbachia pipientis is a member of a classification of bacteria that infects arthropods and nematodes plus cause parthenogenesis and cytoplasmic incompatibility. These microorganisms are abundant in insects and form a monophyletic group, the alpha division of Proteobacteria (Stouthamer, Breeuwer, Luck, & Werren, 1993). These bacterial endosymbionts are cytoplasmically inherited. Alpha Proteobacteria alters host chromosomal behavior by manipulating the early mitotic divisions of the egg and sperm. Cytoplasmic incompatibility bacteria interfere with the paternal chromosomes, and parthenogenesis bacteria prevent segregation of the chromosomes in the egg (Stouthamer, Breeuwer, Luck, & Werren, 1993). *Wolbachia pipientis* infestation uses these methods among others to manipulate the reproduction of arthropods causing cytoplasmic incompatibility, feminization, parthenogenesis, sterilization, and male killing which decreases the number of progeny and skews the male/ female ratios in arthropod populations (Fukui, et al., 2015; Rech, et al., 2020). *Wolbachia pipientis* is known to be a master manipulator of insect reproductive systems in order to enhance its own spread and survival (Weeks & Breeuwer, 2001). Insect hosts attempt to produce an even sex ratio, however, *Wolbachia's* manipulation of insect gametes leads to increased female progeny which results in uneven sex ratios (Stouthamer, 2001). In some species, this has changed mating behaviors. Usually, the male insects compete for female mates. But, in *Acraea encedon*, a Ugandan butterfly, there has been a reversal of mating behavior. Now, the females compete for the males (Jiggins, Hurst, & Majerus, 2000). Extreme ratio reductions, like that of the one cited, can lead to the extinction of a species or restrict habitats (Stouthamer, 2001). This study investigates the interaction between *Wolbachia pipientis*, *Brachystola magna* and *Oxyspirura petrowi*.

STUDY AREA:

Brachystola magna specimens were collected from a five-hectare area (~32.17°N, 107.63°W) at an elevation of ~14,000 meters from the bajada on the north side of the Florida Mountains near Deming, New Mexico. The Florida Mountains are an inactive fault-block range comprised of Paleozoic limestone and dolomite rocks that extend north-south for 38.6 kilometers (Clemons, 1998).

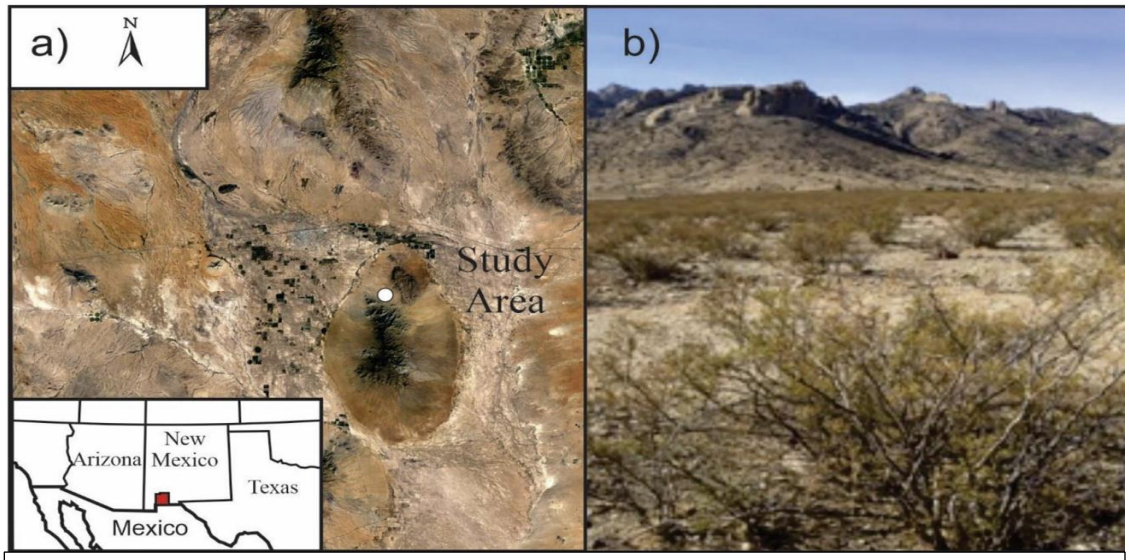

Figure 1. (a) Downloaded satellite image from Google Earth, (b) photograph of the study area.

The *B. magna* emergence began in July of 2021. The specimens were collected between July and November of that year. Many species of flora and fauna inhabit the area. Creosote bushes, *Larrea tridentate* (Coville) are a major component of the area, comprising 98% of the vegetation. They are an invasive species from Argentina arriving approximately 1000 years ago. The bushes are high in nordihydroguaiaretic acid which serves as a repellent for *B. magna.* Wild grasses, Joshua trees, *Yucca brevifolia* (Engelm), honey mesquite trees, *Prosopis glandulosa* (Fabaceae) and cacti in the genus *Opuntia* are interspersed between the creosote bushes (Dodson, 2012; Salisbury & Ross, 1992). The high temperatures for the region average from 35° C to 19.44° C between July and November. The lows are between 19.44° C and 2.22° C. The average rainfall per year is less than 25.4 centimeters (NOAA, 2021).

METHODS: *Oxyspirura petrowi* examination. *Brachystola magna* specimens were kept at 4° C after capture, and then thawed for dissection. Dissection proceeded under a dissecting microscope. Dissecting scissors were used to cut the specimens from the ventral posterior to the ventral anterior. The specimens were eviscerated, and the internal organs were examined for nematode larva. Larva were removed and counted. Finally, the procuticles were inspected for eggs. All results were recorded.

***Wolbachia* and Insect DNA extraction and PCR protocols**. Two millimeters (mm) were removed from the specimen's posterior 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 99°C water bath for 5 minutes. After heating, the tube was opened briefly to release pressure then centrifuged for 8 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 rpm's 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 8 minutes. The supernatant was carefully poured out and the mouth of tube was tapped lightly to remove most of the liquid. The pellet was air dried for 10 minutes. Two-hundred µL of TE/RNase was added. The pellet was disturbed by pipetting and 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; PuReTaq™ Ready-To-Go™ PCR beads were used. The DNA was thawed. Twenty microliters of primer was added to the PCR bead along with 5 µL of extracted DNA. Primer for 16S rDNA was used to identify *W. pipientis*, and primer for the Cytochrome C oxidase gene was used to identify insect DNA. 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. One point two percent agarose electrophoresis gels were run at

150V for 30 minutes. SYBR safe green loading dye was used with lithium bromide buffer. An EDVOTEK TruBlu2 DNA illuminator was used to view the DNA. *Wolbachia pipientis* DNA is identified at 438 kilo-basepairs (kbp) and insect DNA is identified at 708 kbp.

RESULTS: Sixty-nine *B. magna* were randomly captured during their emergence season, 14 males and 55 females. The sex ratio shows a skew of 3.9 females for each male as opposed to a normal 1:1 ratio, which indicated the presence of *W. pipientis*. Thirty-seven of the specimens were tested for *W. pipientis*. Thirty percent of *B. magna* tested were positive for *Wolbachia*. Of that sample, eighty-two percent of the *W. pipientis* infected specimens were female (table 1).

Table 1. W. pipientis & O. petrowi Infection Rates

Specimens	*W. pipientis* Infection	*W. pipientis* & *O. petrowi* Infection
Total Infected	11	7
Male	2	0
Female	9	7

Table 2. Number of Specimens Infected with Eggs and Larva

Specimens	Eggs	Larva
Total – 55	26	27
Male - 7	0	3
Female - 48	26	24

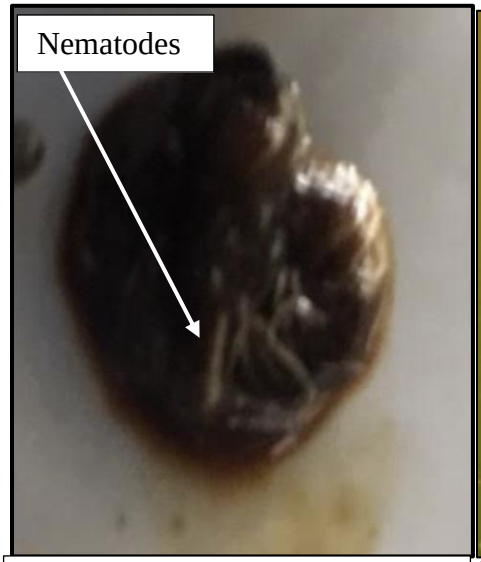

Figure 2. Entrails of *B. magna* infected with *O. petrowi* nematodes

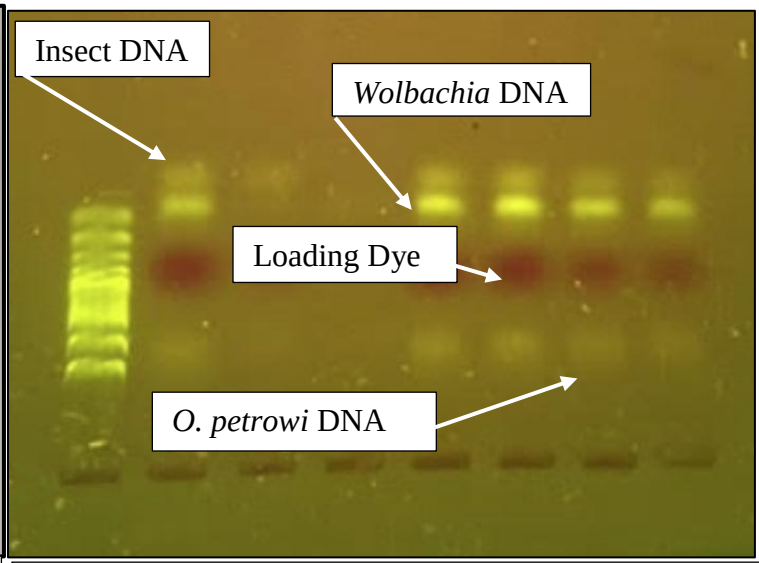

Figure 3. Electrophoresis gel showing infections for specimens 54, 35, 47, 63, and 70. To the left is the DNA 100 bp ladder

These specimens were also tested for *O. petrowi*; seven specimens tested positive for both parasites. *Oxyspirura petrowi* DNA is identified at 218 kbp. All of the specimens that tested positive for both parasites were females.

Fifty-five *B. magna* were tested for *O. petrowi*. Of those specimens, 7 males and 48 females were found to contain either larva, eggs or both (table 2). None of the male *B. magna* contained *O. petrowi* eggs. Three contained larva. However, those 3 were not infected with *W. pipientis*. Twenty-six out of 48 females, or 54% contained *O. petrowi* eggs, and 24 of the 48, or 50% contained larva. Therefore, 44% of the females tested contained both larva and eggs. All of the larva (figure 2) were found in the abdominal entrails (figure 2). The eggs were found attached to the peri-cutical of the *B. magna* specimens. The electrophoresis gel (figure 3) shows the double infection of specimens 54, 35, 47, 63, and 70. Fourteen of the *B. magna* were not tested due to an error in extracting the DNA.

DISCUSSION:

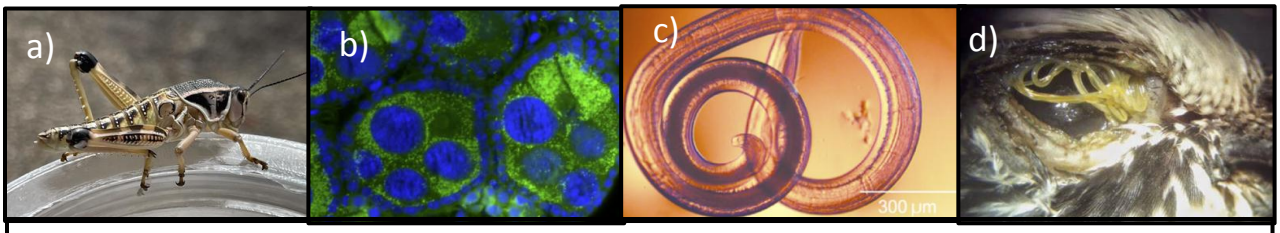

Figure 4. (a) *Brachystola magna*, (b) *Wolbachia pipientis*, (c*) Oxyspirura petrowi*, (d) *Colinus virginanus* infected with advanced eyeworm disease

Oxyspirura petrowi and *W. pipientis* are both endosymbiont parasites. *Oxyspirura petrowi* requires an intermediate and a definitive host, *W. pipientis* does not. The relationship between the two parasites, and their hosts is poorly understood. Our results imply a direct correlation between the rising *W. pipientis* infection rate, and the increase in *O. petrowi* infections that are decimating the Northern Bobwhite Quail (*C. virginianus*).

We propose that the cycle of infection between *B. magna* and *O. petrowi* is exasperated by the increasing infection rates of insects by *W. pipientis*. *Brachystola magna* become infected *W. pipientis* through vertical and horizontal transfer thus skewing the sex ratio of the insects. The grasshoppers are prone to infection via horizontal transfer due to their feeding habits. Females *B. magna* are larger than males and therefore consume an increased amount of biomass strengthening the probability that they will consume *O. petrowi* eggs through cannibalism and engulfing feces. Northern Bobwhite quail have a higher probability of consuming female *B. magna* simply because there are more females due to *W. pipientis* infection, plus the females are larger and more visible. *Colinus virginanus* than consume the grasshoppers and become infected with the nematode. The cycle is self-perpetuating. Much more research is needed to corroborate our findings. *Brachystola magna* will emerge again in 2023, allowing for another avenue of research. At that time, we are planning to capture a larger sample of specimens, test them for both *W. pipientis* and *O. petrowi*, and compare the results to our initial findings.

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