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CORRELATION BETWEEN ABDOMINAL VOIDS FOUND IN *MAGICICADA CASSINI* (HOMOPTERA: CICADIDAE) AND *MASSOSPORA CICADINA* INFECTION RATE

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Abstract: One hundred sixty-two *Magicicada cassini* (Fisher,1851),106 females and 56 males, were captured from the Southwestern area of Ohio in June of 2021. *Magicicada cassini* are periodical cicadas, and previously emerged seventeen years ago. The intent of this study was to test and examine all specimens for *Wolbachia pipientis* (Hertig, 1936) and *Massospora cicadina* (Peck, 1878) which was done. However, a phenomenon of abdominal voids was discovered in the process of testing. Thirty-six percent of the of the male cicadas had no tissue in their abdomens. Sixty-three percent of the males were missing between 81-100% of the tissue normally found in an insect abdomen. Logically, only the females were infected with *M. cicadina*, probably because of the lack of tissue in the male abdomens. Eighty-seven specimens were tested for *Wolbachia pipientis* and seventy-one specimens were tested *Massospora cicadina*. Identification of *W. pipientis* was based on the 16S gene of rDNA. The gene used for insect DNA identification was the cytochrome C oxidase gene. *Wolbachia* is a Rickettsial bacteria that is primarily transmitted vertically from mother to offspring. However, horizontal transmission does occur. Beings the *M. cassini* eggs were laid seventeen years ago, the infection rate should be consistent with the general insect infection rate from 2004. *Massospora cicadina* is a pathogenic fungus in the order Entomophthorales. *Massospora cicadina* only infects periodic cicadas, of which there are three species, *Magicicada cassini, Magicicada. septendecim, Magicicada. septendecula.*

Keywords: *Magicicada cassini, Wolbachia pipientis, Massospora cicadina*, Abdominal voids

INTRODUCTION

Periodical cicadas are only found in eastern North America, and have one of the longest life spans of any insect on Earth (Dybas & Lloyd, 1974). The three species of periodical cicadas alternate between 13- and 17-years life cycles. The cicadas were first described in scientific literature 357 years ago (Oldenburg, 1666). It is hypothesized in this postglacial period, the cicadas long subterrain nymphal stage protected them from harsh temperatures (Ito, et al., 2015). Also, the long life-cycle with the synchronized emergence allows them to escape the build-up of predators (Hoppensteadt & Keller, 1976). Some entomologists have given the 13-year and 17-year cicadas different names even though they are not distinguishable from each

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other with the exception of the time period (Dybas & Lloyd, 1974). However, most entomologists refer to only three species. All species emerge in the same time period and remain reproductively isolated even though their habitats may overlap at times (Dybas & Lloyd, 1974). *Magicicada cassini* inhabit floodplain woods. They are the only periodical cicada represented in the North American southwest, however, the highest concentrations of *M. cassini* previous emergence have been in Ohio. *Magicicada cassini* is easily identifiable by its solid black ventral side of the abdomen (Dybas & Lloyd, 1974). There have been a few specimens that have random small orange spots on the ventral side, but no orange patterned markings which are present in the other two species, *M. septendecium* and *M. septendecula* (Dybas & Lloyd, 1974).

Periodical cicada nymphs emerge from their subterrain habitat at the fifth instar, and enclose into adults (Williams & Simon, 1995). The adults are active from two to four weeks. Adults cloister into “chorus centers”. Males attract females by serenading them with sound producing tymbals (Karban, 1984). After mating, the females lay their eggs in the peripheral twigs of trees causing damage. The outer leaves of the trees die, and slits in the bark caused by oviposition allows pathogens in enter the plant. The eggs hatch in six to eight weeks, and the nymphs fall to the ground where they remain subterrain for 13 or 17 years. Nymphal death rates can be as high as 98% in the first two years (Williams & Simon, 1995). As the nymphs mature, they burrow deeper in the ground feeding on xylem far out of the predators’ paths (Williams & Simon, 1995). During the year before emergence, the physical characteristics, eye and body color, of the nymphs start to change. The mechanisms which determine whether the nymphs emerge at 13 or 17 years is poorly understood (Bryce & Aspinwall, 1975). Photoperiods, soil and air temperature all influence the synchronized emergence. Periodical cicadas live approximately 45 days after emergence (Speare, 1921).

The adults, and nymphal stages of the periodical cicadas feed on the xylem fluids, and have similar digestive systems. Hickernell (1923) found the digestive systems of both sexes to be complete and well organized though complicated by a twisting of the structures. The males do have an air chamber to amply their sounds (English, English, Dukes, & Smith, 2006). The digestive system does change as the insect ages, but does not degenerate (Hickernell, 1923). Cicada nymphs feed only on root xylem, which is unusual due to the lack of nutrients in the xylem. Xylem only contains dissolved minerals and water. This may account for the nymphs’ slow growth (Marlatt, 1898). The nymphs only grow five millimeters in the first four years of development. Periodical cicadas seldom or never feed during adult life. However, the cicada digestive tube has an enlargement that in other Homoptera serves as a food reserve. It is thought that cicadas also use this for a food reserve allowing them to survive adulthood without eating (Kershaw, 1913). Hargitt (1923) found the cicadas' digestive organs to be degenerate or atrophied (Hargitt, 1923) which agrees with Williams and Simon (1995) who described the digestive system as underdeveloped (Williams & Simon, 1995).

Massospora cicadina is a pathogenic fungus that infects periodic cicadas, and is the only predator or pathogen of the periodical cicadas (Speare, 1921). It is capable of "Stage I" and "Stage II" infections. "Stage I" infections produce haploid conidiospores which enter the insect's abdomen (Speare, 1921) and induce wing-flick signaling behavior in males. This behavior is normally seen in females receptive to copulation. However, as the conspecific males are attracted to the wing-flicking males and attempt copulation the infectious conidiospores are spread by contact (Cooley, Marshall, & Hill, 2018). The fungus changes the host behavior to its own advantage. Based on Poulin's definition (Poulin, 1995) for an adaptive parasite to be an "extended phenotype", Cooley, Marshall, and Hill (2018) proposed the *Massospora* fungus changes the behavior of the cicadas to its own advantage and qualifies as an "extended phenotype". The adjective "zombie fungus" has also been used (Nuwer, 2021). *Massospora cicadina* changes the behavior of the periodical cicadas by producing amphetamines and cathinones (Boyce, et. al., 2019; Nuwer, 2021). Conidiospores later induce "Stage II" infections resulting in diploid resting spores, but do not exhibit wing-flicking behavior. Resting spores infect the next generation of cicadas as they emerge after the fifth instar. When the nymphs emerge, they come in contact with resting *M. cicadina* spores, and the fungal life cycle continues (Cooley, Marshall, & Hill, 2018). The nymphs go through their final molt, mate as adults, and die. Periodical cicadas live approximately 45 days after emergence (Speare, 1921).

Wolbachia pipientis is an endosymbiotic rickettsial alpha-proteobacteria found in the gonads of arthropods and filarial nematodes. It is known as a reproductive manipulator and causes cytoplasmic incompatibility, parthenogenesis, male-killing, and feminization in congenital males (Werren, 1997). Researchers have found that *Wolbachia* induced cytoplasmic incompatibility is associated with the maternal transmission of intracellular bacteria (Dobson, 2002) as well as the manipulation of host population by mechanisms such as population replacement using *W. pipientis* as a conduct for the spread of desirable genes in host populations that can potentially lead to a genetic drift in insects (Shropshire, 2020). *Wolbachia* can be transferred both vertically between mother and offspring, and horizontally between predator and prey. In the past *W. pipientis* has been considered a parasite in arthropods, but recently it has been shown to be mutualistic in some insects. Several insect populations have shown resistance to both RNA viral infections and some insecticides when they have high concentration of *Wolbachia* (Cogni, Ding, Pimentel, Day & Jiggins, 2021). Genome manipulation by *Wolbachia* favors the production of females resulting in vertical transmission (Werren, 1997). To this end, male-killing leads to parthenogenesis. One population of moths, *Ostrinia furnacalis*, has been completely feminized by *Wolbachia* (Katsuma, et al., 2022). There is also a positive correlation between high concentrations of mitochondria and *Wolbachia* (Henry & Newton, 2018). Past studies (Stouthamer, et al., 1999) concluded that *W. pipientis* could not survive in a cell-free environment. However, a recent study (Rasgon et al., 2006) successful horizontal transfer depended on a long-term intracellular environment for *W. pipientis*. *Wolbachia pipientis* has been an important factor in the evolution of arthropods and nematodes due to its manipulation of the host's genome (Stouthhamer, et al.,1999). As the infection rate of *Wolbachia* increases, it will continue to have an impact on arthropod and nematode evolution.

METHODS:

Capture Methods: Areas, where a high number of periodical cicadas had been reported, were visited. The cicadas were easy to capture. The individuals were picked up by the wings to reduce possible damage. The cicadas were placed in individual 50 mL centrifuge tubes. The tubes were then labeled with date and location. The specimens were frozen until testing for *Wolbachia pipientis* and *Massospora cicadina.*

Identification: *Magicicada cassini* are slightly smaller than the other two periodical species, and the ventral side of their abdomen is black, with the exception of an occasional yellow/ orange mark. The gender is also easy to identify. Male cicadas have tymbals, sound producing structures, under their wings and females do not.

Massospora cicadina Cultures: *Magicicada cassini* specimens' exoskeletons were swabbed with a wet Q-tip then streaked on Lysogeny Broth (LB) agar and incubated for four days at 30 degrees Celsius. Then, the specimens were severed between the thorax and the abdomen and examined for both abdominal voids and fungal tissue.

Abdominal Tissue Measure

The internal structures of fifty-two specimens were examined. First, the length of the abdomen was recorded. Then using a dissecting probe, the amount of void in the abdomen was measured. The two measurements were used to conclude a percentage of the abdomen that was empty.

DNA Extraction Methods: 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:

STUDY AREA

Table 1. Exact Locations of Capture Sites

Site Number	Site Name	Latitude	Longitude	State
1	N. KY Airport	39^{o}06'56.71"N	84^{o}47'10.90"W	Kentucky
2	Southern Oxford	39^{o}30'03.94"N	84^{o}44'40.80"W	Ohio
3	Brookville Lake	39^{o}30'57.66"N	84^{o}00'22.16"W	Indiana

4	Hueston Woods	39°34’19.59”N	84°45’05.85”W	Ohio
5	Chestnut Fields	39°29’50.80”N	84°44’42.02”W	Ohio
6	Sycamore Bluffs	39°28’58.67”N	84°41’07.68”W	Ohio
7	Milford Cemetery	39°29’37.97”N	84°40’32.14”W	Ohio
8	Valley Thrift Shop	39°21’04.71”N	84°33’16.51”W	Ohio
9	Jungle Jim’s	39°20’03.07”N	84°30’43.96”W	Ohio

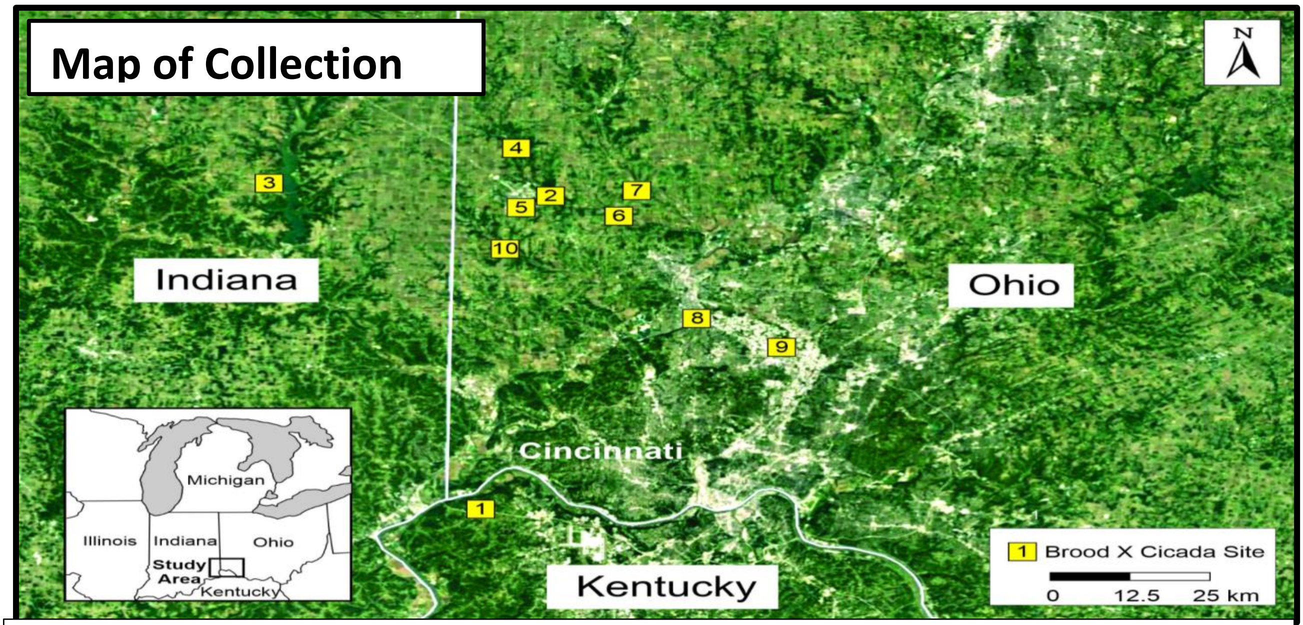

Figure 1. Map of the *Magicicada cassini* collection sites

***Massospora cicadina* Results:**

Table 2. *Massospora cicadina* Infection Rates

Female Specimens Tested	46
Infected Females	17
Non-Infected Females	29
Percentage Infected	37%
Male Specimens Tested	25
Infected Males	0
Non-Infected Males	25
Percentage Infected	0%

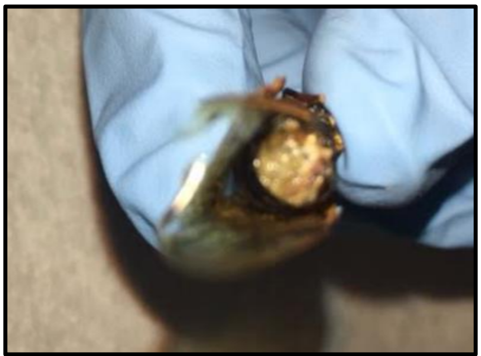

Figure 2. *M. cicadina* fungus growing in the abdomen of female specimen 5-11

Abdominal Void Results:

Sixty-seven *Magicicada cassini*, 32 females and 35 males, were examined for their internal abdominal structures, and the percentage of abdominal void area was determined.

Table 3. Percentage of Abdominal Void Area

Percentage of Abdominal Void	Number of Male Specimens	Percentage of Males Affected	Number of Female Specimens	Percentage of Females Affected	Total Percentage Affected
0%	0	0%	2	6.3%	2.9%
1-20%	0	0%	10	31.3%	4.8%
21-40%	2	5.7%	12	37.5%	20.8%
41-60%	4	11.4%	4	12.5%	11.9%
61-80%	7	10.4%	4	12.5%	16.4%
81-100%	22	62.9%	0	0%	62.9%

Figure 3. Normal Male Abdomen

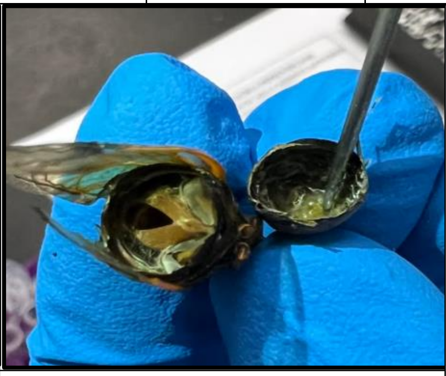

Figure 4. Abdominal Void

Left (Figure 3) is a normal male abdomen and to the right is (Figure 4) is an empty male abdomen. Males had more abdominal voids than the females and no fungus.

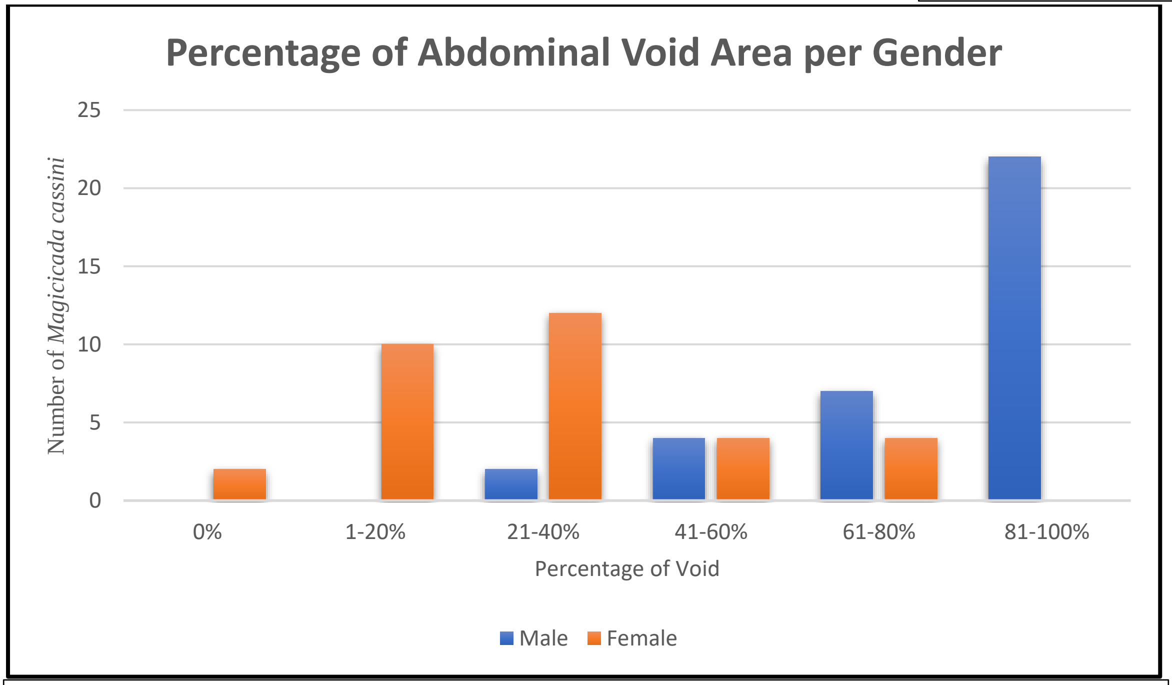

Figure 5. Chart indicating the Contrast between Male and Female Abdominal Voids

Table 4. *Wolbachia pipientis* Infection Rates

Specimens	Number
Male	15
Infected	3
Not Infected	12
Percentage Infected	20%
Females	72
Infected	44
Not Infected	28
Percentage Infected	61%
Total Specimens	87
Infected	47
Percentage Infected	54%

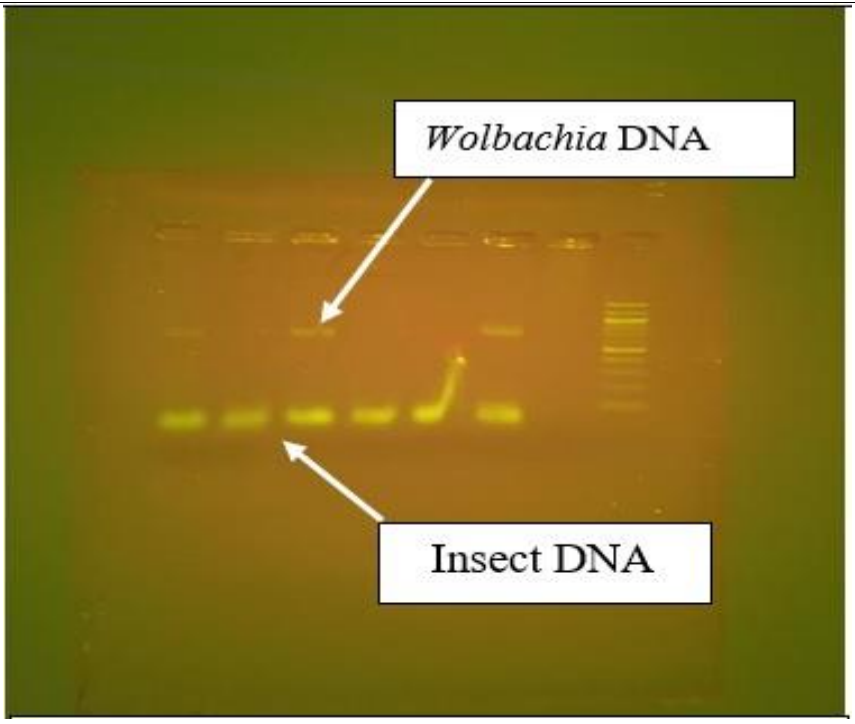

Figure 6. Electrophoresis gel showing *Wolbachia* DNA and Insect DNA

DISCUSSION

One hundred sixty-two *Magicicada cassini* were captured in the Southern Ohio region (table 1, figure 1) at 10 different sites. The cicadas were tested for *Wolbachia pipientis* (table 4) and *Massospora cicadina* (table 2). Even considering that the male cicadas have an air chamber to amplify sound, there was a considerable lack of abdominal tissue in the abdomens which we are calling “abdominal voids” (table 3, figure 4, figure 5). A normal male abdomen is pictured in figure 3. There is a deficit of research on periodical cicadas, probably because they only emerge every thirteen or seventeen years. Since the late 1800’s entomologists have discussed the developmental level of the cicada’s digestive system, which has been described as well-developed, under-developed, atrophied, and degenerate. Our results align with the digestive system being under-dev developed. Many of the specimens, especially th al voids were measured using a probe and millimeter measuring stick. Thirty-six percent of the male specimens had absolutely no tissue in their abdomens. For this reason, it was impossible to test them for *W. pipientis*. The measurements of the voids were divided into six categories, each being approximately 20% of the length of the abdomen (table 3, figure 4, figure 5). Eighty-two point two of the males had voids that measured between 61% to 100% of their abdomens. The abdomens of the females were different. Only 12.5% of the female had abdominal voids in the

61% to 80%, and there were no female abdomens that were totally empty. Generally (figure 5) the female abdominal voids were lower percentage of the abdomens, while the male abdominal voids tended to be in the higher percentage of the abdomens. Our results show a correlation between abdominal voids and *Massospora cicadina* infections. None of the male specimens were infected with *Massospora cicadina* probably because of the lack of abdominal tissue. The fungus uses the abdominal tissue for nutrition; therefore, a lower amount of tissue is not favorable for the fungal growth. Thirty-seven percent of the female specimens were infected with the *M. cicadina* fungus (table 2) and all of these had little or no abdominal voids.

Originally, we had intended to test all of the *M. cassini* specimens for *Wolbachia pipientis*. However, due to the lack of tissue in the males, only 15 males were tested for *Wolbachia* and 3 were infected with *Wolbachia*. The sex ratio of the captured *M. cassini* indicated a *Wolbachia* infection in the population. *Wolbachia* manipulates the host DNA changing males into females, or killing the male embryos. There were 4.8 females for every male captured giving a 4.8:1 ratio. A normal gender ration for an insect population would be close to 1:1. Sixty-one percent of the females were infected with *Wolbachia*, and 20% of the males (table 4). Overall, there was a 54% infection rate. The electrophoresis gel (figure 6) showing an example of *Wolbachia pipientis* DNA and insect DNA.

In conclusion, more research is needed on periodical cicadas, but due to their emergence only one every 13 or 17 years that is unlikely. We found the male cicadas to have atrophied or non-existent digestive structures, while this phenomenon did not affect the females extensively. No males were infected with *M. cicadina* probably due to the lack of abdominal tissue. The sex ratio distortion indicated a *Wolbachia* infection which we found to be true.

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