

## PROBOSCIS VARIATION AND *WOLBACHIA PIPIENTIS* INFECTION RATE OF *HYLES LINEATA* IN THE NORTHERN CHIHUAHUAN DESERT

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**Abstract:** *Hyles lineata* (Fabricius, 1775) is a common moth and native pollinator whose habitat is dispersed throughout Central and North America. The local habitat, the Northern region of the Chihuahuan Desert, experienced an explosive, synchronous emergence of the moth during the first two weeks of July, 2022. One-hundred nine *Hyles lineata* moths were captured in a five-hectare area at the base of the Florida Mountains. The moths are important for several reasons. First, they are edible biomass for many species. Secondly, *Hyles lineata* with short proboscis can be effective native pollinators while those with long proboscis are less effective. Interestingly, eight of moths had forked proboscis. Ninety specimens were tested for *Wolbachia pipientis* (Hertig, 1936). *Wolbachia* is an endosymbiont that manipulates the DNA of arthropods and filarial nematodes. *Wolbachia* DNA was identified by the 16S rDNA gene of the bacterial ribosome. Insect DNA was identified by the cytochrome C oxidase gene.

**Keywords:** *Hyles lineata*, White-lined Sphinx moths, *Wolbachia pipientis*, Proboscis length.

### INTRODUCTION

Thirty-five percent of the world's crops rely on animal pollinators (Klein, et al., 2007). Traditionally, farmers have depended on domesticated honeybees, *Apis mellifera*, for pollination. However, 40% of domestic honeybee colonies in the United States are lost each year to Colony Collapse Disorder (CCD) (Evison, et al., 2012). Domesticated honeybees are not native to North America, and have been constantly inbred reducing their resistance to diseases. Thus, the importance of native pollinators cannot be overstated (Rech, et al, 2020). Studies have shown that an increased number of pollinators increase the production of the crop (Hoehn, Tschardtke, Tylianakis, & Steffan-Dewenter, 2008). Lepidoptera are native pollinators and important to ecosystems. There are approximately 11,500 species in North America. Lepidopterans also have considerable economic impact. Besides being native pollinators, most of the larvae are phytophagous and are a huge pest of cultivated crops. However, they also add a lot of biomass to an ecosystem (Triplehorn & Johnson, 2005). *Hyles lineata*, the white-lined sphinx moth (National Center for Biological Information, 2022) is a common moth in the family Sphingidae. Its geographic range extends from Central America to Canada (Miller,

1981). *Hyles lineata*, are acclimated to their regional climates and, for the most part, resistant to diseases due to out breeding. Being polyphagous, they pollinate a huge diversity of plants, usually at night. *Hyles lineata* are moderately large moths, many having a wing span at, or above 160 mm (Johnson, et al., 2017; Triplehorn & Johnson, 2005). Proboscis length is one variation of the species. Short proboscis *H. lineata* are efficient pollinators due to the density of their setae. The short length of their proboscis requires them to bury themselves in the flower to acquire nectar (Miller, 1997). Moths with a long proboscis are less efficient pollinators. The immature stages of *H. lineata* add a significant amount of biomass to an area supplying ample food for many organisms, especially during explosive outbreaks (Miller, 1981). However, they are voracious herbivores and can decimate crops. During an explosive, synchronous emergence, large tracks of crops can be lost. The pupa over-winter in the ground, and emerge in the spring (Triplehorn & Johnson, 2005). Lepidoptera serves as host for *Wolbachia*. Also, there is evidence of horizontal transfer in this insect order (Ahmed, Brinholt & Kawahara, 2016).

*Wolbachia pipientis* is known as a master manipulator of insect reproductive systems in order to enhance its own spread and survival (Werren, 1997; Weeks & Breeuwer, 2001). It is a Gram-negative alpha-proteobacterium endosymbiont that causes cytoplasmic incompatibility, parthenogenesis, male-killing, and feminization (Werren, 1997). Collectively, these methods of manipulation are known as reproductive parasitism (Perimutter, et al., 2019). These strategies benefit *Wolbachia* in several ways; (1) reducing male and female offspring competition for limited resources, (2) reducing inbreeding, and (3) providing nutrients in cases where infected females cannibalize embryos of the dead males (Perimutter, et al., 2019). However, there are also disadvantages to male-killing; (1) it decreases the genome of the species facilitating less variation in the population which affects a species ability to adapt to a changing environment, and (2) it can cause a shift in male-attracting behaviors (Jiggins, Hurst, & Majerus, 2000). Obviously, *Wolbachia* impacts the evolution of insects (Landmann, 2019). Previously, it was only described as parasitic in insects, but more recently it has been shown to be mutualistic in some insect species (Pimentel, Cesar, Martins, & Cogni, 2021). Hedges, Brownlie., O'Neill and Johnson (2008) determined that two strains of *Wolbachia*, wMelCS and wMelPop, have an antiviral effect in *Drosophila* making them resistant to some RNA viruses (Hedges, Brownlie, O'Neill, & Johnson, 2008). *Wolbachia* is wide spread in the phylum Arthropoda, and infect up to 66% of insect species (Landmann, 2019). This percentage differs slightly per research paper. *Wolbachia* strains are divided into supergroups A through U, but only supergroups A and B

infect insects (Tiago, et al., 2022). *Wolbachia* is typically transferred vertically from mother to offspring, but horizontal transfer from predator to prey does happen (Ahmed, Brinholt & Kawahara, 2016). Actually, horizontal transfer is much more common than previously thought (LePage, et al., 2017). *Wolbachia* infections are also associated with increased mtDNA polymorphism in some moths, suggesting that *Wolbachia* facilitates horizontal transfer in mitochondrial DNA in some species (Zhu, et al., 2023). Several species of bacterial symbionts, including *Wolbachia*, induce male-killing. In *Wolbachia*, male-killing is induced by cytoplasmic incompatibility (CI). Multiple models for CI exist and are currently being researched. Basically, infected males' sperm are lethal to the embryos unless the embryos are rescued by the same strain of *Wolbachia* (Namias, Sicard, Weill, & Charlat, 2022). CI is complex, and differs with both the insect species and the *Wolbachia* strain. Currently, research on CI is ongoing (He, et al., 2019; Namias, Sicard, Weill, & Charlat, 2022). Basically, with CI, there is a disruption of the male pronucleus which mutates the paternal chromosomes. If the oogonia is infected with the same strain of *Wolbachia*, it can repair the damage to the paternal DNA to facilitate fertilization and propagation (Reed & Werren, 1995). Entire populations of the Asian Corn Borer moth, *Ostrinia furnacalis*, have been feminized by *Wolbachia* (Katsuma, et al., 2022). Parthenogenesis is procreation without the direct involvement of a male (Singh, Mal, Gautam & Mukesh, 2019). Parthenogenesis causing *Wolbachia*, facilitates females in some haplodiploid species to produce females from both fertilized and unfertilized eggs, further skewing the sex-ratio of the insect population (Stouthamer, Russell, Vavre & Nunney, 2010).

### **CAPTURE SITE**

The specimens were collected from a 5-hectare area (~32.17°N, 107.63°W) at an elevation of ~1400m from the bajada of the north side of the Floridian Mountains in the Northern Chihuahuan Desert (Rech, 2021). The mountains are an inactive fault-block range comprised of Paleozoic limestone and dolomite rocks (Clemons, 1998). The area receives ~23.37 cm of precipitation each year. The high temperature is 35.5°C, and the low is ~18.3°C. The floras include creosote bushes *Larrea tridentate*, Joshua trees, *Yucca brevifolia*, mesquite trees, *Prosopis glandulosa*, and several species of the genus *Opuntia*, prickly pear cacti (Salisbury & Ross, 1992).

### **METHODS**

Specimen Capture: One-hundred nine moths were captured using insect nets, then transferred to 50 mL centrifuge tubes. The tubes were identified with the date and location. The specimens were stored at 15.5°C until DNA extraction.

Specimen Identification: The species of the moths was determined using the taxonomic key of the National Center for Biological Information (NCBI, 2022). The gender was determined by measuring the width of the abdomen. Male abdomens are approximately 20% thinner than females. Also, the females are slightly larger.

DNA extraction and PCR protocols: Two millimeters (mm) were removed from the posterior of specimen's abdomen. The abdominal segment was then placed in a 1.5 milliliters (mL) microfuge tube with 200 microliters ( $\mu\text{L}$ ) of lysis buffer. The abdominal segment was macerated for 1 minute. Eight-hundred  $\mu\text{L}$  of lysis buffer was added to the microfuge tube then vortexed. The tube was placed in a  $99^{\circ}\text{C}$  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  $\mu\text{L}$  of the supernatant and put into the new tube. Forty  $\mu\text{L}$  of 5.0 M NaCl was added and placed on ice for 5 minutes. Tubes were placed in the centrifuge at the same speed and time as previously stated. Another clean microfuge tube was obtained and 300 $\mu\text{L}$  of supernatant was transferred. Four-hundred microliters of  $6.5^{\circ}\text{C}$  isopropanol was added and then centrifuged at 10,000 rpm 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  $\mu\text{L}$  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 Biorad thermocycler t100. PuReTaq™ Ready-To-Go™ PCR beads were used. The DNA was thawed. Ten microliters of primer was added to the PCR bead along with 10  $\mu\text{L}$  of extracted 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. Two percent agarose electrophoresis gels were run at 150 V for 30 minutes. Five  $\mu\text{L}$  of SYBR safe green was added to each gel. Lithium bromide buffer was used. An Edvotek TruBlu 2 Transilluminator was used to view the DNA.

Proboscis Measurement: Each proboscis was gently straightened with a pin. The moth and the distal end of the proboscis were adhered to the paper with a fast-drying adhesive. Specimens were allowed to dry overnight, and then measured with a SI unit ruler.

## RESULTS

Ninety *Hyles lineata* moths were tested for *Wolbachia pipientis*, and 110 were measured for proboscis length. *Wolbachia* infection rate showed 30 female specimens tested positive, while only 17 male specimens tested positive. One hundred ten moths were measured for proboscis

length. Forty-nine of the moths were male and 61 were female. Fifteen of the specimens had short proboscides, 59 had medium proboscides, and 28 had long proboscides. Three male and five females had forked proboscides. There seemed to be no correlation between gender and proboscis length.

**Table 1.** Proboscis Length

| Specimens        | Male | Female | Total | Percentages |
|------------------|------|--------|-------|-------------|
| Short 27-30 mm   | 7    | 8      | 15    | 15%         |
| Medium 31-36 mm  | 31   | 28     | 59    | 58%         |
| Long 37-42 mm    | 8    | 20     | 28    | 27%         |
| Total            | 46   | 56     | 102   | 100%        |
| Forked Proboscis | 3    | 5      | 8     | 7.8%        |

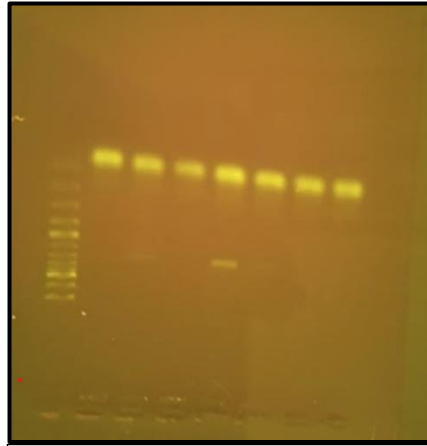


**Figure 1.** (L to R) Specimen 72 with a short proboscis, specimen 58 with a long proboscis, and specimen 100 with a forked proboscis.

**Table 2.** *Wolbachia pipientis* infection rate

| Specimens | Number Tested | Number Infected | Percentage of Infection Rate |
|-----------|---------------|-----------------|------------------------------|
| Total     | 90            | 47              | 52%                          |
| Male      | 44            | 17              | 39%                          |
| Female    | 46            | 30              | 65%                          |

*Wolbachia pipientis* 16S rDNA bacteria ribosomal gene is identified at 438 base pairs (bp) and insect cytochrome C oxidase DNA is identified at 709 bp. The banding in the electrophoresis gel to the left shows specimen 108, a female *H. lineata* is infected with *W. pipientis*. Sixty-five percents of the females were infected, while only 39% of the males were infected.



**Figure 2.** Specimen 108, lane 5, is infected with *W. pipientis*

## DISCUSSION

*Hyles lineata*, the white-lined sphinx, is a common pollinator in the Northern Chihuahuan Desert. In the first two weeks of July, there was an explosive, synchronous emergence of the moth. One-hundred nine moths were captured in a 5-hectare area at the northern base of the Florida Mountain to the East of Deming, New Mexico. All of the moths were measured for proboscis length, and ninety of the moths were tested for *Wolbachia pipientis* infection. The sex ratio of the specimens was 1:1.22 which is almost 1:1. A normal sex ratio in insect population is normally close to 1:1 (Compton & Tu, 2022). This did not indicate a high *W. pipientis* infection rate, but the infection rate was 52%, a lot higher than we anticipated. The females had a much higher infection rate than the males, 65%. The males had an infection rate of 39%. Our findings align with other researchers for the percentage of *Wolbachia* infection. Of course, the infection rates vary from species to species, but generally the average infection rate of insect populations has been approximately 50%. Presently, *Wolbachia* is the fastest spreading endosymbiont on the planet.

Proboscis length is an important factor in pollination. North American *H. lineata* stigma tube lengths range from 0 to 175 mm, whereas the average proboscis length of sphinx moths in North America is between 53 to 60 mm (Grant, 1983). However, comparing our proboscis lengths to Grant's measurements, all of our lengths are much shorter. But, when our lengths are compared to more recent findings (Ahmed, Araugo-Jnr, Welch & Kawahara, 2015) our measurement align with current research. Possibly there has been a change in proboscis lengths of *H. lineata* in the last 40 years. Shorter proboscides facilitate pollination because the moth has to bury itself in the flower to obtain the nectar. The numerous setae on the moth's body

pick up the pollen which is easily transferred when the insect moves to another flower. The opposite is true of longer proboscides.

Eight of the specimens had forked proboscis. One possible explanation for this is desiccation. Perhaps, the structure dried extensively while being frozen. The proboscis consists of two extended concave maxillary galeae and a hollow food tube, which are joined by dorsal and ventral cuticular projections (Zhang, Niu, Hu, & Lu, 2021). In the event of desiccation, the structure might have separated. The freezing process may have facilitated the separation.

Our findings are a data point into the ever-evolving infection rates of *Wolbachia pipientis* and the changing proboscis length of *H. lineata*. The white-lined sphinx moth is an important native pollinator of the Northern Chihuahuan Desert in New Mexico, which is an agricultural state. Presently, farmers rely on *Apis mellifera*, the domesticated honeybee which is not native to Northern America. But the efficiency of the species is limited. It is prone to viral infections mostly due to the vector mite, *Varroa destructor* (Rech, et al., 2020). Native pollinators will and should be promoted to sustain the viability of agricultural.

## References

- [1] Ahmed, M., Araujo-Jnr, E., Welch, J., & Kawahara, A. (2015). *Wolbachia* in butterflies and moths: geographic structure in infection frequency. *Frontiers in Zoology*, 12(16), 1-9.
- [2] Ahmed, M., Breinholt, J., & Kawahara, A. (2016). Evidence for common horizontal transmission of *Wolbachia* among butterflies and moths. *BMC Evolutionary Biology*, 16(118), 1-16.
- [3] Clemons, R. (1998). *Geology of the Florida Mountains southwestern New Mexico*. Socorro: New Mexico Institute of Mining and Technology.
- [4] Compton, A., & Tu, Z. (2022). Natural and Engineered Sex Ratio Distortion in Insects. *Frontiers in Ecology and Evolution*, 10, 1-9.
- [5] Evison, S., Roberts, K., Laurenson, L., Pietravalle, S., Jui, J., Biesmeijer, J., & Hughes, W. (2012). Pervasiveness of parasites in pollinators. *PloS One*, 7(1), 1-7.
- [6] Grant, V. (1983). The systematic and geographical distribution of hawkmoth flowers in the temperate North American flora. *Botanical Gazette*, 144, 439-449.
- [7] He, A., Zheng, O., Yu, W., Fang, Y., Mao, B., & Wang, Y. (2019). How does *Wolbachia* modify the *Drosophila* ovary? New evidences support the "titration-restitution" model for the mechanisms of *Wolbachia* induced CI. *BMC Genomics*, 20, 1-14.
- [8] Hedges, L., Brownlie, J., O'Neill, S., & Johnson, K. (2008). *Wolbachia* and Virus Protection in Insects. *Science*, 322, 702.

- [9] Hoehn, H., Tschardtke, T., Tylianakis, J., & Steffan-Dewenter, I. (2008). Functional group diversity of bee pollinators increases crop yield. *Proceeding of the Royal Society Biology*, 275, 2283-2291.
- [10] Jiggins, F., Hurst, G., & Majerus, M. (2000). Sex-ratio-distorting *Wolbachia* causes sex-role reversal in its butterfly host. *Proceedings of the Royal Society of London*, 267, 69-73.
- [11] Johnson, S., More, M., Amorim, F., Haber, W., Frankie, G., D., S. & Raguso, R. (2017). The long and short of it: a global analysis of hawkmoth pollination niches and interaction networks. *Functional Ecology*, 31, 101-115.
- [12] Katsuma, S., Hirota, K., Matsuda-Imai, N., Fukui, T., Muro, T., Nishino, K., & Kiuchi, T. (2022). A *Wolbachia* factor for male killing in lepidopteran insects. *Nature Communications*, 13(1).
- [13] Klein, A., Vaissiere, B., Cane, J., Dewenter, I., Cunningham, S., Kremen, C., & Tschardtke, T. (2007). Importance of pollinators in changing landscapes for world crops. *Proceedings of the Royal Society*, 274, 303-313.
- [14] Landman, F. (2019). The *Wolbachia* Endosymbiont. *American Society of Microbiology*, 1-15.
- [15] LePage, D., Metcalf, J., Bordenstein, S., On, J., Perimutter, J., Shropshire, D. & Bordenstein, S. (2017). Prophage WO genes recapitulate and enhance *Wolbachia* induced cytoplasmic incompatibility. *Nature*, 543, 243-247.
- [16] Miller, R. (1981). Hawkmoths and the geographic patterns of floral variation in *Aquilegia caerulea*. *Evolution*, 35(4), 763-774.
- [17] Miller, W. (1997). Diversity and Evolution of tongue length in hawkmoths (sphingidae). *Journal of the Lepidopterists' Society*, 51(1), 9-31.
- [18] Namias, A., Sicard, M., Weill, M., & Charlat, S. (2022). From *Wolbachia* genomics to phenotype: molecular models cytoplasmic incompatibility must account for the multiplicity of compatibility types. *Current Opinion in Insect Science*, 49, 78-84.
- [19] National Center for Biological Information. (2022, March 1). *Insect identification*. Retrieved from National Center for Biological Information: <https://www.ncbi.nlm.nih.gov/search/all/?term=insect%20identification>
- [20] Perimutter, J., Bordenstein, S., Unckless, R., LePage, D., Metcalf, J., Hill, T., & Bordenstein, S. (2019). The phage gene wmk is a candidate for male killing by a bacterial endosymbiont. *Plos Pathogens*, 1-29.



- [21] Pimentel, A., Cesar, C., Martins, M. & Cogni, R. (2021). The antiviral effects of the symbiont bacteria *Wolbachia* in insects. *Frontiers in Immunology*, *11*, 1-5.
- [22] Rech, J. (2021, March 15). Geology Professor Miami University. (N. Rech, Interviewer)
- [23] Rech, N., Darrow, A., Lopez, A., Mendoza, D., Nicoll, V., & Paulk, L. (2020). Prevalence of *Wolbachia pipientis* and *Varroa destructor* mites in Africanized Bees in the Deming, New Mexico Area. *International Journal of Science, Environment and Technology*, *9*(2), 274-284.
- [24] Reed, K., & Werren, J. (1995). Induction of paternal genome loss by the paternal-sex-ratio chromosome and cytoplasmic incompatibility bacteria (*Wolbachia*): a comparative study of early embryonic events. *Molecular Reproduction & Development*, *40*, 408-418.
- [25] Salisbury, F. R. (1992). *Plant Physiology, Fourth Edition*. Belmont: Wadsworth Publishing Company.
- [26] Singh, B., Mal, G., Gautam, S., & Mukesh, M. (2019). Parthenogenesis-a potential tool to reproductive biotechnology. *Advances in Animal Biotechnology*, 239-248.
- [27] Stouthamer, R., Russell, J., Vavre, F., & Nunney, L. (2010). Intragenomic conflict in populations infected by parthenogenesis inducing *Wolbachia* ends with irreversible loss of sexual reproduction. *BMC Evolutionary Biology*, *10*, 1-12.
- [28] Tiago, M., Gomes, F., Gabriel, L., Wallau, L., Elgion, L., & Loreto, S. (2022). Multiple long-range host shifts of major *Wolbachia* supergroups infecting arthropods. *Scientific Reports. Nature*, *12*(8131), 1-8.
- [29] Triplehorn, C., & Johnson, N. (2005). *Borror and DeLong's Introduction to the Study of Insects, 7th Edition*. Belmont: Brooks / Cole Cengage Learning.
- [30] Weeks, A., & Breeuwer, J. (2001). *Wolbachia*-induced parthenogenesis in a genus of phytophagous mites. *The Royal Society*, *268*(1482), 2245-2251.
- [31] Werren, J. (1997). Biology of *Wolbachia*. *Annual Review of Entomology*, *42*, 587-609.
- [32] Zhang, C., Niu, Y., Hu, G., & Lu, J. (2021). Ultramorphological comparison of proboscis and associated sensilla of sensilla of *Scotogramma trifolii* and *Protoscutosa* (Lepidoptera: Noctuidae). *Insects*, *992*, 1-15.
- [33] Zhu, X., Liu, T., He, A., Zhang, L., Li, J., Li, T., & Yu, S. (2023). Diversity of *Wolbachia* infection and its influence on mitochondrial DNA variation in the diamondback moth, *Plutella xylostella*. *Molecular Phylogenetics and Evolution*, *182*, 1-7.