Spring 2023

Wolbachia Infection in Gall Associated Insect Communities in Illinois and Indiana

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Abstract

Wolbachia is a maternally inherited, gram-negative bacterium and has been found to be associated with up to 60% of all insects, frequently resulting in male-killing, feminization, and cytoplasmic incompatibility in the hosts. Gall wasps and other gall-forming insects induce on various host plants abnormal tissue growths, or plant galls, with distinct morphology. They are considered as obligatory parasites to their host plant. The presence of *Wolbachia* infection in gall wasp populations in North America has not been extensively documented. Given the strong influence on host reproduction, not knowing *Wolbachia* infection in insect populations prevents the understanding of how populations are being affected in their dynamics and evolution. In this study, I collected from nine locations, within Illinois and Indiana, galls made by several gall wasp and other gall-making insect species. DNA extracted from the gall makers was amplified with a *Wolbachia* specific gene (wsp) to detect *Wolbachia* infection. This allowed me to assess the frequencies and patterns of *Wolbachia* infection in site and species of gall wasps and other gall-making insects in Illinois and Indiana. Of the 101 individuals sampled 31 (30.39%) tested positive for *Wolbachia* infection. Ten of 13 sampled species tested positive for *Wolbachia* infection with populations varying in infection rate with some populations having no presence of *Wolbachia*. These findings were among the first survey of *Wolbachia* infection in these species and locations providing a basis for further studies to monitor the impacts of *Wolbachia* infection on the populations dynamics and reproductive evolution.

Degree Type

Dissertation/Thesis

Degree Name

Master of Science (MS)

Department

Biological Sciences

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Keywords

Wolbachia, Galls, Entomology, Cynipini, Cynipids, Hymenoptera

Subject Categories

Biology | Entomology

Wolbachia Infection in Gall Associated Insect Communities in Illinois and Indiana

by

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Thesis

Submitted for the requirements for the degree of

Master of Science

in Biological Sciences

2023

Eastern Illinois University

Charleston, Illinois

Abstract

Wolbachia is a maternally inherited, gram-negative bacterium and has been found to be associated with up to 60% of all insects, frequently resulting in male-killing, feminization, and cytoplasmic incompatibility in the hosts. Gall wasps and other gall-forming insects induce on various host plants abnormal tissue growths, or plant galls, with distinct morphology. They are considered as obligatory parasites to their host plant. The presence of *Wolbachia* infection in gall wasp populations in North America has not been extensively documented. Given the strong influence on host reproduction, not knowing *Wolbachia* infection in insect populations prevents the understanding of how populations are being affected in their dynamics and evolution. In this study, I collected from nine locations, within Illinois and Indiana, galls made by several gall wasp and other gall-making insect species. DNA extracted from the gall makers was amplified with a *Wolbachia* specific gene (wsp) to detect *Wolbachia* infection. This allowed me to assess the frequencies and patterns of *Wolbachia* infection in site and species of gall wasps and other gall-making insects in Illinois and Indiana. Of the 101 individuals sampled 31 (30.39%) tested positive for *Wolbachia* infection. Ten of 13 sampled species tested positive for *Wolbachia* infection with populations varying in infection rate with some populations having no presence of *Wolbachia*. These findings were among the first survey of *Wolbachia* infection in these species and locations providing a basis for further studies to monitor the impacts of *Wolbachia* infection on the populations dynamics and reproductive evolution.

Introduction

Species interact with each other and form various relationships in a biological community. Parasitism is a relationship between species where one species benefits at the expense of another. Gall making is a unique example of this type of relationship, where the host is a plant species and the gall maker is a parasite. Galls are abnormal growths of plant tissue induced on host plants by another organism to provide that organism's offspring with food and a measure of physical protection during development (Cook et al. 2002; Egan et al. 2018; Stone & Cook 1998). Plant galls can form on leaves, twigs, roots, stems, or flowers of the host plants and vary in shape, color, and size (Bird et al. 2013; Hartley 1998; Stone & Cook 1998; Kot & Rubinowska 2018). The formation and presence of galls alters the host plants' characteristics including the development of the plant itself and can cause harm to the host plant such as loss of productivity in host plant and even host death (Hartley 1998).

One well-known group of organisms that induces galls on plants is gall wasps in the family Cynipidae (Hymenoptera, Cynipoidea), which are responsible for inducing diverse plant gall types (Cornell 1985; Cook et al. 2002; Kot & Rubinowska 2018). Gall wasps are considered obligatory parasites of plants as they cannot complete their life cycle without utilizing a host plant (Cook & Butcher 1999). The host plants provide the developing larvae with shelter as well as nutrients until they pupate and finally emerge as adults (Cornell 1985; Egan et al. 2018; Stone & Cook 1998). There are approximately 1400 species described for gall wasps, all with specific gall morphotypes (Ronquist et al. 2015), appearing as balls, knobs, lumps, or warts. Cynipid galls are characteristic to the gall maker rather than the host plant giving rise to these distinct gall morphotypes (Bird et al. 2013; Stone & Cook 1998). This allows entomologists to reliably

associate many types of galls to a particular gall wasp species based on the distinct gall features (Cornell 1985; Abrahamson and Melika 1998, Stone & Cook 1998, Egan et al. 2018).

Although there are many different types of gall wasps, some of the most notable types are oak gall wasps (Hymenoptera: Cynipidae, tribe Cynipini) which produce the most structurally complex and diverse galls of any gall-inducing organisms (Stone & Cook 1998; Taper & Case 1987). Out of all gall wasps, approximately 70% of them (936 species) are oak gall wasps, and 580 species occur in the United States (Cook et al. 2002; Egan et al. 2018; Medianero & Nieves-Aldrey 2013; Stone & Cook 1998; Taper & Case 1987). A notable phenomenon observed in the oak gall wasps is cyclical parthenogenesis, where there is a regular alternation of sexual and asexual reproducing generations.

In oak gall wasps the observed pattern of cyclical parthenogenesis is particularly unique as the alteration between the sexual and asexual reproducing generations is obligatory. The sexual generation start to emerge in June then mates to produce the asexual generation composed of all females which will emerge in April to produce haploid males and diploid females parthenogenetically (Egan et al. 2018). This pattern of cyclical parthenogenesis differs from most other cyclical parthenogenesis patterns observed in organisms that involve multiple rounds of asexual cloning with one round of sexual reproduction that resets the genetic diversity (Rouger et al. 2016). The obligate alteration between sexual and asexual generation in oak gall wasps is not extensively studied and the cause for this distinct cyclical parthenogenesis is unknown.

While organisms may form symbiotic relationships with other organisms visible to the human eye, there are also interactions at the microscopic level that are equally important. Organisms may also have endosymbionts, such as bacteria and viruses, that play an intricate role in thier evolution, ecology, and behavior. This is seen with the bacterial genus *Wolbachia*, which

include species of gram-negative bacterium that has been associated with male-killing, feminization, parthenogenesis, and cytoplasmic incompatibility in up to 60% of all insects (Pimentel et a. 2021 & Zhu et al. 2021).

While there have been several studies on *Wolbachia* infection patterns and effects in gall wasp populations in other countries there have been few studies conducted in the United States especially the Midwest (Zhu et al. 2021). In this project I collected galls from sites throughout Indiana and Illinois to assess the *Wolbachia* presence within different populations and species of insects making note of their host plant. Given that *Wolbachia* infection presence in populations is not well studied in these ateas, oak gall wasps, non-oak gall waps, and gall midges were chosen for this study to provide insight on whether *Wolbachia* occurs in non-oak gall wasps and gall midges as well as to provide a baseline for future studies.

Materials and Methods

Collection and Rearing

Leaf and stem galls were collected from various sites across Indiana and Illinois. The collection sites within Illinois were Douglas-Hart Nature Center (DH) in Charleston, Eastern Illinois University campus (EIU) in Charleston, Fox Ridge State Park (FR) in Charleston, Lake Charleston (LC) in Charleston, Weldon Springs State Park (WS) in Clinton, and Lincoln Log Cabin (LL) in Lerna. The collection sites within Indiana were Austin Bottoms (AB) Austin, Brown County State Park (BC) Nashville, and Clark State Forest (CF) Henryville. These sites were chosen due to their accessability and upon receiving samples from others. The species collected were hedgehog gall wasp (*Acraspis erinacei*), marble gall wasp (*Andricus kollari*), wool-bearing gall wasp (*Andricus quercuslanigera*), oak petiole gall wasp (*Andricus quercuspetiolicola*), cup plant gall wasp (*Antistrophus jeanae)*, rosinweed gall wasp

(*Antistrophus silphii)*, oak apple gall wasp (*Biorhiza pallida*), horned oak gall wasp (*Callirhytis cornigera*), gouty oak gall wasp *(Callirhytis quercuspunctata*), blackberry knot gall wasp *(Diastrophus nebulosus)*, rough bullet gall wasp (*Disholcaspis quercusmamma*), oak vein pocket gall midge (*Macrodiplosis quercusoroca*), and willow pinecone gall midge (*Rabdophaga strobiloides*). Galls were removed from host plants using a chain drive extendable pole saw & pruner (7'–16') and placed into clear sealed bags that were marked with species, host plant, and site location. Bags were kept outside hung on metal wiring and until insect emergence. Tiny holes were made into the bag to get rid of excess moisture. Upon emergence gall-makers were identified by host gall morphology and distinguished as oak gall wasp, non-oak gall wasp, or gall midge. After identification wasps and midges were placed into separate 1.5mL centrifuge tubes with 100% ethanol in -20°C until DNA extraction.

DNA Extraction

A maximum of six individuals per species (one per gall) collected from each location were randomly selected for DNA extraction to limit oversaturation of samples. Specimens were removed from -20°C storage and placed into a new 1.5 mL microcentrifuge tube. QIAGEN buffers and protocol outlined in the DNeasy Blood & Tissue Kit was used for DNA extraction (QIAGEN, Valencia, CA, USA). Quantified DNA exact from DNeasy kit through Nanodrop-Lite-Spectrophotometer (Fisher Scientific, Waltham, MA) (Table 1). Extracted DNA was stored in -20°C storage until undergoing Polymerase Chain Reaction (PCR) protocols.

PCR Protocol and Primers

Upon retrieval from -20°C storage the isolated DNA samples were thawed to undergo PCR in the SimpliAmp Thermal Cycler (Applied Biosystems, Singapore, Singapore). Extractions from samples were used in up to 4 different PCR protocols with the first protocol

serving as a test protocol and the last three served as control protocols. The test protocol was the WSP protocol that tested the DNA for *Wolbachia* infection (Table 3 & 4). The WSP primer was the selected primer for this experiment because it is ideal for population analyses and is documented to detect the presence of Wolbachia at 1/1000 concentration (Marcon et al. 2011). The three control protocols were conducted on gall makers that were not infected with *Wolbachia* infection to ensure that the negative result in protocol 1 was due to only lack of infection.

The second protocol was the mitochondrial cytochrome oxidase subunit 1 (COI) protocol used on samples that tested negative for *Wolbachia* infection in protocol 1 (Table 3 & 4).

The third protocol was a double COI treatment used on samples that tested negative in the first two protocols (Table 3 & 4). PCR amplifications were performed the same as in the second treatment except 5 µL of PCR product from the second treatment was used instead of 5 µL of template DNA.

The fourth and final protocol was a 16s treatment used in specimens that showed no bands in any of the previous three protocols (Table 3 & 4).

Mini Gel Electrophoresis

Mini agarose gels were made by combining 40µL 1X TBE buffer, 0.6g powder agarose, and 4µL of GelRed dye (Biotium, San Francisco, USA) and microwaving to produce a clear homogenous mixture. The product was poured into a casting tray with the electrophoresis comb used to create eight wells in the mini gel. The finished gel was loaded into a gel electrophoresis buffer chamber with 1X TBE buffer covering the wells. The first well was loaded with a 5 µL of DNA ladder and 1 µL of Blue/Orange loading dye mixture. Wells 2-8 were loaded with 5 µL of sample PCR solution and 1 µL of Blue/Orange loading dye. The gel was run at 75 V until the

dye had made it ~75% down the gel. The casting tray was then removed from the buffering chamber and placed onto a foto/Phoresis UV Ultraviolet Transilluminator (Fotodyne Inc., Hartland, WI, USA) to visualize successfully amplified target DNA fragment present in the samples through black light. Results were photographed to record the presence or absence of bands within the mini gels.

Statistics

Fisher's Exact Tests were used to assess if there an assocation between state of sampled oak gall wasps and *Wolbachia* infection, galling insect type and *Wolbachia* infection, or gall wasp type and *Wolbachia* infection.

Results

A total of 101 individual specimens were collected from nine collection sites in Central Eastern Illinois and South Eastern Indiana (Figure 1, Figure 2, & Figure 3). The collection consists of 13 different species with some species being more represented than others. Of the 101 individuals, DNA were successfully extracted from all 101 individuals (100%) as quantified by NanoDrop Lite – Spectrophotometer with DNA concentration ranging from 0.8 - 48.8 mg/µL (Table 1 & Table 2). Of the 13 species there were 3 taxonomic groups: oak gall wasps, non-oak gall wasps, and gall midges (Table 2).

Wolbachia presence was detected in 31 out of the 101 individuals (30.69%) (Table 5). Among the 13 species of gall making insects represented by these samples, *Wolbachia* was present in 10 species and absent from in the other three (Figure 4). The 10 species with detected *Wolbachia* infection were *Acraspis erinacei* (16.7%), *Andricus kollari* (27.3%), *Andricus quercuspetiolicola* (33.3%), *Antistrophus silphii* on cup plants (40%), *Biorhiza pallida* (14.3%), *Callirhytis comigera* (28.6%), *Callirhytis quercuspunctata* (50%), *Diastrophus nebulosus*

(18.2%), *Disholcaspis quercusmamma* (34.6%), and *Macrodiplosis quercusoroca* (16.7%) (Figure 4).

Wolbachia infection was not detected from all locations where samples were collected. *Wolbachia* was found present in six of the nine collection sites. The six sites were DH (39.3%), EIU (38.5%), FR (14.3%), LC (26.9%), WS (20%), and AB (50%) (Figure 5 and Table 6).

Multiple species were collected from several sites including *Acraspis erinacei, Andricus kollari, Biorhiza pallida, Callirhytis cornigera, Callirhytis quercuspunctata, Diastrophus nebulosus, Dischocaspis quercusmamma, and Macrodiplosis quercusoroca* (Table 7). *Acraspis erinacei* were sampled from DH and LC with an infection rate of 0% and 20% at each site respectively (Figure 6). *Andricus kollari* were sampled from DH, FR, LC, and BC with infection rate of 40%, 0%, 50%, and 0% at each respective site (Figure 7). *Biorhiza pallida* were sampled from LC and WS with an infection rate of 16.7% and 0% respectively (Figure 8). *Callirhytis cornigera* were sampled from FR, LL, and AB with infection rates of 33.3%, 0%, and 33% respectively (Figure 9). *Callirhytis quercuspunctata* were sampled from FR and AB with infection rates of 0% and 75% respectively (Figure 10). *Diastrophus nebulosus* were sampled from DH, EIU, and LC with infection rates of 0%, 50%, and 0% respectively (Figure 11). *Dischocaspis quercusmamma* were sampled from DH, EIU, FR, LC, WS, BC, and CF with infection rates of 83.3%, 50%, 0%, 60%, 33.3%, 0%, and 0% respectively (Figure 12). *Macrodiplosis quercusoroca* were sampled from EIU and LC with infection rates of 20% and 0% respectively (Figure 13).

Infection rate differed among gall making insect taxonomy groups. The oak gall wasp taxonomic group had an infection rate of 30.3%, the non-oak gall wasp taxonomic group had an

infection rate of 22.2%, and the gall midge taxonomic group had an infection rate of 14.3% (Table 8).

Fisher's Exact Tests found no significant assocation between state of sampled oak gall wasps and *Wolbachia* infection ($p = 0.169$), galling insect type and *Wolbachia* infection ($p = 1$), or gall wasp type and *Wolbachia* infection ($p = .757$).

Discussion

The DNA extraction was highly effective with 100% of specimens tested having DNA concentrations reported through the Nanodrop-Lite. Although aimed at assessing the presence of *Wolbachia* in gallwasp populations the methods and reactions used to rear the gall makers, extract DNA, amplify via PCR, and make agarose mini gels provided evidence of not only *Wolbachia* presence but also other bacterial infections. This suggests that these methods would be appropriate for larger scale studies of *Wolbachia* infection in the future.

Although *Wolbachia* infection was low overall it was particularly high in certain populations and individuals. This is seen in *Callirhytis cornigera* in AB with 75% of the screened individuals showing *Wolbachia* infection as well as *Disholcaspis quercusmamma* which had an infection percentage of 83.33%. In all sites *Wolbachia* infection varied (Table 6 and Figure 5). This revealed that there is fluctuation in *Wolbachia* infection and opening room for scientific inquiry in future studies to determine what could be causing this inconsistency. This pilot study was designed to assess the effectiveness of tools and process used to screen for *Wolbachia* infection in populations not extensively studied and is why species were limited to a maximum of six individuals per species at each site to be screen but also included species with as little as 1 individual to provide a baseline of understanding infection. If *Wolbachia* infects only a small percentage of a population or species, a few individuals screened may provide an underestimation of the real percentage of infection within an area thus this study will serve as a minimum estimation of infection in the study species (Jiggins et al. 2001 & Rokas et al. 2002).

Of the 10 species that tested positive for *Wolbachia* infection the most notable are the *Acraspis erinacei, Andricus kollari, Anistrophus jeanae, Diastrophus nebulosus,* and *Macrodiplosis quercusoroca*. Although there were individuals of these species that tested

positive for *Wolbachia* infection the individuals collected at each site had different infection rates in different populations (Table 7). This suggests that environment may play a factor in *Wolbachia* infection and opens the idea for future studies to assess what is causing these uneven infection rates. These population of galling insects also served as the baseline for future studies. *Acraspis erinacei* and *Andricus kollari* unlike in previous studies where they had been found to have no *Wolbachia* infection (Rokas et al. 2002). The *Anistrophus jeanae, Diastrophus nebulosus*, and *Macrodiplosis quercusorocoa* are also notable species in this study because currently this seems to be the first documentation of *Wolbachia* being found in either of these species.

The infection rate amongst the taxonomic groups of the gall associated insect communities were not equal with the oak gall wasp guild having the highest infection rate. This was to be expected as many oak gall wasps have been studied and documented to have *Wolbachia* infection in previous studies (Rokas et al. 2002 & Zhao et al. 2013).

Although there was no significant association found in the populations through Fisher's Exact Test, the results may not realistically reflect the association of *Wolbachia* infection and the listed factors due to the limited sample size, and hence should be taken with caution. Additional studies would be needed to provide more data to allow more in-depth and concrete conclusions on association with infection in populations.

This study not only provided a protocol effective for assessing *Wolbachia* presence in larger projects and set up a preliminary estimation of *Wolbachia* infection in galling insect populations within Illinois and Indiana areas but also documented *Wolbachia* infection in multiple species for the first time. The finite time and funding for this project bounded the intensity of sampling and thus produced a useful, but limited estimation of *Wolbachia* infection

patterns in species and populations. For future studies with additional time and funding, researchers can do more intense sampling to provide a more extensive evaluation of the *Wolbachia* presence in galling insect populations. Future studies could also compare *Wolbachia* infection in male and females to assess if there is a correlation between sex and infection. With this more intese sampling future researchers can concentrate on understanding *Wolbachia* infection patterns either through concentration on species that have wide local representation or variation in fection rate between species as revealed by this study.

Acknowledgements

To start I dedicate this thesis to my beloved cat Abbie Watts. Abbie was with me for almost 13 years until she left this world on May 7th, 2023. May she continue being smart, sassy, kind, and running the show in the afterlife whatever that is for her.

I would like to thank my advisor, Dr. Zhiwei Liu, who not only challenged me but also encouraged and supported me during this project. His advice and guidance helped me throughout this project.

I would like to give thanks to my other committee members, Drs. Barbara Carlsward and Scott Meiners, for their insight and advice in this project.

I am grateful to EIU for awarding me the Research/Creative Activity Grant, which, together with an EIU Impact Grant (to Dr. Z. Liu), helped fund this project.

I thank Dr. Liu, Dr. Liu's spring 2022 Ecology and Evolution class, Kaylee Smith, Savannah Pendleton, and my grandfather James Harmon for helping collect galls for this project.

I would like to give a special thanks to my friends and fellow lab mates Sean Chien and Sunita Mahat for their encouragement and support during this project and my graduate studies.

Finally, I would like to thank my family and friends for their love, encouragement, and support. I could not have done this without them.

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Figures

Figure 1. Map of Indiana with areas of sample locations highlighted with corresponding color dots. Black: Austin Bottoms, Austin, IN. Green: Clark State Forest, Henryville, IN. Brown: Brown County State Park, Nashville, IN.

Figure 2. Map of Illinois counties with areas of sample locations highlighted with corresponding color dots. Red: Weldon Springs State Park, DeWitt County, IL. Blue: Douglas-Hart Nature Center, Eastern Illinois University campus, Fox Ridge State Park, and Lake Charleston, Coles County, IL.

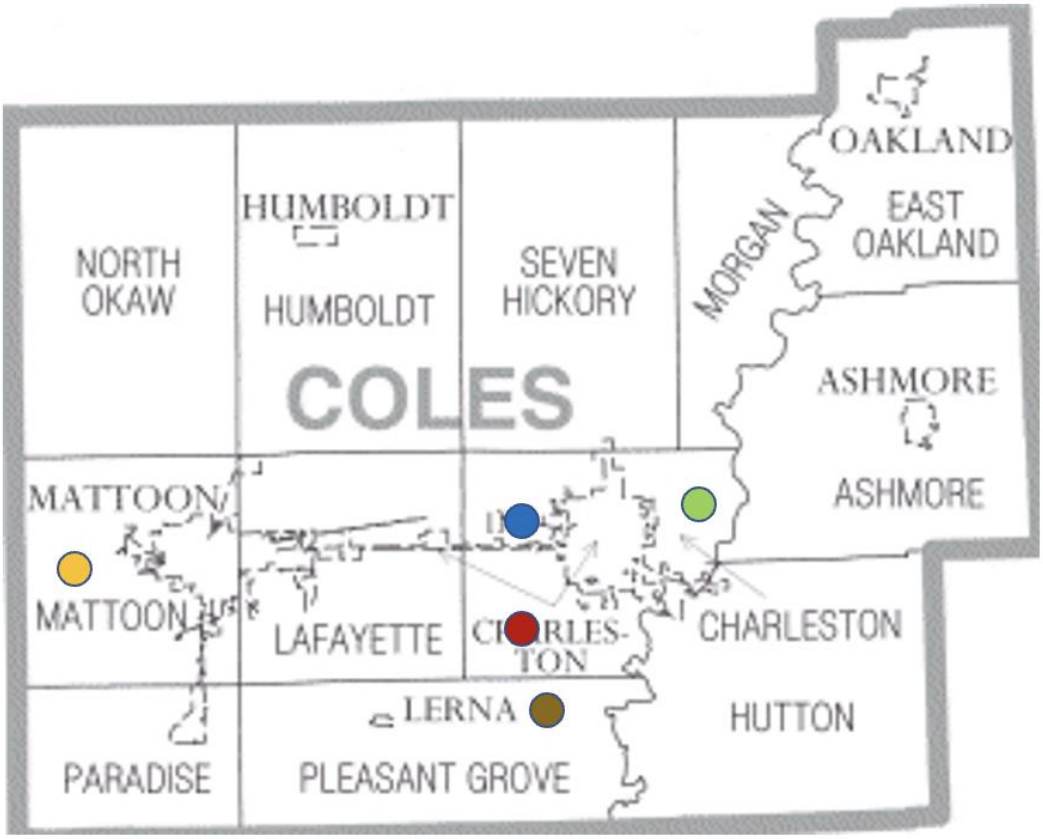

Figure 3. Map of Coles County Illinois with areas of sample locations highlighted with corresponding color dots. Blue: Eastern Illinois University campus. Maroon: Fox Ridge State Park, Charleston, IL. Brown: Lincoln Log Cabin, Lerna, IL. Yellow: Douglas-Hart Nature Center.

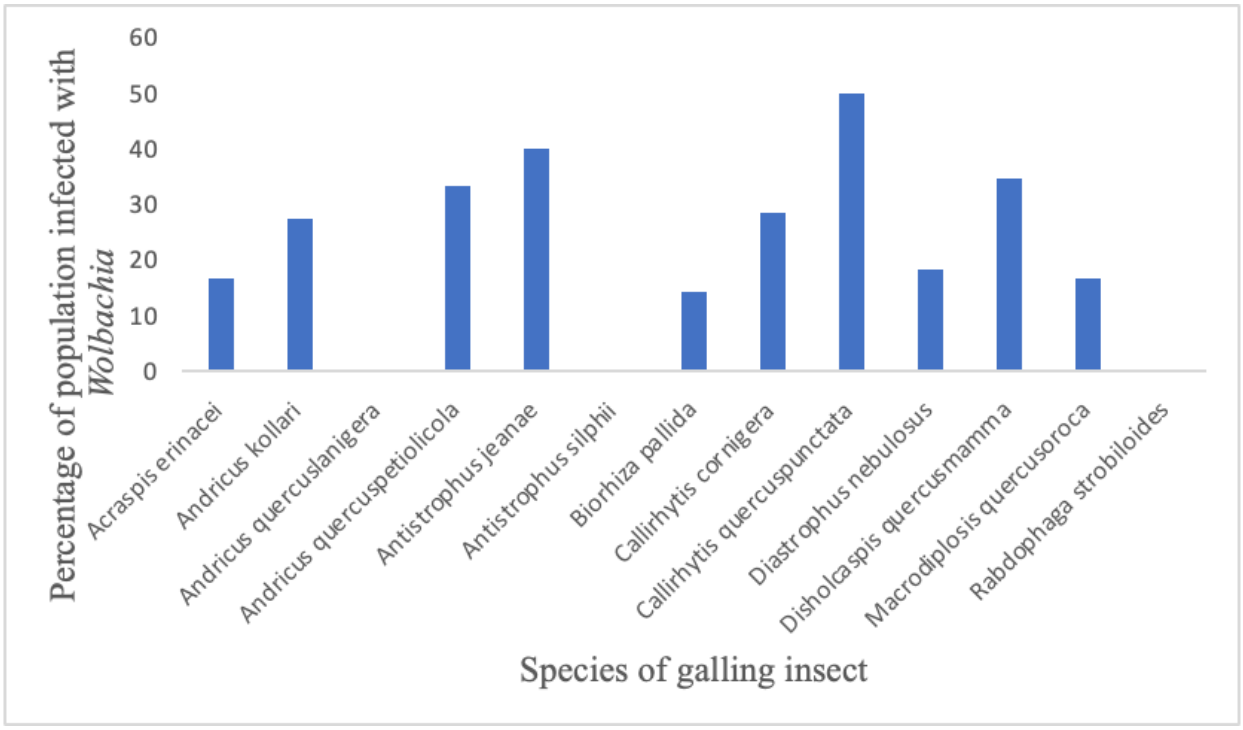

Figure 4. *Wolbachia* infection rate variation gall-inducing species. These species mostly are cynipid gall wasps (Hymenoptera), including *Acraspis erinacei, Andricus kollari, Andricus quercuslanigera, Andricus quercuspetiolicola, Anistrophus jeanae, Anistrophus silphii*, *Biorhiza pallida, Callirhytis corningera, Callirhytis quercuspunctata, Diastrophus nebulosus,* and *Dischlocaspis quercusmamma,* except two species belonging to the gall midge family (Cecidomyiidae, Diptera): *Macrodiplosis quercusoroca, and Rabdophaga stroblioides*.

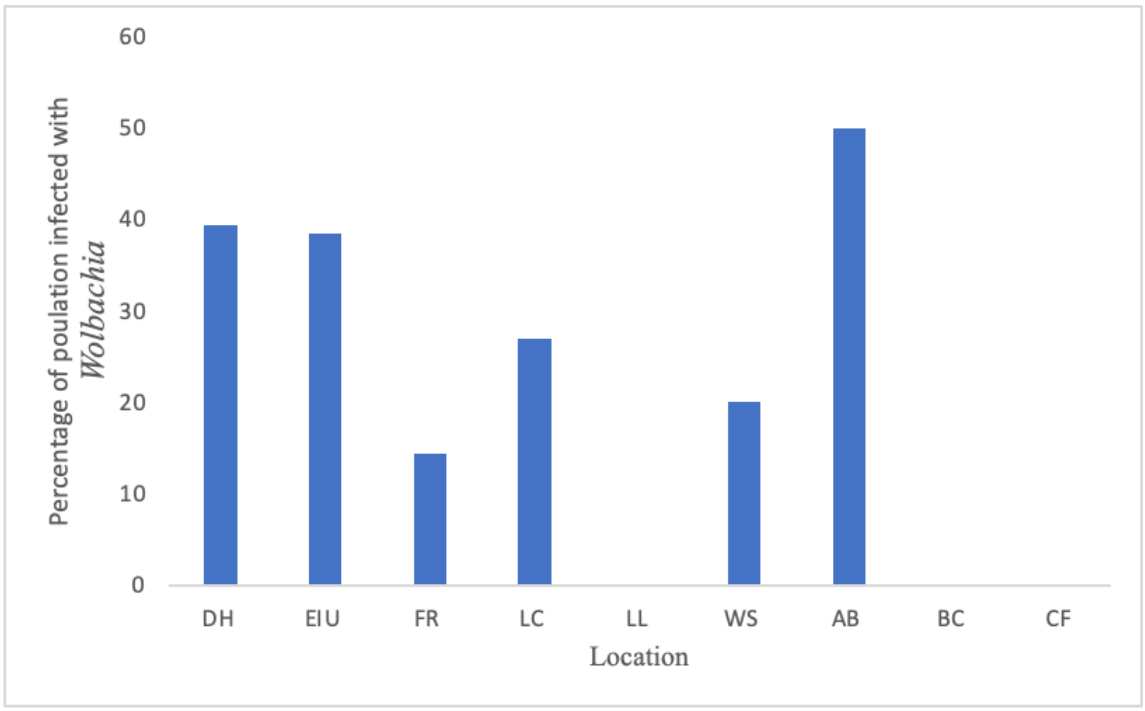

Figure 5. *Wolbachia* infection rate variation among sample locations. DH - Douglas Hart Nature Center, EIU - Eastern Illinois University campus, FR - Fox Ridge State Park, LC - Lake Charleston, LL - Lincoln Log Cabin, WS - Weldon Springs State Park, AB - Austin Bottoms, BC - Brown County State Park, and CF –Clark Sate Forest.

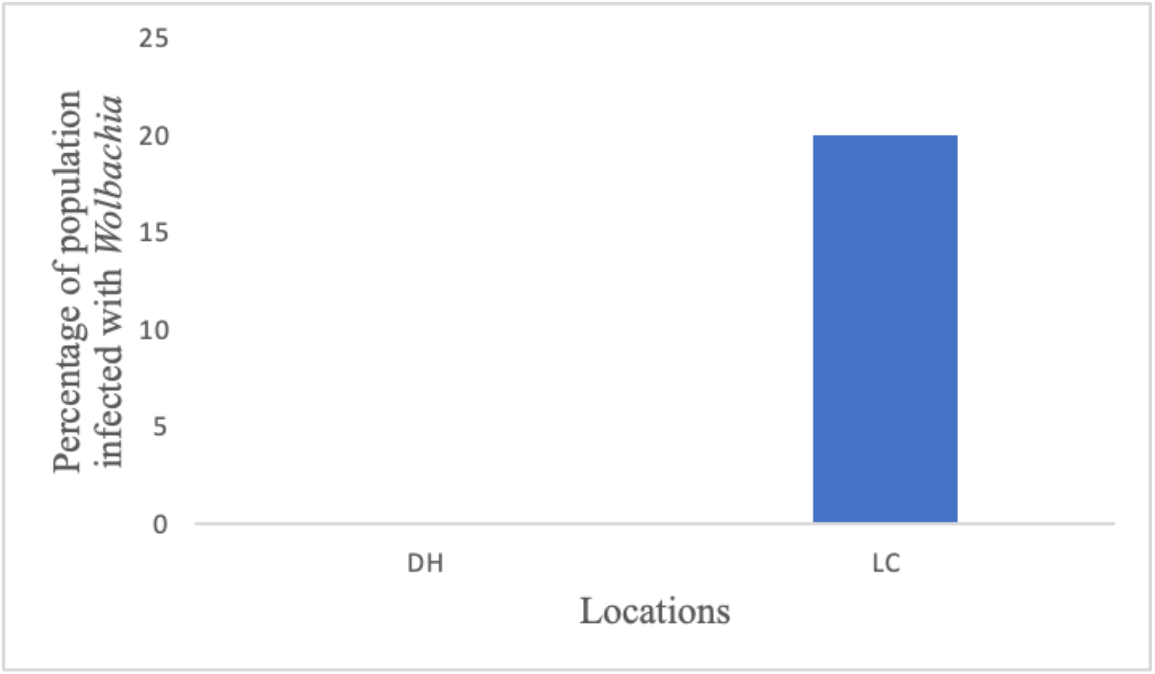

Figure 6. *Wolbachia* infection percentage in sampled populations of *Acraspis erinacei* (Cynipidae, Hym). DH - Douglas Hart Nature Center, Mattoon, IL, LC - Lake Charleston, Charleston, IL.

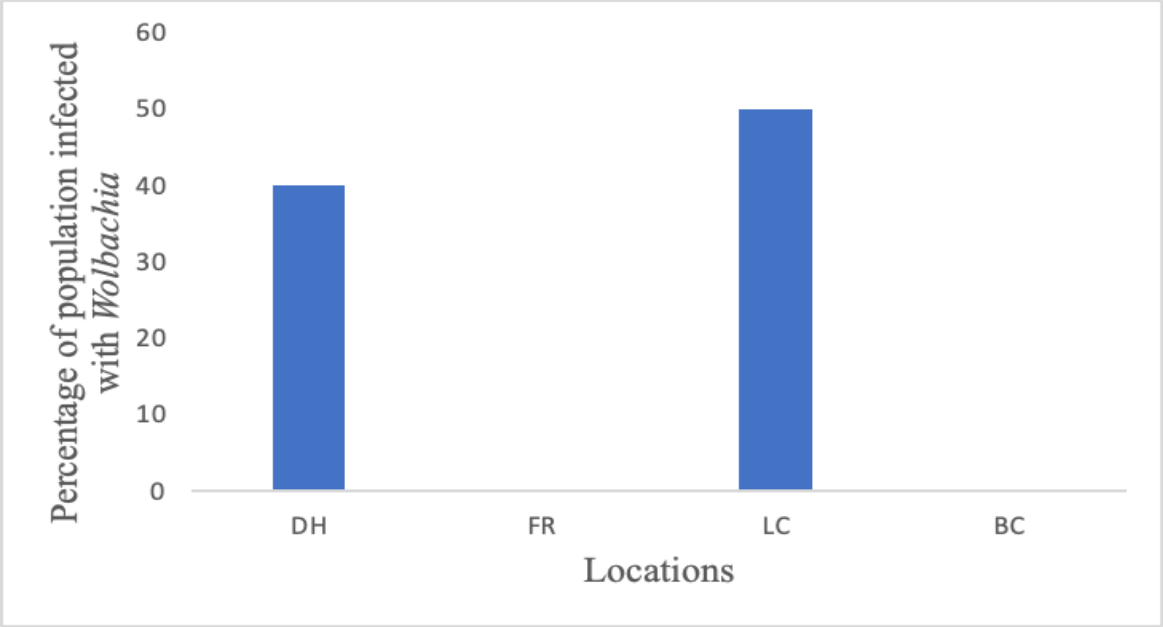

Figure 7. *Wolbachia* infection percentage in sampled populations of *Andricus kollari* (Cynipidae, Hym). DH-Douglas Hart Nature Center, Mattoon, IL, FR- Fox Ridge State Park, Charleston, IL, LC- Lake Charleston, Charleston, IL, BC-Brown County State Park, Nashville, IN.

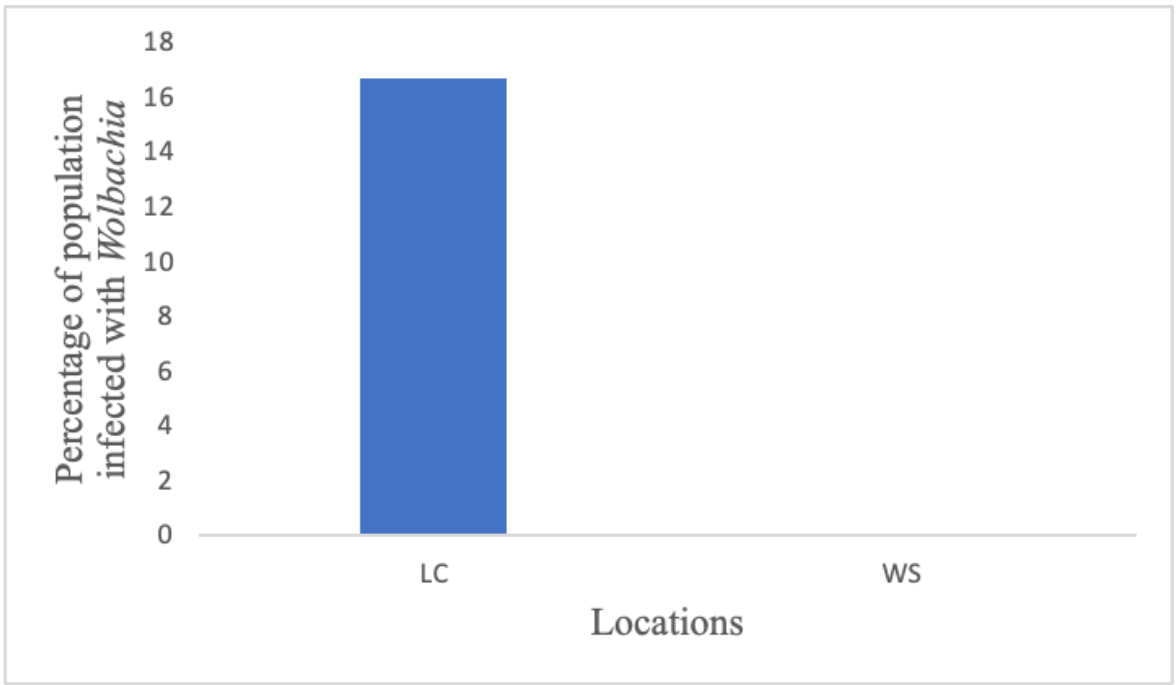

Figure 8. *Wolbachia* infection percentage in sampled populations of *Biorhiza Pallida* (Cynipidae, Hym). LC-Lake Charleston, Charleston, IL and WS-Weldon Springs State Park, Clinton, IL.

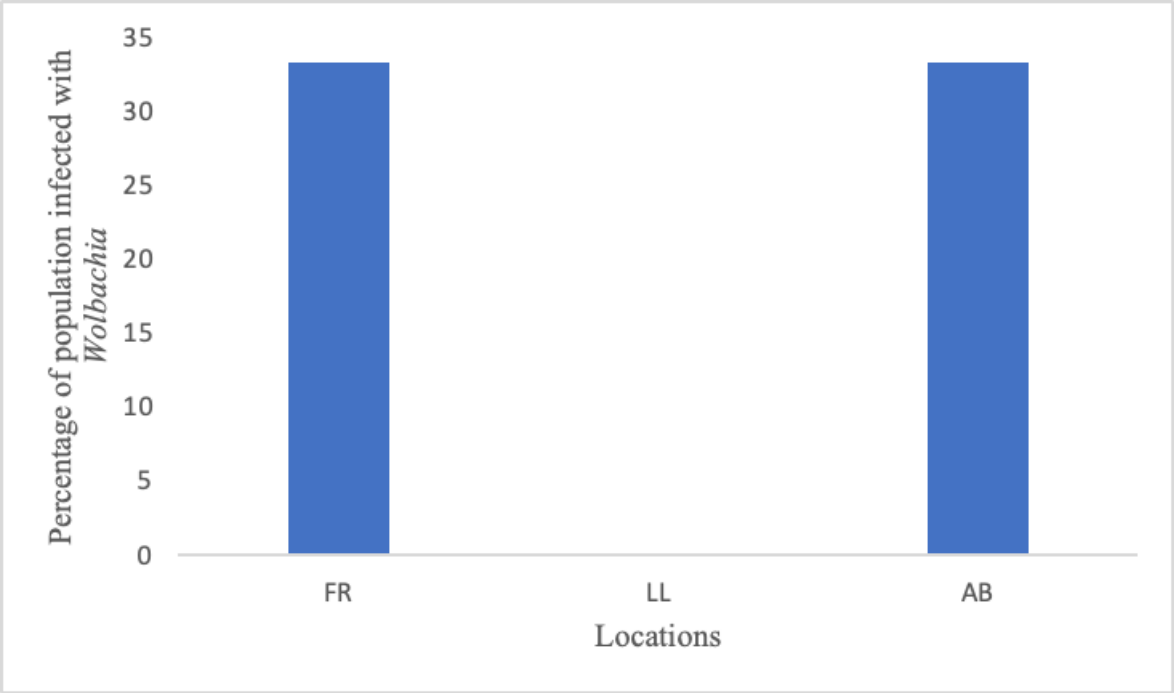

Figure 9. *Wolbachia* infection percentage in sampled populations of *Callirhytis cornigera* (Cynipidae, Hym). FR- Fox Ridge State Park, Charleston, IL, LL-Lincoln Log Cabin, Lerna, IL AB-Austin Bottoms, Austin, IN.

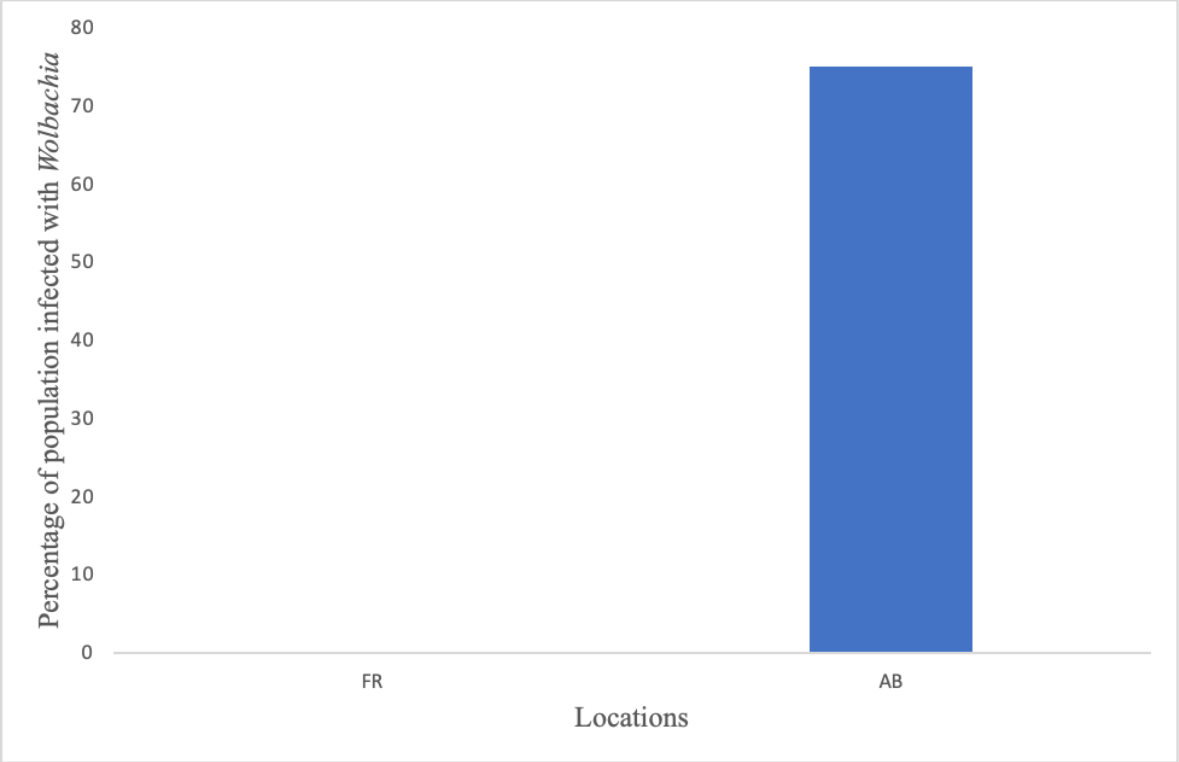

Figure 10. *Wolbachia* infection percentage in sampled populations of *Callirhytis quercuspunctata* (Cynipidae, Hym). FR-Fox Ridge State Park, Charleston, IL and AB-Austin Bottoms, Austin, IN

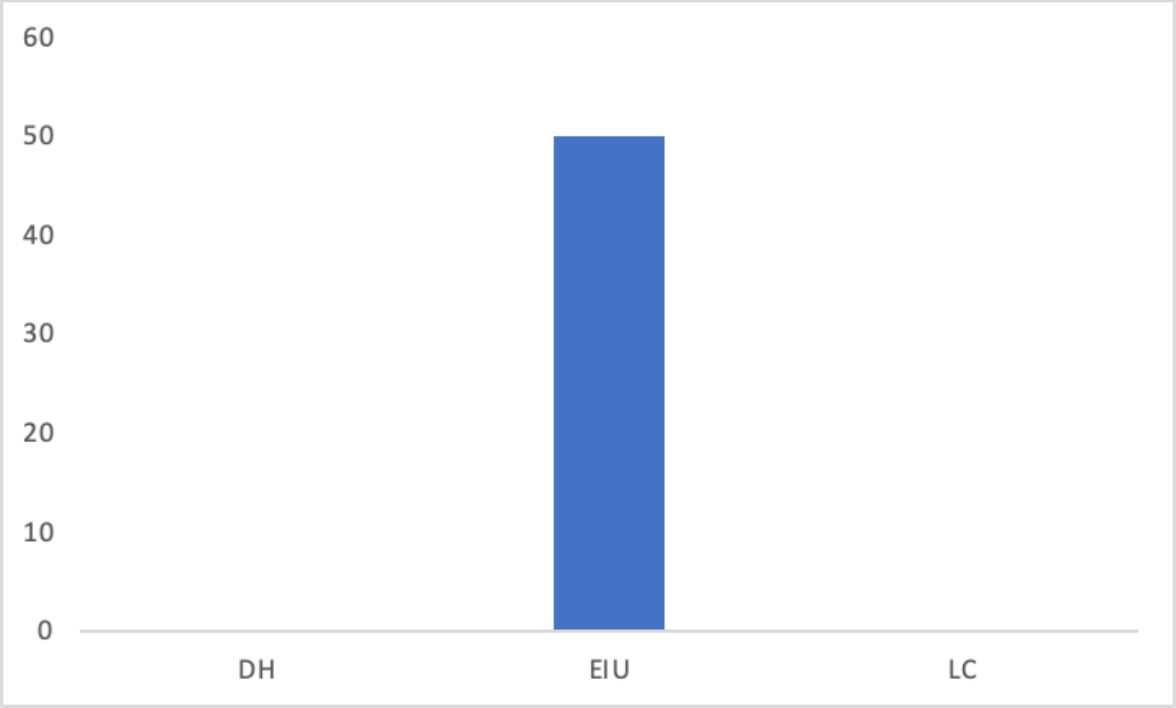

Figure 11. *Wolbachia* infection percentage in sampled populations of *Diastrophus nebulosus (*Cynipidae, Hym). DH - Douglas Hart Nature Center, Charleston, IL, EIU - Eastern Illinois University campus, Charleston, IL, and LC - Lake Charleston, Charleston, IL.

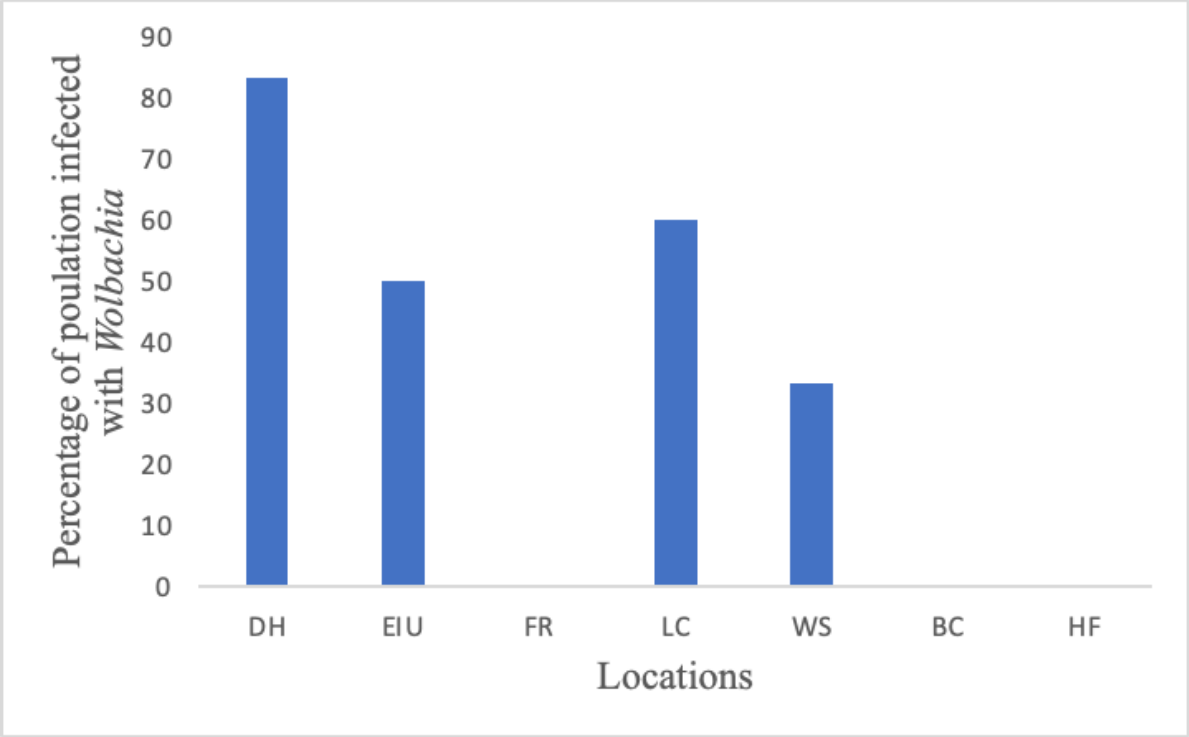

Figure 12. *Disholcaspis quercusmamma* were sampled at seven locations, *but Wolbachia* infection was detected in four of the sampled populations DH-Douglas Hart Nature Center, Mattoon, IL EIU- Eastern Illinois University campus, Charleston, IL, FR-Fox Ridge State Park, Charleston, IL LC-Lake Charleston, Charleston, IL, WS-Weldon Springs State Park, Clinton, IL, BC-Brown County State Park, Nashville, IN and CF-Clark State Forest, Henryville

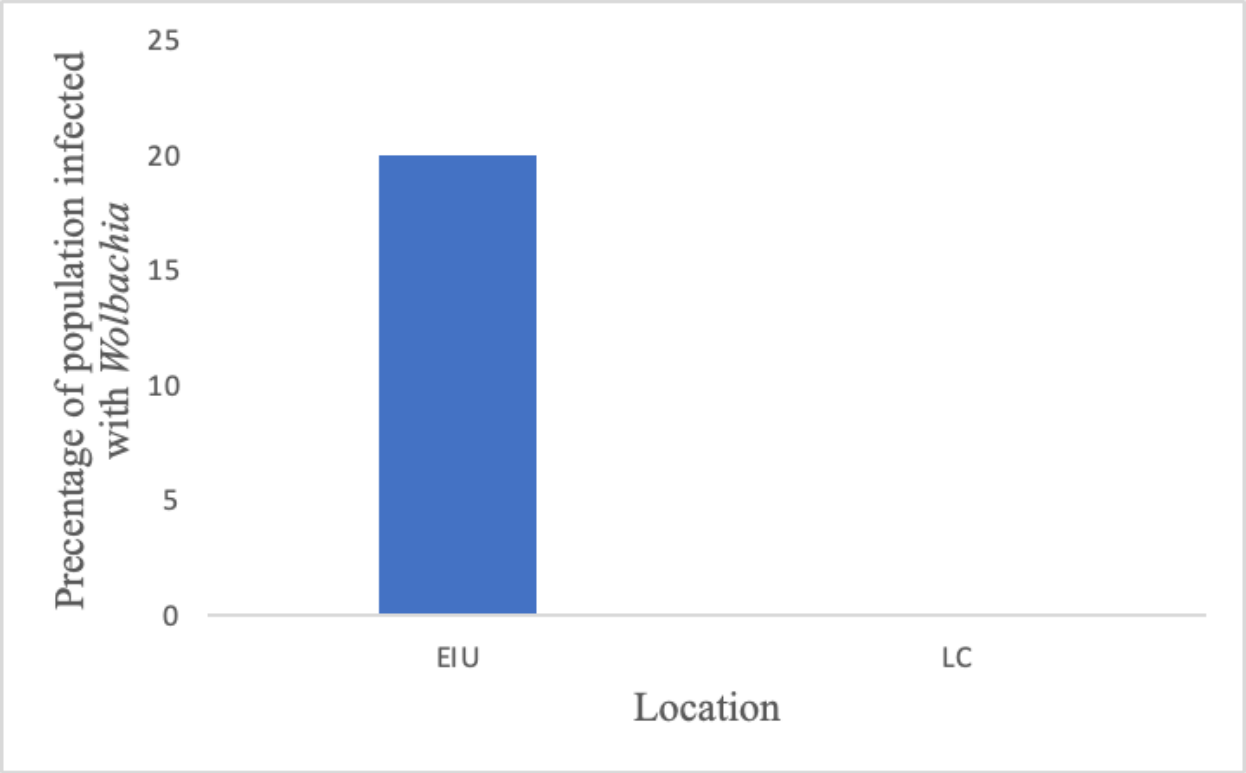

Figure 13. *Macrodiplosis quercusoroca* (Cecidomyiidae, Diptera) were sampled at two locations, but *Wolbachia* infection was detected in one population EIU-Eastern Illinois University, Charleston, IL and LC-Lake Charleston, Charleston, IL.

Tables

Table 1. DNA concentrations of sampled species of gall making insects at Douglas Hart Nature Center, Mattoon, IL (DH), Eastern Illinois University campus, Charleston, IL (EIU), Fox Ridge State Park, Charleston, IL (FR), Lake Charleston, Charleston, IL (LC), Lincoln Log Cabin, Lerna, IL (LL), Weldon Springs State Park, Clinton, IL (WS), Austin Bottoms, Austin, IN (AB), Brown County State Park, Nashville, IN (BC), and Clark State Forest, Henryville, IN (CF).

Sample #	Species	Location	DNA concentration (ng/µL)
1	*Disholcaspis quercusmamma*	FR	0.8
8	*Callirhytis cornigera*	FR	1.6
10	*Andricus quercuspetiolicola*	DH	1.6
14	*Diastrophus nebulosus*	LC	2.3
15	*Diastrophus nebulosus*	LC	5.1
16	*Disholcaspis quercusmamma*	DH	3.5
18	*Andricus quercuspetiolicola*	DH	4.1
19	*Diastrophus nebulosus*	EIU	0.8
20	*Disholcaspis quercusmamma*	DH	3.3
22	*Disholcaspis quercusmamma*	DH	1
23	*Antistrophus jeanae*	DH	1.1
24	*Antistrophus jeanae*	DH	1.4
25	*Disholcaspis quercusmamma*	DH	7.6
27	*Andricus kollari*	LC	2.6
28	*Andricus kollari*	DH	2.6
29	*Andricus quercuspetiolicola*	DH	4.4
30	*Callirhytis cornigera*	JH	5.3
31	*Disholcaspis quercusmamma*	DH	1.8
32	*Callirhytis cornigera*	FR	2.3
33	*Andricus kollari*	DH	5
34	*Callirhytis cornigera*	JH	1.7
35	*Callirhytis cornigera*	FR	7.2
36	*Andricus kollari*	FR	4.3
37	*Antistrophus silphii*	DH	3.8
38	*Callirhytis cornigera*	JH	3.5

39	*Disholcaspis quercusmamma*	FR	2.5
40	*Antistrophus silphii*	DH	48.8
41	*Callirhytis cornigera*	FR	9.9
42	*Antistrophus silphii*	DH	0.8
44	*Callirhytis quercuspunctata*	JH	5.1
45	*Diastrophus nebulosus*	LC	3.8
46	*Rabdophaga strobiloides*	WS	3.3
47	*Andricus quercuspetiolicola*	DH	4.6
48	*Antistrophus silphii*	DH	3
49	*Antistrophus silphii*	DH	2.2
50	*Callirhytis cornigera*	LL	1.7
51	*Disholcaspis quercusmamma*	HI	11.4
52	*Diastrophus nebulosus*	DH	10
56	*Disholcaspis quercusmamma*	DH	41
57	*Callirhytis quercuspunctata*	FR	4.5
58	*Callirhytis quercuspunctata*	FR	6.8
59	*Andricus kollari*	DH	6.4
60	*Andricus kollari*	DH	19.1
61	*Andricus kollari*	DH	10.7
62	*Acraspis erinacei*	DH	15
69	*Callirhytis cornigera*	FR	3.3
79	*Callirhytis cornigera*	LL	4.4
87	*Diastrophus nebulosus*	EIU	10.7
88	*Diastrophus nebulosus*	DH	2.3
89	*Diastrophus nebulosus*	DH	3.5
92	*Disholcaspis quercusmamma*	FR	13.2
104	*Diastrophus nebulosus*	LC	3.7
105	*Diastrophus nebulosus*	LC	8.7
115	*Disholcaspis quercusmamma*	LC	10.2
116	*Disholcaspis quercusmamma*	LC	3.6
117	*Disholcaspis quercusmamma*	LC	12.5

127	*Diastrophus nebulosus*	LC	20.7
143	*Acraspis erinacei*	LC	13.9
145	*Acraspis erinacei*	LC	27.5
146	*Disholcaspis quercusmamma*	BC	9.4
147	*Andricus kollari*	LC	3.2
148	*Disholcaspis quercusmamma*	DH	9.7
149	*Andricus quercuspetiolicola*	DH	7
150	*Andricus quercuspetiolicola*	DH	6
168	*Disholcaspis quercusmamma*	FR	13.1
169	*Callirhytis quercuspunctata*	JH	5.5
170	*Callirhytis quercuspunctata*	JH	4.7
171	*Callirhytis cornigera*	JH	4.1
172	*Callirhytis cornigera*	JH	3.1
177	*Callirhytis cornigera*	JH	5.3
178	*Andricus kollari*	FR	8.1
184	*Callirhytis cornigera*	FR	2.4
193	*Callirhytis quercuspunctata*	JH	21.6
215	*Biorhiza pallida*	WS	3.8
216	*Disholcaspis quercusmamma*	WS	26.9
217	*Disholcaspis quercusmamma*	WS	21.9
218	*Disholcaspis quercusmamma*	WS	17.6
219	*Acraspis erinacei*	LC	13.8
220	*Acraspis erinacei*	LC	3.5
221	*Acraspis erinacei*	LC	4.9
222	*Disholcaspis quercusmamma*	EIU	11
224	*Biorhiza pallida*	LC	3.7
225	*Disholcaspis quercusmamma*	LC	3.4
226	*Disholcaspis quercusmamma*	LC	2.8
227	*Biorhiza pallida*	LC	2.7
233	*Biorhiza pallida*	LC	3.5

234	*Biorhiza pallida*	LC	36.9
235	*Macrodiplosis quercusoroca*	EIU	3.9
236	*Macrodiplosis quercusoroca*	EIU	3
264	*Macrodiplosis quercusoroca*	EIU	12.4
265	*Macrodiplosis quercusoroca*	EIU	16.3
266	*Macrodiplosis quercusoroca*	EIU	7.1
267	*Andricus kollari*	BC	3
302	*Disholcaspis quercusmamma*	EIU	6.6
303	*Disholcaspis quercusmamma*	EIU	7.3
304	*Disholcaspis quercusmamma*	EIU	29.1
305	*Disholcaspis quercusmamma*	EIU	16
306	*Disholcaspis quercusmamma*	EIU	1.6
375	*Andricus quercuslanigera*	LC	5.5
376	*Biorhiza pallida*	LC	4
378	*Macrodiplosis quercusoroca*	LC	16.5

Table 2. Species name, common names, and classification of galling insects sampled in this study.

Species Name	Common Name	Classification
Acraspis erinacei	Hedgehog gall wasp	Cynipini, Cynipidae Hym.
Andricus kollari	Oak Marble gall wasp	Cynipini, Cynipidae Hym.
Andricus quercuslanigera	Wool-bearing gall wasp	Cynipini, Cynipidae Hym.
Andricus quercuspetiolicola	Oak Petiole gall wasp	Cynipini, Cynipidae Hym.
Antistrophus jeanae	Cup Plant gall wasp	Aulacideini, Cynipidae, Hym.
Antistrophus silphii	Rosinweed gall wasp	Aulacideini, Cynipidae, Hym.
Biorhiza pallida	Oak Apple gall wasp	Cynipini, Cynipidae Hym.
Callirhytis cornigera	Horned Oak gall wasp	Cynipini, Cynipidae Hym.
Callirhytis quercuspunctata	Gouty Oak gall wasp	Cynipini, Cynipidae Hym.
Diastrophus nebulosus	Blackberry Knot gall wasp	Diastrophini, Cynipidae, Hym.
Disholcaspis quercusmamma	Oak Rough Bullet gall wasp	Cynipini, Cynipidae Hym.
Macrodiplosis quercusoroca	Oak Vein Pocket gall midge	Cecidomyiidae, Diptera
Rabdophaga strobiloides	Willow Pinecone gall midge	Cecidomyiidae, Diptera

Table 3. PCR amplification reactants and primers in PCR protocols.

Protocol	Amplification reactants	Primers
Protocol 1 (WSP)	5 µL of template DNA, 12.5µL Greenmaster mix, 2.5 µL nuclease-free water, and 5 µL primer	5µL WSP-81F (5'— TGG TCC AAT AAG TGA TGA AGA AAC—3') & 69F (5'— AAA AAT TAA ACG CTA CTC CA—3')
Protocol 2 (CO1)		5µl HymCOI-HCO&LCO-Folm (5'—TAA ACT TCA GGG TGA CCA AAA AAT CA – 3' & 5'— GGT CAA CAA ATC ATA AAG ATA TTG G – 3')
Protocol 3 (CO1 X2)		
Protocol 4 (16S)		16S forward (5'– TAACTGTACAAAGGTAGC– 3') and 16S reverse (5'– TTAATTCAACATCGAGGTC– 3')

Table 4. PCR amplification step specifications.

Protocol	Initial Denature	Cycling	Final Extention
Protocol 1 (WSP)	95°C for 3'	95°C for 30", 52°C for 30", 72°C for 60", repeat for 35 cycles	72°C for 10'
Protocol 2 (CO1)	95°C for 5'	95°C for 60", 48°C for 60", 72°C for 60", repeat for 32 cycles	
Protocol 3 (CO1 X2)			
Protocol 4 (16S)		95°C for 30", 55°C for 30", 72°C for 30", repeat for 35 cycles	

Table 5. *Wolbachia* infection rate of all sampled *Acraspis erinacei*, *Andricus kollari*, *Andricus quercuslanigera*, *Andricus quercuspetiolicola*, *Antistrophus jeanae (ex Cup Plant)*, *Antistrophus silphii (ex Rosinweed)*, *Biorhiza pallida*, *Callirhytis cornigera*, *Callirhytis quercuspunctata*, *Diastrophus nebulosus*, *Disholcaspis quercusmamma*, *Macrodiplosis quercusoroca*, and *Rabdophaga strobiloides* in this study.

Species	Infection rate in populations (percentage)
Acraspis erinacei	16.7
Andricus kollari	27.3
Andricus quercuslanigera	0.0
Andricus quercuspetiolicola	33.3
Antistrophus jeanae (ex Cup Plant)	40.0
Antistrophus silphii (ex Rosinweed)	0.0
Biorhiza pallida	14.3
Callirhytis cornigera	28.6
Callirhytis quercuspunctata	50.0
Diastrophus nebulosus	18.2
Disholcaspis quercusmamma	34.6
Macrodiplosis quercusoroca	16.7
Rabdophaga strobiloides	0.0

Table 6. *Wolbachia* infection rate at Douglas Hart Nature Center, Mattoon, IL (DH), Eastern Illinois University campus, Charleston, IL (EIU), Fox Ridge State Park, Charleston, IL (FR), Lake Charleston, Charleston, IL (LC), Lincoln Log Cabin, Lerna, IL (LL), Weldon Springs State Park, Clinton, IL (WS), Austin Bottoms, Austin, IN (AB), Brown County State Park, Nashville, IN (BC), and Clark State Forest, Henryville, IN (CF).

Location	Infection rate (percentage)
DH	39.3
EIU	38.5
FR	14.3
LC	26.9
LL	0.00
WS	20.0
AB	50.0
BC	0.0
CF	0.0

Table 7. *Wolbachia* infection rates of in sampled populations of gall making insects at Douglas Hart Nature Center, Mattoon, IL (DH), Eastern Illinois University campus, Charleston, IL (EIU), Fox Ridge State Park, Charleston, IL (FR), Lake Charleston, Charleston, IL (LC), Lincoln Log Cabin, Lerna, IL (LL), Weldon Springs State Park, Clinton, IL (WS), Austin Bottoms, Austin, IN (AB), Brown County State Park, Nashville, IN (BC), and Clark State Forest, Henryville, IN (CF).

Species	Location								
	DH	EIU	FR	LC	LL	WS	AB	BC	CF
Acraspis erinacei	0%	-	-	20%	-	-	-	-	-
Andricus kollari	40%	-	0%	50%	-	-	-	0%	-
Biorhiza pallida	-	-	-	16.7%	-	0%	-	-	-
Callirhytis cornigera	-	-	33.3%	-	0%	-	33.3%	-	-
Callirhytis quercuspunctata	-	-	0%	-	-	-	75%	-	-
Diastrophus nebulosus	0%	50%	-	0%	-	-	-	-	-
Dischocaspis quercusmamma	83.3%	50%	0%	60%	-	33.3%	-	0%	0%
Macrodiplosis quercusoroca	-	20%	-	0%	-	-	-	-	-

Table 8. Infection rates of *Wolbachia* within gall taxonomy groups: Oak gall wasps, non-oak gall wasps, and other gall making species sampled

Gall Taxonomic Groups	Infection rate (Percentage)
Oak gall wasps	30.3
Non-oak gall wasps	22.2
Other gall making species	14.3