BIOLOGICAL CONTROL OF JAPANESE BEETLE (*POPILLIA JAPONICA*) THROUGH THE USE OF THE MICROSPORIDIAN PATHOGEN, *OVAVESICULA POPILLIAE*

By

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ABSTRACT

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Three experiments were designed to evaluating the effects of *Ovavesicula popilliae,* a species-specific microsporidian pathogen, on Japanese beetle (*Popillia japonica*) in Michigan.

In the first experiment prevalence of *O*. *popilliae*, in Michigan was determined by collecting host larvae and adults at 47 golf courses or rest areas in 2018 and 2019. Larvae and adults were dissected and assessed for *O. popilliae* infection. Infection and scarab species data from nine of the golf courses visited in 2018 was compared to similar data from the same nine golf courses from 1999. The survey of golf courses and highway rest areas in Michigan documented a significant decline of Japanese beetle in the 20 year-period since the last survey, and the slow spread of *Ovavesicula popilliae*. At introduction sites from the last 12 years *O. popilliae* established and persisted at epizootic levels.

In the second experiment survival of *Ovavesiucla popilliae*-infected larvae was compared with survival of healthy larvae during their overwintering period, from October to May, in two consecutive years of experiments. *Ovavesicula popilliae* infection of larvae at the beginning of the experiment (34.0 and 26.9%, in October 2017 and October 2018, respectively) and soil cores from a site where the pathogen was active had a significant impact on the survival of infected larvae (90 – 100% reduction).

In the third experiment healthy Japanese beetle larvae were inoculated by placing them in soil cores collected from a site where *O. popilliae* had established and became epizootic. Infection and survival of inoculated larvae was compared with the same for healthy Japanese beetle larvae from October to May. There was no difference in percent infection of inoculated larvae (5.0 %) and control larvae (1.8%). This may be because the field plots for this study were placed directly over the top of field plots from the previous year where infected larvae were put into turf and soil cores.

Dedicated to Deborah A. Piombino, Dr. Katie Costanzo and Dr. Malini Suchak.

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PREFACE

Japanese beetle, *Popillia japonica* Newman, an invasive insect from Japan, was first discovered in New Jersey in 1916, and is now present across the Mid-west and East coast of the United States of America (Fleming, 1972; USDA-APHIS, 2018). It continues to spread westward, despite federal quarantine measures, and a few far western outlier infestations like Boulder, Colorado and Portland, Oregon have sparked interest in determining how they got there (Rainwater, 1963; Hungate et al., 2016; USDA-APHIS, 2018). In Portland, Oregon researchers used hydrogen isotope composition (δ^2H) in Japanese beetles trapped near the Portland International Airport to determine that most of the beetles trapped came for the eastern USA, probably on cargo aircraft (Hungate et al., 2016).

Japanese beetle larvae can be identified by their "V" shaped rastral pattern on the ventral surface of the last abdominal segment (Fleming, 1968; Fleming, 1972; Hadley et al., 1934). Adults are recognizable by their stout metallic green body and metallic orange-coloured elytra, along with a row of five white hairs on each side of the abdomen. Typically Japanese beetles have a one year life cycle, but in colder temperatures they have been known to move to a two year cycle (Fleming, 1972; Hadley et al., 1934). Japanese beetle larvae will continue to develop at temperatures within a range of 13° to 35° C (Fleming, 1972) and optimum average temperatures for each life stage are as follows: 30° eggs, 27.5° larvae and 30°-32° for pupae (Fleming, 1972). A typical calendar-year life cycle for Japanese beetle starts with larvae overwintering in January at depths of 4 to 8 inches deep in the soil. In March and April they move upward in the soil to the turf root zone to feed (Hadley et al., 1934). Larvae complete development in late April and May, followed by pupation from May to June (Fleming, 1972). After pupation the adult beetle will begin to emerge around mid-June in Kentucky, late June in southern Ohio, and early July in southern Michigan (Loughrin et. al., 1998). Specifically, in East Lansing and other parts of southern Michigan Japanese beetles have a one-year life cycle, with the first adults emerging the first

week of July in an average year. In northern Michigan a partial or complete 2-year life cycle has been observed, but this needs to be further studied for confirmation. In the northeast U.S., in places such as Massachusetts evidence of a 2 year life cycle of Japanese beetle exists (Vittum, 1986). In Lansing and southern Michigan Japanese beetle adults reach peak activity between the middle of July and middle of August. Some Japanese beetles can still be found and trapped in September, but they become difficult to find by the middle of October.

Adults feed on foliage and fruit of over 260 types of plants from June to September (Hadley et al., 1934). Females can lay multiple batches of eggs with continuous plant feeding in July (Fleming, 1964; Fleming, 1972; Hadley et al., 1934). The average life span of a Japanese beetle is from 30 to 45 days (Fleming, 1972; Fleming, 1964a; Hadley et. al., 1934). Female beetles will oviposit their eggs near where they were feeding; this is normally a favored food source (Schwartz, 1968; Fleming, 1972). This has been recorded numerous times and proven by the fact that more beetles are captured by traps near favorable food sources than further away from favorable food sources (Schwartz, 1968; Fleming, 1972; Loughrin et. al., 1996). Majority of Japanese beetle eggs are oviposited in pastures, lawns, golf courses, and clover, corn and nursery stock. Japanese beetle females tend to prefer pastures for oviposition, but in times of drought are attracted to golf courses and home lawns that are irrigated (Fleming, 1972). Females will mate multiple times and can deposit up to 133 eggs in a lifetime (Fleming, 1972).

Japanese beetle larvae feed on turfgrass roots and are known to be a pest of home lawns and golf courses. Adult Japanese beetles can damage ornamental trees, shrubs as well as crops. Odor is hypothesized to be the primary factor for adult beetle plant selection for feeding, but little to no research has tested this (Fleming, 1972). Based on the study conducted by (Fleming et al., 1934) a fruity odor in combination with sugar content seems to be the most attractive to Japanese beetle adults. Japanese beetles were also found to be more attracted to plants that were damaged overnight via feeding than non-damaged plants (Loughrin et al., 1996). Feeding-induced odors were described to most

likely be the cause of attraction, because they serve as indications that conspecifics have found a suitable host plant (Loughrin et al., 1996). Plants that release blends of volatile compounds are more likely to attract Japanese beetles than plants that produce simpler volatiles (Loughrin et al., 1998).

Japanese beetles are gregarious insects and the presence of a female on a plant attracts many males to the same plant (Smith and Hadley, 1926; Fleming, 1972). Males tend to feed in the early morning before females emerge from the soil and have erratic feeding behavior (Fleming, 1972). Males will either feed on one main plant or move from plant to plant. Both males and females are top down defoliators and have a preference for plants in open sun, rather than shade (Fleming, 1972; Hadley et. al., 1934).

In the United States an estimated amount of more than $460 million dollars is spent every year on control measures for Japanese beetle (Potter and Held, 2002; USDA, 2015). Damage caused by larvae alone is estimated at $234 million per a year, $78 million dollars for control costs and $156 million to replace damaged turf (USDA, 2015). In Michigan alone Japanese beetles damage to golf courses has required superintendents to treat all of their fairways, tees and greens once per year with a neonicotinoid insecticides. Adult beetles gregariously feed on plant and tree vegetation on courses, while larvae feeding on turf roots and root hairs cause turf browning.

Japanese beetle has also caused substantial economic losses to Michigan crops including chestnuts, hops, blackberries, blueberries and grapes (Szendrei et. al., 2005). Michigan produces a substantial amount of highbush blueberries that are impacted heavily by Japanese beetles (Szendrei and Isaacs, 2006). A survey administered to growers conducted in Michigan in 2003 indicated that Japanese beetle was a major pest problem for blueberry growers (Szendrei and Isaacs, 2006). Growers reported that Japanese beetles increase production costs by $72 per acre due to insecticide applications and clean cultivation (Szendrei and Isaacs, 2006). The emergence of Japanese beetles in early July as well as feeding and mating habits through mid-September perfectly align with highbush blueberry harvests in

Michigan (Fleming, 1972; Potter and Held, 2002; Szendrei and Isaacs, 2006). A majority of the cost to growers is not due to fruit damage from feeding, but from contamination of harvested fruit. Adults feeding directly on fruits such as grapes, blueberries and raspberries often cling to the fruits; sometimes even after death. In the past this has resulted in the temporary rejection of Michigan blueberries by major processors of these fruits for yogurts and other products. Not only is Japanese beetle a damaging insect on its own, but in some cases it increases damage from native pests. Feeding by Japanese beetles on grapes has been found to attract native green June beetles (*Cotinis nitidia*: Linnaeus) (Hammons et. al., 2009). When the Japanese beetles feed on grapes they contaminate the fruit with yeasts that elicit volatiles that act as aggregation kairomones for green June beetles (Hammons et al., 2009).

In Michigan, Japanese beetles are the most destructive pest to the nursery industry. A federal quarantine (7 CFR 301.48) prevents the movement of any agricultural product or vehicle infested with Japanese beetles across state borders (Fleming, 1972; Hadley et al., 1934; USDA, 2015). This has affected the nursery industry; making shipping plants from infected states to non-infected states impossible unless the plants have been certified by the Michigan Department of Agriculture and Rural Development as being free of Japanese beetle (Smitley, 1996). This has impacted revenue in the nursery industry.

Most attempts by the USDA to control Japanese beetle by importing natural enemies have not been successful. From 1920-1933 the USDA imported 49 different species of natural enemies of Japanese beetle from Asia into the United States; of these only two established and spread enough to have an impact (Potter and Held, 2002). One is *Tiphia vernalis:* Rohwer, a tiphiid wasp that oviposits in Japanese beetle larvae in spring and the second is *Istocheta aldrichi*: Aldrich, a tachinid fly that parasitizes Japanese beetle adults (Cappaert and Smitley, 2002; Potter and Held, 2002). Vertebrate predators including moles, skunks and raccoons can consume large numbers of Japanese beetle larvae; but unfortunately, they are destructive to turf in the process (Potter and Held, 2002). Starlings, crows,

sandhill cranes and some other birds in the United States are also predators of Japanese beetle, but all of them and cranes in particular can cause significant damage to turfgrass when digging for larvae.

Paenibacillus popilliae Dutky or milky disease was used extensively as a biological control agent of *Popilliae japonica* in the United States in the 1940's (Fleming, 1968; Potter and Held, 2002; Petty, 2013). Milky disease spores are ingested with soil by Japanese beetle larvae while they feed on the turf roots (Stahly and Klein, 1992; Potter and Held, 2002). Infection is initiated when spores adhering to the midgut wall germinate and penetrate epithelial cells (Dunbar and Beard, 1975; Hutton and Burbutis, 1974; Redmond and Potter, 1995). Proliferation of the bacterium in the hemocoel turns it a milk hemocoel white colour. At first it was thought that milky disease was a potential solution to Japanese beetle infestations in the United States, but over time it became clear that the milky disease bacterium did not maintain a high level of virulence at locations revisited five years or more after it was introduced (Redmond and Potter, 1995; Potter and Held, 2002). It has been demonstrated that commercial versions of milky disease produced in large fermentation vats are ineffective the year that they are applied (Redmond and Potter, 1995).

The entomopathogenic nematode *Steinernema glaseri* Steiner was grown in culture and introduced to many locations in several states in the United States in the 1940's (Fleming, 1968; Girth et. al., 1940). Initially it appeared to be a successful biological control agent of *Popilliae japonica*, but persistence was a problem (Potter and Held, 2002; Petty, 2014). Unfortunately the shelf life is short and the nematodes are sensitive to environmental changes such as: soil moisture and temperature (Fleming, 1968; Petty, 2014). Japanese beetles have many natural behaviors that defend against entomopathogenic nematodes too. These include grooming behaviors, avoidance methods and a high midgut pH.

Despite numerous efforts to control Japanese beetle through the use of biological control agents, with traps, and multiple applications of different insecticides, nothing has been effective in

controlling the spread and build-up to crop-damaging populations of Japanese beetles in the US (Cappaert and Smitley, 2002; Petty, 2014; Redmond and Potter, 1995). Japanese beetle has been detected in arriving cargo planes by California Department of Agriculture inspectors and California has an active Japanese beetle eradication program that includes trapping, turf treatment with imidacloprid or chlorantraniliprole, and selective adult sprays (Allsop, 1992; CDFA Quarantine Manual). Recently infested states such as Colorado are now severely impacted by the federal quarantine that regulates interstate shipping of nursery stock. As of December 30, 2016 all nurseries in Front Range counties of Colorado must comply with federal regulations, making sure all stock is beetle-free before transporting stock across non-infested counties or out of state. While numerous studies have discussed the economic damage caused by Japanese beetles over the last 100 years, we have still not identified a reliable set of predators, parasitoids and pathogens that can be introduced to states in the western USA when Japanese beetle is first found, to minimize the number of years when golf courses, nurseries, blueberry and raspberry farms, vineyards, and hop farms suffer significant crop losses and apply large amounts of insecticides (Szendrei and Isaacs, 2006; USDA, 2015).

The objectives of this thesis were to survey *O. popilliae* and scarab species at golf courses and highway rest areas in Michigan in 2018 and 2019. Compare results to the 1999 and 2000 survey conducted by Cappaert and Smitley, (2002) to the data collected in 2018 and 2019. To determine the survival of *O. popilliae*-infected larvae compared 'compared with healthy larvae from October to May', and to inoculate healthy Japanese beetle larvae to more accurately determine the impact of infection on survival.

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CHAPTER 1: Distribution of *Ovavesicula popilliae* (Microsporidia: Ovavesiculidae), a Pathogen of Japanese beetle (*Popillia japonica*), in Michigan

ABSTRACT

The prevalence of *Ovavesicula popilliae*, a species-specific microsporidian pathogen of Japanese beetle (*Popillia japonica*), in Michigan was determined by collecting host larvae and adults at 47 golf courses or rest areas. Larvae were collected in May of 2018 and May of 2019. At each location squares of turfgrass were cut and overturned to search the root system and soil for Scarabaeidae larvae. In addition, adults were collected at each location in late July, 2018 by setting four Japanese beetle traps for one week. Larvae and adults were frozen for later dissection to remove the malpighian tubules and examine them for the presence of *O. popilliae* sporophorous vesicles. Nine of the golf courses were chosen because they were previously sampled in 1999. The density of Japanese beetle larvae found at these nine golf courses in southern Michigan declined by 79% from 1999 to 2018. Also, Oriental beetle was found at three of nine golf courses in 2018, and two in 2019, but were absent at the same golf courses in 1999 and 2000. Asiatic garden beetle was found at one of nine golf courses in 2019. *Ovavesicula popilliae* was present at 22 of 47 sites, a substantial increase in prevalence when compared with the last comprehensive survey in 1999 and 2000 when *O. popilliae* was found at 2 of 50 sites in Michigan. The pathogen was active at seven of the sites where it had been introduced between 1999, 2000, 2002 and 2006. Spread to surrounding areas was slow over the 20 year period since the last survey.

1. Introduction

Japanese beetle (*Popillia japonica*) is an economically important invasive pest in the Eastern United States. Over 300 plant species have been identified as hosts of the adults (Fleming 1972, Held, 2004). Larvae prefer to feed on the roots of turfgrass, but may also feed on the roots of flowers, trees and shrubs; while adults feed on the foliage of a wide variety of flowers, shrubs and trees. Japanese

beetles may defoliate grapes and hops, but even more problematic is the fruit feeding on cherries, blueberries and raspberries (Fleming, 1972; Potter, 1998, Szendrei et al., 2005). Consumer backlash to insect contamination of food products has led to a zero-tolerance policy that puts considerable pressure on growers (Wise et al., 2007).

Ovavesicula popilliae is a microsporidian pathogen of Japanese beetle that was first discovered in Connecticut in 1988 (Hanula and Andreadis, 1988; Hanula, 1990). Up until this time *O. popilliae* has only been found in Japanese beetle and not from other scarab larvae collected from turfgrass in North America (Andreadis and Hanula, 1987; Vossbrinck and Andreadis, 2007; Petty et. al., 2012). However, Petty et al., (2012) were able to infect a few other species of scarab larvae using laboratory inoculations. Spores of *O. popilliae* are ingested by larvae from soil when feeding on turf roots (Petty et. al., 2012). The pathogen develops primarily in the malpighian tubules, which are used for removing waste from hemolymph, among other functions. Infection can carry on through pupation into the adult stage of Japanese beetle (Andreadis and Hanula, 1990). Infected malpighian tubules become swollen and packed with sporophorous vesicles, apparently compromising their function (Andreadis and Hanula, 1990). Infected larvae and adults release spores back into the soil via frass and upon death and disintegration of the body. Past studies have demonstrated that infected adult females produce 50% fewer mature eggs than uninfected females (Hanula, 1990; Smitley et al., 2011).

In 1999 and 2000, a survey of parasitoids and pathogens of Japanese beetle was conducted in Michigan (Cappaert et. al., 2002; Smitley et. al., 1999). A total of 11 golf courses and 24 other turfgrass sites, including blueberry farms and recreational turfgrass were sampled for scarab larvae in spring and fall of 1999 and 2000 and for adults in July and August of the same years (Cappaert et. al., 2002; Smitley et. al., 1999). *Ovavesicula popilliae* was only found at two of 35 sites in 1999 and 2000, both located near Kalamazoo, Michigan. The purpose of this current work is to document any changes in the

geographic distribution of *O. popilliae* in Michigan on golf courses and rest areas over the past decade. A secondary purpose is to see if the density of Japanese beetle larvae and the species composition of scarab larvae have changed in Michigan since 1999 at golf courses.

2. Materials and Methods

In 2018 22 golf courses and ten highway rest areas (Table 1.1, Fig. 1.2) were visited from May 9 to May 24 to collect Scarabaeidae larvae, and again from July 16 to July 19 to collect adults. At each site, turf was sampled for the presence of scarab larvae in May with a hand-held sod cutter by cutting 0.42 m^2 rectangles of turf and soil to a depth of 7.0 cm for examination. The turf was cut or torn into 100 cm^2 sections, shaken and examined for the presence of scarab larvae. At golf course sites, irrigated turf located near a fairway was sampled for one hour or until 50 Japanese beetle larvae were collected. A minimum of two turf rectangles with a total area of 0.84 m^2 was sampled at each site. Rest areas were sampled in the same way, but turfgrass in rest area lawns was low maintenance with no irrigation. Scarab larvae were placed into individual 59.1 ml-capacity paper cups containing 50 cm^3 of moist soil from under the turf rectangle. All cups were then stacked into piles of ten, placed into sealable plastic bags, labeled and put into a cooler for transport back to the laboratory. Once in the lab, the larvae were placed into 25-ml screw-cap scintillation vials (10 larvae per vial), filled with normal saline and stored at -20°C.

In 2019, 37 golf courses were sampled from May 15 to May 22 (Table 1.1, Fig. 1.2), including 22 golf course sites sampled in 2018. All larvae were collected and stored as previously described for 2018. Rest areas were not sampled in 2019 because of the low density of Japanese beetle larvae found at highway rest areas in 2018.

2.1. Detection of O. popilliae in larvae

Frozen vials containing Scarabaeidae larvae were placed into hot tap water (120° C) in a beaker until thawed and patted dry using paper towels. Individual larvae from each site were identified to species under a dissecting microscope by visually observing the rastral patterns (Potter, 1998). All Japanese beetle larvae were dissected so that between three and six malpighian tubules could be removed and mounted on a microscope slide with a drop of normal saline. Mounted malpighian tubules were visually scanned under a phase-contrast compound microscope. A larva was considered infected if sporophorous vesicles of *O. popilliae* were observed inside a tubule (Smitley et. al., 2011; Fig. 1.1). Dissection utensils were cleaned after each individual dissection by wiping them clean with a disposable tissue, then rinsing them sequentially in two separate beakers of normal saline.

2.2 Detection of O. popilliae in adult beetles

Adult Japanese beetles were collected by placing four pole-mounted standard Japanese beetle traps at a height of 128 cm baited with floral lures (phenylethyl propionate, geraniol, and eugenol, 3:7:3) and pheromone (Trécé, Salinas, CA) at each location between July 16 and July 20, 2018. Traps were collected after two weeks; beetles found in each trap were transferred to sealable plastic bags and chilled over ice packs in a cooler until they could be returned to the laboratory. From each trap, 50 beetles were placed in each of two 25-ml screw-cap scintillation vials of saline. Vials were stored at -20°C for pathogen diagnosis.

Each adult beetle was dissected with transverse cuts behind the pronotum and just anterior of the tip of the abdomen (Smitley et al., 2011). The exoskeleton was then cut longitudinally on the dorsal surface and opened. Dissected beetles were bathed in normal saline for optimal viewing of internal organs. Between three and six malpighian tubules were removed using fine forceps and mounted on a slide with a drop of saline. Pathogen diagnosis was determined as previously described for larvae.

2.3 Statistical Analysis

The density of Japanese larvae at nine golf courses (n = 9) in 2018 was compared with the same in 1999 with a paired student's t-test (PROC TTEST) in SAS 9.4 (SAS Institute, Cary, NC). Data were tested for normality, and normally-distributed variables were compared using the same procedure. Four of the scarab species proportions data were not normally distributed. Variables that were non-normal were analyzed with a nonparametric one way ANOVA (PROC NPAR1WAY) to determine differences between years. These same tests were performed for all golf courses sampled in 2018 (n=22) compared to the same golf courses (n=22) sampled in 2019.

3. Results

3.1 Density of Japanese beetle larvae and relative proportions of all scarab species found at golf course and highway rest area sites

Twice as many scarab larvae were collected from golf courses sampled in 2018 compared with 2019. Similarly, Japanese beetle larvae were found at eight of nine golf courses in 2018 and at only four of nine golf courses in 2019. For this reason the density of Japanese beetle larvae and the relative proportion of different species of scarab larvae found at nine golf courses sampled in 1999 were compared with the same in 2018 but not for 2019 (Tables 1.1, 1.2. A., 1.2. B. and 1.3). Nineteen years after a 1999 survey of nine golf courses in southern Michigan Japanese beetle continued to be the most abundant scarab found but the proportion of scarab larvae that were Japanese beetles dropped from 94.1 ± 3.9% in 1999 to 59.4 ± 13.9% in 2018 (P = 0.02, F = 2.79, Tables 1.3 and 1.5. A.). The decreased proportion of Japanese beetles found is associated with a decrease in the density of Japanese beetle larvae from 1999 (8.4 ± 2.1) to 2018 (1.8 ± 0.8) collected per 0.1 m^2, while other species became more abundant (P = 0.01, F = 3.12). Oriental beetle, which was not observed in 1999, was found at three of nine golf courses in 2018 (11.7 ± 8.1 per m^2). Although more European chafer and masked chafer were found in 2018 compared with 1999, the means were not significantly different.

3.2 Detection of O. popilliae at golf courses and highway rest areas in 2018 and 2019 compared with 1999

Japanese beetle larvae were collected in 2018 and 2019, and adults were collected in 2018 to determine presence or absence of *O. popilliae* at 47 sites in the southern half of Michigan. Sites where ten or more Japanese beetle larvae or adults were collected were included in the results of the survey.

Japanese beetle larvae or adults were found to be infected with *O. popilliae* at 22 of the 47 collection sites, a significant increase in distribution compared with a similar survey made in 1999 and 2000 when *O. popilliae* was only found at one location near Kalamazoo, Michigan (Cappaert and Smitley 2002, Figure 1.2).

4. Discussion

If the nine golf courses sampled are representative of all golf courses in southern Michigan, the density of Japanese beetle larvae declined by 79% from 1999 to 2018. During the same time *O. popilliae* has established and become epizootic in Japanese beetle larvae and adults at these sites. Although a negative relationship has been demonstrated between the proportion of the Japanese beetles infected with *O. popilliae* and the density of adult beetles trapped, we do not know if *O. popilliae* has caused the decline of Japanese beetle at these sites or if something else caused population declines during the same period. We did not find more than 2% of Japanese beetle adults or larvae to be infected with any other pathogen during our survey.

As Japanese beetle populations declined by 79% the relative proportion of scarab species found at nine golf courses changed from 1999 to 2018. Most notable was the appearance of Oriental beetle at four of nine golf courses and Asiatic garden beetle at one of nine golf courses, considering that they were not found at any of the same nine golf courses in 1999 and 2000. This is having an impact on golf courses, nurseries and field crops in Michigan because Oriental beetle is an important turfgrass and nursery pest, while Asiatic garden beetle is an important field crop pest (Potter, 1998; Vittum et. al.,

1999; Eckman, 2019). The mean density of Japanese beetle larvae found on all golf course sites in Michigan was greater than the density found in lawns of highway rest areas (Table 1.3). Most likely this is because the area of turfgrass sampled at golf courses was irrigated, while turfgrass at rest areas was not irrigated (Potter, 2002). At the same time more Asiatic garden beetle was found at highway rest areas than on golf courses for the opposite reason, they prefer non-irrigated, low maintenance turfgrass sites (Vittum et. al., 1999). Golf course sites in our survey tended to consist of well-fertilized and irrigated Kentucky bluegrass. Rest areas were not irrigated, and consisted of a mixture of Kentucky bluegrass, fine fescue and broadleaf weeds.

In the 1999 and 2000 survey *O. popilliae* was only recorded at two of the 35 sites sampled while in the present survey *O. popilliae* was recorded at 22 of 47 sites (Table 1.1, Cappaert and Smitley, 2003; Smitley et. al., 1999). *Ovavesicula popilliae* was introduced between 1999 and 2006 at eight of our 47 survey sites. It was found at seven of the eight introduction sites. Although *O. popilliae* was often found at golf courses located within 5 km of an introduction sites, but it has not yet been found at 25 of 47 survey sites, some of which were within 10 km of introduction sites (Figure 1.2). For example, *O. popilliae* was not found at the Hancock Turfgrass Research Center, located 2.9 km away from Beal Botanical Gardens at Michigan State University where it was introduced in 2006.

Overall this survey of golf courses and highway rest areas in Michigan documents a significant decline of Japanese beetle in the 20 year-period since the last survey, and slow spread of *Ovavesicula popilliae*, which was sometimes absent within 10 km of site where it was introduced 12 years earlier. However, *O. popilliae* has established and persisted at epizootic levels at almost every site where it was introduced. This indicates that moving infected beetles or larvae to as many locations as possible will help increase the natural rate of spread which may be as little as 20 km in 20 years.

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APPENDIX

Table 1.1. Michigan golf courses, highway rest areas and other sites sampled in 2018 and 2019 for Japanese beetle larvae and in 2018 for adults. The locations of each site are indicated on a map in Figure 1.2.

Site (Fig. 1)	Location	Years sampled	Site (Fig. 1)	Location	Years sampled
1	*Orchard Lake CC	2018, 2019	25	Carleton Glen GC	2019
2	*Eastern Hills GC	2018, 2019	26	Sycamore GC	2019
3	*Kalamazoo CC	2018, 2019	27	Indian Hills GC	2019
4	*Medalist CC	2018, 2019	28	Willow Wood GC	2019
5	*Willow Metropark GC	2018, 2019	29	Deer Run GC	2019
6	Binder Park GC	2018, 2019	30	*Beal Botanical Garden	2019
7	Pinelake CC	2018, 2019	31	MSU HTRC	2019
8	Olde Mill GC	2018, 2019	32	Baypointe GC	2019
9	*Currie GC	2018, 2019	33	Cedar Creek GC	2019
10	*Crooked Creek GC	2018, 2019	34	Hickory Ridge GC	2019
11	Collegefields GC	2018, 2019	35	Forest Akers East GC	2019
12	Royal Scott GC	2018, 2019	36	Forest Akers West GC	2019
13	Groesbeck GC	2018	37	Woodside GC	2019
14	Pine Valley GC	2018, 2019	38	Turkeyville RA	2018
15	Cracklewood GC	2018, 2019	39	Woodbury RA	2018
16	Grand Ledge CC	2018, 2019	40	Galesburg RA	2018
17	The Emerald GC	2018, 2019	41	Okemos RA	2018
18	Pleasanthill GC	2018, 2019	42	Dewitt RA	2018
19	Pohlcat GC	2018, 2019	43	Howell RA	2018
20	Pineview Highlands GC	2018, 2019	44	Potterville RA	2018
21	Quest GC	2018, 2019	45	Ithaca RA	2018
22	IMA Brookwood GC	2018, 2019	46	Clare Welcome RA	2018
23	Links at Crystal Lake GC	2019	47	Grandledge RA	2018
24	Union Lake GC	2019			

CC country club

GC golf course

RA rest area

* Indicates a site where *O. popilliae* was introduced in 1999, 2000, 2002 or 2006

Table 1.2. A. Density of Japanese beetle larvae and proportion of all species of scarab larvae found in the irrigated rough of nine golf courses in southern Michigan in April or May of 1999 from Smitley and Cappaert (1999) and Cappaert and Smitley (2002).

		Proportions of species of scarab larvae collected in April or May of 1999				
Original golf course sites	Japanese beetle larvae per $0.1m^2$	Japanese beetle (%)	European chafer (%)	Masked chafer (%)	Asiatic garden beetle (%)	Oriental beetle (%)
Medalist Golf Course	17.1	100	0	0	0	0
Binder Park Golf Course	12.4	100	0	0	0	0
Eastern Hills Golf Course	6.3	67.5	27.1	5.2	0	0
Kalamazoo Country Club	2.6	81	1.7	17.2	0	0
Cracklewood Golf Course	2.6	100	0	0	0	0
Pine Valley Golf Course	4.8	100	0	0	0	0
Orchard Lake Country Club	5.1	100	0	0	0	0
Pine Lake Country Club	6.1	98.0	2.0	0	0	0
Willow Golf Course	19.0	100	0	0	0	0

Table 1.2. B. Density of Japanese beetle larvae and proportion of all species of scarab larvae found in the irrigated rough of nine golf courses in southern Michigan in May of 2018 and May of 2019, compared with similar data from Table 1.2. A.

Proportions of species of scarab larvae collected in May of 2018 and 2019

Original golf course sites	JB larvae per $0.1m^2$		Japanese beetle (%)		European chafer (%)		Masked chafer (%)		Asiatic garden beetle (%)		Oriental beetle (%)	
	2018	2019	2018	2019	2018	2019	2018	2019	2018	2019	2018	2019
Medalist Golf Course	1.3	0	32.4	0	67.6	0	0	0	0	0	0	0
Binder Park Golf Course	0.24	0	100	0	0	0	0	0	0	0	0	0
Eastern Hills Golf Course	5.7	1.1	81.4	100	0	0	18.6	0	0	0	0	0
Kalamazoo Country Club	0.4	0	6	0	0	0	94	100	0	0	0	0
Cracklewood Golf Course	0.4	1.4	30	19.7	0	0	0	0	0	0	70.0	80.3
Pine Valley Golf Course	5.1	2.9	91.5	100	6.4	0	0	0	0	0	2.1	0
Orchard Lake Country Club	0.1	0	100	0	0	0	0	0	0	0	0	0
Pine Lake Country Club	0	0	0	0	66.7	0	0	0	0	0	33.3	0
Willow Golf Course	0.3	1.1	93.1	25.7	0	0	6.9	60	0	11.4	0	2.9

Table 1.3. Comparison of the density of Japanese beetle larvae and proportion of all species of scarab larvae found on nine golf courses in 1999 with the same in 2018.

Sites	Japanese beetle larvae per 0.1 m^2	Proportion of scarab species found (%)				
		Japanese beetle	European chafer	Masked chafer	Asiatic Garden beetle	Oriental beetle
Original golf course sites in 1999 (n = 9)	8.4 ± 2.1**	94.1 ± 3.9*	3.4 ± 3.0	2.5 ± 1.9	0 ± 0	0 ± 0
Original golf course sites in 2018 (n = 9)	1.8 ± 0.8**	59.4 ± 13.9*	15.6 ± 9.8	13.3 ± 10.3	0 ± 0	11.7 ± 8.1

* Indicates means for 1999 and 2000 are different at P = 0.05
** Indicates means for 1999 and 2000 are different at P = 0.01

Table 1.4. Comparison of the density of Japanese beetle larvae and proportion of all species of scarab larvae found on twenty two golf courses in 2018 with the same in 2019.

Sites	Japanese beetle larvae per 0.1 m^2	Proportion of scarab species found (%)				
		Japanese beetle	European chafer	Masked chafer	Asiatic Garden beetle	Oriental beetle
Golf courses in 2018 (n = 22)	2.9 ± 0.5 a[1]	68.9 ± 8.3 a	16.1 ± 6.3 a	9.7 ± 5.8 a	0.3 ± 0.2 a	5.4 ± 3.6 a
Golf courses in 2019 (n = 22)	2.0 ± 0.5 a	62.0 ± 9.8 a	4.6 ± 2.5 a	12.5 ± 6.9 a	16.0 ± 7.1 ab	4.9 ± 4.7 a

[1] Means for 'all golf courses in 2018, 'all golf courses in 2019' are not significantly different by Tukey HSD multiple means comparison at P = 0.05 if they are followed by the same letter.

Table 1.5. A. Statistical analysis for comparison of density of Japanese beetle larvae and the proportions of all scarab species found on nine golf courses in Michigan in 1999 with the same in 2018 (in Table 1.3). The factor indicating comparison of 1999 data to 2018 data is 'Year'. Statistics below are for a standard T-test made with SAS 9.4 (SAS Institute).

Variable	**Factor**	**Degrees of Freedom**	**t value**	**Pr (> t)**
Density of Japanese beetle larvae in 1999 and 2018	Year	8	3.12	0.01
Percent Japanese beetle (of all scarabs)	Year	8	2.47	0.04

Table 1.5. B. Statistical analysis for comparison of density of Japanese beetle larvae and the proportions of all scarab species found on nine golf courses in Michigan in 1999 with the same in 2018 (in Table 1.3). The factor indicating comparison of 1999 data to 2018 data is 'Year'. Statistics below are for a Wilcoxon Nonparametric ANOVA made with SAS 9.4 (SAS Institute).

Variable	Factor	Degrees of Freedom	Z value	Pr (> Z)
Percent European chafer (of all scarabs)	year	1	-0.32	0.76
Percent Masked chafer (of all scarabs)	Year	1	-0.67	0.51
Percent Asiatic garden beetle (of all scarabs)	Year	1	0.00	1.00
Percent Oriental beetle (of all scarabs)	Year	1	-1.77	0.10

Table 1.6. A. Statistical analysis for comparison of density of Japanese beetle larvae and the proportions of all scarab species found on twenty two golf courses in Michigan in 2018 with the same in 2019 (in Table 1.4). The factor indicating comparison of 2018 data to 2019 data is 'Year'. Statistics below are for a standard T-test made with SAS 9.4 (SAS Institute).

Variable	**Factor**	**Degrees of Freedom**	**t value**	**Pr (> t)**
Density of Japanese beetle larvae in 2018 and 2019	Year	21	1.73	0.10
Percent Japanese beetle (of all scarabs)	Year	15	0.41	0.69

Table 1.6. B. Statistical analysis for comparison of density of Japanese beetle larvae and the proportions of all scarab species found on twenty two golf courses in Michigan in 2018 with the same in 2019 (in Table 1.4). The factor indicating comparison of 2018 data to 2019 data is 'Year'. Statistics below are for a Wilcoxon Nonparametric ANOVA made with SAS 9.4 (SAS Institute).

Variable	**Factor**	**Degrees of Freedom**	**Z value**	**Pr (> Z)**
Percent European chafer (of all scarabs)	Year	1	-1.16	0.25
Percent Masked chafer (of all scarabs)	Year	1	0.04	0.97
Percent Asiatic garden beetle (of all scarabs)	Year	1	2.12	0.04
Percent Oriental beetle (of all scarabs)	Year	1	-0.82	0.41

Figure 1.1. Mature sporophorous vesicle of *O. popilliae* from the malpighian tubule of a Japanese beetle adult collected in Michigan in 2019.

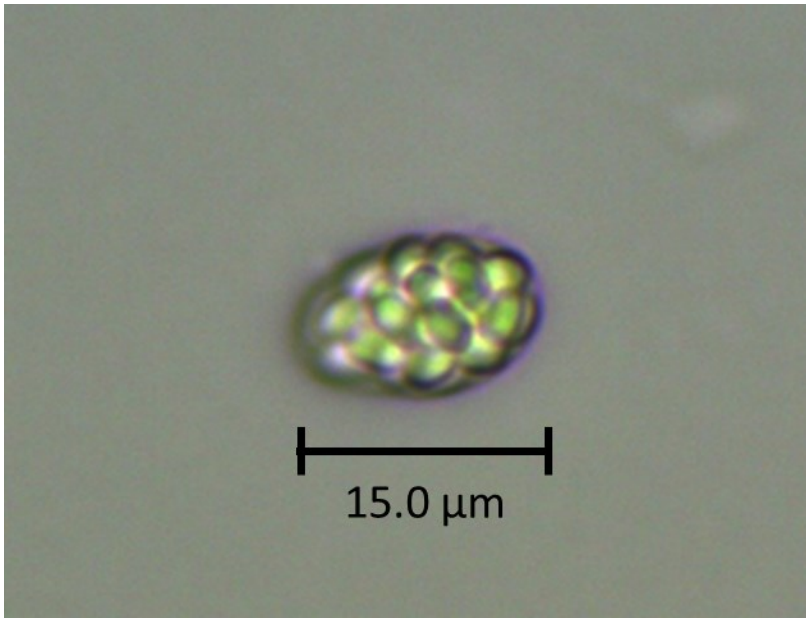
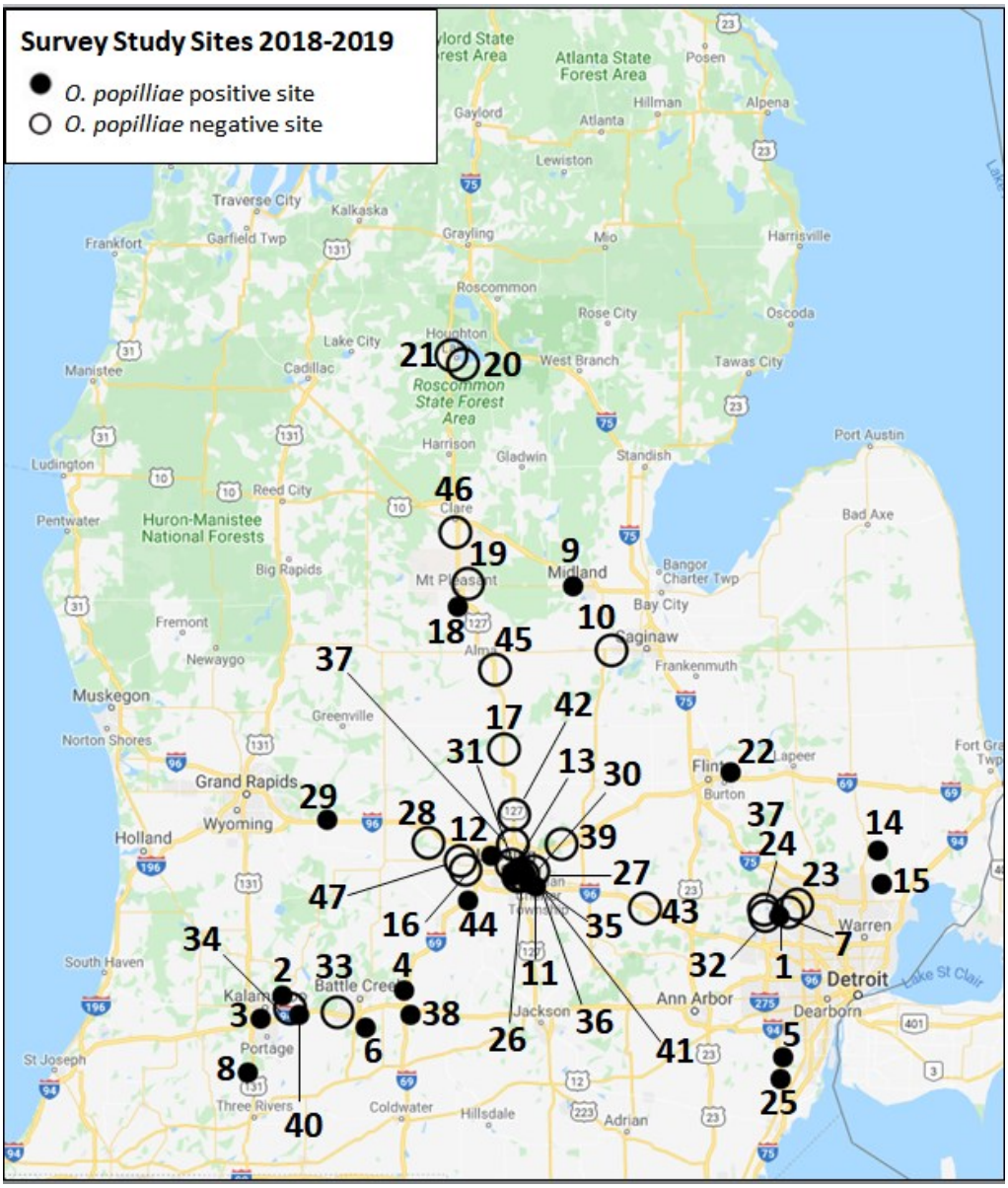


Photo by Lance Forsberg

Figure 1.2. Map of golf courses and highway rest areas visited in MI and sampled for Scarabaeidae larvae in May of 2018 and May of 2019, and for adult Japanese beetles in July or August of 2018. Only sites where more than ten Japanese beetle larvae were collected or more than 100 adult beetles were used to classify a site as positive or negative for *O. popilliae*. If the pathogen was found in any of the larval or adult samples the site was considered 'positive'.



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CHAPTER 2: Survival of Japanese beetle (*Popillia japonica*) larvae infected with *Ovavesicula popilliae (Microsporidia: Ovavesiculidae),* compared with healthy larvae in field plots from October to May

ABSTRACT

Japanese beetle (*Popillia japonica*) is the most important pest of golf courses in the Midwestern United States. Beetle larvae consume the roots of fairway turfgrass, which wilts and dies. This study was conducted to determine the survival of *Ovavesiucla popilliae*-infected larvae compared with healthy larvae during their overwintering period, from October to May. Larvae were placed into plastic sleeve-pots containing 15 cm-diameter cores of turfgrass with roots and soil intact. Larvae were collected from two sites, one where *O. popilliae* was active and one where it had not yet been detected. *Ovavesicula popilliae* infection of larvae at the beginning of the experiment (34.0 and 26.9%, for October 2017 and October 2018, respectively), and the use of soil cores from a site where the pathogen was active had a significant impact on the survival of larvae (90 – 100% reduction). The experiment was repeated two years (October to May of each year) with similar results.

1. Introduction

Ovavesicula popilliae (Andreadis and Hanula, 1987), a microsporidian pathogen of Japanese beetles, was discovered in Connecticut in 1988 (Hanula and Andreadis, 1988; Hanula, 1990)**.** It infects the malpighian tubules of larvae and adults but the impact of infection on the survival of larvae has not been experimentally confirmed. Spores of *O. popilliae* are ingested by Japanese beetle larvae from soil as they feed on turf roots. Infected malpighian tubules become swollen and packed with sporophorous vesicles, compromising their function (Vossbrinck and Andreadis, 2007; Hanula and Andreadis, 1990). Reduced function of the malpighian tubules impairs proper removal of waste products from the hemocoel and likely leads to many adverse health problems. Frass of infected Japanese beetle larvae and adults contain large numbers of spores that persist in soil and may initiate infection when ingested (Petty et. al., 2012; Smitley et. al., 2011).

Between 1999 and 2008 research in Michigan indicated the incidence of *O. popilliae* infection was positively correlated with higher mortality rates of Japanese beetle larvae from October to April in field plots (Smitley et. al., 2011). However no experiments have investigated the survival rate of infected larvae compared with healthy larvae. This study was conducted to determine Japanese beetle mortality due to infection by *O. popilliae*.

2. Materials and Methods

2.1 Collection of Japanese beetle larvae from sites with and without O. popilliae-infected larvae for use in field experiments, and pathogen diagnosis

On 28th September, 2017, ≥ 450 Japanese beetle larvae were collected from Eastern Hills Golf Course, an *O. popilliae* epizootic site near Battle Creek, Michigan. The following day a similar number of larvae were collected from the Hancock Turfgrass Research Center, Michigan State University, in East Lansing, Michigan, where *O. popilliae* had not been detected. At both locations larvae were collected by turning over infested sod and putting larvae into paper cups (59.1 mL, one larva per cup) with an enough soil to cover them. All cups were then stacked into piles of ten, placed into sealable plastic bags, labeled, and put into a cooler over ice packs. These larvae were used the same day or the following day for infesting experimental sleeve pots containing soil cores at both locations.

A subsample of 50 larvae from each site were stored in paper cups with soil as previously described and transported back to Michigan State University where they were placed into 25-mL screw-cap scintillation vials (10 larvae per vial) filled half-way with normal saline and stored at -20°C. On several different occasions the next month, some of the vials containing larvae were placed into hot tap water in a beaker until thawed. Larvae were then gently patted dry using paper towels. Only the number of larvae that could be dissected within two hours of thawing was taken out of the freezer at one time to avoid proliferation of secondary organisms. Larvae were identified to species under a dissecting

microscope by visually observing their rastral patterns (Potter, 1998). Fifty Japanese beetle larvae from each site were dissected and between three and six malpighian tubules removed and mounted on a microscope slide with a drop of normal saline. Mounted malpighian tubules were visually scanned under a phase-contrast compound microscope at 10 X and 45 X magnifications. A larva was considered infected if the sporophorous vesicles of *O. popilliae* were observed inside a tubule (Smitley et. al., 2011). Dissection utensils were cleaned after each individual dissection by rinsing them sequentially in two separate beakers of normal saline and wiping them down with a clean delicate task wipe (Kimwipes) after each soak.

The experiment was repeated the following year, from October 2018 to May 2019. Methods are the same as previously described with the following exceptions. Larvae were collected on (September 21, 2018), and turf cylinders were dug and surviving larvae collected on (May 7, 2019).

2.2 Experimental field plots at sites with and without O. popilliae-infected Japanese beetles

This experiment consisted of six replications of four treatments: (1) larvae collected at the Hancock Center (all healthy larvae) put into turf cores at the Hancock Center, (2) larvae collected at Eastern Hills (at least 25% infected larvae) put into turf cores at the Hancock Center, (3) larvae collected from the Hancock Center put into turf cores at Eastern Hills, and (4) larvae collected at Eastern Hills put into turf cores at Eastern Hills (Table 2.1). Turf and soil cores were dug using a standard-sized golf course cup-cutter. Turf and soil cores were then placed into an 11.53 cm-diameter black plastic flower pot (Greenhouse Megastore Cox) with eight 1 cm diameter holes in the bottom for drainage. The drainage holes were small enough so Japanese beetle larvae could not escape, but wide enough to allow adequate water drainage. Sleeve-pots filled with soil cores were reinserted back into the ground where the core had been removed. A soil sample was collected from both the Hancock Turfgrass Research

Center and Eastern Hills golf course in 2017 and 2018. Both years soil samples were sent to Michigan State University Soil and Plant Nutrient Laboratory for soil analysis.

In order to avoid competition among larvae put into soil cores in sleeve-pots, only three Japanese beetle larvae were placed into each core. Larvae were observed until they tunneled into the soil. Each of the six replicates had a total of 8 pots and two treatments; four pots per each treatment. The total number of turf cores used for the experiment was 96. Larvae collected from the Hancock Center put into plots at the Hancock Center, and larvae collected from Eastern Hills and put into plots at Eastern Hills were collected the same day (September 29, 2017), while larvae from the opposite location were collected the previous day (September 30, 2017). On May 7 or 8, 2018, after Japanese beetle larvae had been in sleeve-pots in the field for seven months, the turf and soil cores were removed from sleeves in the field and placed on a cafeteria tray, where they were examined carefully for the presence of surviving larvae. Larvae from each pot were placed into individual paper cups and brought to Michigan State University and frozen as previously described. Larvae were thawed and dissected as previously described for pathogen analysis.

The same experiment was repeated the following year, from October 2018-to May 2019. Larval collection and plot construction for both sites (Hancock Turfgrass Research Center, Eastern Hills golf course) was repeated as previously described for October of 2017 with the following exceptions. Larvae collected from both sites were identified to species with a hand lens to make sure all of the collected larvae were Japanese beetle. Also, in October of 2018, the six replicates had a total of 10 pots and two treatments; five pots per each treatment for a total of 120 turf cores in the experiment. Larvae collected from the Hancock Center put into plots at the Hancock Center, and larvae collected from Eastern Hills and put into plots at Eastern Hills were collected the same day (September 29, 2018), while larvae from the opposite location were collected the previous day (September 30, 2018). After larvae had been in

sleeve-pots in the field for seven months, the turf and soil cores were removed and examined for surviving larvae on May 7, 2019, as previously described.

2.3 Statistical Analysis

Data from 2017 and 2018 were combined for analysis in SAS 9.4 (SAS Institute; Cary, NC) using the generalized linear mixed model procedure with a binomial distribution. Percent mortality was calculated for each treatment and replication by dividing the number of larvae found at the end of the experiment in May by the number of larvae added to sleeve-pots the previous October, then multiplying the decimal by 100. A two-way ANOVA procedure was used to compare infection by location, by source, and source compared between locations (interactions). We analyzed mortality of larvae based on source of larvae, plot location, and interactions between larvae sources and plot locations were analyzed. Year was found to not be significant and thus was assigned as a random effect during the analysis of both years data pooled together for analysis.

3. Results

3.1 Survival of Japanese beetle larvae in field plots from October 2017 to May 2018

Of the 50 Japanese beetle larvae collected at the Hancock Turfgrass Research Center as a subsample of the larvae used to infest our plots, none were identified as positive for *O. popilliae*. Of the 50 Japanese beetles larvae collected at Eastern Hills golf course as a subsample, three were unable to be dissected thus 47 were dissected and 16 (34%) were positive for *O. popilliae*.

The Hancock turfgrass Research Center had loamy sand like soil: 81.7% sand, 10.9% silt, and 7.4% clay in 2017. In 2018 the soil type was loamy sandy: 79.8% sand, 10.8% silt, and 9.4% clay. Eastern Hills golf course soil type was also primarily sandy loam in 2017: 67.5% sand, 20.9% silt, and 11.6% clay. In 2018 Eastern Hills golf course's soil test came back sandy loam again: 67.8% sand, 19.8 % silt, and

12.4% clay. The soil pH at the Hancock Turfgrass Research Station was 7.7 in 2017 and 2018. The soil pH in 2017 and 2018 at Eastern Hills golf course was 6.9 and 7.5.

When experimental turf cores were dug and examined for surviving larvae in May 2018 (7 months after field-collected larvae were placed into turf cores) at the Hancock Turfgrass Center and at Eastern Hills Golf Course, the proportion of all larvae species found was recorded. The proportion of scarab larvae recovered in May that were Japanese beetle in treatments 1 – 4, as described in Table 2.1 are 87.5%, 61.7%, 100%, and 71.4%, respectively, for the four treatments. The proportions of Masked chafers collected in each treatment are: 0.0%, 27.7%, 0.0%, and 28.6%, respectively. The small proportions of remaining larvae were European chafer and Oriental beetle. Therefore, 62 – 71% of the larvae collected from Eastern Hills Golf Course were Japanese beetle, while 27 – 28% was masked chafer. In contrast, 100% of the larvae collected from the Hancock Center were Japanese beetle. Because we did not know exactly how many Japanese beetle larvae were added to sleeve-pots in October, Japanese beetle survival was calculated as the number of the Japanese beetle larvae found in May divided by the total number of larvae used to infest pots the previous October. Also, only Japanese beetle larvae that weighed more than 0.1g at the end of the 7 month experiment were counted because larvae smaller than that could not have been the same larvae added to pots the previous October. The proportions of surviving Japanese beetle larvae in the four treatments as listed in Table 2.1 are 77.8%, 65.3%, 68.1%, and 48.6%. More of the Japanese beetle larvae collected from the Hancock Center and put into turf cores at both locations survived (66.4 ± 3%), than larvae collected from Eastern Hills golf course and put into turf cores at both locations (42.2 ± 3%, Table 2.1).

3.2 Survival of Japanese beetle larvae in field plots from October 2018 to May 2019

Because some masked chafer were present in areas where larvae were collected in fall of 2017, all of the collected larvae were identified to species using a hand lens in fall of 2018, so that only Japanese beetle larvae were used to infest pots for the experiment. A sub-sample of fifty Japanese

beetle larvae from each location was examined for pathogen infection. Larvae collected from the Hancock Turfgrass Research Center had an infection rate of 0%, while larvae collected from Eastern Hills Golf Course had an infection rate of 26.9%. After seven months in field plots, the mean proportion of surviving Japanese beetle larvae in treatments 1 – 4, as described in Table 2.1 are : 63 ± 4% , 51 ± 4% , 70 ± 3% , and 34 ± 4%, respectively.

3.3 Results of statistical analysis for both years combined

Analysis of combined survival data for both years with a two-way ANOVA revealed that the there was a significant interaction between the source of the larvae and the location of the experiment ($F = 13.91$; df = 1, 57; $P = 0.0004$). Source of the larvae collected for the experiment (either Eastern Hills Golf Course or Hancock Center) significantly affected survival ($F = 55.72$; df = 1, 57; $P<0.0001$), while plot location (at Eastern Hills or at Hancock Center) did not affect survival ($F = 2.41$; df = 1, 57; $P=0.126$).

The interaction of source of larvae with location of the experiment significantly affected infection rates of larvae recovered at the end of the experiment ($F = 6.57$; df = 1, 57; $P < 0.02$). Japanese beetle larvae collected in October from Eastern Hills Golf Course had a much higher infection rate (20%) at the end of the experiment in May than larvae collected from the Hancock Center (1.0 – 7.3 %). The source of larvae was also a significant factor in determining infection rates of larvae recovered at the end of the experiment in May ($F=27.7$; df= 1, 57; $P < 0.0001$). Unlike survival rate, infection rate was also influenced by location of the experiment ($F = 6.5$; df= 1, 57; $P < 0.03$).

4. Discussion

This study is the only research to date that to compare survival of *O. popilliae*-infected and non-infected Japanese beetle larvae. The results show that survival of larvae was driven by the source of larvae used to infest experimental pots and not plot location (locations with and without active *O. popilliae*). However, infection rates at the end of the experiment were impacted by both source of larvae and location of the experiment. Location could have impacted percent infection but not mortality

because infections initiated after the start of the experiment in early October by spores in the soil may not have compromised larvae as much as infections initiated much earlier in the infected larvae used to infest pots.

Previous studies have looked at the spore production of *O. popilliae* in Japanese beetle (Petty et. al., 2012), the route of *O. popilliae* infection of Japanese beetles (Andreadis and Hanula, 1990), decreases in Japanese beetle densities correlated with infection of *O. popilliae*, and reduced fecundity of infected adult females (Hanula, 1990; Smitley et. al., 2011). While these studies increased our understanding of *O. popilliae* disease development, direct evidence of Japanese beetle mortality due to infection by *O. popilliae* was lacking. In the first year of this experiment, October 2017 to May 2018, survival of Japanese beetle larvae with an initial *O. popilliae* infection rate of 34% was 48.6% at Eastern Hills, compared with a survival rate of 68.3 % for larvae with 0 % infection (Table 2.1). In the second year of this experiment, October 2018 to May 2019, the results were similar. Survival of Japanese beetle larvae with an initial infection rate of 26.9 % was 34.4 % at Eastern Hills, compared with a survival rate of 68.8% for larvae with an initial infection rate of 0% (Table 2.2). Therefore larvae that were initially infected with *O. popilliae* at a rate of 27 – 30% in October had an increased rate of mortality (an increase of 20 – 34%) compared with larvae that were not infected in October. This means that most of the larvae that were infected in October must have died before May, and that more larvae became infected between October and May. The results were consistent for both years of the experiment.

An alternative explanation for increase in mortality of larvae collected at Eastern Hills, and that is if larvae were weakened by an undetected pathogen that could have been present. All of the 50 larvae in the sub-sample of larvae used to infest pots were examined for the most important pathogens of Japanese beetle and none were found. By comparing the survival of healthy larvae collected from a location without *O. popilliae* to the survival of infected larvae from a location with active *O. popilliae* we can conclude that most of the increased mortality observed from October to May in larvae from an *O.*

popilliae-active site was likely caused by the pathogen directly or by the pathogen weakening larvae enough to make them susceptible to secondary infections (Table 2.1; Table 2.2). These survival rates also provide evidence that *O. popilliae* could be a successful biological control agent over time. More controlled experiments are needed in the future to eliminate the possibility that other undetected pathogens are playing a role in the results.

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APPENDICES

APPENDIX A. TABLES AND FIGURES

Table 2.1. Survival of un-infected Japanese beetle larvae collected from Hancock Turfgrass Research Center compared with survival of infected larvae collected from Eastern Hills golf course, from October 2017 to May 2018

Treatment	Larvae source	Experimental turf plot location	Percent *O. popilliae* infection of larvae at source in October 2017	Percent *O. popilliae* infection of larvae in May 2018	Percent survival of larvae from Oct 2017 to May 2018 ($\bar{x}$ ± SE)
1	Hancock Turfgrass Center	Hancock Turfgrass Center	0	0	77.8 ± 5.9
2	Eastern Hills Golf Course	Hancock Turfgrass Center	34	27.6	65.3 ± 8.8
3	Hancock Turfgrass Center	Eastern Hills Golf Course	0	0	68.1 ± 6.7
4	Eastern Hills Golf Course	Eastern Hills Golf Course	34	8.0	48.6 ± 10.0

Table 2.2. Survival of un-infected Japanese beetle larvae collected from Hancock Turfgrass Research Center compared with survival of infected larvae collected from Eastern Hills golf course, from October 2018 to May 2019.

Treatment	Larvae source	Experimental turf plot location	Percent *O. popilliae* infection of larvae at source in October 2018	Percent *O. popilliae* infection of larvae May 2019	Percent survival of larvae from Oct 2018 to May 2019 ($\bar{x}$ ± SE)
1	Hancock Turfgrass Center	Hancock Turfgrass Center	0	12.7	61.1 ± 6.57
2	Eastern Hills Golf Course	Hancock Turfgrass Center	26.9	20.0	55.5 ± 7.0
3	Hancock Turfgrass Center	Eastern Hills Golf Course	0	1.8	68.8 ± 5.87
4	Eastern Hills Golf Course	Eastern Hills Golf Course	26.9	16.4	34.4 ± 8.53

APPENDIX B. RECORD OF DEPOSITION OF VOUCHER SPECIMENS

RECORD OF DEPOSITION OF VOUCHER SPECIMENS

The specimens listed below have been deposited in the named museum as samples of those species or other taxa, which were used in this research. Voucher recognition labels bearing the voucher number have been attached or included in fluid preserved specimens.

Voucher Number: 2019-07

Author: Michael A. Piombino IV

Title of Thesis: Biological Control of Japanese Beetle (*Popillia japonica*) through the use of the Microsporidian Pathogen, *Ovavesicula popilliae*

Museum(s) where deposited: Albert J. Cook Arthropod Research Collection, Michigan State University (MSU)

Specimens:

Table A.1 Quantity and preservation method of Scarabaeidae species turned in as voucher specimens to the Albert J. Cook Arthropod Research Collection

Family	Genus-Species	Life Stage	Quantity	Preservation
Scarabaeidae	*Popillia japonica*	Adult	15	Pinned

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CHAPTER 3: Preliminary Research on Inoculation of healthy Japanese beetle (*Popillia japonica*) larvae with *Ovavesicula popilliae (Microsporidia: Ovavesiculidae)* spores in soil

ABSTRACT

In previous research high rates of *Ovavesicula popilliae* infection of Japanese beetle larvae and adults has been correlated with lower densities of Japanese beetle (Smitley et al., 2011). More research is needed to investigate how to inoculate healthy Japanese beetle larvae and adults to more accurately determine the impact of infection on survival. Soil was collected from two locations: Eastern Hills golf course where *O. popilliae* has persisted in the soil for 20 years and the Hancock Turfgrass Research Center where *O. popilliae* is not present. Soil form both locations were evenly distributed into 0.35 l plastic cups and turfgrass was grown in each cup. Three Japanese beetle larvae collected from the Hancock Turfgrass Research Center were placed in every cup and allowed to feed in the two types of soil for 13 d, before being placed into black sleeve pots filled with non-inoculated soil from the Hancock Center and put into an experimental field plot in October 2018. Larvae were dug up in May 2019 and malpighian tubules were removed and assessed under a phase-contrast compound microscope at 10 X and 45 X magnifications to assess infection by *O. popilliae* and for successful inoculation. Survival and infection rates of Japanese larvae from the experimental plot that previously were fed in the laboratory in the two different types of soils was calculated and compared. I did not successfully demonstrate inoculation of Japanese beetle larvae through the use of *O. popilliae* spore ridden soil.

1. Introduction

Insecticides have become the most prevalent way to treat Japanese beetle (*Popillia japonica*) on golf courses and home lawns in the United States (Vittum et al., 1999). Golf courses specifically seek to manage Japanese beetle populations to avoid browning from the larvae feeding on the turf roots and secondary damage from skunks, birds and other rodents that leave holes in the turf seeking larvae for

food (Vittum et al., 1999). In 1986 a survey was conducted that resulted in the reveal that 25% of Japanese beetles at a site in Connecticut were infected with *Ovavesicula popilliae* (Hanula and Andreadis, 1988). *Ovavesicula popilliae* was identified as a species-specific microsporidian, and ruled as a potential candidate for long term control of Japanese beetles, due to the correlation seen when looking at Japanese beetle densities in relation to number of infected larvae (Hanula, 1990). This was confirmed by Smitley et al. (2011) who reported a positive correlation between larval mortality rate of Japanese beetles and incidence of *O. popilliae*.

The mature spore stage of *O. popilliae* is called a sporophorous vesicle (Hanula, 1990). Sporophorous vesicles produce spores that are found in the malpighian tubules of Japanese beetle larvae and adults (Hanula, 1990). Infection occurs when spores of *O. popilliae* are ingested while larvae are feeding on the roots of turf and ingest spore contaminated soil. *Ovavesicula popilliae* infection continues through pupation and into adulthood if the larva does not die from the infection. Adults fly and disperse spores via frass and through their decaying remains. Hanula and Andreadis (1990) described that *O. popilliae* infection in Japanese beetle causes malpighian tubules to swell and distort, which may impact their functionality and impede excretion (Hanula and Andreadis, 1990).

Can Japanese beetle larvae be inoculated by feeding in soil for two weeks that contains spores of *O. popilliae*? I hypothesized that Japanese beetle larvae that fed for two weeks in inoculated soil would have higher infection rates of *O. popilliae* than Japanese beetle larvae that fed in non-inoculated soil.

2. Materials and Methods

2.1 Stadium cups and soil

On 15th and 16th September, 2017, two four-gallon buckets were filled with inoculated soil from Eastern Hills golf course and non-inoculated soil from the Hancock Turfgrass research Center. The soil

was distributed into two different kinds of 0.35 l polypropylene plastic stadium cups, one white and the other clear (polypropylene cups by Berry Plastics; Pro-Kal clear deli containers by Fabri-Kal). White cups received inoculated soil from Eastern Hills golf course an *O. popilliae* epizootic site near Battle Creek, Michigan. The clear cups received non-inoculated soil from the Hancock Turfgrass Research Center, Michigan State University, in East Lansing, Michigan, where *O. popilliae* has not been detected. A total of 120 cups were used in the lab, 60 per treatment. All cups were topped off with 20 ml of a mixture of Kentucky bluegrass and fine fescue grass seed provided by the Hancock Turfgrass Research Center staff. The cups and grass seed were first watered on the 15th and 16th of September, 2018. A soil sample from the plot at the Hancock Turfgrass Research Center was taken and sent over to Michigan State University's Soil and Plant Nutrient Laboratory for analysis.

A total of 410 Japanese beetle larvae were collected from the Hancock Turfgrass Research Center on the 21, September, 2018. Larvae were collected by rolling up the turf by hand from a plot at the Hancock Center and hand collecting larvae. Larvae were identified in the field as Japanese beetles by using a hand lens to look at their raster pattern (Potter, 1998). Japanese beetle larvae were put into paper cups (59.1 mL, one larva per cup) with an enough soil to cover them. All cups were then stacked into piles of ten, placed into sealable plastic bags, labeled, and put into a cooler over ice packs. Once in the lab, three larvae were placed into every cup, with even spacing to minimize competition. The Japanese beetle larvae in both treatments were allowed to feed on the turf roots of the grass in their cups for 13 days. It was hypothesized that larvae feeding in the inoculated soil from Eastern Hills golf course would have higher infection rates and lower survival than larvae that fed in non-inoculated soil from the Hancock Center.

Treatment five was Hancock larvae feeding in non-inoculated soil from the Hancock Center and treatment six were Hancock larvae feeding in inoculated soil from Eastern Hills golf course. All cups were watered evenly every day to proliferate the grass seed. This was done, so that Hancock larvae in

inoculated soil from Eastern Hills golf course while feeding on the turf roots would also ingest soil containing spores of *O. popilliae* and would become infected (Smitley et. al., 2011). Subsamples of 50 larvae were collected to determine the presence or absence of *O. popilliae* in larvae used in the experiment. Larvae placed into 25-mL screw-cap scintillation vials (10 larvae per vial) filled half-way with normal saline and stored at -20°C. Within the next two days, the vials containing frozen larvae were placed into 120° hot tap water in a beaker until thawed. Larvae were then gently patted dry using paper towels before dissection. Only the number of larvae that could be dissected within two hours of thawing was taken out of the freezer at one time to avoid proliferation of secondary organisms. Larvae identifications were checked again during dissection by visually looking at Japanese beetles known raster pattern under a dissecting microscope (Potter, 1998 Fleming, 1968; Fleming, 1972). Three to six malpighian tubules were removed and mounted on a microscope slide with a drop of normal saline. Mounted malpighian tubules were visually scanned under a phase-contrast compound microscope at 10 X and 45 X magnifications. A larva was considered infected if the sporophorous vesicles of *O. popilliae* were observed inside a tubule (Smitley et. al., 2011). Dissection utensils were cleaned after each individual dissection by rinsing them sequentially in two separate beakers of normal saline and wiping them down with a clean delicate task wipe (Kimwipes) after each soak.

2.2. Inoculation of healthy larvae in field plots with O. popilliae spores

A field plot of irrigated Kentucky bluegrass (*Poa pratensis L.)* was reserved at the Hancock Turfgrass Research Center located at Michigan State University. No insecticides had been applied to the plot. The plot was 18.89m by 16.45m and consisted of 120 holes dug by a lever action golf course cup cutter. Each hole was 0.60m apart from all other holes in all directions. Each soil core extracted by the cup cutter was placed back into an 11.43cm diameter black plastic pot (Greenhouse Megastore Cox). The plastic pots all had eight 1 cm diameter small holes for drainage on the underside of the pots. Pots were then placed back into the initial hole dug from the cup cuter. The plot was stratified into ten

columns and twelve rows, and consisted of two treatments: (Non-Infected larvae) Hancock Center larvae that fed in non-inoculated Hancock center soil in the lab and (Inoculated larvae) Hancock larvae that fed in inoculated soil from Eastern Hills in the laboratory. Each row and column was marked using turf markers. Experimental groups and control groups were randomly assigned and alternated. Treatment five was the control group and consisted of Hancock Turfgrass Research Center larvae that were allowed to feed in the lab in non-inoculated soil (Hancock soil) and was designated by the regular black color of the pots. Treatment six was the experimental group, inoculated Hancock Center larvae that were allowed to feed in (Eastern Hills soil) and was designated by spray painting the black pots rims yellow. Treatment five's black pots received three (Non-Infected larvae), while treatment six's yellow-rimmed pots received three (Inoculated larvae) on the 3rd of October, 2018. Do to over-watering, some Japanese beetle larvae died while in the laboratory inside the plastic cups and thus were not placed into field plots. Because of this of the 120 pots in the field; five pots from treatment six received only one larva, while four pots in treatment five received only two larvae. The remaining 111 pots received three larvae each. A total of 346 Japanese beetle larvae were added to the plot.

At the end of the experiment seven months later on May 7, 2019, turf cylinders in the pots were emptied and sifted through for surviving Japanese beetle larvae. Larvae from each pot were placed into individual paper cups and brought to Michigan State University and frozen as previously described. Larvae were thawed and dissected as previously described for pathogen analysis.

2.3. Statistical Analysis

Data was analyzed in SAS 9.4 (SAS Institute; Cary, NC) using the generalized linear mixed model procedure with a binomial distribution. A one-way ANOVA was used to compare infection: by soil source, and survival of the two kinds of larvae (Inoculated vs control). Percent mortality was calculated

for each treatment as 100% minus the ratio of larvae found in May to larvae put into pots in October times 100.

3. Results

3.1 Survival of Japanese beetle larvae

Soil from the Hancock Turfgrass Research Center was loamy sand and composed of: 79.8% sand, 10.8% silt, and 9.4% clay. The soil pH was 7.7. Of the 50 Japanese beetle larvae that were dissected none of them were identified as positive for *O. popilliae*. Therefore the infection rate of larvae collected at the Hancock Center was estimated at 0%. No other species of Scarabaeidae larvae was recovered from the turf cores. When experimental turf cores were dug and examined for surviving larvae on May 7, 2019 (7 months after field-collected larvae were placed into turf cores) at the Hancock Turfgrass Center plot, 69.6% of the larvae in non-Inoculated soil survived. Larvae that fed previously in the inoculated soil from Eastern Hills golf course in the lab before being put into the plot had a survival rate of 66.9% (Table 3.1). At the end of the experiment 1.8% of the beetles that were fed in non-inoculated soil before being placed into the plot were infected with *O. popilliae*, while 5.0% of the beetles that fed in spore infected soil in the laboratory before being placed in the plot were infected (Table 3.1).

Analysis of survival data with a one-factor ANOVA revealed that the type of soil (with or without *O. popilliae* spores) was not significant in determining survival ($F=0.32$; $df= 1, 11$; $P= 0.58$). Healthy larvae that fed on turf grass roots in soil from the Hancock Center had a mean survival of (69.6 ± 4.3%), were as healthy larvae that fed on turf grass roots in Eastern Hills soil and became inoculated had a mean survival of (66.9 ± 4.4%).

The type of soil did not impact the infection rate of larvae based on the one-factor ANOVA ($F= 1.83$; $df= 1, 11$; $P= 0.2$). Healthy larvae exposed to turf grass roots in soil from the Hancock Center (No *O. popilliae* spores) had a mean infection rate of (1.7 ± 1.2%), and healthy larvae exposed to turf grass roots in Eastern Hills soil and became inoculated had an infection rate of (5.0 ± 2.0%).

4. Discussion

This study is not the first to experiment with infection and mortality of *O. popilliae* infected and non-infected Japanese beetle larvae. Petty et. al. (2013) demonstrated that other species of insect larvae could be infected with *O. popilliae* in a controlled laboratory setting through inoculation with *O. popilliae* spores.

The infection rate of Japanese beetle larvae exposed to inoculated soil from Eastern Hills golf course had a slightly numerically higher infection rate (5.0 ± 2.0%) than larvae exposed to non-inoculated soil (1.7 ± 1.2%). However, we were not successful in demonstrating infection of Japanese beetle larvae by putting them in soil with *O. popilliae* spores for two weeks because some infected larvae were also found in control plots. This may be because the field plots were located in the same plot area where a similar experiment was conducted the previous year. We believe this resulted in two larvae becoming infected in control plots. It is also possible that younger Japanese beetle larvae, ingesting spores in August and September could become infected more easily than the larger larvae we used in early September. Future experiments could use younger larvae or field plots established in turf heavily infested with Japanese beetle in location where the pathogen is absent, could be inoculated using homogenated dead-infected beetles.

Though the experiment did not go as anticipated, the salvageable information on *O. popilliae* infection and survival of Japanese beetles is still useful for designing future experiments on how pathogenic *O. Popilliae* is to Japanese beetle larvae and their survival from October to May in Michigan. Determining spore counts in soil used for inoculum or in solutions of ground beetles is also critical for future experiments.

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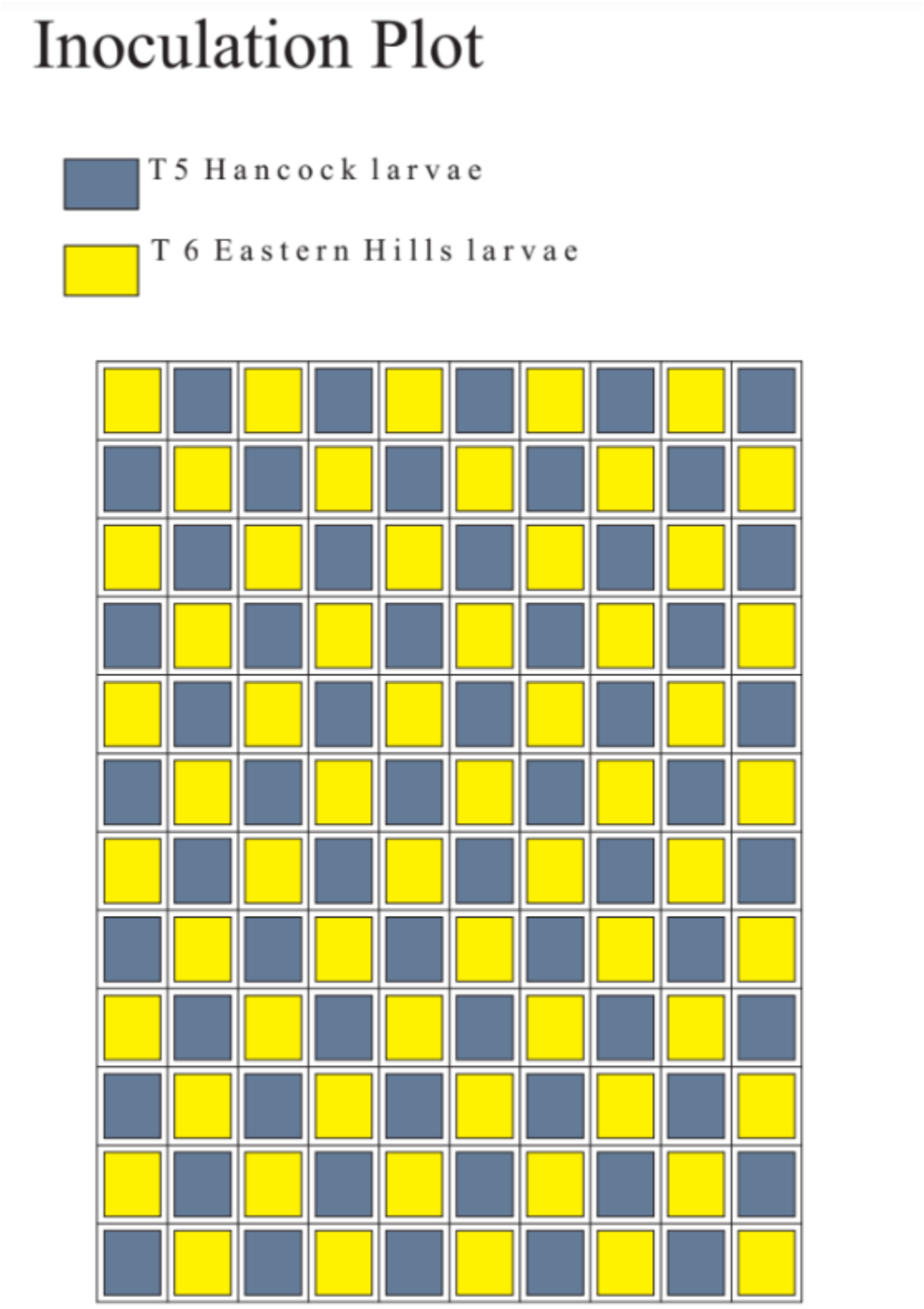
APPENDIX

Table 3.1. Survival of healthy Japanese beetle larvae collected from Hancock Turfgrass Research Center fed in Hancock (Non-inoculated) soil compared with survival of Hancock Center larvae fed in soil from Eastern Hills golf course (Inoculated), from October 2017 to May 2018

Treatment	Larvae source	Soil location in cup while feeding in laboratory	Percent *O. popilliae* infection of larvae at source in September 2018	Percent survival of JB larvae from Oct 2018 to May 2019 ($\bar{x}$ ± SE)	Percent Infected JB larvae in Spring 19
5	Hancock Turfgrass Center	Hancock Turfgrass Center	0.0	69.6 ± 4.3	1.8
6	Hancock Turfgrass Center	Eastern Hills golf course	0.0	66.9 ± 4.4	5.0

Figure 3.1. Research plot at the Hancock Turfgrass Research Center



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CHAPTER 4: Reflection

The main purpose of conducting an updated survey in spring and summer of 2018 and 2019 was to evaluate the presence of *O. popilliae* at golf courses and rest areas in Michigan and observe its spread relative to the data from 1999 and 2000. The secondary purpose was to compare past and present densities of Japanese beetle at the original nine introduction locations of *O. popilliae* in 1999 and 2000 with the current densities in 2018 and 2019. This comparison between the survey of golf courses and highway rest areas in Michigan in 2018 and 2019 and the 1999 and 2000 survey conducted by Cappaert and Smitley (2002) provided insight into the significant decline of Japanese beetles since the original survey, and the subsequent slow spread of *O. popilliae* in Michigan.

In the 1999 and 2000 survey, *O. popilliae* was recorded at two of the 35 sites sampled. In the 2018 and 2019 survey, *O. popilliae* was recorded at 22 of 47 sites, including positive finds at seven of eight introduction sites (Table 1.1, Cappaert and Smitley, 2002). Although *O. popilliae* was often found at nearby golf courses, located within 5 km of an introduction sites, it has not yet been found at 25 of 47 survey sites, some of which were within 10 km of introduction sites (Figure 1.2). For example, *O. popilliae* was not found at the Hancock Turfgrass Research Center, located 2.9 km away from an introduction site: Beal Botanical Gardens at Michigan State University.

By observing infection levels of *O. popilliae* and densities of Japanese beetles at its nine introduction sites in Michigan since 1999, a positive correlation has been established between infection rates and declines in populations of Japanese beetle. However, other possible causes of population decline could not be eliminated. More controlled experiments where *O. popilliae* is used to inoculate plots are needed to confirm the impact of the pathogen on populations of Japanese beetle. Previous research has shown that the fecundity of *O. popilliae* infected female Japanese beetles is reduced by 50% compared to healthy females (Hanula, 1990; Smitley et al., 2011). Infection rates for adult females

ranged from 25 to 60% at sites where *O. popilliae* is well established. This accounts for a 20% reduction in the number of eggs produced by females each year. When this information is combined with a typical infection rate of larvae at 30% in October, and 90% resulting larval mortality between October and May, the total population reduction due to *O. popilliae* can be conservatively estimated at 45–50%.

My results raised new questions regarding *O. popilliae* infection. First, we do not know if there is any mortality of young larvae that become infected in August and early September. Collection of larvae in inoculated and control plots in August and September would help answer this question. Also, we do not know if there is any additional mortality of mature larvae and pupae from late May to early July when adults begin to emerge.

Research is still needed to determine the best way to introduce *O. popilliae* to new locations in order to facilitate spread. *Ovavesicula popilliae* appears to spread slowly and the geographic distribution lags far behind that of Japanese beetle, making refined techniques of human introduction necessary. Collecting infected larvae is labor intensive, but adult collection with Japanese beetle traps is efficient and easy. A new experiment was initiated this fall to determine if infected adult beetles can be homogenized in a blender and then applied as a liquid to turfgrass with a standard sprayer. This will also help determine what is an adequate density of spores to use in spray inoculations because we know the density of *O. popilliae* spores in the spray solution.

All three experiments that were conducted for my master's thesis were designed to pioneer studies involving *O. popilliae* and to try to fill gaps in the knowledge of *O. popilliae's* direct impact on Japanese beetles. More research will be needed to sufficiently deem *O. popilliae* an effective biological control agent of Japanese beetles in North America, and to justify the money and time it will cost to successfully deploy and spread *O. popilliae*. The current body of knowledge on *O. popilliae* points towards a promising future, but further research data and information about the microsporidian will

expand the body of knowledge and allow for easier, cost effective decision making in the future for federal agencies using *O. popilliae*.

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