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Abiotic and Biotic Factors Affecting the Japanese Beetle in Arkansas

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By

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ABSTRACT

Japanese beetles are a relatively new pest to Arkansas. During my Ph.D. research I investigated the pathogens and environmental factors influencing Japanese beetle populations in the state. The prevalence of various pathogens and parasitoids attacking *Popillia japonica* were recorded annually from wild populations. Of specific interest was the microsporidian pathogen *Ovavesicula popilliae*, which I introduced as a biological control agent in the state. Details of the relationship between this pathogen and the Japanese beetle were investigated, including dose response, host range, and spore production. Additionally, annual abundance of the beetle in the region was recorded and tracked over the course of 4 years using soil sampling for larvae and pheromone traps for adults. Outdoor and indoor rearing methods were developed, and the density of these populations were recorded and compared to wild populations and temperature data, allowing us to model climate impact on the beetle.

Results from these studies showed that, naturally occurring pathogens, such as *Stictospora villani*, *Ovavesicula popilliae* and *Adelina* sp., and parasitoids appeared to have minimal influence on the beetle populations in this region. In the case of *O. popilliae*, low levels of the pathogen appeared in the area naturally. This pathogen also has a narrow physiological host range outside of the Japanese beetle, primarily in other scarab larvae. *O. popilliae* also appears to primarily infect Japanese beetle larvae and was incapable of infecting adult beetles. Infected larvae which survived to adulthood remained infected, with the adults capable of producing on average 25 million spores. In comparison, high summer temperatures and lack of rain reduced late summer populations of larvae.

In 2010, *O. popilliae* was released at four locations in Northwest Arkansas. In 2011 it was detected at one of those locations. A second introduction of *O. popilliae* was made in 2012.

The results from this classical biological control effort involving the release of *O. popilliae* require further monitoring to confirm establishment. This information can be used to help establish long term control of this new pest in the Southern United States. This information will be of use to fruit growers, horticulturalists, turf managers, nursery operators, and homeowners.

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Petty, B.M., D.T. Johnson, and D.C. Steinkraus. 2012. Survey of pathogens and parasitoids of *Popillia japonica* (Coleoptera: Scarabaeidae) in northwest Arkansas. J. Invert. Pathol. 111:56-59.

Chapter 10:

Petty, B.M., D.T. Johnson, and D.C. Steinkraus. 2012. Spore production of *Ovavesicula popilliae* in naturally infected adult Japanese beetles. J. Invert. Pathol. 111:255-256.

1 Introduction - Literature review on the life history and control Japanese beetle (*Popillia japonica* Newman) in the United States

The Japanese beetle (*Popillia japonica* Newman) was introduced to the United States in 1916, most likely arriving in the root balls of Japanese irises imported into New Jersey (Fleming 1972). Since then, it has spread to cover the Eastern United States, except Florida, and parts of the Midwest, Midsouth and Southern Canada. Pockets of Japanese beetles have also been found in California, Montana, Oregon, Washington, Utah, New Mexico and Colorado (NAPIS 2013). This spread continues despite internal quarantine measures (Jackson and Klein 2006). In 1997, the Japanese beetle was detected in Arkansas, probably arriving through horticultural imports, and has since become established in both the central and northwest regions of the state (Johnson 2004).

1.1 General biology of the Japanese beetle

Popillia japonica belongs to the family Scarabaeidae (Rutelinae: Anomalini), and the adults are easily recognized by their green body and copper-colored elytra. Each adult beetle has five tufts of white setae on either side of the abdomen and two tufts of white hair on the dorsal surface of the last abdominal segment. Adult size ranges from 8-11 mm long and 5-7 mm wide (Fleming 1972).

The beetle is univoltine, except in the most northern parts of its range where it takes two years to complete its lifecycle. Timing of adult emergence from their soil pupal chambers varies with latitude. In southern regions of the United States, adult emergence begins in late May, but in cooler climates farther north the adult emergence may not start until July (Fleming 1972, Régnière et al. 1981b, Vittum 1986). Males emerge first and will mate with females as soon as they emerge from their pupal chambers (Fleming 1972, Kim and Leal 2000).

Adult females emerge with a full complement of eggs and may mate several times during their life, although they only need to mate once (Ladd 1987a). The Japanese beetle sex pheromone, (R,Z)-5-(-)-(1-decenyl)oxycyclopentan-2-one, also known as (R)-japonilure, is capable of attracting several hundred males for every one female (Tumlinson et al. 1977). Male beetles have twice as many antennal sensilli which respond to this pheromone as compared to females (Adler and Jacobson 1971, Kim and Leal 2000). In areas of high beetle density, an emerging female may attract large numbers of males. The males may form a ball around the female (Ladd 1970).

The male who has most recently mated with a female has his sperm preferentially used for fertilization, which may have given rise to multiple mating attempts in males, as well as the practice of post-copulatory mate guarding (Tigreros and Switzer 2009). During mate guarding, males spend several minutes up to several hours guarding a female to prevent other males from mating by latching onto the female's back (Saeki et al. 2005). Adults feed and mate frequently during their 30 day life-span (Régnière et al. 1981b).

Females may lay 40 – 60 eggs over the course of life. Mated females burrow into the soil, 2-4 cm on average, preferring sandy-loam, to oviposit several eggs and afterwards, the female will fly to nearby vegetation to feed and mate again (Fleming 1972, Allsopp et al. 1992, O'Neil et al. 2008). Eggs vary in shape, but are usually off-white spheres, 1-1.5 mm wide (Fleming 1972).

First instars feed on the plant roots they hatch under. First instars are too small and delicate to move through the soil to any great extent. Second and third instars are larger and more mobile and are capable of moving away from disturbed areas (Villani and Nyrop 1991). In most regions, it is the third instar which overwinters. In the northern most areas of the Japanese beetle

range, it is the second instar which overwinters in the first year followed by a second year in which the third instar will overwinter before emergence (Régnière et al. 1981a).

Japanese beetle larvae are predominantly turfgrass feeders, preferring cool-season turfgrasses to warm-season turfgrasses, but will feed on the roots of other herbaceous plants. Females are also thought to search for certain contact stimuli found on the grasses (Szendrei and Isaacs 2005, Wood et al. 2009). High third instar larval populations are known to do extensive damage to water stressed and non-tolerant turfgrasses (Fleming 1972, Crutchfield and Potter 1995a, Crutchfield and Potter 1995b). In addition because the larvae preferentially feed on turfgrass roots, this can facilitate the growth and spread of weeds (Richmond et al. 2004). The selection of food sources for larvae is influenced by surface sugars. Sucrose, maltose, trehalose, fructose, and glucose have been shown to increase the feeding response of larvae (Ladd 1988). The viability of a food source is also impacted by the presence of endophytic fungi. These fungi do not impact palatability do reduce although survival rates for neonate larvae, most likely by production of toxins (Potter et al. 1992, Davidson and Potter 1995, Richmond et al. 2004).

Adults have been observed feeding on the foliage and fruit of over 300 species of woody and herbaceous plants in both field and laboratory studies (Fleming 1972, Ladd 1987b, Ladd 1989). The beetles predominantly feed on foliage, feeding on interveinal tissue thus skeletonizing the leaves; however, they will also supplement their diet with energy-rich fruit and flowers. Beetles fed a variety of plant hosts lived longer (Held and Potter 2004). Feeding occurs mostly from midmorning through midafternoon with feeding beginning again in the evening. Feeding may be limited by adult flight, which occurs only after ambient temperature is above 27°C (Kreuger and Potter 2001).

Adult beetles probably identify food sources by sugars present on the plant surface. Sucrose, maltose, fructose, glucose and to a lesser extent arabinose, xylose and raffinose, all stimulate feeding in Japanese beetle adults. The ubiquitous presence of these sugars on plants may contribute to the wide host range of Japanese beetles (Ladd 1986). Adult beetles are also attracted a wide array of host plant volatiles. The most attractive of these are phenylacetonitrile and (Z)-jasmone. (Z)-jasmone is associated with flowers and phenylacetonitrile is associated with damaged plant tissues (Loughrin et al. 1998). Beetle-damaged tissue also releases a number of other volatile compounds; phenylpropanoids, terpenoids, fermentation compounds, and aliphatic compounds, which act as a weak aggregation pheromone to adult beetles (Loughrin et al. 1996). The damage related volatiles and fermentation compounds associated with *P. japonica* also act as kairomones for other phytophagous insects such as *Cotinis nitida* (Hammons et al. 2009).

Species and varieties of plants which show susceptibility to feeding by adult beetles appear to be more palatable than closely related resistant lines. Increased feeding may trigger a cascade leading to chemical volatile release, which attracts more feeding beetles and more damage (Spicer et al. 1995, Loughrin et al. 1997). Palatability of vegetation is partially controlled by sugar content of the tissue (Ladd 1986), water content (Gu et al. 2008), leaf pubescence (Miller and Ware 1999, Miller et al. 1999) and age (Zavala et al 2009).

1.2 Chemical and cultural control of the Japanese beetle

In 1917, the U.S. Bureau of Entomology established the Japanese Beetle Laboratory in Riverton, NJ, for the task of basic research on the biology of the beetle and to develop control tactics (Fleming 1976). By 1945, the beetles had moved out of New Jersey and as far south as North Carolina, and by 1954 the beetle was as far west as Illinois. In both locations, eradication

programs using DDT and dieldrin were implemented with limited success. In 1923, geraniol was patented and became available as an attractant of Japanese beetles for the purposes of trapping. In the 1940's, geraniol was mixed with eugenol to improve attractiveness. Later, Japonilure and phenethyl-propionate were added to lures to further increase attractiveness (Ladd et al. 1981). Modern traps are baited with floral lures and the synthetic sex pheromone Japonilure (Ladd et al. 1981, Alm et al. 1996). Trap height and color have also been shown to have an effect on beetle capture rates. White and yellow traps tend to capture more beetles than traps of other colors, and traps close to the ground capture more beetles than traps further above the ground (Fleming 1976, Alm et al. 1994, Alm et al. 1996).

The use of mass trapping has mostly failed to prevent defoliation by adult beetles, often resulting in more damage and higher larval density in areas surrounding the trap (Gordon and Potter 1985, Gordon and Potter 1986). The reason for the traps failing to reduce populations is primarily caused by beetle behavior as described by Switzer et al. (2009). There is a significant "spill-over" effect of beetles being attracted to the area of the trap but never becoming trapped. Females are attracted to the trap general area, but land on nearby plants and feed. Approaching males also land on the plants to feed and mate with the females who did not go to the trap, using visual cues more than pheromones at close range. This may result in significant damage to nearby susceptible plants.

Baited traps have also been used for the auto-dissemination of pathogens in an attempt at biological control of the Japanese beetle. Attractive traps would be impregnated with pathogenic spores. The most common pathogens used for these traps have been *Metarhizium anisopliae*, *Beauveria bassiana,* and pathogenic nematodes (Klein and Lacey 1999, Morris 2009). Traps

containing fungal pathogens have been shown to be effective in disseminating the pathogens, while nematode containing traps failed to cause significant infection (Morris 2009).

Control of *P. japonica* is primarily focused on insecticide use. Most commonly used insecticides to prevent damage by larval Japanese beetle populations in turf include chloranthaniliprole, clothianidin, halofenozide, imidacloprid, and thiamethoxam (Studebaker 2013). These insecticides must be leached through the thatch layer into the root zone to be effective. Insecticides for curative control include trichlorfon and carbaryl (Potter and Held 2002). Preventative turf treatment during the summer and early autumn can reduce larval damage for the rest of the year (George et al. 2007).

Insecticide controls for adult beetles include imidacloprid, spinosad, pyrethrin, esfenvalerate, cyfluthrin, zeta-cypermethrin, bifenthrin, carbaryl, lambda-cyhalothrin, chlorpyrifos + gamma-cyhalothrin, lambda-cyhalothrin + thiamethoxam, zeta-cypermethrin + bifenthrin, imidacloprid + cyfluthrin, and microencapsulated methyl parathion (Studebaker 2013), Potter and Held 2002, Bradley et al. 2011). Chemicals derived from biological sources have also been used to control Japanese beetle populations by either killing individuals or reducing feeding damage by making tissue unpalatable. These include neem extracts, garlic oil, rosemary oil, capsaicin, and other pepper extracts, as well as others. These biological extracts have varying usefulness in control programs (Gupta and Krischik 2007, Ranger et al. 2009).

Treatment thresholds for adult Japanese beetles in agricultural systems are best defined in corn and soybeans. In corn, treatment is recommended if three or more adult beetles are present on silks or if silks are eaten to 1/2 inch in length and pollination is less that 50% complete. In soybeans, treatment is recommended when defoliation reaches 30% before bloom or 20% between bloom and pod fill (Bradley et al. 2011). In corn and wheat, white grubs, including

Japanese beetle larvae, are not tolerated. In turfgrass, damage thresholds vary between eight and ten per square foot (Bradley et al. 2011). Grass species differ in ability to tolerate white grub damage.

Chemical dips and drenches are the most common way for preventing the spread of Japanese beetles via infested nursery stock in contaminated areas. Treatments include imidacloprid, clothianidin, thiamethoxam, halofenozide, imidacloprid + cyfluthrin, chlorpyrifos, and bifenthrin (Oliver et al. 2008a, Oliver et al. 2008b). Drenches and dips are most effective when used to prevent the buildup of larval populations in nursery stock and often fail to cure infestations (Nielson and Cowles 1998, Mannion et al. 2000).

Cultural control of the Japanese beetle in turf primarily consists of limited irrigation and warm-season turfgrasses in place of cool-season turfgrasses. This can limit damage caused by larval densities. Local densities are also impacted by the presence of endophytes, which reduce survival rates for neonate larvae, most likely through the production of toxins (Potter et al. 1992, Davidson and Potter 1995). Cultural controls for Japanese beetles in crop systems include cover crop selection and tilling. Ryegrass, clover rye, and buckwheat ground covers in soybeans, orchards, and other fruit stands are known to increase the desnity of both adults and larvae (Smith et al. 1988, Szendrei and Isaacs 2006) while reduction of border grasses in fields and nurseries reduces the total number of larvae and adults (Smitley 1996). No-till practices, while useful in soil conservation, increase the abundance of larval Japanese beetles. Tilling or disking can kill 30-50% of eggs in summer and larvae during the late summer and early fall (Smith et al. 1988, Tonhasca and Stinner 1991).

1.3 Climatic limitations to beetle spread and survival

Allsopp (1996) predicted the spread of the Japanese beetle west to through Nebraska and Texas, south to the middle of Georgia and Mississippi using rainfall and temperature data and the program CLI-MEX 4.3. Additionally, limited areas of the Pacific-Northwest could be suitable habitat. This prediction has been shown to be mostly accurate, although small, isolated populations have been found as far west as Colorado and Arizona, although these populations are not considered established (NAPIS 2013).

The effects of extreme temperature on development of the Japanese beetle have been studied since early in the U.S. infestation. Ludwig (1928) found eggs would not hatch below 15°C, but had 50% or greater mortality for early instars exposed to temperatures higher than 31°C for prolonged periods. There is also thermal death point of 35°C for first instars and 31°C for second instars. Ludwig (1928) went on to construct rudimentary curves of larval development rates. Régnière et al. (1981a) developed a model for larval instar development based on the Ludwig (1928) data as well as plotted the amplitude of temperature changes in the soil. This model predicted optimum development of eggs at 31-32°C, first instars at 28°C, second instars at 28°C, third instars at 30°C, and pupae at 32°C.

First and second instar *P. japonica* are fragile and minor stresses can cause mortality. Total larval density is largely dependent on the mortality of these first two instars (Dalthrop et al 2000). Dalthorp et al. (2000) found that annual variation in population densities depended primarily on mortality occurring in July and August and not on larval deaths occurring during the winter months. Japanese beetle can tolerate temperatures down to -7°C (Hoshikawa et al. 1988). High summer mortality is likely linked to drought and heat.

Moisture plays a large role in larval Japanese beetle survival and abundance. Ovipositing females prefer wet soils, and if no moist soil is available females will not oviposit (Allsopp et al. 1992). Larvae are capable of development in most soil moisture conditions; however, eggs will delay hatching in extreme moistures, particularly soil saturation, and die when desiccated. Additionally, in low moisture environments (defined as 3% free water in clay or silt soils and 9% free water in sand soils), the larvae which hatch from eggs tend to be smaller than normal, probably from the neonate larva failing to absorb enough water during cuticular sclerotization, although these larvae remain viable (Régnière et al. 1981c).

1.4 Biological control of the Japanese beetle

In 1920, the U.S. Bureau of Entomology began research into the biological control of the Japanese beetle and sent researchers to Japan to identify parasitoids and predators of the beetle (Fleming 1976). At the same time, the Japanese beetle Laboratory began conducting research into *Paenibacillus popilliae* (Dutky), the milky spore bacteria, and pathogenic nematodes. The biological control agents released included the parasitoids *Tiphia vernalis*, *T. popilliavora*, and *Istocheta aldrichi* and the pathogens *P. popilliae*, *P. lentimorbus* and nematodes (Fleming 1976).

1.4.1 Arthropod natural enemies

Research began in Japan to locate possible parasitoids in 1920, and in 1922, this search was expanded to Korea. Several parasitoids were found (King 1931), but only two were imported and established a significant population on the East coast. A fly in the family Tachinidae, *Istocheta aldrichi* Mesnil, was introduced in an attempt to control adult beetles. The fly lays eggs on the pronotum of the adult beetle. When the eggs hatch, the larvae burrow into the flight muscles and then into the abdomen of the adult beetle, where the fly pupae will overwinter. Death of the adult beetle usually occurs within six days (Simões and Grenier 1999).

A wasp in the family Tiphiidae, *Tiphia vernalis* Rohwer was also found to be an effective parasitoid of Japanese beetle larvae. It was released in the United States in 1924 and has spread with the beetle (Rogers and Potter 2004, Ramoutar and Legrand 2007). The adult wasp locates third instar larvae by kairomones. The wasp uncovers the subterranean larva, paralyzes it, and lays an egg on the exterior of the larva. The egg will later hatch and burrow into the host to feed (Rogers and Potter 2004). *Tiphia vernalis* is known to parasitize other invasive white grub species, but is not known to parasitize native species (Jackson and Klein 2006).

Other arthropods account for significant amounts of beetle mortality. Ant species appear to be the primary insect predator of Japanese beetle eggs, larvae and other white grubs. López and Potter (2000) predicted that up to 90% of Japanese beetle eggs were consumed by ants on golf courses in areas where ants were not themselves controlled. This was later confirmed by Zenger and Gibb (2001), who showed *Solenopsis molesta* (Say) was individually responsible for up to 65% of egg predation with many other ant and beetle species contributing to overall mortality.

1.4.2 Bacteria

The most heavily researched bacterial pathogens of the Japanese beetle are *Paenibacillus popilliae* and *P. lentimorbus* (Dutky 1940). Both are non-motile, spore-forming bacteria whose vegetative cells are gram-negative and rod-shaped but may have gram-positive inclusions. The sporangia and presporal bodies are gram positive (Bulla et al. 1978). These species are the causative agents of Type A and B milky spore disease, respectively (Klein and Jackson 1992, Steinkraus 1957a) and are obligate parasites. *P. popilliae* spores contain a refractive parasporal body attached to the primary spore. This characteristic is usually missing in *P. lentimorbus*

(Steinkraus 1957a) but DNA evidence suggests that some subspecies of *P. lentimorbus* do produce these bodies (Rippere et al. 1998).

P. popilliae spores are consumed from the soil by the host, normally a scarab larva (Dingman 1994). The spores germinate and make their way into the hemolymph. In the hemolymph, the vegetative cells multiply until they number in the billions. The bacterial density increases, causing the hemolymph to appear white, giving milky disease its name. At sporulation, the vegetative rods swell and begin an incomplete division. One end becomes the spore, containing a refractive vacuole, and the other end develops into a parasporal body, half the size of the spore (Dutky 1940). Between 50-75% of the vegetative cells form spores (Steinkraus 1957a). Eventually, the host larva dies from bacterial septicemia (Bulla et al. 1978, Sharpe and Detroy 1979). At death, the hemolymph may contain up to 5×10^{10} spores (Bulla et al. 1978). These extremely resistant spores are released from the cadaver into the soil and may persist for long periods of time, showing viability after seven years of storage at room temperature (St. Julian et al. 1978). In field releases, large numbers of infective spores (1.7 billion/kg soil) could be isolated 26 years after initial release as part of a Japanese beetle biological control program (Ladd and McCabe 1967).

Milky spore bacteria are contribute some control of Japanese beetle larvae in natural settings (Hanula and Andreadis 1988, Hanula 1990, Smitley 2008) and are known to cause epizootics after field application (Kaya et al. 1993). However, as a biological control agent, *P. popilliae* has had limited success. No large scale *in vitro* method exists for the production of spores (Redmond and Potter 1995). Current and historic small scale *in vitro* methods, described by Steinkraus (1957b) and Steinkraus and Provvidenti (1957), appear to cause the pathogen to lose virulence. Additionally, multiple sub-strains *P. popilliae* have been identified, often co-

occurring within the same natural infection, each with variable growth and sporulation rates (Sharpe and Bulla 1978). The standard method for the production of virulent spores is to grow the bacteria in vivo and pulverize the infected larvae with talc powder (Hayes and Rhodes 1966). Larvae injected *P. popilliae* spores may require exposure to upwards of one million spores in order to develop infection. Larvae in contaminated soil may require exposure to over three million spores.

P. popilliae was negatively impacted by the use of turf fungicides, limiting its usefulness for biological control of the Japanese beetle in high maintenance turf systems. Spores will not germinate when turf was treated with isofenphos, chlordane and 2-[(4-chloro-*o*-tolyl)oxy]propionic acid plus (2,4-dichlorophenoxy)acetic acid (Dingman 1994). Vegetative cell growth was also inhibited by the use of triadmefon, chlorothalonil, iprodione, pendimethalin and chloropyrifos (Dingman 1994).

Another promising pathogen for Japanese beetle control is *Bacillus thuringiensis* subspecies *japonensis (Btj)* Buibui strain, which is another spore-forming bacterium with parasporal inclusions. The species was isolated from the soil in Japan and shows considerable larvicidal abilities against scarabs. Japanese beetle larvae fed spores of *Btj* exhibited mortality within two days, and 100% mortality was achieved after seven days (Ohba et al. 1992). *Btj* has been shown to be an effective control agent of third instar Japanese beetle larvae in field tests but is more pathogenic against early instars (Koppenhöfer et al. 2000c).

1.4.3 Nematodes

The most common entomopathogenic nematodes belong to the families Heterorhabditidae and Steinernematidae. Entomopathogenic nematodes are considered useful biological control agents under certain conditions (Klein and Georgis 1992, Yeh and Alm 1992,

Simões et al. 1993, Selvan et al. 1994) and have limited impact on non-target arthropods (Georgis et al.1991, Klein and Georgis 1992).

Entomopathogenic nematodes co-occurring with host species must first find hosts. Two strategies are employed: ambush and cruising. Ambush nematodes wait for passing hosts species, stand on their tails (nictation) and attach. These nematodes usually disperse poorly and will only travel shirt distances once in the soil (Gaugler et al. 1997, Lacey et al. 2001) Cruisers actively seek hosts and may respond to either host volatiles or plant volatiles from host feeding (Gaugler et al. 1997, Wang and Gaugler 1998). Cruiser nematodes may disperse 2-8 cm when seeking a suitable host (Lacey et al. 2001). If a host is found and recognized, the nematode will penetrate through the mouth, anus, spiracles or cuticle and release symbiotic bacteria. These bacteria will liquefy the host and allow for nematode reproduction (Gaugler et al. 1997).

Multiple species are known to infect and kill the larval instars of *P. japonica* after entering the body cavity. The most common nematode parasites include *Heterorhabditis bacteriophora* (Poinar), *H. heliorhidas* (Khan, Brooks & Hirschmann), *Steinernema scarabaei* (Stock & Koppenhöfer), *S. kushidi* (Mamiya), *S. glaseri* (Steiner), *S. feltiae* (Flilipjev) and *S. carpocapsae* (Weiser). Japanese beetles are particularly susceptible to *S. glaseri* and *H. bacteriophora*, because both are cruisers which seek sedentary hosts. Japanese beetle larvae are not as susceptible to ambushers like *S. carpocapsae* (Wright et al. 1988, Georgis and Gaugler 1991, Yeh and Alm 1992).

The susceptibility of Japanese beetle larvae is dependent of the species and strain of a nematode (Selvan et al. 1994, Lacey et al. 2001), but is also dependent on the life-stage of the larva. Resistance to infection by *H. bacteriophora* in Japanese beetle larvae increases from first instar to late third instar, and then decreases in pupae. This pattern in resistance doesn't exist for

larval infection by *S. scarabaei*, with no changes in resistance between instars (Koppenhöfer and Fuzy 2004). Additionally, resistance to *S. glaseri* decreases from larval to pupal stages (Lacey et al. 2001).

Differences based on Japanese beetle larval immune response also dictate how successful an invading nematode will be. The nematode *H. bacteriophora* is known to illicit strong immune responses from larvae consisting of melanization and encapsulation but may be able to effectively evade this immune response. *S. carpocapsae* and *S. glaseri*, however, do not illicit much immune response from its host (Wang et al. 1994). At least one factor which controls the intensity of the host's immune response is the surface coat of the nematode. In *S. glaseri*, the surface coat protein SCP3a was found to effectively suppress the melanization and encapsulation immune responses of Japanese beetle larvae against itself, *H. bacteriophora*, and the nematode symbiotic bacteria *Xenorhabdus poinarii* (Wang and Gaugler 1999).

S. carpocapsae, however, has been shown to increase its pathogenicity to scarab larvae when selective pressure is applied (Gaugler and Campbell 1991). In many cases, nematode field treatments produced control of beetle larvae comparable or surpassing insecticide treatments (Koppenhöfer et al. 2000, Koppenhöfer et al. 2004, Power et al. 2009, Villani and Wright 1988, Wang et al. 1995, Yeh and Alm 1995). As opposed to many microbial pathogens, some nematodes actively seek out their hosts for infection (Schroeder et al. 1993). However, nematodes are highly susceptible to desiccation, limiting their overall usefulness in large scale control of Japanese beetle in the absence of irrigation (Downing 1994).

Adult beetles can also be infected with nematodes, as shown by Lacey et al. (1995b) which infected adults with *S. glaseri*. However, infected adults generally died after 4 days post exposure, often times producing a very low number of new nematodes (mean of 238 IJ). Before

dying, adults may also transport nematodes phoretically. Both infected adults and adult beetles in which nematodes are attached exteriorly have been shown to play a role in establishing nematode infection foci and aid in the dispersal of nematodes into difficult to treat areas for purposes of biological control. Auto-dissemination of nematodes via adult exposure has been deemed unfeasible because of problems associated with large scale inoculation and maintaining adults healthy enough to fly (Lacy et al. 1995b).

Entomopathogenic nematodes have also been used as effective curative treatments when combined with pesticides. Synergistic effects have been documented when *H. bacteriophora* and *S. glaseri* have been combined with the neonicotinoid insecticides imidacloprid and thiamethoxam (Koppenhöfer et al. 2000b, Koppenhöfer et al. 2002). Although nematodes can effectively reduce infestations of *P. japonica*, the persistence of control by nematodes from year to year is variable. *H. bacteriophora* sustained control of *P. japonica*, while *S. carpocapsae* only offers temporary control (Klein and Georgis 1992)

The mechanism responsible for the increased effectiveness of nematodes when combined with neonicotinoid insecticides is thought to be a disruption of nerve function, resulting in reduced grooming and evasive behavior in Japanese beetle larvae. The reduction in responsive behavior allows nematodes to attach and penetrate the host at higher rates. This pattern holds for multiple entomopathogenic species (Koppenhöfer et al. 2000a).

Effective nematode application for biological control of *P. japonica* in turf requires high levels of soil moisture. Irrigation following application moves the nematodes 2.5-5 m below the surface and also prevents desiccation. Additionally, application of nematodes to dry soil may also cause desiccation and death, so soils may be need irrigation or rainfall prior to application (Shetlar et al. 1988, Selvan et al. 1994)

1.4.4 Fungi

Of the fungi which infect *P. japonica*, the most widely dispersed fungal pathogens are *Metarhizium anisopliae* (Metchnikoff) Sorokin and *Beauveria bassiana* (Bals.-Criv.) Vuill. Both fungi are generalist insect pathogens (Glare 1992, St. Julian et al. 1982). *M. anisopliae* is the only known *Metarhizium* species to infect scarabs. It has been found in over 200 other insects, as well (Glare 1992). Like *B. bassiana*, *M. anisopliae* is known to infect both larvae and adult beetles (Lacey et al. 1994 and Mannion et al. 2001) although larval infection is low in the wild (Hanula and Andreadis 1988). Mortality in adult beetles exposed to *M. anisopliae* spores can reach 100 percent when adults are exposed at a rate of 10 mg of spores per 100 beetles (Lacey et al. 1994). The fungus also requires several days to kill adult *P. japonica*, and can thus be readily disseminated in populations (Klein and Lacey 1999, Lacey et al. 1995a). Klein and Lacey (1999) have worked on ways to auto-disseminate this pathogen throughout environments via traps containing *M. anisopliae* spores but with limited success.

The presence of *M. anisopliae* spores repels larvae but attracts ovipositing females. It is speculated that spores produce secondary metabolites or increased CO_2 concentration (Villani et al. 1994). The phenomenon of *M. anisopliae* spores changing possible host behavior is documented in other insects as well (Baverstock et al. 2010). Additionally, females are attracted to areas where *M. anisopliae* is present for purposes of oviposition. Again, this phenomenon may be due to other secondary metabolites attracting the female or high CO_2 levels which mimic dense grass roots (Villani et al. 1994).

The genus *Beauveria* is known to infect more than 500 insect species. *B. bassiana* is one of the most common entomopathogenic fungi, found throughout the world (Glare 1992). Laboratory and field studies have shown its effectiveness against both larval and adult Japanese

beetles (Lacey et al. 1994, Mannion et al. 2001). Mortality in adult beetles exposed to spores can reach 100 percent when adults are exposed to as little as 1 mg of spores per 100 beetles (Lacey et al. 1994).

1.5 *Ovavesicula popilliae* and the microsporidia

The microsporidia are a group of obligate intracellular parasites of animals and some protists probably related to the Zygomycetes (Keeling et al. 2000, Lee et al. 2008). Many species are thought to be limited to one or a small group of hosts, with some species requiring a complex of intermediate hosts. The host specificity for individual microsporidian species is highly variable and information is based on both field studies and laboratory host ranges. For most species, this host specificity information is not well known (Sprague et al. 1992). In order for a host to be suitable for a microsporidian to infect, the spore must first be exposed to appropriate germination conditions, such as pH of the gut, penetrate the host, survive the immune system, and find cells in which to develop. Depending on the method by which spores enter a host, some of these problems may be overcome readily (Undeen. and Maddox 1973).

The physiological host range of a pathogen, the range of hosts in which infection can be produced under laboratory conditions, can be used as an initial indicator of what its ecological host range, the range of hosts in which infection will occur in nature, may be (Solter and Maddox 1998). Physiological host range, while wider than ecological host ranges, has previously been used to estimate the safety of microsporidia as biological control agents by examining potential hosts which are both taxonomically related to natural hosts and which share a common habitat (Solter et al. 1997, Solter and Maddox 1998), despite the fact that broad host ranges can be developed depending on inoculation techniques (Undeen and Maddox 1973). However, evidence

exists that the host range of microsporidia can be underestimated by laboratory testing (Solter et al. 2000).

Transmission between hosts is most often horizontal, through a fecal-oral route, but may also occur vertically via contaminated or infected eggs. Once within a host, microsporidia penetrate the host cells through the eversion of a polar filament coiled within the spore that punctures the host cell, allowing the microsporidian to enter (Sprague et al. 1992).

The virulence of microsporidian species is apparently linked on their mode of transmission. Microsporidia which require horizontal transmission show a pattern of increased virulence and host spore load. The increase in spores produced increases the likelihood of passage to a new host. Vertically transmitted microsporidia in females display low virulence, generally produce lower spore loads, and allow for host reproduction. In males, this pattern may not hold. In microsporidia with both transmission routes, virulence will be higher than in species with only vertical transmission (Dunn and Smith 2001).

Ovavesicula popilliae (Andreadis and Hanula) (Microsporidia: Ovavesiculidae) is a microsporidian pathogen of the Japanese beetles. This pathogen primarily infects the Malpighian tubules in both larval and adult stages but can spread to other tissues (Andreadis and Hanula 1987). It was discovered in Connecticut and is thought to be a relatively new *P. japonica* associate because of the intense immune response exhibited by the host (Hanula and Andreadis 1990).

Like other microsporidia, *O. popilliae* penetrates its host cell through the eversion of the polar filament coiled within the sporont, the spore stage, of the cell. The polar filament punctures the host cell membrane, allowing the microsporidian cell to enter. The meront, the vegetative

stage, cell membrane is normally in direct contact with the host cell cytoplasm, and acquires ATP in this way (Hanula and Andreadis 1992).

O. popilliae is one of nine taxa to produce multiple spores from a single sporont (Vossbrinck 2007). *O. popilliae* produces 32 ovoid spores within its sporophorous vesicle, through synchronous, binary fission (Andreadis and Hanula 1987). The spheroid vegetative cell, the meront, has a diplokaryon typical of many Microsporida. The meront is surrounded by both its cell membrane and the endoplasmic reticulum of the host cell (Andreadis and Hanula 1987). A typical Golgi apparatus and mitochondria are lacking in *O. popilliae*. Instead, it has a Golgi zone and mitosomes. This organism also has many free ribosomes and small vacuoles.

The meronts eventually differentiate into sporonts, which begins producing the sporophorous vesicle. In the sporont stage, the diplokaryon separates and the sporont undergoes nuclear division. It forms a plasmodium within the sporophorous vesicle and may have up to 16 unpaired nuclei. The sporogonial plasmodium undergoes synchronous nuclear division again, followed by cytokinesis. This point of the life cycle indicates the formation of the sporoblasts (Andreadis and Hanula 1987). As sporoblasts mature, they develop exospores and in internal polar filament consisting of six coils. Each spore is uninucleate and persists within the sporophorous vesicle. Spores are 2.0-2.5 µm x 1.7-1.8 µm and sporophorous vesicles have an average size of 20.0-21.0 µm x 15.0-15.5 µm (Andreadis and Hanula 1987).

The pathogen is most likely transmitted through a fecal-oral route between larvae and adults to larvae. Disease initially develops in the Malpighian tubules of larvae but may spread to the fat body, epidermis, pericardium and oenocytes (Hanula and Andreadis 1990). An infected larva may die, releasing spores as it decays, or may pupate into an infected adult. Both infected

larvae and adults excrete spores and sporophorous vesicles in the frass (Hanula and Andreadis, 1990) which are persistent in nature (Andreadis and Hanula 1987).

There are no external signs of *O. popilliae* infection in larvae or adults (Andreadis and Hanula 1987). Infected larval Malpighian tubules become swollen and discolored. They are often covered in melanized nodules containing spores. The infection may become systemic in larvae, moving to the fat body, epidermis, and pericardial cells. Tissue that is infected undergoes intense inflammation and hypertrophication. Transstadially infected adults are usually found to have a systemic infection (Hanula and Andreadis 1990). While the pathogen has not been found to infect reproductive tissue, it does reduce fecundity with heavily infected females laying 50-60% fewer eggs than healthy females (Hanula 1990, Smitley 2008).

The pathogen was discovered in Connecticut and has been found infecting Japanese beetle in Southern Michigan (Cappaert and Smitley 2002) and Kentucky (Redmond and Potter 2010). Infection in Connecticut occurred in 25% of the Japanese beetle larval population, on average. Adult populations had infection rates between 20-50%, increasing as the summer progressed (Hanula and Andreadis 1988). In certain locations, infection reached 100 percent (Hanula 1990). In Michigan, infection reached between 3.3- 20% in adults and up to 75% infection in larvae (Cappaert and Smitley 2002, Smitley 2008). Infection is associated with 17% increased winter mortality in larvae at locations with populations heavily infected with *O. popilliae* (Smitley et al. 2011). *Ovavesicula popilliae* would cause a population decline of Japanese beetle at a projected 57% per year (Smitley 2008).

1.6 Thesis and objectives

This project investigates the pathogens and environmental factors influencing Japanese beetle populations in Arkansas. In order to accomplish this, baseline data were established on

the larval and adult abundance of *P. japonica* in northwest Arkansas as well as baseline data on the abundance of pathogens and parasitoids infecting the Japanese beetle. In addition to this baseline data, I sought to establish a biological control program for *P. japonica* using *O. popilliae.* This included the details of this pathogen-host relationship, examining the host range life stage specificity, Japanese beetle dose response to and spore production of *O. popilliae.*

Additionally, sources of *P. japonica* in Arkansas and the mass rearing of *P. japonica* larvae in the field. Difficulties in mass rearing due to hot, drought conditions gave rise to a method of modeling the effects of temperature on *P. japonica* larval mortality in order to better predict population increases and declines.

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2 Changes in abundance of larval and adult *Popillia japonica* (Coleoptera: Scarabaeidae) and other white grub species in relation to temperature

2.1 ABSTRACT

The Japanese beetle is a relatively new pest in Arkansas that damages turfgrasses, horticultural plants, and fruit crops. The annual density of Japanese beetle larvae and native white grub species (Scarabaeidae) in northwest Arkansas was monitored from 2010 to 2012, and adult Japanese beetle abundance was tracked from 2005 to 2012 to determine changes in the local populations. White grub populations (all species) varied from 0.69 larvae/0.1 m^2 in 2010 to 0.15/0.1 m^2 in 2012. Japanese beetle larval populations were significantly lower in 2012 than in 2010 while native white grub species were not. Adult beetle captures in a transect of traps set in Fayetteville, Arkansas, ranged from a mean weekly trap capture of 3974.0 beetles in 2006 to 100.2 beetles in 2012. This pattern of decline in adult Japanese beetle populations was mirrored at 10 other collection locations. Previous studies showed that there were few microbial and parasitoid natural enemies of *P. japonica* in the region. Therefore, fluctuations in Japanese beetle populations are likely due to abiotic factors, such as spring flooding and extremes of summer drought and heat along this invasive species' southwestern range.

Keywords: *Popillia japonica*, Arkansas, white grub, Japanese beetle

2.2 INTRODUCTION

The Japanese beetle (*Popillia japonica* Newman) is an invasive pest species that was introduced to the United States in 1916, most likely through horticultural imports (Fleming, 1972), and now covers most of the Eastern U.S. (NAPIS 2012). In 1997, *P. japonica* was found in northwest Arkansas (Johnson 2004) where it is currently established and spreading. This region, along with northeastern Oklahoma, represents the southwestern extent of the range of Japanese beetle infestation (NAPIS 2012).

Japanese beetles are pests in both their larval and adult stages. Larvae damage turfgrass roots and adults feed on over 300 species of plants (Fleming 1972). Though larval population densities have been relatively low in northwest Arkansas, adult populations have been destructive (Wood et al. 2009). Surveys for *P. japonica* abundance have been conducted in states with long standing infestations and high beetle populations, such as Connecticut (Hanula 1990) and Michigan (Cappaert and Smitley 2002). Recent surveys documented abundance of *P. japonica* as well as other white grub species in Kentucky (Redmond and Potter 2010, Redmond et al. 2012). However, no quantified surveys of *P. japonica* have been published from Arkansas.

The objective of this study was to document yearly changes in the abundance *P. japonica* larvae and adults in northwest Arkansas. This is a relatively new infestation that constitutes part of the current southwestern extent of Japanese beetle expansion. Additionally, population densities of native white grub species were recorded to compare yearly changes in density.

2.3 MATERIALS AND METHODS

2.3.1 Survey of white grub density and adult Japanese beetle abundance

The field sites used were described in Petty et al. (2012); and included two fruit orchards, one horticultural nursery, three golf courses, three city parks, and the University of Arkansas Research and Extension Farm (listed in Table 2.3). In 2012, the peach orchard located in Springdale, AR. was removed as a sampling location.

The methods described are described more fully in Petty et al. (2012). In 2010, ten field sites were selected to collect larval *P. japonica* as well as other white grub species using the soil sampling method adapted from Fleming and Baker (1936). One sample consisted of sifting 0.1 m^2 of soil approximately 8 cm deep. All scarab larvae discovered were brought back to the lab for identification via raster pattern (Vittum et al. 1999). Ten soil samples were analyzed for white grubs from each site once a month during April and May of 2010, 2011, and 2012. In 2010, field sites were also sampled once in September. The September 2010 data was included in with the spring 2011 data, because these beetles were part of the same generation.

From 2005 through 2012, adult Japanese beetle traps were set as a 0.75 km line transect on the University of Arkansas Agricultural Research and Extension Center (AAREC) (Fayetteville, AR). The number of traps in the transect varied between years, ranging from 3 traps in 2005 to 20 traps in 2012. Traps were spaced 19 m apart. Additionally, adult Japanese beetles were collected from the same field sites as larval sampling from mid-June to mid-August in 2010 and 2011, and from mid-May to mid-July in 2012. In 2012, Japanese beetle adults began their flight earlier than previous years. Jumbo Jug (Trécé Inc., Adair, OK) funnel mouth traps (1.9 L) and modified 3.8 L traps were used and baited with floral and sex lures. Traps were in place when the first adult Japanese beetles of the season were found and removed when the adult

trap counts fell into single digits in August or early September. Traps were emptied weekly and the numbers of adults trapped were determined volumetrically (250 beetles/100 ml).

2.3.2 Statistical analysis of abundance data

P. japonica larvae per 0.1 m^2 was used to compare changes in yearly density across all sites as well as years using a one-way analysis of variance (ANOVA) in JMP® 10.0 (SAS Institute Inc.). Additionally, the density of native white grub species per 0.1 m^2 was examined for yearly change across all sites. Mean values were separated using a Student's T-test. The mean weekly trap captures of Japanese beetle adults were compared using analysis of variance to determine changes in beetle abundance. The mean weekly adult trap captures were averaged over the number weeks the adult Japanese beetles were active and the number of traps per site.

2.3.3 Temperature data analysis

To examine the possible effects of temperature and its association with changes in the Japanese beetle population, we examined National Oceanic and Atmospheric Administration (NOAA) archival data from the Drake Field Airport meteorological station in Fayetteville, AR. The mean monthly temperatures from July and August, months in which early instar Japanese beetles are present in soil, for 2004 to 2011 were compared to the mean weekly adult trap captures from the AAREC trap transect line during the same month of the following year and examined for correlation. These correlations were found using Spearman's ρ non-parametric correlation in JMP® 10.0. Additionally, regional precipitation during this time period was also examined.

2.4 RESULTS

2.4.1 Survey of white grub density

In spring 2010, a total of 200 soil samples were examined from the 10 sites. A total of 116 *P. japonica* larvae were collected. In addition, 12 *Phyllophaga* spp. larvae and 10 *Cyclocephala* spp. larvae were collected. Mean white grub density across all locations in spring 2010 was 0.69 larvae/0.1 m^2, with *P. japonica* larval density at 0.57/0.1 m^2, *Cyclocephala* spp. larval density at 0.05/0.1 m^2, and *Phyllophaga* spp. larval density at 0.06/0.1 m^2 (Table 2.1).

During fall 2010 and spring 2011, a total of 300 soil samples were collected. From these samples, 123 *P. japonica* larvae, 14 *Phyllophaga* spp. larvae and 34 *Cyclocephala* spp. larvae were collected. Mean white grub density across all locations in fall 2010/spring 2011 was 0.57/0.1 m^2, with *P. japonica* larval density at 0.41/0.1 m^2, *Cyclocephala* spp. larval density at 0.11/0.1 m^2, and *Phyllophaga* spp. larval density at 0.05/0.1 m^2.

In spring 2012, a total of 200 soil samples were examined from 10 sites. From these samples, a total of 12 *P. japonica* larvae were collected. Seven *Phyllophaga* spp. larvae and zero *Cyclocephala* spp. larvae were collected. Mean white grub density per location in spring 2012 was 0.1 larvae/0.1 m^2, with *P. japonica* larval density at 0.05/0.1 m^2 and *Phyllophaga* spp. larval density at 0.04/0.1 m^2.

Japanese beetle larval densities differed significantly by year. Mean larval density was higher in 2010 and 2011 than in 2012 (α=0.05, P=0.0381). There were no significant differences in *Cyclocephala* spp. or *Phyllophaga* spp. larval population densities between years.

2.4.2 Survey of adult Japanese beetle abundance

At the University of Arkansas Research Extension Farm transect collection site, mean weekly counts of Japanese beetle adults captured by year were: 3528.5 in 2005; 5228.2 in 2006;

908.9 in 2007; 2992.2 in 2008; 2244.9 in 2010; 464.8 in 2011; and 116.3 in 2012 (Table 2.2). Data from 2009 was not included in this analysis because of loss of replication data; however, the mean 2009 trap capture of 4661.6 was used in the temperature data analysis. The number of Japanese beetles caught between the years differed significantly. The number of beetles captured in 2006 was significantly higher than the number of beetles captured all other years (α=0.05, $P<0.0001$). Trapping in 2012 represented the lowest number of beetles captured in our collection period, and was significantly lower the number collected in 2007, 2009 and 2010 and 2011.

Mean trap captures similarly declined in the other locations trapped from 2010 to 2012 (Table 2.3). The irrigated locations (Valley View Golf Course, Lost Springs Golf Course, and Westfork Nursery) experienced less of a decline in 2012 than the other non-irrigated sites. These locations were not compared statistically with the University of Arkansas transect collections because of lack of trap replication, but at all sites a decline of Japanese beetles between years was evident. This decline reflects what was seen in trap captures at the AAREC.

2.4.3 Temperature data analysis

July temperatures fluctuated from 2.6°C below average in 2004 to 3.1°C above average in 2011. August temperatures fluctuated between 3.3°C below average in 2004 to 3.0°C above average in 2011. July precipitation fluctuated from 6.4 cm below average in 2011 to 10.5 cm above average in 2005. August precipitation fluctuated between 3.4 cm below average in 2005 to 12.7 cm above average in 2006 (NOAA 2012). Many of the collection sites were also flooded during the spring of 2011, with a total of 44.2 cm of precipitation above average falling in April and May 2011 (NOAA 2012), possibly reducing larval survival for that year. Mean weekly trap counts at the AAREC from 2005 to 2012 appeared related to the mean temperature during July (Fig. 2.1) and August of the previous year (Fig. 2.2) with mean trap captures having a -0.595

correlation with changes in July temperatures and a -0.714 correlation with changes in August temperatures. Both months show moderate inverse correlations between summer temperatures and the succeeding years Japanese beetle abundance.

2.5 DISCUSSION

Densities of *P. japonica* third instar populations in Arkansas are relatively low compared to larval densities reported from other states. In Connecticut, larval densities ranged from < 1 to 18 larvae/0.1 m^2 (Hanula 1990). In Michigan, densities ranged from 3.7 to 21 larvae/0.1 m^2, with an average of 9.5 larvae/0.1 m^2 (Cappaert and Smitley 2002). We found the Arkansas larval population densities were lower in northwest Arkansas than previous estimates of < 2 larvae/0.1 m^2 (Wood et al. 2009).

The relatively low larval densities in Arkansas make it difficult to explain the high numbers of adult Japanese beetles. The immense area of the cultivated and uncultivated grasslands in northwest Arkansas may result in the emergence of very large adult populations. The adults are then attracted by pheromones and plant volatiles (Fleming 1972, Loughrin et al. 1996) into horticultural and orchard areas, resulting in significant damage to crops.

However, the low density of Japanese beetles in Arkansas compared to other states with established infestations has remained largely unexplained. Northwest Arkansas lacks substantial populations of pathogens or parasitoids of the Japanese beetle that keep the populations low (Petty et al. 2012). However, Arkansas is on the extreme southwestern range of Japanese beetle infestations in the U.S., and a mixture of non-preferred turfgrasses for oviposition and severe weather conditions may create a suboptimum environment for this invasive beetle. Japanese beetle females have reduced oviposition in common bermudagrass (*Cynodon dactylon* [L.] Pers.)

and hybrid bermudagrass in choice tests. Bermudagrass is a common pasture and turf grass in Arkansas (Wood et al. 2009). Additionally, temperatures and precipitation in Arkansas can be extremely variable, especially during the summer, which is the height of Japanese beetle oviposition, hatch, and feeding.

Fluctuations at the AAREC in Japanese beetle abundances were evident between 2005 and 2010, with a rapid and steady decline following 2009. While trap captures at other locations were not replicated within a single year and thus could not be used for statistical comparison, the severe decline of the Japanese beetle abundance from 2010 to 2012 is evident at all locations. Temperatures during July and August were inversely correlated with the abundance of adult Japanese beetles the following year and may be largely responsible for the sharp decline in the Japanese beetle density between 2009 and 2012. This may be due to the fact that eggs and early instars are present during July and August, and these instars are particularly sensitive to heat. First instar Japanese beetles, which have a significant thermal death starting at 35 °C. (Ludwig 1928), may be experiencing significant declines from heat exposure. Multiple extremely hot summers in succession from 2010 to 2012 would have the effect of extreme reduction in the Japanese beetle population, as was observed.

Severe drought and heat during the summers of 2011 and 2012 may have also reduced larval survival. Lack of available moisture is known to increase larval mortality (Régnière et al. 1981), but the effects of drought between individual locations are harder to quantify with the data set. However, field sites which had irrigation, such as the golf courses, vineyard, and the horticultural nursery, produced more adult beetles than locations without irrigation, i.e. city parks (Table 2.3). Drought may also inhibit adult emergence in June and July 2012 by hardening

surface soil, alternatively frequent rain or irrigation events may facilitate adult emergence by softening soil.

Compared to *P. japonica*, native scarab larvae were not significantly impacted by recent increased summer temperatures or drought. These native scarabs may have a tolerance to harsh climatic conditions that the invasive Japanese beetle doesn't have. Should the Japanese beetle continue to expand southwest into the United States, the increasingly warm and dry climate may cause this beetle to become a minor pest, as compared to the severe economic impact the Japanese beetle is known to have in the cooler and wetter climates of the northeastern United States (Potter and Held 2002).

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Table 2.1. Mean (±SE) larval densities of three scarab species, *Popillia japonica*, *Cyclocephala spp.* and *Phyllophaga* spp. per 0.1 m^2 in northwest Arkansas from 2010 to 2012.

	Scarab larvae/0.1 m^2		
	P. japonica	*Cyclocephala* spp.	*Phyllophaga* sp.
2010	0.57 (±0.14) a	0.05 (±0.05) a	0.06 (±0.03) a
2011	0.41 (±0.10) a	0.11 (±0.04) a	0.05 (±0.02) a
2012	0.05 (±0.14) b	0.00 (N/A) a	0.04 (±0.03) a

Lower case letters differing within a column indicate significant differences between densities within a species across years. SE used a pooled estimate. For *P. japonica* (α=0.05, P=0.0381), for *Cyclocephala* spp. (α=0.05, P=0.0867) and for *Phyllophaga* spp. (α=0.05, P=0.8577).

Table 2.2. Mean (± SE) weekly trap counts for adult Japanese beetles from 2005 to 2012 at the University of Arkansas Agricultural Research and Extension Center.

Year	Mean Weekly Capture
2005	3528.5 (±488.5) b
2006	5228.2 (±226.1) a
2007	908.9 (±199.4) d
2008	2992.2 (±218.5) b
2010	2244.9 (±218.5) c
2011	464.8 (±267.5) de
2012	116.3 (±189.2) e

Lower case letters differing within a column indicate significant differences between mean weekly trap captures across years (α=0.05, P<0.0001).

Table 2.3. Mean (±SE) weekly trap count per site for adult Japanese beetles in 2010, 2011, and 2012.

Location	Year		
	2010	2011	2012
Wilson Park	656.9 (±151.0)	238.8 (±70.1)	46.4 (±15.4)
Lewis Park	1,900.6 (±586.6)	321.9 (±122.3)	48.8 (±14.1)
Gulley Park	1,971.7 (±404.3)	384.4 (±120.9)	84.8 (±28.2)
Valley View Golf Course	3,196.7 (±718.8)	1,946.7 (±750.2)	701.8 (±218.7)
Lost Springs Golf Course	4,122.2 (±1411.5)	1,572.3 (±488.9)	1,346.7 (±395.5)
Razorback Golf Course	3,901.4 (±994.1)	784.9 (±238.4)	140.2 (±38.1)
Westfork Nursery	1,908.9 (±441.8)	1,250.4 (±413.2)	644.1 (±251.3)
Fayetteville Apple Orchard	1,952.8 (±449.5)	584.2 (±154.6)	100.8 (±34.8)
Springdale Peach Orchard	5,468.7 (±1101.0)	1,140.8 (±347.6)	N/A

Figure 2.1. Changes in Japanese beetle adult weekly mean trap captures in Fayetteville, Arkansas from 2005 to 2012 in relation (red bars) to the average July temperature of the previous year (blue points) shown as the fluctuation in the average temperature (ΔT) of the month of July as compared to the 30 year historical average for northwest Arkansas.

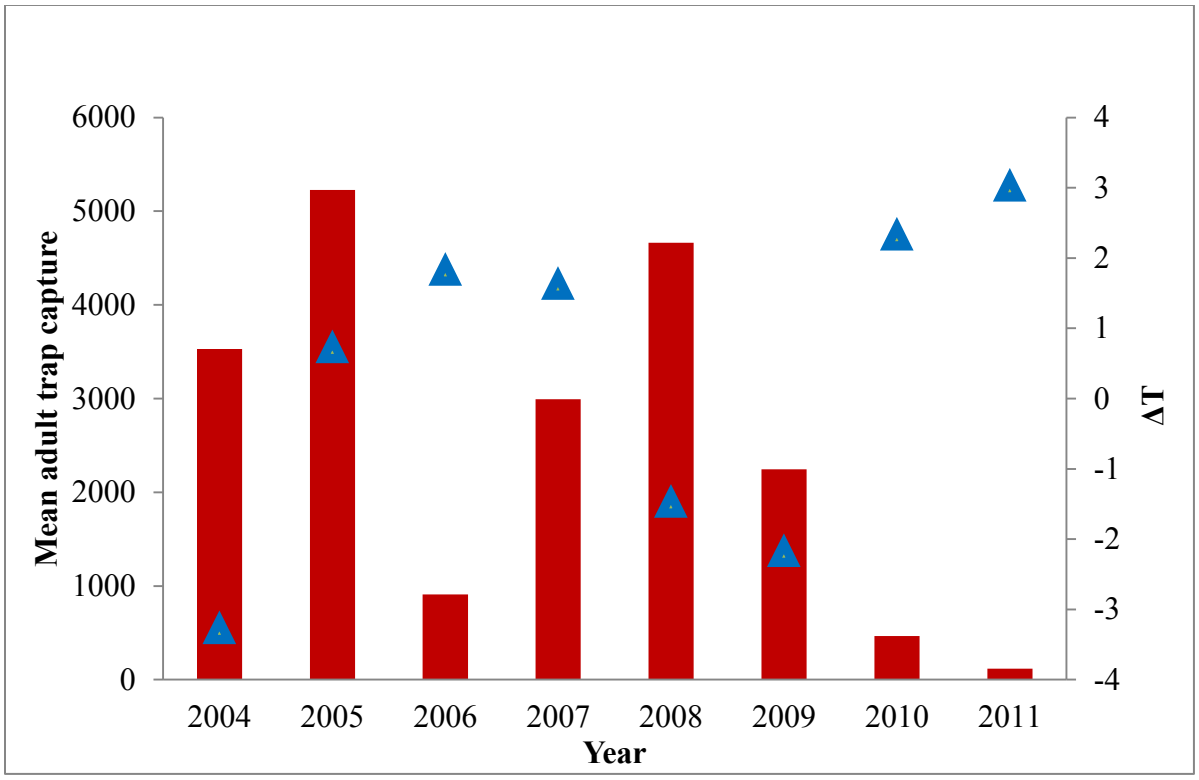
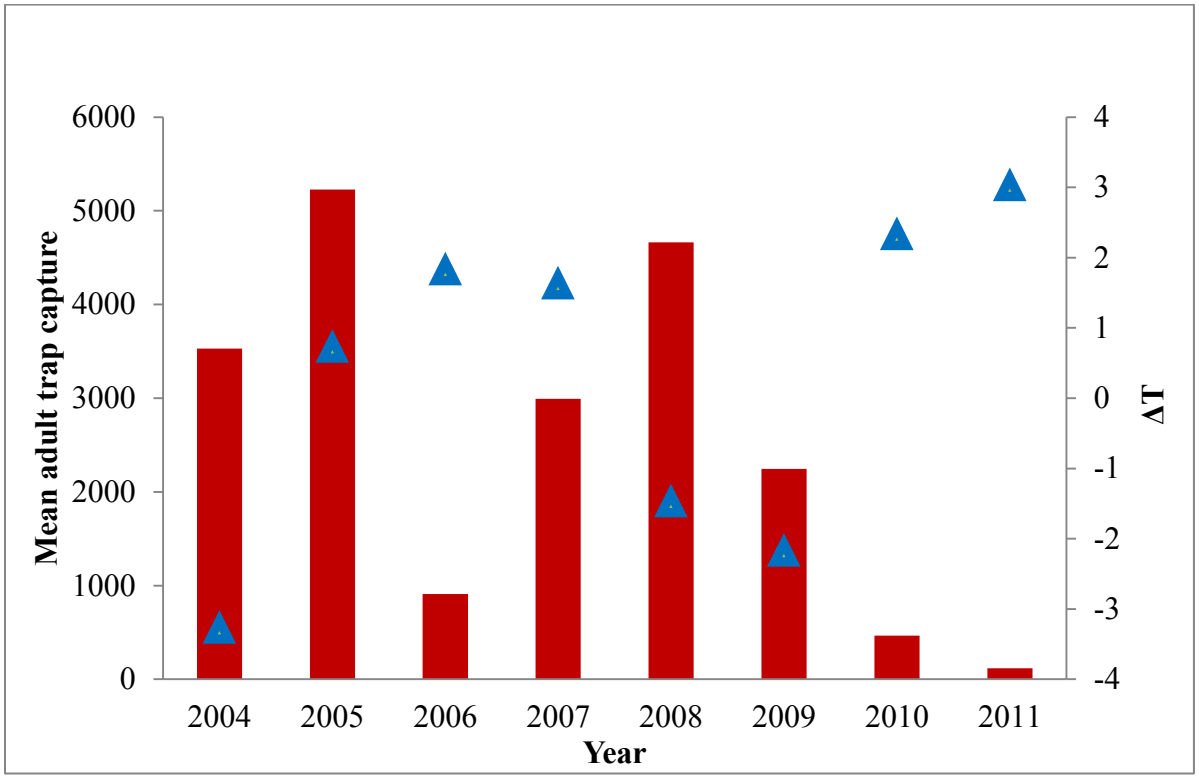


Figure 2.2. Changes in Japanese beetle adult weekly mean trap captures in Fayetteville, Arkansas from 2005 to 2012 in relation (red bars) to the average July temperature of the previous year (blue points) shown as the fluctuation in the average temperature (ΔT) of the month of August as compared to the 30 year historical average for northwest Arkansas.



3 Modeling the effects of temperature on the mortality of early instar *Popillia japonica* (Coleoptera: Scarabaeidae)

3.1 ABSTRACT

The Japanese beetle (*Popillia japonica* Newman) is an invasive species introduced to the United States from Japan in 1916. Since its introduction, the beetle has spread to cover most of the Eastern U.S., parts of the Midwest, and Midsouth. Climatic factors such as rainfall and temperature are thought to limit the range the Japanese beetle is able to spread. Here, a predictive model for temperature dependent mortality of first and early second instars was constructed by using archival data on the range of temperatures the beetle can survive at, field collected data, and established soil thermal models. Model results were compared to mortality ratesof Japanese beetle larvae in outdoor mass rearing cages. Simulations agree to within ≤16% of data collected on first instar mortality from 2009 to 2012; however, mortality of early second instars was under-predicted in all years. This increased mortality for second instars may be associated with problems of failed molting and increased mortality following molting at higher ambient temperatures, which were not accounted for in the model. Simulations well replicate the decrease in larval densities of *P. japonica* during and following extremely hot temperatures and explain the decreasing density with decreasing latitude. Our model has predictive applications and could be used to project adult densities in the succeeding summer.

Keywords: *Popillia japonica,* temperature, climate, mortality, white grubs

3.2 INTRODUCTION

The impact of density-independent factors like climate on insect populations has been a topic of interest for decades. Huffaker and Messenger (1964) proposed that as species moved from favorable to unfavorable environments there would be a high correlation between changes in population density and density-independent factors. In unfavorable environments, factors such as temperature and humidity may impact population density more than natural enemies or food supplies. This idea has played an important role in the introduction of natural enemies for the control of invasive pests in new areas. One of the more well-known examples of this phenomenon was the introduction of *Trioxys complanatus* (Quilis) (Hymenoptera: Braconidae) to control spotted alfalfa aphid in California. The biological control program was temporarily held back by the introduction of an Italian biotype of the wasp, which was adapted to cooler climates and failed to thrive during the hot and dry Californian summers (Flint 1980).

The relationship between insect development, insect mortality, and temperature is well established (Speight et al. 2008). Development rates increase with temperature until they reach a turning point where they begin a precipitous decline, eventually reaching a thermal death point, past which there are no surviving insects. This thermal death point varies between species and even the developmental stages within a single species. Percent survival to adulthood of the population has been shown to increase to a certain upper developmental temperature, which can be experimentally determined, and then decrease sharply above that temperature. The decrease in survival generally occurs well before the decline in development rates (Speight et al. 2008).

The Japanese beetle, *Popillia japonica* Newman, was introduced to the United States nearly a century ago in 1916 (Fleming 1972). Since then, it has spread in range to cover large sections of the U.S. (NAPIS 2013) where it can be a severe pest. The beetle was introduced to

Arkansas in 1997 where it heavily infested the northwestern region of the state (Johnson 2004). This region, along with northeastern Oklahoma, represents the southwestern border of the U.S. Japanese beetle infestation (NAPIS 2013). While the adult population causes significant economic damage, the larval population density in Arkansas rarely exceeds one per 0.1 m^2 (Wood et al. 2009). With a large number of fertile females emerging every summer and a large larval habitat available, the population densities of larvae would be expected to be bigger. Wood et al. (2009) suggested that high summer temperatures and sparse rain paired with large areas of grassland may account for the discrepancy between adult abundance and larval density. Since very few pathogens or parasitoids of *P. japonica* occur in the state (Petty et al. 2012), it is probable that an abiotic factor is limiting the density.

There has been much work investigating the effects of temperature on the development of *P. japonica.* Ludwig (1928) found 50% or greater mortality for early instar Japanese beetles exposed to temperatures higher than 31°C for prolonged periods and a thermal death point of 35°C. Ludwig (1928) went on to construct rudimentary curves of larval development rates. Régnière et al. (1981a) developed a model for first and second instar development based off the Ludwig (1928) data as well as plotted the amplitude of associated temperature changes in the soil. First and second instar Japanese beetles are fragile and minor stresses can cause mortality. Total larval density is largely dependent on the mortality of these first two instars (Dalthrop et al 2000). Dalthorp et al. (2000) found that annual variation in densities depended primarily on mortality occurring in July and August and not on larval deaths occurring during the winter months, because larvae are able to tolerate temperatures as low as -7 °C (Hoshikawa et al. 1988).

In addition to temperature dependent development and mortality in laboratory experiments, Allsopp (1996) predicted the spreading limits of the Japanese beetle to its

outermost borders, which extend west through Nebraska and Texas and south to the middle of Georgia and Mississippi. This prediction was made using rainfall and temperature data and the modeling program CLI-MEX 4.3. The Allsopp (1996) prediction has so far been shown to be accurate, although small, isolated populations have been found as far west as Colorado and Arizona, but these populations are not considered established (NAPIS 2013)

In order to study the effect of temperature on *P. japonica* populations, we constructed a heat transfer model was used to predict subsurface soil temperatures. Using archival data, we developed a temperature dependent mortality rate, which in conjunction with the heat transfer model, was used to predict population decline. Model results were validated by comparing simulations with field and lab data of the change in *P. japonica* populations over four years.

3.3 MATERIALS AND METHODS

In order to simulate the effects of temperature on population decline, we constructed a heat transfer model, which incorporates known temperature-mortality relationships from archival data. Our model, which finds its basis on applications to planetary science (Ulrich et al 2010; Rivera-Valentin et al. 2011; Chevrier and Rivera-Valentin 2012; Kereszturi and Rivera-Valentin 2012), incorporates the known thermal properties of common soil types to replicate the temperature with depth profile. At every studied depth and time, temperature is used as an input in our developed temperature-mortality functions. Simulated population decline is extracted for every diurnal run and compared to our field and lab data.

3.3.1 Thermal model

Surface and subsurface temperatures in the soil column were simulated by solving the one-dimensional thermal diffusion equations, which govern the transport of heat due to temperature gradients. The time and depth dependent thermal gradient is

$$\frac{\partial T}{\partial t} = \frac{k}{\rho C}\frac{\partial^2 T}{\partial z^2}, \quad (1)$$

where k is thermal conductivity, ρ is soil density, C is heat capacity, T is temperature, t is time, and z is depth. The heat flux through the soil column due to the thermal gradient is

$$Q = -k\frac{\partial T}{\partial z}, \quad (2)$$

where Q is heat flux. The thermal diffusion equations were solved numerically using an Euler forward finite element procedure (Edwards et al. 1979; Sally 2002). This discrete approximation to the continuous function has been shown to well replicate planetary temperatures (Mellon and Jakosky 1993; Ulrich *et al.*, 2010; Rivera-Valentin *et al.* 2011; Chevrier and Rivera-Valentin 2012; Kereszturi and Rivera-Valentin 2012). The surface is assumed to follow a diurnal sinusoidal temperature condition bounded by the maximum and minimum temperatures for the studied days, which were obtained from the National Oceanic and Atmospheric Administration (NOAA), such that the surface temperature as a function of local hour (H) is

$$T_s(H) = T_x + \Delta T \cos\left(1.0225\, r_p\,(H-14)-1\right), \quad (3)$$

where T_x is the maximum surface temperature in Fahrenheit, ΔT is the temperature amplitude, and r_p is the rotational period of the earth (Fig. 3.1). Even though there exists perturbations to the sinusoidal surface temperature oscillations, the assumption is fair and a common approximation to temperature variations (Régnière et al., 1981a; Schorghofer, 2007; Schorghofer and Taylor, 2007).

To reach convergence, thermal diffusion is modeled to several times the skin depth (δ),

$$\delta = \sqrt{\frac{kP}{\pi \rho C}}, \tag{4}$$

where P is the period in seconds (Mellon and Jakosky, 1993; Ulrich *et al.*, 2010; Rivera-Valentin et al 2011), which is the e-folding depth of the thermal wave, which describes how it dampens with depth in a material of known thermal properties. For reference, the annual skin depth (the case where P is one year) of dry soils is 1 m (Schorghofer, 2007). Two soil types were used in this analysis, sandy and clay loam. The thermal conductivity of sandy loam is 0.2 W m^{-1} K^{-1} and its volumetric heat capacity is 10625 kJ m^{-3} K (Abu-Hamdeh, 2001; Bristow, 1998) (Table 3.1). Clay loam has a thermal conductivity of 0.36 W m^{-1} K^{-1} and a volumetric heat capacity of 11305 kJ m^{-3} K (Abu-Hamdeh, 2001; Bristow, 1998). Here, thermal properties were assumed constant with temperature.

For our finite difference methodology, we considered a depth of 2 m using 200 elements resulting in an element thickness of 0.01 m. The code runs one day at time increments of 100 seconds 100 times in order to reach a converged temperature profile. Since we model temperatures well below the annual skin depth, which is where the temperature is approximately

the average surface temperature, then the bottom boundary condition is set to the average surface temperature for the studied day.

3.3.2 Mortality rate function

The temperature dependent development rates of *P. japonica* were established using data from Ludwig (1928) and the framework of Briere *et al.* (1999) (Fig. 3.2). The development rate $R(T)$ for *P. japonica* can be expressed as

$$R(T) = \alpha T\left(T - T_{\min}\right)\left(T_{\max} - T\right)^{\frac{1}{m}}, \tag{5}$$

where T_{max} and T_{min} are the upper and lower thermal limits of *P. japonica*, and α and m are variables describing the effect of temperature (Table 3.1). These variables are found for both the first and second instar. The thermal-mortality function, $M(T)$, or rather the percent of the larval population that die when exposed to a steady temperature, was found using the data provided by Ludwig (1928) (Fig. 3.3 and 3.4). Goodness of fit for the data was found using a chi-squared model utility test; however, the first to second instars showed low confidence, so we performed a Student's t-test to show that there exists correlation between mortality and temperature.

The mortality rate, or rather percent of the larval population that die per unit time as a function of temperature, is then found by multiplying the thermal-mortality function by the development rate specific to the life stage such that

$$v_m = R(T) \times M(T), \tag{6}$$

where v_m is the mortality rate. Temperature results as a function of depth and time from our thermal model are inputted per simulated time into the mortality rate in order to achieve a depth profile of the effects of temperature on the larvae. The total percent of the population mortally affected by temperature at the end of one day (Tables 3.2 and 3.3) is then found by numerically integrating the mortality rate for one day:

$$\int v_m \, dt = \int \left(R(T) \times M(T) \right) dt \quad (7)$$

Since the maximum number of days each larva was exposed to temperature in the Ludwig study (1928) was 220 days, the minimum development rate considered here is 0.0045.

3.3.3 Source of adult Japanese beetles

Japanese beetles were collected during the summers of 2009 -2012 at the Arkansas Agriculture Research and Extension Center. Beetles were trapped using Jumbo Jug (Trécé Inc. Adair, OK) funnel mouth traps (1.89 L) with floral and sex lures. Traps were emptied daily and beetles were identified as male or female by apical tibial spur (Fleming 1972). The number of beetles captured in traps each year varied and this determined the numbers for stocking cages. There was an extreme reduction in the numbers of beetles capture din 2011 and 2012, and this greatly impacted the number of beetles added to cages those years.

3.3.4 Production of Japanese beetles in rearing cages for model validation

In order to quantify Japanese beetle larval mortality in relation to temperature, rearing cages were constructed for the production of large, quantifiable populations of larvae.

3.3.4.1 2009

Three 3.8 m × 3.8 m plots were cleared and tilled to a depth of 15 cm. The soil was modified with 0.3 m^3 of sand per plot because of high clay content, as determined by soil analysis by the Washington County Extension Service. Three tents, each measuring 1.8 m × 1.7 m × 1.9 m, were constructed on the plot to delineate rearing cages. The first rearing cage received 29.8 g of KY31E- Fescue seed. The second rearing cage received 4.9 g of Teff seed, and the third rearing cage received 14.9 g Fescue seed and 2.5 g Teff seed. When the grasses were established, adult Japanese beetles were added to each. Each cage received 1,100 females and 1,100 males that were allowed to mate and oviposit. Adults were fed fresh grape leaves and apple slices daily for two weeks while oviposition was occurring. Rearing cages were irrigated as needed to maintain soil moisture.

Our sampling method to determine larval beetle density within each rearing cage was adapted from the field sampling method originally described by Fleming and Baker (1936). One soil core (7.6 cm × 7.6 cm × 12.7 cm) was taken from each rearing cage twice weekly and all eggs and larvae of each instar were counted. Then the soil and all beetle stages were returned to the cages from which they were removed. Samples were taken from 13 July 2009 to 30 October 2009.

3.3.4.2 2010

Japanese beetle collection methods and time frame were the same in 2010 as they were in 2009. A 7.6 m × 7.6 m plot was divided into six sections and each section received 0.1 m^3 of sand. Six rearing cages were constructed and were divided as follows: two rearing cages were planted with mature fescue sod and four rearing cages were each planted with 5 g of Teff grass seed. All rearing cages were irrigated as needed. When grass was established, each rearing cage

received 1,150 female and 650 males. Adult beetles were fed grape leaves and apple slices for two weeks while they mated and oviposited.

Egg, first instar and second instar density was tracked as before, but in 2010 two sampling cores were taken from each rearing cage twice weekly, instead of one core twice weekly. This was done to provide a more accurate estimate of density. Samples were taken from 13 July 2010 to 1 September 2010. Density of each life stage per grass type per week was compared using ANOVA (α=0.01) in JMP® 9.0 to determine any differences in abundance (SAS Institute Inc.).

3.3.4.3 2011

In 2011, outdoor rearing cages were prepared the same as in 2010, with the exception that all cages were seeded with fescue grass, approximately 30 g of KY31E- fescue seed per cage, and the soil type was closer to silt loam. Adult beetles, 720 females and 730 males, were added to each outdoor cage. In addition, four indoor rearing cages were prepared using 13 plastic m^2 swimming pools filled with topsoil, covered in screening, and seeded with fescue, 25 g of KY31E- Fescue seed per pool. Indoor cages were maintained under constant light and 24 °C. Because they were a smaller volume and surface area than outdoor rearing cages, 490 females and 430 males were added to each indoor rearing cage. Larval density and developments was tracked as in 2010. Samples were taken from 14 July 2011 to 26 August 2011.

3.3.4.4 2012

In 2012, outdoor and indoor rearing cages were prepared the same as in 2011**.** Because of an extreme decline in the abundance of wild adult Japanese beetles, only 160 females and 140 males were added to each outdoor cage and 90 females and 80 males to each indoor cage. Larval

density and life stage development was tracked as in 2010. Samples were taken from 3 July 2012 through 9 August 2012.

3.3.5 Comparison of model results and field data

Temperature data for the days that the rearing cages were operational and monitored were acquired from the National Oceanic and Atmospheric Administration (NOAA). For 2009, 2011, and 2012, the temperature record from Drake Airfield in Fayetteville, Arkansas was used, and in 2010 the temperature record from the University of Arkansas Experimental Station was used. Two different locations were used because complete data sets were not available for both years from either location. The two locations are approximately 32 km apart and so large differences in recorded temperatures are not expected.

Temperatures above 31°C were used to determine mortality rates as predicted by the model. Below 31 °C mortality is predicted to be very small and may not be distinguishable from other sources of mortality. Percent mortality of each instar population on days above 31°C was summed in order to predict the range of mortality whereby the upper threshold of the range is found assuming all larvae in the population are immediately below the thatch layer and the lower range is given assuming all larvae in the population are 10 cm below the thatch layer. Estimated mortality in the rearing cages for each instar was also calculated. This was done by comparing the peak population density of each instar and its succeeding instar. This estimate was then compared to the predicted range of mortality, which was constructed using temperatures from every day the instar of interest was present in the rearing cages.

3.4 RESULTS

3.4.1 Production of Japanese beetles in rearing cages for model validation

3.4.1.1 2009

Eggs and first instars could be found in each of the rearing cages by July 13, 2009 (Week 1). Second instars were present by August 5, 2009 (Week 4) and third instars were present by August 27, 2009 (Week 7). Because all three cages had different grass types with no replication, no analyses between the abundance of larval stages or development time were conducted.

3.4.1.2 2010

Eggs and first instars could be found in each of the rearing cages by July 13, 2010 (Week 1). Second instars were present in large numbers in the fescue rearing cages by July 30, 2010 (Week 2) and in the teff rearing cages by August 12, 2010 (Week 4). Third instars were never present, with measurable larval densities in all rearing cages disappearing by September 1, 2010 (Week 6).

There were no significant differences between egg densities between different grass types. During week one, two, four, five and six, there were no significant differences in first instar densities and grass types. During week three, there was a significant difference between the fescue and teff rearing cages for first instar densities ($F=8.2075$, $P=0.0090$) and second instar densities ($F=11.7333$, $P=0.0024$) with both life stages existing in higher densities in the fescue rearing cages. For weeks four and six, there were no significant between second instar densities between grass types but there was a difference between second instars in week five ($F=37.7589$, $P<0.0001$).

3.4.1.3 2011

In the outdoor fescue rearing cages, eggs and first instars could be found by 14 July 2011 (Week 1), second instars were present by 18 August 2011 (Week 4)**,** and third instars were never present, with measurable larval densities in all rearing cages disappearing by 26 August 2011. In the indoor fescue pools, eggs could be found by 14 July 2011 (Week 1)**,** first instars were present by 27 July 2011 (Week 3), and second instars were present by 26 August 2011 (Week 6). Sampling was stopped after this because of a collapse in the larval Japanese beetle populations in the outdoor beds, although larvae were still present in indoor beds.

3.4.1.4 2012

In the outdoor fescue rearing cages, eggs and first instars could be found by 3 July 2012 (Week 1). Second and third instars were never present, with measurable larval densities in all rearing cages disappearing by 27 July 2012 (Week 4). In the indoor fescue pools, eggs and first instars could also be found by 3 July 2012 (Week 1), second instars were present by 18 July 2012 (Week 3), and third instars were present 9 August 2012 (Week 7). Sampling in the outdoor rearing cages stopped after 9 August 2012 because no larvae remained alive. Indoor sampling continued through 24 August 2012 (Week 9). When sampling of indoor beds ceased, first, second and third instar Japanese beetles were still present.

3.4.2 Comparison of model results and field data

From the thermal-mortality function chi-squared model utility test for eggs to first instars, we found a chi value of 0.99 indicating a close relationship, with 99% of variation being predicted by the model. For first to second instars, the chi value was 0.0001, most likely caused by low replication and high variation in the Ludwig (1928) data. However, the Student's t-test

found that to a 95% CI, temperature provides information to the prediction of mortality and that from 10 - 31°C there exists a negative correlation.

Data from NOAA showed nine days above 31°C in July 2009 and eight days above 31°C in August 2009 during the sampling period. In 2010, there were 22 days above 31°C in July, and 28 days above 31°C in August. In 2011, there were 18 days above 31°C in July and 23 days above 31°C in August. In 2012, there were 29 days above 31°C in July and 9 days above 31°C in August. Temperatures above 31°C were paired with their corresponding percent population mortality from our mortality rate equation.

The 2009 combined rearing cages had the following estimated peaks for each instar (Table 3.4): egg density of 259/0.1 m^2, first instar density of 204/0.1 m^2, second instar density of 66/0.1 m^2, and third instar density of 23/0.1 m^2. For 2009, the model predicted population mortality between 21.5 to 36.9% for early first instars eclosing from eggs. We observed a 22% decline from the egg population to the first instar population in the rearing cages during the same period of time. For late first instars molting into second instars, the model predicted population mortality between 7.8 to 10.2% and we observed a population decline of 74%.

The 2010 fescue grass rearing cages had the following estimated peaks for each instar (Table 3.4): egg density of 105/0.1 m^2, first instar density of 60/0.1 m^2, and second instar density of 17/0.1 m^2. There was no measurable density of third instars. The 2010 teff grass rearing cages had the following estimated peaks for each instar: egg density of 140/0.1 m^2, first instar density of 70/0.1 m^2, and second instar density of 3/0.1 m^2. There was no measurable density of third instars. For 2010, the model predicted mortality between 35.1 to 57.3% for early first instars eclosing from eggs. We observed in the fescue rearing cages during the same period of time a 42% decline from the egg population to the first instar population. In the teff rearing cages, this

decline was observed to be 50%. For late first instars molting into second instars, the model predicted population mortality between 31 to 46.3%. We observed a population decline of approximately 72% during that time in the fescue rearing cages and in the teff rearing cages a 96% decline in the population.

The 2011 outdoor fescue rearing cages had the following estimated peaks for each instar (Table 3.4): egg density of 56.2/0.1 m^2, first instar density of 33.1/0.1 m^2, and second instar density of 5.8/0.1 m^2. There was no detectable third instars. The 2011 indoor fescue pools had the following estimated peaks for each instar: egg density of 134.0 /0.1 m^2, first instar density of 134.0/0.1 m^2, and second instar density of 2.2/0.1 m^2. The second instar peak is an artifact of a stop in sampling and not a true peak. Sampling was not continued in indoor pools through third instar development. For 2011, the model predicted mortality between 48.8 to 71.9% for early first instars eclosing from eggs. We observed in the outdoor fescue rearing cages during the same period of time a 41.03% decline from the egg population to the first instar population. For late first instars molting into second instars, the model predicted population mortality between 8.03 to 14.34%. We observed a population decline of approximately 82.6% during that time in the outdoor fescue rearing cages followed by the death of all second instar population. For the indoor fescue pools, we observed a 0% decline from the egg to first instar and a 98.4% decline from first instar to second instar. This percentage is an artifact of a stop of sampling in the second instar population.

The 2012 outdoor fescue rearing cages had the following estimated peaks for each instar (Table 3.4): egg density of 8.6/0.1 m^2 and first instar density of 2.9/ 0.1 m^2. There was no measurable second or third instar density. The 2012 indoor fescue pools had the following estimated peaks for each instar: egg density of 30.3/0.1 m^2, first instar density of 36.8/0.1 m^2,

second instar density of 49.8/0.1 m^2, and third instar density of 12.9/0.1 m^2. For 2012, the model predicted population mortality between 32.68 – 60.8% for early first instars eclosing from eggs. We observed in the fescue rearing cages during the same period of time a 66.7 % decline from the egg population to the first instar population. For late first instars molting into second instars, the model predicted population mortality between 19.55 – 20.45%. We observed a population decline of approximately 100% during that time in the fescue rearing cages, with no second instars being found. For the indoor fescue pools, we observed a 0% decline from the egg to first instar populations and a 0% decline from first instar to second instar populations. Instead, we did find an increase in the number of beetles between subsequent life stages. Since this is impossible without more beetles being added to the system, the increase may be caused by sampling error and aggregations in the larval populations within a rearing cage.

3.5 DISCUSSION

3.5.1 Violations of assumption in the thermal model and mortality rate function

The first assumption is the thermal properties of the soil are constant in both space and time. For simplicity, it was assumed within the model that thermal properties of the soil are constant with temperature and do not vary with soil depth (i.e. a homogeneous soil column). Coupled with this assumption is that there are no microhabitats within the soil with different thermal properties. However, variation in soil properties within the relevant depths considered for mortality are minimal and true values do not deviate much from our assumed values. The second assumption is that diurnal surface temperature follows a sinusoidal curve. Though in general this is an accurate statement, weather patterns do alter this trend, which would then affect the models ability to predict mortality. The third assumption in the model is that mortality rate is

constant for a given temperature. Although it is possible larvae die in a non-constant fashion when exposed to a steady high temperature, rearing cage data corroborates a constant mortality rate with constant temperature as opposed to points of group mortality. Our model also assumes steady soil moisture levels. Effects of low moisture have been previously established in Régnière et al. (1981c).

3.5.2 Production of Japanese beetles in rearing cages for model validation

It is possible that the grasses had no treatment effects on the oviposition habits of the adult female beetles in the 2009 rearing cage setup, but this is not well supported in the literature (Wood et al. 2009). The more likely scenario is there were some treatment effects that were overshadowed by the effects of native grass contamination in the rearing cages, which was problematic in 2009. This may have had the net effect of making all rearing cages roughly "equal" habitats for the beetles. Due to the abundance of native grasses invading the rearing cages in 2009, the three rearing cages were treated as similar treatments when fitted with the model. Additionally, lack of replication in 2009 made analysis of differences impossible.

In 2010, we did see some significant differences from grass types in Weeks 3 and 5. The difference in first and second instar populations were not consistent between weeks, although it is consistent with data showing that Japanese beetle larvae do well on fescue and other cool season turfgrasses (Crutchfield and Potter 1995, Wood et al. 1999). It is possible that the differences in larval densities caused by the grass types were overshadowed by the effects of temperature, which would explain why there is no consistent difference between grass types. Some differences were seen between the 2010 fescue and teff rearing cages that couldn't be seen in 2009. This may be caused by less native grass contamination in the 2010 rearing cages than there was in the 2009 rearing cages. In 2010, some effects, although inconsistent, were seen and

so each treatment was fitted to the data independently. In 2011 and 2012, different grasses were not used. Additionally, in 2011 and 2012 fewer beetles were added to each cage because of a decline in the number of available wild Japanese beetles. This is reflected in the overall lower initial population of Japanese beetles per outdoor and indoor cage.

3.5.3 Comparison of model results and field data

Observed density decline from egg to first instar fit within the model's prediction range in all rearing cages in 2009 and 2010. In 2011, mortality between eggs and first instars was over-predicted by 7.85% and in 2012 mortality was under-predicted by 5.9%. In both 2011 and 2012, there was no observable mortality in indoor climate controlled rearing pools, which was expected. Some differences were expected between predicted and actual mortality, as the model assumes sinusoidal temperature fluctuations and idealized soil properties. Because the largest error between our simulation results and field data is <16%, then out of the set of parameters that can effect mortality, we can safely assume temperature is the largest.

Observed density decline from first instar to second instar was above the range that was predicted by the model in all years. The higher than predicted mortality in second instars may be due to complications with molting associated with high temperatures. For example, in the delphacid *Laodelphax striatellus*, a rearing temperature of 35 °C during nymphal growth has been associated with increased mortality several days to a week after molting in both nymphs and adults (Noda and Saito1979). Data used to construct our model did not make any clear distinctions with regards to this cause of mortality, and the first and second instar Japanese beetles in our rearing cages were often exposed to temperatures higher than 35 °C.

When estimating the percent population mortality in the system, it is difficult to know where in the soil column any given larva is, much less the vertical distribution of the entire

population. For data fitting purposes, the estimated population mortality is between the two points of highest and lowest mortality. Highest mortality corresponds to all larvae within the area being confined to the top of the soil column. The lowest estimate corresponds to all larvae being at the bottom of the soil column at the depth of interest, in this case 10 cm. The true spatial distribution of the larvae in the column will be more randomized and should fit within the range.

Another possible confounding factor in estimating mortality in rearing cages is cannibalism cause by overcrowding. Régnière et al. (1981b) originally reported that high densities of first instar *P. japonica* increased survival of the larvae while increasing densities of second and third instars actually decreased the likelihood of survival, possibly through competition for resources or cannibalism. Crutchfield and Potter (1995) showed that third instar *P. japonica* do not suffer from increased mortality at densities as high as 146/0.1 m^2. Despite the Régnière et al. (1981b) findings of first instars surviving better at higher densities; we observed a sharp decline in our first instar populations. Our second and third instar densities were also well below the reported densities that *P. japonica* larvae are known to survive without increased density dependent mortality in all four years of data gathering. Because of this, the mortality in our cages was probably not caused by limiting resources or cannibalism, except in 2011 indoor rearing cages, in which the grass died from excessive feeding damage by the larvae followed by a precipitous drop in larval numbers between first and second instars.

Considerable research has been done on preferences of *P. japonica* females for cool- and warm-season turfgrasses during oviposition and impacts these grasses have on local populations (Potter and Held 2002, Wood et al. 2009). *P. japonica* females were reported to prefer to oviposit in tall fescue (*Festuca arundinacea* Schreb.) and zoysiagrass (*Zoysia japonica* Steud.), and had reduced oviposition in common bermudagrass (*Cynodon dactylon* [L.] Pers.) and hybrid

bermudagrass in choice tests. In no choice tests, females oviposited most in tall fescue and oviposit in zoysiagrass and common bermudagrass at similar rates. Hybrid bermudagrass underperforms all three (Wood et al. 2009). Data indicates a trend for increased oviposition and therefore an increased density of larval *P. japonica* in cool-season turfgrasses. The effects of cool or warm-season turfgrasses may be the primary limiting factor for the size of an initial population of *P. japonica*, but probably does not drastically affect populations between years. Climatic conditions can be a major cause of mortality within that population for first and second instars and may be responsible for major abundance changes in the Japanese beetle between years.

Larval densities of Japanese beetle vary across the U.S. In Connecticut, density ranged from <1 to 18 larvae per 0.1 m^2 (Hanula 1990). In Michigan, densities ranged from 3.7 to 21 larvae per 0.1 m^2, with an average of 9.5 larvae per 0.1 m^2 (Cappaert and Smitley 2002), and in eastern Massachusetts, fall density of larvae ranged from 12.8 to 27.2 per 0.1 m^2 (Vittum 1986). Whereas, the density of third instars in Arkansas has ranged from 0.05- 0.7 per 0.1 m^2 from 2010 to 2012 (unpublished). Overall, the pattern is a decrease in density with decreasing latitude.

If we return to the idea that as a species moves out of favorable environments their density is increasingly impacted by density-independent factors (Huffaker and Messenger 1964), this pattern should be expected. The native range of *P. japonica* in Japan ranges from Hokkaido in the North to Yokohama in the south (Fleming 1972). The equivalent range in the United States is approximately Maine to North Carolina east of the Appalachian Mountains. Expansion outside of this range would mean expansion into less favorable habitat and a larger impact of climatic factors on population densities. Climatic effects may be increased as the Japanese beetle spreads into the interior of the U.S. as the interior suffers from continental climate effects resulting in

hot, dry summers and cold winters. These effects are caused by the absence of large bodies of water to mediate temperature (Ahrens 2008), a problem not encountered in the beetle's native range.

Major changes in Japanese beetle populations have been observed since the introduction of the beetle to Arkansas in 1997 (Johnson 2004), including a 90% reduction in the adult population since 2009 (unpublished data). The reduction in the population of Japanese beetles in Arkansas has coincided with extreme weather events such as spring flooding and long periods of extreme summer heat and drought. As the Japanese beetle expands its range southward, its ability to thrive will be hampered in areas with extremely hot summer temperatures. This is to be expected when we consider climate as an important density-independent population regulation factor.

Our model can be used to explain mortality rates in juvenile populations of Japanese beetles in warm-climate areas and the adult population fluctuations associated with changes in larval densities. This model is not only explanatory, but has predictive applications. By tracking summer temperatures in a geographical area, this model can predict expected adult populations in the following summer. Although such a predictive model is possible, it will require more investigation.

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Table 3.1. Thermal property constants in two soils and variables describing the effects of temperature on Japanese beetle eggs and first instars.

	Constant	Value	Units
Model Parameter			
	Finite Element Thickness	0.01	m
	Time Step	100	s
Thermal Properties			
Sandy Loam	Thermal Conductivity	0.2	$W\ m^{-1}\ K^{-1}$
	Density	1.25	$g\ cm^{-3}$
	Specific Heat	800	$J\ kg^{-1}\ K^{-1}$
Clay Loam	Thermal Conductivity	0.36	$W\ m^{-1}\ K^{-1}$
	Density	1.33	$g\ cm^{-3}$
	Specific Heat	900	$J\ kg^{-1}\ K^{-1}$
Development Rate Constants			
Eggs	alpha	1.16×10^{-4}	-
	m	2.099	-
First Instar	alpha	9.07×10^{-5}	-
	m	3.5	-

Table 3.2. Sample output of the mortality model showing the percentage population mortality for early first instar Japanese beetles at varying surface temperatures and soil depths in sandy loam.

	Air Temperature (°C)									
Depth (cm)	**31**	**32**	**33**	**34**	**35**	**36**	**37**	**38**	**39**	**40**
1	1.3	1.6	1.8	2.1	2.5	2.8	3	2.6	2.3	2.2
2	1.1	1.3	1.6	1.8	2.2	2.5	2.9	3.2	3.3	2.7
3	0.88	1.1	1.3	1.6	1.9	2.3	2.6	3	3.4	3.7
4	0.74	0.93	1.2	1.4	1.7	2	2.4	2.8	3.3	3.7
5	0.64	0.81	1	1.3	1.5	1.9	2.2	2.7	3.1	3.6
6	0.56	0.72	0.91	1.1	1.4	1.7	2.1	2.5	3	3.4
7	0.5	0.65	0.83	1.1	1.3	1.6	2	2.4	2.8	3.3
8	0.45	0.6	0.77	1	1.2	1.5	1.9	2.3	2.7	3.2
9	0.42	0.56	0.73	0.9	1.2	1.5	1.8	2.2	2.6	3.1
10	0.39	0.53	0.69	0.9	1.1	1.4	1.7	2.1	2.6	3

Table 3.3. Sample output of the mortality model showing the percentage population mortality for second instar Japanese beetles at varying surface temperatures and soil depths in sandy loam.

	Air Temperature (°C)									
Depth (cm)	**31**	**32**	**33**	**34**	**35**	**36**	**37**	**38**	**39**	**40**
1	0.84	0.97	1.2	1.5	1.3	1.1	1.1	1.1	1.1	1.1
2	0.82	0.86	0.89	1.2	1.4	1.6	1.3	1.2	1.2	1.2
3	0.81	0.85	0.89	0.93	1.2	1.5	1.7	1.5	1.4	1.3
4	0.79	0.84	0.89	0.95	1	1.2	1.6	1.9	1.6	1.5
5	0.77	0.83	0.9	0.97	1.1	1.1	1.4	1.7	2	1.7
6	0.75	0.82	0.9	1	1.1	1.2	1.2	1.5	1.8	2.1
7	0.74	0.82	0.92	1	1.1	1.2	1.2	1.3	1.6	2
8	0.73	0.83	0.94	1	1.1	1.2	1.3	1.3	1.4	1.8
9	0.72	0.84	0.94	1	1.1	1.2	1.3	1.3	1.3	1.6
10	0.73	0.84	0.95	1	1.1	1.2	1.3	1.3	1.4	1.4

Table 3.4. Rearing cage type by year and observed changes is rearing cage population of Japanese beetles in Fayetteville, Arkansas compared to predicted changes

Year	Bed Type	# Females	Instar	Predicted Decline	Obs. Decline	Peak Instar Densities / 0.1 m^2
2009	Outdoor - Mixed	1,100	Egg to first	21.5−36.9%	22%	Eggs:259, First instars:204
2009	Outdoor - Mixed	1,100	First to second	7.8−10.2%	74%	First instars:204, Second instars:66
2010	Outdoor - Fescue	1,150	Egg to first	35.1−57.3%	42%	Eggs:105, First instars:60
2010	Outdoor - Teff	1,150	Egg to first	35.1−57.3%	50%	Eggs:140, First instars:70
2010	Outdoor - Fescue	1,150	First to second	31−46.3%	72%	First instars:60, Second instars:17
2010	Outdoor - Teff	1,150	First to second	31−46.3%	96%	First instars:70, Second instars:3
2011	Outdoor - Fescue	720	Egg to first	48.9−71.9%	41.0%	Eggs:56.2, First instars:33.1
2011	Indoor - Fescue	490	Egg to first	48.9−71.9%	0%	Eggs:134.0, First instars:134.0
2011	Outdoor - Fescue	720	First to second	8.1−14.3%	82.6%	First instars:33.1, Second instars:5.8
2011	Indoor - Fescue	490	First to second	8.1−14.3%	98.4%	First instars:134.0, Second instars:2.2
2012	Outdoor - Fescue	160	Egg to first	32.7−60.8%	66.7 %	Eggs:8.6, First instars:2.9
2012	Indoor - Fescue	90	Egg to first	32.7−60.8%	0%	Eggs:30.3, First instars:36.8
2012	Outdoor - Fescue	160	First to second	19.6−20.5%	100%	First instars:2.9
2012	Indoor - Fescue	90	First to second	19.6−20.5%	0%	First instars:36.8, Second instars:49.8

Figure 3.1. Diurnal sinusoidal surface temperature and soil temperatures at varying soil depths.

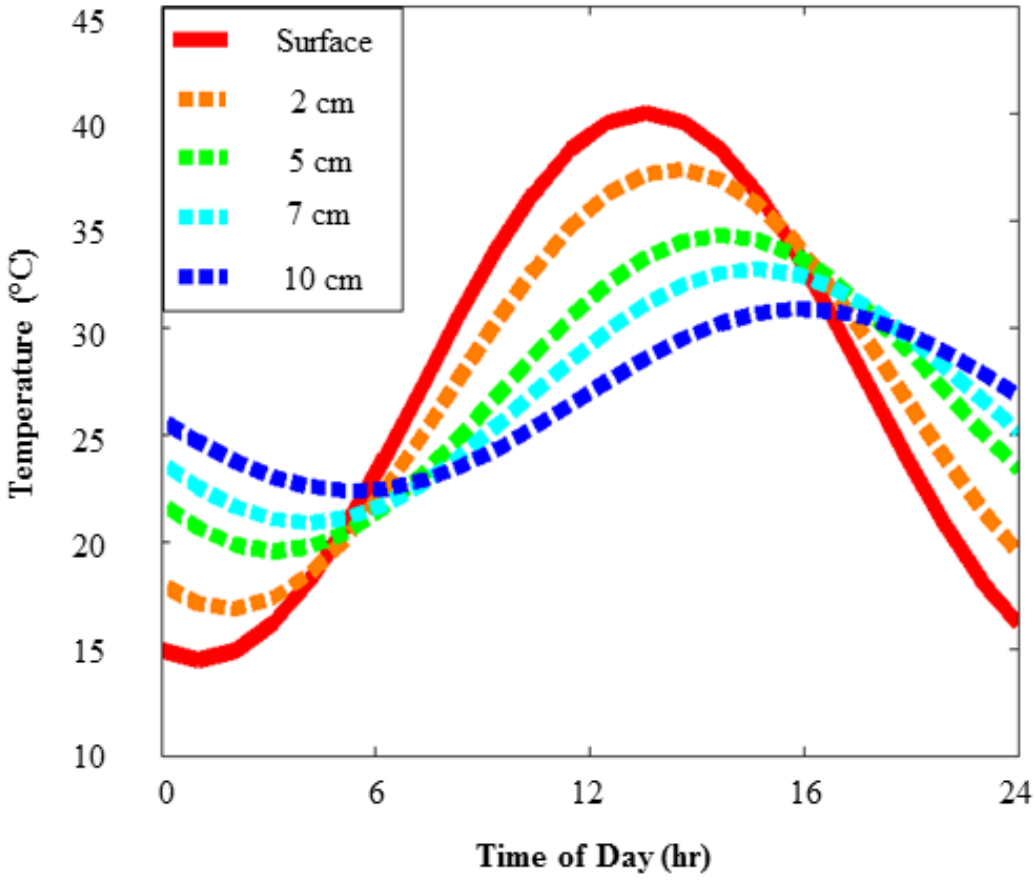


Figure 3.2. Development rates of eggs and first instar *Popillia japonica*. Data points were taken from Ludwig (1928) and lines of best fit created by equation (5).

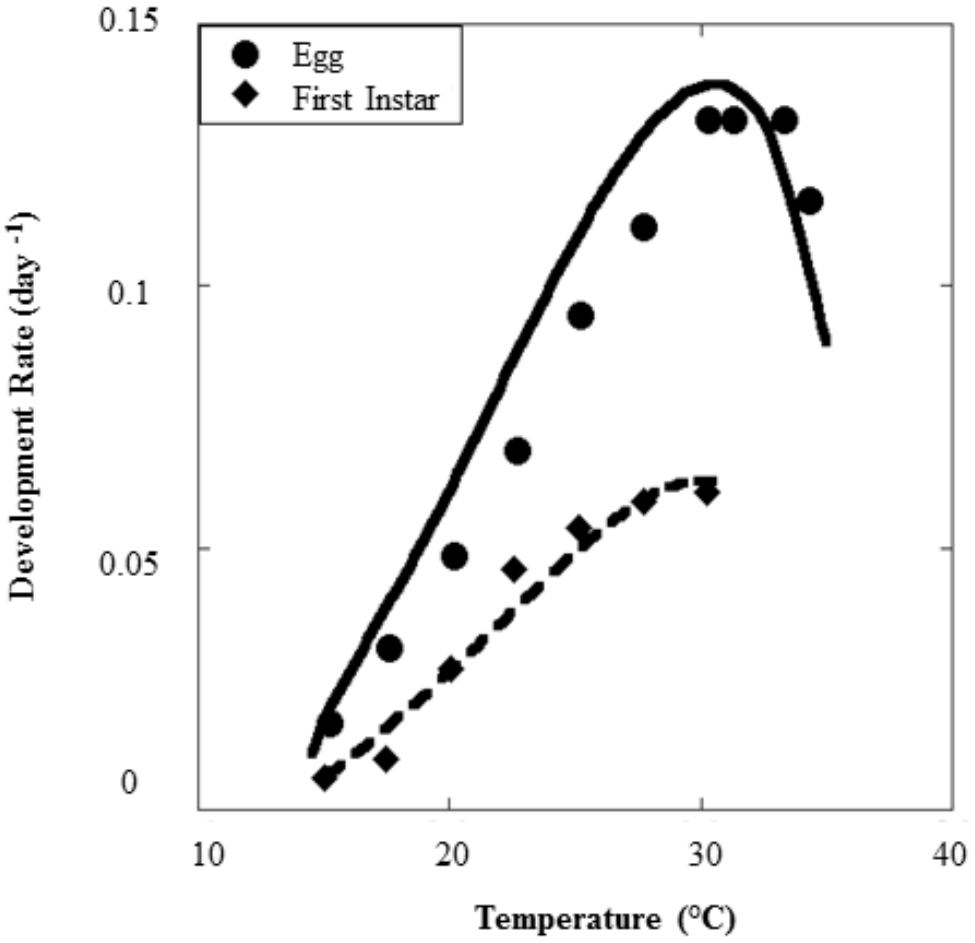


Figure 3.3. The thermal-mortality function of egg to first instars exposed to a steady temperature based on Ludwig (1928) data.

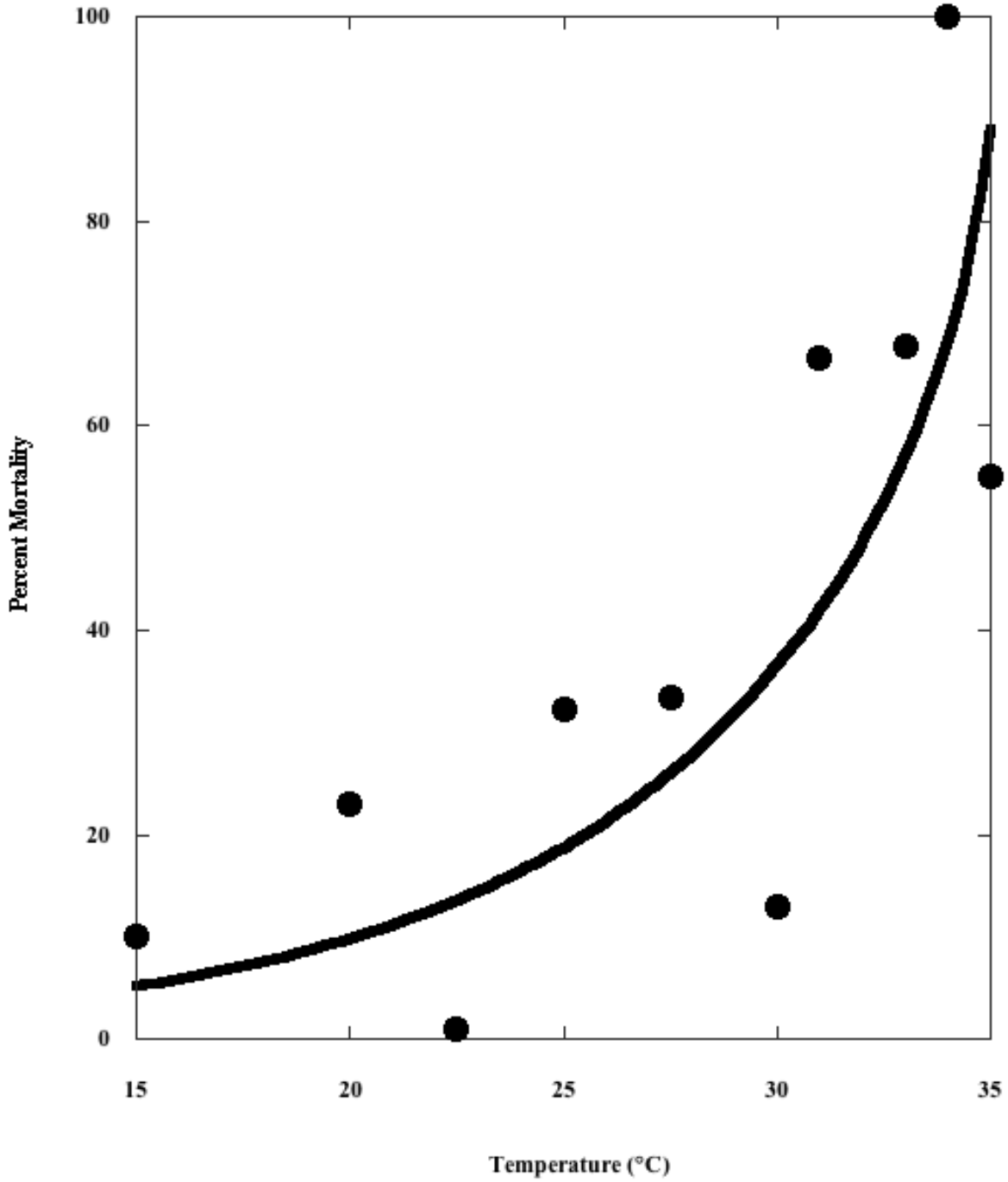
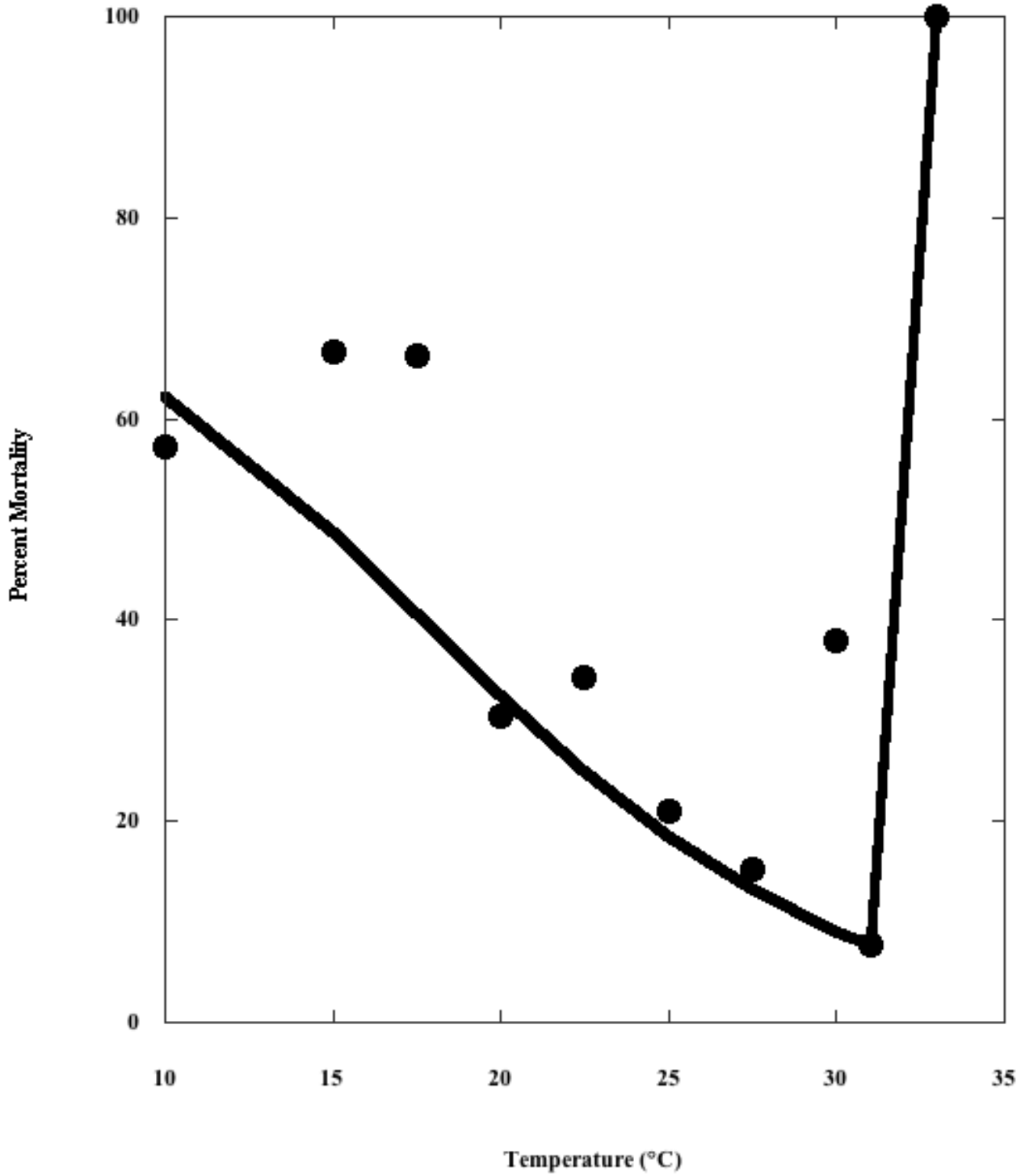


Figure 3.4. The thermal-mortality function of *Popillia japonica* first to second instars exposed to a steady temperature based on Ludwig (1928) data.



4 Survey of pathogens and parasitoids of *Popillia japonica* (Coleoptera: Scarabaeidae) in northwest Arkansas

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4.1 ABSTRACT

The impact of pathogens and parasitoids on the recently established population of *Popillia japonica* Newman in northwest Arkansas has been unknown. In this study, we quantified the prevalence of natural enemies: *Stictospora villani* Hays, *Ovavesicula popilliae* Andreadis and Hanula*, Paenibacillus spp.* (Dingman), nematodes and parasitic Diptera and Hymenoptera in third instar and adult populations in 2010 and 2011. *S. villani* was found in 38.4% and 35.5% of larvae in 2010 and 2011, respectively. *S. villani* was not found in adult beetles. *Paenibacillus* bacteria were not found in either larvae or adults in either year. In 2010, the microsporidian *O. popilliae* was not found in larvae but was present in 0.2% of adults. In 2011, 2.6% of larvae were infected with *O. popilliae*, but the microsporidian was not found in adults. A previously unknown *Adelina* sp. was found infecting 0.4% of adult beetles in 2010 and 1.3% of larvae in 2011. Nematode infections were found in 1.8% of larvae and 0.1% of adults in 2010 and not found in either life stage in 2011. No parasitic Hymenoptera or Diptera were found in either year. Apparently, pathogens and parasitoids currently provide little control of the Japanese beetle population within northwest Arkansas.

Keywords: *Popillia japonica*; *Ovavesicula popilliae*; *Paenibacillus* spp.; *Stictospora villani*; Arkansas

4.2 INTRODUCTION

The Japanese beetle (*Popillia japonica* Newman) was accidentally introduced to North America in 1916 in New Jersey through horticultural imports (Fleming 1972). Since then, it has spread to cover the Eastern United States, parts of the Midwest, Midsouth and Southern Canada (NAPIS 2011).

P. japonica was first found in northwest Arkansas in 1997 (Johnson 2004). *P. japonica* larvae are predominantly turfgrass root feeders and may cause significant damage in high numbers. Adults feed on over 300 species of woody and herbaceous plants (Fleming 1972) causing considerable damage by skeletonizing leaves. Larval populations in turf tend to be relatively low in northwest Arkansas (Wood et al. 2009, unpublished data); however, adults often damage fruits and ornamental plants within the region.

Regions in the U.S. with long-standing infestations have conducted surveys of pathogens and parasitoids (Régnière and Brooks 1978, Hanula and Andreadis 1988, Cappaert and Smitley 2002, Redmond and Potter 2010). There are several pathogens and parasitoids that affect either or both the larval and adult stages. The most commonly reported have been milky disease bacteria *Paenibacillus* spp. (Dingman 2009, Hutton and Burbutis 1974, Cappaert and Smitley 2002), the microsporidian *Ovavesicula popilliae* (Andreadis and Hanula 1987), the gut protist *Stictospora villani* (Hays et al. 2004), a tiphiid parasitoid, *Tiphia vernalis* Rohwer (Rogers and Potter 2004, Ramoutar and Legrand 2007), and the tachinid fly, *Istocheta aldrichi* Mesnil (Fleming 1968). The prevalence of these natural enemies in the Arkansas beetle population has been unknown. Our objective in this study was to determine the prevalence of pathogens and parasitoids affecting *P. japonica* adult and larval populations in northwest Arkansas.

4.3 MATERIALS AND METHODS

4.3.1 Field site selection

Ten field sites were selected for the collection of third instar larval and adult *P. japonica* in 2010 and 2011. Criteria for site selection included established beetle populations, ease of access, soil quality, and pesticide treatment history. The field sites selected included three golf courses, three City of Fayetteville parks, the University of Arkansas Research and Extension Farm, one horticultural nursery and two orchards (Table 4.1).

4.3.2 Survey for pathogens and parasitoids in larvae and adults

Each field site was sampled once per month for larvae during April, May, and September and weekly for adults during June, July and August in each year. Larval sampling occurred from 16 April 2010 to 13 May 2010, and 14 September 2010 to 24 September 2010. In 2011, larval sampling occurred from 7 April 2011 to 16 May 2011. The methodology used was similar to the field sampling method originally described by Fleming and Baker (1936). Ten samples were taken per site on each sampling trip. A sample consisted of sifting 0.1 m^2 of soil approximately 8 cm deep. All scarab larvae found were placed in individual diet cups to be brought back to the lab for identification (Vittum et al. 1999).

Adults were collected from mid-June to mid-August in both 2010 and 2011 using Jumbo Jug (Trécé Inc., Adair, OK) funnel mouth traps (1.9 L) baited with floral and sex lures. The 1.9 L jug traps were later replaced with 3.8 L jugs to accommodate increased beetle volume. Ten to twenty living adult beetles were randomly removed from each weekly trap catch for dissection from June to August.

All larval and adult beetles were washed in deionized water and examined externally for the presence of any pathogens or parasitoids. Hemolymph smears were taken by removing a

prothoracic leg from a larva or making an incision in the dorsal abdominal membrane of an adult. Hemolymph samples were examined for spores of *Paenibacillus* spp. All larvae and adults were then dissected. Portions of the midgut and Malpighian tubules were removed and slide mounted in lactophenol-acid fuchsin for examination and preservation. Midgut tissues were examined for gut pathogens while the Malpighian tubules were examined for *O. popilliae*. Tissues were examined with 400x phase contrast microscopy. All pathogens were identified morphologically (Lacey 1997).

4.4 RESULTS

4.4.1 Larval pathogens and parasitoids

In 2010, a total of 217 third instars were collected and dissected. The most common larval pathogen found was *S. villani*, a Eugregarine pathogen of the gut (Table 4.1, Fig. 4.1). This protist was found at five of the ten larval sampling sites. Larvae collected in the spring and fall exhibited 40.5% and 27.7% infection, respectively, for an average of 37.8%. No *Paenibacillus* spp., *O. popilliae*, or *T. vernalis* were found in any of the larvae sampled. Only 1.8% of the third instars contained nematodes. Three of the nematode specimens belonged to the Steinernematidae and one specimen belonged to the Heterorhabditidae.

In 2011, 76 third instars were collected and dissected. Again, the most common larval pathogen found was *S. villani* (Table 4.1). This pathogen was found at six of the ten larval sampling sites and had a prevalence of 35.5%. *O. popilliae* (Fig. 4.2) was found at one site, the Westfork horticultural nursery, and had an average prevalence of 13.3% in the larvae at this site. No nematodes, *Paenibacillus* spp., or *T. vernalis* were found in any of the larvae. The protist

Adelina sp. (Fig. 4.3) was identified morphologically (Undeen and Vávra 1997) in 1.3% of the larvae. To our knowledge, this is the first report of an *Adelina* species in *P. japonica*.

4.4.2 Adult pathogens and parasitoids

In 2010, a total of 978 adult *P. japonica* were dissected. No *S. villani, Paenibacillus* spp., or *I. aldrichi* were found in any adult beetles. A few adults, 0.2%, were infected with *O. popilliae*. A single nematode belonging to the family Mermithidae was found in one adult. *Adelina* sp. was found infecting the midgut of 0.4% of adult beetles. In 2011, a total of 649 adult *P. japonica* were dissected. No *S. villani, O. popilliae, Paenibacillus* spp., *Adelina* sp., nematodes or parasitoids were found in any 2011 adult beetles.

4.5 DISCUSSION

In 2011, fewer Japanese beetles were dissected than in 2010 because populations were lower in 2011 possibly due to weather. Many of our collection sites were flooded during the spring of 2011, possibly reducing larval survival. In April 2011, the region's rainfall was 27.8 cm above average (38.8 cm total) and in May 2010 the rainfall was 16.4 cm above average (29.2 cm total) (NOAA 2011). In addition, severe drought and heat during the summer of 2011 may have harmed survival. The adult flight ended several weeks earlier in 2011 than in previous years. In June 2011, temperatures were 2.7 °C above average (maximum 35.0 °C), and rainfall was 10.9 cm below average. In July 2011, temperatures were 3.1 °C above average (maximum 38.9 °C), and rainfall was 6.4 cm below average. In August 2011, temperatures were 3.0 °C above average (maximum 43.3 °C), and rainfall was 1.4 cm above average (NOAA 2011).

S. villani was the most prevalent pathogen found in *P. japonica* in Arkansas, similar to results found in some other states (Régnière and Brooks 1978, Hanula and Andreadis 1988,

Cappaert and Smitley 2002, Hays et al. 2004, Redmond and Potter 2010). Host larvae consume spores in the soil. *S. villani*'s life cycle within the host ends with syzygy and the formation of gametocysts which are expelled from the host (Maddox 1987). The life stage we found most frequently was the gamont, although oocysts and trophozoites were also seen. Severity of infections varied. In some specimens, only one *S. villani* could be found within the midgut, while other specimens contained hundreds. Although this pathogen is not known to cause mortality within larval populations, *S. villani* may disrupt gut function or nutrient absorption in cases where a larva has many eugregarines inhabiting its midgut (Maddox 1987).

No *S. villani* were found in adults. There are several possible reasons for this. First, mature larvae empty their guts prior to pupation (Fleming 1972), possibly eliminating *S. villani.* Second, after adults emerge they no longer feed on grass roots in the soil and would be unlikely to ingest *S. villani* gametocysts. While female adults do reenter the soil to lay eggs they do not feed at this time and would be unlikely to ingest *S. villani* gametocysts.

Prevalences of larval pathogens and parasitoids have varied greatly in studies from across the United States. For instance, *Paenibacillus* spp. prevalences in *P. japonica* were 3.7% in Connecticut (Hanula and Andreadis 1988), 7 - 26% in Delaware (Hutton and Burbutis 1974) and <1% in southern Michigan (Cappaert and Smitley 2002) and eastern North Carolina (Régnière and Brooks 1978). In Kentucky, *Paenibacillus* spp. infected 3.6 - 13.7% of the population (Redmond and Potter 2010). However, we did not find any *P. japonica* in northwest Arkansas infected with *Paenibacillus* spp.

The microsporidian *O. popilliae* was first discovered in Connecticut (Andreadis and Hanula 1987). It has been found naturally occurring in low prevalence in other areas (Cappaert and Smitley 2002, Redmond and Potter 2010) and has also been artificially introduced to try to

reduce *P. japonica* populations in Michigan (Cappaert and Smitley 2002, Smitley et al. 2011). *O. popilliae* appears to spread slowly with the expanding Japanese beetle population as indicated by Michigan (Cappaert and Smitley 2002) and Kentucky surveys (Redmond and Potter 2010). Japanese beetles were first documented in 1997 in Arkansas (Johnson 2004). In 2010 we found a prevalence of 0.2% *O. popilliae* in adult beetles and zero in larvae. In 2011, it was found in 2.6% of larvae and no adults were found infected.

Nematode-infected *P. japonica* larvae were uncommon in northwest Arkansas. Similar results were found in southern Michigan (1.4% infected) (Cappaert and Smitley 2002); in eastern North Carolina (10% infected) (Régnière and Brooks 1978), and in Kentucky (0.3% infected) (Redmond and Potter 2010). While nematodes may provide control in areas where they are augmented as a biological control agent, they do not appear to account for much natural mortality in northwest Arkansas.

Adelina sp. is a coccidian pathogen inhabiting the midgut tissues of its host. This genus has been found in Asiatic garden beetles (*Maladera castanea Arrow)* in Connecticut (Hanula and Andreadis 1988). This is the first record of this pathogen in Japanese beetles. In Arkansas, we observed *Adelina* sporocysts in both the larval and adult Japanese beetle midguts.

In our study, no parasitoids were found in either larval or adult stages of Japanese beetles in Arkansas. Similarly, in Michigan surveys no *T. vernalis* or *I. aldrichi* were found (Cappaert and Smitley 2002). In Kentucky, *T. vernalis* was found 19.7% in *P. japonica* larvae in early May (Redmond and Potter, 2010). It is possible we could have missed some *T. vernalis*-parasitized larvae because they are often found deeper than 8 cm (Rogers et al. 2003).

Based on our study, the impact of pathogens and parasitoids on Japanese beetle larvae and adults in Arkansas appears to be minimal at this time. This low impact of pathogens and

parasitoids may justify classical or augmentative biological control efforts for this pest in Arkansas.

4.6 ACKNOWLEDGEMENTS

We thank the golf course managers and private landowners who allowed us to collect samples from their property. We thank the United States Department of Agriculture Specialty Crop Block Grant Program administered by the Arkansas Agriculture Department for funding.

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Table 4.1. Prevalence of pathogens in third instar Japanese beetles in Northwest Arkansas in 2010 and 2011.

		Percentage Infected by Year				
	# Larvae Dissected	*Paenibacillus spp.*	*O. popilliae*	*S. villani*	*Adelina* sp.	Nematodes
Site	2010 / 2011	2010 / 2011	2010 / 2011	2010 / 2011	2010 / 2011	2010 / 2011
University of Arkansas Farm	56 / 6	0 / 0	0 / 0	0 / 16.7	0 / 0	3.6 / 0
Razorback Park Golf Course	32 / 28	0 / 0	0 / 0	50 / 50	0 / 0	0 / 0
Lost Springs Golf Course	32 / 9	0 / 0	0 / 0	81.3 / 77.8	0 / 11.3	6.3 / 0
Valley View Golf Course	13 / 1	0 / 0	0 / 0	69.2 / 0	0 / 0	0 / 0
Wilson Park	41 / 5	0 / 0	0 / 0	65.9 / 60	0 / 0	0 / 0
Gulley Park	8 / 5	0 / 0	0 / 0	0 / 0	0 / 0	0 / 0
Lewis Park	3 / 6	0 / 0	0 / 0	0 / 20	0 / 0	0 / 0
Westfork Horticultural Nursery	20 / 15	0 / 0	0 / 13.3	0 / 6.7	0 / 0	0 / 0
Springdale Peach Orchard	0 / 1	0 / 0	0 / 0	0 / 0	0 / 0	0 / 0
Fayetteville Apple Orchard	12 / 0	0 / 0	0 / 0	33.0 / 0	0 / 0	0 / 0
Total	**217 / 76**	**0 / 0**	**0 / 2.6**	**37.8 / 35.5**	**0 / 1.3**	**1.8 / 0**

Figure 4.1. *Stictospora villani* gamont found in larval Japanese beetle midgut tissue.

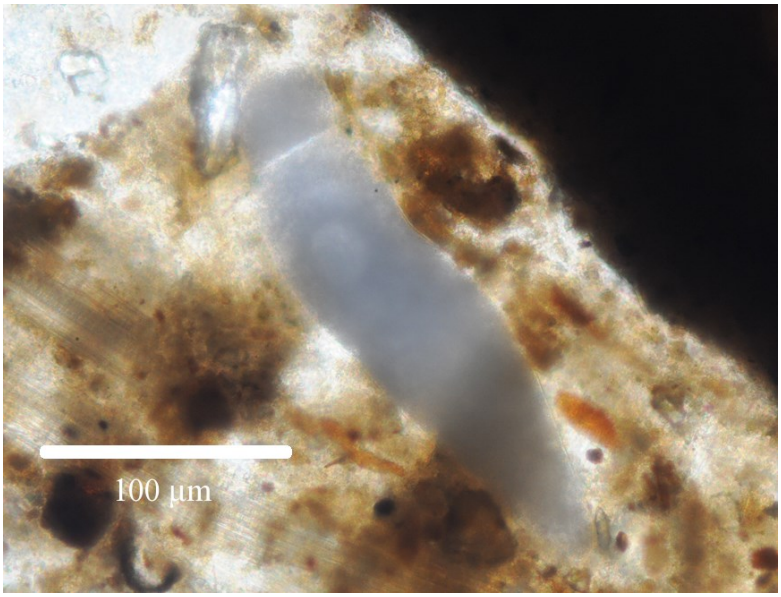


Figure 4.2. Sporophorous vesicles of *Ovavesicula popilliae* in adult Japanese beetle Malpighian tubule.

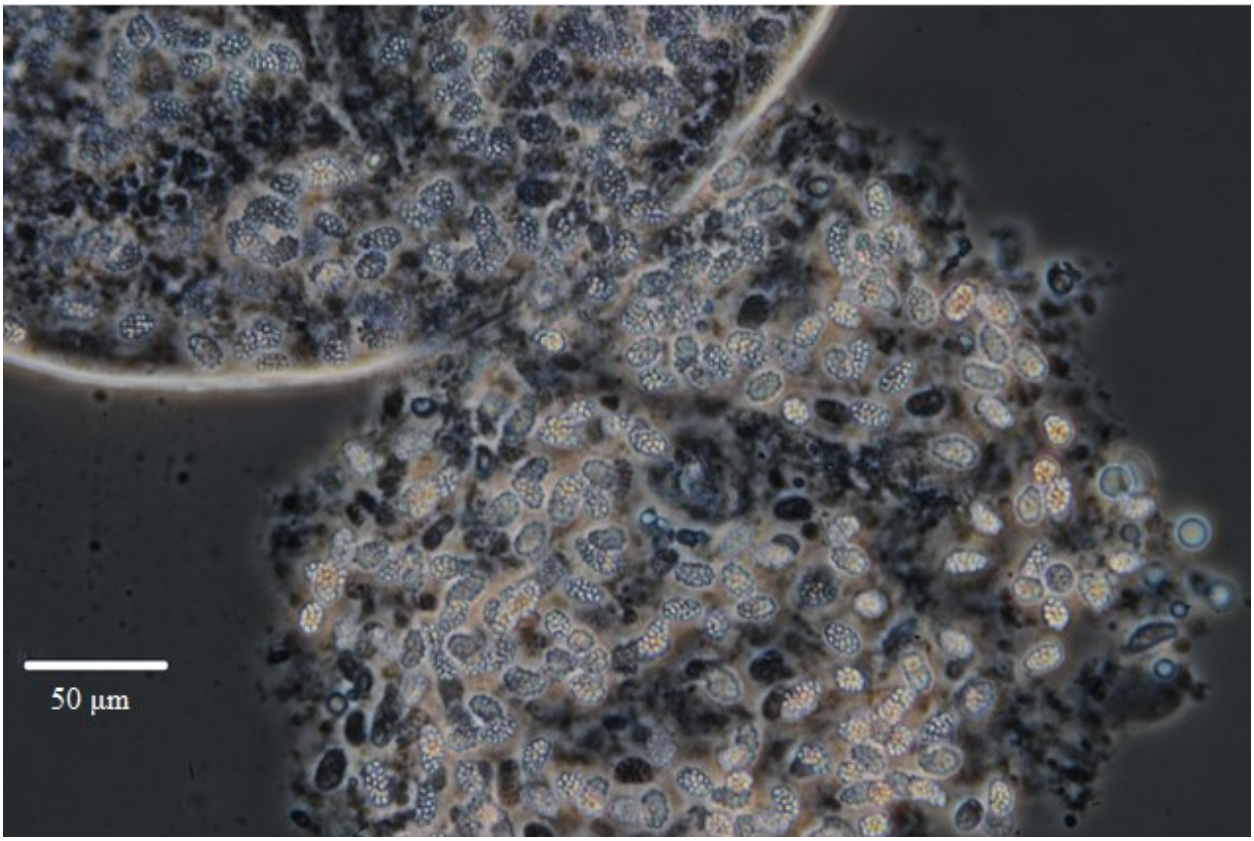
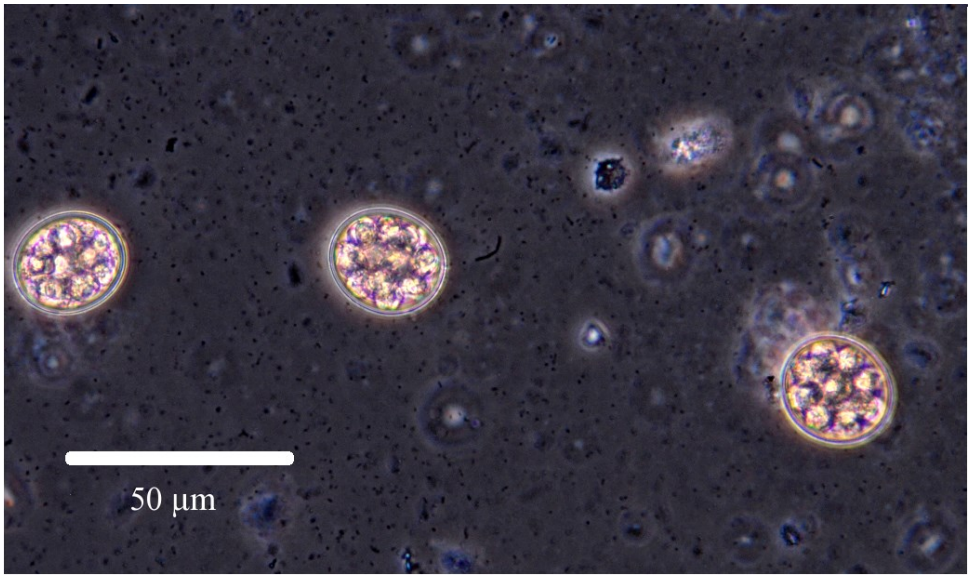


Figure 4.3. *Adelina* sp. oospores from adult Japanese beetle midgut.



5 Appendix A: Publishing agreement and signed documentation of multi-authored chapters

Article:	**Survey of Pathogens and Parasitoids of Popillia japonica (Coleoptera: Scarabaeidae) in Northwest Arkansas**
Corresponding author:	**Mr. B. M. Petty**
Journal:	**Journal of Invertebrate Pathology**
Our reference	**YJIPA6307**
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July 10, 2013

To Whom It May Concern:

Bryan Petty is lead author on the publication:

Petty, B.M., D. T. Johnson, D. C. Steinkraus. 2012. Survey of pathogens and parasitoids of *Popillia japonica* (Coleoptera: Scarabaeidae) in northwest Arkansas. J. Invertebr. Pathol. 111: 56-59.

Mr. Petty conducted more than 51% of the research and writing on this paper as part of his Ph.D. research at the University of Arkansas.

Sincerely,

Dr. Donald C. Steinkraus, Professor of Entomology, steinkr@uark.edu

6 Appendix B: Classical biological control of *Popillia japonica* (Coleoptera: Scarabaeidae) in Arkansas using *Ovavesicula popilliae* (Microspora: Ovavesiculidae)

6.1 ABSTRACT

The Japanese beetle, *Popillia japonica* (Newman), is a serious, invasive pest that was recently established in Arkansas. The adult beetle causes extensive damage to fruits and ornamental plants in Arkansas, and is quickly spreading from established areas in northwest Arkansas to other parts of the state. Japanese beetles in Arkansas have few natural enemies capable of reducing the density. Some states have introduced *Ovavesicula popilliae* to provide long-term biological control of this pest. A biological control program using *O. popilliae* began in 2010 and has continued it through 2012. The pathogen was released in parks, nurseries and golf courses in the region. The establishment of *O. popilliae* was monitored in both larval and adult population over the course of three years. Establishment of *O. popilliae* at release sites was low, which was attributed to a decrease in larval and adult Japanese beetle densities, probably due to extreme high summer temperatures and drought.

Keywords : *Ovavesicula popilliae*, *Popillia japonica*, biological control, microsporidia

6.2 INTRODUCTION

The Japanese beetle (*Popillia japonica* Newman) is a relatively new pest in Arkansas that as adults damages flowers and fruits, defoliates horticultural plants, and as larvae damages turfgrass roots (Fleming 1972). *P. japonica* was first found in northwest Arkansas in 1997 (Johnson 2004), and has since become established in Benton, Washington, Crawford, Pulaski, and White counties and has been found in limited numbers in Madison, Sebastian, Faulkner, Conway, Pope, Prairie, Columbia, Baxter, Sharpe, Randolph, Lawrence, Green, Craighead, Mississippi, Cross, St. Francis, and Crittenden counties (NAPIS 2013). There has been some success in eradication efforts of the beetle, particularly in Lonoke, Garland, Arkansas, and Clay counties (NAPIS 2013) but the beetle largely continues to spread with very little control.

A study of pathogens and parasitoids of adult and larval Japanese beetles showed an extremely low prevalence of pathogen and parasitoid natural enemies in northwest Arkansas. Natural enemies currently present in northwest Arkansas offer little control for the beetle population (Petty et al. 2012). The introduction of additional natural enemies into the Arkansas could help provide long term reduction in Japanese beetle abundance, reducing costs associated with Japanese beetle damage and control.

Michigan has had considerable success with the long term biological control of the Japanese beetle through the introduction and spread of the microsporidian pathogen *Ovavesicula popilliae. O. popilliae* was originally found in Connecticut, infecting beetles at 69% of sites examined and 25% of all Japanese beetle larvae statewide (Hanula and Andreadis 1988). Smitley (2008) reported that after release of *Ovavesicula* in Michigan in 1999, it spread to infect 5% of the local Japanese beetle population within 3 years of release and 10-50% of the larvae by

7 years. Smitley et al. (2011) also found a 17% increase in annual larval mortality at locations where beetles were infected with *O. popilliae*.

Our objective was to introduce and establish *O. popilliae* from infected Michigan stock into turf areas in Arkansas infested with Japanese beetles in an attempt to establish a larger *O. popilliae* presence in Arkansas. We then monitored the establishment and spread of *O. popilliae* at Arkansas release sites over the course of three years.

6.3 MATERIALS AND METHODS

6.3.1 Field Sites in Arkansas

We identified 10 field sites for release of *O. popilliae* in northwest Arkansas. These sites consisted of fruit orchards, golf courses, city parks, and horticultural nurseries with relatively high Japanese beetle populations. These 10 sites were randomly assigned to be either one of four *O. popilliae* release sites or one of six sites with no release to act as controls. AT each site, a release or control plot (15 m x 23 m delimited by GPS coordinates) was prepared.

6.3.2 Release of O. popilliae

In August 2010, we acquired large numbers of infected *P. japonica* adults from the Charles Binder Golf Course, Battle Creek, MI with the assistance of Dr. David Smitley of Michigan State University. These beetles were collected using funnel mouth traps baited with floral and sex lures, then frozen and transported to Arkansas. These frozen beetles were used in a release of *O. popilliae* in northwest Arkansas. Between 1,500 and 2,000 dead, adult beetles were buried per site across four sites. The frozen adult beetles had a 13.7% (n=210) infection rate as determined by morphological identification of spores in a subset of the collected Japanese beetles. The four release sites consisted of two golf courses, one public park and one nursery.

Approx. 50 beetles were buried every 0.1m^2 at each release area by removing soil cores 5 cm in diameter and 8 cm deep, and placing dead adult beetles in the hole and replacing the plug. In July 2012, this process was repeated at the same locations as in 2010, but with fewer infected Japanese beetles. Only 100-150 beetles were released per locations with an infection rate of 25% (n=100).

6.3.3 Monitoring changes in O. popilliae prevalence

From April to September during 2010 to 2012, Japanese beetle larvae and adults were collected from the 10 sites across northwest Arkansas. Ten samples of soil were taken from each site during the months of April, May, and September to collect larvae. A sample consisted of sifting 0.1 m^2 of soil approximately 8 cm deep. All Japanese beetle larvae were brought back to the lab for dissection. From late May until early August, Jumbo Jug (Trécé Inc., Adair, OK) funnel mouth traps (1.89L) each baited with Japanese beetle floral and sex lures were placed at these same 10 field locations. Weekly from June to August, ten living adult beetles were randomly removed from each trap catch and later dissected. All larvae and adults were dissected, and the midgut and Malpighian tubules were removed and slide mounted in lactophenol-acid fuchsin for examination and preservation. The tissues were examined for microsporidian *O. popilliae*. Tissues were examined with 400x phase contrast microscopy. All pathogens were morphologically identified (Lacey 2007).

6.4 RESULTS

6.4.1 Monitoring changes in O. popilliae prevalence

Surprisingly, a very low prevalence of *O. popilliae* was found in the Arkansas Japanese beetle population prior to the release of the pathogen for biological control purposes. The

prevalence of *O. popilliae* in adults in 2010 was 0.2% (Petty et al. 2012) and was zero in larvae. This suggests that *O. popilliae* in Arkansas was providing little, if any control of Japanese beetles. In 2011, after the release of *O. popilliae* as an augmentative biological control agent in August 2010, the mean prevalence rate across sites in larvae was 2.6% of larvae, on average, after it had been introduced to the region as an augmentative biological control agent in August 2010 (Table 6.1). Additionally in 2011, the prevalence of *P. japonica* larvae infected with *O. popilliae* increased at one of the four release sites. At the Westfork Horticultural Nursery, the prevalence of *O. popilliae* increased from 0% in 2010 to 13.3% in 2011. However, the prevalence of *O. popilliae* in the adult *P. japonica* population decreased at the Lost Springs Golf Course, from 0.2% in 2010 to 0% in 2011.

In 2012, due to extreme summer heat and drought, only 12 Japanese beetle larvae were found for dissection across all 10 locations and none were infected with *O. popilliae*. Additionally, a total of 515 adult beetles were dissected from the 10 field locations (Table 6.2). *O. popilliae* was found infecting the Malpighian tubules in 3.2% of adult beetles collected from an irrigated vineyard that was a non-release control location in Hindsville, AR. No *O. popilliae* was found at release sites.

6.5 DISCUSSION

Because of the extremely low beetle population in 2012, it is difficult to determine if the release of *O. popilliae* in 2010 augmented the natural low prevalence. Survey data from 2011 does suggest its presence in the region did slightly increase following release (Petty et al. 2012); however, this could not be substantiated in 2012. If the population of Japanese beetles in

northwest Arkansas remains low in the following years, it may be very difficult to determine if the augmentative releases of *O. popilliae* resulted in establishment.

Releases of *O. popilliae* consisted of burying infected adult beetles in areas of known larval Japanese beetle infestation. This was not an ideal way of releasing *O. popilliae* for two reasons which were not apparent at the time of initial release. The first problem was the density of larval Japanese beetles in known areas decreased drastically from 0.57 larvae/0.1 m^2 in 2010 to 0.05 larvae/0.1 m^2 2012 (unpublished). This decline in Japanese beetle abundance likely hindered widespread establishment of *O. popilliae* infections. This issue cannot be readily fixed in Arkansas, but can be avoided entirely if *O. popilliae* is released in states with high densities of Japanese beetles or at times of severe Japanese beetle infestation.

The second problem was that *O. popilliae* was released into locations with third instar Japanese beetles. Third instar Japanese beetles have reduced susceptibility to infection by *O. popilliae*, requiring relatively high dosages (unpublished). This issue can be corrected by either releasing *O. popilliae* early in the Japanese beetle larval development cycle, particularly targeting first instars, or by releasing live, infected Japanese beetle adults into locations, which will then be free to mate and oviposit, possibly contaminating eggs and first instars in the process.

Further releases of *O. popilliae* in Arkansas and continued monitoring should be implemented for establishment of this biological control agent. Increasing the prevalence of *O. popilliae* in this region could prove beneficial for controlling the impact and spread of the Japanese beetle both across the state of Arkansas and elsewhere. Arkansas represents the leading southwestern edge of the U.S. Japanese beetle infestation. As the beetle continues to move southward ad westward, established *O. popilliae* populations in Arkansas could move with the

beetle. Additionally, surrounding states such as Oklahoma, Tennessee and Missouri are also infested with Japanese beetles and could benefit from the spread of *O. popilliae* from Arkansas.

6.6 ACKNOWLEDGEMENTS

Thanks to David Smitley for facilitating the transfer of *O. popilliae* from Michigan to Arkansas and all land owners who allowed sampling to be conducted on their property.

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Table 6.1. Infection prevalence of *Ovavesicula popilliae* in larval Japanese beetles collected in northwest Arkansas in 2010, 2011 and 2012.

Site	# Larvae Dissected 2010 / 2011 /2012	% Infected by Year 2010 / 2011 /2012
University of Arkansas Farm	56 / 6 / 0	0 / 0 / 0
Razorback Park Golf Course*	32 / 28 / 1	0 / 0 / 0
Lost Springs Golf Course*	32 / 9 / 3	0 / 0 / 0
Valley View Golf Course	13 / 1 / 0	0 / 0 / 0
Wilson Park*	41 / 5 / 0	0 / 0 / 0
Gulley Park	8 / 5 / 0	0 / 0 / 0
Lewis Park	3 / 6 / 0	0 / 0 / 0
Westfork Horticultural Nursery*	20 / 15 / 4	0 / 13.3 / 0
Springdale Peach Orchard	0 / 1 / NA	0 / 0 / NA
Springdale Vineyard	NA / NA / 4	NA/ NA/ 0
Fayetteville Apple Orchard	12 / 0 / 0	0 / 0 / 0
Total	**217 / 76 / 12**	**0 / 2.6 / 0**

*** Denotes release locations of *O. popilliae* in 2010 and 2012**.

Table 6.2. Infection prevalence of *Ovavesicula popilliae* in adult Japanese beetles collected in northwest Arkansas in 2010, 2011 and 2012.

Site	# Adults Dissected 2010 / 2011 /2012	% Infected by Year 2010 / 2011 /2012
University of Arkansas Farm	100 / 82 / 71	1 / 0 / 0
Razorback Park Golf Course*	100 / 70 / 38	0 / 0 / 0
Lost Springs Golf Course*	100 / 70 / 48	1 / 0 / 0
Valley View Golf Course	100 / 65 / 44	0 / 0 / 0
Wilson Park*	90 / 60 / 51	0 / 0 / 0
Gulley Park	100 / 53 / 44	0 / 0 / 0
Lewis Park	100 / 43 / 47	0 / 0 / 0
Westfork Horticultural Nursery*	100 / 68 / 57	0 / 0 / 0
Springdale Peach Orchard	90 / 71 / NA	0 / 0 / NA
Springdale Vineyard	NA / NA / 62	NA/ NA/ 3.2
Fayetteville Apple Orchard	98 / 67 / 53	0 / 0 / 0
Total	**978 / 649 / 515**	**0.2 / 0 / 0.4**

*** Denotes release locations of *O. popilliae* in 2010 and 2012.**

7 Appendix C: APHIS PPQ permit for the release of *Ovavesicula popilliae*

APHIS Animal and Plant Health Inspection Service

Plant Protection & Quarantine

United States Department of Agriculture
Animal and Plant Health Inspection Service
4700 River Road
Riverdale, MD 20737

Permit to Move Live Plant Pests, Noxious Weeds, and Soil

Interstate Movement

Regulated by 7 CFR 330

This permit was generated electronically via the ePermits system

PERMITTEE NAME:	Dr. Donn Johnson	**PERMIT NUMBER:**	P526P-09-03001
ORGANIZATION:	University of Arkansas	**APPLICATION NUMBER:**	P526-090905-001
ADDRESS:	AGRI 320, Department of Entomology Fayetteville, AR 72701	**FACILITY NUMBER:**	N A
MAILING ADDRESS:	AGRI 320, Department of Entomology Fayetteville, AR 72701	**HAND CARRY:**	Yes
		DATE ISSUED:	09 29/2009
PHONE:	(479) 575-2501		
FAX:	(479) 575-2452	**EXPIRES:**	**09/29/2012**
DESTINATION:	AGRI 320, Department of Entomology, Fayetteville, AR 72701		
RELEASE:	AGRI 320, Department of Entomology, Fayetteville, AR 72701		

Under the conditions specified, this permit authorizes the following:

Article Category: Invertebrate Pests - Insects; BioControl Organisms - Pathogens of Invertebrates

Regulated Article	Life Stage(s)	Intended Use	Shipment Origins	Originally Collected	Culture Designation
Ovavesicula popilliae	Adult, Larvae	Release - BiocontrolMI		Originally Collected from Within the Continental U.S.	
Popillia japonica	Adult, Larvae	Release - BiocontrolMI		Originally Collected from Within the Continental U.S.	

Permit Number P526P-09-03001

THIS PERMIT HAS BEEN APPROVED ELECTRONICALLY BY THE FOLLOWING PPQ HEADQUARTER OFFICIAL VIA EPERMITS.	DATE
Robert Tichenor	09/29/2009

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PERMIT CONDITIONS

This permit authorizes the interstate movement of the microorganism Ovavesicula popilliae and the Japanese beetle host, Popillia japonica, from Michigan to Donn Johnson at the University of Arkansas, AGRI 320, Department of Entomology, Fayetteville, AR 72701, and further authorizes the release of Ovavesicula popilliae via infected Popillia japonica grubs and adults into the environment in Arkansas.

1. This permit authorizes the interstate movement of only Ovavesicula popilliae by way of live infected Popillia japonica grubs and adults and does not authorize the interstate movement of live insects of any other species. The permit holder shall include only apparently infected Popillia japonica in containers transported from Michigan to Arkansas.
2. The approved organisms must be shipped in sturdy, secure, sealed (no openings), escape-proof containers.
3. This permit does not authorize interstate movement of containers of infected Popillia japonica that contain plant parts, soil, or any other organisms and contaiminants from the natural environment. When field collecting infected Popillia japonica for interstate transport, the permit holder must remove all such contaminants from the natural environment using a reasonable and effective means prior to shipment.

   Live infected Popillia japonica shall be placed in potting soil which has been sterilized in an autoclave or other appropriate medium that does not contain soil.
4. Except infected Popillia japonica which are released or otherwise used for research purposes, all biological material contained in the shipment, as well as soil or other medium used to transport Popillia japonica grubs, must be double-bagged, incinerated or sterilized in an autoclave prior to disposal.

   Live Popillia japonica life stages retained for research purposes including those kept as preserved specimens and/or samples used for examination and analysis, shall not be kept as live specimens except as needed for proper processing and preparation. Such live specimens and samples must be held in sturdy escape proof containers.
5. The permit holder, and individuals authorized by this permit holder, are further authorized to personally transport approved organisms in motor vehicles.
6. This permit is issued only for the named permit holder at the address(s) identified on this permit. This permit cannot be transferred or assigned nor does it fulfill or modify the requirements of any other federal or state regulatory authority (such as the U.S. Environmental Protection Agency, the U.S. Fish and Wildlife Service, the U.S. Food and Drug Administration, the Centers for Disease Control and Prevention, the Animal Health Protection Act- 7U.S.C. 8301, or your State's Department of Agriculture).
7. The permit holder is solely responsible for ensuring compliance with all statutory requirements and specifically listed permit conditions. Failure to comply with the terms and conditions of this permit is cause for the following: (a) cancellation of this permit, (b) cancellation of other permits issued to the permit holder, (c) seizure and/or destruction of regulated organisms, (d) denial of future permit applications by this permit holder, (e) liability for civil penalties, and (f) criminal prosecution under provisions in the Plant Protection Act.
8. Any alteration, forgery, unauthorized use of this permit and/or associated Federal Forms are subject to civil and criminal penalties including fines and imprisonment.

Permit Number P526P-09-03001

THIS PERMIT HAS BEEN APPROVED ELECTRONICALLY BY THE FOLLOWING PPQ HEADQUARTER OFFICIAL VIA EPERMITS.	DATE
Robert Tichenor	09/29/2009

WARNING: Any alteration, forgery, or unauthorized use of this Federal Form is subject to civil penalties of up to $250,000 (7 U.S.C.s 7734(b)) or punishable by a fine of not more than $10,000, or imprisonment of not more than 5 years, or both (18 U.S.C.s 1001).

9. Importation, interstate movement, possession, and use of strains of genetically engineered regulated organisms (created by use of recombinant DNA technology) are not authorized under this permit.

10. This permit does not authorize movement or use of plant pathogens listed in the Public Health Security and Bioterrorism Preparedness and Response Act of 2002. If any organism listed as a Select Agent is identified from materials associated with this research, the permit holder is required to notify APHIS, Agricultural Select Agent Program (ASAP) immediately by phone at 301-734-5960, and within seven (7) days submit APHIS/CDC Form 4 (Report of Identification of a Select Agent or Toxin in a Clinical or Diagnostic Laboratory) to APHIS, ASAP, 4700 River Rd, Unit 2, Riverdale, MD 20737 (see instructions at: http://www.aphis.usda.gov/programs/ag_selectagent/index.shtml). Failure to comply with this requirement is a violation of the Agricultural Bioterrorism Protection Act of 2002.

11. If organisms that are not authorized in this permit are received, the permit holder must take all prudent measures to contain and destroy or otherwise devitilize the organism(s) and notify the PPQ permit unit by contacting a compliance officer immediately (that is, within one business day) by calling 866-524-5421 or by e-mail to pest.permit@aphis.usda.gov.

12. The permit holder must maintain a valid permit so long as the regulated organisms are alive and in your possession. The permit holder must safeguard and dispose of the regulated organisms during the term of this permit. This permit cannot be extended or renewed. A new permit is required for uninterrupted authorization/use of regulated organisms after this permit expires.

13. The permit holder must take all necessary precautions to prevent the escape of plant pests. In the event of an escape, the permit holder must immediately notify the permit unit, as above. The permit holder must adequately mitigate any and all environmental impacts resulting from unauthorized release of organisms received under this permit.

14. Without prior notice and during reasonable hours, authorized PPQ and/or State regulatory officials shall be allowed to inspect the conditions associated with the regulated organisms authorized under this permit.

15. The permit holder must maintain an official permanent work assignment at the address identified on this permit. If the permit holder ceases assignment/affiliation at the address identified on this permit, or personnel circumstances change in any way, then a compliance officer must be notified at the PPQ permit unit immediately (that is, within one business day) by either (a) email to pest.permits@aphis.usda.gov, (b) fax to 301-734-4300 or 8700, or (c) conventional mail to USDA PPQ Permit Unit, 4700 River Road, Riverdale, MD 20737. The permit holder must destroy all regulated organisms prior to departure unless the permit holder either (a) requests cancellation of this permit and complies with all permit-specific termination conditions, (b) applies for and receives a permit to move the organisms to a new facility, or (c) relinquishes control of the regulated organisms to a qualified individual who obtained a permit for the continued use of these regulated organisms prior to this permit holder's departure.

END OF PERMIT CONDITIONS

Permit Number P526P-09-03001

THIS PERMIT HAS BEEN APPROVED ELECTRONICALLY BY THE FOLLOWING PPQ HEADQUARTER OFFICIAL VIA EPERMITS.	DATE
Robert Tichenor	09/29/2009

8 Host life-stage susceptibility and dose response of *Popillia japonica* (Coleoptera: Scarabaeidae) to *Ovavesicula popilliae* (Microsporidia: Ovavesiculidae)

8.1 ABSTRACT

The Japanese beetle (*Popillia japonica*) is a severe turf and horticultural pest in the eastern half of the United States. One successful modern biological control program for the Japanese beetle has used *Ovavesicula popilliae*, a microsporidian pathogen, to reduce both larval and adult beetle abundance. Our objectives were to determine the life-stage susceptibility of *P. japonica* to infection by *O. popilliae* and determine the dose response of *P. japonica* to *O. popilliae* in susceptible life stages. First, second, and third instars, as well as adult beetles were inoculated *per os* with 50,000 *O. popilliae* spores. Additionally, all larval life stages were inoculated with 0, 100, 1,000, 10,000, and 100,000 spores. All three larval life-stages of the Japanese beetle appear susceptible, with susceptibility to *O. popilliae* decreasing with maturity. Adult beetles, however, were not susceptible to infection. The lack of susceptibility of healthy adult Japanese beetles to *O. popilliae* infection corroborates initial claims that infections in adult Japanese beetles were the product of infections that occurred earlier in life and were carried transstadially.

Keywords: *Popillia japonica*, *Ovavesicula popilliae*, dose response, susceptibility

8.2 INTRODUCTION

The Japanese beetle (*Popillia japonica* Newman) was first introduced to the United States in 1916, and it has since spread its range across most of the eastern United States (NAPIS 2013). Japanese beetle larvae are predominantly turfgrass feeders, preferring cool-season turfgrasses to warm-season turfgrasses, but will also feed on the roots of other herbaceous plants. The adult beetle is a serious pest of agricultural and horticultural crops, causing severe defoliation and damage to fruit and flowers (Fleming 1972).

Extensive research on the natural enemies of *P. japonica* has been conducted, primarily focusing on the milky spore bacterium, *Paenibacillus popilliae* Dutky, and entomopathogenic nematodes; however, there are few examples of widespread biological control programs that have successfully reduced Japanese beetle populations in the U.S. (Fleming 1968). One of the more successful contemporary biological control programs for Japanese beetle has taken place in southern Michigan with the use of *Ovavesicula popilliae* (Andreadis and Hanula), a microsporidian pathogen (Smitley et al. 2011).

O. popilliae is most likely transmitted through a fecal-oral route between larvae, from adults to larvae, or from infected, dead, decomposing larvae to healthy larvae. Infection initially develops in the Malpighian tubules of larvae, but may become systemic, infecting the fat body, epidermis, and pericardial cells (Hanula and Andreadis 1990). Infected larvae that die release spores as they decay. Infected larvae that survive to pupate become adults with systemic infections. Both infected larvae and adults excrete persistent spores in the frass (Hanula and Andreadis 1990). There are no known external signs of *O. popilliae* infection in larvae or adults despite intense internal swelling and melanization of the Malpighian tubules and pericardium (Andreadis and Hanula 1987).

O. popilliae has been found in Connecticut (Andreadis and Hanula 1987), Michigan (Cappaert and Smitley 2002), Kentucky (Redmond and Potter 2010), and Arkansas (Petty et al. 2012a). In Connecticut, 25% of the Japanese beetle larvae collected were infected, with some localities approaching 100% infection (Hanula 1990). Adult populations showed infection rates from 20-50%, increasing as the summer progressed (Hanula and Andreadis 1988). Heavily infected adult females oviposited 50-60% fewer eggs than healthy females, further reducing densities (Hanula 1990). Additionally, infection was associated with 17% higher winter mortality in larvae at locations with beetles heavily infected with *O. popilliae* in Michigan (Smitley et al. 2011).

The life stages of the Japanese beetle that are susceptible to *O. popilliae* infection are unknown, although infections are found in both larvae and adults (Hanula and Andreadis 1990). Although it is known that an infected adult beetle is capable of producing an average of 2.7×10^7 spores during its lifetime (Petty et al. 2012b), it is unknown how many *O. popilliae* spores are needed to cause infection in susceptible life stages of the Japanese beetle. The objectives of this study were to determine the life-stage susceptibility of *P. japonica* to infection by *O. popilliae* through oral inoculation of all feeding life stages. Additionally, we determined the dose response of *P. japonica* to infection by *O. popilliae* in susceptible larval life stages.

8.3 MATERIALS AND METHODS

8.3.1 Collection and identification of O. popilliae

Spores of *O. popilliae* were identified morphologically both *in situ* and in suspension. *O. popilliae* is the only known microsporidian to infect *P. japonica* and is recognizable from a characteristic “raspberry” shape created from multiple spores within one sporophorous vesicle (Andreadis and Hanula 1987, Cappaert and Smitley 2002) (Fig. 8.1). Spores range in size from

2.0-2.5 µm x 1.7-1.8 µm and sporophorous vesicles range in size from 20.0-21.0 µm x 15.0-15.5 µm (Andreadis and Hanula 1987). *O. popilliae* spores were collected from wild beetles from Michigan. To obtain spores, the Malpighian tubules from infected adult beetles were removed, homogenized in deionized water, and the spores separated from debris via centrifugation as outlined by Vossbrinck and Andreadis (2007), counted, and diluted to necessary concentrations as described by Undeen and Vávra (1997).

For confirmation of the identity of collected spores, DNA extractions were conducted on the organs of infected adult Japanese beetle specimens using salting-out extraction protocol described in Sambrook and Russell (2001). The PCR primers 18f: 5'-CACCAGGTTGATTCTGCC-3' and 1492r: 5'-GGTTACCTTGTTACGACTT-3' (Vossbrinck and Andreadis 2007) were used (thermal cycling: denaturation at 94°C for 2 min, 39 cycles of 94°C for 45 s, 49.4°C for 45 s, and 72°C for 84 s, extension at 72°C for 5 min.) Products were purified with 30K centrifugal filters (VWR, Radnor, PA), and were sequenced by University of Arkansas Medical School, Little Rock, AR. Sequences were aligned visually and a BLAST (NCBI) search was conducted with Geneious 6.0.3 (Auckland, New Zealand).

8.3.2 Collection of Japanese beetle larvae and adults

Larval Japanese beetles were collected from grass beds at the University of Arkansas Research and Extension Farm. These rearing beds acted as the primary source of Japanese beetle larvae for the purposes of this study. Adult beetles were collected with pheromone traps on the University of Arkansas Research and Extension Farm from June to August in 2011 and 2012 using Jumbo Jug (Trécé Inc., Adair, OK) funnel mouth traps (1.9 L) baited with floral and sex pheromone lures. Only adults that had been trapped within two hours prior to collection were

used in order to reduce heat related stress. Adult Japanese beetles were identified to sex by their apical tibial spurs (Fleming 1972). Both male and female adult beetles were used.

8.3.3 Life-stage susceptibility of P. japonica to O. popilliae

Second instars, third instars, and the adult beetles were inoculated directly *per os* via a smoothly-rounded hypodermic needle (25 gauge) placed in the pro-oral cavity. Each life stage was inoculated with 50,000 *O. popilliae* spores in suspension as determined with a hemocytometer as described by Undeen and Vávra (1997). First instars could not be inoculated *per os* because of their fragility, and instead were immersed in a 10 µl droplet of spore suspension containing 50,000 spores and allowed to feed. Each larval life stage group had contained 30 inoculated individuals and 30 individuals were given deionized water to act as a control. With the adult beetles, 75 individuals were inoculated and 75 individuals were given deionized water to act as a control.

Larvae were incubated for 14 days at 24 (± 0.5 °C) in darkness in individual diet cups (37 ml) along with a sprouted grass seed and soil. Adults were also incubated for 14 days at 24 °C with a 14:10 light regimen with apple slices and grape leaves. During this time, mortality was monitored, and at the end of the 14 days all larvae and adults were dissected to determine rates of infection. Giemsa staining was used to diagnose tissues in which infections were unclear (Becnel 1997).

8.3.4 Dose Response of P. japonica to O. popilliae

All three larval Japanese beetle instars were used in a dose response study. The dose response study consisted of dosages of 0, 100, 1,000, 10,000, and 100,000 spores measured by hemocytometer as described by Undeen and Vávra (1997). For each life stage, 30 individuals were inoculated per spore rate. Individuals were incubated for 14 days at 24 ± 0.5°C in total

darkness in individual diet cups along with a sprouted grass seed. At the end of the 14 days all individuals were dissected and had their Malpighian tubules examined to determine rates of infection. Giemsa staining was used to diagnose tissues in which infection were unclear. Pertinent infective dosage (ID) rates were determined with PROBIT analysis in SAS 9.3 (SAS Institute Inc.) and were dependent on life stage and data available. This analysis included data from the susceptibility trail, which involved a 50,000 spore dosage. Additionally, larvae that died after the first 48 hours were excluded from analysis because of apparent handling-related mortality.

8.4 RESULTS

DNA sequences from microsporidia collected from adult Japanese beetles in Battle Creek, MI, matched *O. popilliae* 99.9% to the original *O. popilliae* sequence as described by Vossbrinck and Andreadis (2007).

First, second, and third instar Japanese beetles all developed *O. popilliae* infections after being fed spores. One first instar and three second instar larvae became infected when fed the standard dose of 50,000 spores. Third instars did not show infection at the 50,000 spore inoculation dosage, but did show infection at both the 10,000 and both 100,000 inoculation dosages. No adult Japanese beetles became infected when fed 50,000 *O. popilliae* spores.

In all three larval instars, infection rates generally increased as spore dosages increased (Table 8.1). Infection rates in first instars were highest, reaching a peak of 46.7% infection in first instars inoculated with 100,000 spores. Infection rates in second and third instars remained relatively low, with a peak infection rate of 20.3% in second instars fed 100 spores and 20.0% infection in third instars fed 100,000 spores. Additionally, mortality rates in first and second

instar larvae were considerably higher than in third instar larvae. This was true even in control populations, which were only inoculated with deionized water.

PROBIT analysis could not be done on first or second instars, as requirements for analysis weren't met because of high mortality. For third instars, ID_{10} was $4.6x10^4$ spores, ID_{20} was $1.1x10^6$, and the ID_{30} was $1.1x10^7$. Beyond ID_{40}, dosages appeared to be too high to be feasible.

8.5 DISCUSSION

All three larval life-stages of the Japanese beetle appear susceptible to *O. popilliae* when inoculated. Results indicate that early instars of *P. japonica* are susceptible to relatively low dosages of spores and that the number of spores required to infect Japanese beetle larvae tend to increase with maturity. Although third instar Japanese beetles did not develop infection in replicates inoculated with only 50,000 spores, they did develop infection when inoculated with both 10,000 and 100,000 spore dosages. The failure of larvae to develop infection when inoculated with 50,000 spores is likely coincidental. The failure to form visible infection at this rate could also indicate problems with time frame of experiments. Larvae were only incubated for two weeks following inoculation. More time may be necessary in order to accurately diagnose infection visually, allowing for infected individuals to accumulate more spores. The Japanese beetle is a relatively long-lived insect, with a life cycle encompassing an entire year (Fleming 1972). Even mild infections early in development could advance into severe infections by adult emergence. Additionally, generation time for insect microsporidia can be highly variable. *Octosporea muscaedomesticae* is known to have a relatively short generation time of 3-4 days (Kramer 1965). Comparatively, *Nosema whitei* may take 10-19 days per generation (George 1974). Even more extreme, *N. necatrix* is known to take between 4-42 days to complete

a generation, depending on temperature (Maddox 1968). The generation time for *O. popilliae* is currently unknown, making it difficult to estimate necessary incubation time.

Determining infective dosages in first and second instars from oral inoculation of *O. popilliae* was limited by high control mortality in both instars, even in control groups. This increase in mortality may be caused by handling, which seems to be most likely in the case of the first instars. First instar Japanese beetles are exceedingly fragile and small stresses can cause mortality. In second instars, handling may have caused some mortality, but the additional stress or trauma caused by forced oral inoculations probably also contributed to mortality. Trauma from inoculations likely contributed to third instar mortality in some cases as well. Determining infective dosages may have also been limited by our technique of visual diagnosis. As stated earlier, it is possible that mildly infected larvae were misdiagnosed as not having infections.

Results indicate that adult Japanese beetles have a very low susceptibility to *O. popilliae* and that infections acquired in this stage are currently unknown. Susceptibility to microsporidian infections is known to decrease in larval insects as the insect matures (Bauer and Nordin 1988, Vijendravarma et al. 2008), possibly from improved host defenses and immune system. The lack of susceptibility of healthy adult Japanese beetles to *O. popilliae* infection corroborates initial statements that infections in adult Japanese beetles were the product of infections that occurred earlier in life and were carried transstadially (Hanula and Andreadis 1990). Additionally, adults that maintain *O. popilliae* infections from their larval stages are probably the primary vector for the spread of *O. popilliae* across large geographical areas (Petty et al. 2012b).

Despite difficulties in determining the dose response of each larval life stage of the Japanese beetle to *O. popilliae,* it is clear that as larvae become more mature their resistance to infection increases. For biological control purposes, it is probably best to introduce *O. popilliae*

to new areas while Japanese beetle larvae are as young as possible. Soil could be inoculated with *O. popilliae* spores during the mid-to-late summer when first instars are present; however, first instar Japanese beetles are not highly mobile. In order to ensure contact between larvae and *O. popilliae*, high density areas would need to be targeted for release. Additionally, live adult Japanese beetles known to be infected with *O. popilliae* could be released in order to spread the pathogen. Infected adult females could possibly contaminate their eggs with *O. popilliae* spores via defecation while ovipositing, as spores are present in the frass of infected individuals (Hanula and Andreadis 1990). Females may also die near their eggs while ovipositing, resulting in decay and the release of spores. In either case, early instar Japanese beetles would likely be exposed to spores.

8.6 ACKNOWLEDGEMENTS

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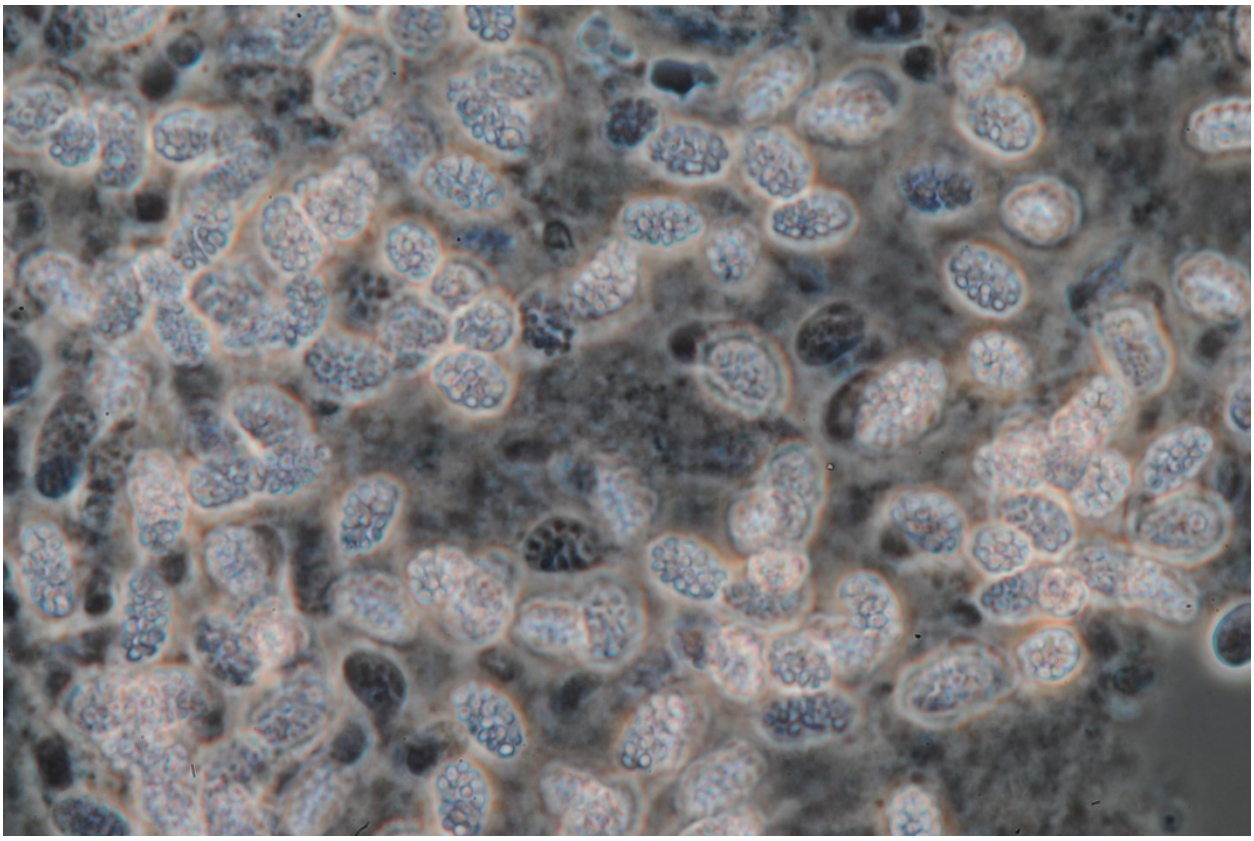
Table 8.1. First, second, and third instar Japanese beetle infection and mortality rates 14 days after being inoculated with *Ovavesicula popilliae*.

	First Instar		Second Instar		Third Instar	
Spore Dosage	% Infection	% Mortality	% Infection	% Mortality	% Infection	% Mortality
0	0	93.3	0	66.7	0	16.7
10	0	83.3	4.3	73.3	0	36.6
100	20	70	20.8	80	3.3	33.3
1,000	16.7	86.7	4	83.3	3.4	43.3
10,000	22.2	83.3	9.1	83.3	7.1	43.3
50,000	6.3	66.7	12	60	0	23.3
100,000	46.7	60	12	60	20	70

Figure 8.1. *O. popilliae* spores in sporophorous vesicles from an adult Malpighian tubule.



9 Laboratory host range for *Ovavesicula popilliae* (Microsporidia: Ovavesiculidae): a microsporidian pathogen of *Popillia japonica* (Coleoptera: Scarabaeidae)

9.1 ABSTRACT

The Japanese beetle (*Popillia japonica*) is a widespread pest across the Eastern United States. Many pathogens have been studied for the biological control of the Japanese beetle, including the microsporidian *Ovavesicula popilliae*. The objective of this study was to evaluate the physiological host range of *O. popilliae,* a biological control agent for the Japanese beetle. *O. popilliae* was inoculated *per os* into selected field collected and lab reared insect larvae of severl species: green June beetles, small hive beetles, lesser mealworms, wax moth larvae, Eastern tent caterpillars, *Phyllophaga* sp., masked chafers, elaterid larvae, yellow-striped armyworms, true armyworms, sod webworms, and black cutworms. A total of 287 insect larvae were inoculated with *O. popilliae* spores, of which nine green June beetle larvae, two Eastern tent caterpillars, two *Phyllophaga* spp. larvae, and one sod webworm developed infections. This investigation showed that *O. popilliae* can be infective to several species; including *Phyllophaga* spp. which were reported to not be able to develop infections. Additionally, *Phyllophaga* spp. and *C. nitida* could support infections and both can be pest species in either turfgrass or fruit systems. The bioassays used in this study only reflected short term disease development.

Keywords: *Popillia japonica*, *Ovavesicula popilliae*, host range, microsporidian pathogen

9.2 INTRODUCTION

The Japanese beetle (*Popillia japonica* Newman) is a widespread pest in the United States, introduced from Japan in 1916. Currently, the beetle ranges from the Eastern U.S. across much of the Midwest and from Southern Canada to Georgia (NAPIS 2012), severely damaging turfgrass, agricultural, and horticultural crops (Fleming 1972). Historically, research on pathogens and parasitoids of *P. japonica* has been conducted in an attempt to suppress density of the beetle with limited success (Fleming 1968). One successful modern biological control agent has been *Ovavesicula popilliae* (Andreadis and Hanula), a microsporidian pathogen found in larvae and adult beetles and which is unknown to infect other insect species (Hanula and Andreadis 1992).

O. popilliae has been found infecting Japanese beetles in Connecticut (Andreadis and Hanula 1987), Michigan (Cappaert and Smitley 2002), Kentucky (Redmond and Potter 2010), and Arkansas (Petty et al. 2012a). Following the release of *O. popilliae* as a biological control agent in Michigan, infection rates ranged from 3.3-20% in adults and up to 75% in larvae (Cappaert and Smitley 2002). *O. popilliae* apparently has caused a decline in density of Japanese beetle in Michigan through reduced female fecundity and increased larval mortality (Smitley et al. 2011).

Like many microsporidia, *O. popilliae* has been thought to be host specific, but observations in this area have been limited. Hanula and Andreadis (1992) showed that *O. popilliae* was absent in naturally occurring populations of Asiatic garden beetle (*Maladera castanea*), Oriental beetle (*Anomala orientalis*), rose chafer (*Macrodactylus subspinosis*) in the northeastern U.S. and also absent in a limited sample of European chafer (*Rhizotrogus majalis*), northern masked chafer (*Cyclocephala borealis*) and an unidentified *Phyllophaga* species

(Hanula and Andreadis 1992). Whether *O. popilliae* has the potential to infect insect species that are not found in the northeastern U.S. or that were not included in the original survey by Hanula and Andreadis (1992) is unknown. Of particular interest is the possibility of a wider host range including scarab pest species such as the green June beetle (*Cotinis nitida* [L.]) and *Phyllophaga* spp.

The range of hosts in which infection can be produced under laboratory conditions, the physiological host range, can be used as an initial indicator of ecological host range, the range of hosts in which infection will occur in nature (Solter and Maddox 1998). Physiological host range, while wider than ecological host ranges, has previously been used to estimate the host range of microsporidia as biological control agents (Solter et al. 1997, Solter and Maddox 1998). The objective of our study was to evaluate the physiological host range of *O. popilliae* as a biological control agent for the Japanese beetle. This was accomplished by comparing infection rates and development in alternate hosts to the Japanese beetle.

9.3 MATERIALS AND METHODS

9.3.1 Collection of O. popilliae spores for inoculation

Infected adult Japanese beetles were collected from the Charles Binder Park Golf Course near Battle Creek, MI with Jumbo Jug funnel mouth traps (1.9 L) (Trécé Inc., Adair, OK) baited with compound floral and sex pheromone lures during July 2009, 2010, and 2011. Spores of *O. popilliae* were identified morphologically *in situ* from infected adult Japanese beetles (Andreadis and Hanula 1987). Spore suspensions for inoculations were prepared by removing the Malpighian tubules from infected adult beetles, homogenizing the tissue in deionized water, and isolating spores via centrifuge as described by Vossbrinck and Andreadis (2007). The volume of

spores in suspension was measured using a hemocytometer as described by Undeen and Vávra (1997).

9.3.2 DNA Sequencing

DNA extractions to confirm the identity of *O. popilliae* were conducted on organs of infected adult specimens using salting-out extraction protocol described in Sambrook and Russell (2001). The PCR primers 18f: 5'-CACCAGGTTGATTCTGCC-3' and 1492r: 5'-GGTTACCTTGTTACGACTT-3' (Vossbrinck and Andreadis 2007) were used to amplify a 1,266 bp region of the small subunit rDNA gene (denaturation at 94°C for 2 min, 39 cycles of 94°C for 45 s, 49.4°C for 45 s, and 72°C for 84 s, extension at 72°C for 5 min.) Products were purified with 30K centrifugal filters (VWR, Radnor, PA), and were sequenced by University of Arkansas Medical School, Little Rock, AR. Sequences were aligned visually and a BLAST (NCBI) search was conducted with Geneious 6.0.3 (Auckland, New Zealand).

9.3.3 Collection of wild and lab reared host insect larvae

Insect larvae were collected from sites around northwest Arkansas. The collection sites include three golf courses, two orchards, three parks, a nursery and the University of Arkansas Research and Extension Farm. The number of species and number of individuals per species collected depended on local densities. All insects collected during this study were identified to species level if possible (Vittum et al. 1999). Insect larvae collected from field sites included yellow-striped armyworms (*Spodoptera ornithogalli*), true armyworms (*Pseudaletia unipuncta*), *Phyllophaga* spp., masked chafers (*Cyclocephala* spp.), Eastern tent caterpillars (*Malacosoma americanum*), black cutworms (*Agrotis ipsilon*), and unidentified sod webworm (*Crambus* spp.) and in elaterid larvae.

In addition to insects collected in the wild, species which are laboratory reared at the University of Arkansas were used. These insects included the green June beetle (*C. nitida*), wax moths (*Galleria mellonella*), the small hive beetle (*Aethina tumida*), and the lesser mealworm (*Alphitobius diaperinus*). All four colonies were maintained at the University of Arkansas. Green June beetle larvae were reared in moist soil containers on decaying alfalfa at room temperature. Small hive beetles and wax moths were reared on honey and pollen in 30°C incubation chambers, and lesser mealworms were reared on apple and fish meal in 30°C incubation chambers.

9.3.4 Feeding bioassays with O. popilliae

Collected larvae were inoculated *per os* via a blunted hypodermic needle and syringe with approximately 50,000 spores of *O. popilliae* or deionized water as a control. Small hive beetle larvae and lesser mealworm larvae were too delicate to inoculate *per os*. For the small hive beetle larvae, 50,000 spores of *O. popilliae* were mixed with 100 mg of pollen and fed to each larva, and for the lesser mealworm larvae, spores were mixed with 100 mg of fishmeal. Larvae were incubated for 14 days at 24°C in individual diet cups during which time mortality was monitored. At the end of the 14 days, all larvae were dissected to determine rates of infection by examining the Malpighian tubules and midgut. Giemsa stain was used to diagnose tissues in which infections were unclear. Infections were characterized further as either "typical" or "atypical". Typical infections resulted in *O. popilliae* completing its life cycle within 2 weeks and successfully forming spores. Atypical infections were characterized by unsuccessful completion of an entire life cycle, with *O. popilliae* usually being present as vegetative cells but no production of spores. The definitions of "typical" and "atypical" infections are similar to definitions used by Solter et al. (1997).

In total, 80 green June beetle larvae, 48 wax moth larvae, 44 small hive beetle larvae, 38 lesser mealworm larvae, 30 Eastern tent caterpillars, 18 *Phyllophaga* sp. larvae, 16 masked chafer larvae, six elaterid larvae, two yellow-striped armyworms, two true armyworms, one sod webworm, and one black cutworm were inoculated with *O. popilliae* and later dissected. In the cases of the small hive beetle larvae, green June beetle larvae, wax moth larvae, eastern tent caterpillars and lesser mealworm larvae, enough individuals were available to also have a control group inoculated with deionized water. In total 42 small hive beetle larvae, 47 lesser mealworm larvae, 30 green June beetle larvae, 46 wax moth larvae, and 15 Eastern tent caterpillars were fed deionized water and later dissected.

9.4 RESULTS

9.4.1 DNA Sequencing

DNA sequences from microsporidia collected from adult Japanese beetles in Battle Creek, MI, matched *O. popilliae* 99.9%. The original *O. popilliae* sequence as described by Vossbrinck and Andreadis (2007) was 1393 bp. Our sequences were truncated to 1266 bp after alignment.

9.4.2 Feeding bioassays with O. popilliae

A total of 205 insect larvae acted as controls and were fed deionized water. The control larvae consisted of 60 small hive beetles, 50 lesser mealworms, 30 green June beetles, 50 wax moths, and 15 eastern tent caterpillars (Table 9.1). Some of the control larvae were found to be missing at the end of incubation period. In total, 42 small hive beetles, 47 lesser mealworms, 30 green June beetles, 46 wax moths, and 15 eastern tent caterpillars were dissected. Control larvae inoculated with deionized water showed signs of *O. popilliae* infection. Not all groups which

were inoculated with *O. popilliae* were represented in the control groups because of lack of specimens.

A total of 287 insect larvae were inoculated with *O. popilliae* spores. Of 80 inoculated green June beetle larvae, 71 remained uninfected, eight individuals developed a typical infection and one larva developed an atypical infection. Of 48 wax moth larvae inoculated, 45 remained uninfected, one larva developed an atypical infection and two larvae developed typical infections (Table 9.2). A total of 44 small hive beetle and 38 lesser mealworms were inoculated but no signs of infection. A total of 30 Eastern tent caterpillars were inoculated with 28 remaining uninfected and two individuals developed typical infections. Of the 18 *Phyllophaga* spp. larvae that were inoculated with *O. popilliae*, two individuals developed typical infections in the Malpighian tubules. None of the 16 *Cyclocephala* spp. larvae or six elaterid larvae inoculated showed infection development. Two yellow armyworms, two true armyworms, and one black cutworm, were inoculated but no infection developed. Only one sod webworm (*Crambus* spp.) was inoculated, which resulted in an atypical infection of the Malpighian tubules and midgut tissue.

9.5 DISCUSSION

Our bioassays show that *O. popilliae* has a limited ability to infect larval insects other than the Japanese beetle, and the insects which were infected did not share close taxonomic relationships. In the majority of cases, no infection developed after two weeks (n=270), although 17 individuals from five of the 12 species inoculated were capable of supporting infection to some degree.

Hanula and Andreadis (1992) reported that *O. popilliae* was not found in scarab populations which co-occurred with infected Japanese beetle populations. We did not attempt to infect most of the species reported in that study, including the Asiatic garden beetle, Oriental beetle, rose chafer, European chafer, and the northern masked chafer. However, Hanula and Andreadis (1992) did not report *O. popilliae* infections in *Phyllophaga* species. Our bioassays showed that *Phyllophaga* spp. were susceptible to *O. popilliae*, although it is unknown which species. Additionally, the green June beetle, *C. nitida*, does appear to support *O. popilliae* to a limited extent and does co-occur with Japanese beetle larvae.

Both *Phyllophaga* spp. and *C. nitida* can be pest species in turfgrass systems when in abundance (Potter and Held 2002) and *C. nitida* and Japanese beetle can cause severe fruit damage when occurring together (Hammons et al. 2008). *O. popilliae* may be a viable biological control agent for all three scarabs and merits further investigation.

A number of Lepidoptera also developed both typical and atypical infections. Both sod webworms and tent caterpillars could hypothetically be exposed to *O. popilliae* spores when passing through turf or by feeding on leaves in which infected adult Japanese beetles have defecated on. Only one sod webworm was inoculated because of the limited number found. Additionally, the individual inoculated only developed an atypical infection, indicating that it is either a sub-optimum host or may require more time for infection development. Wax moths also appeared susceptible to *O. popilliae*. The wax moth is a pest of honey bee colonies, and there is a limited probability for this species to come into contact with *O. popilliae* spores naturally. However, this species is readily mass reared in the laboratory setting. Typical infections do appear to be able to develop in this species. Further investigation is needed, but this species could be ideal for mass rearing large numbers of *O. popilliae* spores in a controlled setting.

The bioassays used in this study only reflect short term disease development. It is possible that *O. popilliae* requires more than two weeks to develop noticeable infection in sub-optimal hosts. There was also some difficulty in determining the number of *O. popilliae* spores present in a dosage. *O. popilliae* was previously reported to contain 32 spores per vesicle and that this number was near constant (Andreadis and Hanula 1987). Our research has shown that 32 spores are not constant and that unbroken vesicles contain as few as 11 spores (unpublished). Our hemocytometer counts estimating *O. popilliae* spore density in a single dosage were based on free spores and a deliberate underestimation of eight spores per vesicle because of the natural inconsistency in spore numbers per vesicle. Additionally, no current test exists to determine the viability of *O. popilliae* spore prior to inoculation, so the proportion of viable to unviable spores in each spore suspension was unknown.

Premature mortality was problematic in insects inoculated with *O. popilliae*. It is possible that a dosage of approximately 50,000 spores was too large, and that germination of the microsporidia disrupted gut function; however, dosages smaller than 50,000 spores may also be too small to cause infection in sub-optimal hosts. One question we didn't answer is what is an optimal spore dosage of this microsporidian to investigate host range?

O. popilliae is able to infect hosts outside the Japanese beetle. The data presented here helps determine the physiological host range of this pathogen, which may not directly reflect the ecological host range of *O. popilliae*. Because *O. popilliae* can infect hosts outside the Japanese beetle, it is necessary to monitor non-Japanese beetle insect larvae in areas where *O. popilliae* is present or has been released for biological control purposes in order to determine if *O. popilliae* is naturally infecting other insects. A possible benefit to *O. popilliae* not being severely restricted in host range is infection of non-Japanese beetle insect pest species. Our data has shown that pest

lepidopteron and scarab larvae are able to support infection, and *O. popilliae* may be beneficial in suppressing populations of multiple, co-occurring pests.

Ecological host range data from Hanula and Andreadis (1992) suggests that *O. popilliae* infection were uncommon in non-Japanese beetle species found in the northeastern U.S.; however, the need for Japanese beetle biological control extends beyond the northeastern U.S. Physiological host range, while wider than ecological host ranges, has previously been used to estimate the host range of microsporidia as biological control agents by examining potential hosts which are both taxonomically related to natural hosts or which share a common habitat (Solter et al. 1997, Solter and Maddox 1998). For a host to be suitable for a microsporidian infection, a number of events must occur. The spore must first occur simultaneously with the host and be exposed to appropriate germination conditions, such as gut pH, penetrate the host, survive the immune system, and find cells in which to develop. Depending on the method by which spores enter a host, some of these problems may be overcome readily (Undeen and Maddox 1973). Ideally, host range for a biological control agent would either be very narrow or limited to pest species. However, evidence exists that the host range of microsporidia can be underestimated by laboratory testing (Solter et al. 2000).

Different groups of possible host species will need to be monitored depending on the location of *O. popilliae* presence or release. It is also worth considering what is the likelihood that other non-Japanese beetle insect larvae will become exposed to a large enough dosage of *O. popilliae* to become infected. Many of the species we inoculated share a turf or soil habitat with Japanese beetle larvae, and horizontal transmission could potentially occur between infected Japanese beetle larvae and other insects. Additionally, infected adult Japanese beetles produce large numbers spores of *O. popilliae* (Petty et al. 2012b) and can also be vectors. The adult

beetles release spores in their frass (Hanula and Andreadis 1990) are predominantly foliage feeders and share this habitant with many other insects, including beneficial predators and pollinators. Further investigation is required concerning the ability of *O. popilliae* to infect other insects in this habitat.

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Table 9.1. Results of larval insects treated with deionized water as a control.

Species	# Infected	# Healthy	# Missing
Small hive beetle	0	42	18
Lesser mealworm	0	47	3
Green June beetle	0	30	0
Wax moth	0	46	4
Tent caterpillars	0	15	0

Table 9.2. Results of larval insects inoculated *per os* with approximately 50,000 spores of *Ovavesicula popilliae*. Typical infection refers to completion of *O. popilliae*'s life cycle 2 weeks. Atypical infection refers to presence of *O. popilliae* but a failure to complete its life cycle.

		Infected	
Species	**Uninfected**	**Atypical**	**Typical**
Green June beetle	71	8	1
Wax Moth	45	1	2
Small Hive beetle	44	0	0
Lesser Mealworm	38	0	0
Tent Caterpillars	28	0	2
Phyllophaga spp.	16	0	2
Cyclocephala spp.	16	0	0
Elaterids	6	0	0
Yellow Armyworm	2	0	0
True Armyworm	2	0	0
Sod webworm	1	1	0
Black cut worm	1	0	0

10 *Ovavesicula popilliae* (Microsporidia: Ovavesiculidae) spore production in naturally infected adult Japanese beetles (Coleoptera: Scarabaeidae)

B. M. Petty, D. T. Johnson, and D. C. Steinkraus

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10.1 ABSTRACT

Ovavesicula popilliae is a microsporidian that infects both Japanese beetle larvae and adults. This is the first study quantifying the number of *O. popilliae* spores produced by Japanese beetle adults. Mean spore production per adult Japanese beetle was 2.67×10^7 (SE ± 4.65 x 10^6) spores with a range of 1.46×10^6 to 1.02×10^8. The number of spores produced per host is similar to other microsporidian species and may help explain the speed with which this pathogen has spread from introduction sites to surrounding areas.

Keywords: *Ovavesicula popilliae*; *Popillia japonica*; Japanese beetle

10.2 INTRODUCTION

Ovavesicula popilliae (Andreadis and Hanula, 1987) is a microsporidian pathogen of the Japanese beetle, *Popillia japonica* Newman, infecting both the larvae and adults. Infection initially develops in the Malpighian tubules of the larvae, but becomes systemic in transstadially infected adults (Hanula and Andreadis, 1990). *O. popilliae* has been used as a biological control agent for the Japanese beetle (Cappaert and Smitley, 2002) and has been shown to be detrimental to both larval and adult beetles through an increase in larval winter mortality (Smitley et al., 2011) and decreased female fecundity (Hanula, 1990).

Japanese beetles become infected with *O. popilliae* when larvae ingest spores. When infected larvae survive to adulthood, the infection may be carried with it through pupation (Hanula and Andreadis, 1990). Adult beetles are capable of traveling over 8 km in a single flight, and thus represent a highly mobile stage of infected hosts (Fleming, 1972). The objective of this study was to determine the number of *O. popilliae* spores that naturally infected Japanese beetle adult hosts can produce and potentially spread.

10.3 MATERIALS AND METHODS

Adult Japanese beetles were collected in Battle Creek, MI, from areas with known establishment of Japanese beetle and *O. popilliae* (Cappaert and Smitley, 2002). In 2010, the prevalence of *O. popilliae* in beetles from this area was estimated to be 13.8% (n=210). Beetles were collected in July 2011 using Jumbo Jug funnel mouth traps (1.9 L) baited with floral and sex pheromone lures (Trécé Inc., Adair, OK). Adult beetles were dissected and a small portion of the Malpighian tubules were removed for microscopic diagnosis of infection. The *O. popilliae* spores were identified morphologically, with packets of spores recognizable by their

characteristic raspberry shape (Cappaert and Smitley, 2002). If an adult beetle was infected with *O. popilliae*, the entire beetle was macerated in 1 ml of deionized water, vortexed, and large debris removed. The first thirty infected adult beetles found were individually homogenized in this way. Individual spores were counted with a hemocytometer as described by Undeen and Vávra (1997). The spore count for each beetle spore suspension was quantified four times in order to give a final average count per beetle. Mean spore count per beetle, variance, and standard error of mean were estimated using JMP® 9.0 (SAS Institute Inc. 2009).

10.4 RESULTS

Mean spore production of *O. popilliae* per adult Japanese beetle host was estimated to be 2.67×10^7 (SE ± 4.65 x 10^6) spores (Fig. 10.1). The number of spores produced per host ranged from 1.46×10^6 to 1.02×10^8. The mean spore count and range were taken from 29 of the original 30 dissected hosts, with one host being excluded as an outlier. The excluded host produced 2.39×10^8 spores, which was more than five standard deviations (SD ± 2.5×10^7) above the mean.

10.5 DISCUSSION

O. popilliae is transmitted horizontally by exposure to spores likely present in the soil surrounding infected but dead Japanese beetle larvae or adults. Infection begins in the Malpighian tubules and spreads throughout the host tissues as the beetle develops. *O. popilliae* spore production numbers in adult Japanese beetles are similar to the range of spore production in other microsporidian species. In comparison, three microsporidia produced similar numbers of spores in larval corn earworms, *Helicoverpa zea* (Boddie): *Vairimorpha necatrix* produced up to

1.67×10^{10} spores per larva under optimal conditions (Fuxa and Brooks, 1979); *Nosema acridophagus* produced 6.51×10^{7} spores per larva; and *N. cuneatum* produced up to 2.17×10^{6} spores per larva (Henry et al., 1979).

Healthy adult Japanese beetles do not appear susceptible to infection with *O. popilliae* when exposed to spores (unpublished data); however, adult beetles which were infected during larval development may be the major vector for the spread of *O. popilliae* over large areas. A single infected adult female may travel several hundred meters over the course of its life, excreting spores in its frass as it travels (Hanula and Andreadis, 1990). If an infected adult beetle dies in the soil it could potentially inoculate the area with several million spores. If a female were to die in the soil during oviposition, her offspring might contact large numbers of spores and become infected.

The spread of *O. popilliae* by the Japanese beetle adults is suggested by biological control studies conducted in Michigan. In southeast Michigan, *O. popilliae* spread at least 400 m from inoculation sites over the course of eight years. Additionally, locations up to 5 km away from inoculation sites were also found to have Japanese beetles infected with *O. popilliae*. Both of these distances were considered too far to be caused by the spread of infected larvae, strongly suggesting deposition of large numbers of spores from infected adult beetles (Smitley et al., 2011). Because adult Japanese beetles can produce large numbers of spores, they are likely to be the primary vector for the spread of *O. popilliae* between distant locations.

10.6 ACKNOWLEDGEMENTS

We thank David Smitley's lab for helping us acquire Japanese beetles infected with *O. popilliae.* This work was funded by the Arkansas Agriculture Department and USDA Specialty Crop Block Grant Program.

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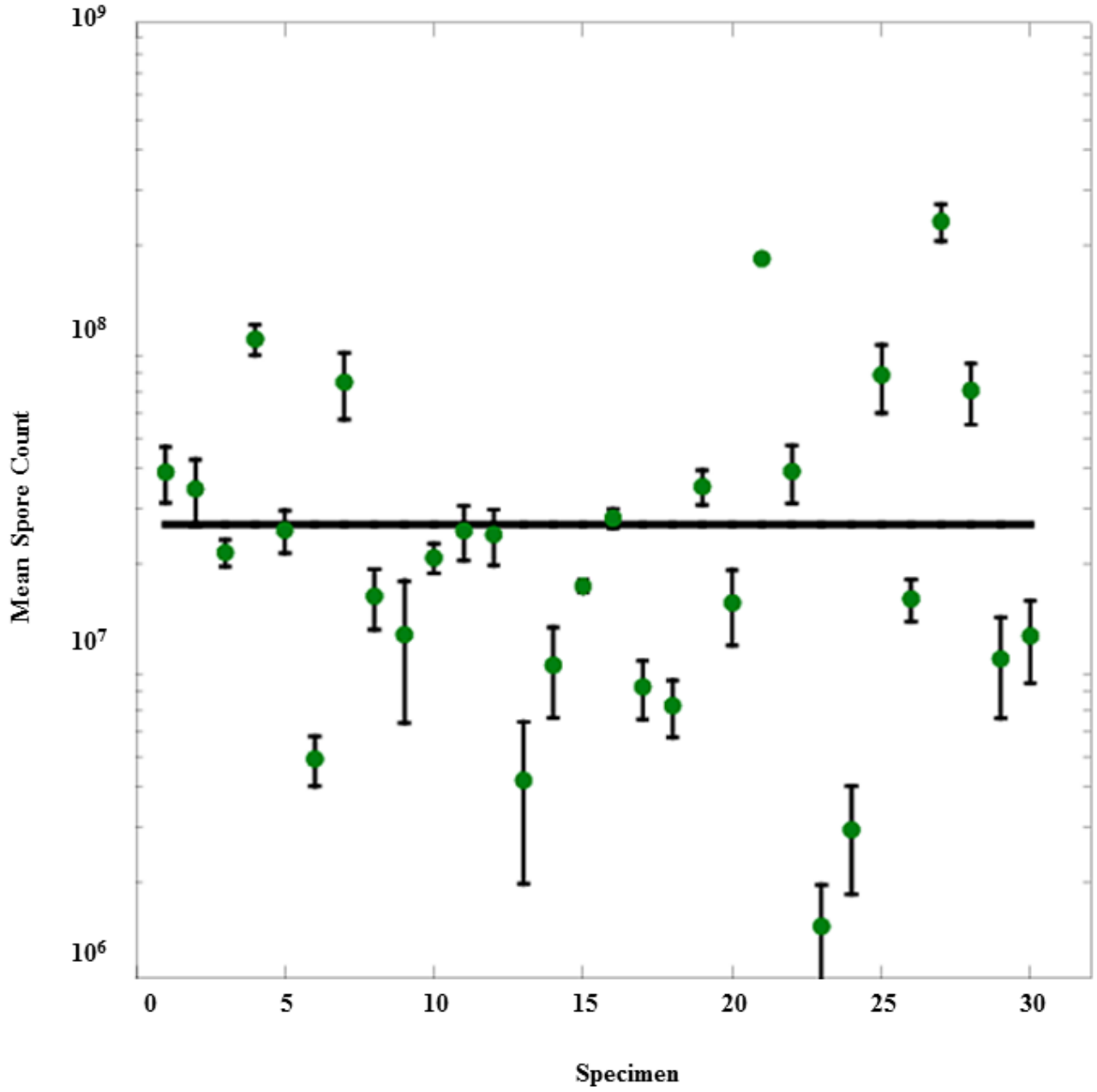
Figure 10.1. Mean spore count distribution from individual beetles (±SE). The overall mean of 2.67×10^7 spores per beetle is represented by the horizontal black line.



11 Appendix D: Publishing agreement and signed documentation of multi-authored chapters

Article:	***Ovavesicula popilliae* (Microsporidia: Ovavesiculidae) spore production in naturally infected adult Japanese beetles (Coleoptera: Scarabaeidae)**
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Our reference	**YJIPA6351**
PII:	**S0022-2011(12)00235-2**
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July 10, 2013

To Whom It May Concern:

Bryan Petty is lead author on the publication:

Petty, B.M., D. T. Johnson, D. C. Steinkraus. 2012. *Ovavesicula popilliae* (Microsporidia: Ovavesiculidae) spore production in naturally infected adult Japanese beetles (Coleoptera: Scarabaeidae) J. Invertebr. Pathol. 111: 255-256.

Mr. Petty conducted more than 51% of the research and writing on this paper as part of his Ph.D. research at the University of Arkansas.

Sincerely,

Dr. Donald C. Steinkraus, Professor of Entomology, steinkr@uark.edu

12 Evaluating spore count and sporophorous vesicle size in *Ovavesicula popilliae* (Microsporidia: Ovavesiculidae) in adult Japanese beetles

12.1 ABSTRACT

Ovavesicula popilliae is a microsporidian pathogen of the Japanese beetle used as a biological control agent. *O. popilliae* reportedly produces 32 spores within a sporophorous vesicle measuring 20.0-21.0 μm x 15.0-15.5 μm. We determined the sizes of sporophorous vesicles and the number of spores contained within vesicles. Sporophorous vesicles had a mean size of 13.3 μm x 9.3 μm and contained 24.7 spores. Varying number of spores may be due to failed divisions of sporonts or death of spores after formation. The differences in vesicle sizes may be due to host effects or be evidence of different pathogen strains.

Keywords: *Ovavesicula popilliae*, *Popillia japonica*, spore, sporophorous vesicle

12.2 INTRODUCTION

Ovavesicula popilliae (Andreadis and Hanula) is a microsporidian pathogen of the Japanese beetle (*Popillia japonica* Newman) used as a biological control agent for this serious pest. This pathogen primarily infects the Malpighian tubules in both larval and adult stages, but can spread to other tissues (Andreadis and Hanula, 1987). *O. popilliae* is most likely transmitted through a fecal-oral route between larvae and adults to larvae. In addition, infected larvae may die releasing spores as they decay, or may pupate into infected adults (Hanula and Andreadis, 1990).

O. popilliae was originally described from Japanese beetles in Connecticut, USA (Andreadis and Hanula, 1987) and has since been found infecting *P. japonica* in Michigan (Cappaert and Smitley, 2002), Kentucky (Redmond and Potter, 2010), and Arkansas (Petty et al., 2012). In Michigan, prevalence of infection was up to 13.9% in adults and 63.7% in larvae after releases of *O. popilliae* during a biological control program (Smitley et al., 2011) and is currently being used as a biological control agent in Arkansas.

O. popilliae is one of nine microsporidian taxa to produce multiple spores from a single sporont (Vossbrinck and Andreadis, 2007), and reportedly produces 32 ovoid spores within a persistent sporophorous vesicle. Andreadis and Hanula (1987) found spores were 2.0-2.5 µm x 1.7-1.8 µm and sporophorous vesicles had a mean size of 20.0-21.0 µm x 15.0-15.5 µm. The objectives of this study were to evaluate reported sizes of *O. popilliae* sporophorous vesicles and the number of spores contained within these vesicles in adult Japanese beetles.

12.3 MATERIALS AND METHODS

Adult Japanese beetles were collected in Battle Creek, MI from the Charles Binder Park Golf Course from July 12 – 17, 2012 with Jumbo Jug funnel mouth traps (1.9 L) (Trécé Inc., Adair, OK) baited with compound floral and sex pheromone lures. *Ovavesicula popilliae* infections were diagnosed by removing a small portion of Malpighian tubule from each beetle and examining them microscopically for sporophorous vesicles (Andreadis and Hanula, 1987). The abdomens of infected beetles were macerated in deionized water to free the sporophorous vesicles for further examination.

Sporophorous vesicles were examined with a phase contrast microscope at 400x magnification and measured with a calibrated ocular micrometer. A total of 125 sporophorous vesicles were examined. Spores within the sporophorous vesicles were also counted at 1000x magnification. Due to overlapping of spores within a single vesicle, vesicles were lightly crushed under their microscope slide covers. Only vesicles that were not ruptured during this process had their spores counted. The spores per sporophorous vesicle in a total of 100 sporophorous vesicles were counted.

12.3.1 DNA Analysis

DNA extractions were conducted on the entire abdominal contents of an infected specimen using a salting-out extraction protocol with in-house reagents (Sambrook and Russell 2001). Each PCR reaction contained 2 µl DNA template, 5 µl 10XThermoPol buffer (New England BioLabs, Ipswich, MA), 4µl dNTP mix (10 mM each nucleotide), 1 µl each primer (20 mM), 1 unit *Taq* polymerase (New England BioLabs, Ipswich, MA), and ultrapure water for a final volume of 50µl. The PCR primers 18f: 5'-CACCAGGTTGATTCTGCC-3' and 1492r: 5'-GGTTACCTTGTTACGACTT-3' (Vossbrinck and Andreadis 2007) were used, which amplify a

1,266 bp region of the small subunit rDNA gene. A negative control sample substituting water for DNA template was also included. Thermal cycling conditions were denaturation at 94°C for 2 min, followed by 39 cycles of 94°C for 45 s, 49.4°C for 45 s, and 72°C for 84 s, followed by a final extension at 72°C for 5 min. PCR products were subjected to electrophoresis and visualized under UV light on a 2% agarose gel stained with ethidium bromide. Amplicons were purified with 30K centrifugal filters (VWR, Radnor, PA), and were sequenced in both directions (UAMS, Little Rock, AR). Sequences were aligned visually and a BLAST (NCBI) search was conducted with Geneious 6.0.3 (Auckland, New Zealand).

12.4 RESULTS

Prevalence of *O. popilliae*-infected Japanese beetle adults collected from Michigan in 2012 was 25% (n=100). Mean sporophorous vesicle length for *O. popilliae* was 13.3 μm (SE ± 0.1) and mean width was 9.3 μm (SE ± 0.1) (Fig. 12.1). The size of sporophorous vesicles ranged from a minimum of 9.6 μm to 16.8 μm long and 7.2 μm to 13.2 μm wide. Mean numbers of spores within each sporophorous vesicle was 24.7 (SE ± 0.8) and ranged from 11 to 64 (Fig. 12.2). Sporophorous vesicles containing 32 spores, reported as the typical number (Andreadis and Hanula, 1987), occurred 27% of the time and was the most common number of spores found per vesicle. Only two sporophorous vesicles contained more than 32 spores.

DNA sequencing produced a 1,266bp amplicon, the entire length of which was unambiguously aligned to the single *O. popilliae* SSU sequence available (GenBank, EF564602). The sequences were identical.

12.5 DISCUSSION

Our results of 24.7 spores per sporophorous vesicle and vesicle size of 13.3 µm x 9.3 µm greatly differs from the original measurements of 32 spores per sporophorous vesicle and a vesicle size of 20.0-21.0 µm x 15.0-15.5 µm reported by Andreadis and Hanula (1987). The original description of *O. popilliae* and its life cycle was made using sporophorous vesicles collected from third instar Japanese beetle larvae from Connecticut (Andreadis and Hanula, 1987). The variation in vesicle size and the number of spores present is noteworthy for two reasons. First, the vesicle size and number of spores differs from the original description used for morphological identification and may cause confusion. Second, it is important to know the average number of spores present per vesicle when planning experimental applications of *O. popilliae*, such as making spore suspensions for inoculation purposes. *O. popilliae*'s sporophorous vesicles are highly persistent, and do not readily break when disturbed. Assuming that 32 spores are present may lead to erroneous estimations of spore density.

One explanation for the difference in size of our sporophorous vesicles and those originally described may be due to the fact that our *O. popilliae* sporophorous vesicles were collected from adult Japanese beetles. The difference in host life stage may have some unknown effect on the vesicle size. Alternatively, the differences in sporophorous vesicle size may be evidence of different strains of the pathogen between what is found in Michigan and what was originally described from Connecticut.

Thirty-two spores per sporophorous vesicle was the most common (27%) configuration for spores within sporophorous vesicles. This is similar to that described by Andreadis and Hanula (1987), but unlike in the original description, variation in the number of spores present was high. The variability in spores per sporophorous vesicle may be due to failed divisions of the

vegetative stages of the microsporidian (Andreadis and Hanula, 1987), an unknown effect of the adult beetle's immune system on the pathogen, death of spores after formation, or some unknown factor.

Number of spores per sporophorous vesicle or size of vesicle may differ by host life stage or strain of *O. popilliae* and these variations should be recognized when working with this pathogen for practical purposes, such as counting spores in suspension or identification purposes.

12.6 ACKNOWLEDGEMENTS

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Figure 12.1. Size distribution of *Ovavesicula popilliae* sporophorous vesicles, with the mean size of 13.3 µm x 9.3 µm (SE±0.1 µm) represented by an open box with cross. Only 23 of 125 points are visible because of clustering.

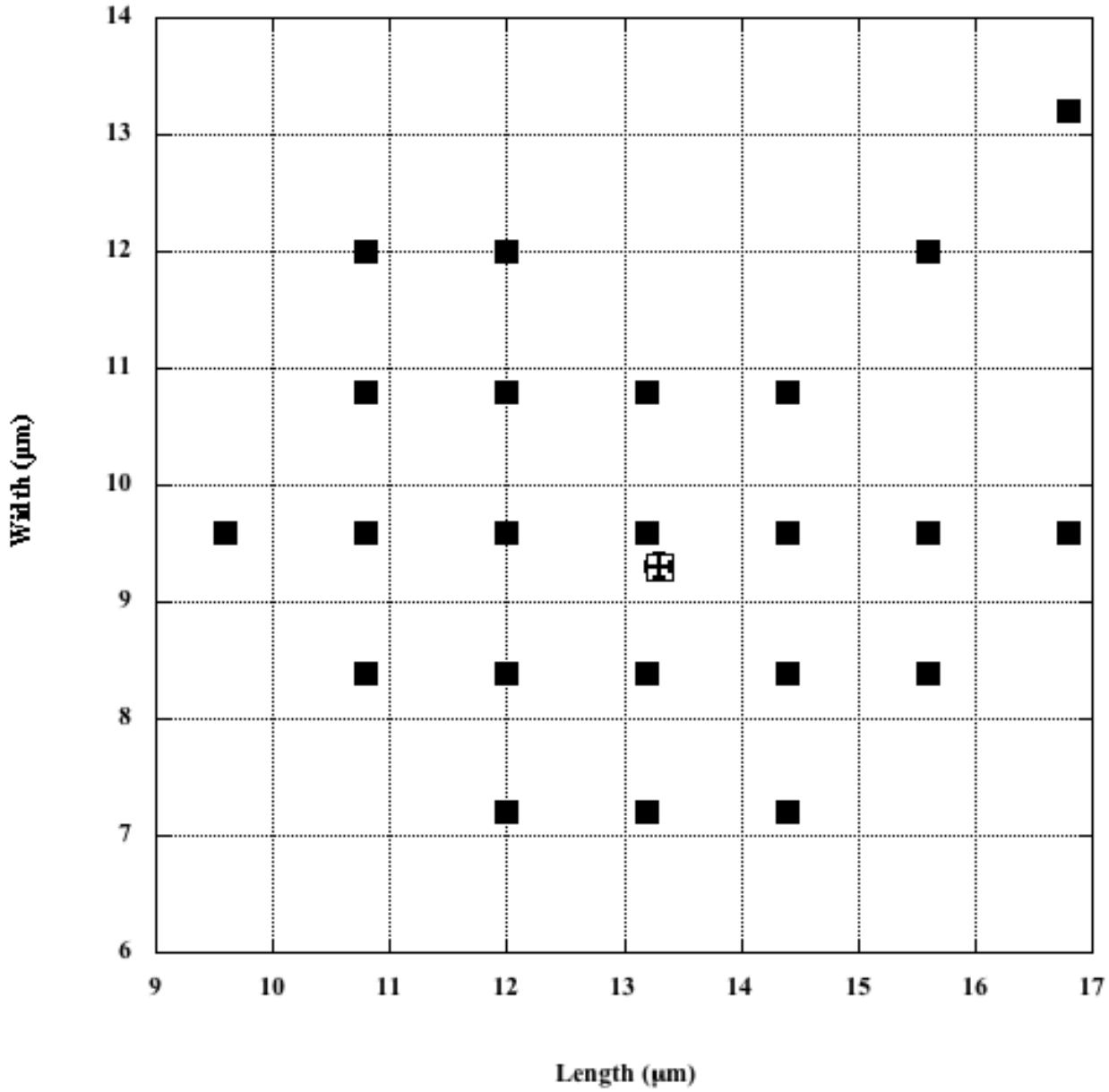
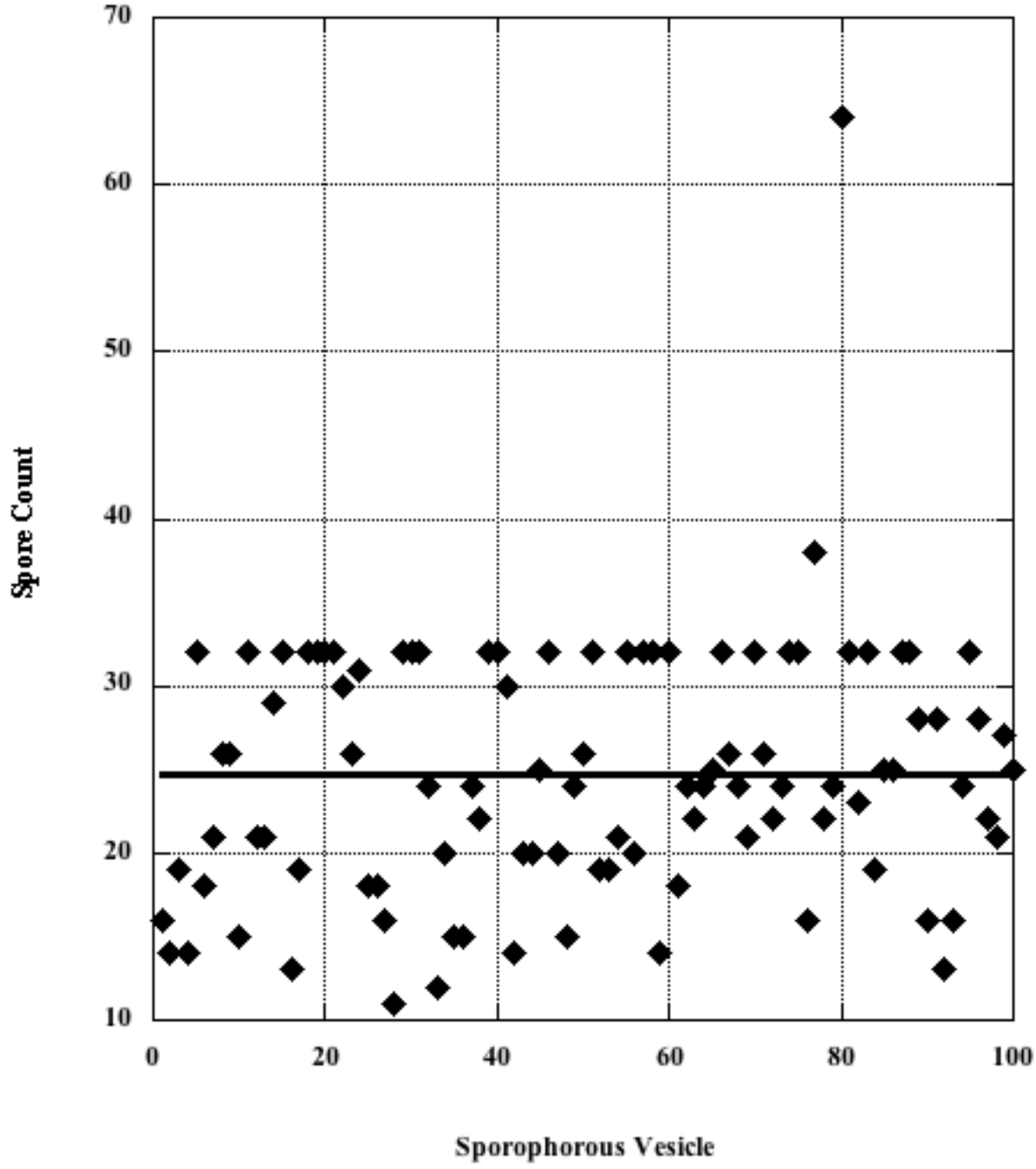


Figure 12.2. Spore count distribution of *Ovavesicula popilliae* with the sporophorous vesicles, with the mean spore count of 24.7 (SE±0.8) represented by the solid bar.



13 Conclusions and future work

The Japanese beetle has been a serious pest in Arkansas and continues to pose a threat. The following sections offer summaries of work already competed and possible future directions for research into the abiotic and biotic factors affecting the Japanese beetle in Arkansas.

13.1 Changes in density of Japanese beetles in Northwest Arkansas and understanding the impacts of temperature on these changes

The low densities of Japanese beetles in northwest Arkansas as compared to other areas of established infestation had remained largely unexplained. Northwest Arkansas lacks any substantial density of pathogens or parasitoids of the Japanese beetle that could keep the density low (Petty et al. 2012); however, this region is on the southwestern border of the Japanese beetle infestation in the U.S., and severe weather conditions may create a suboptimum environment for this invasive beetle.

High temperatures during July and August were inversely correlated with the density of adult Japanese beetles the following year. This may be due to the fact that eggs and early instars occur during July and August (Fleming 1972), and these instars are particularly sensitive to heat. Should the Japanese beetle continue to expand southwest in the United States, the increasingly warm and dry climate may cause this beetle to become a minor pest, as compared to the severe economic impact that the Japanese beetle is known to have in the cooler and wetter climates of the northeastern United States (Potter and Held 2002).

The temperature model proposed has been used to explain mortality rates in juvenile populations of Japanese beetles in warm-climate areas and the subsequent adult density fluctuations associated changes in larval densities. Observed density decline in eggs and first instars fit within the model's predictions in all years when rearing cages were in operation. The

higher than predicted mortality in second instars may be due to complications with molting associated with high temperatures, but may also be due to limited data on second instars available from Ludwig (1928). This model also has predictive applications. By tracking summer temperatures in a geographical area, this model can be used to calculate expected adult densities in the succeeding summer.

The model predicting mortality in early instar Japanese beetles needs further testing and modification. The model should be applied in other infested states which experience high summer heat in order to verify predicted population declines. Additionally, second instar mortality has been much higher than the model predicts. This could be because the model does not include effects of temperature on molting mortality or extended heat exposure across multiple instars. Data from Ludwig (1928) on second instar temperature dependent development also needs further refinement. More temperature related experiments will need to be conducted in order to produce data on these topics which can be incorporated into the model.

If the model is indeed verified across multiple states and develops better predictive powers associated with second instar mortality, it could be used in applied settings to better predict Japanese beetle densities in states which experience high summer heat. A user-friendly model could be made available online which could predict annual density declines from the previous year associated with temperature so as to better anticipate local summer Japanese beetle adult damage. This would require monitoring of temperature changes, available from weather stations, as well as annual monitoring fluctuations of Japanese beetle adult densities in a given region. This can most likely be done through coordination with universities and state extension agencies or citizen scientists.

13.2 Presence of natural enemies of Japanese beetles and the attempted establishment *O. popilliae* in Arkansas as a biological control agent

Based on our study, the impact of pathogens and parasitoids on Japanese beetle larvae and adults in Arkansas appears to be minimal at this time. This low impact of pathogens and parasitoids may justify classical or augmentative biological control efforts for this pest in Arkansas.

S. villani was the most prevalent pathogen found in larval *P. japonica* in Arkansas, but this pathogen is not known to cause mortality within larval populations. Additionally, we did not find any *Paenibacillus* spp. or any parasitoid wasps or flies. In 2010, we found a prevalence of 0.2% *O. popilliae* in adult beetles and zero in larvae. In 2011, it was found in 2.6% of larvae and no adults were found infected. In 2012, *O. popilliae* infection was only found in 0.4% of adult beetles and in no larvae. Nematode-infected *P. japonica* larvae were uncommon in northwest Arkansas. While nematodes may provide control in areas where they are augmented as a biological control agent or with more soil moisture, they do not appear to account for much natural mortality in northwest Arkansas. *Adelina* sp. was also found in for the first time in Japanese beetles. *Adelina* sp. is a coccidian pathogen inhabiting the midgut tissues of its host. This genus has been previously found in Asiatic garden beetles (*Maladera castanea Arrow)* in Connecticut (Hanula and Andreadis 1988).

Additionally, an "unknown" pathogen, possibly an undescribed microsporidian or protist pathogen of the gut, was found infecting adult beetles at nine of the ten locations in 2012 (mean infection of 5.2%). This unknown pathogen was not found in 2010 or 2011 surveys and very little is currently known about it. The prevalence of this pathogen may have increased in the

local Japanese beetle population in northwest Arkansas due to environmental stress on beetles of back to back years of drought and high temperatures.

The establishment of *O. popilliae* may be extremely difficult if populations of the Japanese beetle remain low. It may be more beneficial to try and establish biological control of the Japanese beetle using *O. popilliae* in regions which either: 1) have a history of high larval Japanese beetles abundance 2) have high local abundances of larval Japanese beetles such as golf course infestations. Unfortunately, the density of Japanese beetles in the regions sharply declined prior to the release of *O. popilliae* in 2010.

The introduction of *O. popilliae* may also be easier via live adult Japanese beetle releases. Japanese beetles known to be infected with *O. popilliae* could be released in order to spread the pathogen. Ideally, infected adult females could contaminate their eggs with *O. popilliae* spores via defecation while ovipositing, as spores are present in the frass of infected individuals (Hanula and Andreadis 1990).

Continued releases and monitoring of *O. popilliae* in Arkansas should be implemented should the abundance of Japanese beetles rebound, especially along the expanding border of the infestation in both the northwest region and around Little Rock, AR. It may also be beneficial to coordinate releases of *O. popilliae* into Japanese beetle populations with researchers in Tennessee, Oklahoma and Missouri. Releases at all locations should be delayed until a rebound in the Japanese beetle densities, which has suffered severe decline since 2010 in association with extreme summer heat and drought.

13.3 Host range, host life stage specificity, and dose response of *O. popilliae*

All three larval life-stages of the Japanese beetle appear susceptible to *O. popilliae* when inoculated with 50,000 or more spores. The data also suggests adult Japanese beetles have a very low susceptibility to *O. popilliae* and that infections acquired in this stage are atypical. The lack of susceptibility of healthy adult Japanese beetles to *O. popilliae* infection corroborates initial claims that infections in adult Japanese beetles were the product of infections which occurred earlier in life and were carried transstadially (Hanula and Andreadis 1990). The dose response data shows that only early instars of *P. japonica* are susceptible to relatively low or natural dosages of spores.

The host range bioassays show that *O. popilliae* has a limited ability to infected non-Japanese beetle larval insects. In the majority of cases, no infection developed after two weeks (n=270), although 17 individuals from five of the 12 species tested were capable of supporting infection to some degree. This included green June beetles, wax moth larvae, Eastern tent caterpillars, *Phyllophaga* spp., and sod webworms, which are all pest species. Additionally, wax moth may be a suitable species for the mass rearing of *O. popilliae* spores.

This physiological host range data may not directly reflect the ecological host range of *O. popilliae*. Because *O. popilliae* can infect hosts outside the Japanese beetle, it is probably necessary to monitor non-Japanese beetle insect larvae in areas where *O. popilliae* is present or has been released for biological control purposes in order to determine in *O. popilliae* is naturally infecting other insects. Our data has shown that pest lepidopteron and scarab larvae are able to support infection, and *O. popilliae* may be beneficial in suppressing densities of multiple, co-occurring pests.

Further work needs to continue on mass rearing of *O. popilliae* using insects which can be easily maintained in a laboratory setting. If it is possible to cheaply mass rear *O. popilliae*, this pathogen may be a feasible biological control for widespread use. Although it is unlikely that *O. popilliae* could ever be used as a curative treatment for severe Japanese beetle infestation considering the slow acting and chronic nature of microsporidian infections, the pathogen could help lower densitiess of the Japanese beetles.

13.4 Spore production in *O. popilliae*

O. popilliae spore production numbers in adult Japanese beetles are similar to the range of spore production in other microsporidian species. Healthy adult Japanese beetles do not appear susceptible to infection with *O. popilliae* when exposed to spores; however, adult beetles which were infected during larval development may be the major vector for the spread of *O. popilliae* over large areas. A single infected adult female may travel several hundred meters over the course of its life, excreting spores in its frass as it travels. If an infected adult beetle dies in the soil it could potentially inoculate the area with several million spores. Because adult Japanese beetles can produce large numbers of spores, they are likely to be the primary vector for the spread of *O. popilliae* between distant locations.

Additionally, spore size and sporophorous vesicle size originally reported for *O. popilliae* does not seem to be entirely accurate. Our data of 24.7 spores per sporophorous vesicle and vesicle size of 13.3 μm x 9.3 μm greatly differs from the original measurements of 32 (Andreadis and Hanula 1987). Possible explanations include differences in host effects on the pathogen (i.e. differences in development in adults versus larvae) or geographical strain differences in the pathogen. Number of spores per sporophorous vesicle or size of vesicle may differ by host life

stage or strain of *O. popilliae* and these variations should be recognized when working with this pathogen for practical purposes, such as counting spores in suspension, inoculation, or identification purposes.

13.5 REFERENCES CITED

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