To the Graduate Council:

I am submitting herewith a thesis written by Donald Ray Wimmer entitled "The Adult Japanese Beetle, Popillia japonica Newman, As a Host and Potential Autodisseminator of the Milky Disease Pathogen, Bacillus Popilliae Dutky." I have examined the final copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Entomology and Plant Pathology.

Lyle E. Klostermeyer
L. E. Klostermeyer, Major Professor

We have read this thesis and recommend its acceptance:

Reid R. Leonhardt

Accepted for the Council:

L. Evans Roth
Vice Chancellor
Graduate Studies and Research

THE ADULT JAPANESE BEETLE, *POPILLIA JAPONICA* NEWMAN, AS A HOST AND POTENTIAL AUTODISSEMINATOR OF THE MILKY DISEASE PATHOGEN, *BACILLUS POPILLIAE* DUTKY

A Thesis

Presented for the

Master of Science

Degree

The University of Tennessee, Knoxville

Donald Ray Wimmer

December 1982

ACKNOWLEDGMENTS

The author expresses his gratitude to Dr. L. E. Klostermeyer, Dr. R. R. Gerhardt, and Dr. C. D. Pless for their advice and suggestions through the course of this study. The author also thanks the Tennessee Department of Agriculture for their financial support of this study. Thanks go to Dr. M. G. Klein, Japanese Beetle Research Laboratory, Agricultural Research Service, USDA, Wooster, Ohio, and Ms. R. Galloway, Biological Laboratory, Pesticide and Plant Protection Division, North Carolina Department of Agriculture, Raleigh, North Carolina, for providing the B. popilliae spores used in the study. To Mr. Robert Kelly and Mr. Gary Miller special thanks for the many hours of conversation and encouragement.

The author also thanks Dr. R. F. Harwood of Washington State University for his friendship and encouragement while the author was an undergraduate. Sincere gratitude is extended to Dr. E. C. Burts, Dr. S. C. Hoyt, and Dr. J. F. Brunner of The Tree Fruit Research Center, Washington State University for their friendship and job doing that which the author enjoys most, entomology.

ABSTRACT

Adult Japanese beetles, Popillia japonica, were evaluated for competency as hosts and potential autodisseminators of the milky disease pathogen, Bacillus popilliae. Adults were injected during a series of 20 replicates with either 1.0×10^5, 5.0×10^4, or 1.0×10^4 spores and maintained for 16 days in a controlled environment.

Forty-one percent of the females and 29% of the males were competent hosts. Eighty-four percent of the sporulation had occurred by days 10 and 11 postinjection. Competent beetles contained hemolymph which had an average of 1.6×10^7 spores per µl 16 days postinjection. Beetles injected after mid-July were not competent hosts of the pathogen.

A mark, release, and recapture study was conducted. No injected adults were recaptured out of the 1,200 released. Twenty of 4,000 control beetles released were recaptured within two days.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

Since the detection of the Japanese beetle Popillia japonica Newman in the United States in 1916, numerous insecticides have been used in attempts to control adults and larvae. Possible development of insecticide resistant larval populations (Dunbar and Beard 1975) as well as recent Environmental Protection Agency (EPA) decisions to ban various persistent insecticides have given new importance to the continued use of Bacillus popilliae Dutky as a biological control agent. B. popilliae, the causitive agent of milky disease of the Japanese beetle, was one of the first bacteria to be mass produced in vivo and applied in the field. Dutky (1940) provided evidence that B. popilliae could be effective in control of Japanese beetles in what has become a classic example of biological control. If the United States Department of Agriculture's recently requested (U.S. Federal Register 1982) exemption from the requirement of a tolerance for B. popilliae to be used on pastures is approved by the EPA, the role of B. popilliae will be indeed an important one.

Presently, all commercial spore production is based on Dutky's (1942) in vivo methods. In vivo spore production is expensive because laboratory rearing of Japanese beetles has not been perfected. Feral larvae are collected, injected with spores, and held until they succumb to the milky disease. They are then ground up and mixed with talc to produce the milky disease powder. There have been problems in maintaining and producing viable material. Klein (1981) pointed out that, in the future,

emphasis must be placed on selecting strains of the B. popilliae bacteria which fit target conditions and larval populations. Also, because of increasing in vivo production costs and in hope of increasing production to meet anticipated demands, recent research has been directed towards in vivo spore production (Klein et al. 1976). Results have not been encouraging (Faust 1979, Beegle 1978).

In Tennessee, where the Japanese beetle is now present in some 33 counties (Williams 1981), there are some 1.7 million hectares of pasture, about 16% of the total land area (U.S. Bureau of the Census 1980). This does not include lawns, parks, golf courses and grassy highway right-of-ways, all of which are potential habitat for the larvae.

Langford et al. (1942) suggested that the Japanese beetle could be a natural host of B. popilliae and potentially a major factor in the natural dissemination and establishment of the bacteria. They also suggested that adults could be used for in vivo production of milky disease powder. Klein (1981) has examined the latter more closely.

Adult scarabaeids can be injected with a pathogen and successfully utilized as a host and autodisseminator (Zelanzny 1976, 1978). The objective of this study, which was conducted during the summers of 1981 and 1982, was to evaluate the adult Japanese beetle, injected with B. popilliae spores, as a host and potential autodisseminator of milky disease.

CHAPTER I

LITERATURE REVIEW

I. THE PATHOGEN, *BACILLUS POPILLIAE* DUTKY

Beard (1945), Dutky (1963), Fleming (1968), and Milner (1974) have reviewed the milky diseases of scarabaeid larvae. Klein et al. (1976) have compiled a comprehensive bibliography of the *Bacillus* spp. associated with *P. japonica* and closely related Scarabaeidae.

History

Diseased Japanese beetle larvae were first detected in central New Jersey in 1933. Their hemolymph was milky in appearance and under microscopic examination was confirmed to be filled with bacterial spores, in contrast to the clear hemolymph of healthy larvae (Hawley and White 1935). The disease was caused by two closely related spore-forming bacteria which White and Dutky (1940) designated as Type A and Type B. Dutky (1940) proposed the names *Bacillus popilliae* and *Bacillus lentimorbus* for the pathogens causing Type A and Type B diseases, respectively. The origin of these entomopathogenic bacteria remains unknown but they are believed to be indigenous to North America (Fleming 1968).

Morphology

There are two distinct stages of *B. popilliae*. These are the resting spore and the vegetative rod. Black (1968) described and illustrated both stages. The vegetative stage is characterized by

gram-positive, nonmotile rods. They are approximately 0.9 by 5.2 μm in size (Beard 1945). All species of Bacillus produce endospores. B. popilliae has a centrally located endospore and a parasporal crystal. This crystal is about half the size of the endospore and is located in the wider pole of the cell. Both endospore and parasporal body are enclosed by the sporangium. Unstained spores appear pyriform and, in outline, suggest a footprint. This characteristic shape easily distinguishes B. popilliae from other bacteria or inorganic contaminants. The spore is gram-positive and is about 0.9 by 1.8 μm.

Developmental Cycle

The infection begins with the appearance of the vegetative rods in the hemolymph after injection or ingestion. Rods are present in small numbers after 30 hours at 30° C (Dutky 1940). At this temperature multiplication is extremely rapid. The precise mechanism of sporulation is not known, except that it occurs when the vegetative rods reach high numbers in the hemolymph (Bulla et al. 1978). Sporulation continues until all the cells have reached the definitive spore stage. Spores are sufficiently numerous by the sixth day that the hemolymph is turbid. Seven to ten days after entry into the hemolymph the number of spores reaches the maximum and the hemolymph is distinctly milky in appearance (Dutky 1940).

Dissemination

The pathogen can be disseminated both naturally and artificially (Rivers 1967). Thousands of kilograms of the spore have been applied to

turf in the eastern states where the beetle has become established by state and federal agriculture departments (Fleming 1968).

Natural dissemination. The pathogen is dispersed naturally by the host itself, other insects, birds, mammals and soil movement (Fleming 1968). Both larva and adult P. japonica can act as natural dispersal agents. Larvae have been observed to move as much as three meters horizontally in cultivated soil (Hawley 1935). Langford et al. (1942) concluded that the adult could be a natural host and disseminator of B. popilliae. In field surveys 2.6 diseased individuals per 1,000 adults were found. Each diseased adult was found to have about 5.0×10^8 spores. Ants have been observed moving diseased larvae more than 3 m. Solitary wasps, Tiphia vernalis (Rohwers) and T. popilliavora (Rohwers), have become contaminated with spores while searching for Japanese beetle larvae upon which to oviposit (White 1943).

Artificial dissemination. Various carriers and application methods have been investigated since Dutky (1942) first developed in vivo spore production techniques (Fleming 1968, Beard 1945). Attempts to inoculate soil with lyophilized vegetative cells have met with little success (Linng and McMahon 1969). Virtually all spore production and application continue as originally developed. All commercial spore preparations are powders labeled to contain not less than 1.0×10^8 viable spores per gram of carrier, calcium carbonate. Label instructions are for the contents to be applied to turf at the rate of 51 kg/ha. Application methods vary. For small areas such as lawns it is often applied

by hand using a teaspoon or a can with holes in the bottom. For larger areas such as golf courses, public parks and traffic medians, mechanical dispensers mounted on the back of a motor vehicle are used (Klein 1981).

Langford et al. (1942) observed that the adult could be an artificial disseminator of B. popilliae. They found that they could induce the disease by injecting 1.0×10^6 spores suspended in 2 µl of water per adult. They reported that injected adults readily developed the disease and that most adults remained vigorous and active until the disease was well advanced. They speculated that "collecting, inoculating and liberating adults may prove to be an economical method of supplementing the present method of artificially disseminating the disease."

II. THE ADULT JAPANESE BEETLE

At one time placed in the family Rutelidae, the Japanese beetle, Popillia japonica, is now in the family Scarabaeidae, subfamily Rutelinae. This polyphytophagous pest has been the center of considerable research since it was first discovered near Riverton, New Jersey, in 1916. Fleming's (1972) review of the biology of P. japonica is the definitive reference.

This holometabolous insect is univoltine except in the northern most part of its range where eggs deposited in late August may require two years to complete development (Hawley 1944, Gould 1963). Adults begin to emerge from their earthen cells as early as the third week of May in central North Carolina and as late as the last week of June in

the higher altitudes of the state (Fleming and Hawley 1950). In eastern Tennessee they appear the first week of June (Millington 1980).

During the female's lifespan of 30 to 45 days, she may enter the soil 16 or more times, depositing as many as 133 eggs (Fleming 1972). Normal fecundity is between 40-60 eggs. Oviposition usually occurs at a soil depth of 5-6 cm. Each egg is deposited into a depression made by the ovipositor. This depression becomes a small cell encompassing the egg when the female digs laterally, pushing the loosened soil behind her. She may deposit eggs at three or more places within one area. Ovipositing females may remain in the soil three to four days (Smith and Hadley 1926).

There are many factors which influence the female's selection of an oviposition site. The main factors are (1) the proximity of a suitable site to the plant upon which she is feeding, (2) the nature of the ground cover, (3) the condition of the soil, and (4) site exposure to direct sunlight (Fleming 1968).

III. ENTOMOPATHOGENS AS BIOLOGICAL CONTROL AGENTS

Numerous fungi, protozoans, viruses, and bacteria have been used in the biological control of arthropod pests. Tanada (1959), Steinhaus (1954), Van Der Laan (1967), Allen et al. (1968), and Burges (1981) offer comprehensive reviews of entomopathogens used in pest management.

Entomopathogens can be introduced and colonized in the environment by various methods. Harper (1978) defined introduction as the process of placing an entomopathogen where it did not occur or was not previously

active. He defined colonization as the permanent or long-term establishment of an entomopathogen introduced as a regulatory agent into a host-insect population. Successful colonization is possible when biotic and abiotic factors necessary for transmission and maintenance of the entomopathogen are present. These interrelated factors have been studied by Tanada (1973), Franz (1971), Harshbarger and Faust (1973), and Hall (1974). Three types of introduction of entomopathogens are (1) inoculative release with permanent establishment, (2) inundative release with permanent establishment, and (3) inundative release without permanent establishment.

Bacillus popilliae is applied inoculatively with permanent establishment and colonization intended. As a microbial insecticide, B. popilliae is applied in the same manner as chemical insecticides (Klein 1981). However, bacteria, like other entomopathogens, can be introduced into the environment through autodissemination. Autodissemination is defined as the establishment of foci of infection and reliance upon living insects to spread a pathogen to target populations in a larger area (Soper 1978). Angus (1978) used the term specifically to define the spread, movement, or passage of bacteria as a result of host-insect activity.

Knipling (1960) stated that insects could be used to disseminate pathogens among their own kind. He cited three specific factors which favor autodissemination: (1) Certain pathogens are nonvirulent when present in or on the adults but are highly virulent to the larval form. (2) Inoculum levels needed to cause infection in the early larval stages

are extremely small for some pathogens. (3) The adult of any given species while seeking a mate or oviposition site will naturally find the environment where the pathogen is most likely to survive and establish itself.

Adult scarabaeids, injected with hemolymph suspensions from diseased larvae, can be competent autodisseminators (Zelazny 1976, 1978). The pathogen, Rhabdionvirus oryctes Huger, a baculovirus, was found to occur naturally in both larval and adult populations of the Indian rhinoceros beetle, Oryctes rhinoceros (L.), a serious pest of coconut palms (Huger 1973). Introduction and colonization of this pathogen through autodissemination significantly reduced beetle populations on several South Pacific islands (Zelazny 1978, Gorick 1980).

CHAPTER II

MATERIALS AND METHODS

I. PRELIMINARY INJECTION STUDY, 1981

Various injection techniques, sites of injection, spore suspensions, inoculum levels, collection dates, holding and temperature regimens, and foods were evaluated from 9 June through 27 August 1981. Adults were hand collected from host plants in Happy Valley, Blount County, Tennessee. They were held in plastic dishpans 33 × 27 × 12 mm and maintained on apple, Malus domestica Borkh, slices in the laboratory where daytime temperatures often exceeded 30° C. Beetles were held as long as 48 hours before injection.

Spore Suspensions

Spore suspensions were prepared from slides of dried hemolymph films provided by R. Galloway, Biological Laboratory, Pesticide and Plant Protection Division, North Carolina Department of Agriculture, Raleigh, North Carolina; and M. G. Klein, Japanese Beetle Research Laboratory, Agricultural Research Service, USDA, Wooster, Ohio. All smears contained B. popilliae spores which were at least 6 months old. Spore suspensions were made by wetting the dried hemolymph film. After several minutes the film was gently agitated with a sterile insect pin and washed into a 10 ml test tube. Desired inoculum levels were subsequently made from this stock solution by diluting with sterile distilled water. Final volumes were between 5 and 10 ml. Final inoculum levels were determined

using an Improved Neubauer Ultra Plane^{R} hemacytometer following standard procedures. During the course of the study, nine different inoculum levels were evaluated. They consisted of 1.0×10^{4}, 3.0×10^{4}, 5.0×10^{4}, 7.6×10^{4}, 1.0×10^{5}, 3.0×10^{5}, 1.2×10^{6}, 2.7×10^{6}, and 9.3×10^{7} spores per beetle. These were used for injection for as long as three weeks. All suspensions were held at room temperatures.

Injection Techniques

Beetles were injected in one of two places. During June they were injected through the integument of the cervix between the head and prothorax, dorsal lateral. During July and August injections were through the integument of the pronotum, in which case entry was anterior lateral of the dorsal midpoint. All injections were done with a Hamilton^{R} 705-N, 50 µl syringe mounted in a Hamilton^{R} PB600 Dispenser. Each depression of the dispenser button delivered 1 µl, 1/50 of the syringe volume. During the first half of the summer, beetles were injected with either 2 or 4 µl of inoculum and were held on the needle for the few seconds required for the delivery of the inoculum. During the second half of the summer, beetles were injected with 2 µl and were held on the needle for 30 seconds.

Maintenance and Observations

Beetles were held in plastic 3.76 liter (gallon) jars, paper 0.47 liter (pint) ice cream containers or plastic 70 ml (4 oz.) Solo^{R} souffle cups. Food was either Pennsylvania smartweed, Polygonum pennsylvanium (L.), or apple slices. Replicates of either five, ten, or 20 beetles

per sex were held at room temperature or at a constant 30° C in a Percival^{R} I-35 incubator.

Observations were taken at one, two, or three day intervals after injection. The number of dead beetles of each sex in each treatment was recorded.

II. HOST COMPETENCY, EVALUATION IN A CONTROLLED ENVIRONMENT, 1982

Host competency, the ability of the host to survive until completion of pathogen development, was evaluated in a controlled environment in 1982. All studies were carried out on the Agricultural Campus, The University of Tennessee, Knoxville, between 14 June and 28 July 1982. All beetles used were hand picked from host plants at the Plant Science Farm, Knoxville, and held in the laboratory for at least 18 hours at about 21° C. Only active, normal-appearing adults, assumed free of collection trauma and pesticide exposure, were used in the study.

Spore Suspensions

All spore suspensions were prepared from slides of dried hemolymph smears provided by M. G. Klein, Japanese Beetle Research Laboratory, Agricultural Research Service, USDA, Wooster, Ohio. All slides were designated as containing only B. popilliae spores from milky-diseased larvae and dated 28 October 1980.

Dried hemolymph containing spores was scraped from the surface of the slide with a sterile surgical blade. The material was then transferred to a disposable, 1.5 ml microcentrifuge tube containing

0.5 ml sterile distilled water, pH 7.1. After initial mixing in a Vortex-Genie^{R} vortex mixer for 30 seconds, the spore suspension sat for one hour to permit complete wetting of the hemolymph. One hundred seconds of mixing then followed. Then an aliquot was removed from the suspension using a disposable 25 µl micropipet and loaded into the counting chamber of a hemacytometer. Once in position on the stage of a compound microscope the loaded hemacytometer remained undisturbed for at least three minutes. The number of spores per µl of suspension was then calculated following standard hemacytometer counting procedures. Sterile distilled water, pH 7.1, was added to obtain the desired inoculum level.

Injection Procedures

Adults were injected using a Hamilton^{R} 705-N, 50 µl syringe with a 22 S gauge needle (0.71 mm outside diameter) mounted in a Hamilton^{R} PB600 dispenser. Each depression of the dispenser button delivered 1/50 of the syringe barrel volume. Separate syringes of the same type were used for the spore inoculum and sterile distilled water treatments.

Injection was performed as follows:

1. The syringe was filled with a treatment suspension and placed in the dispenser.
2. Held between thumb and index finger of one hand, the beetle was brought into contact with the needle which was held firmly with the other hand. The point of entry was always anterior and lateral of the midline of the pronotum.
3. The beetle was then gently forced onto the needle point.

4. Once the integument was pierced, the beetle was gently rotated so that its long axis was in the alignment axis of the needle. Thus, the needle point moved towards the caudal portion of the prothorax, avoiding the dorsal vessel.
5. The beetle was then forced further onto the needle until the entire needle opening was within the hemocoel.
6. Two 1 µl droplets were then delivered in succession.
7. The beetle remained on the needle point for 30 seconds to allow the action of the hemolymph to disperse the droplets away from the needle tip.
8. The injected beetle was then removed from the needle.

Treatments

Two µl of each of the following treatments were injected into each beetle. (1) 10×10^4 spores, (2) 5.0×10^4 spores, (3) 1.0×10^4 spores, (4) sterile distilled water control, and (5) uninjected control. One replicate consisted of these five treatments administered to each of five adults per sex. There were 20 replicates over time.

Maintenance

All injected adults were held in 70 ml souffle cups; five bettles per cup. Males and females were maintained separately. Apple slices, 0.5 cm thick and approximately 4 g, were provided for food. Cups were cleaned, new apple slices exchanged for old, and dead beetles removed every 24 hours. All beetles were maintained in an incubator at 24° C

from 0900h to 2000h and at 19° C from 2000h to 0900h. Photophase was 0600h to 2100h. Relative humidity was maintained at about 75%.

Observations

Observations were taken every 24 hours and the number of days survival after injection was recorded for each beetle. Beginning at 10 days postinjection (DPI), a sample of hemolymph was extracted from each beetle by reopening the injection wound with a sterile number 2 insect pin. A glass slide was then brought into contact with hemolymph droplet and a smear made. Spore presence was determined microscopically at 400×.

Sixteen days after injection 1 µl of hemolymph was removed from one beetle per treatment, when available. The injection wound was reopened or, if necessary, a new hole was made. As the droplet of hemolymph exuded from the beetle, a 1 µl disposable Microcap^{R} microcapillary tube was touched to it immediately and was filled through capillary action. If the Microcap failed to fill to capacity because of insufficient hemolymph, the extent of load fill was marked on the surface of the tube and the length was measured. Volume was then calculated as a proportion of length of a full load. The contents of the microcapillary tube were then discharged into 25 to 250 µl of sterile distilled water in a disposable, 1.5 ml microcentrifuge tube and mixed for 30 seconds in a vortex mixer. An aliquot was then removed with a disposable 25 µl pipet and loaded into the counting chamber of a hemacytometer.

III. MARK, RELEASE AND RECAPTURE STUDY, 1982

Eight mark, release and recapture experiments were conducted at the Plant Science Farm, Knoxville, Tennessee. Beetles were hand collected from host plants at the farm and used immediately. Each beetle was injected with 5.0×10^4 spores in 2 µl sterile distilled water. Fifty males and 50 females were injected, marked and released every other day from 18 June through 16 July. Uninjected controls (500 males and 500 females) were marked and released on 22, 24, 26 and 28 June.

All beetles were marked with Day-Glo^R colors; Arc Yellow, Signal Green and Rocket Red fluorescent pigment. Pigments were applied to the ventral surface of each beetle with a fine camel-hair brush. For additional marking, scratches were made on the pronotum with an insect pin at the time of injection.

Immediately after injection and marking, the beetles were released at a point midway along a slight draw which diagonally crossed a 0.5 ha area of mowed fescue-clover turf. Deciduous trees and shrubs, including favored hosts, surrounded this clearing. Ten Ellisco^R Japanese Beetle Traps baited with Lure N Kill^R dual lure baits were placed on metal rods 1.3 m above ground level. Traps were placed at 30 m intervals along the edge of the clearing, 40 to 80 m from the release point. Traps were emptied once every 48 hours, contents returned to the laboratory and frozen. Trap catches were weighed and numbers estimated by subsample weights of 100 beetles. All beetles were then viewed in darkness under ultraviolet light and checked for the presence of fluorescent pigments.

CHAPTER III

RESULTS AND DISCUSSION

I. PRELIMINARY INJECTION STUDIES, 1981

Thirty-seven percent of the 39 beetles injected with spores on 9 June survived 10 days postinjection (DPI) (Table 1). None of the males or females injected with spores on 14 and 16 June survived beyond 2 DPI. In comparison, 52 and 44%, respectively, of the sterile-distilled-water injected males and females in the 14 June and none in the 16 June treatments survived 10 DPI. Survival to 10 DPI among uninjected males and females was 68 and 75%, respectively, on 14 June and 48 and 50%, respectively, on 16 June treatment dates. When beetles were injected with 4 µl of solution, fluid exuded from the wound. This was not observed when only 2 µl were injected. Five percent of the males and 30% of the females injected with spores on 8 July survived 10 DPI. More females than males, uninjected or injected with sterile distilled water, on 8, 10, 12, and 13 July survived. After 13 July no beetles, regardless of treatment, survived more than 5 DPI.

The variable survival of early June and early July spore-injected beetles and the premature death of beetles injected with spores during the remainder of June, July, and August are not understood. One reason might have been contamination of the needle during injections and of the stock spore suspensions. Stock spore suspensions were held at room temperature for as long as 2 weeks. Daily preparation of the suspensions

Table 1. Survival of Japanese beetles injected with Bacillus popilliae spores, 1981.

Injection Date	Number of Beetles Treated	Treatment[a] per Beetle	Percent Survival 10 DPI[b] Male		Female
9 June	27	3.0×10^5[c]	--	48[d]	--
9	12	3.0×10^5	--	25[d]	--
11	20	2.7×10^7[c]	50		--
11	25	2.7×10^7[c]	--		16
14	50	9.3×10^7[c]	0		0
14	50	0	52		44
14	50	uninjected	68		75
16	100	7.6×10^4	0		0
16	50	0	0		0
16	50	uninjected	48		50
8 July	20	2.8×10^5	5		5
8	20	1.1×10^5	0		35
8	20	8.0×10^4	5		40
8	20	0	65		85
8	20	uninjected	50		45
10	20	2.8×10^5	0		0
10	20	1.1×10^5	0		10
10	20	8.0×10^4	5		20
10	20	0	5		35
10	20	uninjected	70		80
12	10	2.8×10^5	0		0
12	10	1.1×10^5	0		0
12	10	8.0×10^4	0		0
12	10	0	50		50
12	10	uninjected	0		90
13	10	2.8×10^5	0		0
13	10	1.1×10^5	0		0
13	10	8.0×10^4	0		0
13	10	0	20		0
13	10	uninjected	0		90

[a]Spores injected in 2 µl sterile distilled water.

[b]Days postinjection.

[c]Spores injected in 4 µl sterile distilled water.

[d]Sex not determined.

and frequent needle sterilization might have prevented contamination. Another factor in beetle death might be the number of beetles held together before and after treatment. Overcrowding resulted in fecal contaminated food as well as possible stress on the beetles.

II. HOST COMPETENCY, EVALUATION IN A CONTROLLED ENVIRONMENT, 1982

First observance of spores occurred on 10 and 11 DPI in 84% of the 153 beetles found to have spores in their hemolymph (Figure 1). After day 12 few additional beetles with spores were found.

Microscopic examination of the hemolymph extracted from adults 16 days after injection verified previous observations of spore presence. Spore concentrations averaged 15.9×10^6 spores per µl of hemolymph for males and 16.2×10^6 spores per µl of hemolymph for females (Table 2). More females were still alive than males at 16 DPI.

Beetles injected with spores after 10 July, regardless of sex or inoculum level, were not capable of surviving 10 days after treatment (Figure 2). After 13 July, none of the control beetles, regardless of sex or treatment, survived until 10 days after treatment. All beetles injected with spores on 25 June died within 48 hours, possibly because of needle contamination.

Host competency, the ability to survive pathogen development, was determined for male and female Japanese beetles in 1982 (Table 3, Table 4). Among spore-injected females, 36, 47, and 41% were competent hosts after being injected with 10×10^4 , 5×10^4 , or 1×10^4 spores, respectively (Table 4). Overall host competency of spore injected males was 29% as

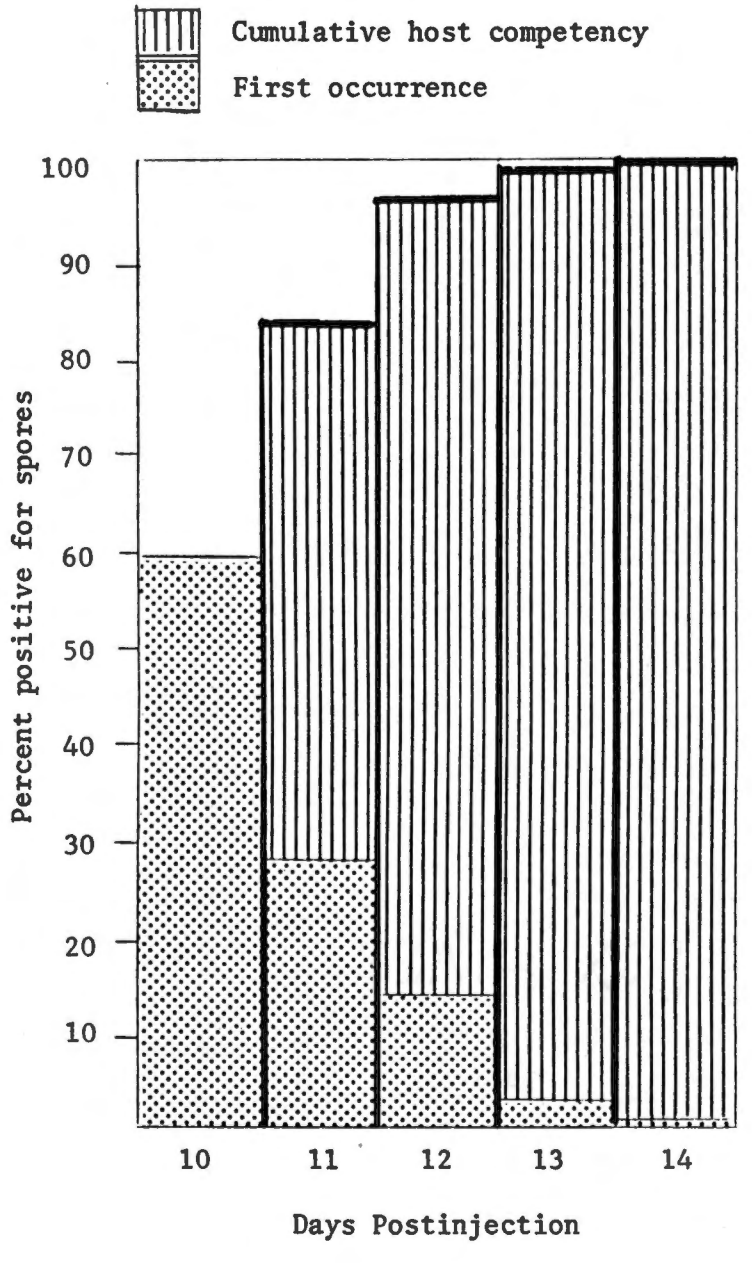

Figure 1. First occurrence of B. popilliae spores in hemolymph of Japanese beetles and cumulative host competency, 1982. Composite of 3 levels of spore inoculum over 5 weeks for 153 beetles.

Table 2. *Bacillus popilliae* spore concentration in hemolymph of selected Japanese beetles at 16 days after injection.

Injection Date		Spore Concentration ($\times 10^6$/µl) in Hemolymph[a] Inoculum Level ($\times 10^4$ spores/2 µl) 10	5	1
		Male		
14 June		15	16	16
19		15	17	17
23		16	15	--
25		--	--	--
27		--	15	17
27		--	--	16
29		--	16	--
29		--	16	15
1 July		16	16	17
1		16	17	15
3		--	--	16
5		--	16	--
7		--	--	--
	Mean	15.6×10^6	16.0×10^6	16.1×10^6
		Female		
14 June		17	16	15
19		17	15	16
23		17	16	18
25		--	--	--
27		15	16	16
27		16	17	16
29		15	--	--
29		15	16	17
1 July		16	17	17
1		15	15	17
3		17	17	17
5		--	16	--
7		17	16	16
	Mean	16.1×10^6	16.1×10^6	16.5×10^6

[a]Hemolymph extracted from 1 beetle per treatment when available.

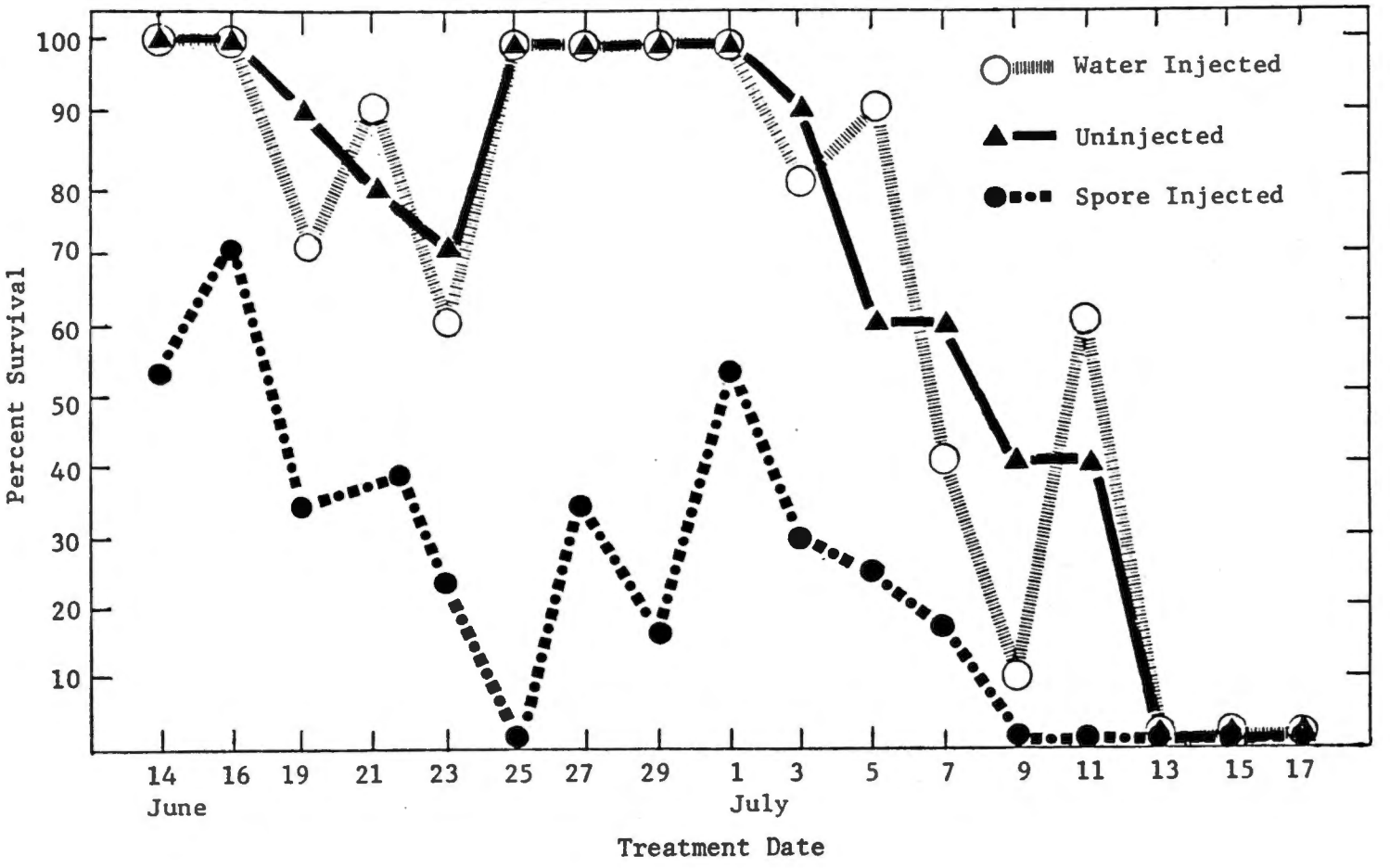

Figure 2. Effect of injected Bacillus popilliae spores on survival of injected Japanese beetles at 10 days after injection. Spores injected is a composite of three levels of spore inoculum.

Table 3. Host competency of B. popilliae spore injected male Japanese beetles and survival among control beetles, 1982.

Injection Date	Percent Host Competency[a] Inoculum Levels[b] 10	5	1	Percent Survival[a] Water[c] Injected	Uninjected
14 June	60	60	60	100	100
16	80	60	60	100	100
19	20	20	20	80	80
21	40	60	40	80	100
23	40	20	0	60	80
25	0	0	0	100	100
27	0	40	60	100	100
27	0	20	20	100	100
29	0	0	0	100	100
29	0	40	0	100	100
1 July	40	40	60	100	100
1	20	20	80	100	100
3	0	0	40	60	80
5	0	40	60	80	20
7	0	0	20	40	40
9	0	0	0	0	40
11	0	0	0	60	40
13	0	0	0	0	0
15	0	0	0	0	0
17	0	0	0	0	0
Mean[d]	21	30	37		

[a]At 10 days after treatment.

[b]Spores ($\times 10^4$) in 2 µl sterile distilled water injected per adult, five beetles per sex per treatment per date.

[c]Sterile distilled water (2 µl).

[d]Results from 25 June, and 9, 11, 13, 15, 17 July injections omitted from means.

Table 4. Host competency of B. popilliae spore injected female Japanese beetles and survival among control beetles, 1982.

Injection Date	Percent Host Competency[a] Inoculum Level[b] 10	5	1	Percent Survival[a] Water[c] Injected	Uninjected
14 June	40	60	40	100	100
16	80	60	80	100	100
19	40	60	40	60	100
21	20	40	20	100	60
23	20	40	20	60	60
25	0	0	0	100	100
27	40	40	80	100	100
27	40	20	40	100	100
29	20	20	0	100	100
29	40	60	40	100	100
1 July	60	80	100	100	100
1	60	20	60	100	100
3	20	80	40	100	100
5	0	60	0	100	100
7	20	40	20	40	80
9	0	0	0	20	40
11	0	0	0	60	40
13	0	0	0	0	0
15	0	0	0	0	0
17	0	0	0	0	0
Mean[d]	36	47	41		

[a]At 10 days after treatment.

[b]Spores ($\times 10^4$) in 2 µl sterile distilled water injected per adult, five beetles per sex per treatment per date.

[c]Sterile distilled water (2 µl).

[d]Results from 25 June, and 9, 11, 13, 15, 17 July injections omitted from means.

compared to an overall host competency of spore injected females of 41%. None of the water injected or uninjected control adults were found to host the pathogen when their hemolymph was examined at 16 DPI. Host competency ranged from a high of 100% among the 5 females injected 1 July with 1.0×10^4 spores (Table 4) to a low of 20% among males and females injected with any of the three inocula (Tables 3 and 4). No single inoculum level was observed to be consistently superior or inferior.

The maximum longevity observed among spore-injected beetles was 21 days after injection. Two females survived the hemolymph extractions on 10 and 16 DPI and continued to be active and live an additional 5 days. Two males survived for 20 days after injection.

Earlier and continued monitoring of hemolymph beginning on at least day 8 after injection would have provided a more precise date of first occurrence of spores. Dutky (1942) reported sporulation in 14 days at 17.2° C, 11 days at 21.1° C, and 9 days at 25.0° C in the hemolymph of spore injected P. japonica larvae. In this study, the beetles were held at 24° C for 11 hours and at 19° C for 13 hours daily, for an average daily temperature of 21.3° C. The 84% competency in adults by 11 DPI agrees with that found by Dutky (1942) in larvae. If host beetles were held at higher temperatures, spore production would occur faster but in 1981 beetles were observed to be more restless and fed less at the higher temperatures and had lower survival to 10 DPI.

Differences in size, weight, and hemolymph volumes between males and females could account for differences in host competency. Females weight about 30% more than males and presumably have more hemolymph.

A larger volume of hemolymph may be advantageous to females in surviving the disease cycle longer.

The steady decline in percent survival and host competency observed among treated beetles began with 1 July-treated adults and continued until 13 July when no survival to 10 DPI occurred. A similar decline during early July was noted in 1981. Decreased survival apparently precedes peak population of adults in East Tennessee by about a week. Senescence may be a factor in the decreased survival beginning in mid-July. Because age is difficult to determine in feral beetles, injection should be done before mid-July. Langford et al. (1942) reported that adults injected with 1.0×10^6 spores and held at room temperature for 15 to 30 days resulted in 56%, 60%, and 93% host competency. Klein (1981) injected 1×10^4 and 10×10^4 spores per beetle and reported 53 and 95% competency, respectively, within 14 days. Their average adult host competencies were about twice as high as reported in this study. Neither Langford et al. (1942) nor Klein (1981) reported the collection or injection dates.

Both studies reported spore counts of 5.0×10^8 per beetle. Because hemolymph volume has not been determined for adult Japanese beetles, it is difficult to compare spore concentration with total number of spores per beetle.

III. MARK, RELEASE AND RECAPTURE STUDY, 1982

No injected adults were recaptured. Three males and 13 females of the control group which were released 26 June were recaptured on

30 June. Four males from 28 June were recaptured on 1 July. About 40,000 beetles were collected from all traps every 48 hours. Heavy rains on 26 June and 8 July were responsible perhaps for reducing 28 June and 10 July counts by about half.

Most beetles, injected and uninjected, were observed to fly away from the release point. Those that did not were observed digging into the turf. Failure to recapture any of the injected beetles might be attributed to death shortly after injection, dispersal beyond the range of the traps or the limited number of released adults. Failure to recapture more than 20 of the 4000 control adults also can be attributed to dispersal beyond effective trapping range and limited number of released beetles. Polivka (1949) and Millington (1980) reported low recapture rates also.

The results from this study should not eliminate spore injected P. japonica adults as potential autodisseminators of B. popilliae spores. Extensive field trails involving many injected adults over a period of several summers could determine their effectiveness. Survey of larval populations before and after the release of injected adults would demonstrate the establishment and spread of the pathogen. Further field studies are needed to determine where injected adults die. If the adults die in areas of low larval populations such as under host plants, then the usefulness of the adult as an autodisseminator would be diminished.

CHAPTER IV

CONCLUSIONS

Popillia japonica adults injected with *Bacillus popilliae* spores are competent to survive the time necessary for completion of the milky disease cycle. Throughout the 1982 injection study, the three inoculum levels were capable of eliciting competent adults. Inoculum levels between 1×10^4 and 10×10^4 spores are suitable for injection purposes. The critical factor seems to be date of collection and injection. Adults injected prior to the second week of July in East Tennessee are potentially competent hosts. More females than males survived to 10 days after injection and are probably more competent hosts.

Because there are no environmental or economic reasons to inject only females, they are the better candidates as host and autodisseminator of *B. popilliae* spores. Females routinely enter the soil for oviposition and remain there as long as four days. Therefore, an injected female could die while in the soil. Death could be the result of the same factors that were assumed to cause mortality during experimentation: abundance of spores and beetle senescence. Subsequent disintegration of the host's body would result in the liberation of approximately 500 million viable spores into the soil. Adults which do not die fortuitously in the soil would still be valuable in establishing foci of infection if they die in grassy areas.

The Japanese beetle is a serious pest and poses a potential threat to Tennessee agriculture, particularly the nursery industry, as it

advances westward. The expense of milky disease powder and difficulty of monitoring spore concentration and virulence before and after application justify new and innovative methods of introducing B. popilliae into the environment. Autodissemination is one such method. Adults injected with spores and released into new areas of beetle invasion could create foci of inoculum. This could be important in reducing or delaying the buildup of extremely high beetle populations in these new areas.

REFERENCES CITED

REFERENCES CITED

Allen, G. E., C. M. Ignoffo, and R. P. Jacques eds. [1978]. Microbial control of insect pests. NSF-USDA-Univ. Fla. Workshop, 10-12 July 1978. 290 pp.

Angus, T. A. [1978]. Autodissemination of entomopathogens: bacteria. pp. 66-8. In G. E. Allen, C. M. Ignoffo, and R. P. Jacques eds., Microbial control of insect pests. NSF-USDA-Univ. Fla. Workshop, 10-12 July 1978.

Beegle, C. C. 1978. Use of entomogenous bacteria in agroecosystems. Devel. Indust. Microbial. 20: 97-104.

Beard, R. L. 1945. Studies on the milky disease of Japanese beetle larvae. Conn. Agric. Exp. Stn. Bull. 491: 505-82.

Black, S. H. 1968. Cytology of milky disease bacteria. I. Morphogenesis of Bacillus popilliae in vivo. J. Invertebr. Pathol. 12: 148-57.

Burges, H. D. ed. 1981. Microbial control of pests and plant diseases 1970-1980. Academic Press, New York. 949 pp.

Bulla, L. A., Jr., R. N. Costilow, and E. S. Sharpe. 1978. Biology of Bacillus popilliae. Adv. Appl. Microbiol. 23: 1-18.

Dunbar, D. M., and R. L. Beard. 1975. Japanese and Oriental beetles in Connecticut. Conn. Agric. Exp. Stn. Bull. 757. 5 pp.

Dutky, S. R. 1940. Two new spore-forming bacteria causing milky diseases of the Japanese beetle larvae. J. Agric. Res. 61: 57-68.

_____. 1942. Method for the preparation of spore-dust mixtures of type A milky disease of Japanese beetle larvae for field inoculation. USDA, Bur. Entomol. Pl. Quar., ET-192, 15 pp.

_____. 1963. The milky diseases. pp. 75-115. In E. A. Steinhaus ed. Insect Pathology, an advanced treatise. Vol. 2. Academic Press, New York. 689 pp.

Faust, R. M. 1969. Pathogenic bacteria of pest insects as host-vector systems. Nat. Inst. Health Tech. Bull. 2: 1-8.

Fleming, W. E. 1968. Biological control of the Japanese beetle. USDA Tech. Bull. 1383. 78 pp.

Fleming, W. E. 1972. Biology of the Japanese beetle. USDA Tech. Bull. 1449. 129 pp.

Fleming, W. E., and I. M. Hawley. 1950. A large scale test with DDT to control the Japanese beetle. J. Econ. Entomol. 43: 586-90.

Franz, J. M. 1971. Influence of environment and modern trends of crop management on microbial control. pp. 407-44. In H. D. Burges and N. W. Hussey, eds., Microbial control of insects and mites. Academic Press, New York.

Gorick, B. D. 1980. Release and establishment of the baculovirus disease of Oryctes rhinoceros (L.) (Coleoptera: Scarabaeidae) in Papua, New Guinea. Bull. Entomol. Res. 70: 445-53.

Gould, G. E. 1963. Japanese beetle damage to soybeans and corn. J. Econ. Entomol. 56: 776-81.

Hall, I. M. 1974. Dispersion of pathogens. pp. 591-598. In F. G. Maxwell and F. A. Harris eds., Proceedings of the summer institute on biological control of plant insects and diseases. Univ. Press of Mississippi, Jackson.

Harper, J. D. [1978]. Introduction and colonization of entomopathogens. pp. 3-13. In G. E. Allen, C. M. Ignoffo, and R. P. Jacques eds., Microbial control of insect pests. NSF-USDA-Univ. Fla. Workshop, 10-12 July 1978.

Harshbarger, J. C., and R. M. Faust. 1973. Environmental factors internal to the host that affect the success of microbial insecticides. pp. 131-40. In L. A. Bulla ed., Regulation of insect populations by microorganisms. Ann. N. Y. Acad. Sci. 217.

Hawley, I. M. 1935. Horizontal movement of larvae of the Japanese beetle in field plots. J. Econ. Entomol. 28: 656.

_____. 1944. Notes on the biology of the Japanese beetle. U.S. Bur. Entomol. and Plant Quar. E-615. 19 pp.

Hawley, I. M., and G. F. White. 1935. Preliminary studies on the diseases of larvae of the Japanese beetle (Popillia japonica Newman). J. N. Y. Entomol. Soc. 43: 407-12.

Huger, A. M. 1966. A virus disease of the Indian rhinoceros beetle, Oryctes rhinoceros (L.) caused by a new type of insect virus, Rhabdionvirus oryctes gen. n. sp. n. J. Invertebr. Pathol. 8: 38-51.

Klein, M. G. 1981. Advances in the use of Bacillus popilliae for pest control. pp. 183-92. In H. D. Burges ed., Microbial control of pests and plant diseases 1970-1980. Academic Press, New York.

Klein, M. G., C. L. Johnson, and T. L. Ladd, Jr. 1976. A bibliography of the milky disease bacteria (Bacillus spp.) associated with the Japanese beetle, Popillia japonica, and closely related Scarabaeidae. Bull. Entomol. Soc. Amer. 22: 305-10.

Knipling, E. F. 1960. Use of insects for their own destruction. J. Econ. Entomol. 53: 415-20.

Langford, G. S., R. H. Vincent, and E. N. Cory. 1942. The adult Japanese beetle as host and disseminator of type A milky disease. J. Econ. Entomol. 35: 165-9.

Linng, A. J., and K. J. McMahon. 1969. Survival of lyophilized Bacillus popilliae in soil. Appl. Microbiol. 17: 718-20.

Millington, W. M. 1980. Population survey and bacterial control of the Japanese beetle in Happy Valley, Blount Co., Tennessee. Unpublished M. S. Thesis. Univ. Tennessee. 57 pp.

Milner, R. J. 1974. The in vivo production of Bacillus popilliae var. rhopaea spores. J. Invertebr. Pathol. 23: 289-96.

Polivka, J. B. 1949. The use of fluorescent pigments in a study of the flight of the Japanese beetle. J. Econ. Entomol. 42: 818-21.

Rivers, C. F. 1967. The natural and artificial dispersion of pathogens. pp. 252-63. In P. A. Van Der Laan ed., Insect pathology and microbial control. North Holland Pub. Co., Amsterdam. pp. 360.

Steinhaus, E. A. 1954. The effects of disease on insect populations. Hilgardia 23: 197-261.

Sharpe, E. S., and R. A. Rhodes. 1973. The pattern of sporulation of Bacillus popilliae in colonies. J. Invertebr. Pathol. 21: 9-15.

Smith, L. B., and C. H. Hadley. 1926. The Japanese beetle. USDA Circ. 363. 67 pp.

Soper, R. S. [1978]. Autodissemination of entomopathogens: fungi. pp. 63-5. In G. E. Allen, C. M. Ignoffo, and R. P. Jacques eds., Microbial control of insect pests. NSF-USDA-Univ. Fla. Workshop, 10-12 July 1978.

Tanada, Y. 1959. Microbial control of insect pests. Annu. Rev. Entomol. 4: 277-302.

U.S. Bureau of the Census. 1980. 1978 Census of Agriculture. Vol. 1 Part 42 Tennessee. p. 1.

U.S. Federal Register. 1982. Bacillus popilliae; proposed tolerance. Fed. Reg. 47 (130): 29575-6. July 7, 1982.

Van Der Laan, P. A. 1967. Insect pathology and microbial control. North-Holland Pub. Co., Amsterdam. 360 pp.

White, R. T. 1943. Effect of milky disease on tiphia parasites of Japanese beetle larvae. J. N. Y. Entomol. Soc. 51: 213-8.

White, R. T., and S. R. Dutky. 1940. The effect of the introduction of milky diseases on populations of Japanese beetle larvae. J. Econ. Entomol. 33: 306-9.

Williams, H. E. 1981. The Japanese beetle and its control. Univ. Tn. Agric. Serv. Pub. No. 946. 14 pp.

Zelazny, B. 1976. Transmission of a baculovirus in populations of Oryctes rhinoceros. J. Invertebr. Pathol. 27: 221-7.

_____. 1978. Methods of inoculating and diagnosing the baculovirus disease of Oryctes rhinoceros. Pl. Prot. Bull. F. A. O. 26: 163-8.

VITA

Donald Ray Wimmer was born in Spokane, Washington, on 15 December 1948. He attended Davenport High School, Davenport, Washington. In 1972, 1975, and 1979 he received the B. A. degree in general studies, the B. A. degree in anthropology, and the B. S. degree in entomology, respectively, from Washington State University, Pullman. He entered the Department of Entomology and Plant Pathology at The University of Tennessee, Knoxville, in the fall of 1980 where he served as a graduate research assistant. In December 1982 he received the M. S. degree with a major in entomology and plant pathology.

Donald Wimmer is a member of the Tennessee Entomological Society, the Entomological Society of America, the Entomological Society of Pennsylvania, the Florida Entomological Society, the Southeast Biological Control Working Group, and the agricultural honor society, Gamma Sigma Delta.