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Interactions between two gypsy moth (Lymantria dispar L.) pathogens, nuclear polyhedrosis virus and Entomophaga maimaiga (Entomophthorales: Zygomycetes).

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INTERACTIONS BETWEEN TWO GYPSY MOTH (LYMANTRIA DISPAR L.) PATHOGENS - NUCLEAR POLYHEDROSIS VIRUS AND ENTOMOPHAGA MAIMAIGA (ENTOMOPHTHORALES: ZYGOMYCETES)

A Dissertation Presented

by

RAKSHA D. MALAKAR

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DEDICATION

To my dear friends

Alun and Pushpa

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ABSTRACT

INTERACTIONS BETWEEN TWO GYPSY MOTH (LYMANTRIA DISPAR L.) PATHOGENS - NUCLEAR POLYHEDROSIS VIRUS AND ENTOMOPHAGA MAIMAIGA (ENTOMOPHTHORALES: ZYGOMYCETES)

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The gypsy moth, Lymantria dispar L., is one of the most damaging pests of the deciduous forests in the United States. It was accidentally introduced from Europe in 1868 by an amateur naturalist in eastern Massachusetts. High density gypsy moth populations are regulated primarily by a nuclear polyhedrosis virus (LdNPV). LdNPV is transmitted by feeding the LdNPV contaminated foliage or the contaminated egg chorion on the way out from the egg by a larva. In 1989, an entomophthoralean fungus, Entomophaga maimaiga Humber, Shimazu et Soper was discovered in the northeastern United States, which caused massive epizootic in both low and high density gypsy moth populations. My study focused on the interactions between E. maimaiga and LdNPV. Laboratory bioassays in which I inoculated gypsy moth larvae with LdNPV and E. maimaiga at the same time indicated that the majority of dually inoculated larvae die from E. maimaiga because of the shorter incubation period of E. maimaiga (5-7 days) compared to LdNPV (14 days) at 20°C. When the larvae were inoculated with E. maimaiga, 10 days after LdNPV inoculation, there was an apparent synergistic effect of E. maimaiga with LdNPV. Dually inoculated larvae died producing LdNPV propagules, 1-2 days earlier than the

larvae inoculated with LdNPV alone. Small-scale field experiments conducted in mesh-bags showed that artificial rainfall increases the E. maimaiga transmission. In a naturally occurring, moderate density gypsy moth population, I found that the LdNPV infection level was little affected by the presence of E. maimaiga. Host heterogeneity is suspected as one of the factors leading non-linear LdNPV transmissions. I showed that the host heterogeneity cannot explain the E. maimaiga epizootic observed in low density populations. I experimentally demonstrated this by comparing the E. maimaiga infection rates in feral (experienced the E. maimaiga/ LdNPV epizootic in their parental generations) and laboratory reared (with no epizootic experience) larvae. This is probably due to the short period to which the North American gypsy moths have been exposed to E. maimaiga, so these gypsy moths have not had chance to develop resistance against E. maimaiga.

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INTRODUCTION

Gypsy moth (Lymantria dispar L.) larvae are one of the major pests of the deciduous trees of the northern hemisphere (Elkinton and Liebhold 1990). Gypsy moth was accidentally introduced from Europe by an amateur naturalist, Leopold Trouvelot, in Medford, Massachusetts in 1868. Gypsy moths remain at innocuous level for several years and suddenly rise to high densities and defoliate many types of trees. Several parasitoids and small mammals regulate the low density gypsy moths, whereas a nuclear polyhedrosis virus (LdNPV) is the main factor regulating high density populations. In 1989 an obligate fungal pathogen of gypsy moth larvae, Entomophaga maimaiga Humber, Shimazu et Soper was discovered in the seven northeastern states of the United States (Andreadis and Weseloh 1990, Hajek et al. 1990a). Since then several fungal epizootics have been noted in both high and low density populations of gypsy moth.

LdNPV infection starts with the feeding of LdNPV-occlusion bodies-contaminated egg chorion or foliage by a gypsy moth larva (Doane 1970, Murray and Elkinton 1989). The occlusion body (OB) is environmentally resistant and consisted of several virions embedded in a crystalline protein matrix called a polyhedron or an occlusion. After ingestion by a gypsy moth larva, the protein matrix dissolves in the alkaline mid-gut lumen of the larva releasing the virions (Murphy et al. 1995). Each virion consists t of multiple helicals of nucleocapsids surrounded by an envelope. The virions, also known as the occlusion-derived virus (ODV) initiate the infection of the mid gut epithelial cells by fusing with the apical, microvillar membrane of the columnar cells (Granados and Lawer 1981, Horton and Burand 1993). Budded virus (BV) is the second phenotype of NPV, is produced in the mid gut cells. BV first infects the tracheole cells and from there the systemic infections spread to the other larval tissues (Engelhard et al. 1994). These tissues later produce both BV and

polyhedra (Volkman et al. 1996). The polyhedra are released into the environment, when these tissues undergo lysis liquefying the infected larva (Engelhard and Volkman 1995). It takes about 2 weeks to complete LdNPV infection cycle in the field. The first batch of the larval mortality observed among the first or second instar producing a large number of virus occlusion bodies. These occlusion bodies become a source of inoculum for the other susceptible larvae, which die as older instars (Woods and Elkinton 1987). In high density populations, the cadavers of the young instars become the source of the inoculum for the older instars, a second cycle of viral epizootic is observed.

The life cycle of E. maimaiga involves production of two types of spores, a resting spore or azygospore and asexual conidium (Hajek and Shimazu 1996) . The primary infection starts with a germ-conidium produced by an over-wintered, resting spore in the spring time. When a passing by larva contacts a germ conidium, it penetrates the larval integument. Once inside the larva, the fungus reproduces vegetatively, producing rod-shaped to amorphous protoplasts. Hyphae are produced at the later stage of infection and penetrate the vital organs of the larvae just before the death. Early instars (1st - 4th) succumb to E. maimaiga, which produce externally visible conidia, a short-lived, infective stage (Soper et al. 1988). Conidia are passively transmitted to other larvae by wind or when a passing susceptible larva comes into contact with a cadaver on which the fungus is sporulating or a substrate on which a spore has landed. A larva dies 4-7 days after contacting an E. maimaiga spore(s) depending upon the temperature and the larval stage (Hajek et al. 1993, Hajek and Shimazu 1996). Double-walled, sphere-shaped resting spores are produced in older instars. Resting spores need to over-winter before becoming infective in the spring time (Shimazu and Soper 1986) and experimentally they are shown to be infective even after six years, when they were left under soil cover (Weseloh and Andreadis 1997). The resting spores germinate throughout the period

when gypsy moth larvae are available (Hajek and Roberts 1991). E. maimaiga has become successful in establishing itself to the leading edges of the gypsy moth occurring areas in the Northeast and the mid-Atlantic region of the United states within 6 years after its first appearance (Hajek et al. 1996, Smitley et al. 1995).

The main focus of my study is on the effect of Entomophaga maimaiga on the gypsy moth nuclear polyhedrosis virus (LdNPV) transmission dynamics. Yerger and Rossiter (1996) speculated that the E. maimaiga could be a possible reason for the virtual absence of LdNPV-induced neonate mortality from the eggs collected from the sites where there were E. maimaiga epizootics in the previous years. The research described in this dissertation was conducted in attempt to gain an understanding of the effect of a gypsy moth fungal pathogen, E. maimaiga on gypsy moth nuclear polyhedrosis virus transmission.

The objectives of the study were to understand:

(1) the interactions between the pathogens within the host,

(2) the effect of pathogen density and rainfall on virus and fungus transmissions in small-scale field studies,

(3) to predict the virus- and fungus-induced mortalities in naturally occurring gypsy moth larvae using a host-pathogen model, and

(4) to determine if there were differences in variation in susceptibility of gypsy moth to the two pathogens and whether these account for the observed differences in density dependence in natural populations.

CHAPTER I

WITHIN-HOST INTERACTIONS OF LYMANTRIA DISPAR L. (LEPIDOPTERA: LYMANTRIIDAE) NUCLEAR POLYHEDROSIS VIRUS (LdNPV) AND ENTOMOPHAGA MAIMAIGA HUMBER, SHIMAZU *et* SOPER (ZYGOMYCETES: ENTOMOPHTHORALES)

Abstract

We studied the interaction of two gypsy moth (Lymantria dispar L.) pathogens, - a nuclear polyhedrosis virus (LdNPV) and a fungus (Entomophaga maimaiga), by assessing mortality among dually inoculated hosts. When fourth and fifth instar gypsy moths were inoculated with a range of dosages of LdNPV and a fixed dosage of E. maimaiga on the same day, the majority of larvae died from E. maimaiga infections regardless of the dosage of LdNPV. When the larvae were inoculated with E. maimaiga 10 days after LdNPV, there was an apparent increase in mortality of hosts induced by LdNPV. Among the fourth instars, the mortality due to LdNPV in the presence of E. maimaiga was significantly higher when insects were reared at 25°C than at 20°C. At 25°C, the lethal dose (LD_{50}) of LdNPV for fifth instars was 2-fold greater than that of fourth instars. For those larvae that died from LdNPV, the median survival time (ST_{50}) of dually inoculated fourth and fifth instars was ca. 1 day shorter than those inoculated with LdNPV alone. The number of LdNPV occlusion bodies produced in the cadavers of the dually inoculated larvae was lower than those inoculated with LdNPV alone.

Keywords: Dose response; Entomophaga maimaiga; Lymantria dispar; Gypsy moth; interactions; LD_{50}; Nuclear polyhedrosis virus; ST_{50}; temperature.

Introduction

Gypsy moth, Lymantria dispar L. (Lepidoptera: Lymantriidae) is one of the most damaging defoliators of deciduous forests of the northeastern United States. Gypsy moth outbreaks have been observed at intervals of approximately 8-10 years (Elkinton and Liebhold 1990). Gypsy moth nuclear polyhedrosis virus (LdNPV) is the major pathogen responsible for the decline of gypsy moth outbreaks (Campbell 1981, Doane 1970). LdNPV epizootics have been recorded in North America since the early 1900's (Glaser and Chapman 1913, Doane 1970). In nature, when a gypsy moth larva consumes LdNPV-contaminated foliage, it becomes infected and dies in ca. 2 weeks (Woods and Elkinton 1987) producing a large number of polyhedral occlusion bodies (OBs). These OBs become a source of inoculum for other susceptible larvae. The OB is environmentally resistant and consisted of several virions embedded in a crystalline protein matrix called a polyhedron or an occlusion. After ingestion by the gypsy moth larva, the protein matrix dissolves in the alkaline mid-gut lumen of the larva releasing the virions (Murphy et al. 1995). Each virion consists t of multiple helicals of nucleocapsids surrounded by an envelope. The virions, also known as the occlusion-derived virus (ODV) initiate the infection of the mid gut epithelial cells by fusing with the apical, microvillar membrane of the columnar cells (Granados and Lawer 1981, Horton and Burand 1993). Budded virus (BV) is the second phenotype of NPV, is produced in the mid gut cells. BV first infects the tracheole cells and from there the systemic infections spread to the other larval tissues (Engelhard et al. 1994). These tissues later produce both BV and polyhedra (Volkman et al. 1996). The polyhedra are released into the environment, when these tissues undergo lysis liquefying the infected larva (Engelhard and Volkman 1995).

The gypsy moth fungal pathogen, Entomophaga maimaiga Humber, Shimazu et Soper appeared unexpectedly in the U.S. in 1989 (Andreadis and Weseloh 1990, Hajek et al. 1990a, 1995). Since then E. maimaiga has decimated gypsy moths from both low and high density populations (Elkinton et al. 1991, Hajek et al. 1996, Weseloh and Andreadis 1992a). E. maimaiga is an obligate fungal pathogen of gypsy moth larvae. Over-wintering resting spores (azygospores) germinate in the spring and through out the summer (Hajek and Roberts 1991, Weseloh and Andreadis 1992b). When a larva comes in contact with the germinating spore, it penetrates the larval integument. Once inside the larva, the fungus reproduces vegetatively, producing rod-shaped to amorphous protoplasts and hyphal bodies (Balazy 1993). Early instars (1st - 4th) succumb to E. maimaiga, which produce externally visible conidia, a short-lived, infective stage (Soper et al. 1988). Conidia are passively transmitted to other larvae by wind or when a larva comes into contact with a cadaver on which the fungus is sporulating or a surface on which actively ejected conidia have landed (Hajek unpublished data). A larva dies 4-7 days after contacting an E. maimaiga spore(s) depending upon the temperature and the larval stage (Hajek et al. 1993). Double-walled, sphere-shaped resting spores are mostly produced in the cadavers of older instars and these resting spores need to over-winter before becoming infective (Shimazu and Soper 1986, Hajek and Shimazu 1996).

Simultaneous occurrence of both LdNPV and E. maimaiga in natural populations of gypsy moths have been reported (Elkinton et al. 1991, Hajek and Roberts 1992, Weseloh and Andreadis 1992a). When we collected a large number of naturally occurring gypsy moth larvae and reared them in an outdoor insectary, a small proportion of the larvae died from mixed infections of LdNPV and E. maimaiga (Malakar et al. in Prep.). Mixed infections due to an interaction between two pathogens are not uncommon among insects in nature and may result in independent coexistence of the pathogens in the host. Alternatively they each may complement

(synergize), or interfere with (antagonize) the development of the other (Tanada and Fuxa 1987). These kinds of interactions are known between microsporidia and viruses (Cossentine and Lewis 1988, Fuxa 1979); bacteria and nematodes (Bari and Kaya 1984, Koppenhofer and Kaya 1997); fungi and nematodes (Barbercheck and Kaya 1990, Timper and Brodie 1995); viruses and nematodes (Agra Gothama et al. 1995); viruses and fungi (Ferron and Hurpin 1974, Koyama and Katagiri 1967); and among viruses (Benz 1971, Ritter and Tanada 1978, Tanada 1959). The present study is the first report of interactions between a gypsy moth fungal pathogen and LdNPV at individual level of the hosts that illustrated the outcomes of the competition between these two pathogens, which in turn is an important factor governing the population dynamics of the gypsy moths.

Yerger and Rossiter (1996) reported that the gypsy moth larvae hatched from egg masses collected from Massachusetts in 1991 had very low mortality from LdNPV compared to the larvae from egg masses collected from other locations. They speculated that this difference might be due to the presence of E. maimaiga at these locations in the previous years. Smitley et al. (1995) and Weseloh and Andreadis (1992a) found a higher mortality of gypsy moths from E. maimaiga than from LdNPV in the field collected samples. Thus it seems likely that the interactions between these two pathogens may influence the prevalence and the transmission dynamics of both pathogens. We have conducted a series of laboratory and field studies since 1992 to determine the effect of E. maimaiga on LdNPV. In this paper, we present the outcomes of joint inoculations of LdNPV and E. maimaiga in gypsy moth larvae in the laboratory. The main purpose of the study was to determine whether the mortality induced by LdNPV would be affected by the presence of E. maimaiga inoculated at the same time or at a later stage of LdNPV infection and to determine whether the time to death of LdNPV infected insects would be affected in the presence of E. maimaiga.

Previous studies have shown that rearing temperature has a direct effect on the mortality of larvae inoculated with LdNPV or E. maimaiga. Gypsy moth larvae infected with LdNPV died sooner at 29°C than at lower rearing temperatures but LdNPV yields and virus activity were similar across all temperatures (Shapiro et al. 1981a). In contrast, the optimal temperature for E. maimaiga infection is 20°C (Shimazu and Soper 1986). To observe whether the rearing temperature would affect the results from sequential inoculations with LdNPV and E. maimaiga, we reared the inoculated larvae at 20°C, which is optimal for E. maimaiga growth, and at 25°C, which is optimal for LdNPV infection and gypsy moth are usually reared at this temperature (Shapiro et al. 1981a). As larval age has a great influence on the type of E. maimaiga spore formation (Hajek and Shimazu 1996), we used two larval stages, fourth and fifth instars, in the study.

Materials and Methods

Insects

Gypsy moth larvae used in the experiments were reared from egg masses of the New Jersey Standard laboratory strain (USDA, APHIS, Methods Development Center, Otis, MA). To disinfest them, egg masses were submerged in 5% formaldehyde solution for 1 hour and then rinsed under running tap water for 2 hours. Neonates were reared at 25±1°C on artificial medium (Bell et al. 1981) in groups of 15 per 180 ml diet cup until they started to molt to fourth or fifth instars. Same-aged (molted within 12 h), similar-sized larvae, held without diet for 12 h were used for inoculations. The similar-sized and same-aged larvae were chosen in order to reduce the variation in bioassay results (Burges and Thompson 1971).

Inoculation of LdNPV

The LdNPV used in this experiment was a plaque-purified, wild type, G2 clone of gypsy moth nuclear polyhedrosis virus, originally obtained from Dr. H. Alan Wood's Laboratory, Boyce Thompson Institute, Ithaca, NY. LdNPV OBs were stored at 4°C, prior to use. Six different concentrations of LdNPV OBs were suspended in blue colored food dye (FD & C Blue #1, Werner-Jenkinson Co., St. Louis, MO) and distilled water by serial dilution of stock solution of OBs. The blue dye was used as a visual cue to distinguish an inoculated diet cube from the uninoculated ones. The OB suspensions contained $1x10^3$, $1x10^4$, $1x10^5$, $1x10^6$, $1x10^7$ and $1x10^8$ OBs/ml The concentrations of OBs used here were based upon a preliminary dose-response study of LdNPV and include a range that killed 5-100% of gypsy moth larvae. For each larva a 1-mm^3 diet cube was cut from the freshly made artificial diet (Bell et al. 1981). The diet cube was placed in a 30 ml empty diet cup and 5 µl of one of the LdNPV suspensions were added. The suspensions were vortexed for one minute before inoculating the diet cubes. Individual larvae were placed in the cups immediately after the diet inoculation. Only those larvae which completely finished the diet cubes within 12 h were used for subsequent analyses. A negative control group of larvae was fed diet cubes inoculated with a mixture of blue dye and distilled water. The larvae were reared at 20°C until fungal inoculations.

Inoculation of E. maimaiga

E. maimaiga used in this study was originally collected by JSE and AEH from a field plot near Northampton, MA and was stored in liquid nitrogen in the form of protoplasts in the ARS Collection of Entomopathogenic Fungi (ARSEF) USDA, Ithaca, NY. Three days prior to the injections, a vial of protoplasts stored in liquid nitrogen was thawed at 37°C and then cultured on 95% Grace's insect culture medium

and 5% fetal bovine serum (Sigma Chemicals, St. Louis, MO) at 20°C and total darkness (Hajek et al. 1990b).

To deliver a precise concentration of inoculum, we injected protoplasts of E. maimaiga into the test insects. The concentration of protoplasts was fixed at $1x10^5$ protoplasts per ml of Grace's insect growth medium, on the basis of a preliminary dose-response test of E. maimaiga protoplasts. This concentration of protoplasts caused 90% mortality in gypsy moth larvae at 20°C. Five µl of $1x10^5$/ml protoplasts were injected per larva, as described by Hajek et al. (1990c) using a sterile 23 gauge microsyringe (Becton Dickinson & Co., NJ) fixed into a microinjector (Model - 4700 Superior, Instrumentation Specialties Co. Inc., Lincoln, NE). After injection, larvae were reared individually in 30 ml cups with artificial medium at 20°C or 25°C. The negative control groups were inoculated with only Grace's medium.

Simultaneous inoculations of LdNPV and E. maimaiga

Larvae inoculated with different concentrations of LdNPV, as described above were divided into two groups. One of the groups was inoculated with 5 µl of $1x10^5$ protoplasts/ml of E. maimaiga, right after the insects had finished their LdNPV-inoculated diet cubes. The other group of insects, inoculated with LdNPV alone, served as the LdNPV positive control group. Both groups of larvae were reared in the groups of 10 per 180 ml cup at 20°C on artificial medium. The mortality was recorded every day. Dead individuals were removed immediately to minimize transmission of either pathogen to other live individuals. The cause of death was determined by examining a drop of fluid extracted from a cadaver, under a compound microscope (Woods and Elkinton 1987).

Sequential inoculations of LdNPV and E. maimaiga

In this experiment, the larvae inoculated with different doses of LdNPV were reared at 20°C on an artificial medium in groups of ten for 10 days. These larvae were then divided into two groups. One of the groups was injected with E. maimaiga protoplasts at rate of 5 µl of 500 protoplasts/ml per larva as in the simultaneous inoculation experiment (Fig. 1) and the other group of LdNPV inoculated larvae was kept as the LdNPV positive control group. Both groups of larvae were then reared individually in 30 ml cups at 20 or 25°C on artificial medium. This experiment was repeated twice. Altogether, we used 2136 larvae in these experiments. The mortality was recorded and the cause of mortality was determined as described above.

LdNPV progeny production

To determine the number of LdNPV pathogen progeny produced, we weighed the cadavers (within 12 h after death of the larvae), ground them individually using a rounded tip glass rod and sonicated them. One mg of macerated tissue was suspended in 1 ml of distilled water. One hundred µl of the suspension were transferred to 900 µl of distilled water and the number of OBs and conidia were counted using a hemocytometer. The statistical difference between the virus progenies produced among larvae inoculated with both pathogens or with LdNPV alone was determined by two-sample t-tests. There were very few number of hyphae and resting spores in the cadavers of dually inoculated larvae. We were not sure whether those hyphae we saw on hemocytometer were pieces from one hypha or not. Therefore, we noted such cadavers having both LdNPV and E. maimaiga as died from both agents, but did not use the fungal propagule numbers for any analysis.

Data analysis

We pooled the data from the two experiments. The mortality score was based upon the visible evidence of the pathogens under microscopic examinations of the cadavers. Microscopic examinations of larval smears to confirm the presence of LdNPV have been a standard practice used in field studies (Hajek and Roberts 1992, Murray and Elkinton 1989, Woods and Elkinton 1987). We did not have any mortality in our negative control groups, however, we had some mortality due to unknown causes in LdNPV positive control groups . We adjusted for unknown mortalities using a modified Abbott's correction (1925):

$$d_V = \frac{N_{all} - N_{un}}{T_i - N_{un}} \qquad (1)$$

where d_V is the proportion of larvae that died from LdNPV, N_{all} is the total number of larvae that died from all causes, N_{un} is the number of larvae that died from unknown causes and T_i is the initial number of larvae.

Although we have no direct way of knowing which pathogen is the real cause of death of the dually inoculated insects, we assumed that all cadavers that contained large number of occlusion bodies, would have died from LdNPV, whether or not they also contained E. maimaiga spores. We used three ways of calculating mortality from LdNPV to account for those that were killed instead by E. maimaiga. Such mortality among the dually inoculated insects was calculated as (a) crude mortality (without Abbott's correction), (b) with Abbott's correction and (c) the marginal rates under the assumption of proportional hazards. Applying the standard Abbott's correction (1925), which assumes that E. maimaiga (or any other agent) kills the larvae first and LdNPV mortality is calculated as the proportion dying from LdNPV among those that survive E. maimaiga. As pointed out by Elkinton et al. (1992), this assumption may be unwarranted. Some of the dually infected larvae may die from E. maimaiga and

some from LdNPV. Elkinton et al. (1992) proposed an alternate calculation based on the assumption of proportional hazards which assumes that dually infected individuals will die from each mortality agent in proportion to the rates of infection by that agent. The formula of the mortality rate under the assumption of proportional hazards caused by LdNPV is:

$$m_V = 1 - (1 - d)^{\frac{d_v}{d}} \qquad (2)$$

where d is the observed death rate of gypsy moth larvae due to all mortality agents, d_V is the death rate due to LdNPV.

The LdNPV-induced mortality data were analyzed using a software PC-POLO (LeOra Software 1987). A logit model was fitted to the data. The slope of the resulting logit line is the inverse of the standard deviation of the tolerance distribution (Finney 1971). The mean of the tolerance distribution is the median lethal dose (LD_{50}). We compared LD_{50}s of LdNPV in both treatment groups - one with LdNPV alone and the other with both LdNPV and E. maimaiga. To determine whether the presence of E. maimaiga affected the mortality of gypsy moth due to LdNPV, we compared the slopes of the logit lines for the both treatment groups using a Z - test statistic (Kleinbaum and Kupper 1978). The median survival time (ST_{50}) due to LdNPV was determined using VISTAT (Hughes 1991), which is based upon the logit model of Bliss (1937).

Results

Simultaneous inoculation of LdNPV and E. maimaiga

The total mortality of the dually inoculated group was higher than the LdNPV alone inoculated group. Among the dually inoculated group of the insects, most of the

larvae that died contained E. maimaiga conidia or resting spores or both. The mortality due to E. maimaiga in dually inoculated larvae occurred at the same time as in those larvae inoculated with E. maimaiga alone (5-7 days in fourth instars and 6-9 days in fifth instars) (Fig. 1.1). Mortality due to LdNPV was observed 14 days after inoculation. There was some mortality due to mixed infections and we suspect that this is due to a secondary infection from the conidia produced by the insects which died earlier from E. maimaiga, because these larvae were reared in groups of 10 after inoculation (Fig. 1.1A). Although, we removed any cadavers we found daily, E. maimaiga spores from the cadavers could have probably infected other larvae.

Sequential inoculation of LdNPV and E. maimaiga

Unlike the simultaneously inoculated larvae, the sequentially inoculated larvae were reared individually after E. maimaiga inoculation so that no secondary infection was possible. The total mortality in the dually inoculated larvae in all dosages of LdNPV was higher than the total mortality in the corresponding LdNPV alone treated groups. The LD_{50} of LdNPV in the dually inoculated group was significantly lower than the LD_{50} of the insects inoculated with LdNPV alone (Table 1.1). At the lower doses of LdNPV, there was higher mortality from LdNPV among the dually inoculated groups than the larvae inoculated with LdNPV. However as the dose of LdNPV increased above the LD_{50} level, the LdNPV-induced mortality did not differ between two groups (Fig. 1.2). In all cases, except the fifth instars reared at 25°C, the standard deviation of the tolerance distribution is higher among the dually inoculated insects (Table 1.1) and the slopes of the logit lines are higher for the LdNPV inoculated insects than for dually inoculated groups (Table 1.2).

Slope tests showed a significant difference only between the LdNPV only and dually inoculated fourth instars reared at 20°C ($p = 0.001$), and fifth instars reared at

25°C (p=0.031). This suggests that LdNPV and E. maimaiga interaction is temperature and larval stage dependent. The median lethal dose (LD_{50}) of LdNPV was lower for both fourth and fifth instars at 20°C than at 25°C. Although we observed E. maimaiga within the cadavers of dually inoculated insects reared at 25°C, we did not find any fourth instars dying of E. maimaiga (positive control groups). Except for one insect, all of the fifth instars inoculated with E. maimaiga alone and reared at 25°C pupated and later all of them died producing resting spores in the abdominal intersegmental region of pupae.

Proportional hazard rates

The calculated marginal rates based on the assumption of proportional hazards of LdNPV showed a lower LD_{50} for LdNPV in the dually inoculated larvae than in the larvae inoculated with LdNPV alone (Table 1). There was a significant increase in LdNPV mortality among the sequential treatment group of fourth instars inoculated with low doses of LdNPV (< 500 OBs fed/larva).

Survival time

The median survival time (ST_{50}) of dually inoculated larvae with sequential inoculation, that died from LdNPV was significantly different from that of the larvae inoculated with LdNPV alone (Table 1.3). The ST_{50} of fourth instars (whether dually inoculated or not) was significantly shorter than that of fifth instars, when insects were reared at 20°C. Among the simultaneously dually inoculated groups, the majority of larvae died from E. maimaiga at similar times like the E. maimaiga positive control groups. Similarly, the time to death was similar in the LdNPV positive control group and the larvae that died and contained OBs in the dually inoculated group. In contrast, the sequentially inoculated groups had LdNPV-induced

mortality 1-2 days earlier than the mortalities in the LdNPV positive control groups (Table 1.3). At 25°C, we observed either no or very few deaths from E. maimaiga both in the positive control and the dually inoculated groups.

LdNPV progeny production

The OBs were counted from the cadavers of fifth instars only. We noted the presence or absence of the fungal propagules, because we were not sure whether the pieces of hyphae we observed were from a single hypha or not. The number of OBs produced per mg body weight of cadavers was higher in the positive control groups than in sequentially dually inoculated groups ($t = 2.56$, d.f. = 6, $p = 0.04$) (Table 1.4). This suggests that the presence of E. maimaiga lowers the LdNPV production in the cadavers of the larvae inoculated sequentially with LdNPV and E. maimaiga.

Discussion

Larvae inoculated with both LdNPV and E. maimaiga simultaneously, when died, showing a large number of visible E. maimaiga, they looked similar to the cadavers of larvae inoculated with E. maimaiga only. These cadavers had a whitish fungal mat on the body surface and when such cadaver was dissected and examined its tissue under a light microscope, a large number of hyphae and conidia or sometimes even the double-walled resting spores were observed. On the other hand, the larvae which were inoculated with LdNPV first and E. maimaiga 10 days later when died with large number of E. maimaiga propagules, they did not show any external evidences of fungal infection. However, when such cadaver's tissue was examined under a microscope, a large number of hyphae and conidia or resting spores were observed. Dually inoculated (simultaneously or sequentially) larvae which

exclusively had LdNPV occlusion bodies upon their death were similar to the larvae killed by LdNPV alone. The cadavers having both LdNPV and E. maimaiga propagules, externally did not look different than the LdNPV alone killed cadaver, however, the former contained both OBs and hyphae, but no conidia or the resting spores.

The higher total mortality among the insects inoculated with the both LdNPV and E. maimaiga indicates the additive effect of these pathogens. Such additive effect had been reported by Koppenhofer and Kaya (1997), when grubs were treated with Bacillus thuringiensis japonensis seven days before the nematodes Steinernema glaseri or Heterorhabditis bacteriophora. Similarly, when Melolontha melolontha grubs were treated with Beauveria bassiana in peat soil, one month after Entomopoxvirus melolonthae, Ferron and Hurpin (1974) observed a higher mortality among the grubs than when they were treated separately with the pathogens. These observations are compatible with our observations with LdNPV and E. maimaiga.

When larvae were inoculated with E. maimaiga and LdNPV on the same day, E. maimaiga alone was observed in the majority of cadavers. This is probably because of the shorter incubation time of E. maimaiga (5-7 days, Shimazu and Soper 1986) than that of LdNPV (ca. 2 weeks in natural populations, Woods and Elkinton 1987). Among sequentially inoculated fourth instars reared at 20°C or 25°C, the LD_{50} of LdNPV decreased significantly compared to the larvae inoculated with LdNPV alone (Table 1.1), suggesting that the presence of E. maimaiga, enhanced the insects' susceptibility to LdNPV. The same was true among the fifth instars when they were reared at 20°C. However at 25°C, the confidence intervals of LD_{50} of LdNPV for the two treatment groups overlap. These findings suggest that E. maimaiga may respond to fourth and fifth instars differently at different temperature, possibly due to changes in the larval sizes and their physiological conditions. Physiological differences among LdNPV-infected fourth and fifth instars of gypsy

moth have been reported by Park et al. (1993) and Burand et al. (1996). Orally baculovirus-fed lepidopteran larvae become increasingly resistant to the virus-infection as such larvae become older (Engelhard and Volkman 1995). Shimazu and Soper (1986) reported that a majority of older instars infected with E. maimaiga produce resting spores and younger instars produce conidia. Our observations are compatible with their findings.

The calculated proportional hazard rates of LdNPV showed that deaths of the larvae from the sequential experiments are similar to the proportion of larvae that died from LdNPV calculated using Abbott's correction (1925). The proportional hazards calculations are based upon the assumption that when two mortality agents are present at the same time, the outcome from their interactions depends upon which kills the larva first. Thus the evident lowering of LD_{50} of LdNPV with dual infections is not due to the assumptions regarding priority of cause of death embodied in Abbott's formula.

E. maimaiga development is temperature dependent and the optimal growth temperature is 20°C (Hajek et al. 1993, Shimazu and Soper 1986), whereas, 25°C is more optimal for the LdNPV replications (Shapiro et al. 1981a). We observed a very little mortality from E. maimaiga among the positive control group of fifth instars at 25°C. This is similar to the findings of Shimazu and Soper (1986). However, we observed sporulation and a significant number of mixed infections of E. maimaiga with LdNPV among the dually inoculated larvae at this temperature indicating that E. maimaiga could infect larvae at higher temperatures in the presence of LdNPV, or LdNPV enhanced E. maimaiga infections or vice versa.

The number of LdNPV OBs produced per mg of body weight of fifth instars cadavers of the LdNPV positive control group was similar to those numbers reported by Shapiro et al. (1981b). The number of LdNPV progeny produced per mg body weight of cadavers of dually inoculated larvae was lower than those of cadavers of

larvae inoculated with LdNPV alone (Table 1.4). It is possible that the dually inoculated larvae may have died before the LdNPV production reached the levels required to kill the larvae inoculated with LdNPV alone. We suspect that E. maimaiga might have interfered with the LdNPV OBs production. The presence of E. maimaiga decreased the survival time of the dually inoculated insects by 1-2 days (Table 1.3) compared to the insects inoculated with LdNPV alone.

All the mortality scores presented in this paper are based upon the visible evidence of the pathogens in the cadavers. It is not possible to determine which agent is the cause of death of the larva. We could have used molecular techniques like DNA hybridization (Keating et al. 1989) and ELISA (Hajek et al. 1991) to detect and quantify LdNPV and E. maimaiga, but these molecular techniques are no more accurate than the microscopic examinations of cadaver smears (Hajek et al. 1991, Keating et al. 1991).

In conclusion, most larvae simultaneously inoculated with both E. maimaiga and LdNPV will actually die from E. maimaiga just due to the shorter incubation time of E. maimaiga. On the other hand subsequent infection of LdNPV inoculated larvae by E. maimaiga appears to enhance the likelihood that such larvae will die containing a large number of LdNPV occlusion bodies in their body tissues. However, the numbers of such occlusion bodies produced in dually inoculated larvae are fewer than the numbers produced in the cadavers inoculated and killed by LdNPV alone.

Table 1.1. Mortality of gypsy moth larvae due to LdNPV among the fourth and fifth instars inoculated with LdNPV or LdNPV and 10 days later Entomophaga maimaiga, at 20°C and 25°C.

Instar/ Temperature	Treatment	n	LD_{50} (95% C.I.) OBs/larva w/ Abbott's correction	S.D. of LD_{50} (w/ Abbott's correction)	LD_{50} (95% C.I.) OBs/larva w/o Abbott's correction	LD_{50} based on Prop. hazard (95% C.I.)[1]
4th 20°C	LdNPV alone	212	856.17 (439.54-1660.03)	0.56		
	LdNPV+EM	261	10.60 (0.01-114.46)[2]	1.00	296.35 (121.15-634.67)	44.26 (8.35-132.43)
4th 25°C	LdNPV alone	75	1015.02 (382.99-2784.58)	0.58		
	LdNPV+EM	100	2.63 (0.05-9.183)[2]	0.68	2.65 (0.06-9.20)[2]	3.95 (0.11-12.94)
5th 20°C	LdNPV alone	280	991.88 (499.39-2107.86)	0.60		
	LdNPV+EM	359	71.74 (25.98-163.98)	0.61	2969.70 (1162.40-11067.00)	384.32 (167.47-860.10)

Contd...

Table 1.1 Contd...

5th 25°C	LdNPV alone	391	2792.50 (932.74-11603.26)	0.92		
	LdNPV+EM	458	513.02 (291.01-1085.73)	0.68	1081.34 (569.00-2390.39)	657.49 (365.91-1430.87)

LD_{50} = dose of inoculum required to kill 50% of the population, higher the LD_{50} value, higher the number of inoculum required to kill the larvae.
1 marginal rates based upon proportional hazards (eq. 9 of Elkinton et al. 1992)
2 90% confidence interval

Table 1.2. Comparison slopes of the LdNPV dose response curves of fourth and fifth instars inoculated with LdNPV alone or a sequential inoculations of LdNPV and E. maimaiga, at 20°C and 25°C.

Instar	Temp.	Pathogen	Slope	SE	p
4th	20°C	LdNPV only	1.78	0.26	
		LdNPV + EM	1.00	0.03	0.0014
	25°C	LdNPV only	1.72	0.37	
		LdNPV + EM	1.48	0.57	0.3632
5th	20°C	LdNPV only	1.66	0.21	
		LdNPV + EM	1.64	0.32	0.4801
	25°C	LdNPV only	1.09	0.12	
		LdNPV + EM	1.48	0.17	0.0307

Table 1.3. Comparison of mean survival time (ST_{50}) for deaths due to LdNPV of fourth and fifth instars inoculated with LdNPV alone or with E. maimaiga, at 20°C and 25°C.

Instar/ Temperature	Treatment[1]	ST_{50} (±S.E.) in days	Slope (±S.E.)
Simultaneous inoculation:			
4th	LdNPV alone	14.38(±0.32)	26.83(±7.74)
20°C	LdNPV+Em	14.49(±0.50)	39.49(±16.81)
5th	LdNPV alone	18.03(±0.44)	20.05(±4.93)
20°C	LdNPV+Em	17.39(±0.52)	20.85(±6.52)
Sequential inoculation:			
4th	LdNPV alone	15.01(±0.28)	38.78(±15.07)
20°C	LdNPV+Em	13.04(±0.30)	25.62(±7.69)
4th	LdNPV alone	13.28(±0.47)	15.79(±4.45)
25°C	LdNPV+Em	11.86(±0.15)	41.74(±12.33)
5th	LdNPV alone	19.38(±0.32)	36.35(±10.54)
20°C	LdNPV+Em	14.03(±0.59)	10.39(±2.26)
5th	LdNPV alone	9.61(±0.20)	15.72(±2.56)
25°C	LdNPV+Em[2]	11.39(±0.26)	13.63(±2.13)

1. Larvae were inoculated with 5,000 OB/larvae, i.e. closest to the LD_{50} value
2. Larvae inoculated with 500 OB/larva

Table 1.4 Comparison of the production of occlusion bodies produced from the cadavers of the fifth instars inoculated with LdNPV alone or inoculated with LdNPV and E. maimaiga 10 days later.

No. of LdNPV fed to 5th instars	*Mean ± SE of LdNPV OBs x 10^6/mg body wt. of the cadavers*	
	LdNPV only	LdNPV+E. maimaiga
$5x10^2$	3.20±0.35	1.80±0.26
$5x10^3$	3.25±0.38	1.97±0.21
$5x10^4$	3.03±0.44	3.04±0.59
$5x10^5$	3.17±0.31	2.74±0.31

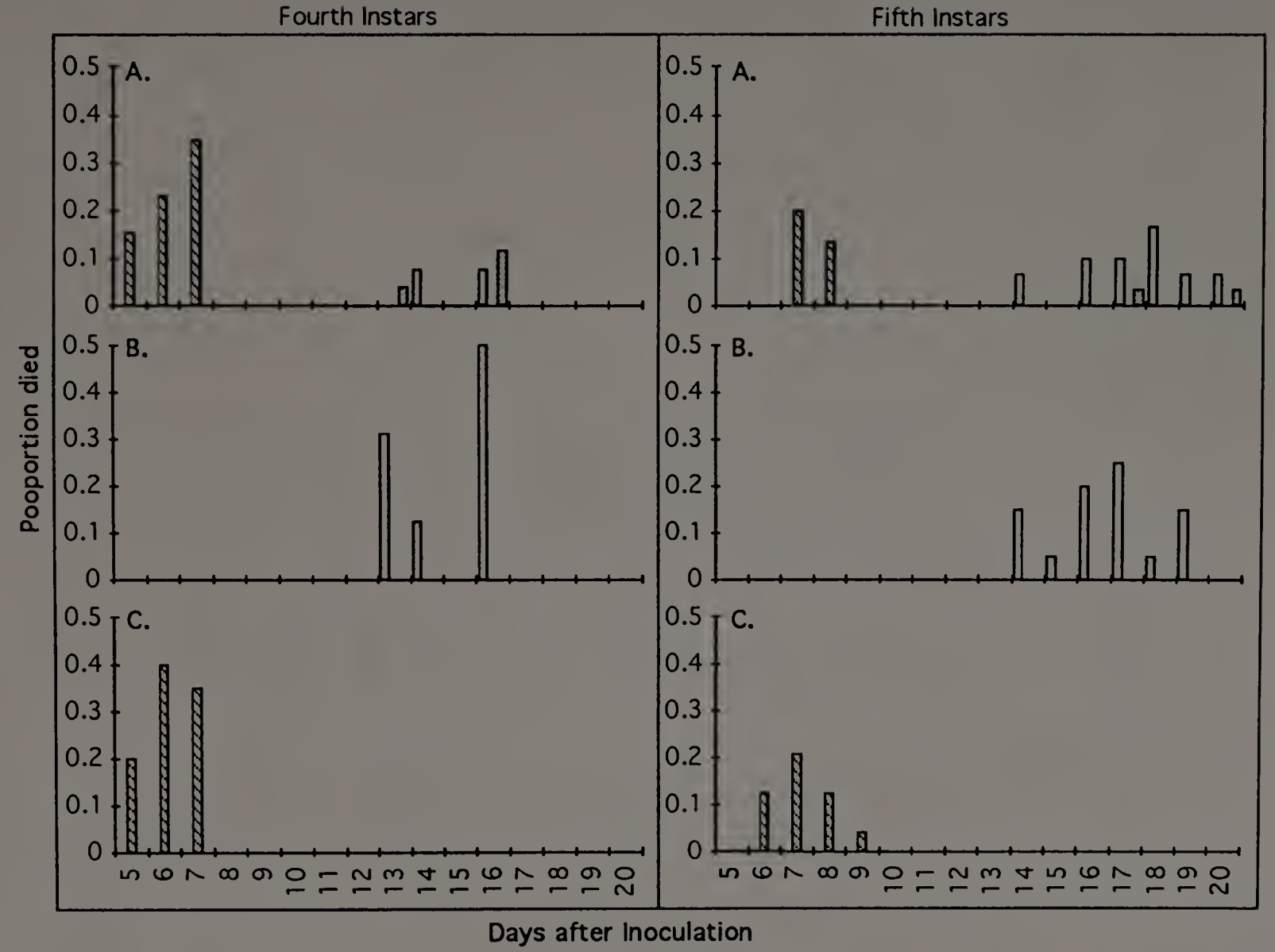

Fig 1.1 Proportion of gypsy moth fourth and fifth instars died producing the visible evidences of LdNPV, Entomophaga maimaiga, or both. The blank bars indicate the proportion of the larvae died showing the LdNPV only, the bars with hatch crossing are the larvae died producing the visible evidence of E. maimaiga and the bars with dots indicate the proportion of larvae died with both pathogens. (A) larvae inoculated with LdNPV and E. maimaiga simultaneously, (B) larvae inoculated with LdNPV only, and (C) larvae inoculated with E. maimaiga only.

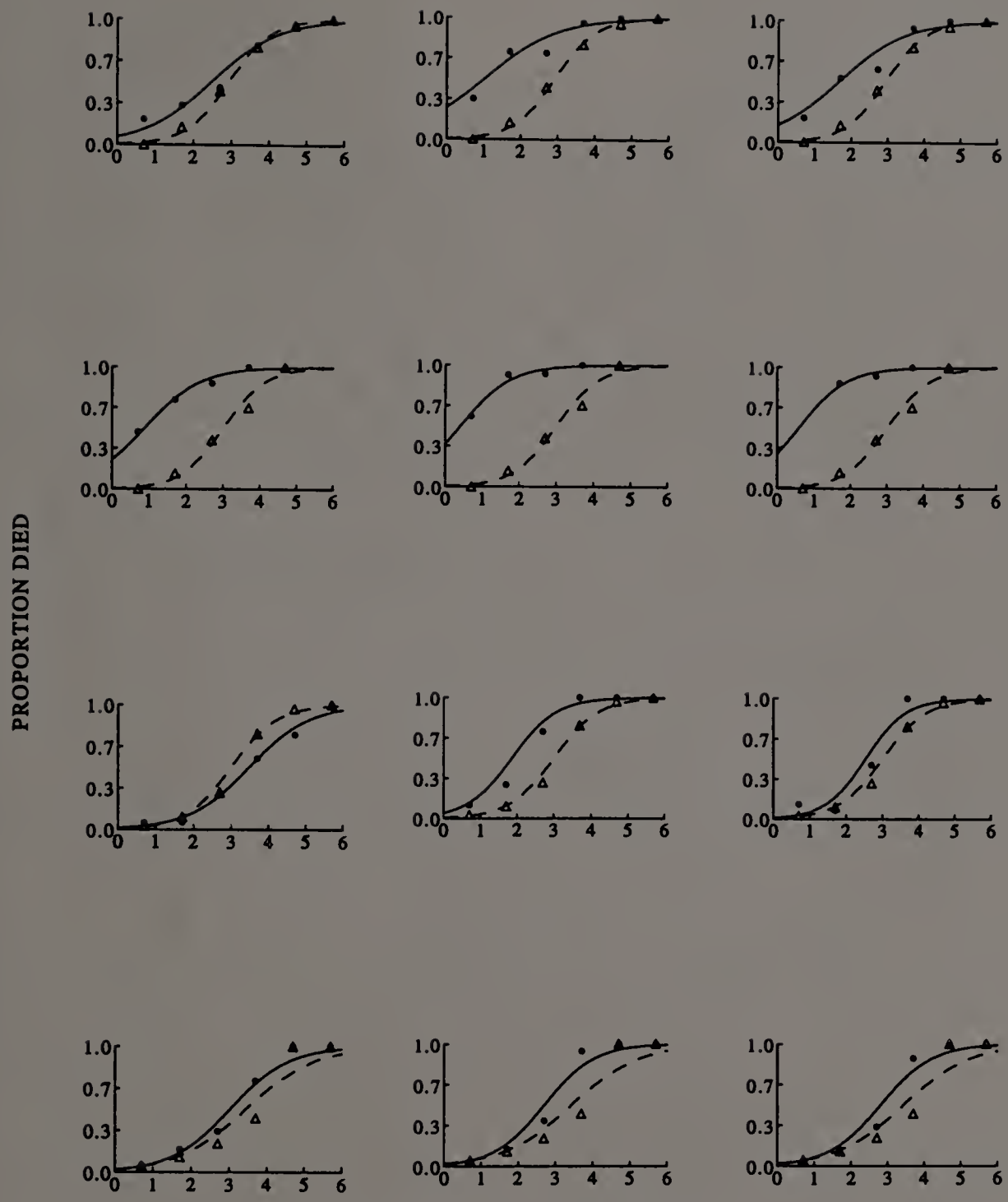

Fig. 1.2 LdNPV-induced mortality of fourth and fifth instars inoculated with LdNPV at day 1 and Entomophaga maimaiga on day 10 after their molting. The dark circles are the observed mortality due to LdNPV among the insects inoculated with both LdNPV and E. maimaiga, the open triangles are the LdNPV mortality in the larvae inoculated with LdNPV alone. The dark solid line is fitted logit curve for LdNPV mortality in dually inoculated group and the dashed line is for the LdNPV mortality in LdNPV only group. The first column indicates the crude observed mortality due to LdNPV without any corrections, the second columns with Abbott's corrections and the third column represents the mortality rate based on the assumptions of proportional hazards (Elkinton et al. 1992). The first and second rows are for fourth instars reared at 20°C and 25°C, third and fourth rows are for fifth instars at 20°C and 25°C respectively.

CHAPTER II

EFFECTS OF ENTOMOPHAGA MAIMAIGA (ZYGOMYCETES: ENTOMOPHTHORALES) ON TRANSMISSION OF NUCLEAR POLYHEDROSIS VIRUS OF GYPSY MOTH (LEPIDOPTERA: LYMANTRIIDAE) IN SMALL-SCALE FIELD EXPERIMENTS

Abstract

A study of interactions of two gypsy moth pathogens - the fungus, Entomophaga maimaiga and gypsy moth nuclear polyhedrosis virus (LdNPV) - was conducted to determine whether E. maimaiga infections would affect the level of LdNPV-infections. We enclosed fourth instar gypsy moths on oak foliage in mesh-bags with cadavers of the larvae infected with LdNPV and E. maimaiga in combination or separately. There was no consistent trends across treatments in the effect of E. maimaiga on the fraction dying or estimated fraction infected with LdNPV. The fraction of larvae that died and contained visible evidence of both pathogens was lower than the estimated fraction containing dual infections, because most of dually infected larvae will die from one of the pathogens without visible evidence of the other. In one experiment, simulated rainfall was applied to half the bags of each treatment and the other half was protected from rainfall. Simulated rainfall increased the mortality of gypsy moths induced by both pathogens, LdNPV and E. maimaiga, when they were placed separately. In another experiment, we varied the density of larvae infected with either pathogen, but this had no effect on pathogen transmission and subsequent mortality from either pathogen.

Keywords: density, Entomophaga maimaiga, fungus, gypsy moth, infection rate, interaction, LdNPV, proportional hazard, rainfall, virus.

Introduction

Gypsy moth (Lymantria dispar L.), is one of the most damaging forest defoliators in the northeastern United States. The population dynamics of gypsy moth are characterized by periods of high and low densities with a brief transition period in between (Campbell 1975). In North America, high density populations of gypsy moth usually collapse due to epizootics of a nuclear polyhedrosis virus (LdNPV) (Campbell 1963, 1976, Doane 1970). In 1989, a fungal pathogen, Entomophaga maimaiga Humber, Shimazu et Soper, was discovered in the northeastern United States, which caused an extensive gypsy moth larval mortality in the region (Andreadis and Weseloh 1990, Hajek et al. 1990a). This fungus is genetically indistinguishable from isolates of E. maimaiga from Japan (Hajek et al. 1990a). Recently, E. maimaiga has spread to the southern limit of the gypsy moth range in the northeastern U.S. (Hajek et al. 1996) and has been introduced to Michigan (Smitley et al. 1995). Gypsy moth larvae acquire LdNPV by consuming LdNPV contaminated foliage (Woods and Elkinton 1987), whereas E. maimaiga is acquired by contacting a sporulating cadaver, by conidia deposited on a surface or on the larva, or germ-conidia produced by over-wintered, resting spores (Shimazu and Soper 1986).

Rainfall affects the movement of LdNPV in the field (D'Amico and Elkinton 1995) and also the NPV of Douglas-fir tussock moth (Thompson 1978). Rainfall or abundant free water is necessary for the germination of resting spores of E. maimaiga (Hajek and Roberts 1991, Hajek et al. 1993, Weseloh and Andreadis 1992a). The greatest number of conidia are produced on cadavers on days with rainfall (Weseloh and Andreadis 1992a). Weseloh and Andreadis (1992b) found a significantly higher transmission of E. maimaiga among the caged third and fourth instar gypsy moth larvae in irrigated plots than in non-irrigated plots. Rainfall is known to influence the

infection rates of many fungal pathogens. For example, moderate amounts of rainfall in May and June were found to favor the spread of smut (Ustilago zeae) infection in corn (Coffman et al. 1926) and downy mildew epidemics of hops in Washington State were correlated with the rainfall in April and May (Johnson et al. 1994). Similarly, mycoses due to Pandora neoaphidis of cereal aphids Diuraphis noxia and Sitobion avenae increased with the frequent rainfall during late May and June (Feng et al. 1991).

Pathogen density in the environment is considered the principal factor affecting transmission (Podgwaite et al. 1979, Entwistle et al. 1983, Dwyer 1991) in host-pathogen models. These models typically assume that the transmission rate is proportional to the density of infected individuals (Anderson and May 1980). However, D'Amico et al. (1996) and Dwyer et al. (1997) have shown that the transmission rate of LdNPV is a non-linear function of pathogen density.

Yerger and Rossiter (1996) collected gypsy moth egg masses from various locations in the northeastern U.S. with or without previous exposure to E. maimaiga. They found almost no mortality from LdNPV among the neonates from the egg masses collected, where there were E. maimaiga epizootics in the previous years. They suggested that previous exposure to E. maimaiga might be the cause of this difference. Yerger and Rossiter's research suggests that the presence of E. maimaiga may interfere with the transmission of LdNPV. Resting spores of E. maimaiga germinate in synchrony with the hatching of the overwintering gypsy moth eggs, and the spores are available throughout the larval season (Hajek and Roberts 1991, Weseloh and Andreadis 1992b). The incubation time of E. maimaiga (4-5 days, Shimazu and Soper 1986) is shorter than that of LdNPV (1-2 weeks, Woods and Elkinton 1987). As a result, death from E. maimaiga infection may supersede death from LdNPV, when coinfection occur (Chapter I). Here, we present a study of an interaction between these two gypsy moth pathogens occurring contemporaneously in

small scale field experiments. We tested whether rainfall would affect the transmission of both LdNPV and E. maimaiga and also tested whether either pathogen has any antagonistic effect on the other by manipulating the density of both. We carried out these experiments in small mesh-bags in which we could manipulate the density of pathogen (in the form of infected larvae) and apply artificial rainfall easily.

Materials and Methods

Gypsy moth larvae

Gypsy moth egg masses from the standard New Jersey laboratory strain were obtained from USDA-APHIS, Methods Development Center, Otis Air Force Base, MA. Larvae were reared in the laboratory at 28°C on wheat germ diet (Bell et al. 1981) until they became fourth instars.

Inoculation with LdNPV

Newly molted fourth instars were inoculated with a plaque-purified G2 clone of LdNPV using a modified diet cube method (Boucias et al. 1980). Each larva was dosed with $5x10^4$ occlusion bodies (OBs), six days prior to placement in the field. Inoculated larvae were reared on wheat germ diet, at 28±1°C, to insure they would be dead by the time the healthy test larvae contacted them in the field.

Rearing and inoculation of E. maimaiga

Protoplasts of E. maimaiga isolate ARSEF 2779 (originally obtained from a gypsy moth population at Northampton, MA in 1989) were obtained from USDA, Agricultural Research Service Collection of Entomopathogenic Fungi, Ithaca, NY. These protoplasts were maintained in liquid nitrogen. Five days prior to injecting the

larvae, a 2.0 ml vial of protoplasts was thawed and a1.0 ml aliquot was transferred to a tissue culture tube with 9.0 ml of Grace's insect culture medium (SIGMA Chemical Co. St. Louis, MO). Three days later, 1 ml of the culture was transferred to another tissue culture flask with 9.5 ml of Grace's insect culture medium, and 0.5 ml of bovine's fetal serum (SIGMA Chemical Co. St. Louis, MO). The protoplast culture was maintained at 20°C under constant darkness.

Four days prior the deploying them in the field, a group of newly-molted fourth instars were injected intrahemoceolically with cultured protoplasts at a rate of $5x10^2$ protoplasts per larva (Hajek et al. 1990b), so that they would die in the field, producing infective conidia to inoculate the test larvae. We infected fourth instars rather than fifth and sixth instars to assure that the fungus would produce conidia rather than resting spores (Shimazu and Soper 1986).

Study sites

The study was conducted in July and August of 1993 at the edge of a woodlot in the University of Massachusetts, Amherst campus. We used red oak trees (Quercus rubra L.) for both experiments. The density experiment was conducted between July 2 and 9, 1993. The simulated rainfall experiment was conducted between August 9 and 16, 1993. Many previous experiments along these lines in our laboratory have shown that the time of the year makes little difference in LdNPV transmission in experiments like these (D'Amico et al. 1997).

Placement of infected and healthy larvae on foliage

On each tree, inoculated larvae were placed on a branch with approximately 40 leaves. Branches were enclosed in a finely woven polyester bag (Kleen Test Products, Brown Deer, WI). The previously-inoculated larvae were counted and

transferred to leaves and then 25 newly-molted, healthy, fourth instar test-larvae were added to the bag. The resulting density of test larvae per bag corresponds to 500 larvae per m^2 of leaf area, which lies within the range of density of insects observed in gypsy moth outbreaks (Campbell 1981). The mouth of each bag was secured by a cable tie and duct tape.

Simulated rainfall experiment

We exposed bags with inoculated and uninfected test larvae to artificial rain in the form of water from a garden hose to determine the impacts of rainfall and E. maimaiga on transmission of LdNPV. The treatment groups consisted of (a) 5 LdNPV inoculated larvae per bag, (b) 5 E. maimaiga inoculated larvae per bag, (c) 5 LdNPV and 5 E. maimaiga inoculated larvae per bag, and (d) control without any inoculated larvae. Each treatment was replicated 12 times. Six replicates from each treatment were then exposed to artificial rainfall and six replicates were not. A garden hose with a spray nozzle was used to create the simulated rain as described by D'Amico and Elkinton (1995). Six liters of water were sprayed approximately 3 m above each bag for 1 m for three times during a week. The bags were completely drenched with water. To protect the experimental foliage from natural rainfall, the mesh-bags were covered by plastic garbage bags if rain was imminent. The plastic bags were removed after the rain had stopped. There were three natural rainfalls during this experimental period.

Density experiment

We varied the density of inoculated larvae in the bags to determine the effect of density of pathogens on the transmission of disease to the healthy insects. The treatments consisted of (a) 5 (= low density) or (b) 20 (= high density) LdNPV

inoculated larvae per bag; (c) 5 or (d) 20 E. maimaiga inoculated larvae per bag; (e) 5 LdNPV and 5 E. maimaiga inoculated larvae per bag; (f) 20 LdNPV and 20 E. maimaiga inoculated larvae per bag and (g) control without any inoculated larvae. Each treatment was replicated five times.

In our density experiment, we did not protect our bags from natural rainfall and during the period when the bags were out in the field, we had three rainfall events (total rainfall = 1.08 cm, NOAA, 1993).

Calculation of mortality rates

Branches with the bags were removed after 7 d and brought to the laboratory. The 7 d period was selected because we wanted to collect test larvae before they died in the bags. We were able to separate the test larvae from the few larvae that survived pathogen inoculation in the laboratory, because any of the previously-inoculated larvae that did not die were in their late fifth instar stage, whereas test larvae were late fourth or early fifth instars. The numbers of dead and live larvae were counted. Live larvae were transferred to individual 30 ml plastic cups with wheat germ diet and reared at room temperature (25±1°C) until they died or pupated. Test larvae were checked daily to record the mortality. Dead larvae were autopsied and the cause of death was determined on the basis of visible presence of LdNPV or E. maimaiga propagules under a light microscope (400x magnification). In the few cases when cadavers contained both LdNPV OBs and E. maimaiga spores, we categorized half of them as having died from LdNPV and half died from E. maimaiga for the purpose of the statistical analysis.

Data analysis

For statistical analysis we used a two-way factorial ANOVA to test the differences between the treatments on the proportion of test insects that died from LdNPV or E. maimaiga (Statistix 4.0, Analytical Software 1992). For this analysis, the proportion of larvae that died from LdNPV or E. maimaiga was transformed to arcsine square root (Sokal and Rohlf 1981).

To further explore the effect of artificial rainfall on the two pathogen treatments, we compared the proportions of test larvae that died from LdNPV within rainfall treatments (a) LdNPV only: rainfall and (b) LdNPV with E. maimaiga: rainfall); and within no rainfall treatments (c) LdNPV only: no rainfall and (d) LdNPV with E. maimaiga: no rainfall, using the Mann-Whitney U test. The proportion of larvae that died from E. maimaiga was tested in the same way.

To further explore the effect of E. maimaiga on LdNPV, we compared the proportions of test larvae that died from LdNPV from low density treatments (a) LdNPV only: low density and (b) LdNPV with E. maimaiga: low density; and from high density treatments (C) LdNPV only: high density and (d) LdNPV with E. maimaiga: high density using a Mann Whitney U-test. The same test was performed to compare the proportion of larvae that died from E. maimaiga.

Estimation of joint infection

A small proportion of the cadavers of test larvae contained visible evidence of both LdNPV OBs and E. maimaiga hyphae or spores. We compared the observed proportion of jointly infected cadavers and the estimated infection rates or marginal rates (Royama 1981) from the joint infection of LdNPV and E. maimaiga. The estimated probability of being infected by both agents is a product of infection rates of LdNPV and E. maimaiga (Elkinton et al. 1992). We used three methods of calculating the infection rates (Elkinton et al. 1992). The first method assumes that

half the number of the individuals infected by both pathogens will die from LdNPV and other half from E. maimaiga. The infection rate for LdNPV is m_V and for E. maimaiga is m_F respectively:

$$m_V = \frac{b - \sqrt{b^2 - 4cd_V}}{2c} \qquad (1.a)$$

$$m_F = \frac{d_F}{(1 - cm_V)} \qquad (1.b)$$

where $b = [c(d_V+d_F) + 1-d_F]$, d_V and d_F are the observed fraction of larvae that died and contained visible evidences of LdNPV and E. maimaiga respectively, c = 0.5 and is the competition coefficient.

The second method assumes that the first agent to initiate the infection will be the cause of death of the host.

$$m_V = 1 - (1 - d_V - d_F)^{\frac{d_V}{d_V+d_F}} \qquad (2.a)$$

$$m_F = 1 - (1 - d_V - d_F)^{\frac{d_F}{d_V+d_F}} \qquad (2.b)$$

The third method assumes that a particular agent will always be the cause of death of the host (Elkinton et al. 1992: p. 37) and is identical with Abbott's correction (Abbott 1925). In calculating the infection rates from the third method, we assumed that E. maimaiga is always the cause of death of gypsy moth, because of its shorter incubation time, so the infection rate for E. maimaiga is equal to the observed death rate i.e., $m_F = d_F$, and the infection rates for LdNPV is given by $d_V/(1-m_F)$.

Calculation of transmission coefficients

The transmission coefficients are parameters used in host-pathogen models such as those developed by Anderson and May (1981). We calculated the transmission coefficient 'ν' for LdNPV or E. maimaiga using an equation derived by Dwyer and Elkinton (1993) and modified by D'Amico et al.(1996). Each bag contained 40 ±3 red oak leaves, which are equivalent to a leaf area of ca. 0.05 m^2 (Dwyer and Elkinton 1993). Therefore, the density of pathogens, either LdNPV OBs or E. maimaiga conidia (produced by the cadavers of the insects inoculated with pathogens in the laboratory) is expressed in terms of number of pathogens per total leaf area in the bag.

$$\nu = -\frac{\mu}{(1-e^{-\mu t})P_0}\ln(\frac{S_t}{S_0}) \qquad (1)$$

where S_0 = initial density of uninfected test larvae

S_t = number of test larvae died from LdNPV or E. maimaiga by the end of the experiment

P_0 = number of polyhedral inclusion bodies or conidia per 0.05 m^2

μ = decay rate of OBs (0.15 OB/day, D'Amico et al. 1996) or conidia (one spore per day Hajek et al. 1993)

t = Number of days when the uninfected test larvae were with infected cadavers in the bag. In our experiment t = 7.

We took the average proportion infected from the bags for each treatment to calculated the ν for each treatment (see D'Amico et al. 1996). The difference between two transmission coefficients was estimated by test statistic - Z = D/SE(D),

where D is the difference between the means of two transmission coefficients, SE(D) is the standard error attached to D (Buonaccorsi and Elkinton 1990);

$$SE(D) = \sqrt{SE(v_A)^2 + SE(v_B)^2 - 2\$} \qquad (4)$$

Here $\$$ is the estimated covariance between v_A and v_B, the transmission rate of two pathogens, A and B (in our case LdNPV and E. maimaiga).

Results and Discussion

Effect of each pathogen on the mortality of the other

There was no consistent pattern across treatments in the effect of E. maimaiga on LdNPV-induced gypsy moth mortality. The total mortality from both pathogens was higher than mortality from LdNPV alone in two out of four treatments (Table 2.1 a and b). In all four treatments, mortality from E. maimaiga was lower when the two pathogens occurred together than when E. maimaiga occurred alone. When two or more sources of mortality compete for the same life stage of a host, deaths from one agent will usually reduce the fraction dying from the other agent (Royama 1981, Buonaccorsi and Elkinton 1990, Elkinton et al. 1992). There was no consistent effect on the fraction infected with either pathogen due to the presence of the other. In the absence of synergistic or antagonistic interactions, one would expect the estimated fraction infected in the mesh-bags with both pathogens to equal the fraction that died in the bags with only one pathogen for each of the two pathogens.

The estimated joint-infection rates were higher than the observed proportion of larvae that died and contained the visible evidence of both pathogens (Fig. 2.1). This was observed from all three methods we used for calculating the joint infection

rates. Therefore, it is likely there are more joint-infections in nature than those what we observe directly by dissecting or rearing the larvae that we bring from the field.

Rainfall experiment

The rainfall significantly increased the rate of transmission of E. maimaiga (Z = 2.17, p = 0.02). The transmission rate of LdNPV was also higher in rain treated groups than without rain (Table 2.1). The results from the factorial two-way ANOVA showed that there was a positive effect of rainfall on E. maimaiga-induced mortality (F = 8.01; df = 1, 15; P = 0.01; Table 2.3). For LdNPV there was a marginally significant interaction between the rainfall and the presence or absence of E. maimaiga (F = 3.92; df = 1, 15; P = 0.07) due to the higher LdNPV mortality in LdNPV only treatment(with rainfall) than in LdNPV with E. maimaiga treatment applied with simulated rainfall.

Density experiment

The results of two-way factorial ANOVA (Table 2.4) indicate that there is no significant effect of density of either pathogen and that mortality from LdNPV was not affected by the presence of E. maimaiga and vice versa. As we did not observe interactions between the pathogen density and the presence or absence of E. maimaiga, we tested the main effects using the Mann-Whitney U test. There was a marginally significant increase in mortality of gypsy moths due to E. maimaiga at high density of E. maimaiga (P = 0.075). We did not find a density effect at the two densities tested in this study on the proportion of test larvae that died from LdNPV.

In subsequent mesh-bag experiments covering a wider range of densities (Chapter IV), we have shown that mortality increases with density of both pathogens. Our failure to observe a density effect in the results presented here may be due to a

non-linear rate of transmission of LdNPV (D'Amico et al. 1996, Dwyer 1997) so that the pathogen transmission did not increase with the increase in the pathogen density in a linear fashion. In the case of E. maimaiga, there was a high variability among the mortality observed in the bags (% mortality ± SE = 26 ± 19) with the 5 infected cadavers/bag treatment.

Transmission coefficients

Our calculated transmission coefficients for LdNPV at both low and high pathogen densities (mean $\nu = 2.59 \times 10^{-12}$ m^2/day) are similar to those of D'Amico et al. (1996) (mean $\nu = 9.82 \times 10^{-12}$ m^2/day) and Dwyer and Elkinton (1993) (mean $\nu = 1.45 \times 10^{-12}$ m^2/day) under similar conditions, except they used first instars as LdNPV inoculum and third or a mixture of third and fourth instars as the healthy test insects. The proportion of larvae secondarily infected and dying from E. maimaiga with mean transmission coefficient $\nu = 1.37 \times 10^{-8}$ m^2/day (Table 2.2) was higher in our case than reported by Hajek et al. (1993) (in their case, the maximum level of transmission was 16.7%). It may be due to the higher probability of encountering the fungus-killed cadavers by the test larvae in our small mesh bags than in large cages used by Hajek et al. (1993).

The non-linearity of transmission is illustrated by the substantial difference in transmission coefficients between the two LdNPV killed cadaver densities tested. At higher host density the transmission coefficient is smaller for LdNPV, confirming earlier findings (D'Amico et al. 1996, Dwyer et al. 1997). Here we show the same trend for E. maimaiga. Knell et al. (1996) experimentally demonstrated that the transmission coefficient of Bacillus thuringiensis decreased when pathogen density increased. It appears that non-linear pathogen transmission is a typical phenomenon in insect-pathogen systems.

In conclusion, there is little evidence that either pathogen had a significant impact on mortality from the other. Artificial rainfall significantly increased the fungus-induced mortality. The estimated dual-infections from both pathogens was higher than the observed proportion of larvae dying with joint infections. Therefore the observed dual-infection rate is not a good way to estimate the dual-infections, when there are more than one mortality agents present.

Table 2.1. Simulated rainfall experiment: proportion died and estimated infection rates (m_V or m_F) of gypsy moth larvae from nuclear polyhedrosis virus (LdNPV) and E. maimaiga (EM) and the transmission coefficients of LdNPV and E. maimaiga when they occurred separately.

Treatment/ Cause of mortality	Prop. died (Mean±S.E.)	Estimated[a] m_{V1} or m_{F1} (Mean±S.E.)	Estimated[b] m_{V2} or m_{F2} (Mean±S.E.)	Estimated[c] m_{V3} or m_{F3} (Mean±S.E.)	Transmission coefficient m^2 per day
Rainfall:					
1) LdNPV alone	0.80±0.03				$1.81x10^{-12}$
2) EM alone	0.31±0.09				$1.18x10^{-08}$
3) LdNPV + EM (total)	0.78				
3.a) LdNPV	0.64±0.05	0.73±0.08	0.73±0.07	0.76±0.08	
3.b) EM	0.14±0.05	0.24±0.08	0.38±0.15	0.14±0.05	
No rainfall:					
1) LdNPV alone	0.57±0.10				$9.81x10^{-13}$
2) EM alone	0.06±0.03				$2.96x10^{-09}$
3) LdNPV + EM (total)	0.76				
3.a) LdNPV	0.72±0.07	0.75±0.08	0.75±0.08	0.76±0.08	
3.b) EM	0.04±0.01	0.07±0.02	0.09±0.03	0.04±0.01	

1 = When there is only one pathogen present, the proportion died = the estimated infection rate.
a = m_V (LdNPV infection rate) and m_F (EM infection rate) calculated using eqs. 1a and 1b
b = m_V and m_F calculated using the eqs. 2a and 2b
c = m_V and m_F calculated using the Abbott's correction

Table 2.2. Pathogen density experiment: proportion died and estimated infection rates (m_V or m_F) of gypsy moth larvae from nuclear polyhedrosis virus (LdNPV) and E. maimaiga (EM) and the transmission coefficients of LdNPV and E. maimaiga when they occurred separately.

Treatment/ Cause of mortality	Prop. died (Mean±S.E.)	Estimated[a] m_{V1} or m_{F1} (Mean±S.E.)	Estimated[b] m_{V2} or m_{F2} (Mean±S.E.)	Estimated[c] m_{V3} or m_{F3} (Mean±S.E.)	Transmission coefficient m^2 per day
Low pathogen density:					
1) LdNPV alone	0.83±0.08[1]				2.08×10^{-12}
2) EM alone	0.26±0.19[1]				1.74×10^{-08}
3) LdNPV + EM	0.77				
3.a) LdNPV	0.62±0.15	0.67±0.15	0.67±0.15	0.69±0.14	
3.b) EM	0.15±0.05	0.20±0.06	0.23±0.06	0.15±0.05	
High pathogen density:					
1) LdNPV alone	0.84±0.05[1]				5.14×10^{-13}
2) EM alone	0.54±0.09[1]				1.01×10^{-08}
3) LdNPV + EM	0.96				
3.a) LdNPV	0.76±0.04	0.95±0.03	0.94±0.04	0.95±0.03	
3.b) EM	0.20±0.05	0.38±0.10	0.71±0.18	0.20±0.05	

1 = When there is only one pathogen present, the proportion died = the estimated infection rate.
a = m_V (LdNPV infection rate) and m_F (EM infection rate) calculated using eqs. 1a and 1b
b = m_V and m_F calculated using the eqs. 2a and 2b
c = m_V and m_F calculated using the Abbott's correction

Table 2.3. Two-way ANOVA of arcsine square root transformed proportions of fourth instar gypsy moths that died from LdNPV and E. maimaiga, when exposed to artificial rainfall

Source	LdNPV			E. maimaiga		
	df	F	P	df	F	P
Bag (A)	5	0.11	0.99	5	2.05	0.13
Rain vs. No rain (B)	1	0.62	0.44	1	8.01	0.01
Pathogen type (C)	1	0.00	0.97	1	2.62	0.12
Interaction B x C	1	3.92	0.07	1	1.30	0.27

Note: Pathogen type = LdNPV only X LdNPV and E. maimaiga

Table 2.4. Two-way ANOVA of arcsine square root transformed proportions of fourth instar gypsy moths died from LdNPV and E. maimaiga when exposed to low (5) and high (20) densities of the cadavers of the larvae infected with LdNPV and E. maimaiga

Source	LdNPV			E. maimaiga		
	df	F	P	df	F	P
Bag (A)	4	0.62	0.66	4	1.70	0.22
Density low vs. high (B)	1	0.18	0.68	1	2.05	0.18
Pathogen type (C)	1	2.95	0.11	1	3.08	0.10
Interaction B x C	1	0.63	0.44	1	0.75	0.40

Note: Pathogen type = LdNPV only X LdNPV and E. maimaiga

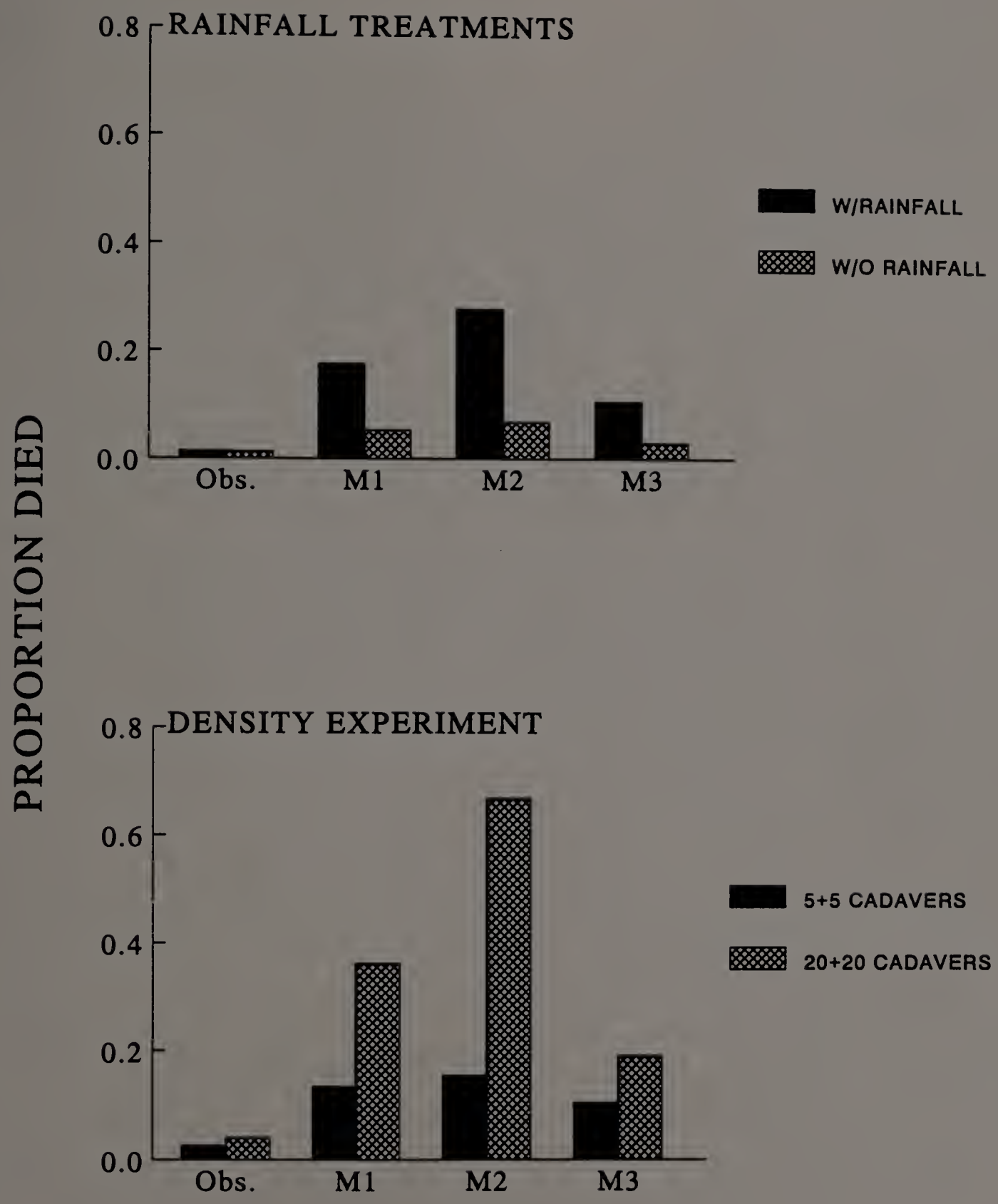

Fig. 2.1 Observed and estimated joint infections in rainfall and density treatment groups. In rainfall treatments, we applied artificial rainfall on half of the treatment bags and in density treatments, we had 5 LdNPV infected and 5 E. maimaiga infected or 20 LdNPV infected and 20 E. maimaiga infected larvae as inoculum for 25 healthy test larvae. M1, M2, and M3 are the estimated joint infections based on the three methods of infection rate calculations (Elkinton et al. 1992).

CHAPTER III

INTERACTIONS BETWEEN TWO GYPSY MOTH (LEPIDOPTERA: LYMANTRIIDAE) PATHOGENS: NUCLEAR POLYHEDROSIS VIRUS AND ENTOMOPHAGA MAIMAIGA HUMBER, SHIMAZU ET SOPER (ZYGOMYCETES: ENTOMOPHTHORALES) IN THE FIELD

Abstract

The sudden appearance of a gypsy-moth-fungal pathogen, Entomophaga maimaiga Humber, Shimazu et Soper, in the natural gypsy moth (Lymantria dispar L.) populations in the United States, raised a question that whether it will have any effect on the naturally occurring gypsy moth nuclear polyhedrosis virus (LdNPV). To determine the impacts of E. maimaiga on LdNPV-induced larval mortality, gypsy moth larvae were collected from seven 0.04 ha plots in 1992 and four 0.04 ha plots in 1994. Two of the plots in 1994, supplemented with artificial rain had a higher E. maimaiga-induced gypsy moth mortality (seasonal cumulative mortality = 80%) than in the non-irrigated plots (66%). However, the levels of LdNPV mortality were similar in both irrigated and non-irrigated plots (seasonal cumulative mortality 34% and 30% respectively). To elucidate the impact of E. maimaiga on LdNPV-induced mortality, we developed a host-pathogen model and fitted our observed data to it. The model predicted that at a moderate densities of gypsy moths, as in our plots, the mortality induced by LdNPV would not be very different in the presence of E. maimaiga than when it is absent. This occurred because gypsy moth mortality from E. maimaiga reaches high levels only when the older instars are present.

Keywords: Entomophaga maimaiga, LdNPV, gypsy moth, epizootic, interactions, model.

Introduction

Gypsy moth, Lymantria dispar (L.) is the most damaging defoliator of deciduous forests in the northeastern United States. Gypsy moth populations typically remain at low densities for several years due to parasitoids and small mammal predation (Campbell et al. 1977, Gould et al. 1990, Elkinton et al. 1996) and suddenly increases to outbreak levels and causes extensive defoliation (Campbell 1981, Elkinton and Liebhold 1990). Epidemic populations usually collapse from naturally occurring epizootics of the gypsy-moth nuclear polyhedrosis virus (LdNPV) and high mortalities from LdNPV sometimes persist in the year following such population collapses (Doane 1969, 1970). LdNPV infection starts when larvae emerge from egg masses laid on surfaces contaminated with LdNPV (Murray and Elkinton 1990). LdNPV has several virus particles (or virions) occluded inside a polyhedral-shaped protein-coat and each virion contains multiple nucleocapsids (Harrap 1972) and it is called an occlusion body (OB). It replicates in the host cell nucleus and releases viral progenies rupturing the host cell membrane. These viral progenies in turn invade other cells and tissues. In ca. two weeks, the infected larva dies, releasing millions of LdNPV occlusion bodies (OBs) into the environment and thus becomes a source of inoculum for other healthy larvae (Woods and Elkinton 1987).

Entomophaga maimaiga, Humber, Shimazu et Soper, a fungal pathogen of gypsy moth, decimated gypsy moth populations throughout New England for the first time in 1989 (Andreadis and Weseloh 1990, Hajek et al. 1990a). This fungal pathogen was introduced from Japan in 1910, to an area near Boston, MA, but it was never recovered from gypsy moth in North America prior to 1989. The reasons for its sudden reappearance in 1989 are unknown (Andreadis and Weseloh 1990, Hajek et al. 1995). Overwintering E. maimaiga resting spores start to germinate in April

(Weseloh and Andreadis 1992a). If a gypsy moth larva comes in contact with a germinating spore, that spore penetrates the larval integument and starts protoplast production in the insect hemolymph. Infected larvae die within 7 days (Shimazu and Soper 1986). Hyphae and conidia or resting spores are produced in cadavers. Conidia are short-lived infectious stages and are mostly produced on younger instars. Resting spores are generally produced in older instars, and these spores need to overwinter before they become infective (Shimazu and Soper 1986, Hajek et al. 1993). E. maimaiga was observed in both high and low density gypsy moth populations in 1989 and during subsequent years throughout Massachusetts (Hajek et al. 1990a, Elkinton et al. 1991). We have observed several high density gypsy moth populations which experienced E. maimaiga epizootics, but nevertheless rebounded to high density the following year (JSE's personal observations). Yerger and Rossiter (1996) reported that gypsy moth larvae hatched from eggs collected from several high density populations in Massachusetts had very low levels of LdNPV infections compared to the larvae collected in other locations. They speculated that the presence of E. maimaiga in these locations might have caused this difference. These observations suggest that E. maimaiga may, in some manner, suppress or interfere with LdNPV mortality and thus allow the gypsy moth populations to rebound. Both agents co-occur in field populations (Andreadis and Weseloh 1990, Hajek and Roberts 1992, Weseloh and Andreadis 1992a), but we know very little about the manner in which the two pathogens interact.

To explore the possible interactions between LdNPV and E. maimaiga in naturally occurring gypsy moth populations, we measured the levels of mortality caused by both pathogens in 1992 and 1994. We attempted to manipulate experimentally the level of E. maimaiga infection by applying artificial rainfall on two of the four experimental plots in 1994. We developed a host - pathogen model to simulate the mortality caused by LdNPV in the presence and absence of E. maimaiga.

This host-pathogen model is an extension of the host-pathogen model of Dwyer and Elkinton (1993), developed for LdNPV epizootics.

Materials and Methods

Experimental plots and estimation of initial densities of insects and virus inocula

We established seven 20 m x 20 m plots in 1992 and four plots in 1994 in Holyoke Range State Forest in Amherst, MA. Plots were separated by at least 200 m. Red oak (Quercus rubra) and chestnut oak (Q. prinus) dominate the forest canopy and witch hazel (Hamamelis virginiana) dominates the understory. Gypsy moth larvae defoliated most of the trees on these sites each year from 1990 to 1993 (J.S.E., personal observations). We estimated the density of egg masses in each plot by conducting a complete census of all egg masses on the ground, understory vegetation and trees within the plots in mid-April, prior to egg hatch.

To estimate the percent of egg hatch and virus infection among the neonates (Table 3.1), we collected 10 egg masses from the vicinity of each plot. We removed egg masses from tree boles with a sterilized knife and transferred them individually into 60 ml empty diet cups, with a piece of wet dental wick. The larvae that hatched from each egg mass were counted and reared in groups of 15 on artificial diet (Bell et al. 1981) in 180 ml cups. They were held for two weeks at room temperature and monitored for mortality every other day. We examined tissues of each cadaver at 100-400x under a compound microscope to determine the cause of death (Hajek and Roberts 1992). We estimated the number of larvae per m^2 of ground area in each plot by multiplying the total number of egg masses in a plot by the average number of larvae that hatched from egg masses and dividing by the area of the plot.

Measuring disease mortality in the field

To estimate the mortality due to diseases of gypsy moth larvae, we collected about 50 larvae per plot each week from 25 May to 7 July in 1992. Similarly, in 1994, we collected ca. 100 larvae from each plot per week, starting 17 May, when most larvae were first instars. We continued the collections until 8 July, when most of the survivors had pupated. Early instars were collected from understory foliage and later instars were collected from burlap bands wrapped around the tree trunks (McManus and Smith 1984, Murray and Elkinton 1992). Larvae were collected individually into 60 ml diet cups and reared for a week in an outdoor insectary. We checked mortality on alternate days and autopsied dead larvae to determine the cause of death. We tabulated the fraction of gypsy moth larvae that died within one week of collection and contained visible LdNPV or E. maimaiga, or both.

Effect of rainfall on mortality of gypsy moths due to E. maimaiga

In 1994, we attempted to manipulate the levels of E. maimaiga infection by applying artificial rain to two of the four plots. Each plot was divided into four 10 m x 10 m subplots. The artificial rain was applied for 30 min in each subplot with a hose affixed to a rotary garden sprinkler so that all the understory vegetation in the plot was completely soaked. We calculated that this was equivalent to 2.1 mm of rainfall twice a week. We knew from previous research (Weseloh and Andreadis 1992a) that such applications might have little detectable impact on E. maimaiga, but we could think of no better way to manipulate E. maimaiga under field conditions.

The model

We modified Dwyer and Elkinton's (1993) LdNPV epizootic model to incorporate the behavior of LdNPV dynamics in the presence of E. maimaiga. This model is essentially a "within-generation" version of the model of Anderson and May

(1981) which additionally incorporates a time delay between infection and death of gypsy moth larvae from each pathogen. The rate of change of susceptible (uninfected) host density due to the LdNPV and E. maimaiga is given by :

$$\frac{dS}{dt} = -(\nu_F F + \nu_V V)S \qquad (1)$$

where S is the density of susceptible gypsy moth larvae, F is the density of E. maimaiga inoculum, V is the density of LdNPV inoculum and ν_F and ν_V are the transmission coefficients of E. maimaiga and LdNPV, respectively. The rate of change of E. maimaiga infected host density is:

$$\frac{dI_F}{dt} = \nu_F F_t S_t - \nu_F F_{(t-\tau_F)} S_{(t-\tau_F)} \qquad (2)$$

where I_F is the density of larvae infected by E. maimaiga, and τ_F is the incubation period of E. maimaiga in the host. The infected insects are produced from the transmission of pathogens to the susceptible hosts $\nu_F F_{(t)} S_{(t)}$, but before they die, the pathogen incubates within the infected hosts, which is shown by $\nu_F F_{(t-\tau_F)} S_{(t-\tau_F)}$. The rate of change of LdNPV infected host density is:

$$\frac{dI_V}{dt} = \nu_V V_t S_t - \nu_V V_{(t-\tau_V)} S_{(t-\tau_V)} \qquad (3)$$

where I_V is the density of larvae infected by LdNPV, and τ_V is the incubation period of LdNPV in the host. The rate of change of E. maimaiga conidial density in the environment is:

$$\frac{dF}{dt} = \Lambda_F \nu_F F_{(t-\tau_F)} S_{(t-\tau_F)} - \mu_F F_t \qquad (4)$$

Here, Λ_F is the number of conidia produced by a cadaver that succumbed to E. maimaiga and μ_F is the decay rate of conidia in the environment. The rate of change of density of LdNPV occlusion bodies in the environment is:

$$\frac{dV}{dt} = \Lambda_V \nu_V V_{(t-\tau_v)} S_{(t-\tau_v)} - \mu_V V_t \qquad (5)$$

Here, Λ_V is the number of occlusion bodies produced by a cadaver that succumbed to LdNPV and μ_V is the decay rate of occlusion bodies in the environment.

The values of the LdNPV related parameters, ν_V, Λ_V, μ_V and τ_V were taken from Dwyer and Elkinton (1993). The values of the E. maimaiga related parameters, Λ_F, and τ_F were taken from Hajek et al. (1993) (Table 3.2). The initial density of the host population, S(0), was estimated as number of larvae present per m^2 of ground area using the total number of egg masses present in the plot and the average number of larvae that hatched from the egg masses (see above). The initial fraction of larvae hatching with LdNPV infections ($I_V(0)$) was estimated from the proportion of larvae that died from the egg masses collected before they hatched in the field. The density of E. maimaiga infected larvae ($I_F(0)$) was estimated from the proportion of the larvae, collected in the first week, that died from E. maimaiga. However, in 1992, we did not observe any E. maimaiga-induced mortality until the fourth collection week, so we used that information as $I_F(0)$ in the model. In each case we adjusted the transmission parameters ν_F so that model predictions for E. maimaiga fit the observed data. In nature it is probable that ν_F will vary depending upon the natural rainfall (Hajek et al. 1993). Our purpose was thus to model the impact of the observed levels of E. maimaiga on mortality of larvae to LdNPV, rather than predicting mortality from E. maimaiga.

Modeling cross-infection of LdNPV and E. maimaiga

In earlier studies, we detected some cadavers that contained both LdNPV occlusion bodies and E. maimaiga conidia or resting spores. Based on our laboratory work (Chapter I), we know that larvae infected with either pathogen can become infected with the other and the cause of death is determined by the timing of infection with respect to the relative incubation times of each (7 days for E. maimaiga and 14 days for LdNPV) pathogen. Thus, in the simulation, all larvae coinfected with both pathogens died from E. maimaiga, unless they had been infected with LdNPV more than 7 days prior to infection with E. maimaiga, in which case they died from LdNPV. The model was implemented in the Pascal language with a 0.01 day time step. We used Euler's method (Haefner 1996) to compute the number of new infections and number dying in each time step.

Results

Initial density of gypsy moth larvae and LdNPV inoculum

The egg mass counts in the plots (Table 3.1) correspond to high density population of gypsy moths (Campbell 1981). However, the hatch rate was lower in 1992 than in 1994 and was lower than in a typical outbreak population (Campbell 1981) . We did not see any E. maimaiga-induced mortality in the larvae that hatched from the egg masses collected in either year. In 1992, 25% of the hatched insects from the collected egg masses died from LdNPV and in 1994, 10-12% died from LdNPV.

Mortalities of gypsy moth larvae due to viral and fungal diseases in the field

In both years, we started the larval collections when 97-99% insects were first instars. Among the larvae collected in the first week of 1992, 2-6% died from LdNPV, but there was no mortality from E. maimaiga. In 1994, there was 3-5% mortality from LdNPV and 1-7% mortality from E. maimaiga among the insects from the first week of collection. The overall cumulative mortality due to LdNPV in 1992 was higher than in 1994 and E. maimaiga mortality was higher in 1994 (Table 3.2).

Effect of rainfall

According to the National Oceanic and Atmospheric Administration (NOAA), the total rainfall of May and June 1989 was the highest recorded in the last 30 years. The total rainfall in May 1994 was also much higher than the average, whereas the rainfall in May of 1992 was close to the 30-year average (Fig. 3.1). The higher amount of rainfall in May 1994 compared to May 1992 presumably explains the observation of higher mortality from E. maimaiga in 1994. In both 1992 and 1994, rainfall in June was very close to the 30 year average.

In our two artificial rainfall experimental plots, we applied 0.84 cm of artificial rain in each plot in May and 1.68 cm in June, 1994. The cumulative weekly mortality due to E. maimaiga was significantly higher in watered plots than the mortality in the unwatered plots ($\chi^2 = 4.86$, d.f. = 1, P = 0.028). In contrast, we did not see any significant differences in LdNPV mortalities among the watered and controlled plots ($\chi^2 = 0.99$, d.f. = 1, P = 0.32) (Table 3.2).

Model predictions

We predicted the mortality of gypsy moth larvae due to LdNPV in the presence and absence of E. maimaiga using our simulation model. The overall impact of E. maimaiga on LdNPV mortality was minor in our simulations at densities

represented by our field data (Fig. 3.2). When we included the effects of cross-infection in our model, in which we allowed larvae previously infected with LdNPV to become infected and die from E. maimaiga, the effect of E. maimaiga on the mortality due to LdNPV was noticeable only at the very end of the larval season (Fig. 3.3).

Discussion

According to our model, E. maimaiga had only limited impact on LdNPV mortality at the larval densities represented by our field data, despite the competitive advantage of faster incubation time of E. maimaiga over LdNPV. This occurred, because E. maimaiga did not become a major source of mortality until the insects became fifth or sixth instars. The model predictions closely matched the levels of LdNPV mortality actually observed on our plots. The weekly mortality from E. maimaiga increased steadily throughout the larval stage of gypsy moth. This result agrees with earlier studies of E. maimaiga in field populations which showed high mortality only among late instars (Weseloh and Andreadis 1992a). Mortalities that peak at the end of the larval stage are also typical of LdNPV (Campbell 1967, Woods and Elkinton 1987), because the number of infectious particles, and hence the number of larvae becoming infected, increases exponentially with each cycle of the pathogen in the population.

Although the density of egg masses was high, the number of eggs per mass were very low, so the larval populations in our research plots were only moderate in both years (Table 3.1). We observed a very small second peak of mortality due to LdNPV in our plots (Fig. 3.3 and 3.4). According to our model, these larval densities were not sufficient to create a large second wave of LdNPV mortality, even if E.

maimaiga were absent (Fig. 3). The model predictions from the 1994 data are supported by the egg mass counts from the subsequent year. In spring 1995, we counted an average of 112.5 egg masses/ha in unwatered plots and whereas in watered plots we found only 37.5 egg masses/ha. When we ran a simulation with a much higher density of susceptible insects, we found increases in mortality rates from both LdNPV and E. maimaiga and the second 'wave' of LdNPV was mostly eliminated. In all of our simulations, the combined mortality from LdNPV and E. maimaiga was always higher than the mortality from LdNPV when it was present alone.

In a laboratory study, we showed that E. maimaiga was able to reproduce in those larvae which were already infected with LdNPV. Gypsy moth larvae simultaneously inoculated with both LdNPV and E. maimaiga, usually succumbed to E. maimaiga (Chapter I); this is likely due to the shorter incubation time of E. maimaiga (4-7 days, Hajek et al. 1993, Shimazu and Soper 1986) compared to the ca. 14-day incubation period for LdNPV (Woods and Elkinton 1987). Thus, in nature, it is likely that some of the larvae infected with LdNPV will become infected with and subsequently die from E. maimaiga instead. Even without this within-host interaction, larvae dying as early instars from E. maimaiga will reduce the density of larvae available to die subsequently from LdNPV, thereby reducing the LdNPV inoculum required to cause the second wave of LdNPV mortality among late instars (Woods and Elkinton 1987). This is probably the mechanism by which applications of Bacillus thuringiensis suppresses of LdNPV-induced mortality in gypsy moths (Woods et al. 1988). The effect of B.t. was much larger than the effect we showed here for E. maimaiga, presumably because the B.t. was applied at a very early larval stage (second instars) which suppressed the density of gypsy moths that would die from LdNPV and thus the inoculum that triggers the second wave of LdNPV mortality (Woods and Elkinton 1987) among late instars. However, there is one

difference - B.t. was inundatively released in the sites, while E. maimaiga we referred here is a naturally occurring pathogen.

The greatest effect of E. maimaiga on LdNPV may be on the level of LdNPV in the environment at the time of pupation and hence on the inoculum present to be transmitted to the next generation. Environmental contamination is thought to be the principal route of transmission of LdNPV in the next generation via egg masses deposited on LdNPV-contaminated surfaces (Murray and Elkinton 1990). The model indicates that the fraction dying from (4.5%) LdNPV in the presence of E. maimaiga was about half that without E. maimaiga (9.4%) in the week just before pupation (Fig. 3.3). Predicted amounts of LdNPV inoculum left were 8.63×10^9 OBs and 1.48×10^{10} OBs per m^2, with and without E. maimaiga respectively. This negative impact of E. maimaiga on LdNPV contamination might help to explain why gypsy moth populations sometimes appear to rebound following E. maimaiga epizootics. Perhaps the results reported by Yerger and Rossiter (1996) in which they found a less than 1% LdNPV-induced mortality of neonate gypsy moth larvae which were collected as egg masses from the coastal and central Massachusetts in summer 1991. E. maimaiga was present in their collection sites.

Previous studies had shown a positive correlation between rainfall and the mortality rates of gypsy moths from E. maimaiga (Elkinton et al. 1991, Hajek and Roberts, Smitley et al. 1995, Weseloh and Andreadis 1992a, b, Weseloh et al. 1993). Secondary transmission via conidia is considered to be the major source of disease spread among the later instars, and it depends upon the pattern of rainfall (Weseloh and Andreadis 1992b). Our study plots received a much higher than average natural rainfall in May 1994. This probably explains the higher cumulative mortality of gypsy moths from E. maimaiga in 1994 compared to 1992 (Table 3.2). These observations support Weseloh and Andreadis's (1992b) conclusion that high rainfall in May was more important than rainfall in June in causing epizootics of E.

maimaiga. We found a higher cumulative mortality due to E. maimaiga in watered plots compared to the control plots, which is similar to the findings of Hajek et al. (1996). However, there was a little effect of watering on the mortality induced by LdNPV.

In conclusion, E. maimaiga at these gypsy moth larval densities has little effect on gypsy moth mortality induced by LdNPV in the same generation of gypsy moth. However, it may lower the probability of LdNPV inoculum production for infection of the next generation.

Table 3.1. Estimates of egg mass density, larvae hatched, and the percent initially infected by LdNPV in the research plots of 1992 and 1994

Location/Year	No. of Plots	Mean no. of egg masses/ ha(±SE)	Mean no. of larvae hatched/egg mass(±SE)	Initial no. of larvae/m^2 (±SE)	Mean % died from NPV (±SE)
Holyoke '92 (Not Watered Plots)	7	6260.0 (±1368.2)	26.0(±1.6)	17.5(±4.7)	25.2(±4.0)
Holyoke '94 (Watered Plots)	2	2100.0 (±425.0)	128.7(±13.5)	27.0(±6.2)	10.2(±2.2)
Holyoke '94 (Not Watered Plots)	2	2612.5 (±187.5)	105.2(±10.6)	27.5(±2.3)	12.3(±3.5)

Table 3.2. Cumulative % mortalities, model predicted total survivors at the end of 1992 and 1994 gypsy moth seasons

Year	Cum. Observed % mortalities due to		Model Predicted Total survivors/ha	
	LdNPV	E. maimaiga	Without E. maimaiga	With E. maimaiga
1992	59.00	27.00	157.75	52.75
Unwatered Plots, 1994	29.50	65.70	307.25	91.75
Watered Plots, 1994	32.70	81.30	349.75	19.75

Table 3.3. Values of parameters from equations 1-5, which were used in the model

ν_F	transmission rate of the EM	(2.5×10^{-7} m^2 /day)
ν_V	transmission rate of the LdNPV	(1.45×10^{-12} m^2/day)
τ_F	EM incubation time	(7 days)
τ_V	LdNPV incubation time	(14 days)
Λ_F	no. of conidia produced	(2.12×10^5/cadaver)
Λ_V	no. of occlusion bodies produced	(2×10^9/cadaver)
μ_F	rate at which conidia break down in the environment	(3×10^{-3}/day)
μ_V	rate at which occlusion bodies break down in the environment	(3×10^{-3}/day)

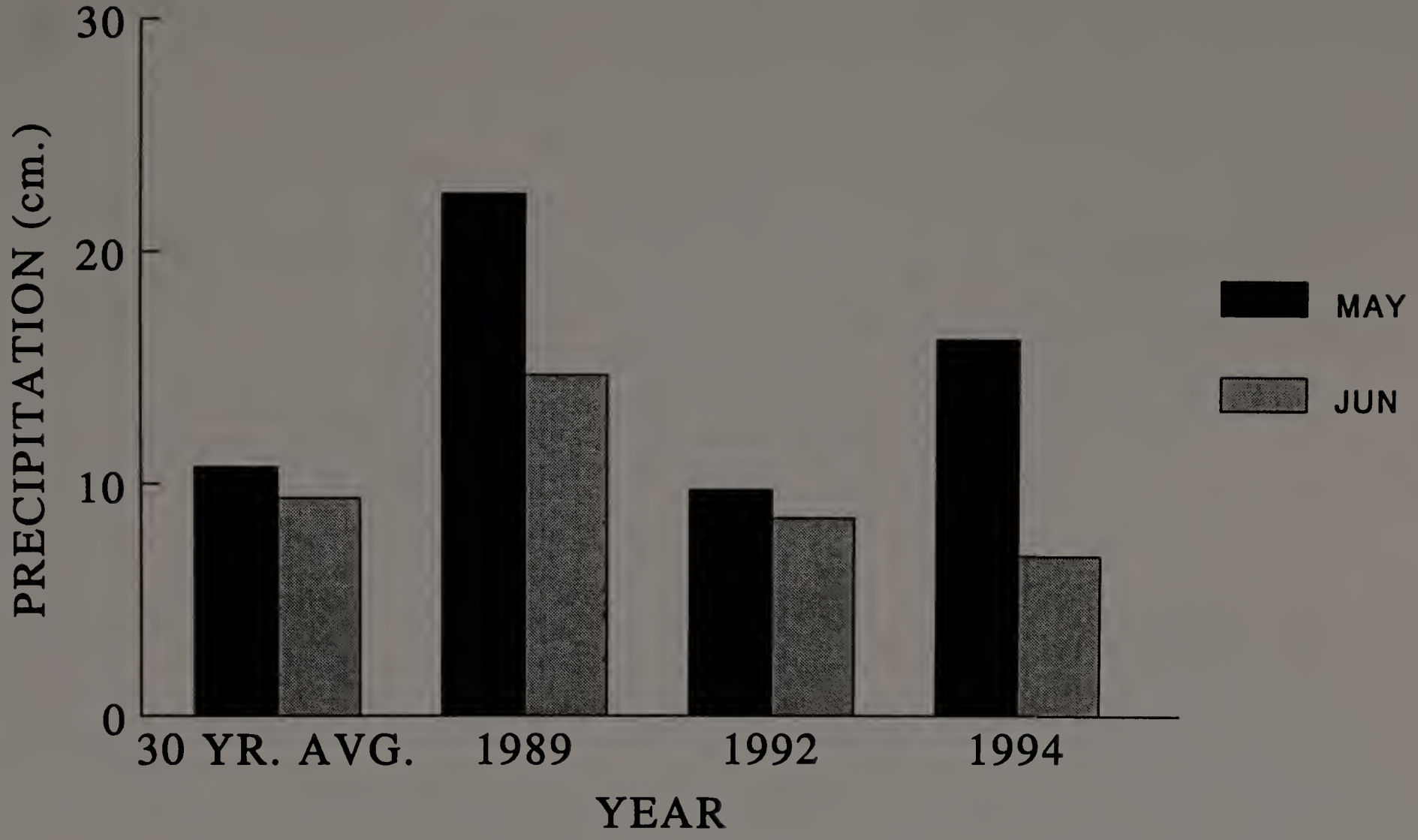

Fig. 3.1 Total recorded rainfall (cm) in Amherst, MA area in the months of May and June.

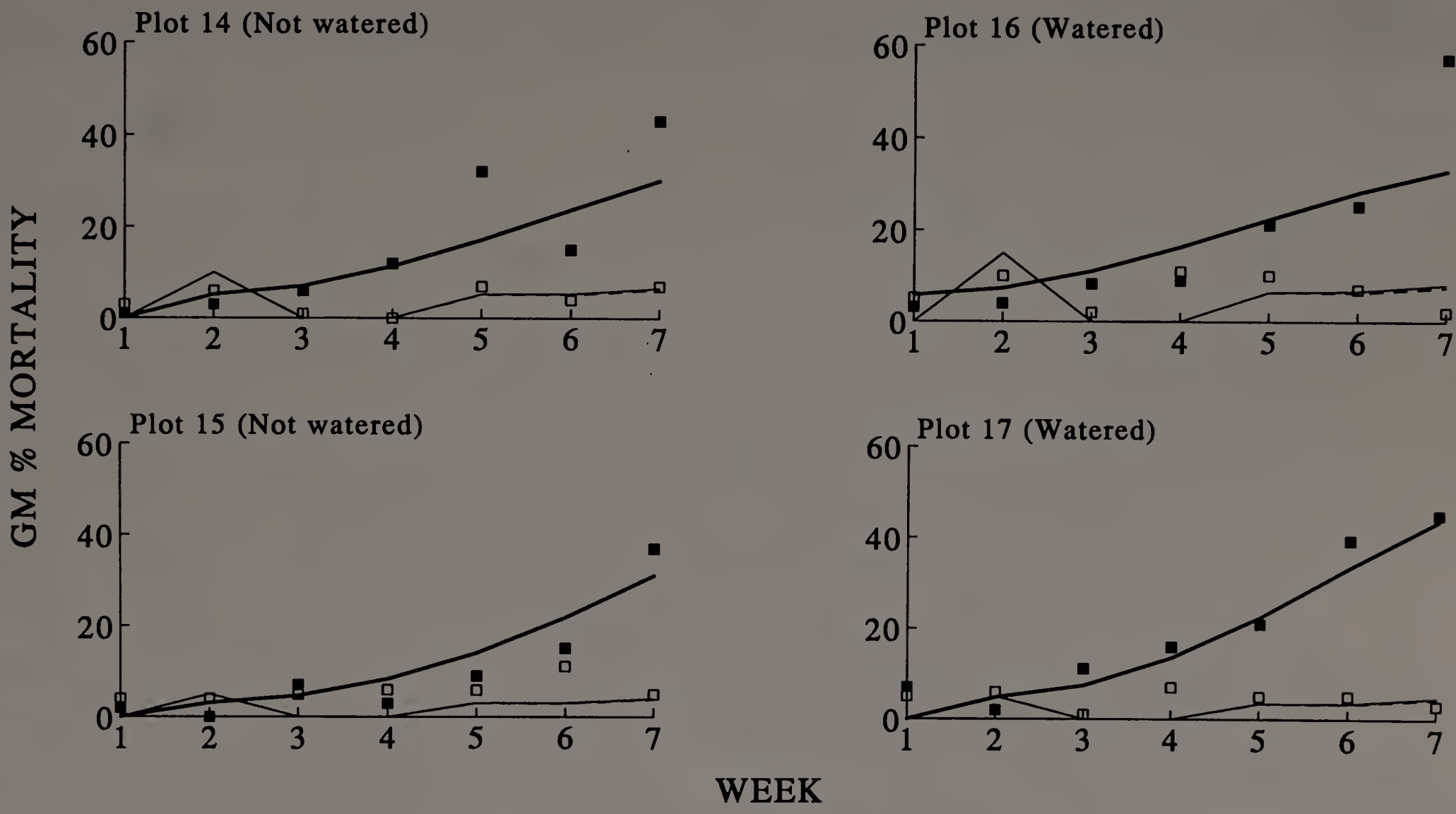

Fig. 3.2 Gypsy moth's observed weekly mortality in four research plots in Holyoke mountain, MA in 1994 and the predictions from the host-pathogen model without cross-infection. The solid squares are the observed E. maimaiga-induced larval mortality and open squares are LdNPV mortality. The thick solid line is the predicted E. maimaiga mortality from the model, the thin solid line is predicted LdNPV mortality in the presence of E. maimaiga and dotted line is LdNPV mortality in the absence of E. maimaiga.

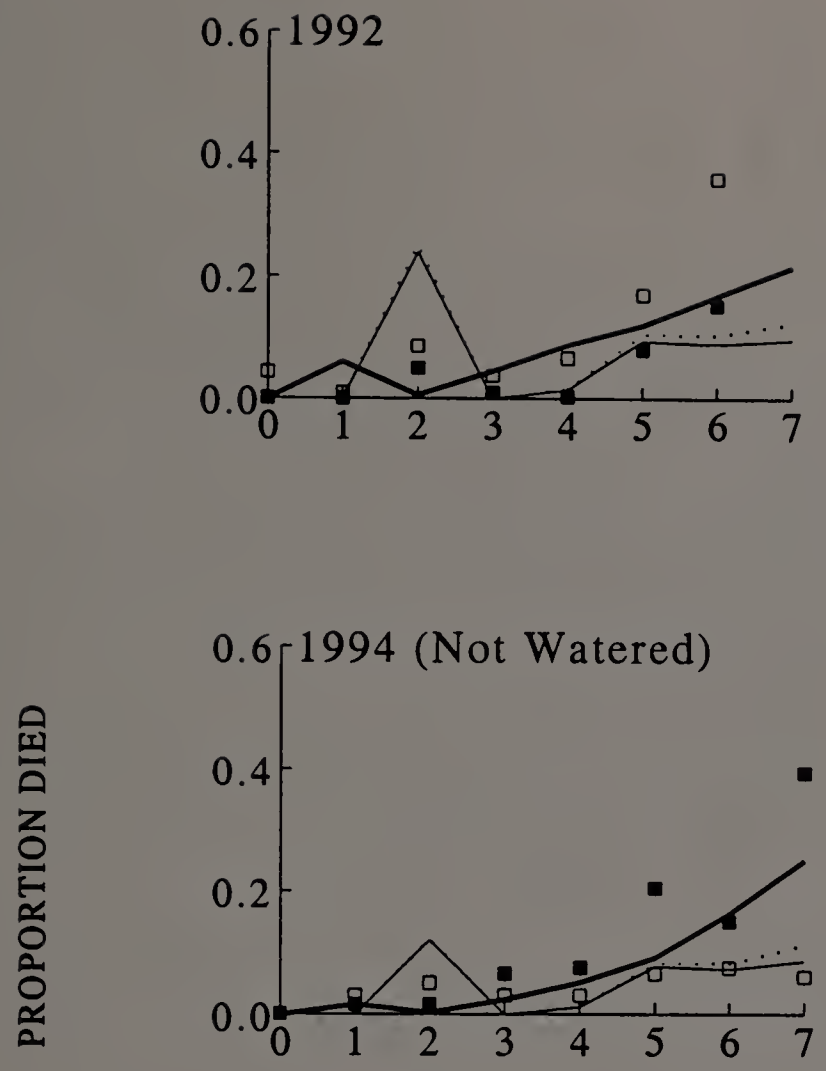

Fig. 3.3 Weekly gypsy moth mortality recorded from the research plots in Holyoke Range Mountain, MA in 1992 and 1994 and the mortality predictions from two-pathogen interaction model with cross-infection. The first graph indicates the mortality data from 1992, pooled from 7 of 0.01 ha plots the second graph is the pooled mortality data from two non-irrigated, 0.01 ha plots and the third graph is from two irrigated and two plots were not. The filled squares represent the observed fungus mortality and unfilled squares represent the virus mortality and The thick solid line is the predicted E. maimaiga mortality from the model, the thin solid line is predicted LdNPV mortality in the presence of E. maimaiga and dotted line is LdNPV mortality in the absence of E. maimaiga.

CHAPTER IV

COMPARISON OF HETEROGENEITY IN GYPSY MOTH SUSCEPTIBILITY TO TWO PATHOGENS: DOES IT EXPLAIN DIFFERENCES IN DENSITY DEPENDENCE?

Abstract

The population dynamics of gypsy moth (Lymantria dispar L.), are strongly affected by the occurrence of its two pathogens, the nuclear polyhedrosis virus (LdNPV) and the entomophthoralean fungus, Entomophaga maimaiga. LdNPV epizootics only occur in high densities of gypsy moths whereas E. maimaiga epizootics occur in both high and low density populations. Recent theoretical work on the dynamics of insect diseases has shown that variation in host susceptibility can strongly reduce the degree to which pathogen prevalence increases with host density. Here we show that differences in heterogeneity of susceptibility are not adequate to explain the observed difference in density dependence of the two pathogens. Laboratory bioassays and mesh-bag experiments indicate that gypsy moths are less heterogeneous in susceptibility to E. maimaiga than to LdNPV

Keywords: density, Entomophaga maimaiga, Gypsy moth, heterogeneity, LdNPV, pathogen, transmission, variation in host susceptibility.

Introduction

Gypsy moth, Lymantria dispar L. (Lepidoptera: Lymantriidae), has been a serious forest defoliator of eastern North America since its introduction into Massachusetts from Europe in 1868 (Elkinton and Liebhold 1990). At present, it has spread as far south as North Carolina, west to Wisconsin, and north to Quebec (Hajek et al. 1996). There are two major pathogens of gypsy moth, a nuclear polyhedrosis virus (LdNPV) and a fungus, Entomophaga maimaiga Humber, Shimazu et Soper. LdNPV epizootics have long been associated only with high density gypsy moth populations (Doane 1970, Woods and Elkinton 1987, Woods et al. 1991) and usually cause the decline of population outbreaks. E. maimaiga, in contrast, causes epizootics in both low and high density populations (Hajek et al. 1990a, 1993, Elkinton et al. 1991, Weseloh and Andreadis 1992a). Here we ask whether this difference in density dependence between the two pathogens is caused by differences in the levels of variability in host susceptibility and explore how the variability in the gypsy moth's susceptibility to E. maimaiga affects the likelihood of E. maimaiga epizootics at different gypsy moth densities. Our approach is to use a mixture of laboratory bioassays; small-scale transmission experiments on cut foliage, and then explain the consequences of host variability in susceptibility for the effects of density on the likelihood of epizootics.

Conventional models of infectious disease dynamics assume that the pathogen transmission is directly proportional to the densities of host and pathogen i.e., the transmission of pathogen increases with the density of susceptible hosts and infected individuals, which would release infective pathogens when they die (Anderson and May 1979, 1981, Dwyer 1991, Hochberg and Holt 1990). However, recent theoretical work has shown that under some conditions, the transmission of pathogens

increases with the density of infected insects at a decreasing rate, so that pathogen transmission becomes a non-linear function of density (Liu et al 1987, Hochberg 1991, Anderson and May 1992, Knell et al. 1996). This non-linearity in disease transmission may be caused by different biological factors such as, genetic diversity among the hosts (Anderson et al. 1982) and parasites (Forsyth et al. 1989), host behavior (Anderson and May 1992) and pathogen contact rates (Woolhouse et al. 1991). In our laboratory, we have experimentally shown that LdNPV transmission is a non-linear function of pathogen density (D'Amico et al. 1996). Furthermore, Dwyer et al. (1997) have demonstrated that an important part of this non-linearity is due to heterogeneity in host susceptibility to LdNPV. In this paper, we will discuss whether such non-linearity observed in LdNPV transmission also occurs for E. maimaiga transmission.

We used a linear model as a kind of null hypothesis about the dynamics of pathogens in gypsy moth populations. This basic model assumes that all host individuals are homogeneous in their susceptibility to pathogens and the rate of transmission depends upon density of the host and the pathogen. Mortality of host under such assumptions can be represented as:

$$-\ln \frac{S_t}{S_0} = \nu P_0 t \qquad (1)$$

where ν is the transmission coefficient, S_0 the number of healthy insects before they were exposed to the pathogens, S_t number of the insects that survived at the end of the experiment, P_0 pathogen density in the form of pathogen-killed cadavers and t is the length of the time that the experiment lasted (7 days). Here, the mortality rate of the host due to the pathogens ($-\ln \frac{S_t}{S_0}$) is a linear function of density of the pathogens.

In our modified model, we incorporated variability in the host susceptibility by introducing two new parameters, $\overline{v}$, and k. The $\overline{v}$ is a mean of the transmission rates and the k is the inverse of the squared coefficient of variation of transmission. Now the mortality rate of the host can be expressed as:

$$-\ln\frac{S_t}{S_0} = k\ln\left[1+\frac{\overline{v}}{k}P_0 t\right] \qquad (2)$$

where all the symbols have the same meaning as in equation (1) except the transmission coefficient ν is now replaced by the mean transmission rate, $\overline{v}$, and the 'k' value is an inverse measure of the host heterogeneity in susceptibility. As heterogeneity in susceptibility of the hosts increases, the parameter k decreases i.e., the variation in transmission rate is high. Here, we have kept mathematical details to a minimum. For a thorough mathematical analysis, we suggest readers to refer Dwyer et al. (1997).

Testing for an effect of host variability in susceptibility is equivalent to testing whether the mortality rate of the host, expressed as $(-\ln\frac{S_t}{S_0})$, is a non-linear function of the pathogen density. By manipulating host variability, we can test whether host variability is the mechanism that underlies any non-linearity in transmission. We attempted to manipulate the host variability by varying the strain of the healthy host insects in our experiment, i.e., laboratory vs. feral (wild) insects. We hypothesized that the feral larvae would be more heterogeneous in their susceptibility than those from laboratory colonies, because the feral larvae were taken from different populations that experienced variable recent exposure to LdNPV or E. maimaiga epizootics. In contrast, larvae bred in the laboratory colony had not been exposed to LdNPV or E. maimaiga for at least 42 generations.

Materials and Methods

Insects

Feral egg masses were collected in the winters (in the months of Feb. and March) of 1994 from MA and VA and in 1995 from MA and WV and stored at 4°C. The laboratory-reared, New Jersey strain of gypsy moth was obtained from APHIS-USDA, Methods Development Center, Otis Air National Guard Base, MA. Hereafter the laboratory strain larvae will be referred to as Otis larvae. In the beginning of the summer in 1995 and 1996, we surface sterilized both feral and Otis eggs with 5% formalin solution. Sterilized eggs were left in empty 180 ml diet cups with a piece of water-soaked dental wick, at 28°C until hatch. The neonates from the eggs were transferred to an artificial diet (Bell et al. 1981) and reared at 28°C with a light: dark cycle of 16:8 hours until they became fourth instars. The time of hatching was adjusted by transferring the eggs from 4°C to 28°C in such a way that the neonates from the Otis eggs hatched on the same day as the feral eggs. In order to minimize discrepancies in infecting methods, we inoculated the feral and Otis insects at the same time using the same stock and same concentration of inoculum.

Pathogens

For LdNPV treatments, we used the plaque purified G2 clone virus, kindly provided by Dr. John P. Burand from the University of Massachusetts. E. maimaiga protoplasts were kindly provided by Dr. Ann E. Hajek of Cornell University. This fungus was originally collected from Virginia in 1994 and was then maintained in the laboratory at -80°C as protoplasts. E. maimaiga conidia were produced by inoculating healthy fourth instars with E. maimaiga protoplasts.

Laboratory studies

E. maimaiga bioassay

E. maimaiga conidium collection: E. maimaiga protoplasts were injected into freshly molted fourth instars (Otis larvae) at a rate of 500 protoplasts per larva, as described by Hajek et al. (1990b). Larvae were subsequently reared on artificial diet at 20°C under constant darkness. The number of protoplasts injected was high enough to kill more than 90% of the injected larvae. To collect conidia, cadavers that were just beginning to show some conidial spores were moved to a clean wire platform with a sterile tooth-pick. The cadavers were placed in a covered casserole dish with 50 ml of Atmos 300/Tween 80 solution (1.25 ml of Atmos, 0.5 ml of Tween in 500 ml of distilled water) and held at room temperature in a dark corner. The conidia ejected by the cadavers were collected by centrifuging (at 9000 rpm for 6 minutes) the suspension from the casserole dish every 2 hr for 8 hr. After each collection, the supernatant was reused for conidia collection. The pellet of conidia in 1 ml of Atmos/Tween solution was stored at 4°C while collecting the conidia. All collected conidia were pooled together at the end of the collection period and immediately used them to inoculate the test larvae.

Conidial showering: Conidial concentration was determined by counting the conidia on a hemocytometer under a light microscope. A series of conidial suspensions ($1x10^2$, $1x10^3$, $1x10^4$ and $1x10^5$ conidia per ml) was made by diluting the stock with Atmos/Tween solution (0.125 ml of Atmos, 0.05 ml of Tween in 500 ml of distilled water). Approximately 25 freshly molted fourth instars, either from feral or Otis strains, were individually dipped into each concentration of the conidial suspension and briefly dried on a paper towel as described by Hajek et al. (1991). The control groups were dipped in Atmos/Tween solution. All of the larvae were held in parafilm sealed petri-dishes with 3 moist filter papers for 2 days and then they were

transferred to artificial diet and reared at 20°C. Mortality was checked every day and cause of death was determined by autopsy of the cadavers.

LdNPV bioassay

Newly molted fourth instars of feral and Otis insects were inoculated with LdNPV occlusion bodies (OBs) by the modified diet cube method of Boucias et al. (1980). The concentrations of OBs used were 0.5, 5, 50, $5x10^2$, $5x10^3$ and $5x10^4$ OBs/larva in 1995 and 2.5, 25, $2.5x10^2$, $2.5x10^3$, $2.5x10^4$, $2.5x10^5$ OB/larva in 1996. Approximately 25 larvae of either feral or Otis insects were treated with each concentration of OBs and the control groups were fed with diet cubes treated with distilled water. Those larvae that did not completely finish the inoculated diet cube within 24 hr were discarded (Chapter I). The inoculated larvae were reared on artificial diet at 28°C. Mortality was checked every day and cause of death was determined by autopsy of the cadavers.

Mesh-bag experiments

To initiate transmission in mesh-bag experiments, we used LdNPV or E. maimaiga - infected larvae. The Otis larvae were inoculated with viral occlusion bodies ($5x10^5$ per larva) by feeding or with E. maimaiga protoplasts ($5x10^2$ per larva)by injection, six days and four days, respectively, before deploying them in the bags. These days were selected in such a way that infected insects would not die before we put them in the bags, but they would die within a day or two afterwards and the OBs or the conidia would be available to infect the healthy (test) larvae we put in the bags. We varied the density of pathogens by selecting the appropriate number of infected insects in each bag. The density of virus-infected insects per bag were 1, 5,

30 and 60 per bag (in 1995) or 1, 5, 10, 20, 30 and 60 (in 1996) and 1, 5, 10, 20, 30 and 60 E. maimaiga infected insects per bag in both years. Each treatment was replicated 4 times in 1995 and 7 times in 1996.

Freshly cut branches of red oak (Quercus rubra) trees with 40 (±3) leaves were brought in the laboratory. All the branches used in the experiments were from trees located on the campus of University of Massachusetts, Amherst. The branches were sterilized by soaking them in 10% Clorox for 10 minutes and rinsing with tap water. Each branch was fixed in a clean one-gallon plastic jug with water. The mouth of the jug was closed with duct tape. The branch was covered with a polyester fine-mesh-bag (Kleen Test products, Brown Deer, WI), size 60x60x55 cm^3. The required density of infected insects and 25 uninfected larvae were placed in each bag. The mouth of each bag was secured with a cable tie and duct tape. The bags were left for 7 days in an incubator room maintained at 20°C with light and dark cycle of 16:8 hours and 90% relative humidity. Water was sprinkled twice a day at 9 AM and 6 PM on the top of the bags. On the eighth day, the live insects from the bags were transferred individually into 60 ml cups with artificial diet and reared at room temperature (22°C) for three weeks. Mortality was checked every other day.

Data analysis

Dose-mortality data from lab bioassays were analyzed with probit analysis (Finney 1971) using PC POLO (LeOra Software, 1987). The variations among the feral and Otis insects with respect to LdNPV or E. maimaiga response were tested by comparing their slopes with D-statistics, $D = \frac{b_F - b_O}{\sqrt{{s_F}^2 + {s_O}^2}}$, where b_F and b_O are the slopes of the probit lines for feral and Otis insects and s^2_F and s^2_O are the standard

errors of the corresponding slopes. The D statistics have an approximately normal distribution with mean = 0 and standard deviation = 1.

The variability among the larvae in terms of their susceptibility to either LdNPV or E. maimaiga, was measured by the variation in the transmission coefficient (ν) of that pathogen as in Dwyer et al. (1997). The transmission coefficients have a gamma distribution (Dwyer, unpublished) with a mean = $\overline{\nu}$ and the variance = $\frac{\overline{\nu}}{k}$, where k is the inverse of the square of the coefficient of variation. The mortality rate of the susceptible insects were determined using the equation (1) for the linear model and the equation (2) for the non-linear model. To test which model gives the best fit to the experimental observations, we used the Akaike Information Criterion (AIC, Akaike (1973)):

$$AIC = - 2 \log(L_i) + 2n_i, \qquad (3)$$

where L_i is the likelihood and n_i is the number of parameters in model i. The model with lowest AIC value is considered to be a better model. The differences between the host strains were determined by comparing the k parameters. Lower values of k indicate higher levels of heterogeneity in transmission.

Results

Dose-response tests in the laboratory

Both feral and Otis larvae were markedly more heterogeneous in their response to different dosages of LdNPV than E. maimaiga, as indicated by the slopes of the dose-response curves (Fig. 4.1). The median lethal concentration of E. maimaiga conidia (LC_{50}) for both feral and Otis insects were similar in 1995, but in

1996, the LC_{50} was higher for feral insects than for Otis insects (Table 4.1). However, the slopes of the probit lines for both feral and Otis larvae were not significantly different for both years 1995 (D= 1.31, df = 1, p =0.09) and 1996 (D = 0.17, df = 1, p =0.43). Similarly, the lethal dose (LD_{50}) of LdNPV required to kill 50% of the feral larvae was higher than that of Otis larvae in 1996, but the slopes of the probit lines are not statistically different between two strains of the larvae in both years (Table 4.2). The slopes of the probit lines are the indicators of heterogeneity among the test larvae; the smaller the slopes, the higher the heterogeneity (Finney 1971).

Mesh-bag experiments

The response of larvae to LdNPV was much more heterogeneous than to E. maimaiga as indicated by the low k-values (Table 4.3) for LdNPV compared to E. maimaiga. The lines shown in the Fig. 2 are the fitted linear and non-linear models (eqs. 1 - 2). According to the AIC test statistics, the non-linear model is the best to describe the observed LdNPV data of 1996, whereas the linear model was the best-fitted model to describe the E. maimaiga data for all the host strains and experimental years. Linear models indicate very low heterogeneity. The overall mortality due to E. maimaiga was higher in 1995 than in 1996. The mortality of Otis insects due to E. maimaiga as well as LdNPV was higher than feral insects in both years.

The LdNPV k-values calculated for the feral larvae were lower in both years than for Otis larvae (Table 4.3) which indicates that the feral larvae are more heterogeneous than the Otis larvae. For E. maimaiga, however, we found lower *k* for feral larvae than for Otis larvae in 1996, but we had the opposite effect in 1995. In other words, there were no consistent differences in heterogeneity between Otis and feral larvae in response to E. maimaiga.

Discussion

Our laboratory dose-response data as well as mesh-bag transmission data for two years indicate that gypsy moth larvae are consistently more heterogeneous in their susceptibility to LdNPV than to E. maimaiga. Our results for LdNPV transmission is consistent with Dwyer et al.'s (1997) findings, i.e., the LdNPV transmission did not increase linearly with the increase of LdNPV density at least in 1996. In contrast, there was little heterogeneity in susceptibility of gypsy moths to E. maimaiga infection, which is supported by the smaller *k* values for LdNPV than for E. maimaiga. These results are consistent in both years. The higher the heterogeneity among the larvae the more strongly the fitted curve departs from the linearity. E. maimaiga had high k values suggesting that E. maimaiga transmission is a linear process, because of low heterogeneity of gypsy moth to E. maimaiga. The feral insects also had lower k (calculated from the transmission experiments) in both years and thus had a higher non-linearity in transmission. Our LdNPV results are consistent with the findings of Dwyer et al. (1997).

Weseloh and Andreadis (1992a) observed higher mortality from E. maimaiga transmission in high vs. low density gypsy moth populations. Density dependent fungal infections have been reported in Choristoneura fumiferana (Vandenberg and Soper 1978). Our mesh-bag results are consistent with these previous reports. On the other hand, E. maimaiga epizootics occur in both high and low density populations (Hajek et al. 1990a, 1993, 1996, Elkinton et al. 1991, Weseloh and Andreadis 1992a) whereas LdNPV epizootics are confined to high density populations (Doane 1970, Campbell 1981, Woods and Elkinton 1987). Thus, in nature, E. maimaiga is only weakly density dependent. However, this lack of density dependence in E. maimaiga dynamics compared with LdNPV in the naturally occurring populations cannot be

explained by differences in variation in susceptibility, because lower variation leads to stronger not weaker density dependence (Dwyer et al. 1997). The lack of density dependence for E. maimaiga must be caused by something else, perhaps the wind-borne nature of E. maimaiga conidia transmission (Weseloh and Andreadis 1992b) which can carry E. maimaiga spores from high to low density populations of gypsy moth. These spores may be able to travel a long distances as indicated by the rapid spread of E. maimaiga in Pennsylvania in 1990 and 1991 (Elkinton et al. 1991) and Virginia in 1992 (Hajek et al. 1996).

Larval behavior may also influence the density dependence of E. maimaiga. In low density to moderate gypsy moth populations, older instars leave the forest canopy during daylight hours and rest in the litter (Lance et al. 1987) and/or dark, cryptic habitats (Campbell et al. 1975) and, during that time, there is a greater chance of coming into contact with the germinating resting spores or sporulating cadavers (Hajek et al. 1990a). In high density populations gypsy moth larvae remain in the canopy day and night and do not seek resting locations on the forest floor.

The low heterogeneity of gypsy moth to E. maimaiga may be due to the very short time period to which North American gypsy moth has been exposed to E. maimaiga as opposed to LdNPV. E. maimaiga has not been recorded in N. America prior to 1989 (Andreadis and Weseloh 1990, Hajek et al. 1990a) and has not been described from European populations, which were the origin of North American gypsy moths. In contrast LdNPV epizootics have always been associated with the outbreak densities of gypsy moths both in N. America and Europe (Doane 1970).

Table 4.1. Dose mortality response of feral and Otis fourth instar gypsy moths to E. maimaiga conidial shower

Year	Larval strain	n	Intercept ±SEM	Slope ±SEM	LC_{50} (95% C.I.)
1995	Feral	130	-6.46±1.05	1.87±0.30	2795.59a
	Otis	127	-5.01±0.82	1.45±0.23	2913.23 (1589.67-5140.99)
1996	Feral	114	-5.74±1.07	1.66±0.30	2869.22 (946.96-7316.62)
	Otis	109	-5.41±1.61	1.91±0.54	680.06 (285.49-1232.81)

a = C.I. could not be calculated

Table 4.2 Dose mortality response of feral and Otis fourth instar gypsy moths to LdNPV occlusion bodies consumed on diet cubes in laboratory bioassays

Year	Larval strain	n	Intercept ±SEM	Slope ±SEM	LD_{50} (95% C.I.)
1995	Feral	132	-1.29±0.33	0.81±0.14	37.98 (1.09-213.50)[b]
	Otis	127	-1.74±0.35	0.75±0.12	216.39 (73.67-529.23)
1996	Feral	112	-2.45±0.40	0.79±0.12	1203.28 (107.32-21313.86)
	Otis	120	-1.58±0.31	0.72±0.12	161.64 (10.52-1250.88)

b = 90% C.I.

Table 4.3. Estimated k and the transmission coefficient (ν)

Year	Larval type	ν	$\bar{V}$	k	AIC Lin	AIC Nlin
	LdNPV					
1995	Feral	3.87×10^{-3}	8.31×10^{-3}	0.88	12.71*	14.64
	Otis	9.38×10^{-3}	1.96×10^{-2}	2.05	10.73*	10.92
1996	Feral	5.55×10^{-3}	1.85×10^{-2}	0.54	58.99	45.13*
	Otis	7.27×10^{-3}	1.34×10^{-2}	1.29	53.25	48.44*
	E. maimaiga					
1995	Feral	1.70×10^{-3}	1.69×10^{-3}	172.83	108.97*	111.44
	Otis	2.66×10^{-3}	2.69×10^{-3}	13.46	16.11*	18.57
1996	Feral	6.81×10^{-4}	4.85×10^{-4}	26.01	29.27*	43.21
	Otis	1.06×10^{-3}	8.50×10^{-4}	110.32	38.63*	49.78

* = best model

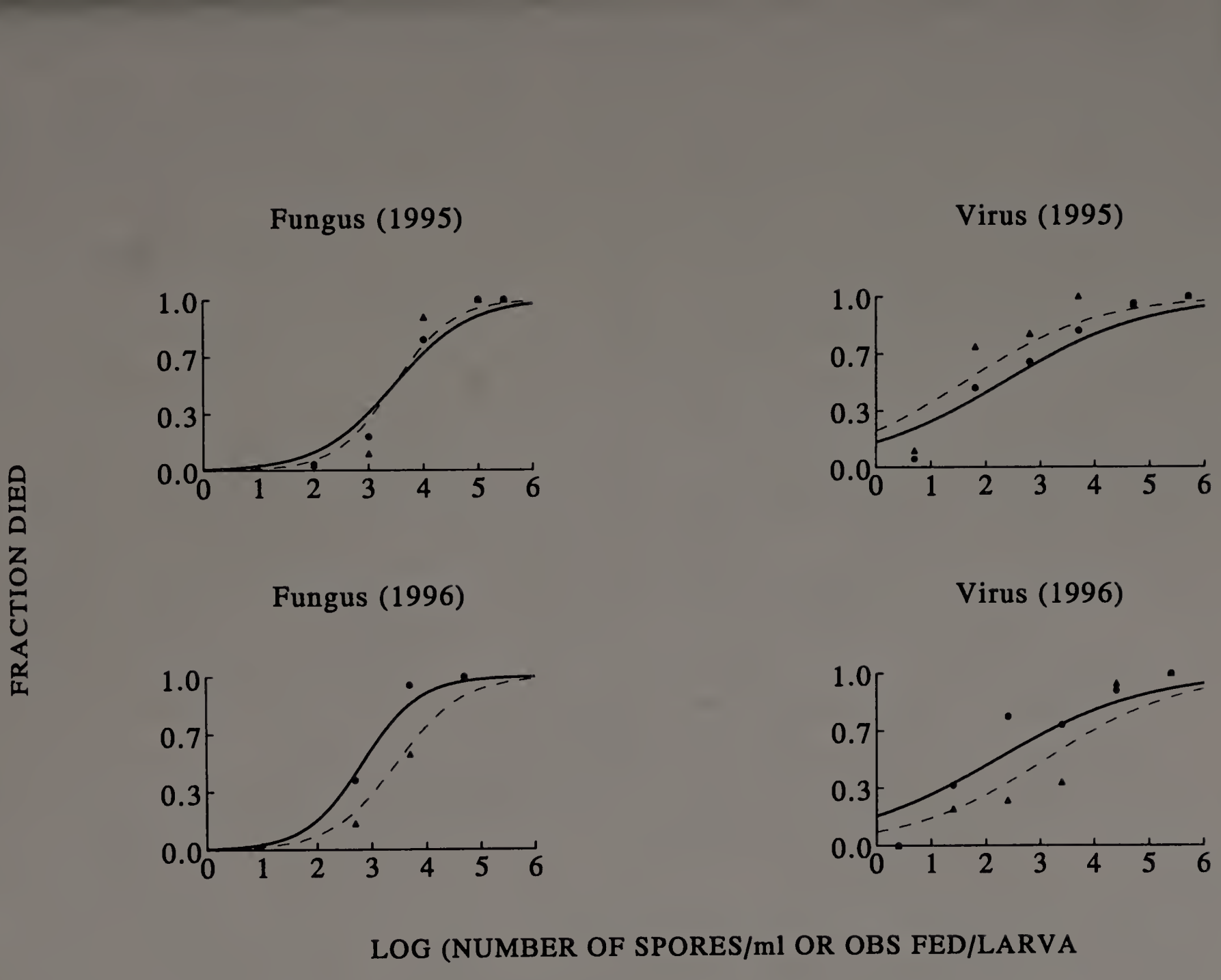

Fig. 4.1 Comparison of susceptibility of feral and Otis (laboratory reared) larvae to Entomophaga maimaiga and LdNPV. The dark circles represent the observed mortality in the Otis larvae and the triangles are the observed mortality among the feral larvae. The solid line is the fitted probit mortality for Otis larvae and dashed line for the feral larvae.

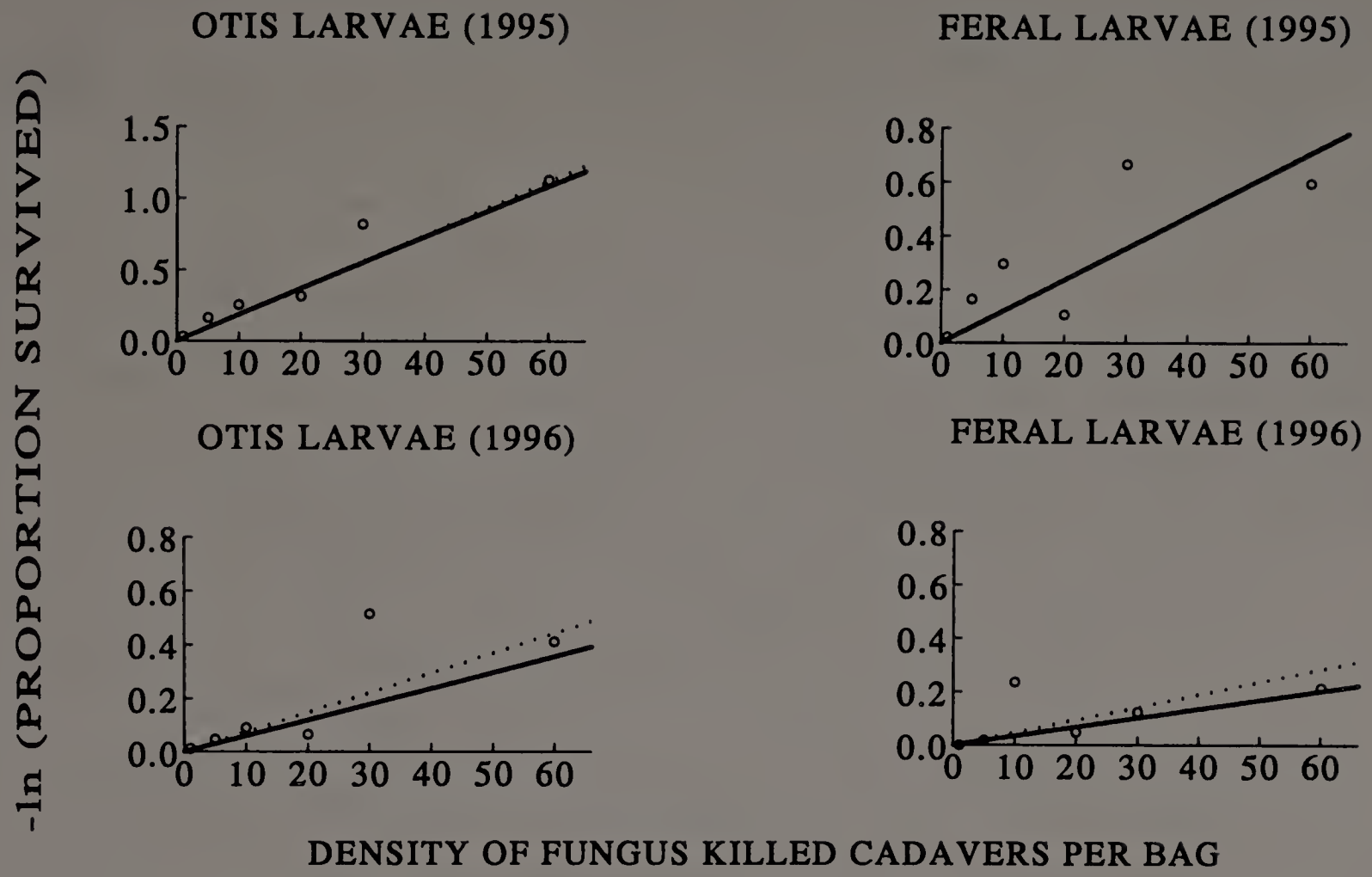

Fig. 4.2. Entomophaga maimaiga-induced mortality among the feral and Otis larvae exposed to different densities of E. maimaiga killed cadavers and comparison of a linear vs. non-linear model fitted into the observed data. The open circles are the observed mortality rate expressed in the terms of the negative natural log of the proportion of the larvae survived from E. maimaiga infection. The dotted lines are the linear model predictions and the dark solid line is the non-linear model fitted to the observed data.

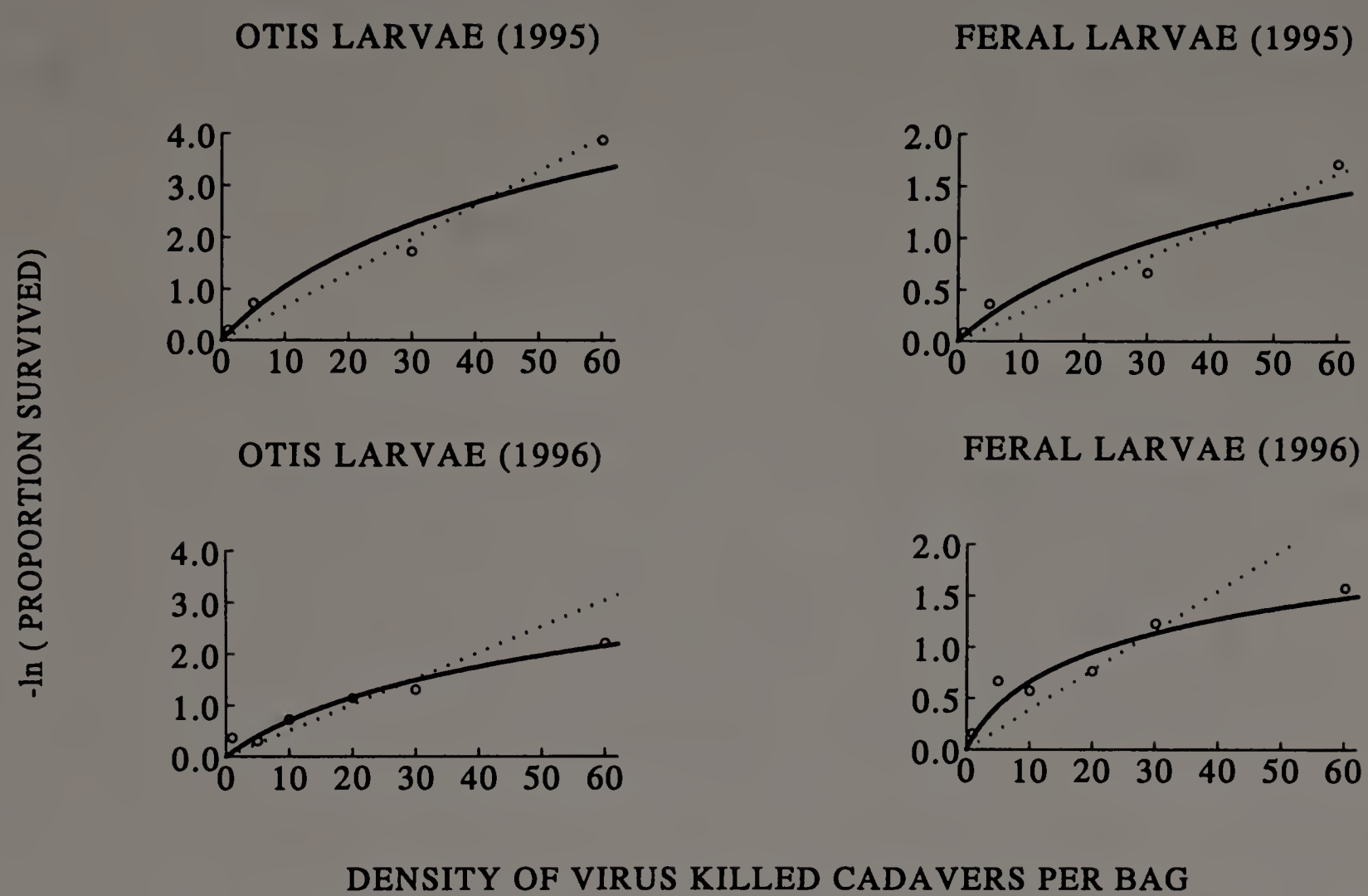

Fig. 4.3. LdNPV-induced mortality among the feral and Otis larvae exposed to different densities of LdNPV killed cadavers and comparison of a linear vs. non-linear model fitted into the observed data. The open circles are the observed mortality rate expressed in the terms of the negative natural log of the proportion of the larvae survived from LdNPV infection. The dotted lines are the linear model predictions and the dark solid line is the non-linear model fitted to the observed data.

CONCLUSION

The population dynamics of gypsy moth are driven by a complex of environmental factors including the naturally occurring pathogens. LdNPV has long been considered the most important naturally occurring pathogen that causes the collapse of high density gypsy moth populations. Recent discovery of a new fungal gypsy moth pathogen, Entomophaga maimaiga among North American gypsy moths has created a new interest and concern about its impact on the epizootiology of LdNPV. An understanding of interactions between these pathogens will be necessary for the successful use of these pathogens in the gypsy moth pest management programs.

In the laboratory studies, when I inoculated gypsy moth larvae with both pathogens simultaneously, I found that the fungus is more efficient in killing the larvae because of its shorter incubation period compared to that of the virus. However, the fungus-induced mortality depends upon the temperature and moisture or rainfall, whereas, virus-induced mortality is almost independent of abiotic environmental factors. If fungal protoplasts were inoculated later than the virus, the mortality of the gypsy moth larvae from LdNPV occurred 1-2 days earlier than when the larvae were inoculated with virus alone. In the presence of fungus, a lower lethal dose of LdNPV was required to kill the larvae, and many of these dually inoculated larvae died producing the visible evidence of virus occlusion bodies or a combination of virus and fungus propagules. Although, there was higher mortality among the dually inoculated larvae, the virus progeny production among the cadavers of these larvae was lower than in the cadavers of the insects inoculated with LdNPV alone.

In small-scale field experiments, conducted in mesh-bags, I demonstrated that fungus-induced mortality increased significantly with the addition of artificial rain and also there was a weak fungus pathogen density-dependent larval mortality. I was

unable to show a clear density dependent mortality with the virus densities I used in this experiment. When both pathogens were present in the same mesh-bag, the total gypsy moth mortality was higher only in two treatments - one in which the bags were secured from any natural or artificial rainfall or when there was a higher density of both pathogens. I used the infection rates based upon the assumptions of the proportional hazards to estimate the mortality of gypsy moths caused by co-infections of LdNPV and E. maimaiga. These estimates showed that there is a higher rate of co-infections in the populations than in the observed co-infected larvae.

Modeling is becoming a popular tool for the estimation of the effects of pathogens or parasitoids on the host populations. I estimated some of the critical parameters for a host-pathogen model and incorporated those parameters in the model to predict the impact of the E. maimaiga on LdNPV-induced mortality in a naturally occurring gypsy moth population of the central Massachusetts. I estimated egg mass density, egg hatch rate, initial virus load and weekly census of larval mortality covering two gypsy moth larval seasons. I found out that the LdNPV mortality rate was not affected by the presence of E. maimaiga, at least in the moderately dense gypsy moth population. This occurred because the E. maimaiga mortality became highest only at the end of the larval season. The virus mortality is initiated by the consumption of virus contaminated egg chorion and foliage by the early instars. As I had only a moderate density of gypsy moth larvae, I did not have enough larvae that would have died from virus when they were young and become the source of inoculum for other susceptible larvae to create the "second wave" of virus mortality in the study populations. Using a host-pathogen simulation model, I showed that the rate of virus mortality would not be different in the current density of gypsy moth larvae, even if there was an absence of fungus. In the second experimental year , the fungus killed more than 80% of the larvae and the total egg mass counts in the subsequent year was very low.

In another study, I demonstrated that both feral and laboratory strain of gypsy moths showed greater variation in susceptibility to LdNPV than to E. maimaiga. Recent theoretical work shows that high variation in susceptibility causes weaker density dependence in host-pathogen systems. Since we found low variation in susceptibility of E. maimaiga compared to LdNPV, host heterogeneity can not explain the occurrences of fungal epizootics in both low and high density gypsy moth populations.

Many ecological factors affect the interaction of two or more pathogens in the same host. Although, there are several questions to be answered to understand the transmission of both virus and fungus, I hope that this study will open many avenues for further research on the ecology of these pathogens and that the results from this dissertation will be useful in gypsy moth management using the microbes.

REFERENCES CITED

Abbott, W.S. 1925. A method of computing the effectiveness of an insecticide. J. Econ. Entomol. 18: 265-267.

Agra Gothama, A.A., P.P. Sikorowski, and G.W. Lawrence. 1995. Interactive effects of Steinernema carpocapsae and Spodoptera exigua larvae. J. Invertebr. Pathol. 66: 270-276.

Akaike, H. 1973. Information theory and an extension of the maximum likelihood principle. pp 267-281. In B.N. Petrov and F. Csaki [eds.], Second International Symposium on Information Theory and Control. Akademiai Kiado, Budapest.

Anderson, R.M. and R.M. May. 1979. Population biology of infectious diseases: Part I. Nature. 280: 361-367.

Anderson, R.M. and R.M. May. 1980. Infectious diseases and population cycles of forest insects. Science. 210: 658-661.

Anderson, R.M. and R.M. May. 1981. The population dynamics of microparasites and their invertebrate hosts. Philosophical Transactions of the Royal Society, B, 291: 451-524.

Anderson, R.M. and R.M. May. 1992. Social heterogeneity and sexually transmitted diseases. pp 228-303. In "Infectious diseases of humans: dynamics and control." Oxford University Press.

Anderson, R.M., V. Capasso, A.D. Cliff, K. Dietz, F. Fenner, R.N. T-W-Fiennes, Z. Grossman, H. Knolle, P.G. Mann, L. Molineaux, G. Schad, and D. Schenzle. 1982. pp 76-85. Transmission: patterns and dynamics of infectious diseases, group report. In Anderson, R.M. and R.M. May, [eds.], Population biology of infectious disease. Springer-Verlag, NY.

Andreadis, T.G. and R.M. Weseloh. 1990. Discovery of Entomophaga maimaiga in North American gypsy moth, Lymantria dispar. Proc. Natl. Acad. Sci., USA. 87: 2461-2465.

Balazy, S. 1993. "Flora of Poland: Fungi (Mycota)," Vol. XXIV, Entomophthorales. Polisi Academy of Sciences, W. Szafer Institute of Botany, Krakow.

Barbercheck, M.E. and H.K. Kaya. 1990. Interactions between Beauveria bassiana and the entomogenous nematodes, Steinernema feltiae and Heterorhabditis heliothis. J. Invertebr. Pathol. 55: 225-234.

Bari, M.A. and H.K. Kaya. 1984. Evaluation of the entomogenous nematode Neoaplectana carpocspsae (= Steirnernema feltiae) Weiser (Rhabditida: Steinernematidae) and the Bacterium Bacillus thuringiensis Berliner var. kurstaki for suppression of artichoke plum moth (Lepidoptera: Pterophoridae). J. Econ. Entomol. 77: 225-229.

Bell, R.A., C.D. Owens, M. Shapiro, and J.R. Tardiff. 1981. Mass rearing and virus production. pp 599-655. In C.C. Doane and M.L. McManus, [eds.]. The gypsy moth: research towards integrated pest management. U.S. For. Serv. Tech. Bull. 1584.

Benz, G. 1971. Synergism of micro-organisms and chemical insecticides. pp 327-355. In H.D. Burges and N.W. Hussey, [eds.], Microbial control of insects and mites. Academic Press.

Bliss, C. I. 1937. The calculation of the time-mortality curve. Ann. Appl. Biol. 24: 815-852.

Boucias, D.G., D.W. Johnson, and G.E. Allen. 1980. Effects of host age, Virus dosage and temperature on the infectivity of a nuclear polyhedrosis virus against velvet bean caterpillar, Anticarsia gemmatalis larvae. Environ. Entomol. 9: 59-61.

Bremermann, H.J. and J. Pickering. 1983. A game-theoretical model of parasite virulence. J. theo. Biol. 100: 411-426.

Buonaccorsi, J.P. and J.S. Elkinton. 1990. Estimation of contemporaneous mortality factors. Res. Popul. Ecol. 32: 151-171.

Burand, J.P., E.J. Park, and T.J. Kelly. 1996. Dependence of eddysteroid metabolism in host larvae on the time of baculovirus infection and the activity of the UDP-glucosyl transferase gene. Insect Biochemistry and Molecular Biology. 26: 845-852.

Burges, H.D. and E.M. Thompson. 1971. Standardization and assay of microbial insecticides. pp 591- 622. In H.D. Burges and N.W. Hussey, [eds.], Microbial control of insects and mites. Academic Press.

Campbell, R.W. 1963. The role of disease and desiccation in the population dynamics of the gypsy moth Porthetria dispar (L.) (Lepidoptera: Lymantriidae). Can. Entomol. 95: 426-434.

Campbell, R.W. 1967. The analysis of numerical change in gypsy moth populations. For. Sci. Monogr. 15.

Campbell, R.W. 1975. The bimodality of gypsy moth, Porthetria dispar (L.) (Lepidoptera: Lymantriidae) populations (abstract) J.N.Y. Entomol. Soc. 83: 287-88.

Campbell, R.W. 1976. Comparative analysis of numerically stable and violently fluctuating gypsy moth populations. Environ. Entomol. 5: 1218-1224.

Campbell, R.W. 1981. Population Dynamics. pp. 65-216. In C.C. Doane and M.L. McManus [eds.], The gypsy moth: research toward integrated pest management. U.S. For. Serv. Tech. Bull. 1584.

Campbell, R.W., D.L. Hubbard, and R.J. Sloan. 1975. Patterns of gypsy moth occurrence within a sparse and numerically stable population. Environ. Entol. 4: 535-542.

Campbell, R.W., R.J. Sloan, and C.E. Biazak. 1977. Sources of mortality among late instar gypsy moth larvae in sparse populations. Environ. Entomol. 6: 865-871.

Coffman, F.A., W.H. Tisdale, and J.F. Brandon. 1926. Observations on corn smut at Akron, Colorado. Agron- J. 18(5): 403-411.

Cossentine, J.E. and L.C. Lewis. 1988. Impact of Nosema pyrusta, Nosema sp., and a nuclear polyhedrosis virus on Lydella thompsoni within infected Ostrinia nubilalis hosts. J. Invertebr. Pathol. 51: 126-132.

D'Amico, V. and J.S. Elkinton. 1995. Rainfall effects on transmission of gypsy moth (Lepidoptera: Lymantriidae) nuclear polyhedrosis virus. Environ. Entomol. 24: 1144-1149.

D'Amico, V., J.S. Elkinton, G. Dwyer, J.P. Burand, and J.P. Buonaccorsi. 1996. Virus transmission in gypsy moths is not a simple mass action process. Ecology 77: 201-206.

D'Amico, V., J.S. Elkinton, G. Dwyer, R.B. Willis, and M.E. Montgomery. 1997. Foliage damage does not affect within-season transmission of an insect virus. Ecology (in press).

Doane, C.C. 1969. Trans-ovum transmission of a nuclear polyhedrosis virus in the gypsy moth and the inducement of virus susceptibility. J. Invertebr. Pathol. 14: 199-210.

Doane, C.C. 1970. Primary pathogens and their role in the development of an epizootic in the gypsy moth. J. Invertebr. Pathol. 15: 21-33.

Dwyer, G. 1991. The roles of density, stage and patchiness in the transmission of an insect virus. Ecology 72: 559-574.

Dwyer, G. and J.S. Elkinton. 1993. Using simple models to predict virus epizootics in gypsy moth populations. J. Ani. Ecol. 62: 1-11.

Dwyer, G., J.S. Elkinton, and J.P. Buonaccorsi. 1997. Host heterogeneity in susuceptibility and disease dynamics: test of a mathematical model. (submitted).

Elkinton, J.S. and A.M. Liebhold. 1990. Population dynamics of gypsy moth in North America. Ann. Rev. Entomol. 35: 571-596.

Elkinton, J.S., A.E. Hajek, G.H. Boettner, and E.E. Simons. 1991. Distribution and apparent spread of Entomophaga maimaiga (Zygomycetes: Entomophthorales) in gypsy moth (Lepidoptera: Lymantriidae) populations in North America. Environ. Entomol. 20: 1601-1605.

Elkinton, J. S., J.P. Buonaccorsi, T.S. Bellows, Jr., and R.G. van Driesche. 1992. Marginal attack rates, k-values and density dependence in the analysis of contemporary mortality factors. Res. Popul. Ecol. 34: 29-44.

Elkinton, J.S., W.M. Healy, J.P. Buonaccorsi, G.H. Boettner, A.M. Hazzard, H.R. Smith, and A.M. Liebhold. 1996. Interactions among gypsy moths, white-footed mice, and acorns. Ecology 77(8): 2332-2342.

Engelhard, E.K. and L.E. Volkman. 1995. Developmental resistance of fourth instar Trichoplusia ni orally inoculated with Autographa californica M Nuclear Polyhedrosis virus. Virology 209: 384-389.

Engelhard, E.K., L.N.W. Kam-Morgan, J.O. Washburn, and L.E. Volkman. 1994. The insect tracheal system: A conduit for the system spread of Autographa californica M Nuclear Polyhedrosis virus. Proc. Natl. Acad. Sci. USA 91: 3224-3227.

Entwistle, P.F., P.H.W. Adams, H.F. Evans, and C.F. Rivers. 1983. Epizootiology of a nuclear polyhedrosis virus (Baculoviridae) in European spruce sawfly (Gilpinia hercyniae): spread of disease from small epicenters in comparison with spread of baculovirus diseases in other hosts. J. Appl. Ecol. 20: 473-487.

Feng, M.G., J.B. Johnson, and S.E. Helbert. 1991. Natural control of cereal aphids (Homoptera: Aphididae) by entomopathogenic fungi (Zygomycetes: Entomophthorales) and parasitoids (Hymenoptera: Braconidae and Encyrtidae) on irrigated spring wheat in southwestern Idaho. Environ. Entomol. 10, 1699-1710.

Ferron, P. and P. Hurpin. 1974. Effects of simultaneous or successive contamination by Beauveria tenella and by Entomopoxvirus melolonthae of Melolontha melolontha larvae (Coleoptera: Scarabaeidae). Ann. Soc. ent. Fr. (N.S.) 10, 771-779.

Finney, D.J. 1971. Probit analysis. pp 20-49. Cambridge University Press.

Forsyth, K.P., R.F. Anders, J. Cattani, and M.A. Alpers. 1989. Small area variation in prevalence of an S-antigen serotype of Plasmodium falciparum in villages of Madang, Papua New Guinea. Am. J. trop. Med. Hyg. 40: 344-350.

Fuxa, J.R. 1979. Interactions of the microsporidium Vairimorpha necatrix with bacterium, virus, and fungus in Heliothis zea. J. Invertebr. Pathol. 33: 316-323.

Glaser, R.W. and J.W. Chapman. 1913. The wilt disease of gypsy moth caterpillars. J. Econ. Entomol. 6: 479-488.

Gould, J.R., J.S. Elkinton, and W.E. Wallner. 1990. Density-dependent suppression of experimentally created gypsy moth, Lymantria dispar (Lepidoptera: Lymantriidae), populations by natural enemies. J. Ani. Ecol. 59: 213-233.

Granados, R.R. and K.A. Lawler. 1981. In vivo pathway of infection of Autographa californica baculovirus invasion and infection. Virology 108: 297-308.

Haefner, J.W. 1996. Modeling biological systems, Principles and Applications. Chapman and Hall.

Hajek, A.E., R. A. Humber, J.S. Elkinton, B. May, S. R.A. Walsh, and J.S. Silver 1990a. Allozyme and restriction fragment length polymorphism analyses confirm Entomophaga maimaiga responsible for 1989 epizootics in North American gypsy moth populations. Proc. Natl. Acad. Sci., USA. 87: 6979-6982.

Hajek, A.E., R.I. Carruthers, and R.S. Soper. 1990b. Temperature and moisture relations of sporulation and germination by Entomophaga maimaiga (Zygomycetes: Entomophthoraceae), a fungal pathogen of Lymantria dispar (Lepidoptera: Lymantriidae). Environ. Entomo. 19: 85-90.

Hajek, A.E., R.A. Humber, and M.H. Griggs. 1990c. Decline in virulence of Entomophaga maimaiga (Zygomycetes: Entomophthorales) with repeated in vitro subculture. J. Invertebr. Pathol. 56: 91-97.

Hajek, A.E. and D.W. Roberts. 1991. Pathogen reservoirs as a biological control resource: introduction of Entomophaga maimaiga to North American gypsy moth, Lymantria dispar, populations. Biol. Control 1: 29-34.

Hajek, A.E., T.M. Butt, L.I. Strelow, and S.M. Gray. 1991. Detection of Entomophaga maimaiga (Zygomycetes: Entomophthorales) using enzyme-linked immunosorbent assay. J. Invertebr. Pathol. 57: 1-9.

Hajek, A.E. and D.W. Roberts. 1992. Field diagnosis of gypsy moth (Lepidoptera: Lymantriidae) larval mortality caused by Entomophaga maimaiga and the gypsy moth nuclear polyhedrosis virus. Environ. Entomol. 21: 706-713.

Hajek, A.E., R.I. Carruthers, T.M. Larkin, and R.S. Soper. 1993. Modelling the dynamics of Entomophaga maimaiga (Zygomycetes: Entomophthorales) epizootics in gypsy moth (Lepidoptera: Lymantriidae) populations. Environ. Entomol. 22: 1172-1187.

Hajek, A.E., R.A. Humber, and J.S. Elkinton. 1995. The mysterious origin of Entomophaga maimaiga in North America. American Entomologist. 41: 31-42.

Hajek, A.E. and M. Shimazu. 1996. Types of spores produced by Entomophaga maimaiga infecting the gypsy moth Lymantria dispar. Can. J. Bot. 74: 708-715.

Hajek, A.E., J.S. Elkinton, and J.J. Witcosky. 1996. Introduction and spread of the fungal pathogen Entomophaga maimaiga (Zygomycetes: Entomophthorales) along the leading edge of gypsy moth (Lepidoptera: Lymantriidae) spread. Environ. Entomol. 25: 1235-1247.

Harrap, K.A. 1972. The structure of Nuclear polyhedrosis virus. II. The virus particle. Virology 50: 124-132.

Hochberg, M.E. 1991. Non-linear transmission rates and the dynamics of infectious disease. J. theor. Biol. 153: 301-321.

Hochberg, M.E. and R.D. Holt. 1990. The coexistence of competing parasites. 1. The role of cross-species infection. Am. Nat. 136: 517-541.

Horton, H.M. and J.P. Burand. 1993. Saturable attachment sites for polyhedron-derived baculovirus on insect cells and evidence for entry via direct membrane fusion. J. Virol. 67: 1860-1868.

Hughes, P.R. 1991. ViStat, Statistical package for the analysis of baculovirus bioassay data. Boyce Thompson Institute, Ithaca, N.Y.

Johnson, D.A., J.R. Alldredge, and J.R. Allen. 1994. Weather and downy mildew epidemics of hop in Washington state. Phytopathology 84: 524-527.

Keating, S.T., J.P. Burand, and J.S. Elkinton. 1989. DNA hybridization assay for detection of gypsy moth nuclear polyhedrosis virus in infected gypsy moth (Lymantria dispar L.) larvae. Appl. Environ. Microbiol. 55: 2749-2754.

Keating, S.T., J.S. Elkinton, J.P. Burand, J.D. Podgwaite, and C.S. Ferguson. 1991. Field evaluation of a DNA hybridization assay for nuclear polyhedrosis virus in gypsy moth (Lepidoptera: Lymantriidae) larvae. J. Econ. Entomol. 84: 1329-1333.

Kleinbaum, D.G. and L.L. Kupper. 1978. Applied regression analysis and other multivariable methods. Duxbury Press.

Knell, R.J., M. Begon, and D.J. Thompson. 1996. Transmission dynamics of Bacillus thuringiensis infecting Plodia interpunctella: a test of the mass action assumption with an insect pathogen. Proc. R. Soc. Lond. B. 263: 75-81.

Koppenhofer, A.M. and H.K. Kaya. 1997. Additive and synergistic interaction between entomopathogenic nematodes and Bacillus thuringiensis for scarab grub control. Biological Control. 8: 131-137.

Koyama, R. and K. Katagiri. 1967. Abst. Proc. Joint U.S.-Japan Semin. Microbial control of insect pests. U.S.- Japan Committee Sci. Cooperation. Panel 8. Fukuoda. pp 53-54. (Cited in A. Krieg 1971. Interactions between pathogens. In H.D. Burges and N.W. Hussey, [eds.], Microbial control of insects and mites. Academic press.

Lance, D.R., J.S. Elkinton, and C.P. Schwalbe. 1987. Behavior of late-instar gypsy moth larvae in high and low density populations. Ecol. Entomol. 12: 267-273.

Levin, S. and D. Pimentel. 1981. Selection of intermediate rates of increase in parasite-host systems. Am. Nat. 117: 308-315.

Liu, W.M., H.W. Hethcote, and S.A. Levin. 1987. Dynamical behavior of epidemiological models with nonlinear incidence rate. J. Math. Biol. 25: 359-380.

McManus, M.L. and H.R. Smith. 1984. Effectiveness of artificial bark flaps in mediating migration of late instar gypsy moth larvae. U.S. Dept. Agric. For. Serv. Res. NE-16.

Murphy, F.A., C.M. Fauquet, D.H.L. Bishop, S.A. Ghabrial, A.W. Jarvis, G.P. Martelli, M.P. Mayo, and M.D. Summers. 1995. Virus taxonomy: classification and nomenclature of viruses. Sixth report of the international committee on taxonomy of viruses. Springer-Verlag, New York.

Murray, K.D. and J.S. Elkinton. 1989. Environmental contamination of egg masses as a major component of transgenerational transmission of gypsy moth nuclear polyhedrosis virus (LdMNPV). J. Invertebr. Pathol. 53: 324-334.

Murray, K.D. and J.S. Elkinton. 1990. Transmission of nuclear polyhedrosis virus to gypsy moth (Lepidoptera: Lymantriidae) eggs via contaminated substrates. Environ. Entomol. 19: 662-665.

Murray, K.D. and J.S. Elkinton. 1992. Vertical distribution of nuclear polyhedrosis virus infected gypsy moth (Lepidoptera: Lymantriidae) larvae and effects on sampling for estimation of disease prevalence. J. Econ. Entomol. 85: 1865-1872.

National Oeanic and Atmospheric Administration (NOAA). 1964-1994. Climatological Data. Massachusetts.

Nowak, M.A. and R.M. May. 1994. Superinfection and the evolution of parasite virulence. Proc. R. Soc. London Ser. B. 255: 81-89.

Park, E., J.P. Burand, and C.M. Yin. 1993. The effect of baculovirus infection on ecdysteroid titer in gypsy moth larvae (Lymantria dispar). J. Insect Physiol. 39: 791-796.

Podgwaite, J.D., K.S. Shields, R.T. Zerillo, and R.B. Bruen. 1979. Environmental persistence of the necleopolyhedrosis virus of the gypsy moth, Lymantria dispar. Environ. Entomol. 8: 528-536.

POLO-PC. A user's guide to Probit or Logit analysis. LeOra Software. 1987. Berkeley, CA.

Ritter, K.S. and Y. Tanada. 1978. Interference between two nuclear polyhedrosis viruses of the armyworm, Pseudoletia unipuncta. Entomophaga. 23: 349.

Royama, T. (1981). Evaluation of mortality factors in insect life table analysis. Ecol. Monogr. 51: 495-505.

Shapiro, M., R.A. Bell, and C.D. Owens. 1981a. In vivo mass production of gypsy moth nucleopolyhedrosis virus. pp 633-655. In C.C. Doane and M.L. McManus, [eds.]. The gypsy moth: research towards integrated pest management. U.S. For. Serv. Tech. Bull. 1584.

Shapiro, M., C.D. Owens, R.A. Bell, and H.A. Wood. 1981b. Simplified, efficient system for in vivo mass production of the gypsy moth nucleopolyhedrosis virus. J. Econ. Entomol. 74: 341-343.

Shapiro, M., J.R. Robertson, and R.A. Bell. 1986. Quantitative and qualitattive differences in gypsy moth (Lepidoptera: Lymantriidae) nucleopolyhedrosis virus produced in different-aged larvae. J. Econ. Entomol. 79:1174-1177.

Shimazu, M. and R.S. Soper. 1986. Pathogenicity and sporulation of Entomophaga maimaiga Humber, Shimazu, Soper & Hajek (Entomophthorales: Entomophthoraceae) on larvae of the gypsy moth, Lymantria dispar L. (Lepidoptera: Lymantriidae). Appl. Ent. Zool. 21: 589-596.

Smitley, D.R., L.S. Bauer, A.E. Hajek, F.J. Sapio, and R.A. Humber. 1995. Introduction and establishment of Entomophaga maimaiga, a fungal pathogen of gypsy moth (Lepidoptera: Lymantriidae) in Michigan. Environ. Entomol. 24: 1685-1695.

Sokal, R.R. and F.J. Rohlf. 1981. Biometry the principles and practice of statistics in biological research. W.H. Freeman & Co., N.Y.

Soper, R.S., M. Shimazu, R.A. Humber, M.E. Ramos, and A.E. Hajek. 1988. Isolation and characterization of Entomophaga maimaiga sp. nov., a fungal pathogen of gypsy moth, Lymantria dispar, for Japan. J. Invertebr. Pathol. 51: 229-241.

Statistix 4.0. User's Manual. 1992. Analytical Software. Talahassee, FL.

Tanada, Y. 1959. Synergism between two viruses of the armyworm, Pseudaletia unipuncta (Haworth) (Lepidoptera: Noctuidae). J. Invertebr. Pathol. 1: 215-231.

Tanada, Y. and J.R. Fuxa. 1987. The pathogen population. pp 113-157. In J.R. Fuxa and Y. Tanada [eds.], Epizootiology of Insect. John Wiley & Sons.

Thompson, C.G. 1978. Nuclear polyhedrosis epizootiology. pp124-138. In Brookes, M.R., R.W. Stark and R.W. Campbell (eds.), The Douglas-fir tussock moth: a synthesis. USDA Tech. Bull. 10585, USDA, Washington, D.C.

Timper, P. and B.B. Brodie. 1995. Interaction of the microbivorous nematode Teratorhabditis dentifera and the nematode - pathogenic fungus Hirsutella rhossiliensis. Biol. Cont. 5: 629-635.

Vandenberg, J.D. and RS Soper. 1978. Prevalence of Entomophthorales mycoses in populations in spruce budworm, Choristoneura fumiferana. Environ. Entomol. 7: 847-853.

Volkman, L. K. Storm, V. Aivazachvili, and D. Oppenheimer. 1995. Overexpression of actin in AcMNPV-infected cells interferes with polyhedrin synthesis and polyhedra formation. 1996. Virology 225: 369-376.

Weseloh, R.M. and T. G. Andreadis. 1992 a. Epizootiology of the fungus Entomophaga maimaiga, and its impact on gypsy moth populations. J. Invertbr. Pathol. 59: 133-141.

Weseloh, R.M. and T. G. Andreadis. 1992 b. Mechanisms of transmission of the gypsy moth (Lepidoptera: Lymantriidae) fungus, Entomophaga maimaiga (Entomophthorales: Entomophthoraceae) and effects of site conditions on its prevalence. Environ. Entomol. 21: 901-906.

Weseloh, R.M., T. G. Andreadis, and D.W. Onstad. 1993. Modeling the influence of rainfall and temperature on the phenology of infection of gypsy moth, Lymantria dispar, larvae by the fungus Entomophaga maimaiga. Biol. Cont. 3: 311-318.

Weseloh, R.M. and T.G. Andreadis. 1997. Persistence of resting spores of Entomophaga maimaiga, a fungal pathogen of the gypsy moth, Lymantria dispar. J. Invertebr. Pathol. 69, 195-196.

Woods, S.A. and J.S. Elkinton. 1987. Bimodal patterns of mortality from nuclear polyhedrosis virus in gypsy moth (Lepidoptera: Lymantriidae) populations. J. Invertebr. Pathol. 50: 151-157.

Woods, S.A., J.S. Elkinton, and M. Shapiro. 1988. Effects of Bacillus thuringiensis treatments on the occurrence of nuclear polyhedrosis virus in gypsy moth (Lepidoptera: Lymantriidae) populations. J. Econ. Entomol. 81: 11706-1714.

Woods, S., J.S. Elkinton, K.D. Murray, A.M. Liebhold, J.R. Gould, and J.D. Podgwaite. 1991. Transmission dynamics of a nuclear polyhedrosis virus and predicting mortality in gypsy moth (Lepidoptera: Lymantriidae) populations. J. Econ. Entomol. 84: 423-430.

Woolhouse, M.E.J., C.H. Watts, and S.K. Chandiwana. 1991. Heterogeneities in transmission rates and the epidemiology of schistosome infection. Proc. R. Soc. Lond. B. 109-114.

Yerger, E.H. and M. Rossiter. 1996. Natural causes and rates of early larval mortality in gypsy moths (Lepidoptera: Lymantriidae) sampled from field populations in different density states. Environ. Entomol. 25: 1002-1011.