THE ECOLOGY OF NUCLEOPOLYHEDROVIRUS TRANSMISSION IN THE GYPSY MOTH (*LYMANTRIA DISPAR*)

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Insect nucleopolyhedroviruses (NPVs) are among the most important pathogens that regulate insect populations. Although these pathogens have been the subject of a great deal of research, many aspects of their transmission remain poorly understood. The transmission of NPV is controlled by a complex set of interactions between host, virus, and the environment. I investigated the effects of three different factors on the transmission of LdNPV in the gypsy moth, (*Lymantria dispar*), using laboratory and field experiments, DNA analysis, and mathematical modeling. The three effects examined were: 1) the effect of density-dependent disease resistance, 2) the effect of vectoring by avian predators, and 3) the potential occurrence of vertical transmission of the virus. My main results are as follows: 1) The resistance of gypsy moth larvae to NPV decreased as larval density increased, perhaps due to the stress associated with high densities, and this relationship is predicted to have a stabilizing effect on population dynamics. 2) In aviary cage experiments, bird species showed differential effectiveness in spreading the virus from infected to healthy larvae on a local scale. This difference was due more to differences in prey processing behaviors among the birds than to differences in the amount of NPV or speed at which NPV passed through the bird guts. The highest level of transmission was achieved by red-eyed vireos, largely because of their strategy of beating larvae against a branch before ingestion, resulting in rupture of the larval cuticle and scattering of viral occlusion bodies.

3) We found no evidence for vertical transmission of lethal NPV infection in the gypsy moth. Although low levels of virus were detected in the tissues of adult female moths by real-time PCR, there was no organ that showed consistently positive levels of NPV. Furthermore, the putative virus from female tissues was not infectious to larvae in our studies, and therefore may represent either latent virus or non-occluded virions. Together, these results contribute significantly to our understanding of the processes governing NPV transmission in gypsy moths and are directly relevant to the host-pathogen dynamics of many other systems.

BIOGRAPHICAL SKETCH

James Reilly was born in Richmond, VA on October 29, 1980, the eldest of three children. He spent his childhood busily catching frogs, exploring the marsh near his home, and raising caterpillars. His parents encouraged him in all his pursuits, and his earliest memory is sitting patiently with his mother, waiting for a muskrat to come back to its hole. He has very broad interests, and a fascination with all aspects of the natural world, but he retains a special fondness for the natural history of the Virginia piedmont and mountains where he grew up. For James, studying ecology and evolutionary biology is not simply a career, but a way of life. He is very interested in birds, and has extensive experience identifying them by sight and song and assisting his father, Bob Reilly, with bird-banding operations in Virginia. However, he also indulges a healthy obsession with observing and photographing moths, and has learned to recognize at least a thousand species by sight. This combination of interests is unusual, since birders often get up at dawn and moth-ers often stay up all night! In the rest of his spare time, James enjoys filming wildlife, measuring big trees, and canoeing (even at night). He is an avid catch-and-release fisherman, an amateur carpenter and electrician, and a web programmer for his website mothguide.com. There was never any question that James would become a biologist (except in middle school, when he wanted to be a cartographer). He graduated from high school as valedictorian in 1999, and enrolled in the biology program at the College of William and Mary in Williamsburg, VA. As an undergraduate, he worked with Shandelle Henson on the mathematical modeling of insect population dynamics. He met his future wife Sarah through their shared biology classes at William and Mary. James graduated in May 2002, promptly got married, and joined the Entomology department at Cornell University that fall. His research has focused on disease ecology and

investigating the interactions between the gypsy moth, its nucleopolyhedrovirus, and its avian predators. He and his wife Sarah (also a graduate student at Cornell) had a baby boy, Sam, in September 2007, and are expecting a second boy in December 2008. James is actively applying for jobs, but for now he is spending a lot of time reading books about trucks and playing with blocks while his wife finishes her own dissertation.

Dedicated to Sarah, Mom, and Dad

ACKNOWLEDGMENTS

A Ph.D. takes a long time and a lot of work. Luckily, help and support have come readily from all sides. I would first like to thank my advisor, Ann Hajek, without whom this project would not have been possible, and who has provided tireless encouragement, excellent advice, and a smooth-running lab with a gypsy moth colony. Thanks are also due to the other two members of my committee, Steve Ellner and Gary Blissard, who have been extremely helpful and constructive during this whole process. There are scores of other people and funding sources who have been instrumental to this project and my development as a scientist, including the past and present members of the Hajek lab, and many others. For a complete accounting, please refer to the acknowledgements section at the end of each chapter. Perhaps most of all, I would like to thank my wife, Sarah, who enrolled in a Ph.D. program at the same time I did. We worked together to figure this thing out at every step of the way and by now we both feel like we've done two Ph.D.s. Also, I owe a lot to my dad for a long correspondence through which I was able to flesh out many of my ideas, and who has been there for me every time. I should also thank my one-year-old son, Sam, who sometimes played by himself or took decent naps so I could finish writing. At this point, I would also like to say no thanks to six long New York winters, which have been comparable to Napoleon's invasion of Russia in the eyes of this southern boy, and which I probably wouldn't even mention if it weren't so cold outside right now.

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PREFACE

The gypsy moth (*Lymantria dispar*) is one of the most important forest defoliators in eastern North America. It was introduced from Europe to Massachusetts in 1869 and has since spread south to Virginia and west to Wisconsin (Wu 2008). The preferred food of the larvae is oak, but they also feed on the leaves of many other trees. In the Northeast, larvae hatch in late April to mid May, feed throughout the spring and early summer, and pupate in late June and July. In North America and Europe, females of the gypsy moth are flightless (though females of the Asian subspecies fly).

Gypsy moth larvae are covered in stiff hairs, which are thought to be a deterrent to predators. However, there are several native North American birds that readily prey on the larvae, including vireos, catbirds, chickadees, cuckoos, orioles, and blue jays (Forbush and Fernald 1896). Mice are effective predators of the pupae (Smith and Lautenschlager 1981). However, the most important regulators of gypsy moth populations are its two major diseases, a virus and a fungus. The *Lymantria dispar* nucleopolyhedrovirus (LdNPV) has been present in North American gypsy moth populations since at least 1907, and was probably introduced with parasitoids released for biocontrol (Hajek et al 2005). NPV is thought to be most important when larvae reach high densities. The fungus, *Entomophaga maimaiga*, is a relatively recent arrival, having just appeared in North America in 1989 (Hajek 1999). *E. maimaiga* has since been responsible for a large portion of larval mortality throughout the gypsy moth's range.

For my dissertation research, I investigated three different aspects of the transmission of LdNPV to gypsy moth and explored the effects of different avenues of transmission on host-pathogen dynamics. The three factors forming the basis of the

three chapters in the dissertation are 1) density-dependent disease resistance, 2) vectoring by avian predators, and 3) vertical transmission of the virus.

Nucleopolyhedroviruses (NPVs) are double-stranded DNA viruses that primarily infect the larvae of leaf-feeding Lepidoptera and sawflies. Generally, different strains of NPV are host-specific and infect only one or a few closely related species. This holds true for LdNPV, *Lymantria dispar* nucleopolyhedrovirus, which infects only the gypsy moth. When a larva dies from NPV infection, the external cuticle of the cadaver ruptures and millions of viral occlusion bodies (OBs) are released. Occlusion bodies (OBs) are proteinaceous structures that encase and protect the virions from degradation in the environment, and are the primary means of horizontal transmission of the disease. Typically, the OBs stick to the surfaces of leaves where they may be ingested by healthy larvae. Infection begins when the occlusion bodies dissolve in the alkaline gut and the virions infect the midgut cells of the new host. In gypsy moth larvae, infection spreads throughout the insect and kills it within 1 to 3 weeks.

Interestingly, the susceptibility of larvae to NPV is influenced by both developmental and environmental factors. One well-known phenomenon is the increase in resistance to infection with progressive larval instars (e.g. Boucias and Nordin 1977). Resistance may even change within an instar (Grove and Hoover 2007). There is also an expanding research interest in the effect of host density on resistance to disease. In some systems, host resistance has been found to increase as a plastic response to high host density (e.g. Wilson and Reeson 1998). In other systems, resistance may decrease as a result of the stress associated with high host density (e.g. Reilly and Hajek 2008), or there may be no relationship (e.g. Pie et al 2005). Since NPV transmission is density-dependent (transmission increases with both host and pathogen density) (e.g. d'Amico et al 1996), the relationship between host density and

resistance to disease is particularly relevant to population dynamics. I investigated the relationship between host density and resistance of *L. dispar* to LdNPV, and explored the implications for population dynamics in a mathematical model. These studies are described in Chapter 1.

Although there is no evidence for biological vectoring of NPVs (where the vectored pathogen reproduces in the body of the vector), the durability of the occlusion body creates the opportunity for mechanical vectoring by a wide variety of organisms including predators such as birds and mammals (e.g. Lautenschlager and Podgewaite 1979), and parasitoids that could spread NPV on their ovipositors (e.g. Raimo et al 1977). By eating larvae and passing intact OBs through their guts, predators such as birds may play a major role in NPV transmission through long-distance dispersal (e.g. Entwistle et al 1993), and by spreading NPV on a local scale. I investigated the relative ability of three important species of bird predators to spread LdNPV by passing it through their guts and by breaking open infected larvae during prey processing. These studies are described in Chapter 2.

NPV does not require a vertical transmission pathway for successful persistence across generations, since the OBs generated from dead larvae remain infectious over the following seasons (e.g Thompson et al 1981). Nevertheless, there is accumulating evidence that vertical transmission of NPV occurs in some systems, and may play an important role in disease dynamics. In the gyspy moth, the literature on vertical transmission of LdNPV is especially unclear, although this issue is critical to understanding the initiation of epizootics. I describe my investigation of the potential for vertical transmission of LdNPV in the gypsy moth in Chapter 3.

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CHAPTER 1

DENSITY-DEPENDENT RESISTANCE OF THE GYPSY MOTH *LYMANTRIA DISPAR* TO ITS NUCLEOPOLYHEDROVIRUS, AND THE CONSEQUENCES FOR POPULATION DYNAMICS

Abstract

The processes controlling disease resistance can strongly influence the population dynamics of insect outbreaks. Evidence that disease resistance is density-dependent is accumulating, but the exact form of this relationship is highly variable from species to species. It has been hypothesized that insects experiencing high population densities might allocate more energy to disease resistance than those at lower densities, because they are more likely to encounter density-dependent pathogens. In contrast, the increased stress of high-density conditions might leave insects more vulnerable to disease. Both scenarios have been reported for various outbreak Lepidoptera in the literature. We tested the relationship between larval density and disease resistance with the gypsy moth (*Lymantria dispar*) and one of its most important density-dependent mortality factors, the nucleopolyhedrovirus (NPV) LdNPV, in a series of bioassays. Larvae were reared in groups at different densities, fed the virus individually, and then reared individually to evaluate response to infection. In this system, resistance to the virus decreased with increasing larval density. Similarly, time to death was faster at high densities than at lower densities. Implications of density-resistance relationships for insect-pathogen population dynamics were explored in a mathematical model. In general, an inverse relationship between rearing density and disease resistance has a stabilizing effect on population dynamics.

Introduction

Pathogens are some of the most important agents in the regulation of outbreaking insect populations. This regulation is achieved through density-dependent transmission processes, whereby higher host density translates to higher probability of infection (Anderson and May 1981). Thus, an insect's level of resistance to a pathogen may be more important at high density than at low density. Indeed, there is evidence that lepidopteran larvae of gregarious species tend to have more virus resistance than solitary species (Hochberg 1991a). Especially in species capable of outbreaking, any individual larva could experience very different conditions depending on whether the population is at high or low density.

For species that exhibit such fluctuations in density, there are two hypotheses that predict the relationship between population density and disease resistance. It has been hypothesized that insects experiencing high population densities might allocate more energy to disease resistance than those at lower densities, because they are more likely to encounter density-dependent pathogens (Kunimi and Yamada 1990; Goulson and Cory 1995; Reeson et al. 1998). Alternatively, the increased stress associated with high density conditions might compromise the insect immune system and leave the insect more vulnerable to disease (Steinhaus 1958).

Recently, much evidence has been accumulating in support of the first hypothesis, termed density-dependent prophylaxis (Wilson and Reeson 1998), whereby resistance to disease increases as insect density increases. This evidence comes mainly from bioassays in which larvae are reared at different densities, then checked for resistance level by exposure to a pathogen. Insects reported to show such a response are the African armyworm (*Spodoptera exempta*) to *S. exempta* NPV (Reeson et al. 1998; 2000) and to eggs of the ectoparasitoid *Euplectrus laphygmae* (Wilson et al. 2001), the Egyptian cotton leafworm (*Spodoptera litturalis*) to

Beauvaria bassiana (Wilson et al. 2001), the Oriental armyworm (*Mythimna separata*) to *M. separata* NPV and *Mythimna unipuncta* granulovirus (Kunimi and Yamada 1990), the cabbage armyworm (*Mamestra brassicae*) to *M. brassicae* NPV (Goulson and Cory 1995), the desert locust (*Schistocerca gregaria*) to *Metarhizium anisopliae* var. acridum (Wilson et al. 2002), and the mealworm beetle (*Tenebrio molitor*) to *M. anisopliae* (Barnes and Siva-Jothy 2003). Among insects exhibiting this type of response, high density often seems to be associated with other characteristics such as cuticular melanization, smaller size, and faster development. Melanism in particular may be closely linked to heightened disease resistance, possibly through the phenoloxidase system (e.g., Wilson et al. 2001; Armitage and Siva-Jothy 2005). However, wild-caught individuals of the yellow form of the mountain stone weta *Hemideina maori* were found to have greater immune response to injection with Sephadex beads than individuals of the black form, which occurred at lower population densities (Robb et al. 2003).

Under the stress hypothesis, resistance should decrease with increasing density. Evidence for this relationship was first reported by Steinhaus (1958) from experiments on the resistance of the alfalfa caterpillar (*Colias philodice*) and the buckeye caterpillar (*Junonia coenia*) to natural pathogens. Many subsequent studies have also shown that crowded insects are more likely to become diseased, but this pattern has rarely been tested in a way that separates density-dependent transmission from density-dependent resistance. Tests of the stress hypothesis require that the insects are kept isolated after exposure to the pathogen.

In fact, the density-dependent prophylaxis and stress hypotheses are not mutually exclusive. In *M. brassicae*, Goulson and Cory (1995) found that resistance to *M. brassicae* NPV rose from low to medium densities, but dropped again at the highest density. Another possibility for some species is that there is no relationship

between density and resistance to a particular disease. A recent experimental paper reported this result for the relationship between the damp wood termite (*Zootermopsis angusticollis*) and *M. anisopliae* (Pie et al. 2005). Indeed, a density-independent relationship is often assumed in mathematical models of disease transmission (e.g., Dwyer et al. 1997, 2000; but see White and Wilson 1999; Hochberg 1991b).

We tested the relationship between larval density and disease resistance for the gypsy moth (*Lymantria dispar*) and its nucleopolyhedrovirus (NPV) LdNPV, in a series of bioassays. We present the results of these bioassays, interpret them as evidence favoring one of the competing hypotheses discussed above (see Figure 1.1), and contrast the implications of the supported hypothesis against those of the competing hypotheses using a mathematical model of host–pathogen dynamics.

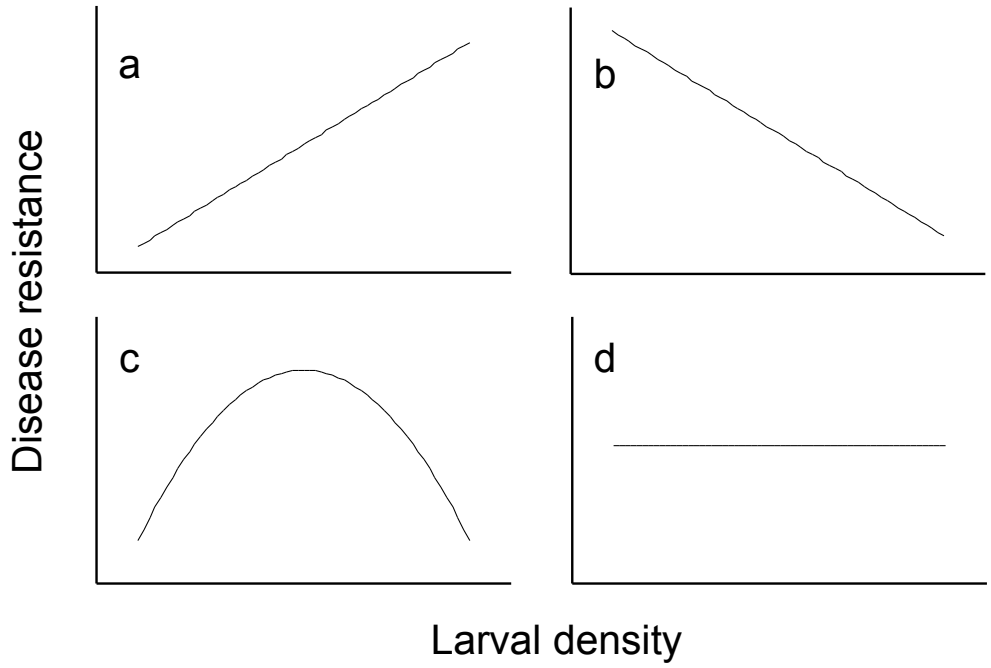

Figure 1.1. Competing hypotheses for the relationship between larval host density and resistance to disease. a) density-dependent prophylaxis b) stress-related decline in immune function c) combination of the previous hypotheses d) density-independent relationship

Methods

Rearing of larvae

Gypsy moth neonates and egg masses of the New Jersey Standard Strain were obtained from the USDA-APHIS, Otis Methods Development Center. All larvae were reared on artificial diet (Bell et al 1981), and all studies were conducted at 25^{o}C. Larvae were maintained in groups of approximately 100 in 240 ml plastic cups with cardboard lids at 10^{o}C up to the 2nd instar and then transferred to 30 ml clear plastic cups containing diet with translucent lids and maintained at particular densities (from 1 to 20 per 30 ml cup). Larvae were reared at the required densities until the 3rd or 4th instar (approximately 7 days). In nature, densities of 4th instar gypsy moths can range from 0 to 10^{7} larvae/ha (Campbell 1978). While we wanted to represent the natural range of caterpillar densities in our experiments, natural densities are not directly comparable to cup densities because "crowding" encompasses many different related aspects, such as number of larvae per unit space, number of contacts per unit time, and degree of competition for food or molting and pupation sites, among other things. We chose 20 larvae per cup as our highest density because it is near the maximum number of larvae that can be reared to the 4th instar in a 30 ml cup without significant mortality and without running out of food. At this density, the 4th instar larvae cannot avoid frequent contact, which we suggest is consistent with conditions larvae would experience during a severe outbreak.

Virus inoculation

The virus used in all studies was the Hamden, CT strain of the *Lymantria dispar* nucleopolyhedrovirus (LdNPV) distributed as "Gypchek" by the USDA Forest Service. Virus suspension (3 µl of the desired concentration) was pipetted onto the surface of small squares (4×4×2 mm) of artificial diet. The inoculated squares were

offered individually to 3rd or 4th instar larvae in clean 30 ml plastic cups. This amount of diet could be easily consumed by a larva in a single day. Larvae that failed to eat the entire diet square within 24 hrs were not included in the experiments. All larvae from all cups were inoculated.

The effect of density on mortality rate

Larvae were reared at five densities ranging from low to high: 1, 5, 10, 15, and 20 larvae per 30 ml cup. Numbers of larvae reared at each density were: 90 larvae at densities 1, 5, 10, and 15 per cup, and 100 larvae at density 20 per cup. Larvae were not very crowded at the second instar in any treatment because larvae at this stage are very small, but larvae in the high-density treatments experienced extreme crowding by the end of the 3rd instar. Food was continually available to all larvae; no cups of larvae exhausted their food supply during the experiment. In this study, we chose to inoculate all larvae on the same day, regardless of whether they were in the 3rd or 4th instar. Since there is variation in development time, not all larvae of the same age will molt on the same day. If each larva is inoculated on the day it reaches the 4th instar, then those larvae reaching the 4th instar later may appear to be more resistant, if resistance increases with age, as is often reported (e.g., Boucias and Nordin 1977). On the other hand, if all larvae of a given age are inoculated on the same day, then not all larvae will be in the same instar. Using larvae of the same age (but not necessarily the same instar) more closely approximates the conditions of a natural epizootic, in which larvae are exposed to the pathogen without regard to instar.

Larvae were fed diet squares inoculated with 1×10^4 occlusion bodies (OBs) per larva, a dosage that consistently produced intermediate levels of mortality (30%-64%) during preliminary experiments. After consuming the inoculated diet squares, each larva was transferred to an individual 30 ml cup and monitored daily. The experiment

ended when all larvae had either pupated or died. Larvae were dissected to search for occlusion bodies at 400× with a phase-contrast compound microscope if cause of death was in doubt, and only larvae dying from viral infections were included in further analyses.

Data were analyzed by both categorical and continuous logistic regression as a generalized estimating equation (GEE) model with a binomial distribution and a logit link function (PROC GENMOD, SAS Institute, Inc.). We controlled for cup (within-cluster correlation) as a random effect. The GEE method allows for varying numbers of observations per cluster (cup) and is robust to small numbers of observations per cluster. The intra-cluster correlation coefficient for cup was estimated from a generalized linear mixed model with the XTLOGIT prodecure in STATA (StataCorp). Overall error rates for all pairwise comparisons were adjusted with a Bonferroni correction.

Effect of density on survival time and larval mass

A second set of experiments was conducted to investigate the effects of density on survival time of the virus and on larval mass. Larvae were reared in three densities ranging from low to high: 1, 10, and 20 larvae per 30 ml cup. Numbers of larvae reared at each density were: 45 larvae at density 1 per cup, 90 larvae at density 10 per cup, and 180 larvae at density 20 per cup. Over the treatment period, larvae likely experienced a transition from lower to higher crowding as the larvae in a cup grew, with the exception of larvae reared singly. Food was continually available to all larvae; no cups of larvae exhausted their food supply during the experiment.

In contrast to the previous study, we chose to inoculate all larvae within 24 hours after molting into the 4th instar. Thus, some larvae were slightly older than others at the time they were infected. However, waiting for each larva to reach the 4th

instar allowed us to compare the weights at a standardized point in development. Since weight is correlated with survival and fecundity (e.g. Hough & Pimentel 1978, Honek 1993), this measure gives us some idea of the physiological effects of the different densities. After weighing, larvae were fed diet squares inoculated with 5×10^6 OBs per larva, a dosage that was expected to produce nearly 100% mortality. Larvae were monitored for infection and diagnosed as in the previous study. Weight data were analyzed with a linear mixed model in PROC MIXED (SAS Institute, Inc.) with cup as a random effect. Means tests are least squares adjusted means comparisons (LSMEANS). Overall error rates were controlled with Tukey's method. Survival time data (censored at pupation) were analyzed with a categorical Cox regression model in PROC TPHREG (SAS Institute, Inc.). Overall error rates for relative risk comparisons were controlled with a Bonferroni adjustment. In addition, the Cox regression model was used to test the effect of larval weight on survival time of larvae within a density.

Mathematical Model

A simple mathematical model was developed to examine the potential implications for host-pathogen dynamics under competing hypotheses for the relationship between resistance and larval density. Model structure draws on within-season/between-season models of gypsy moth-virus interactions (Dwyer and Elkinton 1993, Dwyer et al 1997, Dwyer et al 2000), but with the probability of developing resistance to the virus modeled as a function of the larval density at the beginning of a season. An important distinction between the Dwyer et al (1997, 2000) models and the one employed here concerns the treatment of simple genetic variation in (heterogeneity of) larval susceptibility to its viral pathogen. The cited models treat individual susceptibility as determined at birth, heterogeneous across individuals, and

unresponsive to physiologically limiting environmental factors or their associated cues, including larval rearing density. With the purpose of focusing on potential density-dependent effects, our model specification simplifies the set of individual heterogeneity to two states, low or high resistance, and makes the determination of that resistance state a probabilistic function of larval rearing density. The change in "resistance" in this model is simply an alteration of the transmission efficiency of the pathogen, or in other words how strong an effect the virus density P has on the joint probability of exposure, infection, and death. We suggest that this definition of resistance is appropriately flexible, and can include behavioral resistance, midgut barriers to pathogen infection, as well as systemic resistance. Our model also includes a delay between the start of the season and the density-dependent, probabilistic development of the high resistance state. Consistent with Dwyer et al (2000), a delay between infection and death is incorporated as well. For simplicity, and again consistent with Dwyer et al (2000), within-season mortality from non-viral causes is ignored. The length of a single season is normalized to 1. There is no spatial clumping function (e.g. Briggs & Godfray 1996, White & Wilson 1999) in our model, and so the disease transmission probability is linear rather than sublinear with respect to pathogen density (see Figure 1.2a). Host and pathogen were defined as extinct if their value dropped below 0.01. Delay-differential equations were solved in MATLAB with the dde23 algorithm (The Mathworks, Inc., Natick, MA).

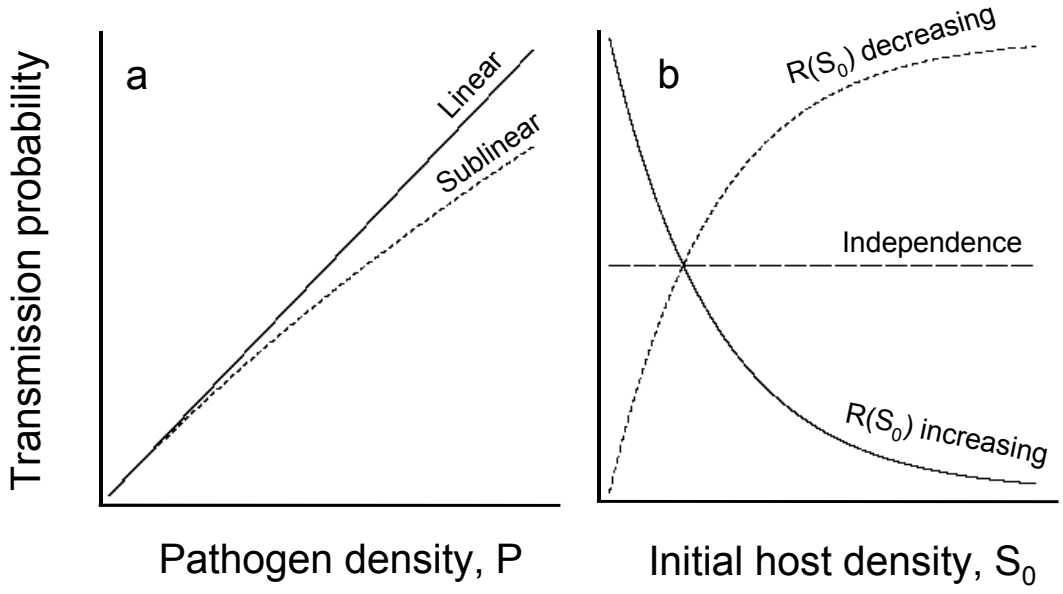

Figure 1.2. Disease transmission probability plotted against a) pathogen density showing linear transmission (as in our model, ——) and sublinear transmission (as in Briggs & Godfray 1996 baseline model, ------) and b) initial host density showing independence of transmission probability and initial larval density (as in our model with *c=d* and in the baseline model of Briggs & Godfray 1996, – – –); transmission probability as an increasing (c>d, ------) or decreasing (c<d, ——) function of larval density. The probability of developing resistance, $R(S_0)$, is inversely related to the transmission probability. Refer to Table 1.1 for explanation of abbreviations.

Variable and Parameter Definitions

δ_i mass action disease transmission parameter:

$\delta_i = \delta_L$ for low resistance larvae, $\delta_i = \delta_H$ for high resistance larvae, with $\delta_L > \delta_H$

$S_i(t)$ number of larvae at within-season time t whose susceptibility to disease is captured by transmission parameter δ_i

$P(t)$ pathogen density at within-season time t

Λ pathogen replication in a single infected host

μ_{PW} within-season instantaneous decay rate of the pathogen

μ_{PB} between-season decay rate of the pathogen

τ delay between host infection and release of the pathogen at host death (expressed as a fraction of a season)

t_R fraction of a season that elapses prior to the development of increased larval resistance

S_0 number of larvae at the start of the season, i.e., for $S_i(t) = S_L(t = 0)$. By definition all larvae at time $t = 0$ have transmission parameter δ_L

$R(S_0)$ resistance function specifying the probability of a larva acquiring high resistance at time t_R as a function of the larval density at the start of the season. The functional form employed is: $R(S_0) = c + (d - c)(1 - e^{-bS_0})$ where b, c, and d are shape parameters whose values are restricted to cases where $0 \le R(S_0) \le 1$. Specifically, $b > 0$, $0 \le c \le 1$, and $0 \le d \le 1$. See Figure 1.3 for representative forms of $R(S_0)$ associated with selected combinations of the shape parameters.

λ discrete between-season fecundity of a surviving larva. λ is assumed net of mortality during the pupal, adult, and egg stages.

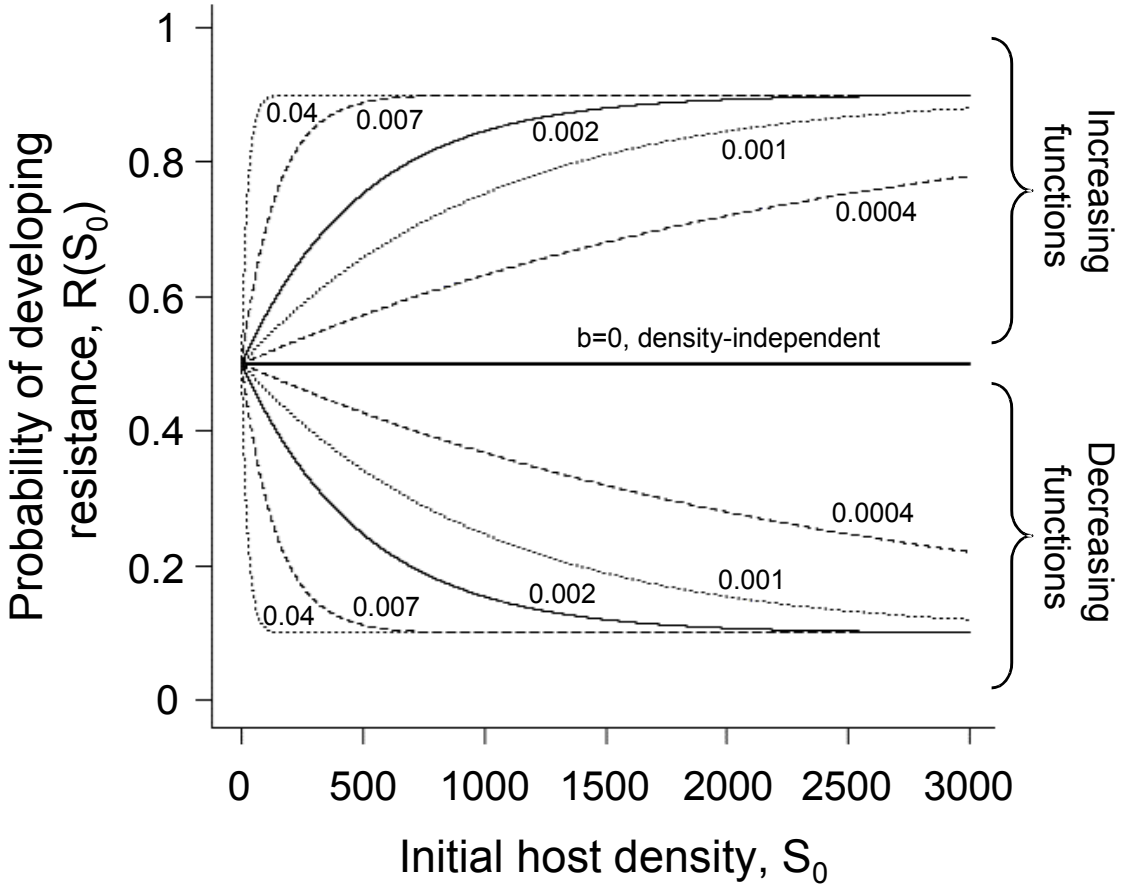

Figure 1.3. Functional relationships between host density S_0 and the probability of developing resistance $R(S_0)$ used in the model. $R(0)=c$ and $\lim R(S_0) = d$ as $S_0 \rightarrow \infty$. Increasing functions (c=0.5, d=0.9) and decreasing functions (c=0.5, d=0.1) are shown for b=0.0004, 0.001, 0.002, 0.007, and 0.04; the b values for each curve are labeled on the graph. The density-independent function (c=d=0.5) is the solid line across the center.

Within-season dynamics ($t = 0$ through $t = 1$)

$$\frac{dS_i(t)}{dt} = -\delta_i P(t) S_i(t) \qquad \text{for } i = L, H \tag{1}$$

$$\frac{dP(t)}{dt} = \begin{cases} -\mu_{PW} P(t) & \text{when } t < \tau \\ \Lambda P(t-\tau) [\, \delta_L S_L(t-\tau) + \delta_H S_H(t-\tau) \,] - \mu_{PW} P(t) & \text{when } t \geq \tau \end{cases} \tag{2}$$

a delay differential equation. Note that all larvae are assumed to die (after a delay of τ) and at time $t = t_R$ (the time at which a fraction of the larva acquire high resistance),

$$\begin{aligned} S_H(t_R) &= R(S_0) S_L(t_R) \\ S_L(t_R) &= [\, 1 - R(S_0) \,] S_L(t_R) \end{aligned} \tag{3}$$

Thus the fraction of uninfected larvae that achieve transmission parameter δ_H at time t_R is a function of the initial rearing density S_0. The remaining fraction $1 - R(S_0)$ continue with the low resistance transmission parameter δ_L.

Between-season dynamics

$$\begin{aligned} S_{H,\,T+1}(t = 0) &= 0 \\ S_{L,\,T+1}(t = 0) &= \lambda [\, S_{L,\,T}(t = 1) + S_{H,\,T}(t = 1) \,] \end{aligned} \tag{4}$$

$$P_{T+1}(t = 0) = (1 - \mu_{PB}) P_T(t = 1) \tag{5}$$

Representative parameter values used in the simulations are given in Table 1.1. These values were chosen to efficiently illustrate key behavioral transition points within the model structure.

Table 1.1. Representative parameter values used in model simulations.

Parameter	Value	Description
δ_L	0.0003	Transmission parameter for low resistance
δ_H	0.00001	Transmission parameter for high resistance
S_0	275	Initial number larvae
P_0	1430	Initial amount of pathogen
Λ	46	Pathogen replication per infected host
μ_{PW}	0.6	Within-season pathogen decay rate
μ_{PB}	0.6	Between-season discrete pathogen decay rate
τ	0.2	Delay between infection and death
t_R	0.3	Delay before potential resistance develops
λ	5	Fecundity of surviving larvae

Results

Effect of density on mortality rate

The proportion of larvae surviving inoculation with the virus decreased as larval density increased (Figure 1.4). The proportions of larvae surviving at each density were 0.67 at density 1, 0.66 at density 5, 0.63 at density 10, 0.48 at density 15, and 0.33 at density 20. Tests of means (after Bonferroni adjustment for 10 pairwise comparisons) suggest that resistance remained relatively constant from low to medium density; means for densities 1, 5, and 10 were not significantly different (χ^2_1 <0.28, P = 1). Resistance at densities 1, 5, and 10 was significantly higher than resistance at density 20 ($P < 0.001$). There were non-significant differences between resistance levels at densities 15 and 10 (χ^2_1 =4.58, $P = 0.320$) and between densities 15 and 20 (χ^2_1 =4.67, $P = 0.310$). Without the Bonferroni adjustment, these two differences become significant ($P < 0.032$). Under logistic regression with density as a continuous variable, the probability of survival was negatively related to rearing density (Z=5.14, $P < 0.001$), and estimated to drop from 0.72 at a density of 1 to 0.37 at a density of 20. Thus our evidence supports the hypothesis of a negatively-sloped relationship between rearing density and the likelihood of developing increased resistance. The XTLOGIT procedure in STATA estimated the intracluster correlation coefficient (between cup variability / between and within cup variability) as rho = 9.3×10^{-8}, or approximately 0. This indicates that the effect cup is not affecting this analysis.

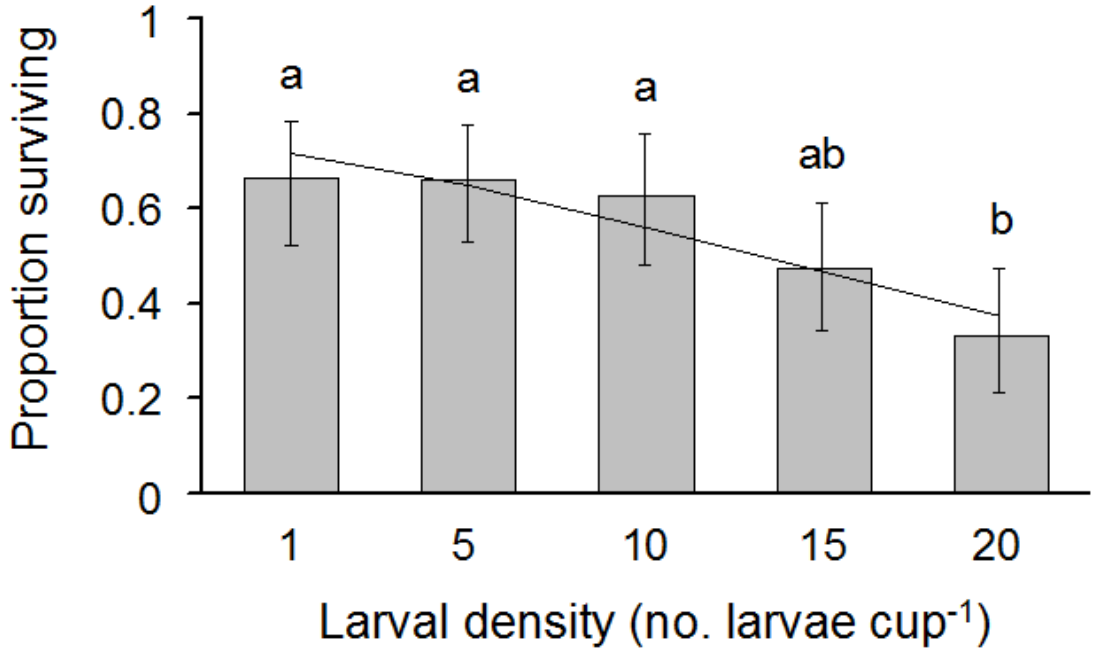

Figure 1.4. Proportion of larvae surviving inoculation with 1×10^4 viral occlusion bodies (OB) for rearing densities of 1, 5, 10, 15, and 20 larvae per 30 ml cup (means ± 95% CI from categorical logistic regression). Different letters indicate significantly different means at α=0.05 after Bonferroni adjustment. Sample sizes for larval densities 1, 5, 10, 15, 20 are n = 90, 90, 89, 87, 93, respectively. The continuous logistic regression curve is plotted in black.

Effect of density on survival time

The dosage of 5×10^6 OBs produced 98.9% mortality in the inoculated larvae. The time between inoculation with virus and death decreased as larval density increased (Figure 1.5). In the Cox regression model, the risk ratio between densities is assumed to be constant over time. Cox regression analysis estimated the relative risk of death at any particular time to be 1.68 times greater at density=10 than at density=1 (χ^2_1=7.47, P = 0.06) and 1.89 times greater at density=20 than at density=1 (χ^2_1=13.09, P < 0.001). The relative risk of death was 1.13 times greater at density=20 than at density=10, but this difference was not significant (χ^2_1=0.701, P = 1).

Using the Cox regression model, there was a statistically significant positive correlation between weight and survival time at densities 10 (χ_1^2=5.00, P = 0.025) and 20(χ_1^2=9.32, P = 0.002). However, there was a non-significant negative correlation between larval weight and survival time in larvae reared singly (χ_1^2=0.95, P = 0.33) (Figure 1.6).

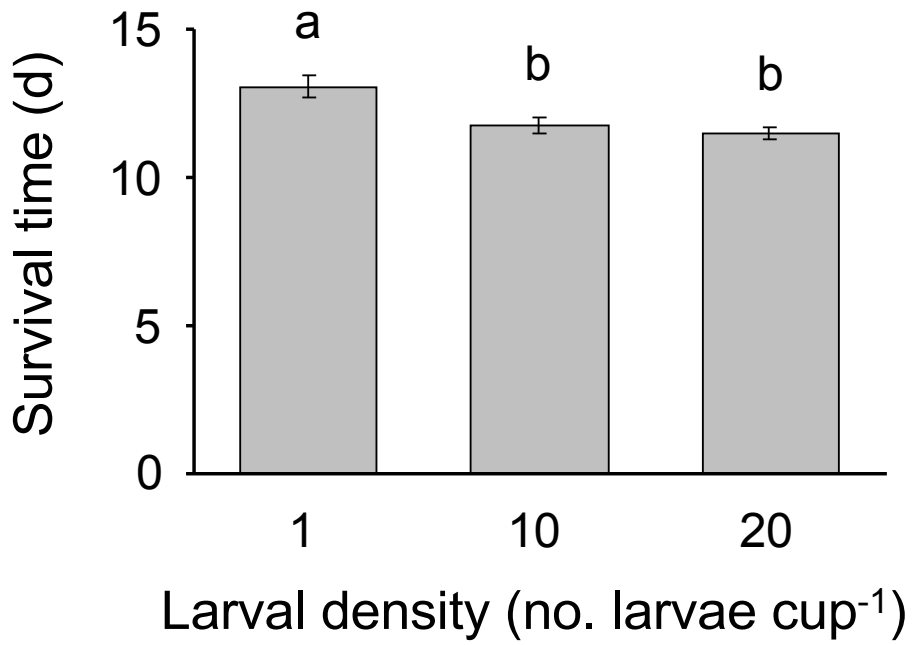

Figure 1.5. Survival times (days post infection) of fourth instar larvae inoculated with 5×10^6 viral OBs (means ± SE) for rearing densities of 1, 10, and 20 larvae per 30 ml cup (n=44, 81, 145, respectively). Different letters indicate significantly different means at α=0.05 after Bonferroni adjustment

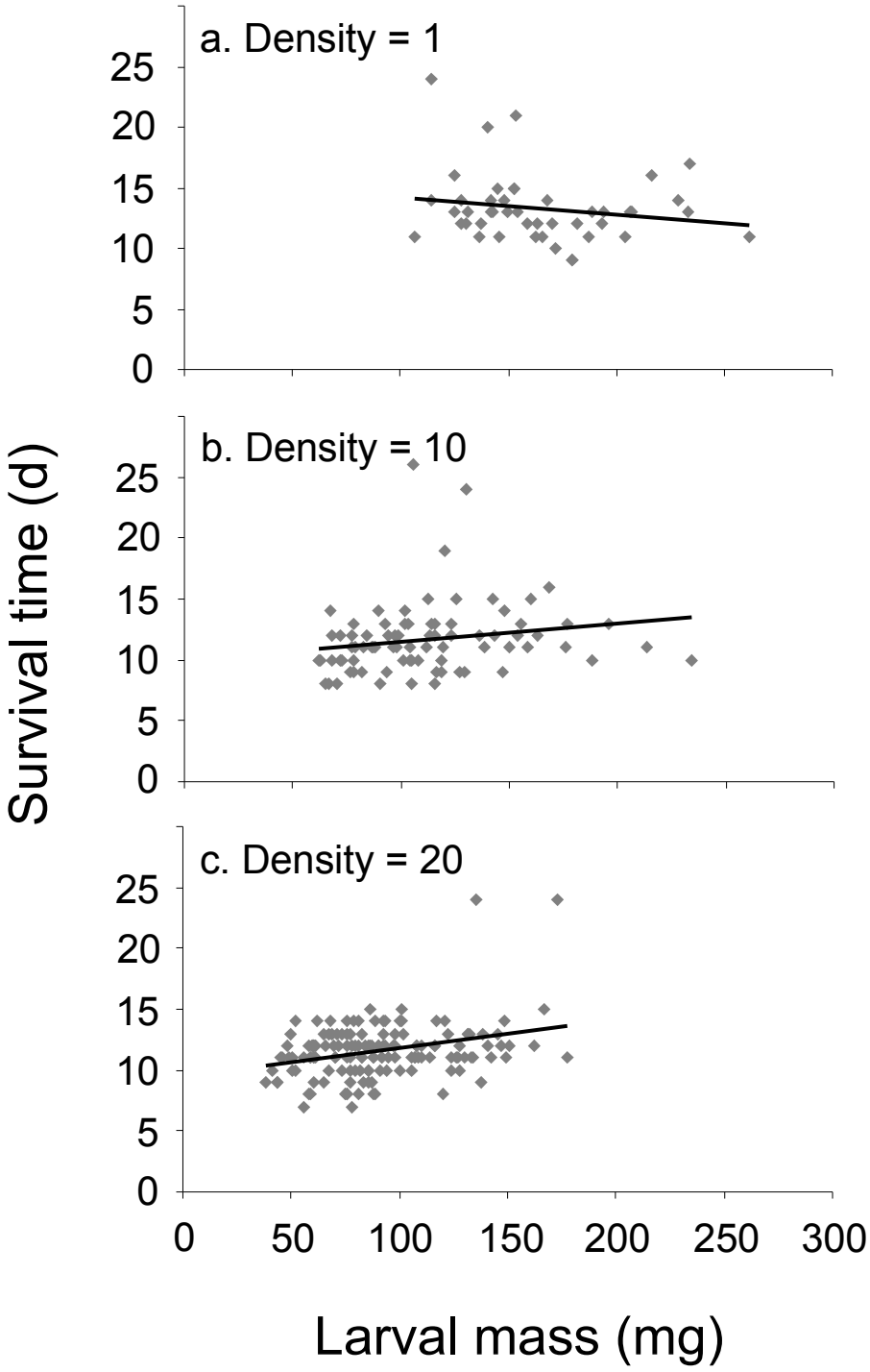

Figure 1.6. Survival time (days post infection) as a function of larval weight for larval densities of a) 1, b) 10, and c) 20 larvae per 30 ml cup (n = 44, 81, 145 respectively). Linear trendlines are plotted in black. R^2 values associated with panels a, b, and c are 0.03, 0.03, and 0.10 respectively

Effect of density on larval mass

Larval mass decreased as larval density increased (see Figure 1.7). Weight differences between all density pairings were significant (t_{238}>4.98, $P < 0.001$). In the mixed model, the effect of cup (random effect) accounted for only 2.3% of the variation in weight and was not significant (χ^2_1=2.6, $P = 0.11$).

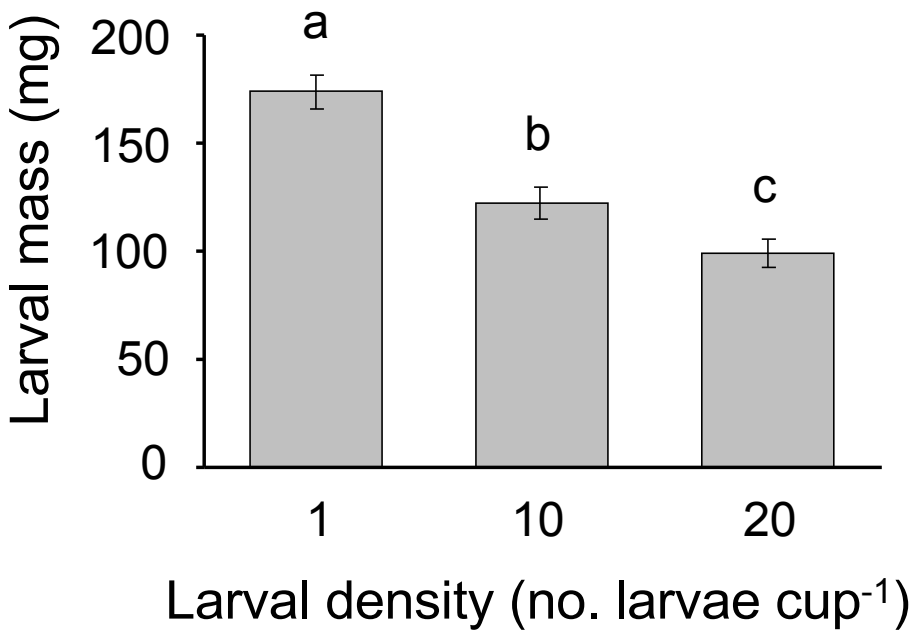

Figure 1.7. Weights of fourth instar larvae inoculated with 5×10^6 viral OBs (means ± SE) for rearing densities of 1, 10, and 20 larvae per 30 ml cup (n=44, 81, 145, respectively). Different letters indicate significantly different means at α=0.05 after Bonferroni adjustment.

Model Results

In model simulations, decreasing functions for the relationship between larval density and disease resistance ($c>d$ in the shape parameters) consistently produced more stable dynamics than increasing functions ($c<d$). Density-independent functions ($c=d$) produced patterns of intermediate stability, though the dynamics were generally much closer to those produced by increasing functions than decreasing functions. Stable steady states only occurred in simulations with a decreasing density-resistance function (Figure 1.8). At parameter combinations where decreasing functions yielded stable steady states (see time series and phase portrait in Figure 1.9a), increasing functions produced unstable cycles of increasing amplitude and increasing period, terminating in extinction of host and pathogen (Figure 1.9c). Note that for these parameter values, the density-independent function also yielded unstable cycles of increasing amplitude and period, but the cycles go extinct slightly more slowly (Figure 1.9b). Similarly, at parameter combinations where decreasing functions yielded stable multi-year cycles (Figure 1.10a), increasing and density-independent functions again produced outbreaks followed by extinction (Figure 1.10b,c). At parameter combinations where decreasing functions produced unstable cycles (Figure 1.11a), the cycles produced by increasing and density-independent functions reached extinction more quickly (Figures 1.11b,c). No regions of parameter space could be found for which stable cycles or stable steady states exist with increasing or density-independent functions (Figure 1.8). Flattening the density-resistance function (e.g., going from b=0.002 to b=0.0004, see Figure 1.3) tended to destabilize the dynamics. This suggests that systems where higher densities are needed to trigger the change in resistance may be prone to more unstable dynamics. This is most clearly seen by examining the stability diagrams (Figure 1.8), where the dynamics associated with the decreasing function produce larger areas of steady states and cycles at b=0.007 and

b=0.002 than at b=0.001. At b=0.0004 there was no stability found for any functional form. Stability is also reduced for steep R(S_0) functions (see Figure 1.8d: b=0.04). This appears to be because the function takes on density-independent properties as the steepness becomes large (i.e. R approaches d (regardless of S_0) as b approaches infinity). Note that in this model there are three ways to achieve a density-independent form: $b \to \infty$, b=0, and c=d.

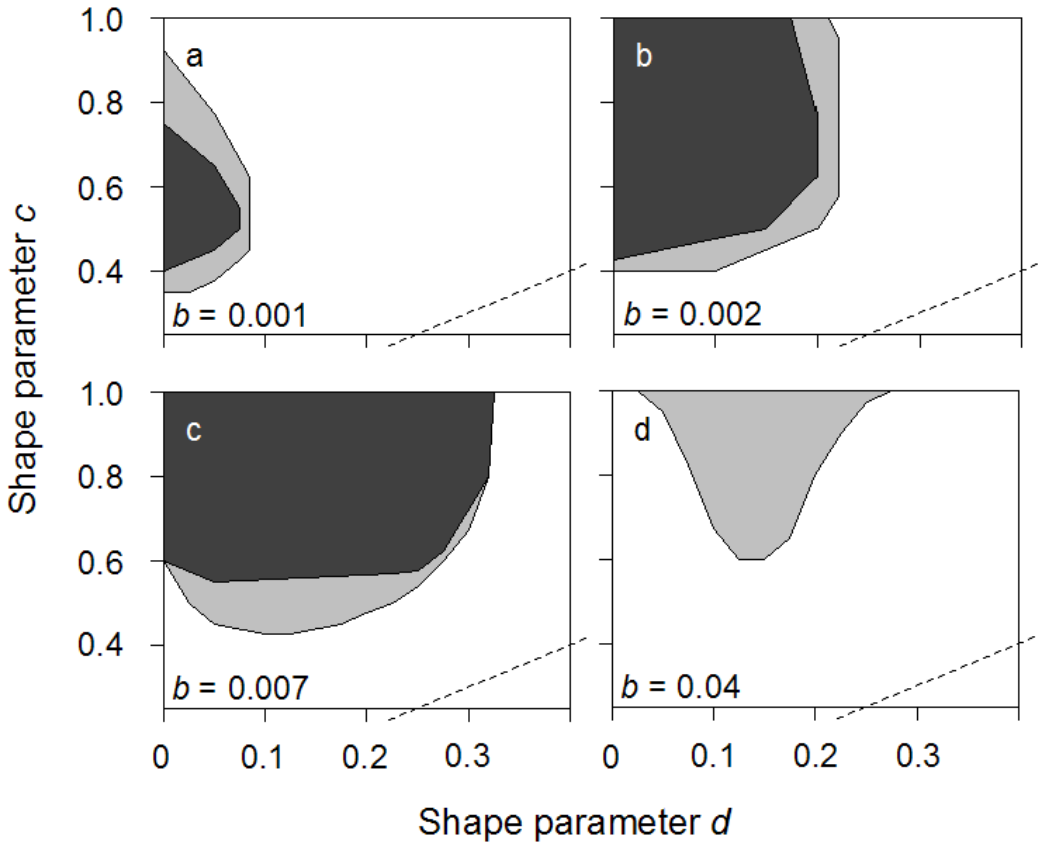

Figure 1.8. Stability diagrams for shape parameters c and d. Stable steady states are plotted in dark gray, cycles are plotted in light gray, and unstable regions are shown in white. The dashed line is c=d. Above the line resistance decreases with larval density, i.e., R(S_0) is a decreasing function. Below the line R(S_0) is an increasing function

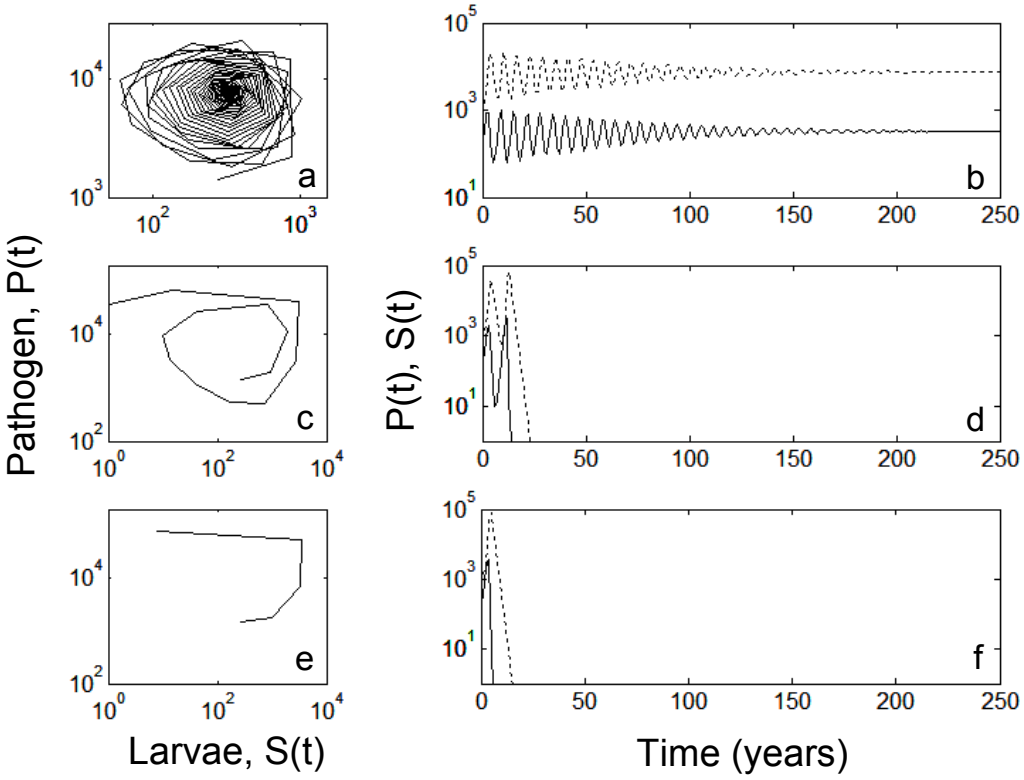

Figure 1.9. Phase portraits (a, c, e) and time series (b, d, f) for (a, b) a decreasing $R(S_0)$ function where shape parameters c=0.5, d=0.025; (c, d) a density-independent function where c=0.5, d=0.5; and (e, f) an increasing function where c=0.5, d=1.0. In the time series graphs, the number of larval hosts at time t, S(t) is the solid line and the amount of pathogen at time t, P(t) is the dotted line. For all three simulations, shape parameter b=0.001, S_0=275, and P_0=1430

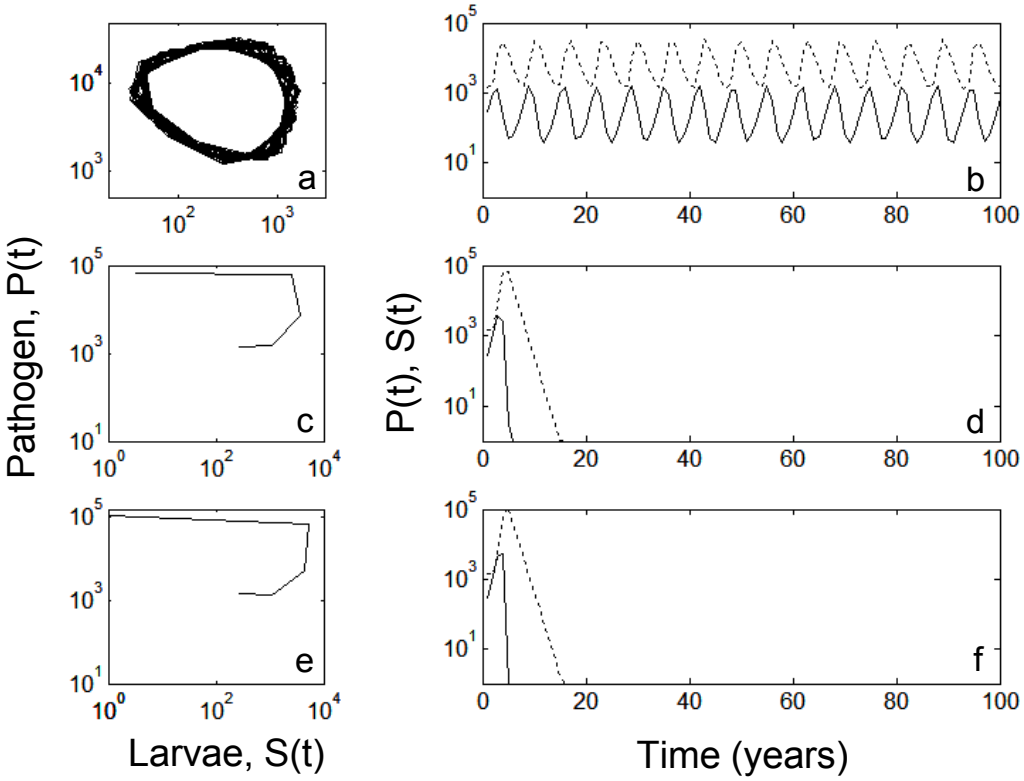

Figure 1.10. Phase portraits (a, c, e) and time series (b, d, f) for (a, b) a decreasing $R(S_0)$ function where shape parameters c=0.8, d=0.025; (c, d) a density-independent function where c=0.8, d=0.8; and (e, f) an increasing function where c=0.8, d=1.0. In the time series graphs, the number of larval hosts at time t, S(t) is the solid line and the amount of pathogen at time t, P(t) is the dotted line. For all three simulations, shape parameter b=0.001, S_0=275, and P_0=1430

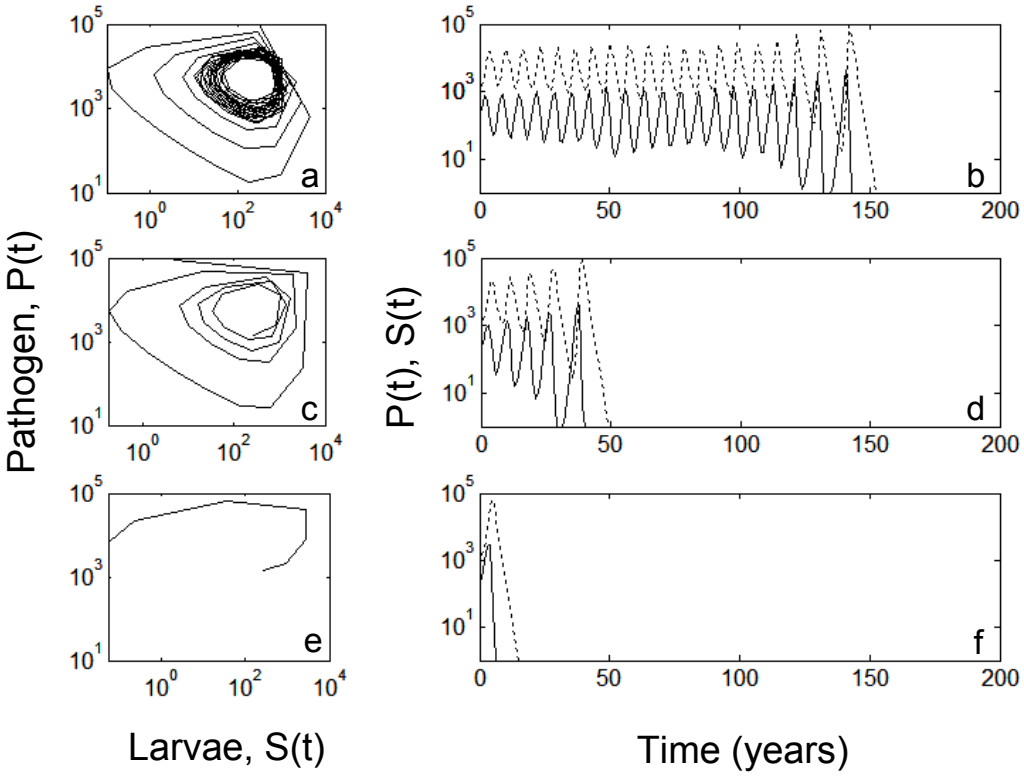

Figure 1.11. Phase portraits (a, c, e) and time series (b, d, f) for (a, b) a decreasing $R(S_0)$ function where shape parameters c=0.3, d=0.025; (c, d) a density-independent function where c=0.3, d=0.3; and (e, f) an increasing function where c=0.3, d=1.0. In the time series graphs, the number of larval hosts at time t, S(t) is the solid line and the amount of pathogen at time t, P(t) is the dotted line. For all three simulations, shape parameter b=0.001, S_0=275, and P_0=1430

Discussion

The results of this study showed density-dependent resistance to LdNPV in the gypsy moth. As larval density increased, resistance to the virus decreased. The relationship between larval density and time from infection to death was also negative; larvae succumbed to infection and death more quickly at higher densities. While Steinhaus (1958) studied the link between density-induced stress and disease resistance, these studies did not isolate larvae after infection to prevent transmission, and were not designed to control dosage. Our study represents the first example of a strictly negative density-resistance relationship between an insect and pathogen where density-dependent resistance is separated from density-dependent transmission. Our results are most consistent with the hypothesis that the stress associated with high densities weakens the immune system, preventing crowded larvae from fighting off disease (Steinhaus 1958). Another possibility is that resistance is plastically abandoned in response to high density in anticipation of the increased competition for food that is typically associated with crowded conditions. At high densities, because of stress and/or an energetic cost of living at high density, maximum resistance is not maintained. Either scenario could be consistent with an energetic cost of resistance (in addition to a cost of density), but probably only at high density and among small larvae. The relationship between larval density and larval mass was also negative. Prior to inoculation, fourth instar larvae from higher density treatments were smaller than larvae raised at lower densities. Since size is usually correlated with survival and fecundity, especially in species with non-feeding adults (e.g. Hough & Pimentel 1978, Honek 1993) such as the gypsy moth, these differences in weight are likely to represent density-related fitness costs. Campbell (1978) found that gypsy moths in high density natural populations laid fewer eggs than those at lower densities, and that the effect of density was apparent even at fairly low levels of defoliation. This is

consistent with our finding that larval size and resistance declined despite the continued presence of food.

The lower resistance observed at high density is not likely to represent an effect of smaller larvae receiving higher doses relative to body size than larger larvae. Within an instar, developmental resistance of the gypsy moth to LdNPV has been found to be unrelated to larval weight (Hoover et al 2002, Grove & Hoover 2007). In our study, no evidence was found of a relationship between larval size and disease resistance in the absence of crowding. Indeed, there was a small but statistically non-significant negative relationship between larval weight and time to death when larvae were reared singly, i.e. in the absence of crowding. Only when substantial crowding was introduced at the 10 and 20 density levels did a small but statistically significant positive correlation develop between weight and time to death (Figure 1.6).

In outbreak insects (e.g. *Spodoptera*, *Mamestra*, *Schistocerca*, etc.), density-dependent resistance is often associated with density-dependent color-phase polyphenism (Kunimi & Yamada 1990, Goulson & Cory 1995, Wilson et al 2001, Armitage & Siva-Jothy 2005). More melanized individuals are often more resistant. Gypsy moths are outbreak insects, but their larvae do not exhibit increased melanization at high density, although adult males reared in crowded conditions appear lighter in color (Leonard 1968), and crowded larvae may be lighter as well (Leonard 1981). Therefore, our results are not inconsistent with the pattern that increased melanism is linked to greater resistance (e.g. Wilson et al 2001, but see Robb et al 2003). However, there are other disease resistance mechanisms that do not involve melanin, so we cannot necessarily attribute the lack of plasticity for increasing disease resistance to the lack of melanization. Testing whether other outbreak insects

without density-dependent phase polyphenism, such as *Malacosoma disstria* or *Hyphantria cunea*, show a similar density-resistance response to that of the gypsy moth would help test this association.

Many researchers have modeled host pathogen and host parasite dynamics using modifications of the basic model structure first proposed by Nicholson & Bailey (1935). The Nicholson-Bailey model employed linear transmission of the pathogen (see Figure 1.2a, solid line), and was inherently unstable (i.e., stable steady states and persistent cycles were not possible). Hassell and May (1973) reviewed subsequent work based on the Nicholson-Bailey model, and concluded that the instability in such models can only be overcome by the addition of certain types of stabilizing mechanisms. They found, for example, that density-dependent searching efficiency of parasitoids could be stabilizing. In a further modified version of the model as a host-pathogen system, and incorporating within-season dynamics, Briggs & Godfray (1995) found that spatial clumping of the pathogen, vertical transmission, and pathogen reservoirs could have stabilizing effects through their introduction of sublinearity in the relationship between transmission probability and viral density (see Figure 1.2a, dotted line). In our model, which is a modification of the Briggs & Godfray (1995) baseline model, the stabilization instead comes from an increasing transmission probability with increasing initial larval density (i.e., decreasing resistance with increasing initial larval density) (see Figure 1.2b, dotted line). Such a stabilizing effect could not be produced by an increasing or density-independent function.

Ours is not the first model to incorporate density-dependent resistance. In a between-season-only model, White and Wilson (1999) included increasing density-dependence through a probabilistic host reproduction function in which higher end-of season densities increased the likelihood of producing offspring that were completely

resistant at birth. However, this function did not allow stable dynamics without clumping of the virus and the addition of a cost of resistance, either in the form of higher resistant mortality or a sufficiently restricted net birth rate for new resistant larvae. By contrast, in our within and between-season model we found that density-dependent resistance can act as a stabilizing mechanism by itself if the relationship is decreasing with density. This could be an important mechanism helping to regulate the stability of natural systems. In systems where host and pathogen have a decreasing density-resistance function, we would predict that, aside from other factors, the pathogen has the potential to be an important regulator of the host population. In systems with increasing or density-independent relationships, regulatory factors of other types are likely to be important, or population cycles may display unregulated behavior. Furthermore, in systems where the switch in resistance occurs at lower host densities, the interaction between host and pathogen is similarly predicted to be more stable. These predictions are relevant to host-pathogen modeling and biological control efforts. Since density-dependent resistance can strongly influence host-pathogen dynamics, we suggest that attempts to understand the population dynamics for any particular species should be informed by an experimental investigation to see if density-dependent resistance is present.

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CHAPTER 2

PREY-PROCESSING BY AVIAN PREDATORS AFFECTS VIRUS TRANSMISSION IN THE GYPSY MOTH

Abstract

Virus dissemination by avian predators is thought to be an important transmission pathway in the dynamics of insect-baculovirus ecology. Under semi-natural conditions in an aviary enclosure, we found that the level of nucleopolyhedrovirus (NPV) transmission that occurred between gypsy moth larvae (*Lymantria dispar*) was strongly enhanced by the presence of three predatory bird species, and differed among bird species. We experimentally tested two hypotheses to explain differences in virus transmission brought about by different bird species: effect of birds on spread in the gypsy moth: 1) that differences in the physiology of bird digestion produce differential transmission 2) that differences in bird behavior such as caterpillar processing method are responsible. Video analysis of bird feeding behavior indicated that *Poecile atricapilla* (Black-capped Chickadee), *Vireo olivaceus* (Red-eyed Vireo), and *Dumetella carolinensis* (Gray Catbird) differed in caterpillar-processing behavior and that this variation strongly affected virus transmission. Real-time PCR quantifications of the amount of NPV present in bird feces over time suggested that differences in gut physiology were less important to transmission at the local scale. In our study the red-eyed vireo, a species restricted to mature forest habitat, was most effective at spreading the virus locally, highlighting the need to consider predator behavior in studies of population dynamics as well as the need to conserve such species for their ecosystem role in facilitating viral epizootics in outbreaking insects.

Introduction

Diseases are often the most important factors regulating insect populations. In the gypsy moth (*Lymantria dispar*), an invasive forest pest in eastern North America, population dynamics are often driven by the *Lymantria dispar* nucleopolyhedrovirus (LdNPV). (Podgwaite 1981, Elkinton and Liebhold 1990) The transmissible stage of this virus consists of infectious virions encased in a protective protein matrix called the occlusion body (OB). When an infected insect dies, its cuticle ruptures, releasing millions of OBs that adhere to the surfaces of leaves where they may be ingested by susceptible hosts. Once eaten, the OB protein dissolves in the alkaline conditions of the caterpillar midgut, the individual virions are released, and the insect can be infected when the virus enters midgut cells (e.g. Fuxa 2004).

Numerous researchers (e.g. Bird 1955, Entwistle et al 1993) have suggested that birds can play a role in the transmission of insect viruses as a consequence of their predation on infected hosts. They hypothesized that when an infected larva is eaten, the viral OBs can pass through the gut of the bird unharmed and will then be deposited with the feces on the surfaces of leaves. Healthy larvae then ingest these OBs when eating leaves and become infected in the normal manner.

So far, evidence supporting this hypothesis comes mainly from two lines of inquiry: 1) OBs detected in birds during viral epizootics (from either stomach dissections or fecal samples) and 2) OBs measured in the feces of birds fed virus under artificial conditions. The first evidence that birds could facilitate NPV transmission was collected during an outbreak of the European pine sawfly (*Neodiprion sertifer*) and an associated NPV epizootic. Bird (1955) found that the dissected stomach contents of catbirds (*Dumetella carolinensis*) and cedar waxwings (*Bombycilla cedrorum*) contained NPV, and that this virus retained its ability to infect larvae. In a similar study, Entwistle et al. (1977a) found infectious NPV in the feces of 82 out of

96 birds of 15 different species captured in a Welsh forest during an outbreak of the European spruce sawfly (*Gilpinia hercyniae*). Entwistle et al. (1977b) also found NPV in the feces of 17 out of 19 birds of 6 species captured in the same location during the winter, suggesting that birds might eat cached larvae from the previous summer. Entwistle et al. (1993) later found NPV in the feces of 18 bird species during an outbreak of the pine beauty moth (*Panolis flammea*) in Scotland. However, in a different outbreak of the European spruce sawfly and associated NPV epizootic, Buse (1977) detected no NPV in the feces of 23 birds randomly caught in mist nets or in the stomachs of 19 birds collected by shooting. Hostetter & Biever (1970) found infectious *Trichoplusia ni* NPV in bird feces collected from a cabbage field in Missouri. During an epizootic of *L. dispar* NPV in Pennsylvania, Lautenschlager and Podgwaite (1980) found infectious NPV in 2/2 blue jays (*Cyanocitta cristata*), 1/2 Eastern towhees (*Pipilo erythrophthalamus*), but 0/2 American robins (*Turdus migratorius*). Entwistle et al (1978) and Lautenschlager and Podgwaite (1979) detected and visually counted OB concentrations in the feces of birds that were hand-fed the virus. In total, the data from these studies indicate that birds often eat larvae harboring NPV infections, and that this interaction facilitates the dissemination of occlusion bodies into the environment.

We hypothesized that birds could aid in virus transmission through a second process: by breaking open the larval cuticle and scattering the virus during prey-processing. Under this hypothesis, virus transmission would not be determined by gut passage dynamics, but rather by the different feeding and caterpillar-processing behaviors employed by different bird species. In this paper, we experimentally test the relative ability of these two mechanisms to explain bird-mediated virus transmission in a semi-natural aviary cage setting. We show that differences in virus transmission are best explained by different processing behaviors among bird species.

Methods

Preparation of infected larvae

Lymantria dispar larvae were reared on artificial diet in groups of 10 larvae per 30 ml cup (Bell et al 1981) until the 4th instar, and then transferred to individual 30 ml cups. Larvae were then individually fed 3 mm^3 cubes of artificial diet on which a 3 µl droplet containing approximately 1×10^8 OBs of *Lymantria dispar* NPV had been placed. The virus used in all studies was the wild-type Hamden, CT strain of the *Lymantria dispar* nucleopolyhedrovirus (LdNPV) distributed as "Gypchek" by the USDA Forest Service. This dosage produced 100% mortality in control larvae infected in the 4th instar. After consuming the diet cube, larvae were placed in individual cups with fresh diet and incubated at 25ºC for at least 6 days, by which time they had molted into the 5th instar, but had not yet died. Only living larvae were used in experiments.

Capturing birds

Birds were captured between May 14, 2006 and August 26 2007 in mist nets at 3 locations in Tompkins County, NY: the woodlot behind Liddell Laboratory on Freese Rd in Ithaca, McGowan Woods on Game Farm Rd in Ithaca, and the woods along Fall Creek in Freeville. We focused on 3 species that are abundant in this area and are known to be predators of *L. dispar* larvae: the Gray Catbird (*Dumetella carolinensis*), the Black-capped Chickadee (*Poecile atricapilla*), and the Red-eyed Vireo (*Vireo olivaceus*). Catbirds were captured primarily by passive mist netting, whereas chickadees and vireos were lured using audio recordings of their songs. All birds were banded with numbered aluminum bands to assure that each individual bird was tested only once. After capture, birds were held in white cloth bags for a maximum of 20 minutes prior to testing. Our experimental protocols were approved by the Cornell University Institutional Animal Care and Use Committee (IACUC).

Measuring bird-mediated transmission

In order to examine the effect of differences in foraging among bird species, we constructed a semi-natural foraging area. We built an outdoor aviary cage 5 m × 2.5 m × 2.5 m high containing an artificial red oak 'tree'. The tree consisted of a 2 m PVC pole (25 mm diameter) with four perpendicular dowels inserted through it at various angles as perches (1, 1.5, 2, and 2.5 m above ground). A freshly cut branch of Northern red oak (*Quercus rubra*) with approximately 25 leaves was inserted into a 30 ml vial filled with water and affixed to the artificial tree. We then placed 20 5th instar *L. dispar* larvae infected with NPV ≥ 6 d before on leaves throughout the branch, and released a bird into the cage. We recorded the time until the bird began feeding on the caterpillars, then recorded the bird's feeding behavior for a further 3 hours using a digital video camera (Canon XL2). Five individuals of each bird species were tested. After the trial, any remaining larvae were removed from the leaves, and the branch was placed into a small cage (0.5 m^3) with 30 healthy 4th instar *L. dispar* larvae. After 7 days, these larvae were transferred to individual 30 ml cups with artificial diet and monitored daily until death or pupation. Animals broke into one of the small cages and ate all the healthy larvae during one catbird trial, thus n=4 for the transmission data for this species. In control trials, the experiment was performed in exactly the same way except no bird was put into the cage and the infected larvae were left on the branch for 5 hours before the branch was caged with healthy larvae. In interpreting the results of this experiment, we assume that higher mortality in the healthy larvae translates to more effective transmission. There was no NPV-caused mortality in the control groups. Data were analyzed by categorical logistic regression with a binomial distribution and a logit link function (PROC GENMOD, SAS Institute, Inc., Cary, NC), with the effect of individual bird controlled for as a random effect.

Measuring gut passage differences

To examine separately the potential effect of differences in NPV passage through the bird gut, we fed birds a known amount of NPV: About 25 NPV-killed *L. dispar* cadavers were homogenized and strained through cheesecloth to remove hairs, resulting in about 10 ml of thick brown liquid. The concentration of viral occlusion bodies in this liquid, counted using a hemocytometer, was 1.84×10^6 per µl. We pipetted 50 µl of this solution (9.2×10^7 OBs) into the lower beak of each bird. Birds readily swallowed the solution. We transferred the birds to separate small covered cages (15 × 30 × 15 cm) with floors made of rubber-coated hardware cloth. Aluminum foil was placed underneath the cage and changed every 10 minutes to collect feces. Chickadees and vireos were held for 1.5 hrs. Catbirds were held for 2 hrs to be sure that the trial did not end before peak virus passage occurred, but the extra time for catbirds proved unnecessary. Four individuals of each species were tested, and no individual bird was used more than once. Viral loads in fecal samples were estimated by quantifying LdNPV DNA using real-time PCR. Differences in peak passage time of the virus and total virus recovered were analyzed as a general linear model (PROC GLM, SAS Institute, Inc.).

Real-time PCR

Fecal samples were transferred to 2 ml microcentrifuge tubes and mixed with 1500 ul dilute alkaline saline (0.1 M NaCl + 0.1 M Na_2CO_3, pH 10) to dissolve occlusion body protein, pelleted (16000 g, 5 min), and resuspended in 400 ul buffer (0.1 M KCl, 0.01 M Tris, 0.01 M EDTA, pH 7.5). Samples were then sonicated for 30 seconds, treated with 50 ul 10% SDS and 20 ul proteinase K, and incubated overnight at 50 C. We then added 200 ul 99.5% ethanol, and pipetted the resulting mixture (~700 ul) into a DNeasy spin column (Qiagen, Inc., Germantown, MD). DNA was extracted using the columns and wash buffers from the Qiagen DNeasy Blood and

Tissue kit. The procedure resulted in clean DNA eluted into 400 ul buffer AE. Real-time quantitative PCR, using the Applied Biosystems 7900 HT Sequence Detection System, was performed with the extracted DNA. Forward and reverse primers and a FAM-TAMRA labeled probe were designed for the G22 gene, ORF 7, which is unique to the LdNPV genome (Blischoff & Slavicek 1995, J. Slavicek pers. comm.). The nucleotide sequences of the primers and probe are as follows:

FWD: GCGCTTCTCCGTGACCAA

REV: AATCCCTGCACATGCCTGTCAT

PROBE: ACCCGTGCCTGTTCATGTTCAAGGA

Estimating absolute numbers of occlusion bodies from relative quantities of LdNPV DNA in fecal samples was performed using a standard curve, which was created by extracting DNA from pure solutions of known occlusion body concentration, and then serially diluting the extracted DNA. Final estimates for each sample were averaged from at least two replicates of the PCR reactions. Minimum detection was approximately 10^3 OBs per sample.

Results

Bird-mediated transmission

In the aviary, virus was transmitted more effectively in trials with the red-eyed vireo than with other birds ($P<0.001$, see Figure 2.1). Black-capped chickadees were responsible for higher transmission than gray catbirds ($P=0.050$). On average, 51 ± 6% of healthy larvae exposed to leaves from red-eyed vireo feeding trials died of LdNPV, with individual trials varying from 38% to 72% infection. Individual chickadee and catbird results varied from 14-48% and 8-29% infection, respectively. Only birds eating larvae were included in this experiment.

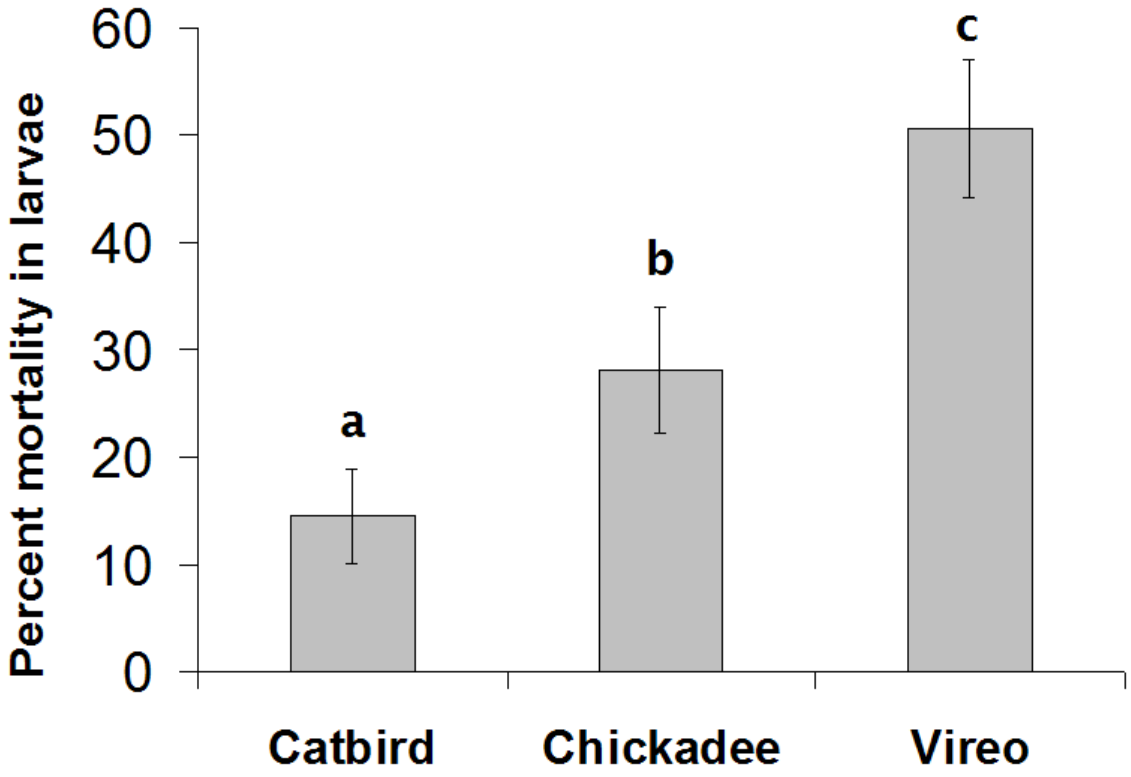

Figure 2.1. Mean percentage of healthy *L. dispar* larvae dying of LdNPV in the transmission experiments associated with catbirds, chickadees, and vireos. Healthy larvae were placed on foliage that had been beneath birds while they fed on infected gypsy moth larvae.

Foraging behavior

All three bird species were willing to eat gypsy moth larvae in the aviary. Chickadees took the least time to begin eating larvae once released into the cage (P=0.005, see Figure 2.2), and a significantly higher percentage of chickadees were willing to eat larvae in the aviary cage compared with the other bird species (P<0.001). All 5 chickadees captured ate larvae, whereas only 56% of 9 vireos and 36% of 14 catbirds ate larvae. Chickadees ate more total caterpillars than the other bird species within the three hour period (P=0.014, see Figure 2.3). Two chickadees ate all 20 larvae provided, an accomplishment shared by no other birds.

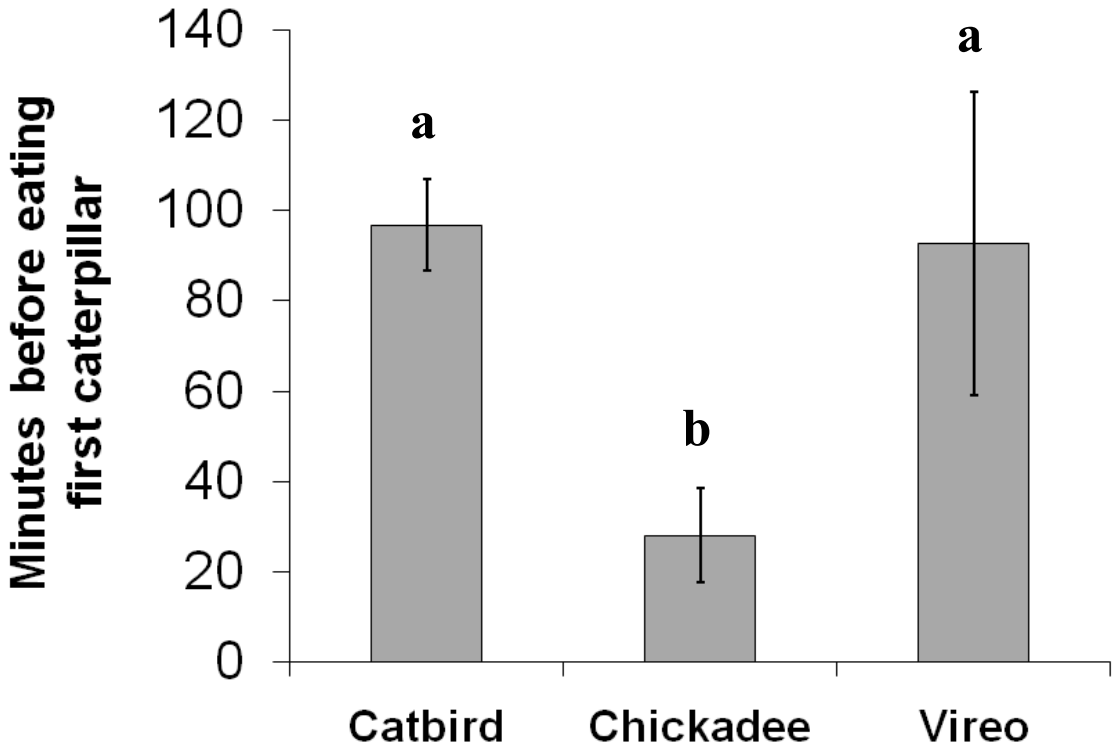

Figure 2.2. Mean time elapsed before first larva was eaten by individual catbirds, chickadees, and vireos (n = 5 birds of each species). Letters indicate significantly different means.

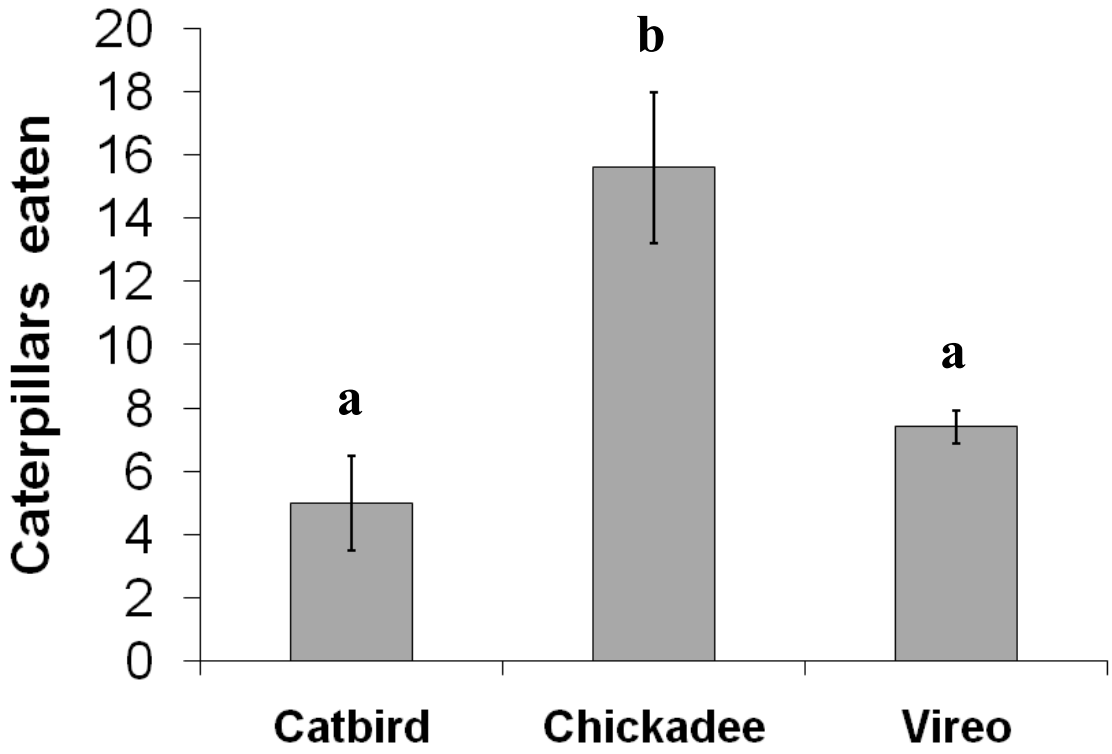

Figure 2.3. Mean number of caterpillars consumed by individual birds of each of the three bird species (n = 5 birds of each species). This data does not include birds that declined to eat any larvae. Letters indicate significantly different means.

Birds employed dramatically different methods for processing the hairy *L. dispar* larvae. Catbirds often swallowed the larva whole with little processing, whereas chickadees and vireos spent considerable time preparing the larva (see Video 1, electronic supplementary material). Chickadees tended to pick larvae apart delicately, tossing aside hairs and small pieces of cuticle, and inserting their bills into the torn end of the larva to carefully extract the internal tissues bit by bit (see Video 2). Vireos tended to beat the larva vigorously against a twig or perch dowel until most of the hairs were broken off and the caterpillar was soft (see Video 3). Vireos would then swallow the softened larva, whereas chickadees never swallowed a larva whole. All three species of birds occasionally resorted to beating a caterpillar against the perch, but vireos employed this behavior far more often ($P<0.001$, Figure 2.4), on average beating each caterpillar 107 ± 15 times. Interestingly, we observed that after a few hard strikes against a perch, caterpillars became leaky. Droplets of hemolymph and liquefied tissues could be seen clearly spraying from the larva as it was swung around and when it contacted the perch (see Figure 2.5). There was a significant positive relationship between the average number of times larvae were beaten and viral transmission by individual birds ($P=0.003$, Figure 2.6), suggesting that this behavior aided in spreading the virus.

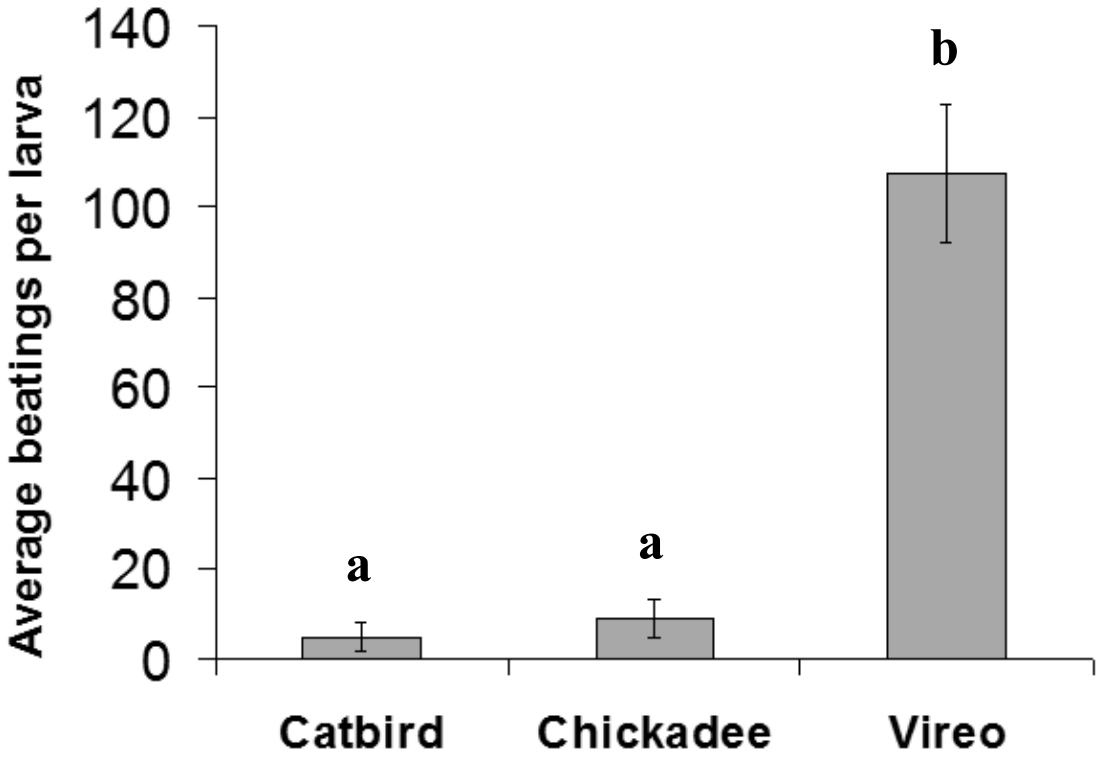

Figure 2.4. Mean number of times infected larvae were beaten by birds during prey-processing, averaged by bird species (n = 5 birds for each species).

Figure 2.5. Red-eyed Vireo (*V. olivaceus*) processing *L. dispar* larva. In this frame from a typical video sequence, virus-bearing liquid can be seen spraying outward from the damaged larva as the bird beats it against the perch.

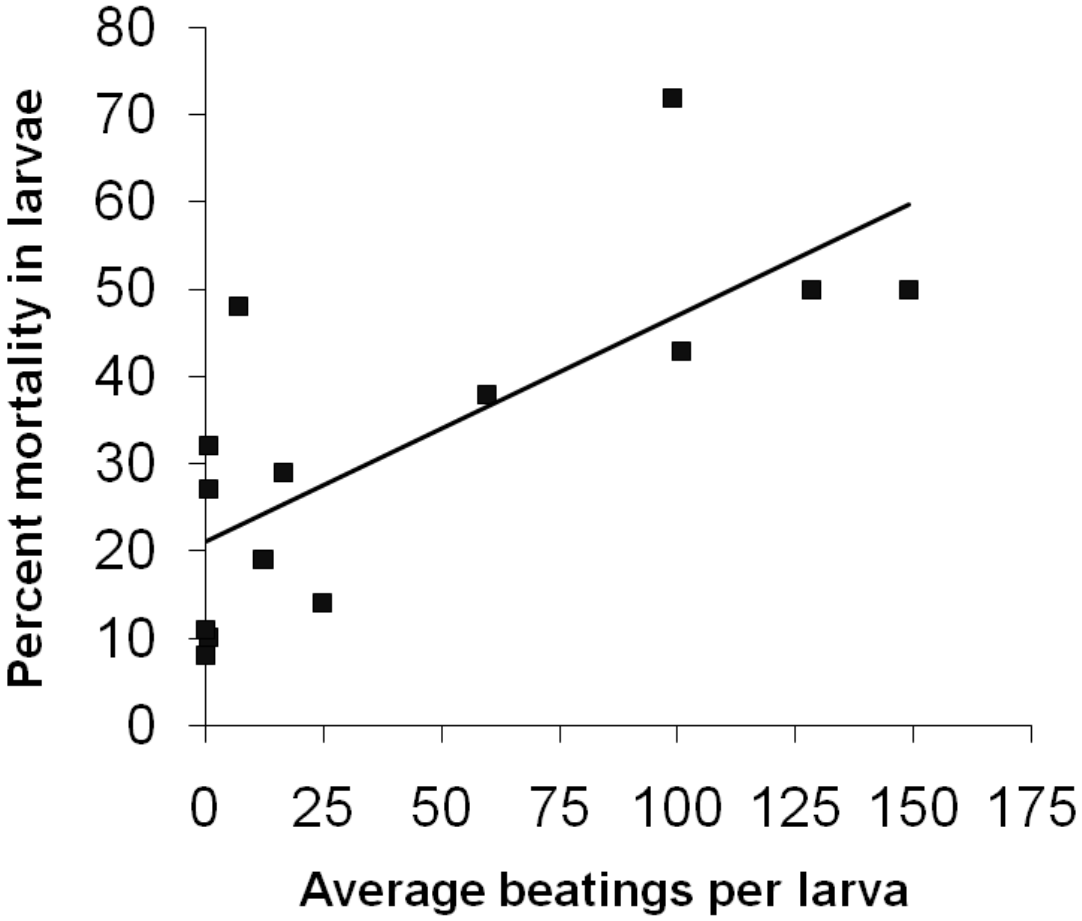

Figure 2.6. Relationship between the average number of times infected larvae were beaten against a perch by a particular bird and the NPV-induced mortality in the transmission experiment associated with that bird (n = 14 birds).

Gut passage differences

For all birds tested, some virus was detected in feces within 10 or 20 minutes, and continued to be detected throughout the trial. The timing of peak virus passage through bird guts and the total amount of virus passed were quite variable among individual birds, though species-level differences were found. Peak virus passage occurred faster in chickadees than in vireos (P=0.026, Figure 2.7). On average, virus passage through catbird guts occurred more slowly than the other species, but this difference was not significant due to high variation among individual catbirds (P>0.249). On average, total virus passage within 90 minutes post ingestion was numerically greatest in chickadees, but not significantly greater than vireos (P=0.672, Figure 2.8). Catbirds passed significantly less total virus than the other bird species (P=0.002).

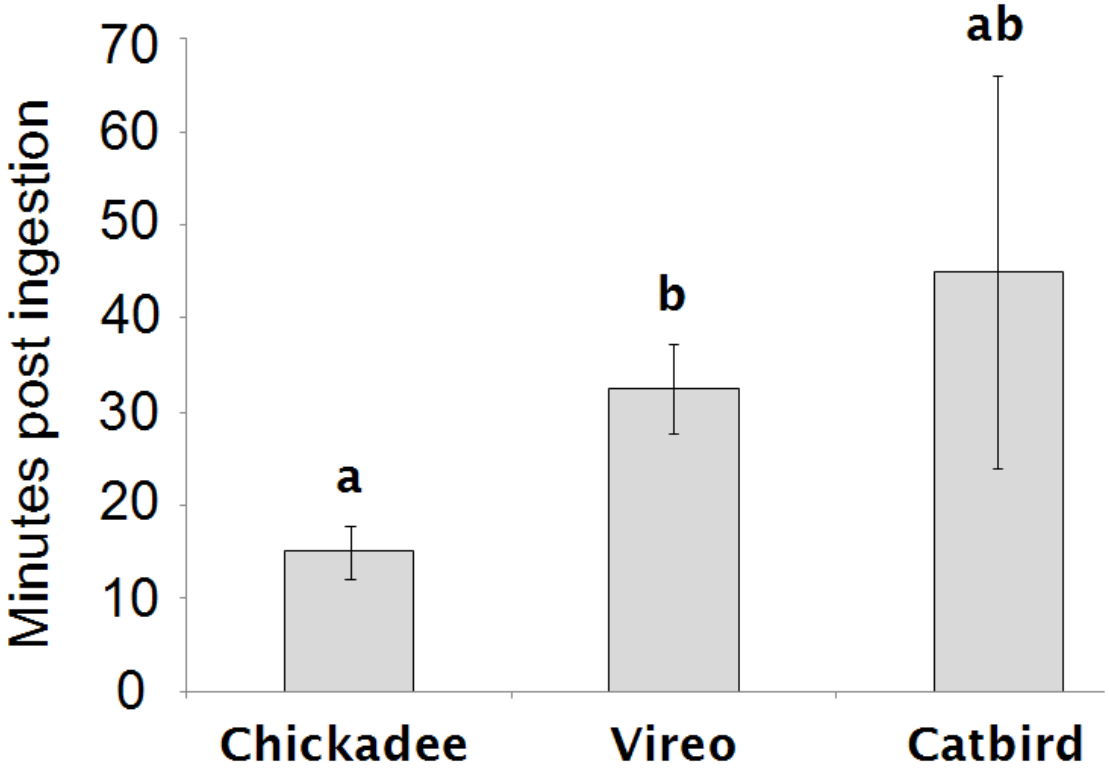

Figure 2.7. Mean peak passage times of LdNPV through the guts of the three bird species as estimated by real-time PCR (n = 4 birds for each species).

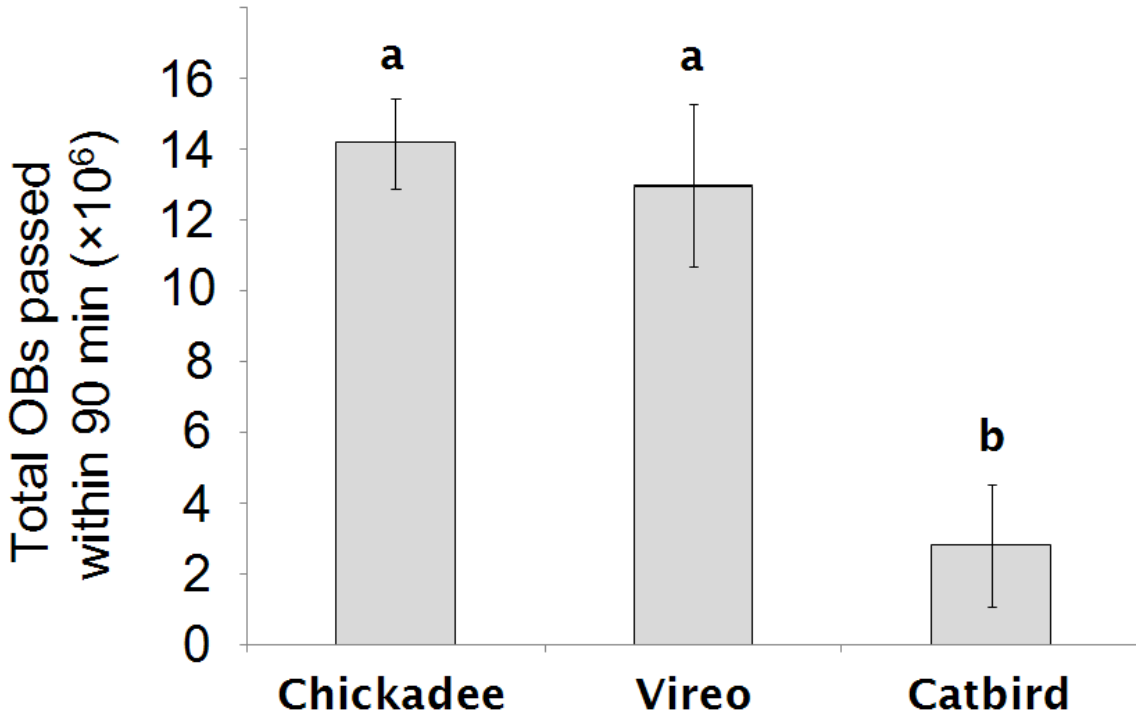

Figure 2.8. Mean total LdNPV OBs passed through the guts of the three bird species within 90 minutes post ingestion as estimated by real-time PCR (n = 4 birds for each species).

Discussion

Initially, the black-capped chickadees seemed most likely to be effective vectors of NPV occlusion bodies for two reasons: 1) chickadees had the fastest gut passage times and passed at least as much total virus as vireos, and 2) chickadees ate more caterpillars within the time limit of the aviary experiment than other birds. In our transmission experiment, chickadees did spread significant amounts of NPV, but the red-eyed vireo was found to be considerably more effective than the chickadee at transmitting the virus. Catbirds were least effective at facilitating transmission. The greater effectiveness of the vireo cannot be explained solely based on the differences in the speed or efficiency of NPV passage through guts of different birds. Rather it is best explained by differences in caterpillar-processing behavior. The vireo's technique of beating larvae against a branch to remove hairs and soften the body had the consequence of breaking open the larval cuticle and scattering virus-contaminated tissues onto the nearby leaves. This virus had the added advantage of being delivered to the larvae without any potential degradation that might have occurred in the bird's gut. We were able to detect differential NPV loads in fecal samples among the three bird species, implying that the gut-passage route also contributed to differences in viral transmission, but this effect was overridden by the larger effect of the behavioral differences. It is likely that both processes play an important role in natural NPV transmission. The breaking open of infected larvae and the scattering of virus was very effective in aiding the spread of NPV at the local scale. This activity by birds has the effect of releasing virus into the environment that would not ordinarily escape until the death of the caterpillar. Gypsy moth larvae may die of NPV in as few as 7 days or as many as 26 days post infection (Reilly & Hajek 2008), whereas occlusion body production begins as early as 24 hours post infection (Blissard and Rohrmann 1990).

In general, the interaction between predator, prey/host, and pathogen enhances viral transmission in two ways: 1) by freeing infectious OBs contained within infected larvae before their natural time of release, and 2) by spreading the OBs in space. The prey-processing mechanism relates primarily to the first effect, as its range is limited to the distance of sprayed droplets after a short flight to an appropriate processing branch. The passage of NPV through the bird gut, however, is probably more important for transmission over larger scales, and is likely to be influenced by factors such as the number and frequency of larvae eaten as well as the conduciveness of the guts of different bird species to LdNPV survival. Studies in which birds were artificially fed NPV have allowed researchers to measure passage times of OBs through the bird's gut. Unfortunately, these studies have often chosen bird species that were easy to catch rather than species that are important predators of lepidopteran or sawfly larvae. Entwistle et al (1978) determined that peak passage times for blue tits (*Parus caerulus*) and starlings (*Sturnus vulgaris*) were between 25 and 50 minutes post ingestion, and that OBs were not discernable in feces by optical microscopy after 2.5 hrs but feces remained infective to larvae for up to 7 days. In our study, peak passage times averaging between 15 and 45 minutes for the three species are consistent with Entwhistle's results. It is interesting to note that approximately 15% of virus administered to chickadees and vireos was recovered within 90 minutes, while only 3% was recovered from catbirds in this time period (Figure 2.8). The remaining 85+% would likely be divided between virus that passes at a later time and virus that is destroyed. In viability tests, approximately 70% of bird-passed OBs retain infectivity (J. Reilly unpublished data). Lautenschlager & Podgwaite (1979) reported that infectious LdNPV was passed by red-winged blackbirds (*Agelaius phoeniceus*), house finches (*Carpodacus mexicanus*), and mourning doves (*Zenaida macroura*) after ingestion of an aqueous solution of occlusion bodies, and that the larger birds (*Z.*

macroura) passed the occlusion bodies more slowly and less effectively than did the smaller species (*C. mexicanus*). However, only one individual of the intermediate-sized species (*A. phoeniceus*) was tested, and these results were not consistent with the pattern from smallest to largest. Furthermore, none of these three species are likely to be important predators of *L. dispar* (see Forbush & Fernald 1896). With those qualifications, our results showed a similar trend to that predicted by Lautenschlager and Podgwaite, in that peak passage time increased with bird size, although there was considerable variation in passage times especially in the largest species (Figure 2.7).

Our results are likely to be applicable to an important set of outbreak caterpillar species in addition to *L. dispar*, including other *Lymantria* species, browntail moth (*Euproctis chrysorrhoea*), fall webworm (*Hyphantria cunea*), tent caterpillars (*Malacosoma* spp.) and other lasiocampids (e.g. *Dendrolimus* spp.), and douglas fir tussock moth (*Orgyia pseudotsugata*). Like the gypsy moth, these caterpillars are hairy and probably elicit similar prey-processing behaviors in birds. They are also known to have important interactions with an NPV. Given the large variation in disease transmission produced among different bird strategies for processing and eating hairy caterpillars, it would be interesting to test how the size of hairy larvae at different instars affects processing mode. Early-instar caterpillars have been observed to be swallowed whole by bird species (e.g the Scarlet Tanager, Forbush 1907) observed to process larger larvae. However, the larvae of many other species of Lepidoptera and sawflies are not excessively hairy, so we would expect these species to be processed differently by birds and would therefore hypothesize the influence of birds on NPV transmission rates to depend more strongly on gut-passage. The role of birds, particularly the red-eyed vireo, in facilitating NPV epizootics may be an important ecosystem service highlighting the need to conserve these bird species. Although the red-eyed vireo is currently thought to be the most common

forest bird in eastern North America (Rich et al 2004), increased habitat fragmentation and the loss of the mature forests that are required by such species could result in decreased bird populations, which may result in increased forest susceptibility to outbreaking defoliators.

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CHAPTER 3

ASSESSING THE POTENTIAL FOR VERTICAL TRANSMISSION OF *LYMANTRIA DISPAR* NUCLEOPOLYHEDROVIRUS (LDNPV)

Abstract

In the gypsy moth-NPV system, attempts to identify the primary mechanism of transmission between generations have produced conflicting results. Sublethal infection of larvae leading to vertical transmission from adult females to their offspring has been claimed by some researchers to be important, whereas external contamination of egg masses with virus from the environment has been identified as the primary mechanism in other studies. Although recent models of gypsy moth population dynamics have incorporated only the external contamination pathway, the presence of a female-offspring transmission pathway would be an important addition to modeling the initiation of NPV epizootics. I conducted a detailed laboratory investigation of the process of vertical transmission from female to offspring with the goal of determining whether adult females exposed to OBs as larvae retain any virus that could be passed to their offspring. Adult female moths that were exposed to virus as larvae were dissected to look for a route by which NPV could pass from adult to larva via contamination of the egg or egg mass at some stage in the process of egg formation and oviposition. Real-time quantitative PCR and bioassays were used to analyze virus concentrations in different parts of the adult female moth. Although low levels of virus were detected in the tissues, there was no location that showed consistently positive levels. Furthermore, this virus did not appear to be infectious to larvae, and therefore may represent either latent virus or non-occluded virions.

Introduction

Vertical transmission is one of the less understood pathways for the spread of insect nucleopolyhedroviruses (NPV). In this type of transmission, virus passes directly from an infected adult female (or male) to its offspring. In the case of NPV, a female must survive exposure to the virus as a larva, but retain virus in her tissues in a location that eventually comes in contact with the eggs. This is very different than the standard horizontal transmission route for NPV, in which the cuticle of a dead infected larva ruptures, releasing millions of environmentally-resistant viral occlusion bodies (OBs) onto the nearby foliage where they can be ingested by other larvae. Unlike some other insect viruses such as Hz-2v (Rallis and Burand 2002), NPV does not require the vertical transmission pathway for successful persistence across generations, since the OBs generated from dead larvae remain infectious over the following seasons (e.g Thompson et al 1981). Nevertheless, an alternative vertical transmission route could strongly affect disease dynamics when the host and virus are at low levels and horizontal transmission is weak.

Interestingly, relatively little theoretical work has explored the consequences of a vertical transmission pathway for NPV, despite its potential importance to our understanding of the ecology of this disease (e.g. Briggs and Godfray 1996, Dwyer et al 2000, but see Boots et al 2003). This avoidance by the modeling literature is perhaps due to the confused state of the experimental literature on vertical transmission in NPV. The relative ability of NPVs to be vertically transmitted from parent to offspring appears to be specific to each host-pathogen system, and no general pattern has been identified thus far (reviewed in Kukan 1999). Even within individual systems, the importance of vertical transmission has often been difficult to demonstrate convincingly due to a number of factors including flawed study designs, infection in controls, and low sensitivity of detection techniques. Interpreting the

results of such studies is further complicated because a virus can potentially use any of several vertical transmission routes, passing inside or outside of the egg, and either in the form of occlusion bodies, non-occluded virions, or as DNA incorporated into the host genome. The occurrence of vertical transmission has been convincingly documented for a few species including *Spodoptera frugiperda* NPV (Fuxa and Richter 1991, Fuxa 2004), and *Mamestra brassicae* NPV (Goulson and Cory 1995, Hughes et al 1993, 1997, Burden et al 2003), but remains unclear for most other systems.

The gypsy moth, *Lymantria dispar*, is a good example of a host with a large but conflicted literature dealing with vertical transmission of NPV. These studies have used four general approaches to generate evidence for vertical transmission: 1) examining the progeny of adults that survive infection as larvae for the presence of NPV, 2) examining the eggs or egg masses of adults that survive infection as larvae for the presence of NPV, and 3) examining the tissues of adults that survive infection as larvae for the presence of NPV. Any one of these methods could potentially provide conclusive results, assuming proper study design. The fourth approach involves attempting to activate latent viral infection by exposing larvae to various stressors. This approach has some inherent problems which we will discuss later.

Four studies have looked for NPV-induced mortality in the progeny of adults that survived infection as larvae. Shapiro and Robertson (1987) infected late 2nd instar larvae using low doses (10-90% mortality level) and the diet surface inoculation method (see Lewis et al 1981), then homogenized the surviving adults and fed the suspensions to healthy 2nd instar larvae in bioassays. Medium mortality levels were observed in progeny of infected females (4-12%), with females surviving higher doses producing progeny with higher larval mortality levels. Murray et al (1991) suggests that the diet surface inoculation method, which involves spreading virus onto the diet

surface where larvae will walk around on it throughout their development, probably resulted in external adult contamination. Even though the control larvae (from females never exposed to virus) in Shapiro and Robertson's study showed 0% infection, such a result is not sufficient to rule out the possibility that the adults merely acted as mechanical vectors of the virus that infected their offspring. In a study by Murray and Elkinton (1989), low-level mortality (<2%) was recorded in the offspring of females inoculated as larvae. However, the authors suspected this result was probably due to inadvertent contamination since the same mortality level also occurred in progeny of undosed control females. Murray et al (1991) infected larvae as 4th instars (44% mortality level) using the droplet-feeding method (see Lewis et al 1981). No NPV-induced mortality was recorded in the progeny of surviving females dosed with NPV as larvae. This result is believable given the lack of contamination by this infection method. A recent study by Myers et al (2000) found very low-level (0.05%) transmission from 2 out of 13 females surviving infection as 5th instar larvae using the diet cube inoculation method (see Lewis et al 1981). This study also avoided problems with contamination, but the results conflict with those of Murray et al (1991). Although the sample size in Murray et al (1991) was about twice as large as that of Myers et al (2000), it is still possible that vertical transmission represents a very unlikely event and by chance this occurred during Myers' study but not Murray's.

Several studies have examined egg masses as likely reservoirs for vertically transmitted virus. Early research by Doane (1969) found that NPV-caused mortality of larvae hatching from field-collected egg masses could be drastically reduced by surface sterilization with sodium hypochorite, which destroys occlusion bodies. He suggested that this result was evidence for transovum transmission. Unfortunately, studies of field-collected egg masses cannot rule out contamination by OBs from

larvae that died near the area where the egg mass was laid. Later, using sets of larval bioassays, Doane (1975) determined that the most infectious part of the egg mass was the female abdominal hairs in which the eggs are packed. In lab studies, Murray et al (1991) used scanning electron microscopy to examine the eggs from females sublethally infected as 4th instar larvae, and did not find any OBs. In combination, these results suggest that OBs reached the egg masses by a route other than vertical transmission from female tissues to the egg surface: either adult females walked across contaminated substrates before oviposition, or OBs reached the surfaces of eggs directly from the environment. Murray et al (1991) suggested that contamination of the egg masses from environmental virus sources is more likely than direct vertical transmission. High larval mortality rates (up to 46%) have been found when egg masses from uninfected females were oviposited on trees in the site of an epizootic (Murray and Elkinton 1989). Furthermore, Murray and Elkinton (1990) showed that egg masses laid on tree bark artificially treated with NPV suffered high mortality in the hatching larvae. A more recent study by Charpentier et al (2003) analyzed field collected egg masses for LdNPV using PCR and DNA hybridization. They found that the DNA signal was drastically reduced when egg masses were subjected to sterilization with sodium hypochlorite prior to PCR, and interpreted this result as evidence against a transovarial transmission route. In contrast, Oberemok (2008) has reported detection of LdNPV DNA from the interior of field-collected gypsy moth eggs using the RAPD PCR technique.

Another approach for assessing the potential for vertical transmission is looking for OBs or other evidence of virus in the tissues of surviving adult females. Doane (1967) reported OB-like particles in the tissues of adult female *L. dispar* using optical microscopy. Shapiro and Robertson (1987) also detected OBs in adults surviving low dose infection (~1-4×10^{6} OBs per insect) by light microscopy, but at

lower levels than they found in pupae. However, Murray et al (1991) did not find any OBs in female tissues observed by light microscopy. Furthermore, they also did not detect NPV in adult tissues by DNA hybridization, although some viral DNA was detected in pupae. Based on these results, Murray et al (1991) believed that NPV infection may be disrupted at metamorphosis (e.g. Stairs 1965). On the other hand, Tsuey and Ma (1993) infected 5th instar larvae and looked at the tissues of surviving adults using transmission electron microscopy (TEM) and labeled antibodies. They reported OBs from various non-reproductive tissues of virus-challenge surviving adult female moths (fat body, trachea, ganglia, brain, prothoracic gland), but also from the ovary. Clearly, the results from this area of research have been highly variable, and are difficult to interpret. While it may be impossible to make a certain diagnosis of NPV using optical microscopy, other methods (such as immunoassays or DNA analysis) should be reliable if there has been no contamination. If Tsuey and Ma (1993) were correct, then it is difficult to understand why Murray et al (1991) found no virus in any of their vertical transmission trials.

A different approach that has relevance to vertical transmission is attempting to trigger latent baculoviruses in seemingly uninfected hosts. Studies typically begin with a colony of insects that shows no evidence of disease, then attempts to activate a latent virus by subjecting the larvae to various stressors. In a study by Longworth and Cunningham (1968), NPV mortality in gypsy moths was higher and occurred sooner when larvae were inoculated with *Aglais urticae* NPV, to which the gypsy moth is not susceptible. This result was interpreted as evidence for activation of latent LdNPV. However, given that 10% of their control larvae died of NPV, it seems just as likely that the foreign NPV simply acted as a stressor which decreased the resistance of the larvae to whatever external NPV they were being exposed to (e.g Steinhaus 1958, Reilly and Hajek 2008). Numerous similar studies have been performed with a great

variety of other stressors, including chemicals, poor nutrition, low temperature, high humidity, crowding, etc. (reviewed in Il'inykh and Ul'yanova 2005). A recent study by Ilyinykh et al (2004) showed that high mortality could be induced from sterilized field-collected egg masses if larvae were treated with copper sulfate. However, this study likewise cannot separate latent infection from environmental contamination given 1) that external sterilization of field-collected egg masses is rarely 100% effective (e.g. Doane 1969), 2) that there was low mortality in control larvae, the cause of which could either be contamination or spontaneous activation of latent virus, and 3) that the higher mortality in the treatment groups could be explained by decreased virus resistance due to stressors. Ilyinykh et al (1997) tested for and confirmed LdNPV DNA in larvae from field collected egg masses that were treated with a different virus. Unfortunately, this study suffers from the same problems in design. At this point, it would seem tempting to conclude that there is very little evidence for latent or persistent viral infections in the gypsy moth, except that such infections are beginning to be convincingly demonstrated in other systems (reviewed in Cory and Myers 2003), particularly through the use of DNA techniques to identify the virus inside all life stages of the host.

Myers et al (2000) and other authors have called for studies of the implications of vertical transmission for *L. dispar* population dynamics. Although recent models of gypsy moth population dynamics have incorporated only horizontal transmission pathways (e.g. Dwyer et al 2000), the presence of a female-offspring transmission pathway would be an important addition to models of the initiation of NPV epizootics, and to the dynamics of NPV when host populations are low. In this paper I report the results of a detailed laboratory investigation of the process of vertical transmission

from female to offspring with the goal of determining whether adult females fed OBs as larvae retain virus in their tissues, whether the virus is in a location where it could be contacted by the eggs, and whether the virus is in an infectious form.

Methods

Preparation of infected larvae

Lymantria dispar larvae were reared on artificial diet (Bell et al 1981) in groups of 10 larvae per 177 ml cup until the required instar, and then transferred individually to 30 ml cups. We performed our experiments both with larvae inoculated with virus as 3rd instars and larvae inoculated as 5th instars, in case the developmental stage of larvae at the time of infection influences the potential for vertical transmission. The virus used in all studies was the wild-type Hamden, CT strain of the *Lymantria dispar* nucleopolyhedrovirus (LdNPV) distributed as "Gypchek" by the USDA Forest Service. Newly-molted third instar larvae were individually fed cubes (3 mm^3) of artificial diet (Bell et al 1981) on which a 3 µl droplet containing approximately 1×10^2 OBs of LdNPV had been placed. Newly-molted fifth instar larvae were inoculated by the same procedure, but with 1×10^4 OBs per diet cube. Relatively low doses were chosen to produce some larval mortality (our doses yielded ~20% mortality) yet minimize the potential for contamination. After consuming the diet cube, larvae were placed in individual cups with fresh diet and incubated at 25ºC with a 16:8 light:dark cycle until they pupated. Pupae were transferred to empty cups with a piece of moist paper towel until they became adults. Adult female moths were dissected, and checked for viral occlusion bodies using phase contrast microscopy, real-time PCR, and larval bioassays.

Dissection of female moths

The reproductive and digestive tracts of adult female gypsy moths were investigated by dissection to visually identify the organs encountered by the egg along its path during oviposition and to choose targets for NPV quantification procedures. To the best of our knowledge, the most recent published diagrams specific to the gypsy moth reproductive tract are those by Forbush and Fernald (1896), and these are incomplete. Therefore, we have included a labeled photograph of a typical dissection (see Figure 3.1). After leaving the ovary, the egg passes through the lateral oviduct, into the common oviduct, and exits the body through the oviporus. As it moves through the common oviduct, the egg would pass the openings to the spermatheca, bursa copulatrix (via seminal duct), accessory glands, and finally the rectum. The bursa copulatrix also connects to the exterior of the abdomen at the vulva. Thus the presence of virus particles in any of these structures would likely contribute to transovum transmission. We found no obvious differences in the morphology of the reproductive tract between dosed and undosed females (see Rallis and Burand 2002).

Optical microscopy

The reproductive and digestive structures of dissected female moths were examined by phase-contrast microscopy, and OB-like particles were counted at 400× using a hemocytometer. Potential OBs were tested for OB-like behavior with the KOH test (see Lacey and Brooks 1997).

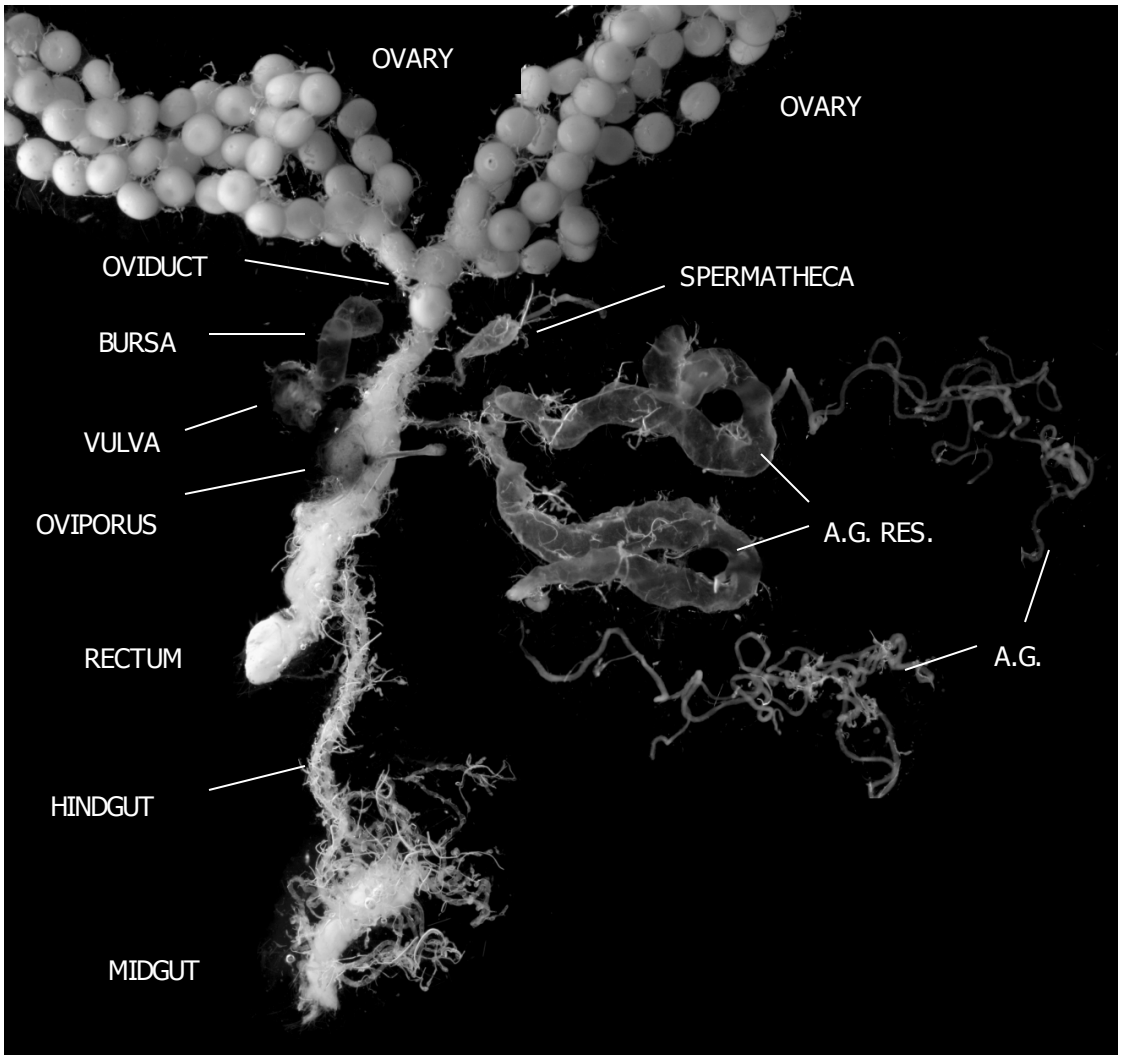

Figure 3.1. Typical dissection of reproductive and digestive structures in the abdomen of an adult female gypsy moth. A.G.=accessory gland, A.G. RES.= accessory gland reservoir. The accessory glands (not including reservoirs) are approximately 65mm long. A small accessory gland also attaches to the end of the spermatheca.

Real-time PCR

Dissected reproductive structures from adult female gypsy moths were homogenized, transferred to 2 ml microcentrifuge tubes, mixed with 500 ml dilute alkaline saline (0.1 M NaCl, 0.1 M Na_2CO_3, pH 10) to dissolve occlusion body protein, pelleted, and resuspended in 400 ml buffer (0.1 M KCl, 0.01 M Tris, 0.01 M EDTA). Samples were then sonicated for 30 seconds, treated with 50 ul 10% SDS and 20 ul proteinase K (600 mAU/ml), and incubated overnight at 50 C. We then added 200 ul 99.5% ethanol, and pipetted the resulting mixture (~700 ul) into a DNeasy spin column from a Qiagen DNeasy Blood and Tissue Kit (Qiagen, Inc., Germantown, MD). DNA was extracted using the spin column and the wash buffers from the kit, and eluted into 400 ul buffer AE. Real-time quantitative PCR, using the Applied Biosystems 7900 HT Sequence Detection System, was performed with the extracted DNA. Forward and reverse primers and a FAM-TAMRA labeled probe were designed for the G22 gene, ORF 7, which is unique to the LdMNPV genome (Blischoff & Slavicek 1995, J. Slavicek pers. comm.). The nucleotide sequences of the primers and probe are as follows:

FWD: GCGCTTCTCCGTGACCAA

REV: AATCCCTGCACATGCCTGTCAT

PROBE: ACCCGTGCCTGTTCATGTTCAAGGA

Reaction volume was 25 ul, including 5 ul of DNA template. In controls, PCR was performed using 5 ul of sterile water. Estimating absolute numbers of occlusion bodies from relative quantities of LdNPV DNA was performed using a standard curve, which was created by extracting DNA from pure solutions of known occlusion body concentration, then serially diluting the extracted DNA. Final estimates for each

sample were averaged from at least two replicates of the PCR reactions. We used adult females that had been inoculated as both 3rd instar larvae (n=3 females) and 5th instar larvae (n=3 females).

Bioassay of LdNPV in reproductive tissues

We tested the infectivity of LdNPV in the tissues of adult female gypsy moths with larval bioassays. Female tissues (ovary, oviduct, gut/rectum, accessory gland, spermatheca, bursa, and external cuticle) were individually homogenized with 30 ul of sterile water, then 1 ul tissue suspensions were pipetted onto the surface of small cubes of artificial diet (1 mm^3). Thirty newly-molted (within 24 hrs) 2nd instar larvae were each fed one inoculated cube, then reared individually and checked daily for death by NPV until pupation. We used adult females surviving infection that had been inoculated as both 3rd instar larvae (n=3 females) and 5th instar larvae (n=3 females). We generated a standard curve by inoculating newly-molted 2nd instar larvae with known doses of OBs ranging from 0 to 500 OBs per larva.

Results

Optical microscopy

Interior spaces of the adult midgut, hindgut, and rectum contained high concentrations of OB-like particles under the phase contrast microscope (see Figure 3.2), and these particles also responded positively to the KOH test (i.e. darkened and dissolved). However, there was no visibly obvious infection in the cells themselves, and such particles were also found in control females.

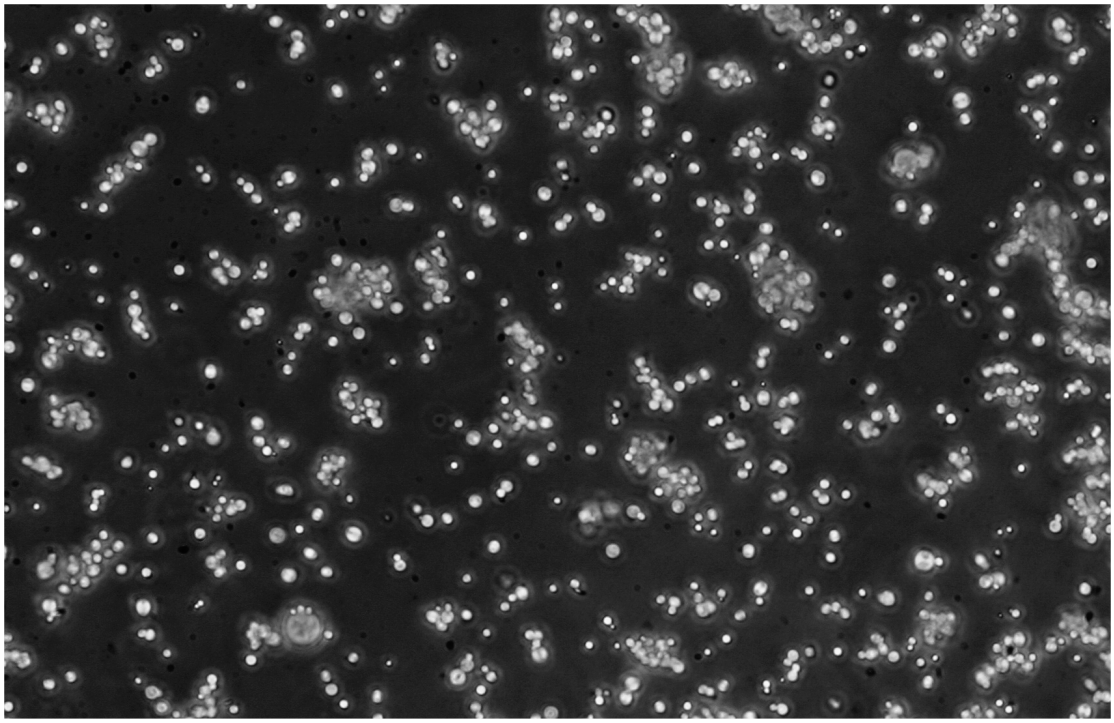

Figure 3.2. An example of OB-like particles from the rectum (meconium fluid) of a female gypsy moth.

Real-time PCR

We detected low levels of LdNPV in multiple tissues of female gypsy moths including hindgut/rectum, ovary, oviduct, bursa copulatrix, spermatheca, accessory gland, and cuticle (see Figure 3.3). Interestingly, there was high variability in the location of virus among individual females that were infected as 3rd instar larvae. However, females infected as 5th instar larvae showed a more uniform and slightly higher level of virus.

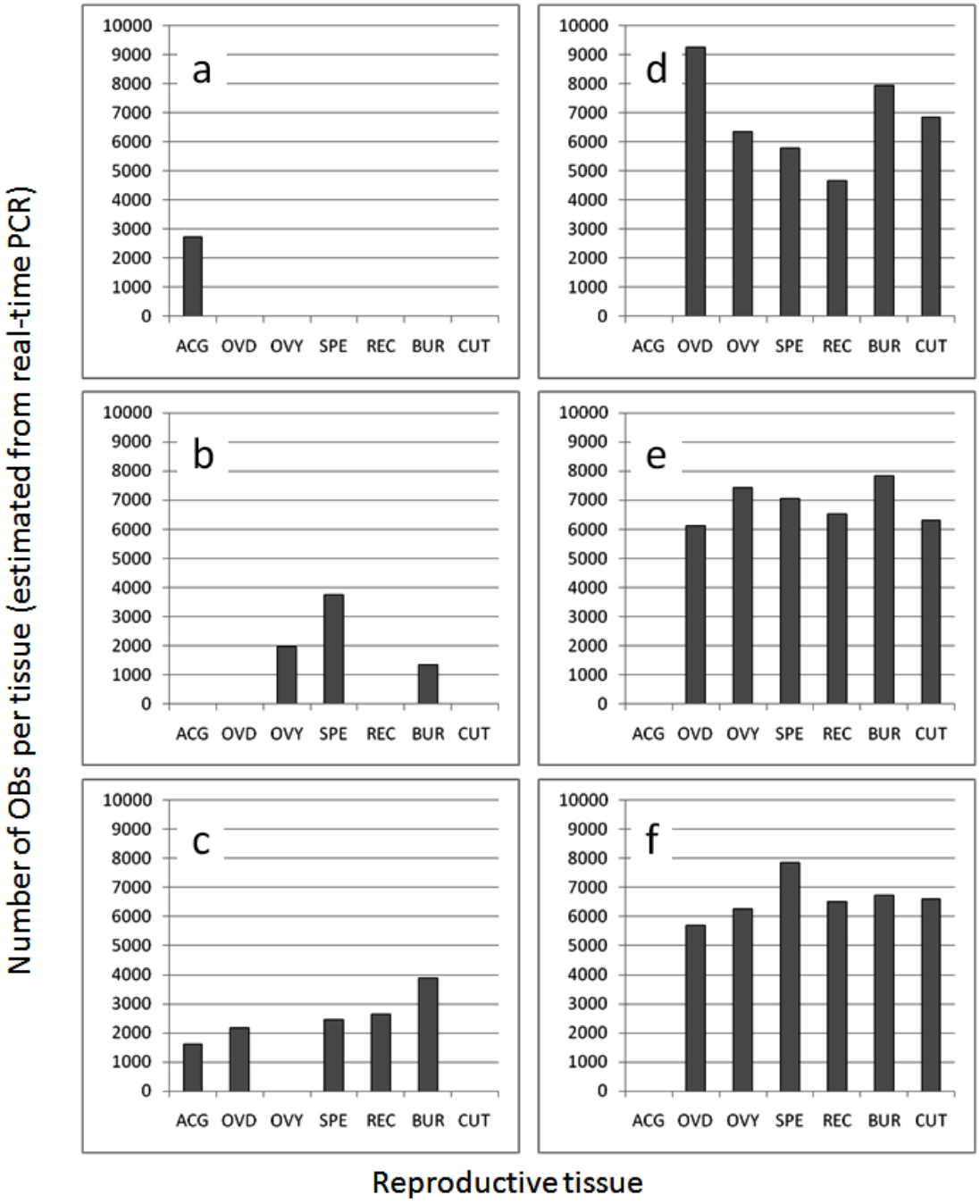

Figure 3.3. Estimated LdNPV OB amounts detected by real-time PCR of individual females inoculated with 1×10^{2} OBs as 3rd instar larvae (panels a-c) or 1×10^{4} OBs as 5th instar larvae (panels d-f). Each panel summarizes the results from one female dissection. ACG=accessory glands and reservoirs, OVD=oviduct, OVY=ovary and eggs, SPE=spermatheca, REC=rectum and hindgut, BUR=bursa copulatrix, CUT=ventral cuticle and body hairs.

Bioassay of LdNPV in reproductive tissues

We found 0% NPV-induced mortality among newly-molted 2nd instar larvae inoculated with tissue suspensions on diet cubes. This was true for all of the female reproductive and digestive structures we tested. We ran an additional set of tests in which 45 newly-molted 2nd instar larvae were fed 1 ul doses of undiluted fluid from the rectums of adult females, since this structure contained high levels of OB-like particles. These larvae similarly suffered no mortality. Among larvae inoculated with known OB concentrations for the standard curve, there was at least partial NPV mortality at all non-zero dosages tested (see Figure 3.4).

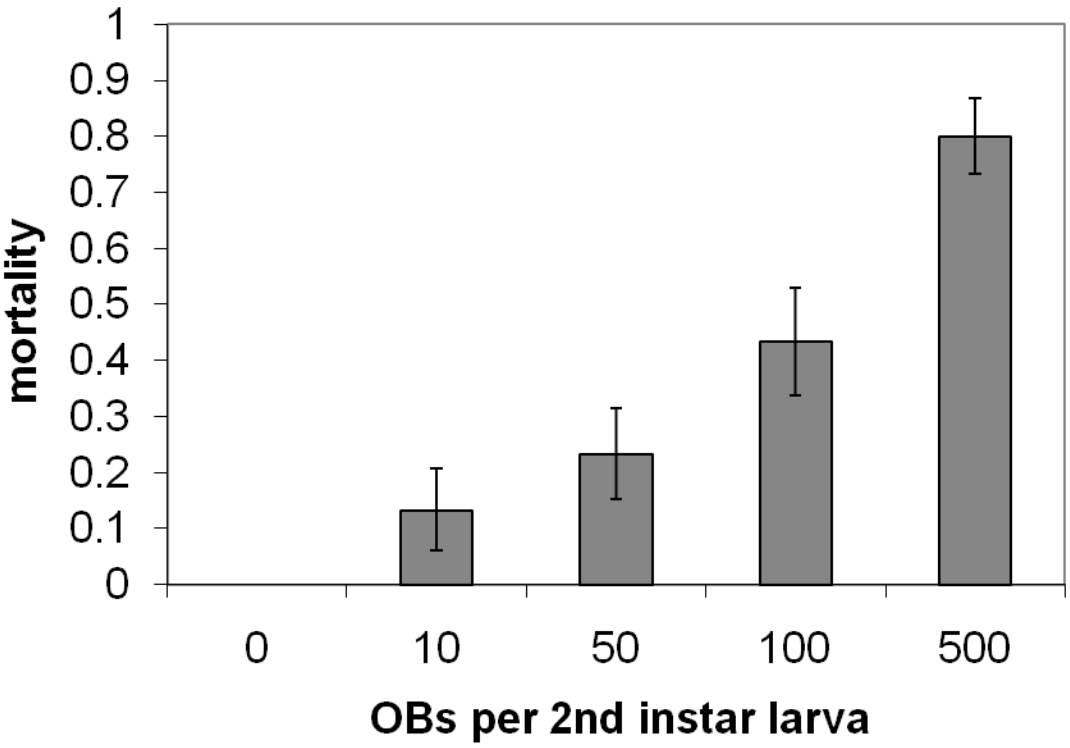

Figure 4. Proportion of newly-molted 2nd instar larvae dying of LdNPV after inoculation with 0, 10, 50, 100, and 500 OBs. N=90 per dosage level.

Discussion

In this study, we did not detect evidence of vertical transmission of NPV occlusion bodies in the gypsy moth. However, real-time PCR did reveal the presence of low amounts of viral DNA in various tissues of adult females sublethally infected as larvae. Particles resembling occlusion bodies were detected in gut tissues by optical microscopy, but these results were not fully consistent with our PCR results, and more importantly the particles were not infective at the doses used. Visual methods cannot reliably quantify OBs for several reasons, including a lack of characters for separating OBs from other crystals, and the possible occurrence of empty OB protein shells lacking virions (e.g Fuxa et al 1992).

In our bioassays, no tissue from any surviving adult female produced infection in larvae, whereas in our infectivity tests, doses as low as 10 OBs were sufficient to produce some infection among 2nd instar larvae. Thus, it seems reasonable to conclude that essentially no viable occlusion bodies were available for passage from female to offspring. If vertical transmission is only fostered at certain larval instars (e.g. Rallis and Burand 2002) or with certain dosages, it is possible that our experimental conditions missed this critical window; however, our results were the same (0%) for adult females sublethally infected as both 3rd and 5th instar larvae with doses of 1×10^{2} and 1×10^{4} per larva, respectively.

These results are different than those we obtained by PCR, which did register low levels of virus in some tissues, although no specific tissue was always a reservoir of the LdNPV. Possibly the reorganization of tissues during metamorphosis could help explain the seemingly random locations of LdNPV signatures that we found in females examined by real-time PCR. An infection of the genital imaginal disc of a larva might be carried through metamorphosis and result in a scattered infection pattern after differentiation into the separate reproductive organs (e.g. Chen and Baker

1997). By detecting some virus, our DNA tests are in apparent conflict with those of Murray et al (1991), but this discrepancy may be explainable by the inherently greater sensitivity of PCR compared with DNA hybridization, coupled with low overall virus amounts.

Extremely low amounts of LdNPV (1-9 × 10^3 OBs per positive structure) detected in tissues might not be expected to produce high mortality when dispersed among numerous progeny, especially if not all of the LdNPV contained within the structure was available to the eggs or if the LdNPV was present in a tissue more distant from the path of the egg during oviposition. Furthermore, NPV amounts detected by PCR could include non-occluded virus or damaged occlusion bodies that might not be infectious when ingested by larvae. There is also a slight possibility that digested fragments of the LdNPV genome could be carried over in the gut from the inoculation event in the larval stage. This seems unlikely since detection was not more common in gut samples (see figure 3.3).

Overall, our results are not consistent with widespread vertical transmission of active infection (e.g. Shapiro and Robertson 1987). Our use of the diet plug method for inoculation avoids having larvae walk on virus-laced diet throughout their development, which is a major source of potential contamination. Given that we experienced no mortality in our controls, our methods appear to have been successful. Myers et al (2000) found extremely low-level (0.05%) transmission from 2 out of 13 females sublethally infected as 5^{th} instars. Such results may be due to contamination or to rare combinations of events.

We do not rule out the possibility that rare developmental conditions may allow low transmission by some females, since our experiments utilized relatively small numbers of adults. Perhaps in rare cases enough OBs accumulate in/on one egg to produce infection when the larva emerges, or perhaps a larva may rarely become

infected by ingesting non-occluded virions which have been protected in the egg mass. Levels of vertical transmission appear to be so low that they may not be of importance to population-level dynamics of LdNPV. However, even low levels might be enough to allow virus persistence in low-density populations, as suggested by Myers et al (2000). Models are needed to explore this issue.

Alternatively, it is possible that our PCR results are evidence of latent or persistent infection, in which the virus is not transmitted via occlusion bodies, but rather as non-occluded virus that happens to infect the egg cell. Persistent infection has been shown to occur in laboratory and field populations of *Mamestra brassicae* NPV (Burden et al 2003). Such a scenario would also be consistent with our bioassay results since virus in this form would not be directly infectious to larvae, and might also be consistent with the results of Oberemok (2008) who reported evidence of transovarial transmission of LdNPV.

Despite the difficulties in testing for vertical transmission, evidence does seem to be accumulating in some systems that vertical transmission and/or latency of baculoviruses may be more common than once believed (e.g. Il'inykh and Ul'yanova 2005, Cory and Myers 2003). If this is the case, then there is a pressing need for research on why the horizontal transmission route continues to be the major driver of disease dynamics.

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