Note

Phenotypic Variation in Overwinter Environmental Transmission of a Baculovirus and the Cost of Virulence

Arietta E. Fleming-Davies[1,2,*] and Greg Dwyer[1]

1. Department of Ecology and Evolution, University of Chicago, Chicago, Illinois 60637; 2. Biology Department, Radford University, PO Box 6931, Radford, Virginia 24141

Submitted November 10, 2014; Accepted July 17, 2015; Electronically published October 15, 2015

Online enhancement: appendix. *Dryad data:* http://dx.doi.org/10.5061/dryad.p1k27.

abstract: A pathogen's ability to persist in the environment is an ecologically important trait, and variation in this trait may promote coexistence of different pathogen strains. We asked whether naturally occurring isolates of the baculovirus that infects gypsy moth larvae varied in their overwinter environmental transmission and whether this variation was consistent with a trade-off or an upper limit to virulence that might promote pathogen diversity. We used experimental manipulations to replicate the natural overwinter infection process, using 16 field-collected isolates. Virus isolates varied substantially in the fraction of larvae infected, leading to differences in overwinter transmission rates. Furthermore, isolates that killed more larvae also had higher rates of early larval death in which no infectious particles were produced, consistent with a cost of high virulence. Our results thus support the existence of a cost that could impose an upper limit to virulence even in a highly virulent pathogen.

Keywords: environmental transmission, gypsy moth (*Lymantria dispar*), host-pathogen interactions, pathogen polymorphism.

Introduction

Within-species variation in pathogens is widespread, and yet the mechanisms that maintain this diversity are still poorly understood (Galvani 2003; Alizon et al. 2009). One well-studied mechanism to promote pathogen coexistence is the trade-off theory of the evolution of virulence (Levin and Pimentel 1981; Anderson and May 1982). In this theory, trade-offs between different traits that affect pathogen fitness lead to strains with similar fitness despite differing phenotypes, thus promoting coexistence of multiple pathogen strains. By introducing a cost to high virulence, trade-offs can also lead to an upper limit to virulence, defined as the harm caused by a pathogen to its host. For example, although high virulence may increase transmission, if it is also associated with high removal rates due to host mortality, then evolution of intermediate virulence may be predicted (Levin and Pimentel 1981; Anderson and May 1982; Bremermann and Thieme 1989; Bull 1994). Although most work on trade-off theory has focused on trade-offs between transmission and removal rates, trade-offs between any two pathogen traits that affect fitness may lead to pathogen diversity, much as trade-offs in the ability to use different resources can lead to coexistence of competing species in community-ecology theory (Tilman 1977).

Variation in environmental transmission rates has been proposed as a mechanism promoting coexistence of multiple pathogen strains (Handel et al. 2014). Ability to persist in the environment is an ecologically important trait for any pathogen with environmental transmission at some stage in its life cycle. Pathogens with environmental transmission often exhibit higher virulence, or harm to the host, than pathogens with direct transmission (Walther and Ewald 2004), as they are able to persist outside of the host and thus do not require its survival for transmission (Ewald 1987; Walther and Ewald 2004). Mathematical models of pathogens with environmental transmission suggest that those with very long-lived infectious particles may be particularly virulent ("curse of the pharaoh" hypothesis; Bonhoeffer et al. 1996). However, this association of long environmental persistence and virulence depends on specific assumptions of the models, such as whether populations are at their equilibrium densities and whether multiple infections are allowed (Bonhoeffer et al. 1996; Gandon 1998; Caraco and Wang 2008). In the case of pathogens with both environmental and direct transmission, pathogen polymorphism may arise if one strain evolves to specialize in environmental transmission (and thus evolves higher virulence) and the other

* Corresponding author; e-mail: arietta.flemingdavies@gmail.com.

Am. Nat. 2015. Vol. 186, pp. 797–806.
0003-0147/2015/18606-55884$15.00.
DOI: 10.1086/683798

specializes in direct transmission (Roche et al. 2011; Boldin and Kisdi 2012). Trade-offs between the two types of transmission could thus promote pathogen polymorphism.

In addition to their potential to help explain standing variation in pathogens, estimates of environmental persistence rates are also necessary to predict pathogen population dynamics, including whether outbreaks will occur (Breban et al. 2009; Fuller et al. 2012). Despite this ecological importance, there are relatively few empirical estimates of pathogen persistence or, conversely, decay. This scarcity may be due in part to the logistical difficulties of estimating pathogen persistence in the environment, because of the need to disentangle persistence of existing infectious particles from accumulation of new infectious particles. Most prior empirical work has focused on survival time of human pathogens on inert surfaces, such as those found in hospitals (reviewed in Kramer et al. 2006). Often these experiments test only for the presence of a pathogen after a given length of time and do not provide quantitative estimates of half-life or pathogen decay rate.

Like many other nucleopolyhedroviruses (Cory and Myers 2003), the baculovirus that infects gypsy moth (*Lymantria dispar*) larvae exhibits high levels of within-species polymorphism. Field-collected isolates vary in their speed of kill (Kennedy et al. 2014), mortality in laboratory dose-response experiments (Kennedy et al. 2014), transmission rates in field experiments, and variability in transmission (Fleming-Davies et al. 2015). In this study, we estimate environmental overwinter transmission in 16 isolates, in order to ask whether trade-offs with other virus traits might promote coexistence of multiple pathogens in a single population.

In the gypsy moth–baculovirus system, environmental persistence is required at two different stages of transmission. First, horizontal transmission within a single season from younger to older larvae requires short-term pathogen persistence on leaf material (Woods and Elkinton 1987; Fuller et al. 2012). Second, gypsy moths have only one generation per year, so overwinter transmission across host generations requires viral persistence on tree bark and gypsy moth egg masses until larvae hatch the following spring (Doane 1969; Murray and Elkinton 1989). Both overwinter persistence and within-season persistence affect long-term gypsy moth dynamics, including the threshold population required for an epizootic to occur and the likelihood of population cycles (Dwyer et al. 2000; Fuller et al. 2012). The 16 isolates used in this study have been found to vary in their within-season environmental persistence rates (Fleming-Davies et al. 2015). Here, we focus on the overwinter transmission process, which includes both overwinter persistence and infection of neonates the following spring.

Baculovirus virions are contained in protein-coated occlusion bodies that can persist outside of the host until broken down by ultraviolet (UV) light and rain (Ignoffo et al. 1977; D'Amico and Elkinton 1995). On leaves, purified virus has an average persistence time of approximately 0.9 days, while virus-killed cadavers persist more than twice as long, an average of 2.5 days (Fuller et al. 2012). In contrast, overwinter persistence occurs over a much longer time period of 8–9 months. The virus overwinters primarily on tree bark on which late-instar larvae have died from virus, and virus particles are protected from UV light by gypsy moth egg masses laid on bark, allowing for longer persistence times (Murray and Elkinton 1989). Other mechanisms, such as soil reservoirs (Fuxa and Richter 2007) or direct transovarian (within-egg) transmission from sublethally infected mothers to offspring (Myers et al. 2000), may also contribute to vertical transmission, but the magnitude of these effects appears to be very small compared to that of external (transovum) contamination of egg masses (Woods and Elkinton 1987; Kukan 1999).

In this study, we estimated environmental overwinter transmission of 16 field-collected isolates of *L. dispar* multicapsid nucleopolyhedrovirus (LdMNPV). We asked whether isolates vary in this ecologically important trait and whether overwinter transmission rate correlates with other virus traits in a way that might promote coexistence of multiple pathogen types. We observed a cost of high virulence: although more virulent isolates have increased overwinter infection rates, they also have higher rates of early larval death in which no infectious particles are produced.

Material and Methods

Study System

In our study system, LdMNPV infects gypsy moths only as larvae, so hosts are available only from approximately early May until mid-July, when larvae pupate. Gypsy moths overwinter as eggs, so the baculovirus must persist in the environment from late summer until the following spring, when the next generation of larvae hatches. Females oviposit by rubbing their abdomens over the surface of tree branches and trunks, and virus contamination is thought to occur during this process. Hairs from the female's abdomen are rubbed off to cover egg masses during oviposition, protecting the virus from exposure to UV light and rain (Murray and Elkinton 1989). The following spring, neonatal larvae consume contaminated egg material as they hatch and thus become infected (Doane 1969; Murray and Elkinton 1989). This causes the start of a new epizootic, with transmission occurring when the infected neonates die on leaves of host trees that are later consumed by later-instar larvae (Woods and Elkinton 1987).

Vertical transmission from mother to offspring in LdMNPV appears to result primarily from contamination of the surface of eggs (transovum transmission; Murray and

Elkinton 1989; Kukan 1999). Other mechanisms of long-term baculovirus persistence appear to be rare in this species, including vertical transmission of the pathogen within eggs (transovarian transmission; Kukan 1999). Transovarian transmission requires presence of viral DNA in the mother, either because of previous sublethal infection (Myers et al. 2000) or from persistent latent or "covert" infections present in a population (Burden et al. 2003). We have observed little evidence for transovarian transmission in LdMNPV. When field-collected egg masses are surface-sterilized with 4% formalin before hatching, virus-induced death in neonates is extremely low (0% mortality observed in approx. 100,000 lab-raised larvae; A. E. Fleming-Davies and G. Dwyer, personal observations). It is possible that sublethal infections are present in these larvae, but these would have little effect on epizootic dynamics, as horizontal transmission occurs only after the death of the host. Rather than transovarian transmission, there is strong evidence for environmental contamination of egg masses (transovum transmission) as an overwintering mechanism that leads to transmission from generation to generation (Doane 1969; Murray and Elkinton 1989; Fuller et al. 2012).

In order to better detect differences among pathogen isolates, we attempted to minimize host variability in our experiment by using gypsy moth larvae from the USDA-APHIS (Animal and Plant Health Inspection Service) colony at Otis Air Force Base (Buzzards Bay, MA). This population consists of the New Jersey standard strain and has been bred under laboratory conditions for more than 40 generations (Doane and McManus 1981).

Field Experiment

Overwintering depends on exposure to environmental conditions, and thus it is necessary to conduct experiments in the field. To replicate the natural overwintering process in a controlled setting, we first infected laboratory-raised fourth-instar larvae with 16 naturally occurring baculovirus isolates ($n = 15$ individuals/isolate) by placing larvae in cups of artificial diet (Doane and McManus 1981) in which one virus isolate had been spread on the surface of the diet (virus concentration $= 3 \times 10^6$ infectious particles/mL) and allowing them to feed for approximately 2 weeks. The virus causes dissolution of the cuticle, resulting in the release of infectious particles from the cadaver into the environment (Miller 1997). After death in the laboratory but before disintegration of the cadaver, each larva was transferred to the center of a piece of red oak bark approximately 10 cm × 10 cm. A 7.5-cm circle centered around the cadaver was drawn to mark the future area of oviposition. All bark pieces were taken from the trunk of a single mature red oak (*Quercus rubra*) tree (lightning-killed local red oak donated by Care of Trees, Chicago). Red oak is a preferred host plant of gypsy moth larvae (Barbosa et al. 1979), and egg masses are often found on red oak branches or trunks (Doane and McManus 1981). Bark pieces with virus-killed cadavers were placed in a dark room for 1 week until cadavers had "melted" on the bark. We then used the software ImageJ to estimate cadaver size from photographs. Size was measured as cadaver area (cm^2) on bark, which correlates well with larval mass and thus viral load (A. E. Fleming-Davies and G. Dwyer, unpublished data).

To expose the virus-contaminated bark pieces to natural environmental conditions that contribute to virus decay, all bark pieces were then transferred to our field site at the Lux Arbor Reserve of the Kellogg Biological Station, in southern Michigan. Bark pieces were hung from string tied around red oak tree trunks at approximately 1.5 m from the ground, with 5–8 pieces per tree. Virus isolates were distributed randomly among trees. Controls consisted of 10 bark pieces without virus-killed cadavers, to test for background contamination of the bark in the field, as well as to account for any experimental contamination or prior virus presence on the bark pieces. Previous measures of virus overwintering in this system found no differences in infection rate between egg masses left in the field for the entire winter and those removed after 3 days of exposure to virus-contaminated bark (Murray and Elkinton 1989), suggesting that the critical exposure period occurs immediately around the time of oviposition. This is likely because egg masses block sunlight and rain from reaching the bark surface, thus protecting virus particles. We therefore left cadavers in the field for 1 week in mid-August and then brought the bark pieces back to the lab for the remainder of the experiment.

In the laboratory, we mated a pair of lab-raised adult gypsy moths on each contaminated bark piece, in a 7.5-cm-diameter paper cup secured to the bark with tape. The cup was placed on the marked 7.5-cm-diameter circle centered on the virus-killed cadaver. Cups were removed after 3 days, and the location of each egg mass was recorded, in case location affected infection probability. In cases where part or all of the egg mass was laid on the cup rather than the bark, the egg mass was scraped off the cup and placed in the center of the bark. Bark pieces with unfertilized egg masses were discarded at this stage. Bark pieces were then stored individually in bags at 25°C for 1 month, then held at 4°C until the following March, in order to replicate the obligate winter diapause in this species.

In March 2014, bark pieces were returned to 25°C to induce hatching, and neonatal larvae were transferred to 6-oz (177.4-mL) cups of artificial diet upon hatching. After approximately 1 week, when all individuals had either molted to the second instar or died, larval fates were recorded in order to estimate an infection probability for each replicate egg mass. The baculovirus interferes with

molting by inhibiting ecdysteroid hormones (O'Reilly and Miller 1989; Park et al. 1993), so individuals reaching the second instar were assumed to have survived virus exposure. It is possible that sublethal infections were present in surviving neonatal larvae in our experiment, as we did not check for presence of viral DNA in survivors. Sublethal infections in neonates would not lead to transmission of the baculovirus, and therefore we focused on infections causing larval mortality. The total sample size for all isolates was 26,425 neonatal larvae from 170 egg masses, because of failed hatching of some egg masses (see fig. 1 for sample sizes per isolate and mean number of larvae per egg mass). Experimental data are deposited in the Dryad Digital Repository: http://dx.doi.org/10.5061/dryad.p1k27 (Fleming-Davies and Dwyer 2015).

Cause of death was determined first by visual inspection, to check whether larvae exhibited a typical "melted" phenotype indicating dissolution of the cuticle by the baculovirus. Virus-induced death was then confirmed for a subset of individuals via necropsies to look for occlusion bodies, which are visible under a light microscope at 400 × magnification. In addition to these virus-induced deaths, we also recorded another phenotype, which we termed "early death." These larvae typically died several days earlier than the first confirmed death from virus and were characterized by small, shriveled cadavers. Occlusion bodies were not found in any

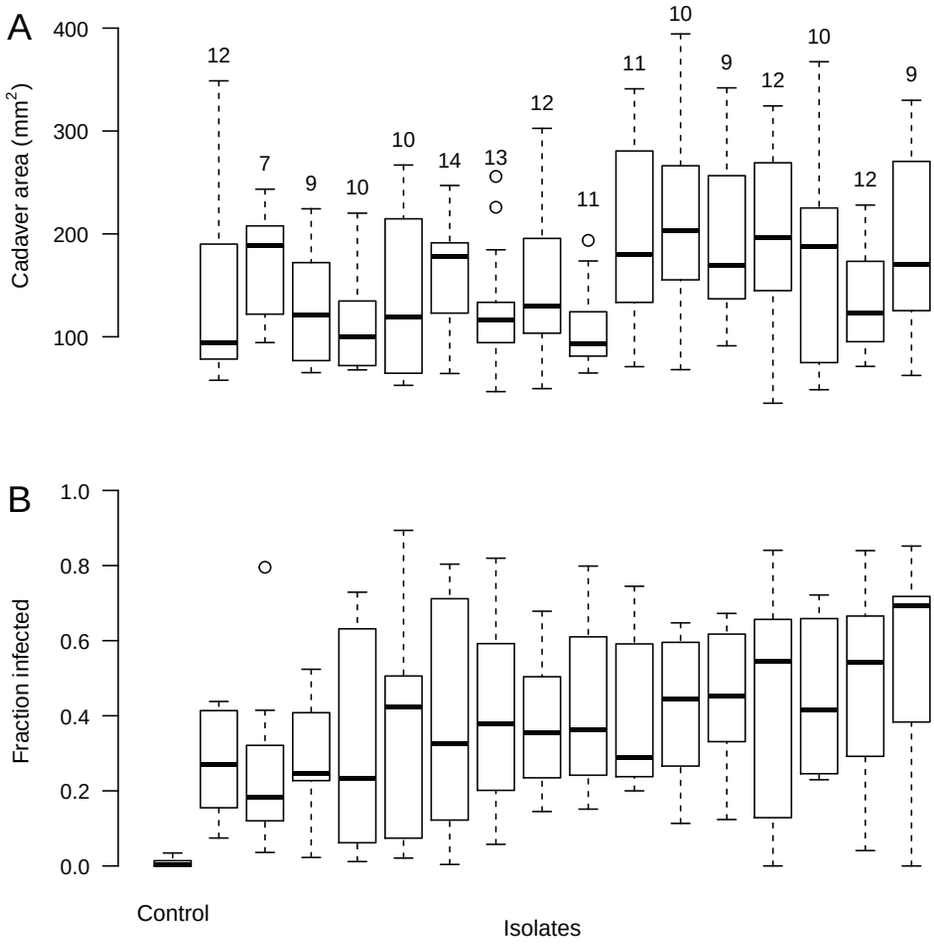

Figure 1: Effects of virus isolate on fourth-instar cadaver size (*A*) and fraction of neonatal larvae infected (*B*). Field-collected virus isolates are ordered by increasing mean fraction infection in both plots. Numbers above box plots in *A* give the number of replicate egg masses or cadavers per isolate (each egg mass was exposed to one cadaver, and thus the sample sizes are the same for *A* and *B*). Although cadaver size correlates with viral load and helps to predict infection risk (see table A1, available online), cadaver size alone does not explain among-isolate differences in fraction infected (*B*). The box plots in *B* show the distribution of the fraction infected among egg masses. Fraction infected per egg mass was estimated as a raw proportion of number of virus-killed larvae over total individuals per egg mass (mean ± 1 SD: 155 ± 53 individuals/egg mass). The leftmost plot is of controls on uncontaminated (but not sterilized) bark pieces. Open circles denote outliers.

of these individuals. This phenotype has been previously observed in gypsy moth larvae at very high doses of LdMNPV, in particular in neonates (G. Dwyer, unpublished data).

Statistical Analysis and Model Fitting

To test for effects of isolate, cadaver size, and oviposition site on infection risk, we fitted general linear models with infection state as a binomial response variable and a logit link function (R function glm). To account for the nonindependence of individual larvae within an egg mass, we treated each egg mass as an independent replicate, with the numbers of infected and uninfected individuals per egg mass as the response variables. In this analysis, the "uninfected" category included both larvae surviving to the second instar and those dying early without evidence of infectious particles. We included early-dying individuals in the "uninfected" category because no infectious particles are produced in early-dying larvae and thus overwinter transmission of the virus is 0, the same as in surviving individuals. We also fitted multinomial models to test whether early death of larvae correlated with the same factors as confirmed virus death (in which occlusion bodies were produced). These multinomial models again used larval fate as the response variable, but with three possible outcomes: survival to second instar, early death, or death from virus. We again tested for effects of isolate, cadaver size, and oviposition site (package nnet in R), and the unit of replication was again an egg mass.

To test whether the binomial was an appropriate distribution for our data, we checked for overdispersion by calculating the variance inflation factor as $\hat{c} = \chi^2/\mathrm{df}$ (Cox and Snell 1989). The χ^2 goodness of fit and residual degrees of freedom (df) were computed from the general linear model fitted to our infection data as a binomial response variable. A variance inflation factor $\hat{c}$ greater than 1 indicates that data are overdispersed. In that case, either a different distribution, such as beta-binomial, should be used or the likelihood values must be corrected by the factor $\hat{c}$ (Burnham and Anderson 2002).

Gypsy moths exhibit genetic variation in their resistance to LdMNPV (Elderd et al. 2008; Páez et al. 2015), and thus variance among replicate bark pieces within an isolate is likely to be due in part to variance in resistance among gypsy moth egg masses, each of which is a full-sibling family. To take into account this genetic variance among families, we also fitted general linear mixed-effects models, which add random intercepts for egg masses (R package lme4). Multiple replicate egg masses per isolate allowed us to distinguish host genetic effects from virus isolate effects.

The Akaike information criterion (AIC) was used to select the best models (Akaike 1981). This metric, calculated as $\mathrm{AIC} = -2L + 2n$, uses the log-likelihood value L to take into account goodness of fit while also penalizing model complexity with n, the number of parameters. We also considered a null model with only an intercept and no other fixed effects. We used a likelihood ratio test to test for a significant effect of isolate not explained by the cadaver size effect. The Markov chain Monte Carlo (MCMC) method (R package MCMCglmm; Hadfield 2010) was used to fit the mixed-effects multinomial models, with noninformative normal and inverse Wishart priors for the fixed-effect coefficients and the random-effect variance-covariance matrix, respectively. The multinomial models were compared by means of the deviance information criterion (DIC), which is similar to the AIC but can be easily computed from MCMC chains (Spiegelhalter et al. 2002).

We also used larval infection data to estimate the overwinter transmission for each virus isolate, using maximum likelihood methods (appendix, available online). We scaled this overwinter transmission rate to match previous population models of gypsy moths and baculovirus (Dwyer et al. 1997, 2000), in order to compare our results with measurements from natural gypsy moth outbreaks (appendix). All maximum likelihood code was written in the statistical software R, with likelihood functions maximized with the R function optim.

Results

Infection rates of neonatal larvae after overwintering differed among virus isolates (fig. 1*B*; table A1, available online). Control infection rates were close to 0 (mean $\pm$ 1 SE among egg masses $= 0.009 \pm 0.005$, $n = 6$ egg masses; 5 of 947 larvae infected in total), suggesting that the influence of background contamination in the field was very small compared to the effects of experimentally placed infectious cadavers. Low control rates of infection also suggest that covert infection leading to transovarian transmission was not a primary mechanism of larval infection in this experiment. Infection data were not overdispersed (variance inflation factor $\hat{c} = 0.24$), and thus use of a binomial model was appropriate.

Isolates varied substantially in the size of the virus-killed fourth-instar cadavers, with some isolates producing cadavers more than twice as large as those produced by other isolates (fig. 1*A*, linear regression of cadaver area in cm^2 by isolate; adjusted $R^2 = 0.08$, $P = .02$, $\mathrm{df} = 153$). Increased cadaver size was correlated with increased probability of virus infection (cadaver area coefficient $= 0.40891$, logistic regression of infection probability; see table A1 for model fitting), suggesting that larger cadavers resulted in a higher virus dose consumed by the neonates. However, differences in cadaver size did not fully explain among-isolate differences in infection probability (logistic regression; isolate + cadaver size model compared to model with cadaver

size alone: likelihood ratio = 1,192, df = 1, 15, $P < .0001$). The best-fitting model included both cadaver size and isolate as well as an interaction between the two (table A1).

Female moths varied in oviposition site, ovipositing directly on the bark 16.4% of the time and on the enclosing cup 48% of the time, with the remainder of egg masses laid partly on bark and partly on cups (35.6%, $n = 177$ egg masses total). Oviposition site did not differ by virus isolate or with cadaver size; a multinomial model with oviposition site as the response variable did not support an isolate (AIC = 367) or cadaver-size effect (AIC = 345) when compared to a null model (AIC = 342; best-fitting model). Larval infection probability was slightly but not significantly higher for egg masses laid on bark than for those laid on the enclosing cup (among–egg mass mean proportion mortality ± 1 SE = 0.415 ± 0.042 for bark only, 0.406 ± 0.029 for bark and cup, 0.380 ± 0.028 for cup only).

The fraction of larvae that died early, with no observed production of occlusion bodies, also varied with virus isolate (fig. 2*A*). Similarly to results for virus-killed larvae, the best model to explain early-dying larvae also incorporated an isolate-specific term, a positive coefficient for cadaver size (0.2830 × cadaver area in cm^2), and an interaction between the two (multinomial models in table A1).

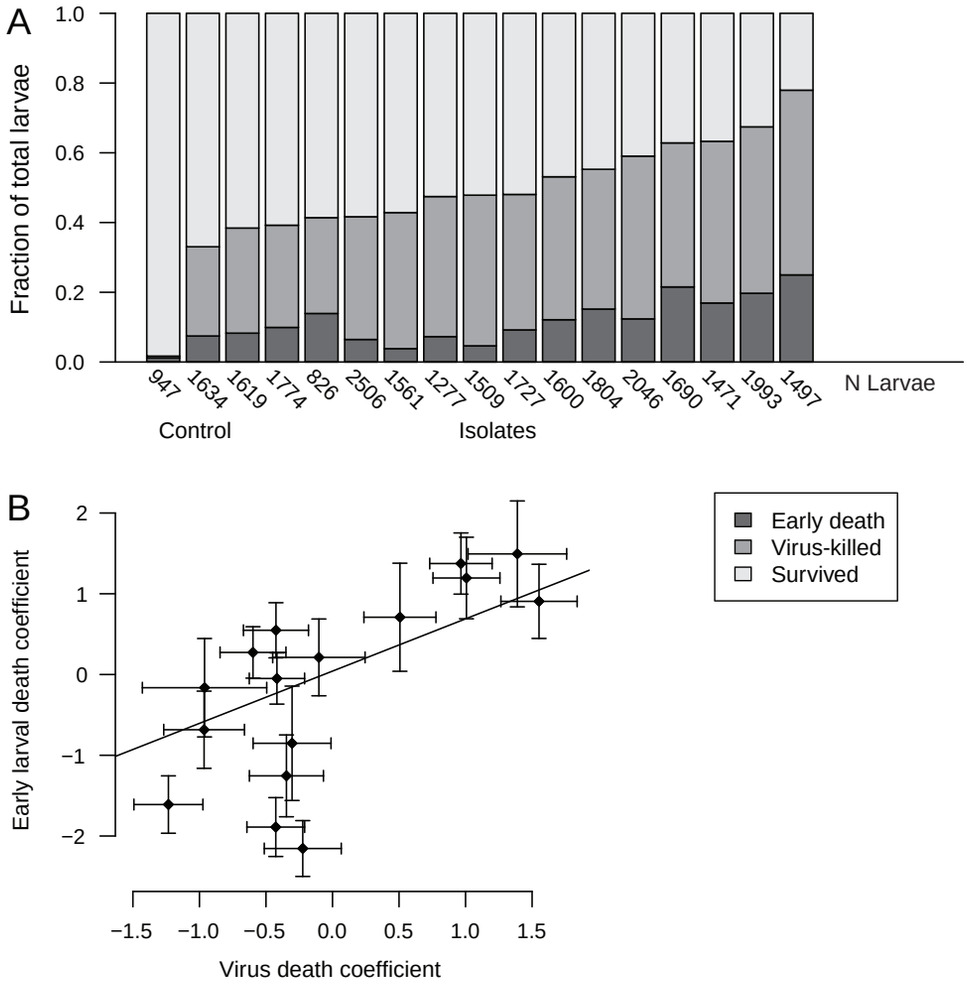

Figure 2: Higher overwinter viral morality is associated with higher early larval death in which no infectious particles are produced. Raw data are shown in *A*. Control larvae, exposed to bark with no virus-killed cadavers, are shown in the leftmost bar. All other bars show raw proportions of fates of all larvae exposed to a particular isolate, ordered by total larval death per isolate. Total numbers of larvae per isolate are given below each bar, summing all egg masses here for clarity (range: 6–14 egg masses per isolate). In *B*, we plot the per-isolate coefficients from a multinomial model that takes into account the nonindependence of probability of early mortality and virus-induced mortality within an egg mass. *X* and *Y* error bars represent the 95% confidence intervals (CIs) for these coefficient estimates. Each point in *B* represents a virus isolate. The regression line comes from a major-axis regression to allow for error in both independent and dependent variables; slope = 0.647, 95% CIs = 0.320, 1.114.

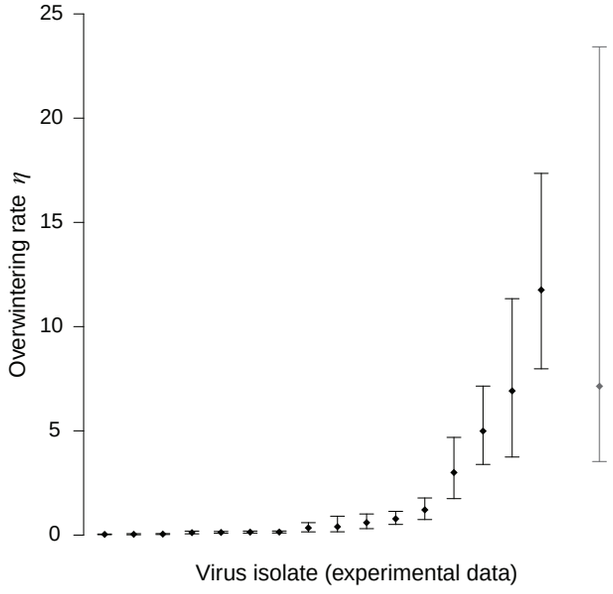

Figure 3: Pathogen overwinter transmission varies among 16 field-collected virus isolates. Points represent maximum likelihood estimates of overwinter transmission rates (1/(days × cadavers/m² leaf)) with bootstrapped 95% confidence intervals. Overwinter transmission rates were estimated by using each egg mass as a replicate (see appendix, available online), and therefore the ordering corresponds to the mean fraction infected, as in figure 1*B*, rather than to the ordering of figure 2*A*, in which all egg masses were pooled. For comparison, the overwinter transmission η for a natural population is also provided (rightmost point; estimate taken from Fuller et al. 2012).

In addition, higher frequency of early death also correlated with a higher frequency of virus death across isolates (fig. 2*B*). In the multinomial model, the Pearson correlation between isolate coefficients for virus death and their coefficients for early death was 0.7068 (95% confidence interval [CI] from parametric bootstrap = 0.5791–0.7789). A nonlinear model with a quadratic term did not fit the data better than the linear model (linear model: AIC = 44.42, model with (virus death)^2 term: AIC = 46.24). If early death is caused by exposure to other pathogens present on bark, correlations between virus death and early death could be due to genetic variation in immune strength among egg masses, which represent full-sibling families. However, a general linear mixed-effects model accounting for random egg mass effects also supports an isolate effect on both virus death and early death (multinomial model with isolate effect: DIC = 35,759; null model with only egg mass effects: DIC = 35,762), suggesting that differences among isolates are real and not an artifact of variation in resistance among egg masses. In addition, rates of early death in control larvae were quite low (mean ± 1 SE among egg masses = 0.011 ± 0.007, n = 6 egg masses; 12 of 947 larvae total; fig. 2*A*), consistent with the hypothesis that baculovirus exposure increases early death.

Differences among virus isolates in postwinter infection rates led to different estimates of overwinter transmission rates among those isolates (fig. 3). Using a linear transmission model for the overwinter infection process (eq. [A3], available online), we found that a model with different overwinter transmission rates η for each virus isolate (n = 16 parameters) fitted the data much better than a one-parameter model assuming the same overwinter transmission for all isolates (ΔAIC = 575). The mean overwinter transmission rate among isolates was lower than an estimate from a natural population in a previous study (among-isolate mean = 1.92, in units of 1/[days × (cadavers/m^2)]); natural population estimate = 7.14, from Fuller et al. 2012). The four highest-overwintering virus isolates did not differ from the estimate of overwinter transmission in a natural population, as indicated by the overlap of their bootstrapped 95% CIs; fig. 3).

Discussion

Our results provide further evidence that environmental contamination of egg masses is the primary mechanism of between-season transmission of gypsy moth baculovirus. Larval infection rates of control egg masses were almost 0, suggesting that the role of covert infections leading to transovarian transmission from mother to offspring is small. In addition, infection rates correlated positively with cadaver size, suggesting that experimentally placed cadavers were the primary source of infections. The parents used to produce egg masses in this study were raised in a clean laboratory environment, and none were exposed to virus. Thus, any transovarian transmission occurring would be due to preexisting covert infections and not due to sublethal infections of the parental generation.

Previous studies arguing for the importance of transovarian vertical transmission in gypsy moths estimated the mortality rate in offspring of infected mothers to be 0.005 (Myers et al. 2000), which is similar to the mortality observed in our control egg masses (mean ± 1 SD = 0.009 ± 0.014). In that study, the two egg masses in which larval infection occurred were not surface sterilized, suggesting that environmental contamination cannot be ruled out. We believe that experimental contamination is a more conservative explanation for the observed virus-caused mortality in our control egg masses. However, even if it were entirely due to transovarian vertical transmission, this control infection rate was still dwarfed by the mortality caused by transovum transmission due to environmental contamination of egg masses, which produced infection rates ranging from 0.2 to 0.7.

Larvae used in this experiment were from an experimental colony where gypsy moths have been raised for more than 40 generations (Keena and ODell 1994), and thus prior existing covert infections might be less likely for this population than in the field, where presence of viral DNA has been found at high rates in other baculoviruses and lepidopteran species such as *Mamestra brassicae* (Noctuidae; Burden et al. 2003). However, a laboratory strain of *M. brassicae* in the same study was found to have even higher rates of covert infection than most field populations (Burden et al. 2003), suggesting that covert infections may occur even in larvae that have been bred in the laboratory for generations. While transovarian transmission and other long-term persistence mechanisms may play a role in gypsy moth epizootics in nature, the high rates of neonate infection in this experiment suggest that environmental contamination of egg masses is sufficient for virus persistence across seasons, confirming prior evidence of this mechanism (Doane 1969; Murray and Elkinton 1989; Kukan 1999).

Our results support the existence of a cost of high virulence in a baculovirus. Isolates that killed more larvae also had higher rates of larval death in which no occlusion bodies were produced, independent of host family. If larvae die before protein-coated occlusion bodies are produced, then pathogen transmission to the next host is 0, thus imposing a high cost. We consider two possible mechanisms for how the baculovirus may cause larval mortality without infectious-particle production. First, early death of larvae might be caused by infection with other pathogens present in the field or on bark pieces. Increased exposure to virus might tax the larva's immune system, such that the individual is less able to defend itself from other pathogens.

Alternatively, early death might be directly caused by the baculovirus if it kills before occlusion-body production, which occurs in the last stages of infection (Miller 1997). In this and other baculoviruses, very high doses are associated with fast kill times and reduced virus yield (van Beek et al. 1988; Hodgson et al. 2002; White et al. 2012; Kennedy et al. 2014), thought to result from reduced time for growth of the host (White et al. 2012). A very high dose of virus might quickly kill a large number of host cells, causing premature death of the host even before occlusion-body production. In other words, if the dose is extremely high, speed of kill is so fast that virus yield essentially approaches 0. Exposure to larger cadavers, which would be expected to deliver a higher dose of virus, is associated with increased early death, also supporting this hypothesis. However, in contrast to our results, previous studies of nucleopolyhedrovirus speed of kill have observed that kill time levels off to a minimum time at the highest doses, in gypsy moth (Kennedy et al. 2014) and other lepidopteran species (van Beek et al. 1988). In the case of Kennedy et al. (2014), larvae that died without visible occlusion-body production were excluded from the data, which would explain the discrepancy between that study's results and our data.

Regardless of the mechanism causing larval mortality without infectious-particle production, the observed correlation of increased early mortality with increased infection rates among isolates has clear implications for pathogen fitness. Baculoviruses are often considered to be an exception to theories of the evolution of virulence, because of their high lethality and the fact that transmission occurs only after host death (Cory and Myers 2003). While host mortality in most pathogens signals the end of transmission, thus constraining the evolution of higher virulence, in baculoviruses and other environmentally transmitted pathogens this constraint is considered to be lifted. However, in any pathogen there must be some upper limit to virulence. If more virulent isolates also experience higher early mortality without infectious-particle production, this trade-off could create an upper limit to virulence. An upper limit was not observed in our field-collected isolates; the most virulent isolates had the highest amount of early death but also the highest mean overwinter transmission, and thus the highest fitness was not observed at intermediate virulence. It is possible that more virulent isolates have been selected against and thus were not observed in the population or that complex trade-offs with other virus traits may play a role in the evolution of virulence in this baculovirus. In addition, the two separate rounds of transmission, between and within seasons, may select for different baculovirus traits and thus constrain its evolution. While very high virulence may increase within-season transmission to larger late-instar larvae, it may come with a cost in the between-season stage of transmission due to the higher susceptibility of neonatal larvae.

Acknowledgments

Funding was provided by National Institutes of Health grant R01GM096655 to G.D., V. Dukic, and B. Rehill. Research was conducted under USDA-APHIS permit P526P-12–01466 to G. D. Bark from a lightning-killed mature red oak tree was provided by Care of Trees, Chicago, IL. We thank D. J. Páez, C. J. Geoga, A. Hudson, B. Muñoz, and K. Smith for their help with field and laboratory experiments.

Literature Cited

Akaike, H. 1981. Likelihood of a model and information criteria. Journal of Econometrics 16:3–14.

Alizon, S., A. Hurford, N. Mideo, and M. van Baalen. 2009. Virulence evolution and the trade-off hypothesis: history, current state

of affairs and the future. Journal of Evolutionary Biology 22:245–259.

Anderson, R., and R. May. 1982. Coevolution of hosts and parasites. Parasitology 85:411–426.

Barbosa, P., J. Greenblatt, W. Withers, W. Cranshaw, and E. Harrington. 1979. Host-plant preferences and their induction in larvae of the gypsy moth, *Lymantria dispar*. Entomologia Experimentalis et Applicata 26:180–188.

Boldin, B., and E. Kisdi. 2012. On the evolutionary dynamics of pathogens with direct and environmental transmission. Evolution 66: 2514–2527.

Bonhoeffer, S., R. E. Lenski, and D. Ebert. 1996. The curse of the pharaoh: the evolution of virulence in pathogens with long living propagules. Proceedings of the Royal Society B: Biological Sciences 263:715–721.

Breban, R., J. M. Drake, D. E. Stallknecht, and P. Rohani. 2009. The role of environmental transmission in recurrent avian influenza epidemics. PLoS Computational Biology 5(4):e1000346. doi:10.1371/journal.pcbi.1000346.

Bremermann, H., and H. Thieme. 1989. A competitive exclusion principle for pathogen virulence. Journal of Mathematical Biology 27:179–190.

Bull, J. 1994. Perspective: virulence. Evolution 48:1423–1437.

Burden, J. P., C. P. Nixon, A. E. Hodgkinson, R. D. Possee, S. M. Sait, L. A. King, and R. S. Hails. 2003. Covert infections as a mechanism for long-term persistence of baculoviruses. Ecology Letters 6:524–531.

Burnham, K. P., and D. R. Anderson. 2002. Model selection and multimodel inference: a practical information-theoretic approach. Springer, New York.

Caraco, T., and N. Wang. 2008. Free-living pathogens: life-history constraints and strain competition. Journal of Theoretical Biology 250:569–579.

Cory, J., and J. Myers. 2003. The ecology and evolution of insect baculoviruses. Annual Review of Ecology, Evolution, and Systematics 34:239–272.

Cox, D. R., and E. J. Snell. 1989. Analysis of binary data. Monographs on Statistics and Applied Probability 32. CRC, Boca Raton, FL.

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

Doane, C. C. 1969. Trans-ovum transmission of a nuclear-polyhedrosis virus in the gypsy moth and the inducement of virus susceptibility. Journal of Invertebrate Pathology 14:199–210.

Doane, C. C., and M. L. McManus, eds. 1981. The gypsy moth: research toward integrated pest management. Forest Service Technical Bulletin 1584. USDA, Economic Research Service, Washington, DC.

Dwyer, G., J. Dushoff, J. Elkinton, and S. Levin. 2000. Pathogen-driven outbreaks in forest defoliators revisited: building models from experimental data. American Naturalist 156:105–120.

Dwyer, G., J. Elkinton, and J. Buonaccorsi. 1997. Host heterogeneity in susceptibility and disease dynamics: tests of a mathematical model. American Naturalist 150:685–707.

Elderd, B. D., J. Dushoff, and G. Dwyer. 2008. Host-pathogen interactions, insect outbreaks, and natural selection for disease resistance. American Naturalist 172:829–842.

Ewald, P. 1987. Pathogen-induced cycling of outbreak insect populations. Pages 269–286 *in* P. Barbosa and J. C. Schultz, eds. Insect outbreaks. Academic Press, San Diego, CA.

Fleming-Davies, A. E., V. Dukic, V. Andreasen, and G. Dwyer. 2015. Effects of host heterogeneity on pathogen diversity and evolution. Ecology Letters. Forthcoming. doi:10.1111/ele.12506.

Fleming-Davies, A. E., and G. Dwyer. 2015. Data from: Phenotypic variation in overwinter environmental transmission of a baculovirus and the cost of virulence. American Naturalist, Dryad Digital Repository, http://dx.doi.org/10.5061/dryad.p1k27.

Fuller, E., B. D. Elderd, and G. Dwyer. 2012. Pathogen persistence in the environment and insect-baculovirus interactions: disease-density thresholds, epidemic burnout, and insect outbreaks. American Naturalist 179:E70–E96.

Fuxa, J. R., and A. R. Richter. 2007. Effect of nucleopolyhedrovirus concentration in soil on viral transport to cotton (*Gossypium hirsutum* L.) plants. BioControl 52:821–843.

Galvani, A. P. 2003. Epidemiology meets evolutionary ecology. Trends in Ecology and Evolution 18:132–139.

Gandon, S. 1998. The curse of the pharaoh hypothesis. Proceedings of the Royal Society B: Biological Sciences 265:1545–1552.

Hadfield, J. D. 2010. MCMC methods for multi-response generalized linear mixed models: the MCMCglmm R package. Journal of Statistical Software 33(2):1–22. http://www.jstatsoft.org/v33/i02.

Handel, A., C. Lebarbenchon, D. Stallknecht, and P. Rohani. 2014. Trade-offs between and within scales: environmental persistence and within-host fitness of avian influenza viruses. Proceedings of the Royal Society B: Biological Sciences 281:20133051. doi:10.1098/rspb.2013.3051.

Hodgson, D., A. Vanbergen, S. Hartley, R. Hails, and J. Cory. 2002. Differential selection of baculovirus genotypes mediated by different species of host food plant. Ecology Letters 5:512–518.

Ignoffo, C., D. Hostetter, P. Sikorowski, G. Sutter, and W. Brooks. 1977. Inactivation of representative species of entomopathogenic viruses, a bacterium, fungus, and protozoan by an ultraviolet light source. Environmental Entomology 6:411–415.

Keena, M. A., and T. M. ODell. 1994. Effects of laboratory rearing on gypsy moth (Lepidoptera: Lymantriidae). USDA, Forest Service, Northeastern Forest Experiment Station, Radnor, PA.

Kennedy, D. A., V. Dukic, and G. Dwyer. 2014. Pathogen growth in insect hosts: inferring the importance of different mechanisms using stochastic models and response-time data. American Naturalist 184:407–423.

Kramer, A., I. Schwebke, and G. Kampf. 2006. How long do nosocomial pathogens persist on inanimate surfaces? a systematic review. BMC Infectious Diseases 6:130. doi:10.1186/1471-2334-6-130.

Kukan, B. 1999. Vertical transmission of nucleopolyhedrovirus in insects. Journal of Invertebrate Pathology 74:103–111.

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

Miller, L. K., ed. 1997. The baculoviruses. Springer, New York.

Murray, K., and J. Elkinton. 1989. Environmental contamination of egg masses as a major component of transgenerational transmission of gypsy month nuclear polyhedrosis virus (LdMNPV). Journal of Invertebrate Pathology 53:324–334.

Myers, J. H., R. Malakar, and J. S. Cory. 2000. Sublethal nucleopolyhedrovirus infection effects on female pupal weight, egg mass size, and vertical transmission in gypsy moth (Lepidoptera: Lymantriidae). Environmental Entomology 29:1268–1272.

O'Reilly, D., and L. K. Miller. 1989. A baculovirus blocks insect molting by producing ecdysteroid UDP-glucosyl transferase. Science 245:1110–1112.

Páez, D., A. Fleming-Davies, and G. Dwyer. 2015. Pathogen exposure affects life history variation in the gypsy moth (*Lymantria dispar*). Journal of Evolutionary Biology. Forthcoming. doi:10.1111/jeb.12699.

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

Roche, B., J. M. Drake, and P. Rohani. 2011. The curse of the pharaoh revisited: evolutionary bi-stability in environmentally transmitted pathogens. Ecology Letters 14:569–575.

Spiegelhalter, D. J., N. G. Best, B. P. Carlin, and A. van der Linde. 2002. Bayesian measures of model complexity and fit. Journal of the Royal Statistical Society B 64:583–639.

Tilman, D. 1977. Resource competition between plankton algae: an experimental and theoretical approach. Ecology 58:338–348.

van Beek, N. A., H. A. Wood, and P. R. Hughes. 1988. Quantitative aspects of nuclear polyhedrosis virus infections in lepidopterous larvae: the dose-survival time relationship. Journal of Invertebrate Pathology 51:58–63.

Walther, B. A., and P. W. Ewald. 2004. Pathogen survival in the external environment and the evolution of virulence. Biological Reviews 79:849–869.

White, S. M., J. P. Burden, P. K. Maini, and R. S. Hails. 2012. Modelling the within-host growth of viral infections in insects. Journal of Theoretical Biology 312:34–43.

Woods, S., and J. Elkinton. 1987. Biomodal patterns of mortality from nuclear polyhedrosis virus in gypsy moth (*Lymantria dispar*) populations. Journal of Invertebrate Pathology 50:151–157.

Associate Editor: Pej Rohani
Editor: Susan Kalisz

To experimentally replicate overwinter transmission, virus-killed gypsy moth caterpillars (*Lymantria dispar*) were attached to bark from an oak tree and then placed on oak trees in the forest. Photograph by Arietta Fleming-Davies.