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POPULATION ECOLOGY - ORIGINAL PAPER

Density-related variation in vertical transmission of a virus in the African armyworm

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Abstract Larvae of the African armyworm, *Spodoptera exempta*, are darker and more resistant to baculovirus infection when reared in groups (gregarious form) compared to being reared singly (solitary form). Lepidoptera that survive virus challenge as larvae could potentially retain a sublethal virus infection which is then transmitted vertically to the next generation. Here we examine whether gregarious and solitary forms of the armyworm differ in the costs of surviving virus infection and in their capacity to transmit an active baculovirus infection to their offspring. Pupae of larvae reared gregariously that survived virus challenge weighed significantly less than uninfected individuals, but this was not so for those reared solitarily. This did not, however, translate into differences in fecundity, at least under laboratory conditions. As found in previous studies, pre-oviposition period was shorter for solitary than gregarious insects, and it was also shorter for females that had been challenged with virus as larvae. Both the prevalence of egg batches containing larvae that died from nucleopolyhedrovirus (NPV) infection and the proportion of infected larvae within each egg batch were significantly increased (approximately doubled) when parental moths were previously challenged with the virus during their larval state. This demonstrates that horizontal transmission in one generation can elevate vertical transmission to the next generation. Moreover, prevalence of overt infection in the offspring generation was two to three times greater when parental moths were reared solitarily as larvae than when reared gregariously. Disease prevalence and proportional infection were both independent of the sex of the infected parent and whether or not the egg batch was surface-sterilized to remove potential contaminants. This suggests that the eggs are infected internally (transovarial) rather than externally (transovum). These results help to shed light on the observed temporal pattern of virus epizootics in eastern Africa.

Keywords Costs of resistance · Gregariousness · Phase polyphenism · Sublethal effects · Transovarial transmission

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Introduction

Many pathogens can be transmitted both horizontally (transmission between individuals of the same cohort) and

vertically (from parent to offspring). The evolution and consequences of these different pathogen transmission strategies have been investigated from a theoretical perspective (e.g. Lipsitch et al. 1995; Lively et al. 2005). Empirical studies have addressed the factors that promote horizontal and vertical transmission in a range of animal, plant and microbial systems and, in particular, its impact on the evolution of virulence (e.g. Turner et al. 1998; Dunn and Smith 2001; Stewart et al. 2005). The outcome of pathogen challenge is often condition dependent (e.g. Møller et al. 1998; Duckworth et al. 2001; Lee et al. 2006) and there is limited evidence that the condition of the host could also provide constraints on vertical and horizontal transmission (Kaltz and Koella 2003). Thus, in order to be able to predict the evolution of vertical and horizontal modes of parasite transmission and their relative impact within populations, we need to understand how they are influenced by host condition and other factors that produce differential susceptibility.

One of the more dramatic examples of phenotypic plasticity is the density-dependent phase change seen in many insects, particularly members of the Orthoptera (e.g. locusts and grasshoppers) and the Lepidoptera (e.g. armyworms). Individuals in both taxa can exist in solitary (low-density) and gregarious (high-density) forms, and it is well documented that these two forms can have significant differences in physiology, behaviour and ecology (Applebaum and Heifetz 1999). For example, the high-density form tends to be more conspicuously coloured, more active, and shows a number of adaptations for dispersal or migration, such as stronger flight. The gregarious forms that live at high population densities are expected to have a greater risk of infection from parasites as transmission rates tend to increase with population density (e.g. Anderson and May 1981). There is, however, increasing evidence that insects in the different phases vary in their ability to fight off pathogen challenge. For example, gregarious Orthoptera (e.g. locusts) and Lepidoptera larvae are more resistant to infection than those in the solitary stage (Reeson et al. 1998; Wilson et al. 2001a, reviewed by Wilson and Cotter 2007). The larvae of gregarious Lepidoptera are usually darker than their solitary counterparts due to increased levels of melanin in their cuticle. This has, in part at least, been shown to relate to alterations in the insect's innate immune function (Wilson et al. 2001a, 2001b; Cotter et al. 2004); gregarious insects invest more in prophylactic disease resistance mechanisms than solitary larvae. The consequences of these differences have been studied in terms of susceptibility to infections that are transmitted horizontally (Reeson et al. 2000); however, differences between the different phases in their propensity to transmit pathogens vertically has yet to be addressed.

The African armyworm, *Spodoptera exempta* (Walker) (Lepidoptera: Noctuidae), is a major pest in eastern Africa, and it feeds on a wide range of graminaceous host plants; both cultivated and pasture species (Rose et al. 2000). Its biology is characterised by irregular outbreaks during the rainy season, which in some years produces huge numbers of gregarious larvae and causes major damage to pasture and crops, followed by periods during the dry season when the insect is solitary and found at very low densities (Rose et al. 2000). In natural populations, insect outbreaks are frequently associated with baculovirus infection (*S. exempta* nucleopolyhedrovirus (SpexNPV)), which often reaches very high levels later in the armyworm outbreak season (>90% prevalence; Brown and Swaine 1965; Swaine 1966). Baculoviruses are usually highly pathogenic, with systemic infection almost invariably being fatal to the feeding larval stages of Lepidoptera. The symptoms are very obvious, with the caterpillar essentially turning into a bag of millions of whitish virus particles. Pupae can only die of NPV infection if the insect is infected during the larval stage; adults cannot be infected directly or die from infection. Although horizontal transmission is thought to be the major route of baculovirus transmission, vertical transmission of both overt (active and fatal) and sublethal forms of the virus has been widely reported, but the process is poorly understood (Cory and Myers 2003). There are few studies where direct comparisons among species have been made, but the propensity for an NPV to be transmitted vertically as an overt infection appears to vary with different host–virus combinations (Kukan 1999). If there is any trend in patterns of vertical transmission, it would appear that higher levels are seen in more mobile (and often tropical) species, particularly those within the genus *Spodoptera* (Swaine 1966; Abul-Nasr et al. 1979; Smits and Vlak 1988; Fuxa and Richter 1991).

Vertical transmission is defined here as the passage of an active, overt virus infection from one generation to the next, i.e. the degree to which insects in the next generation die from NPV infection after their parents have survived virus challenge. Insects can also pass baculoviruses vertically from parents to their young in a covert or sublethal state (Burden et al. 2003), and this can occur in individuals surviving virus challenge (Burden et al. 2002). It is also thought that this covert virus can be converted to an active infection, but it is not known how this occurs, nor has it proved possible to identify consistent triggers (Cory and Myers 2003). Surviving pathogen challenge can also have other consequences in terms of costs, including altered development rate and longevity and reduced fecundity (Rothman and Myers 1996). These costs have yet to be addressed in species that have both solitary and gregarious forms.

Previous work has demonstrated that horizontal transmission of SpexNPV is lower in gregarious than solitary *S. exempta* larvae (Reeson et al. 2000). However, it is not known whether, or how, these differences in susceptibility are associated with levels of disease expression in the next

generation. Several scenarios can be envisaged: because gregarious larvae are more resistant to fatal NPV infection, this could also mean that they are more resistant to the likelihood of vertical transmission, as it must be related to the capacity for an insect to suppress invading virus particles. This should result in lower vertical transmission for gregarious insects (compared to solitaries). Alternatively, because more gregarious larvae survive NPV challenge (if the larvae are given an equal dose), a higher proportion of survivors are likely to retain NPV that can be vertically transmitted; if the larvae are challenged with a higher dose (to achieve equal effect), vertical transmission will be greater because they may carry more virus particles. In both of these cases we would predict increased vertical transmission in gregarious larvae. In this study we address two questions:

(a) Are the fitness costs of surviving NPV challenge different for solitary and gregarious larvae?
(b) Does the vertical transmission and expression of overt NPV infection in the next generation differ between solitary and gregarious larvae?

The answers to both of these questions have relevance, not only for our understanding the natural ecology and epidemiology of this baculovirus and its host, but also for exploring the opportunities and consequences of biocontrol by baculoviruses.

Material and methods

Insect culture

The experiment was carried out using larvae from a culture initiated from *S. exempta* insects collected in Tanzania in 2002. The insects were maintained in continuous culture on a wheatgerm-based semi-artificial diet, a modification of tobacco hornworm diet from Hunter et al. (1984) at 28 (±1) °C with a 12/12 h light/dark cycle. The egg batches were routinely decontaminated by soaking in a 1% sodium hypochlorite for 10 min. The larvae were reared together until the second instar when insects used for the main culture were separated and maintained singly until pupation. Adults were provided with a 10% honey solution and left to mate in groups of 50–100 in cages supplied with strips of filter paper for egg laying.

In order to prepare insects for the bioassay, a subset of larvae were reared together until late in the first instar and they were then separated into two groups; one in which they were maintained singly, the solitary treatment, and the other in which they were reared in groups of several hundred, the gregarious treatment. In both cases the larvae were reared with artificial diet provided ad libitum.

Virus stocks

The *Spodoptera exempta* NPV was originally isolated in 1974 from field-collected *S. exempta* larvae in Tanzania and subsequently amplified in *S. exempta* larvae. To extract the virus, infected larvae were macerated in sterile distilled water, followed by filtration through muslin and then pelleted for 30 min at 3,500×*g*. After two washes with water, the resuspended pellet was centrifuged at 70,000×*g* at 4 °C for 90 min on a discontinuous 50–60% (w/w) sucrose gradient. The virus was harvested, washed and pelleted three times in sterile distilled water at 3,500×*g*. Viral occlusion bodies (OBs) were resuspended in a small volume of water, counted and stored in aliquots at −20 °C. Purified OB suspensions were counted (six replicates), usually at a 1 in 10 or 1 in 100 dilution, using an Improved Neubauer haemocytometer (B.S. 748, Weber, Teddington, England).

Bioassay design

When the insects reached the fourth instar, 575 solitary and 510 gregarious larvae were selected for the experiment. We refer to these insects as the parental generation. The insects were divided into four groups: 100 larvae of both types were designated as untreated control insects, 275 solitary and 210 gregarious larvae were denoted as treated insects, and the remaining 200 insects from each group were designated as "stock" insects and were maintained in the same manner as the untreated controls. The stock larvae remained untreated and were later used to mate with insects from the treatment groups to allow the contribution of each sex to be monitored. As the severity of the infection influences speed of kill and could influence sublethal effects and the vertical transmission of virus, the two groups of larvae were given virus doses intended to produce roughly equivalent levels of insect mortality. Thus, the more resistant gregarious larvae were inoculated with a dose of 45,000 OBs per insect and the solitary larvae were given a dose of 4,000 OBs (based on earlier assays with the same insect culture). The treated insects were inoculated individually with a 1 µl dose of virus on a small (approx. 2 × 1 mm^3) plug of artificial diet. The control insects were given 1 µl of deionized water on a diet plug. Insects that had ingested the dose after 24 h were transferred to individual 30 ml pots each containing approximately 4.5 ml of artificial diet and reared at 28 °C. The larvae were monitored daily until death or pupation.

The untreated control insects and insects that survived virus challenge and pupated successfully were sexed and weighed. The pupae were monitored daily until they emerged as adults and they were then paired with a moth of the opposite sex from the stock insects from the appropriate treatment (solitary or gregarious). Thirty pairs of insects per

treatment were set up in plastic tubs (approximately 10 cm diameter and 6 cm depth) with a 10% honey source. Eggs, laid on paper strips, were collected and counted on a daily basis for nine days. In order to ascertain whether active virus infection was present in the next generation, the eggs from ten randomly chosen pairs of insects from each of the four treatments were taken. Half the eggs from each batch were surface-sterilized in dilute bleach (1% sodium hypochlorite) and the remainder were left untouched. Virus can be passed from parent to offspring in two ways: on the outside of the egg (transovum), probably via contamination, and within the egg (transovarial), which is likely to be more indicative of active infection persisting within the insects themselves. Surface-sterilization should eliminate the transovum component, i.e. any virus (or other pathogen) contaminating the exterior of the eggs. A subset of up to 96 larvae were reared individually on artificial diet with both surface-sterilized and unsterilized egg batches and monitored daily for virus mortality. We refer to these insects as the offspring generation, and we compare their survival rates with the treatments imposed on their parents in the previous experiment (the parental generation).

Virus extraction and DNA analysis

In order to verify that offspring of SpexNPV-challenged parents died of SpexNPV infection, virus was extracted from a subsample of larvae that died and the identity of the progeny virus was determined. Baculovirus infection is readily identified by a characteristic suite of symptoms, including a pale cuticle, flaccidness and the production of a thick white or beige fluid when the body wall ruptures. DNA was extracted from a subsample of infected larvae using a modification of the technique described by Smith and Crook (1988). Each larva was homogenized separately in a glass homogenizer with a small volume of 0.1% sodium dodecyl sulfate (SDS). The homogenate was centrifuged at low speed (400 g) for 5 min to pellet the cellular debris. The supernatant was saved and the process repeated. The supernatants were combined and centrifuged for 20 min at 3,500×*g* to pellet the virus OBs and the pellet was resuspended in 100–200 µl milli-Q water. The virus particles were released from the OBs by adding 5 µl 1 M sodium carbonate followed by incubation at 37 °C for 15−30 min, or until the supernatant became opaque. This was followed by the addition of 10% SDS (10 µl per 100 µl virus suspension) and incubation at 37 °C for 30 min. DNA was purified by sequential phenol and chloroform and isoamyl alcohol extractions. The samples were then dialysed against a large volume (2–3 l) of TE buffer over 36 h, with several changes of buffer, at 4 °C. As the amount of DNA from each insect was small, dialysis was carried out in the tops of 1.5 ml Eppendorf tubes (as described in Smith and Crook 1988). Restriction enzyme analysis of viral DNA was carried out using *Eco*RI. DNA was digested using the conditions recommended by the suppliers and the resulting fragments separated using 0.6% agarose gels, run in 1× TBE containing 150 µl of 10 mg/ml ethidium bromide at 30 V overnight.

Statistical analysis

In order to determine any costs associated with surviving virus challenge, time to pupation, pupal weight and preoviposition period were analysed using general linear models, with sex, larval phase (solitary or gregarious), virus treatment (infected or not) and their interactions considered as potential explanatory terms in the maximal model (S-Plus 6.0, Insightful, Seattle, WA, USA). The contribution of each explanatory term was then tested sequentially, starting with the highest-order interactions, and nonsignificant terms removed from the model to produce the minimal model following standard stepwise deletion protocols (e.g., Crawley 2002; Wilson and Hardy 2002).

Data on virus infection in larvae of the offspring generation were analysed using generalized linear models with binomial errors and a logit link function. For assessing the prevalence of larval deaths within egg batches, the dependent variable was binary (i.e., whether or not an egg batch showed signs of overt viral infection), and the sample size was the number of egg batches that were reared through to the larval stage. For the proportion of infected larvae, the dependent variable was the proportion of larvae in each egg batch dying of NPV infection, and the sample size was the number of egg batches that were reared through as larvae, weighted by the number of larvae in each egg batch that was screened for infection. Since these data were overdispersed, an empirical scale parameter was used and *F*-values calculated (e.g., Crawley 2002; Wilson and Hardy 2002). Potential explanatory terms considered in these models were: larval phase of the parents (solitary or gregarious), virus treatment of parent (challenged or not), sterilization status of the egg batch (sterilized or not), and all interaction terms. As before, nonsignificant terms were sequentially removed from the model starting with the highest-order interactions.

Results

Mortality in the parental generation

In the virus treatment groups, 69/275 (25%) solitary larvae died of NPV, compared with 80/210 (38%) gregarious larvae (chi-square test: $\chi^2_1 = 8.86$, $P = 0.003$). There was also a significant difference in the number of deaths due to other

causes, such as bacterial and fungal infections, and pupation failure, but in the opposite direction (solitary: 31/275; gregarious: 10/275; $\chi^2_1 = 11.14$, $P = 0.001$). Overall, a similar proportion of solitary (36%) and gregarious (43%) larvae died ($\chi^2_1 = 1.84$, $P = 0.17$). Mortality due to other causes generally occurred after virus death would have been expected, i.e. in late-larval or early pupal stages, and thus they were still included in the totals for analysis. It is possible that in solitary-reared larvae these other deaths were precipitated by the viral challenge, even though the NPV was not the ultimate mortality factor. Alternatively, gregarious larvae that would otherwise have died of opportunistic bacterial or fungal infection, may have instead died of NPV. In the control treatment groups (i.e. those not challenged with NPV), five solitary and 20 gregarious larvae were lost as larvae during handling. Of the remainder, background mortality was similar in solitary (13/95 = 14%) and gregarious (12/80 = 15%) larvae ($\chi^2_1 = 0.01$, $P = 0.98$). However, in the solitary group, there were no NPV deaths (most died of fungus or bacteria), whereas in the gregarious group, there were six deaths due to NPV and six due to bacteria. Mortality rates in the "stock" cultures were not quantified, but were of a similar magnitude.

Costs of surviving virus challenge

Insects that survived virus challenge (168 solitary; 114 gregarious) were compared with those in the untreated control groups (80 solitary; 66 gregarious). Insects were only treated as alive or dead; there was no category where an insect could be both infected and alive. A few insects in each treatment group died in the pupal stage and did not emerge as adults; these were not included in the final total. No significant variation occurred in the time taken for surviving insects to pupate (mean ± standard error, virus treatment: infected = 8.57 ± 0.08 d, uninfected = 8.64 ± 0.08 d; $F = 0.37$, $df = 1,426$, $P = 0.54$; larval phase: solitary = 8.65 ± 0.08 d, gregarious = 8.52 ± 0.08 d; $F = 1.40$, $df = 1,426$, $P = 0.24$; or pupal sex: male = 8.50 ± 0.88 d, female = 8.69 ± 0.07 d; $F = 2.58$, $df = 1,426$, $P = 0.11$). However, pupal weight varied with both sex (female pupae = 233.3 ± 2.5 mg; male pupae = 204.4 ± 2.5 mg; $F = 67.51$, $df = 1,423$, $P < 0.0001$) and larval phase (solitary = 229.7 ± 2.4 mg; gregarious = 206.7 ± 2.8 mg; $F = 43.46$, $df = 1,423$, $P < 0.0001$), and there was also a significant interaction between larval phase and infection treatment ($F = 22.81$, $df = 1,423$, $P < 0.0001$). This arises because pupae from virus-challenged, gregarious larvae weighed less than their untreated controls (gregarious insects only: effect of virus treatment: $F = 15.34$, $df = 1,178$, $P = 0.0001$), whereas there was no such effect of virus treatment for the solitary insects (solitary insects only: $F = 1.87$, $df = 1,246$, $P = 0.172$) (Fig. 1).

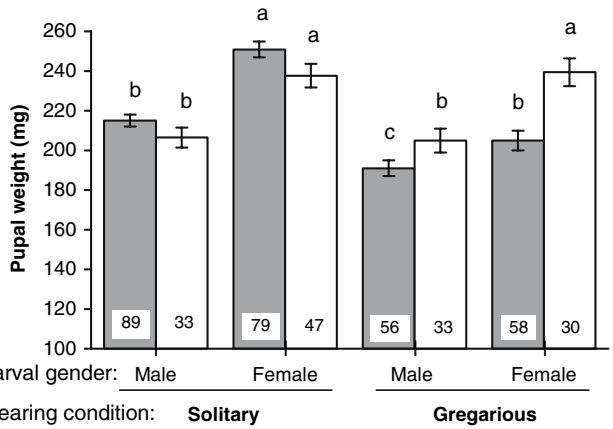

Fig. 1 Weights of male and female pupae (mean ± s.e.) resulting from African armyworm (*Spodoptera exempta*) larvae reared in solitary or gregarious conditions that survived inoculation with *S. exempta* nucleopolyhedrovirus, NPV (challenged, *shaded bars*) or had no virus exposure (control, *open bars*). Numbers at the base of each bar indicate sample sizes. Data analysed via general linear models and contrasts determined by model simplification (critical $P < 0.05$)

The duration of the pre-oviposition period (POP) is an important trait in migratory insects such as *S. exempta*, as it determines the amount of time available for the insect to migrate; the longer the POP, the further the insect can migrate (e.g., Wilson and Gatehouse 1993). As observed in previous studies, gregariously reared individuals delayed oviposition longer than solitary insects (uninfected insects: solitary females = 3.67 ± 0.38 d; gregarious females = 4.93 ± ± 0.43 d; solitary males = 4.54 ± 0.56 d; gregarious males = 6.54 ± 0.39 d; $F = 22.81$, $df = 1,91$, $P < 0.0001$). The insect's infection status did, however, influence POP as oviposition was significantly delayed when the female of a pair had been challenged with virus (infected insects: solitary females = 4.58 ± 0.61 d; gregarious females = 5.90 ± 0.48 d; solitary males = 4.00 ± 0.44 d; gregarious males = 5.64 ± 0.41 d); although pre-oviposition period was not affected by the infection status of the male, as reflected in a significant interaction between sex and infection ($F = 6.32$, $df = 1,91$, $P = 0.014$).

Mating success was high, with around 80% of pairs across treatments producing viable eggs. Mating success was not affected by larval rearing density (solitary = 82%; gregarious = 78%; log-linear model: $\chi^2_1 = 0.21$, $P = 0.64$) or virus treatment (challenged = 75%; unchallenged = 85%; $\chi^2_1 = 1.89$, $P = 0.17$). As expected, female fecundity was positively related with pupal weight (slope ± standard error = 2,945 ± 1,224; $F = 4.60$, $df = 1,93$, $P = 0.035$) and was higher in solitary than gregarious females (solitary = 1,335 ± 92 eggs, gregarious = 1,143 ± 72 eggs; $F = 9.78$, $df = 1,93$, $P = 0.0024$). However, female fecundity was not significantly influenced by whether or not the insect had received a virus challenge as a larva

(infected = 1,297 ± 93 eggs, uninfected = 1,179 ± 77 eggs; $F = 2.11$, $df = 1,92$, $P = 0.15$).

Vertical transmission of virus

Fatal NPV infection was present in the offspring generation in all treatment groups, i.e. there was overt expression of disease. Virus infection could only have originated from their parents, as these larvae were not exposed to SpexNPV. In addition, the results should reflect the treatment that their parents received, as all larvae in the offspring generation were reared singly, thus removing any influence of the gregarious treatment. Virus prevalence was analysed in two ways: firstly, infection was compared among egg batches, thus a single larva dying from NPV infection would mean that an egg batch was recorded as being positive for virus. This gives some indication of the infection status of the parents (as well as the larvae), and is important because the more widespread the virus infection amongst egg batches, the more likely that a virus epizootic will build up over a large area. Secondly, the level of infection within each egg batch was compared, first including all egg batches, and then including only those that contained infected larvae. This gives us an indication of the local intensity of infection.

For each breeding pair, their egg batch was divided in two and one half was surface-sterilized. The prevalence of virus infection in the larvae that emerged from these egg batches was independent of whether or not the egg batch had been surface-sterilized (sterilized = 18/40–45%, unsterilized = 16/40–40%; logistic regression: $F = 0.20$, $df = 1,78$, $P = 0.66$). Therefore, in subsequent analyses, information from both the sterilized and unsterilized egg batches was combined to avoid pseudo-replication across breeding pairs. Virus infection was significantly more prevalent among egg batches from moths that had been reared solitarily as larvae than in those that had been reared gregariously (solitary = 17/20–85%, gregarious = 8/20–40%; $F = 9.06$, $df = 1,37$, $P = 0.0046$). In addition, the prevalence of overt larval infection was higher in egg batches from moths that were challenged with virus as larvae compared to those that were not (challenged = 16/20–80%, unchallenged = 9/20–45%; $F = 7.86$, $df = 1,37$, $P = 0.008$). There was no significant interaction between these two main effects ($F = 1.19$, $df = 1,36$, $P = 0.28$) (Fig. 2a) and there was no effect of whether the challenged moth was male or female (male = 11/17–65%, female = 14/23–61%; $F = 0.05$, $df = 1,36$, $P = 0.82$).

The proportion of fatally infected larvae in each egg batch also varied. However, there was no effect of surface-sterilization on the mean proportion of larvae in each egg batch infected (coefficient ± standard error = 0.316 ± 0.057; sterilized = 6.3%, unsterilized = 3.4%; logistic regression: $F = 1.480$, $df = 1,77$, $P = 0.23$; coefficient ± standard error = 0.316 ± 0.057), and so data from both the surface-sterilized and unsterilized eggs of each pair were combined in subsequent analyses to avoid pseudo-replication across breeding pairs. The proportion of larvae developing overt NPV infection was affected by both the rearing density of the parent moths (solitary = 8.6%; gregarious = 0.7%; $F = 29.84$, $df = 1,37$, $P < 0.0001$; coeff = 1.345 ± 0.115) and whether or not those moths were challenged with virus as larvae (challenged = 7.1%; unchallenged = 2.2%; $F = 10.97$, $df = 1,37$, $P = 0.0021$; coeff = 0.624 ± 0.067) (Fig. 2b). However, there was no significant interaction between these two terms ($F = 0.20$, $df = 1,36$, $P = 0.66$; coeff = −0.231 ± 0.188). The proportion of infected larvae was independent of whether the infected moth was male or female (male = 4.8%, female = 4.5%, $F = 0.01$, $df = 1,36$, $P = 0.93$; coeff = 0.017 ± 0.058).

Surface sterilization did not affect the proportion of larvae dying in infected egg batches (sterilized = 13.7%, unsterilized = 8.5%; logistic regression: $F = 1.24$, $df = 1,32$, $P = 0.27$; coeff = 0.283 ± 0.059), and so data from both the surface-sterilized and unsterilized eggs of each pair were

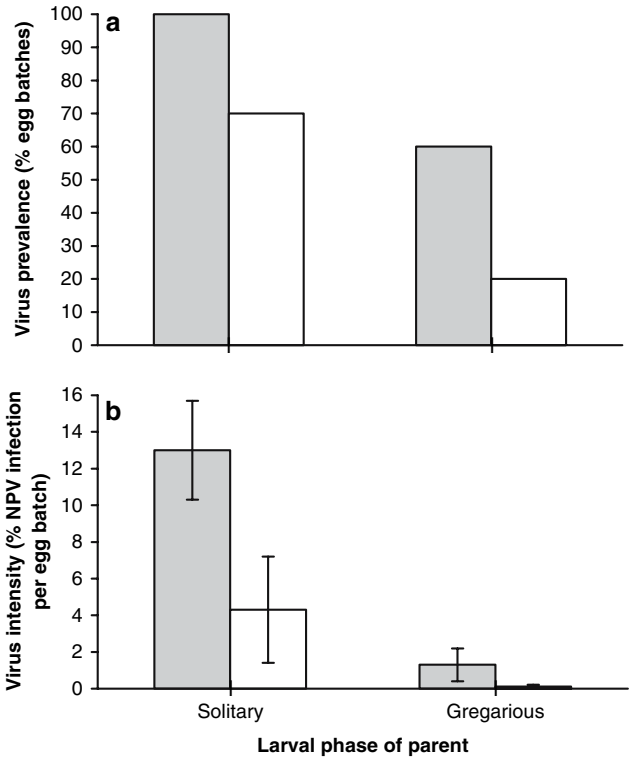

Fig. 2a–b Viral (**a**) presence and (**b**) intensity in egg batches laid by *S. exempta* parents that were reared in solitary or gregarious conditions and survived NPV inoculation (challenged, *shaded bars*) or were never exposed to the virus (control, *open bars*). Values are derived from a logistic regression model. $n = 10$ in all cases. In **a**, an egg batch was considered infected if it contained one (or more) individuals that died from NPV infection

combined. Amongst those breeding pairs whose (sterilized or unsterilized) egg batches included at least one larva dying of viral infection ($n = 25$), the proportion of larvae that became overtly infected with virus was affected only by the larval phase of the parent (logistic regression: $F = 8.73$, $df = 1,23$, $P = 0.007$; coefficient ± standard error = 0.968 ± 0.116), with the proportion of larvae infected being higher amongst egg batches from solitarily reared parents (10.1%) than from gregariously reared parents (1.7%). The infection treatment of the parent had a marginally nonsignificant effect on virus levels in the offspring (challenged = 8.9%, unchallenged = 4.9%; $F = 3.78$, $df = 1,22$, $P = 0.065$; coeff = 0.420 ± 0.067). The proportion of larvae infected per infected egg batch was independent of which sex was challenged with virus (male = 7.4%, female = 7.5%; $F = 0.08$, $df = 1,22$, $P = 0.78$; coeff = -0.063 ± 0.058) and there were no significant interaction terms.

Confirmation of virus identity in the offspring generation

Restriction endonuclease (REN) analysis of insects that died of virus infection in the offspring generation confirmed that these insects died of SpexNPV (Fig. 3). It did not prove possible to isolate sufficient virus DNA from the NPV-infected offspring of gregarious insects that had been challenged with virus. The wild-type SpexNPV used to challenge the larvae in the study is known to contain numerous genetically distinct variants and thus the DNA profile for this virus (Lane 2) contains bands of varying intensities, indicative of a mixed isolate. Although there were slight differences in REN profiles, the virus isolated from the offspring generation larvae all showed bands that were present within the profile of the original SpexNPV inoculum. However, in virtually all cases, fewer bands were evident in the progeny virus, suggesting reduced diversity, although no specific isolate appeared to dominate.

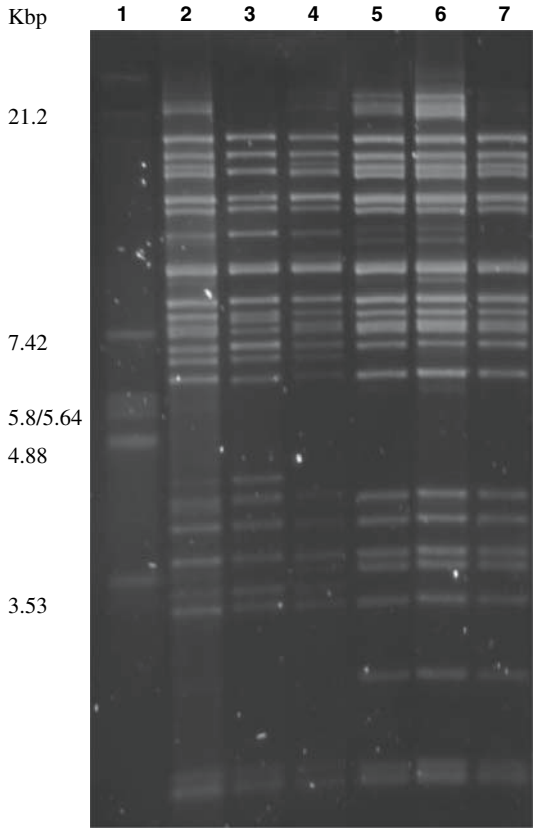

Fig. 3 Restriction endonuclease profile of virus DNA cut with *Eco*RI extracted from *S. exempta* larvae that died in the offspring generation. From left to right, *lane 1* lambda DNA (cut with *Eco*RI), *2* stock SpexNPV, *3* gregarious control unsterilized, *4* solitary "infected" sterilized, *5* solitary "infected" sterilized, *6* solitary "infected" unsterilized, *7* solitary "infected" unsterilized

Discussion

The clear result from this study is that larval phase in armyworms has a major impact on the likelihood of transmission of overt virus infection from one generation to the next, both in terms of the proportion of challenged insects transmitting virus to the next generation and the level of infection within family groups. Interestingly, this result was unaffected by the sex of the insect challenged or by whether the egg batches were surface-sterilized, a process that would remove any pathogens contaminating the outside of the egg. Both these results suggest that an active virus infection was transmitted within the egg (transovarial infection), possibly indicating a link to infection within the gonads of both sexes.

Gregarious and solitary armyworms differ in several key features that are presumably adaptations to their role in the armyworm life cycle. For example, solitary pupae are larger and thus more fecund, allowing rapid population build up, whereas gregarious forms have a longer pre-oviposition period, which allows them to migrate longer distances (Wilson and Gatehouse 1993). As already discussed, gregarious larvae are also considerably more resistant to baculovirus infection, presumably related to the greater risk of infection in the gregarious life style (Reeson et al. 1998). We explored whether challenge with virus affected any of these relationships, and whether it indicated any hidden costs of disease resistance. There was some evidence that the costs of surviving virus challenge varied between the two phases and were potentially higher in the gregarious insects, possibly indicating a trade-off between disease resistance and growth. Females exposed to NPV as larvae had delayed pre-oviposition periods as adult moths. This may indicate a cost of infection (delayed sexual maturation) or may be an adaptation facilitating greater migration away from the source of the virus (Wilson and Gatehouse 1993).

Virus-treated gregarious larvae also had lower pupal weights than uninfected larvae. However, despite a positive relationship between pupal weight and fecundity, this difference did not translate into significant reductions in fecundity, at least within a benign laboratory setting. It does suggest, however, that the costs of surviving virus challenge might be exacerbated in the gregarious form, and it would be interesting to investigate the impact on other life history traits, such as those related to flight capacity, longevity and fecundity in the field.

Despite the possibly higher costs of resistance in gregarious larvae, the results also indicated that their greater capacity to resist fatal virus infection also reduces their likelihood of transmitting active NPV infection vertically to their offspring. The mechanistic bases of vertical transmission of virus, in its various forms, and disease resistance are not well-understood in Lepidoptera. In numerical terms, the gregarious larvae were challenged with a higher number of infectious virus propagules than the solitary larvae. If the site of the superior resistance of gregarious larvae occurs at the gut level [probably the main barrier to infection for baculoviruses (Washburn et al. 1996)]; lower levels of infection might simply be the result of fewer virus particles gaining access to the larval body via the mid-gut cells and thus lower levels of vertical transmission for gregarious larvae. It is also possible that differences in susceptibility relate to post-gut defence mechanisms, as crowding and gregariousness also produce differences in immune factors (e.g. phenoloxidase levels) in different tissues (mid-gut, cuticle), in addition to the haemolymph (Wilson et al. 2001a). The ability to transmit an infection vertically is likely to be determined by a combination of the size of the initial pathogen challenge, the rate at which a larva can clear or slow down baculovirus infection before it reaches an irreversible systemic state (e.g. by encapsulation or the sloughing off mid-gut cells; Keddie et al. 1989; Washburn et al. 1996, 1998), and the timing of the infection in relation to the developmental stage of the insect, as levels of resistance change (decrease) both between and within growing instars (Hoover et al. 2002). Limited evidence suggests that the likelihood of vertical transmission increases in individuals infected as later instars. This supports the notion that time to clear the infection and increasing developmental resistance are important to the process (Fuxa and Richter 1991; Kukan 1999). Correlations with virus dose levels are less apparent.

Infection (death) in the offspring of challenged insects reared solitarily reached an average of 13% of larvae per egg batch (range = 0–29%), which is well within the range found for other Lepidoptera, including *Spodoptera* species, where NPV infection levels of up to 48% have been recorded (Kukan 1999). Adult Lepidoptera cannot be infected directly by baculoviruses, and persistent infections initiated during the larval period cannot develop into fatal infections in moths. Thus, it would appear that the changes undergone during metamorphosis are sufficient to curtail virus replication on a significant scale. OBs are only relevant to host-to-host horizontal transmission of infection and within-host transmission occurs via nonoccluded (budded) virus. The most obvious (and effective) site for vertical virus transport is within the lepidopteran reproductive system. It is difficult to envisage successful vertical transmission that is neither the result of external contamination of the eggs before, during or after oviposition nor related to the reproductive organs. The limited data on this issue are equivocal in that granulovirus (GV) infection in the flour moth *Plodia interpunctella* was shown to persist from one generation to the next after virus challenge with high levels of GV DNA occurring both in the gonads and in the remainder of the adult body of both sexes (Burden et al. 2002). However, what is clear from the data is that virus infection must be transmitted during copulation as challenged males are capable of transmitting overt infection to untreated female moths. The mechanism for this is as yet unclear, but it does indicate that the propensity for vertical transmission in this species is high and must, in part, be related to the gonads rather than external contamination, as surface sterilization of eggs had limited impact.

There was a low level of background virus infection in the unchallenged control insects in the parental generation; the culture of *S. exempta* used in the experiment had only recently been collected from the field in Tanzania prior to the start of the study (six months in culture) and was known to support a persistent virus infection (L. Vilaplana, K. Wilson, E.M. Redman and J.S. Cory, submitted). This infection is covert, in that the insects appear healthy, without any disease symptoms. Covert infections can occasionally be triggered into overt infections; however, neither the nature of covert infection nor what triggers it into a full blown infection is understood in any baculovirus system. It is thus possible that some of the overt infection seen in the offspring generation was the result of activated covert virus; this should approximate to the levels of viral mortality recorded in the untreated controls (<4%). All of the insects used in the experiment originated from the same culture, so would have started with the same genetic background. As all insects in the offspring generation were reared singly, any differences in disease expression are thus related to experimental treatments. It is difficult to make meaningful comparisons between background mortality in the parental and offspring generations, as mortality in the parents refers to mortality after the fourth instar, whereas virus death in the offspring generation relates to mortality post egg hatch. However, the results do indicate that a low level of background virus infection may be transmitted by a relatively large number of apparently healthy individuals. This agrees

with field data; levels of persistent, covert NPV appear to be high in field populations of *S. exempta* (L. Vilaplana, K. Wilson, E.M. Redman and J.S. Cory, submitted). Thus, these data should give a more realistic estimate of what happens in the field and the possible role of vertical transmission in virus persistence and the initiation of disease epizootics.

The relationship of larval crowding to vertical transmission may facilitate a better understanding of the ecology of SpexNPV in natural populations of *S. exempta.* Early studies by Swaine and colleagues (Brown and Swaine 1965; Swaine 1966), as well as our own field observations, suggest that NPV epizootics are generally low or absent from armyworm populations at the beginning of the rainy season, while later in the season >90% viral prevalence can occur. Vertical transmission provides a potential mechanism for this pattern, as it appears that SpexNPV can persist through the dry season, when *S. exempta* densities are too low for horizontal transmission to be common. Thus, overt NPV in the initial armyworm build-up, at the start of the rainy season, will be low. It is likely that a small proportion of larvae in these first outbreaks will die from NPV passed on from their "solitary" parents, but that this will be insufficient to trigger a widespread virus epizootic. However, some larvae from these outbreaks will inevitably receive a sublethal dose of NPV from these initial deaths. These will carry the infection with them when they migrate to new locations as adult moths to initiate the next generation of outbreaks.

We have shown that, following sublethal infection as larvae, moths of both sexes are capable of vertically transmitting overt NPV infections to their offspring, especially those experiencing low conspecific densities (solitary larvae), similar to conditions prevalent on the periphery of outbreaks. Thus, the proportion of larvae dying of overt NPV infection will be greater in this second generation of armyworm than in the first. Larval densities and the prevalence of overt NPV infections may both be sufficient at this stage to generate an NPV epizootic within these high density populations. Alternatively, another generation of sublethal infections leading to higher levels of vertical transmission of overt NPV may be required. This positive feedback mechanism is likely to lead to a build-up of overt NPV infection in armyworm outbreaks as the season progresses, in line with the observations of Swaine and others, despite the greater NPV resistance expressed by gregarious larvae. Ultimately, however, this cycle will be broken, as the rainy season comes to an end and larval densities decline, reducing the opportunity for horizontal transmission of NPV. Under these circumstances, vertical transmission once again becomes the main route of virus transfer between insects, allowing the virus to persist in low-density armyworm populations through the dry season. A challenge for the future is to incorporate our knowledge of the natural ecology of this virus into the design of biocontrol programmes utilizing SpexNPV.

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