Evol Ecol (2010) 24:147–160
DOI 10.1007/s10682-009-9296-2

ORIGINAL PAPER

Pathogen persistence in migratory insects: high levels of vertically-transmitted virus infection in field populations of the African armyworm

Luisa Vilaplana · Kenneth Wilson · Elizabeth M. Redman · Jenny S. Cory

Received: 30 April 2008 / Accepted: 6 February 2009 / Published online: 28 February 2009

Abstract Pathogens face numerous challenges to persist in hosts with low or unpredictable population densities. Strategies include horizontal transmission, such as by the production of propagules that persist in the environment, and vertical transmission from adults to offspring. While many pathogens are capable of horizontal and vertical transmission little is known of their relative roles under realistic conditions of changing population densities. Insect baculoviruses can be transmitted both horizontally and vertically, although much of the work on baculovirus transmission has focussed on horizontal transmission that can be effective at high host densities. Here, we examine the prevalence of a vertically-transmitted, covert infection of nucleopolyhedrovirus (NPV) in field populations of the African armyworm, *Spodoptera exempta*, in Tanzania. African armyworm is a major pest of graminaceous crops in Africa and despite its migratory nature and boom and bust dynamics, NPV epizootics are common and can be intense at the end of the multi-generation armyworm season. We found that virtually all the insects collected in the field

L. Vilaplana · E. M. Redman · J. S. Cory
Molecular Ecology and Biocontrol Group, NERC Centre for Ecology and Hydrology, Mansfield Road, Oxford OX1 3SR, UK

K. Wilson
Department of Biological Sciences, Lancaster Environment Centre, Lancaster University, Lancaster LA1 4YQ, UK

Present Address:
L. Vilaplana
Department of Physiology and Molecular Biodiversity, Institut de Biologia Molecular de Barcelona, CSIC, 08034 Barcelona, Spain

Present Address:
E. M. Redman
Institute of Comparative Medicine, Faculty of Veterinary Medicine, University of Glasgow, Bearsden Road, Glasgow G61 1QH, UK

J. S. Cory (✉)
Biological Sciences, Simon Fraser University, 8888 University Drive, Burnaby, BC, Canada
e-mail: jsc21@sfu.ca

were positive for *S. exempta* NPV (*Spex*NPV) DNA and 60% of these insects had transcriptionally active virus. This suggests that *Spex*NPV is transmitted vertically at extremely high levels in field populations of *S. exempta* and can maintain a persistent infection without obvious symptoms. Similarly high levels of virus DNA and RNA were detected in a *S. exempta* colony that had been maintained in continuous culture for 5 years. This study provides an insight into mechanisms of pathogen persistence in migratory populations where hosts are unpredictable and indicates that covert infection may be more common and more relevant in disease dynamics of insects than had previously been thought.

Keywords Covert infection · Latency · NPV · Persistence · Sublethal · Epizootic

Introduction

Simple mathematical models predict that highly virulent parasites and pathogens will rapidly become extinct following epidemic invasion of a naïve host population due to the exhaustion of the pool of susceptible hosts ('fade-out'; Keeling and Grenfell 1997; Earn et al. 1998; Swinton et al. 1998). This does not usually happen; however, primarily because pathogens have evolved a number of mechanisms to ensure persistence in the face of low or variable host densities. These strategies include the production of resistant life stages to facilitate long-term survival in the environment, the use of alternative hosts and vertical transmission within the host or even within the host genome.

Vertically transmitted parasites and pathogens (i.e. those transmitted directly from parent to offspring) rely on the survival of their hosts for persistence. They therefore need to adapt to the life cycle of their host(s) or even manipulate their hosts to ensure transmission and survival (Moore 2002). Vertically transmitted pathogens are predicted to be less pathogenic than horizontally transmitted forms and to enhance host survival and reproduction in order to maximise their chances of being passed to the next generation (Lipsitch et al. 1995, 1996). It has even been suggested that strategies for persistence could include vertically transmitted pathogens preventing super-infection (i.e. re-infection of an already infected host) by more virulent horizontally transmitted parasites to prevent the second pathogen utilizing or killing the host (Lively et al. 2005). Some pathogens are capable of both vertical and horizontal transmission and provide the opportunity to test predictions about differing virulence demands of each route (Turner et al. 1998; Messenger et al. 1999; Stewart et al. 2005). While such studies are invaluable in the study of the evolution of virulence and its potential impact on disease outbreaks and epidemiology, field-based investigations are needed to ascertain the patterns and prevalence of horizontal and vertical modes of pathogen transmission under realistic conditions of changing population densities and varying environmental conditions.

One group of pathogens that is particularly interesting regarding the interplay between horizontal and vertical routes of transmission are the insect baculoviruses; a group comprising the nucleopolyhedroviruses (NPVs) and the granuloviruses (GVs). Baculoviruses are particularly common in Lepidoptera and infect the larval feeding stage. They are generally highly pathogenic and once infection becomes systemic, host death is almost inevitable. In baculoviruses the within-host spread of infection is accomplished by virus particles, whereas for horizontal transmission between hosts one or many virus particles are enclosed in a proteinaceous matrix (the occlusion body). Baculovirus occlusion bodies

are thought to be the major route of virus transmission at high host densities and they can survive for considerable periods of time outside the host in environmental reservoirs (e.g. tree bark, soil). However, the significance of these reservoirs for the between-generation persistence of the virus remains unclear (Cory and Myers 2003). Baculoviruses are rapidly inactivated by ultraviolet irradiation and their persistence in exposed environments with high levels of sunlight is likely to be greatly reduced. These constraints on virus persistence will be compounded if the host is highly mobile or migratory, as it is less likely that a naïve host will encounter viable baculovirus occlusion bodies remaining in the environment from previous epizootics. Despite this, baculovirus epizootics occur in migratory species, several of which live in the tropics where ultraviolet levels are likely to be particularly strong, e.g. the African armyworm, *Spodoptera exempta* (Brown and Swaine 1965). This indicates that alternative mechanisms of virus persistence and transmission, such as vertical transmission, may be important in the pathogens of species with this type of life history.

Spodoptera exempta is an oligophagous noctuid moth found throughout eastern Africa and some parts of western Africa. Its biology is characterized by outbreaks that can cause devastating, wide-scale damage to host plants (wild and cultivated grasses), followed by periods when the insect occurs at extremely low densities (Rose et al. 2000). The adult stage is highly mobile, with moths migrating hundreds of kilometres each generation. Most armyworm outbreaks are characterized as being single-generation build-ups at locations with no previous history of armyworm for several years (Rose et al. 2000). Thus, any environmental reservoir of baculovirus would probably have to persist for years to be ecologically relevant. Local re-distribution of the virus by biotic (e.g. predators) or abiotic (e.g. rain) mechanisms could contribute to the persistence of the virus in the insect population during the outbreak season. However, during the dry-season armyworm densities are so low and ultraviolet levels so high, that it is highly unlikely that NPV could persist via horizontal transmission alone. Despite this, widespread *S. exempta* NPV (*Spex*NPV) infections are extremely prevalent (e.g. 63% in one study Brown and Swaine 1965) and NPV has been known to cause up to 98% mortality at outbreak sites towards the end of the armyworm season (Rose et al. 2000). NPVs from other, closely-related *Spodoptera* species either have very narrow host ranges or are entirely species-specific (Simón et al. 2004). Moreover, as no alternative hosts have been found for *Spex*NPV (J. S. Cory, unpublished data) the utilization of a reservoir species as a means of persistence seems highly unlikely. We thus predict that the most likely mechanism of persistence is in a covert form of virus that spreads by vertical transmission.

Good evidence exists that baculoviruses can be vertically transmitted from adults to their offspring, both via surface contamination of eggs (transovum transmission) and within the egg itself (transovarial transmission). Transovum and transovarial transmission can be separated in laboratory studies by decontaminating the surface of the eggs before the larvae hatch. Such studies indicate that, while most vertical transmission is usually transovum, transovarial transmission appears to occur at low levels (Kukan 1999; Myers et al. 2000). In early studies, the vertical transmission of baculoviruses was inferred by the 'spontaneous' expression of overt viral infection (i.e. obvious symptoms of virus infection leading to death) in larvae that have not themselves been exposed to the pathogen (Longworth and Cunningham 1968; Jurkovicova 1979; Kukan 1999). To avoid possible confusion over terminology, we follow previous authors here (Burden et al. 2002, 2003) and define *latent* viral infections as those in which the virus is essentially dormant (non-replicating), as opposed to *persistent* viral infections, in which the virus is replicating and a range of viral genes are expressed at low levels. Both latent and persistent viral infections

may be classed as covert, in that neither results in obvious external symptoms of disease. However, it is thought that both latent and persistent infections can be 'triggered' into an overt, lethal infection that could result in subsequent horizontal transmission of virus (see below).

Although covert baculovirus infections have been discussed in the literature for decades, it is only recently that molecular techniques have been developed to detect specific viral DNA sequences and to demonstrate transcriptional activity in apparently healthy host insects. There is evidence from a laboratory culture of the cabbage moth, *Mamestra brassicae* (L.) that NPV can exist as a low-level, persistent infection, i.e. RNA transcripts from the virus can be detected and thus it is not truly latent (Hughes et al. 1997). Recently, it has been demonstrated that adults in field populations of *M. brassicae* in the UK also support a low-level, persistent infection (Burden et al. 2002, 2003). To determine if SpexNPV is persisting in a covert form in *S. exempta*, we address three questions: (1) are covert *Spex*NPV infections present in armyworm populations; (2) if present, is there any evidence that this virus is replicating within its host, and (3) is this virus transmitted vertically to subsequent host generations?

Materials and methods

Insects

Field collection of insects

Collections of late instar *S. exempta* larvae and pre-pupae were made during an armyworm outbreak in February and March 2002 from pastureland on Lucky Lucky farm and the neighbouring M'ringa Estate (S 03 20 25 0 E 36 37 24 5) near Arusha in northern Tanzania and returned to the UK as pupae. The adults that originated from the field-collected insects were used to initiate a laboratory colony and then frozen at −70°C for later PCR analysis to assess virus prevalence in natural populations. Subsequent generations were reared continuously on a wheatgerm-based, semi-artificial diet (Hunter et al. 1984) at 27 (±1)°C with a 12/12 h light/dark cycle. At each generation, pairs of adults were placed in 100 ml plastic pots containing a source of 5% sucrose solution and tissue paper on which eggs were laid and collected on a daily basis. A random selection of eggs was retained and 1–2 days post hatching, 30–60 larvae from each pair were reared solitarily in 25 ml pots of artificial diet. These were reared through to adulthood and were used to initiate the next generation. At each generation (up to seven), an arbitrary selection of larvae and adult moths were frozen at −70°C for viral analysis.

Laboratory cultures

Additional studies were conducted on a culture of *S. exempta* that had been maintained at the NERC Centre for Ecology and Hydrology in Oxford for 5 years (approximately 70 generations). These insects originated from a colony maintained at the Natural Resources Institute, Chatham, UK that was initiated from insects collected in Tanzania in 1996. The insects were maintained in continuous culture on a wheatgerm-based semi-artificial diet (Hunter et al. 1984) at 28 (±1)°C with a 12/12 h light/dark cycle. The egg batches were routinely decontaminated to remove potential surface virus, initially with 10% formalin for 40 min, and in the last year with 1% sodium hypochlorite for 10 min (Bergold 1953). The

Table 1 Covert NPV infection in the African armyworm, *Spodoptera exempta* detected using PCR and RT-PCR

Life stage	Template	% PCR positive[a] (n)[c]	% RT-PCR positive[b] (n)
Field collected			
1st gen. adults	Whole insect	97 (33)	60 (10)
2nd gen. larvae (2nd instar)	Whole insect	78 (9)	–
2nd gen. adults	Whole insect	90 (10)	50 (10)
7th gen. larvae (2nd instar)	Whole insect	100 (8)	–
7th gen. larvae (5th instar)	Whole insect	100 (8)	50 (8)
7th gen. adults	Whole insect	100 (8)	25 (8)
Laboratory colony			
Larvae (2nd instar)	Whole insect	87 (15)	
Larvae (5th instar)	Whole insect	91 (11)	54.5 (11)
Adults (stock culture)	Whole insect	93 (14)	38.5 (13)

Starting material for the PCR was 1.5 ng of sample and for the RT-PCR, 1.5 μg of sample. Percentage of laboratory and field-collected *S. exempta* insects at different life stages positive for *Spex*NPV *polyhedrin* and *lef 8* DNA[a] and RNA[b] by nested PCR. [c]*n* = total number of insects tested

larvae were reared together until the second instar when stock insects were separated and maintained singly in 25 ml pots containing diet until pupation. Adults were provided with a 10% honey solution in a small container with a cotton wool wick and were left to mate in groups of 50–100 in cages. Adults, second and fifth instar larvae were analysed for covert virus infection (sample sizes in Table 1).

DNA and RNA extraction from insects

In order to detect covert levels of *Spex*NPV both DNA and RNA were extracted from different stages of the insect. While positive DNA data can indicate the presence of the virus, confirmation of RNA indicates that the virus is not being passively carried and that at least a subset of genes are being transcribed. This is particularly relevant in adult insects as this is a life stage that cannot be infected directly by baculoviruses, thus the virus can only have been acquired at an earlier larval stage (or passed vertically for many generations). Total genomic DNA was isolated from second and fifth instar larvae and adults of *S. exempta,* both from the insects collected in Tanzania and the laboratory colonies. Insects were either freshly killed or stored at −70°C until processing. Individual cadavers were ground in liquid nitrogen using a mortar and pestle. Between each sample the equipment was washed with sterile water followed by 70% ethanol and RNAseZAP (Ambion), and finally baked in an oven at 240°C. Second instar larvae were used for virus DNA analysis only, using a DNeasy Tissue Kit (Qiagen). Fifth instar larvae and adults were divided into two; half the sample was used for DNA extraction, as described above, and the other half was used for RNA extraction with an RNeasy Mini Kit (Qiagen). RNA samples were then treated with DNase and stored afterwards at −70°C. For the total RNA extraction, all equipment and reagents (unless from a kit) were treated with RNaseZAP (Ambion) to remove RNases. Reverse transcription reactions were carried out on total RNA using an Omniscript Reverse Transcription Kit (Qiagen), after first checking the integrity and concentration of the RNA on a denaturing agarose gel. The reverse transcription reaction mixture was prepared following the manufacturer's protocol adding 8.5 μl of total sample

RNA (approximately 1.5 μg) and using the oligo (dT) 12–18 primers supplied with the kit (0.5 μg/μl Gibco-BRL). The reaction mixture was incubated at 42°C for 1 h, followed by 5 min at 93°C to inactivate the reverse transcriptase. At this stage, the samples were subjected to a two-step nested PCR reaction following the protocol described below.

PCR amplification of the *S. exempta* NPV polyhedrin and *lef-8* genes

Specific viral genes were targeted using PCR in order to detect virus presence at very low levels. As the state of vertically transmitted baculoviruses is unknown, for example, are they defective and do they lack certain genes that are not necessary for persistence in vivo, we opted to work with two *Spex*NPV genes; one a highly expressed, non-essential structural protein and the other an essential gene required for virus replication. As the whole genome sequence of *S. exempta* NPV (*Spex*NPV) was not available at this point, the design of specific primers was based on partial sequences of two genes, polyhedrin (*polh*) and *lef-8* (Herniou 2003). The polyhedrin gene codes for the major matrix protein of the NPV polyhedra. Additionally, polyhedrin is a highly conserved gene, which means that there is a possibility of cross-hybridization with polyhedrin sequences from other baculovirus species. The second gene, *lef8,* is required in late gene transcription (Funk et al. 1997; Lu and Miller 1995; Titterington et al. 2003). Nested PCR reactions were performed for both genes in order to increase PCR sensitivity. Therefore, two sets of specific primers were designed for each of the genes using the Primer 3 program (Whitehead Institute, Centre for Genome Research, Cambridge, UK). For the first amplification step, the primer sequences for the *polyhedrin* gene were: Sepolhf-5′ AGC GGC AAA GAG TTT CTC AG 3′, Sepolhr-5′ ACT CTT CGA AGC TGT TGG 3′, and for the *lef-8* gene; Selef8f-5′ CAT GGT GAA ATG ACT GTG GC 3′and Selef8r-5′ GGC GAA CAT TGA AAG ATG GT 3′. For the nested PCR, internal primers were designed, for polyhedrin; Sepolhf2-5′ GTT ACA GAT TCC TCG CAC AA 3′, Sepolhr2-5′ GGT GTA CTC GGA ATG CAG GT 3′ and *lef-8*, Selef8f2-5′ GAC CCA CGA TTT ACC AGT GC 3′ and Selef8r2-5′ TTG GAC ATT AGC TGC GAC AC 3′. The sizes of the nested products obtained were approximately 170 bp for *polyhedrin* gene and 295 bp for the *lef8* gene.

Reaction parameters and conditions were optimized initially on purified *Spex*NPV DNA extracted using a standard phenol:chloroform protocol. The *Spex*NPV positive control originated from populations of *S. exempta* in Tanzania in the 1970s and has since been amplified in laboratory populations of *S. exempta*. Amplification of target DNA by the PCR was performed in a total reaction volume of 50 μl containing 5 μl of 10× PCR buffer (Sigma, containing 15 mM $MgCl_2$), 1 μl dNTP stock (10 mM), 1.25 μl BSA (20 mg/ml, Sigma), 0.5 μl of each primer (10 pmol/μl), 0.2 μl REDTaq DNA polymerase (1 U/μl; Sigma) and 2 μl of template DNA. A negative control, containing all the PCR reagents except the template (which was replaced with sterile water), was always included, together with a positive control of purified *Spex*NPV DNA. In addition, a PCR reaction was carried out directly on the RNA samples to confirm that they were not contaminated with virus DNA. The cycling parameters for PCR were as follows: 1 cycle of 95°C for 5 min, 52°C for 1 min and 72°C for 1 min, followed by 28 cycles of 95°C for 1 min, 52°C for 1 min and 72°C for 1 min and a final extension cycle ending with a 5 min incubation at 72°C (Hybaid Touchdown Thermal Cycler). For the nested amplification, 1.5 μl of the first step PCR product was used as the DNA template for the adults and fifth instar larvae and 2 μl of product for the second instar larvae (as less material tended to be produced for the smaller larvae), with the same reaction mixture as in the first step. The reaction conditions were the same as for the first amplification except that the middle step was reduced from 28 to 23

cycles. PCR products were visualized by electrophoresis of 20 μl of the reaction on 1.1% agarose gels containing ethidium bromide.

Sensitivity and specificity analysis

As we wanted to be able to estimate the amount of *Spex*NPV that might persist in a host we also carried out a sensitivity analysis to assess the lower detection limits of the PCR assay. We also checked the specificity of the primers as it is possible that baculoviruses other than *Spex*NPV could be detected if there was sufficient homology between the areas of the genes targeted. The sensitivity of the reaction was estimated using tenfold serial dilutions of purified *Spex*NPV DNA using the nested PCR described above. The range of DNA-template concentrations varied from 50 ng to 0.01 pg. A specificity analysis was also carried out using 1.5 ng DNA from a range of other baculoviruses from congeneric and more distantly related species [*Spodoptera exigua* NPV, *S. littoralis* NPV, *M. brassicae* NPV, *Autographa californica* NPV and *Plodia interpunctella* granulovirus (GV)]. A negative control (sterile distilled water) was used in each PCR investigation.

DNA sequencing

As we found a low level of cross-reactivity of the PCR primers to an NPV from a closely related *Spodoptera* species, we also confirmed the identity of the PCR amplification product by sequencing it. A subset of PCR products was sequenced after being extracted from a 1.1% agarose gel using a QIAquick Gel extraction Kit (Qiagen). After quantification using agarose gels and spectrophotometry, the double-stranded DNA obtained was sequenced using an ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction Kit (PE Biosystem) and Applied Biosystems 370A automated sequencer. The pairwise sequence alignments and the analysis of the sequences obtained were performed with the GCG Gap program. For each gene, multiple amino acid sequences were aligned with ClustalW using default parameters.

Results

Detection of SpexNPV DNA and RNA in larval and adult life stages

Field samples and field-derived culture

*Spex*NPV DNA was detected in almost 100% of the adult moths that were derived from larvae and pre-pupae collected directly from the field in Tanzania (generation 1, Table 1). No difference existed between the detection of the two genes (Fig. 1). The progeny of these insects were maintained in culture, and larvae and adults were again probed for the *Spex*NPV *polyhedrin* and *lef-8* genes at the second and the seventh generation. The level of detection of virus DNA remained high in all samples, ranging from 78% detection in second instars from the second generation up to 100% in the adults from the seventh generation. These data indicate that the proportion of insects with covert infections of *Spex*NPV did not vary between life stages. A large proportion (up to 60%) of the original field-collected insects was also found to be positive for *Spex*NPV RNA, although the level was consistently lower than that found for viral DNA detection (Table 1). The proportion of RNA positive insects decreased in the seventh generation, with only 25% of adults being

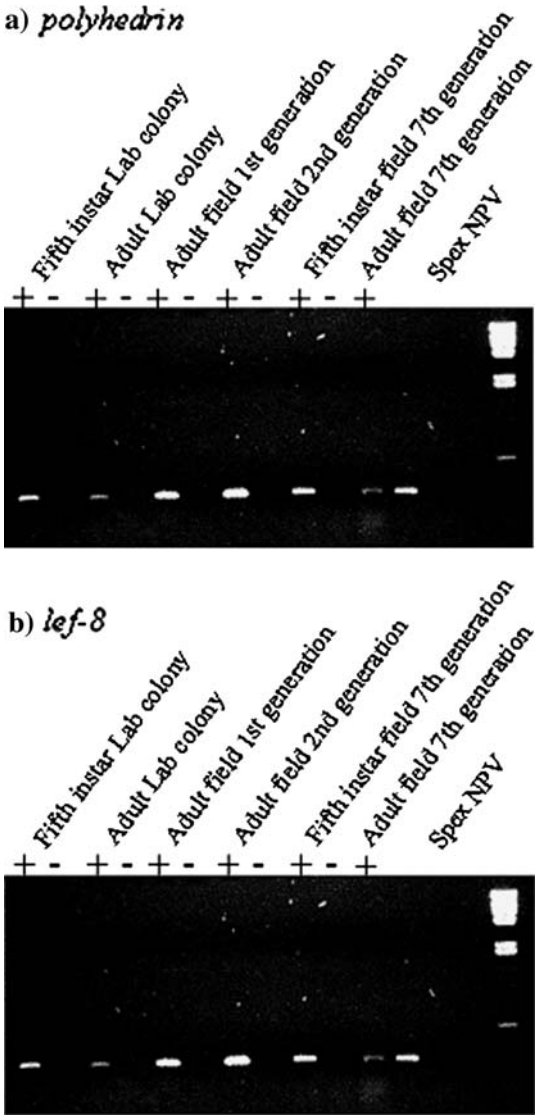

Fig. 1 Reverse transcription-polymerase chain reaction (*RT-PCR*) amplification of *S. exempta* NPV RNA from laboratory and field-collected insects. Specific primers were used to detect: **a** *S. exempta* NPV *polyhedrin* cDNA and **b** *S. exempta* NPV *lef-8* cDNA. *Negative symbols* indicate water only controls. The starting amount of total RNA was approximately 1.5 μg in each case

virus positive, but because of the small sample size this trend was not significant (generalized linear model with binomial errors and logit link: $F_{1,2} = 68.5$, $P = 0.077$). However, 50% of fifth instars sampled from the same generation were found to be RNA positive. In all cases, insects were positive for both genes and in no instances were insects RNA positive and DNA negative.

Laboratory stock

Similar results were found in the *S. exempta* insects that had been in culture for at least 70 generations. *Spex*NPV DNA was detected in a high proportion (87–93%) of second instars, fifth instars and adults (Table 1). In all cases, both genes were detected (Fig. 1). Detection

of *polyhedrin* and *lef-8* RNA was performed on a subset of fifth instars and adults. For fifth instars, although at least 90% were positive for DNA, only 54% were positive for virus RNA. In the adults, transcript detection decreased further to 38%. No samples were positive for RNA alone and both genes were always amplified.

Sensitivity and specificity of the nested PCR assay

The sensitivity of the nested PCR for *Spex*NPV DNA was determined using serially diluted viral DNA. It was estimated that as little as 100 femtograms of *Spex*NPV DNA could be detected for both *polyhedrin* and *lef-8* (data not shown). If the genome size of *S. exempta* NPV is estimated to be approximately 137 Kb, assuming that it is similar to the phylogenetically closely related species *S. exigua* NPV and *S. litura* NPV (IJkel et al. 1999; Pang et al. 2001), this would correspond to the detection of 665 genome copies.

In the specificity tests the polyhedrin primers generated the expected product from the *Spodoptera littoralis* NPV DNA. The *Autographa californica* NPV polyhedrin gene was detected also, but the intensity of the PCR product observed was very faint (data not shown). No PCR products were generated from any of the other viruses. For *lef 8*, no bands were produced that were as intense as those produced with *Spex*NPV; however, faint bands of the predicted size were seen with *Ac*MNPV and *S. littoralis* NPV (data not shown).

Sequencing of PCR products

To confirm that the virus detected with PCR and RT-PCR was *Spex*NPV, PCR products obtained from adults and fifth instar larvae from the laboratory samples and from the field-collected insects were sequenced and compared with the data for purified *Spex*NPV DNA. The alignments confirmed that the virus amplified was *Spex*NPV, although there were minor sequence variations (Fig. 2).

Discussion

The results demonstrate that persistent infections by *Spex*NPV are extremely common in field populations of *S. exempta*, at least in the region studied (northern Tanzania). Both viral DNA, and to a lesser extent RNA, were found in the insects collected directly from the field. The presence of viral DNA and RNA in the adult moths is particularly important; adults cannot be directly infected by baculoviruses, nor are they thought to support extensive (if any) viral replication (Federici 1997). Thus, positive identification of *Spex*NPV in this life stage provides strong support for the possibility of vertical transmission of the virus. The prevalence of viral DNA in the field populations was unexpectedly high, with essentially all of the adult population being infected. The levels of viral RNA detected were lower and found in approximately 50% of the population. Similar results were found with both the genes targeted, indicating that the vertically transmitted virus is likely to be a low level persistent infection, as found for *M. brassicae* NPV (Burden et al. 2003). It is unclear whether the difference in DNA and RNA results reflects a genuine difference, i.e. only a proportion of the virus population within the host is actively producing RNA transcripts. Alternatively, this may indicate that the level of RNA being produced is borderline for the detection methods used. There was no indication that the prolonged rearing of the insects collected in Tanzania in the laboratory reduced the prevalence of vertically transmitted virus DNA within the population. Eggs in the field-

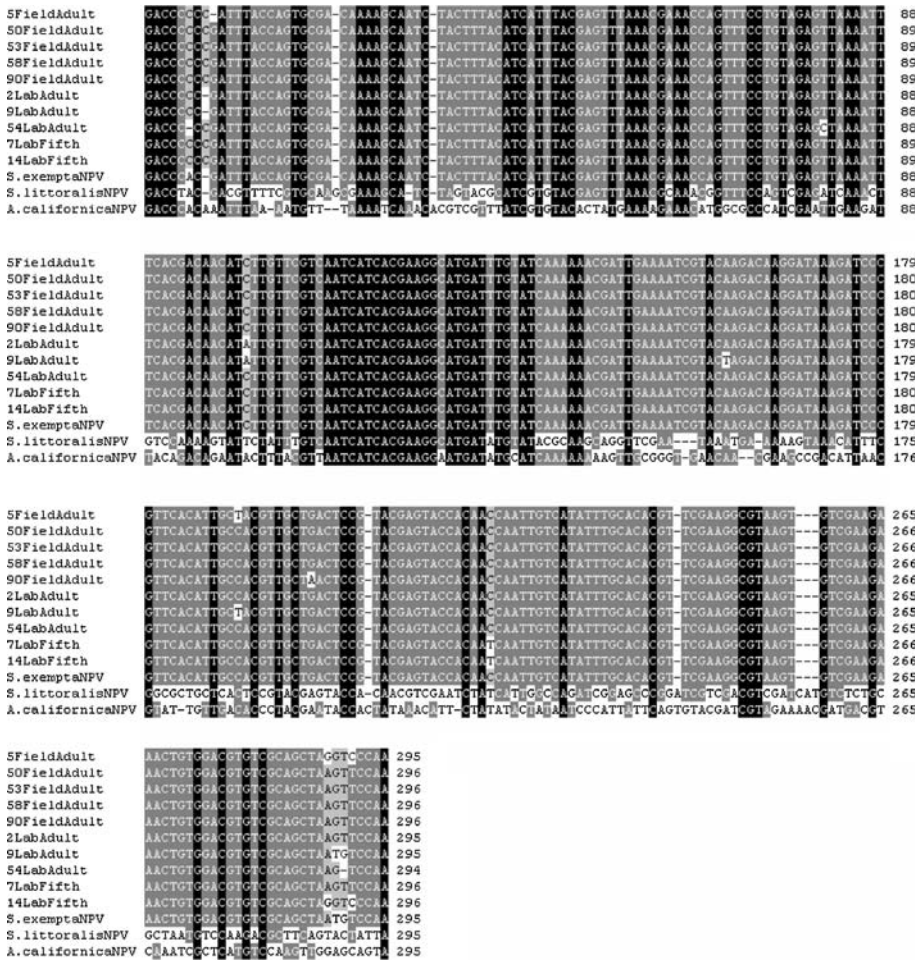

Fig. 2 Sequence alignments for 295 nucleotides of SpexNPV *lef-8*. DNA was amplified by nested PCR from adult and larval *Spodoptera exempta* from a laboratory culture and from insects collected from field populations in Tanzania. The sequence is compared with homologous sequences from the closely related *S. littoralis* MNPV and *Autographa californica* MNPV. The number before each sequence refers to an individual insect. Alignments were carried out with ClustalW at the nucleotide level

derived culture were not surface-sterilized, however, larvae dying of overt disease in this culture were very rare and other studies on the same *S. exempta* culture also indicated that vertical transmission of the virus (as an overt infection) was not influenced by whether the eggs were surface-sterilized or not (Vilaplana et al. 2008). The long-term persistence of the virus in the insects was also supported by the results on a second *S. exempta* colony that had been in continuous culture for many generations (including regular surface-sterilization of the eggs), yet still retained almost 100% covert infection levels within adult moths. The process by which persistent infections develop is unknown. Research using another system, the Indian meal moth, *Plodia interpunctella* (Hubner), and its GV, has started to shed light on this process. Analysis of the survivors of a viral challenge in final instar larvae demonstrated that viral RNA was present in 30% of the adults (of both sexes) and in

up to 80% of the larvae in the next generation (Burden et al. 2002). These data indicate that persistent infections are likely to result from surviving virus challenge.

The continued presence of viral RNA in both cultures clearly demonstrates that persistent virus is not lost from *S. exempta* populations, even after continuous culture. This agrees with the Burden et al. (2003) study which showed the continued presence of NPV in long-term *M. brassicae* cultures. These results imply that low level, persistent infections are maintained in a relatively stress–free laboratory situation. Short-term costs associated with surviving baculovirus challenge are well documented (Rothman and Myers 1996) and in *S. exempta* include reduced pupal weight and altered pre-oviposition period (Vilaplana et al. 2008). These results suggest that it is likely that these costs (sublethal effects) are associated with the initial cost of fighting off infection (e.g. reduced feeding due to damaged gut cells, other tissue damage, increased immune activity etc.) rather than a cost of retaining a persistent infection, although it is possible that the response to covert infection changes over time.

Two outstanding questions emerge from this study: what is the nature of the persisting virus, and, from an evolutionary perspective, under what conditions is vertical transmission the superior strategy for pathogen transmission? Parallels, and perhaps an indication of possible mechanisms, can be drawn from another persistent insect virus, *Heliothis zea*–1, a non-occluded virus found in Lepidoptera. Unlike the baculovirus studies, research on this virus has demonstrated that only one viral gene is transcriptionally active during persistent infection in insect cells, the persistence-associated transcript-1 (Chao et al. 1992). This virus was once thought to be a member of the baculovirus group; however, recent analysis of its complete genome indicates that it is only distantly related to the Baculoviridae, although genes that are baculovirus homologues are found in *Heliothis zea*–1 (Cheng et al. 2002). Various stressors, including high humidity, crowding and infection with heterologous viruses, have all been shown to activate overt baculovirus infection in covertly infected insects (Hughes et al. 1993; Fuxa et al. 1999; Cooper et al. 2003). Persistent *Heliothis zea*–1 virus can be activated by both homologous virus (*Heliothis zea*–1) and heterologous baculoviruses (Kelly et al. 1981), although, interestingly, cells with persistent *Heliothis zea*–1 are also more resistant to super-infection by *Heliothis zea*–1 (Lee and Chao 1998). Clearly, more genes are transcriptionally active in persistent baculovirus infections, although this might also be related to differences between persistence in cells and in the more complex insect body. However, it is not yet clear whether persistent infections of baculoviruses are actively maintained by a number of viral genes, are the result of suppression by the host, or whether they persist by some other more passive mechanism.

Why has persistent infection evolved in insect baculoviruses? The most likely explanation is that persistent baculoviruses are the superior competitors (as compared to highly pathogenic horizontally transmitted variants) under certain scenarios. Models of truly latent viruses, such as herpes, suggest that such viruses could exhibit a bet-hedging strategy to cope with environmental stochasticity (Stumpf et al. 2002). Thus, in situations where conditions fluctuate, for example, in the availability of susceptible hosts, a persistent virus that can reactivate at some point into an active infection that will outcompete non-persistent forms of the virus over time. While this model has been developed for longer lived host species, it is not difficult to envisage a scenario where this argument could also apply to baculoviruses that infect sporadic or migratory hosts, such as the African armyworm. The simplicity of this approach is that the virus does not require a particular trigger for reactivation, just a time lag between initial infection and later reactivation: it need not be related purely to host density (Stumpf et al. 2002). Other modelling approaches, with

different assumptions and more specifically geared to insect viruses suggest that persistent infections could create a variety of host dynamics, including host-pathogen cycles and endemic persistent interactions where the healthy host stages are lost from the interaction (Bonsall et al. 2005). However, whether the susceptibility of persistently infected insects to super-infection is greater or less than the susceptibility of healthy insects is crucial in this particular model, as is the rate of reactivation of the persistent virus. As yet there is no indication that persistently infected *S. exempta* larvae are more resistant to super-infection, (although this is difficult to test as persistent virus infections cannot be cured). The high level of covert virus infection found in field populations of *S. exempta* suggests that the populations studied are close to an endemic situation in which virus-free insects have been lost. Reactivation rates of NPV infection in the field are unknown, as are the potential triggers.

In conclusion, this study indicates that very high levels of covert baculovirus infection are found in field populations of a migratory insect, the African armyworm and this is likely to contribute to the vertical transmission of disease. Further investigations are needed to ascertain how widespread this phenomenon is and what role it plays in the ecology and evolution of host and virus interactions in natural field populations.

Acknowledgments We would like to thank Tim Carty for rearing the laboratory colony of *S. exempta* and for producing insect diet and John Burden for advice on PCR. We are also very grateful to Wilfred Mushobozi and colleagues in Arusha, Tanzania, for their input and support in the field and David Gryzwacz for his encouragement and support throughout the project. We would also like to thank David Grzywacz and Judy Myers for their comments on the manuscript and Hilary Lauzon for help with Fig. 2. This work was funded by NERC grant NER/A/S/2000/01141 to JSC and KW and partly funded by the United Kingdom Department for International Development (DFID) for the benefit of developing countries. The views expressed are not necessarily those of the DFID R7954 Crop Protection Research Programme. JSC would also like to acknowledge the support of the NSERC Canada Research Chair program.

References

Bergold GH (1953) Insect viruses. Adv Virus Res 1:91–139. doi:10.1016/S0065-3527(08)60463-7

Bonsall MB, Sait SM, Hails RS (2005) Invasion and dynamics of covert infection strategies in stage-structured insect-pathogen populations. J Anim Ecol 74:464–474. doi:10.1111/j.1365-2656.2005.00991.x

Brown ES, Swaine G (1965) Virus disease of the African armyworm, *Spodoptera exempta* (Walker). Bull Entomol Res 56:671–684

Burden JP, Griffiths CM, Cory JS et al (2002) Vertical transmission of sublethal granulovirus infection in the Indian meal moth, *Plodia interpunctella*. Mol Ecol 11:547–555. doi:10.1046/j.0962-1083.2001.01439.x

Burden JP, Nixon CP, Hodgkinson AE et al (2003) Covert infections as a mechanism for long term persistence of baculoviruses. Ecol Lett 6:524–531. doi:10.1046/j.1461-0248.2003.00459.x

Chao Y-C, Lee S-T, Chang M-C et al (1992) Differential expression of Hz-1 baculovirus genes during productive and persistent infections. J Virol 63:1442–1448

Cheng C-H, Liu S-M, Chow T-Y et al (2002) Analysis of the complete genome sequence of the Hz-1 virus suggests that it is related to members of the *Baculoviridae*. J Virol 76:9024–9034. doi:10.1128/JVI.76.18.9024-9034.2002

Cooper D, Cory JS, Theilmann DA et al (2003) Nucleopolyhedroviruses of forest and western tent caterpillars: cross-infectivity and evidence for activation of latent virus in high density field populations. Ecol Entomol 28:41–50. doi:10.1046/j.1365-2311.2003.00474.x

Cory JS, Myers JH (2003) The ecology and evolution of insect baculoviruses. Annu Rev Ecol Evol Syst 34:239–272. doi:10.1146/annurev.ecolsys.34.011802.132402

Earn DJD, Rohani P, Grenfell BJ (1998) Persistence, chaos and synchrony in ecology and epidemiology. Proc R Soc Lond B Biol Sci 265:7–10. doi:10.1098/rspb.1998.0562

Federici BA (1997) Baculovirus pathogenesis. In: Miller LK (ed) The baculoviruses. Plenum Press, NY, pp 301–339
Funk CJ, Braunagel SC, Rohrmann GF (1997) Baculovirus structure. In: Miller LK (ed) The baculoviruses. Plenum Press, NY, pp 7–32
Fuxa JR, Sun J-Z, Weidner EH et al (1999) Stressors and rearing diseases of *Trichoplusia ni*: evidence of vertical transmission of NPV and CPV. J Invertebr Pathol 74:149–155. doi:10.1006/jipa.1999.4869
Herniou EA (2003) Use of comparative genomics and phylogenetics to study the evolution of the Baculoviridae. Imperial College, University of London, Unpublished Ph.D. thesis
Hughes DS, Possee RD, King LA (1993) Activation and detection of a latent baculovirus resembling *Mamestra brassicae* nuclear polyhedrosis virus in *M. brassicae* insects. Virology 194:604–615. doi:10.1006/viro.1993.1300
Hughes DS, Possee RD, King LA (1997) Evidence for the presence of a low-level, persistent baculovirus infection of *Mamestra brassicae* insects. J Gen Virol 78:1801–1805
Hunter FR, Crook NE, Entwistle PF (1984) Viruses are pathogens for the control of insects. In: Grainger JM, Lynch JM (eds) Microbiological methods for environmental biotechnology. Academic Press, NY, pp 323–347
IJkel WFJ, van Strien EA, Heldens JGM et al (1999) Sequence and organization of the *Spodoptera exigua* multicapsid nucleopolyhedrovirus genome. J Gen Virol 80:3289–3304
Jurkovicova M (1979) Activation of latent virus infections in larvae of *Adoxophyes orana* (Lepidoptera: Tortricidae) and *Barathra brassicae* (Lepidoptera: Noctuidae) by foreign polyhedra. J Invertebr Pathol 34:213–223. doi:10.1016/0022-2011(79)90066-1
Keeling MJ, Grenfell BT (1997) Disease extinction and community size: modelling the persistence of measles. Science 275:65–67. doi:10.1126/science.275.5296.65
Kelly DC, Lescott T, Ayres MD et al (1981) Induction of a non-occluded baculovirus persistently infecting *Heliothis zea* by *Heliothis armigera* and *Trichoplusia ni* nuclear polyhedrosis virus. Virology 112:174–180. doi:10.1016/0042-6822(81)90623-1
Kukan B (1999) Vertical transmission of nucleopolyhedrovirus in insects. J Invertebr Pathol 74:103–111. doi:10.1006/jipa.1999.4873
Lee J-C, Chao Y-C (1998) Apoptosis resulting from superinfection of *Heliothis zea* virus 1 is inhibited by *p35* and is not required for virus interference. J Gen Virol 79:2293–2300
Lipsitch M, Nowak MA, Ebert D et al (1995) The population dynamics of vertically and horizontally transmitted parasites. Proc R Soc Lond B Biol Sci 260:321–327. doi:10.1098/rspb.1995.0099
Lipsitch M, Siller S, Nowak MA (1996) The evolution of virulence in pathogens with horizontal and vertical transmission. Evol Int J Org Evol 50:1729–1741. doi:10.2307/2410731
Lively CM, Clay K, Wade MJ et al (2005) Competitive co-existence of vertical and horizontally transmitted parasites. Evol Ecol Res 7:1183–1190
Longworth JF, Cunningham JC (1968) The activation of occult nuclear-polyhedrosis viruses by foreign nuclear polyhedra. J Invertebr Pathol 10:361–367. doi:10.1016/0022-2011(68)90094-3
Lu A, Miller LK (1995) The roles of eighteen baculovirus late expression factor genes in transcription and DNA replication. J Virol 69:475–482
Messenger SL, Molineaux IJ, Bull JJ (1999) Virulence evolution in a virus obeys a trade-off. Proc R Soc Lond B Biol Sci 266:397–404. doi:10.1098/rspb.1999.0651
Moore J (2002) Parasites and the behaviour of animals. Oxford University Press, Oxford
Myers JH, Malakar R, Cory JS (2000) Sublethal nucleopolyhedrovirus infection effects on female pupal weight, egg mass size and vertical transmission in gypsy moth (Lepidoptera; Lymantriidae). Environ Entomol 29:1268–1272
Pang Y, Yu JX, Wang LH et al (2001) Sequence analysis of the *Spodoptera litura* multicapsid nucleopolyhedrovirus genome. Virology 287:391–404. doi:10.1006/viro.2001.1056
Rose DJW, Dewhurst CF, Page WW (2000) The african armyworm handbook: the status, biology, ecology, epidemiology and management of *Spodoptera exempta* (Lepidoptera: Noctuidae), 2nd edn. Natural Resources Institute, Chatham
Rothman LD, Myers JH (1996) Debilitating effects of viral disease on host Lepidoptera. J Invertebr Pathol 67:1–10. doi:10.1006/jipa.1996.0001
Simón O, Williams T, Lopez-Ferber M, Caballero P (2004) Virus entry or the primary infection cycles are not the principal determinants of host specificity of *Spodoptera* spp. nucleopolyhedroviruses. J Gen Virol 85:2845–2855. doi:10.1099/vir.0.80179-0
Stewart AD, Logsdon JM, Kelley SE (2005) An empirical study of the evolution of virulence under both horizontal and vertical transmission. Evol Int J Org Evol 59:730–739
Stumpf MPH, Laidlaw Z, Jansen VAA (2002) Herpes viruses hedge their bets. Proc Natl Acad Sci USA 99:15234–15237. doi:10.1073/pnas.232546899

Swinton J, Harwood J, Grenfell BJ et al (1998) Persistence thresholds for phocine distemper virus infection in harbour seal *Phoca vitulina* metapopulations. J Anim Ecol 67:54–68. doi:10.1046/j.1365-2656.1998.00176.x

Titterington JS, Nun TK, Passarelli AL (2003) Functional dissection of the baculovirus late expression factor-8 gene: sequence requirements for late gene promoter activation. J Gen Virol 84:1817–1826. doi:10.1099/vir.0.19083-0

Turner PE, Cooper VS, Lenski RE (1998) Tradeoff between horizontal and vertical modes of transmission in bacterial plasmids. Evol Int J Org Evol 52:315–329. doi:10.2307/2411070

Vilaplana L, Redman EM, Wilson K et al (2008) Density-related variation in vertical transmission of a virus in the African armyworm. Oecologia 155:237–246. doi:10.1007/s00442-007-0914-9