Host-range testing of a mixture of two nucleopolyhedroviruses of *Choristoneura fumiferana* (Lepidoptera: Tortricidae)

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Abstract—The host range of a mixture of *Choristoneura fumiferana* (Clemens) nucleopolyhedroviruses (CfMNPV and CfDefNPV) was investigated using a *per os* bioassay of larvae of 29 species of Lepidoptera and adult males of *Megachile rotundata* (F.) (Hymenoptera: Megachilidae). Using a whole-genomic DNA probe, positive results were obtained in 8 of 10 Tortricidae: *Archips cerasivorana* (Fitch), *Choristoneura fractivittana* (Clemens), *C. fumiferana*, *Choristoneura occidentalis* Freeman, *Choristoneura pinus pinus* Freeman, *Choristoneura rosaceana* (Harris), *Clepsis persicana* (Fitch), and *Cydia pomonella* (L.); one Crambidae: *Ostrinia nubilalis* (Hübner); one arctiine Erebidae: *Estigmene acrea* (Drury); and two Noctuidae: *Oligia illocata* (Walker) and *Pyrrhia exprimens* (Walker). Mortality rates were highest among *C. fumiferana*, *C. occidentalis*, *C. pinus pinus*, *A. cerasivorana*, and *C. pomonella*. Sequenced polymerase chain reaction (PCR) amplicons from infected individuals from several species confirmed that the primer sets amplified the target viruses. CfMNPV was consistently found in virus-fed *C. fumiferana*; whereas, CfDefNPV was present only occasionally. The presence of CfMNPV and CfDefNPV in *A. cerasivorana* was confirmed by PCR and DNA sequencing. Significant treatment-mortality rates were induced in the noctuids *P. exprimens* and *Acronicta impleta* Walker; PCR determined that both viruses were present in treated *P. exprimens* but only CfMNPV was present in *A. impleta*. No virus was detected in *M. rotundata*.

Résumé—Nous avons étudié la gamme des hôtes d'un mélange de virus de la nucléopolyhédrose (CfMNPV et CfDefNPV) de *Choristoneura fumiferana* (Clemens) au moyen de bioessais par voie orale chez des larves de 29 espèces de lépidoptères et des mâles adultes de *Megachile rotundata* (F.) (Hymenoptera : Megachilidae). À l'aide d'une sonde ADN de génome entier, nous avons obtenu des résultats positifs chez 8 de 10 tortricidés, *Archips cerasivorana* (Fitch), *Choristoneura fractivittana* (Clemens), *C. fumiferana*, *Choristoneura occidentalis* Freeman, *Choristoneura pinus pinus* Freeman, *Choristoneura rosaceana* (Harris), *Clepsis persicana* (Fitch) et *Cydia pomonella* (L.) ; un crambidé, *Ostrinia nubilalis* (Hübner) ; un érebidé arctiiné, *Estigmene acrea* (Drury) ; et deux noctuidés, *Oligia illocata* (Walker) et *Pyrrhia exprimens* (Walker). Les taux de mortalité sont maximaux chez *C. fumiferana*, *C. occidentalis*, *C. pinus pinus*, *A. cerasivorana* et *C. pomonella*. Les amplicons séquencés de l'amplification en chaîne par polymérase (PCR) provenant d'individus infectés appartenant à plusieurs espèces confirment que les jeux d'amorces amplifient les virus ciblés. Le CfMNPV se retrouve constamment chez les *C. fumifera* nourris de virus, alors que le CfDefNPV n'apparaît qu'occasionnellement. La présence du CfMNPV et du CfDefNPV chez *A. cerasivorana* est confirmée par la PCR et le séquençage d'ADN. Des taux de mortalité significatifs reliés au traitement ont été provoqués chez les noctuidés *P. exprimens* et *Acronicta impleta* Walker; la PCR a déterminé que les deux virus sont présents chez les *P. exprimens* traités, mais seul CfMNPV apparaît chez *A. impleta*. Aucun virus n'a été décelé chez *M. rotundata*.

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Introduction

In the 1980s, forest insect pest management in Canada underwent a dramatic shift from the broadcast use of synthetic chemical insecticides to a heavier reliance on pathogens and compounds derived from natural sources (Ennis and Caldwell 1991). Since then, there has been a legislated move to alternative control products that are efficacious, economically attractive, and have few or no impacts on non-target forest insects.

Nucleopolyhedroviruses (NPVs) are baculoviral pathogens of various Lepidoptera, Hymenoptera, and Diptera (Jehle *et al.* 2006). Currently, two baculoviruses of lymantriine (Lepidoptera: Erebidae) forest insect pests are registered in Canada and the United States of America (*Lymantria dispar* MNPV and *Orgyia pseudotsugata* MNPV); two other baculoviruses are registered in Canada only (*Neodiprion lecontei* NPV and *Neodiprion abietis* NPV) (United States Environmental Protection Agency 2010; Pest Management Regulatory Agency 2010). Additional registrations of baculoviruses for pests of agriculture or horticulture have been made worldwide (Moscardi 1999).

There has been little research on the effects of an NPV of *Choristoneura fumiferana* (Clemens) (Lepidoptera: Tortricidae) (CfMNPV), except on the main host and some of its close relatives. CfMNPV caused limited mortality (2 out of 500) upon first passage in *Estigmene acrea* (Drury) (Lepidoptera: Erebidae), increasing to 100% by the third passage (Shapiro *et al.* 1982). Similarly, Stairs *et al.* (1981) demonstrated low virus-caused mortality upon first passage followed by increased pathogenicity after second passage in both *Trichoplusia ni* (Hübner) (Lepidoptera: Noctuidae) and *Galleria mellonella* (L.) (Lepidoptera: Pyralidae). Otherwise, replication of CfMNPV has been reported in other species of *Choristoneura* Lederer (*Choristoneura pinus pinus* Freeman (Stairs 1960), *Choristoneura occidentalis* Freeman (Cunningham *et al.* 1983*a*, 1983*b*), *Choristoneura rosaceana* (Harris) (Lucarotti and Morin 1997)) and in *Archips cerasivorana* (Fitch) (Lepidoptera: Tortricidae) (Valéro 1990). In contrast, Burke and Percy (1982) were unable to find any NPV in field collections of larvae of *Choristoneura conflictana* (Walker).

CfDefNPV, a "companion" of CfMNPV, occurs in wild populations of *C. fumiferana* and is not capable of infecting its presumed host on its own—CfMNPV serves as a "helper virus" enabling CfDefNPV to co-infect *C. fumiferana* (Lauzon *et al.* 2005). Although CfDefNPV has been characterized as "defective" (Arif *et al.* 1994), its genome is unlike other known defective NPVs, which have clear deletions and are thus smaller than their whole counterparts (Lauzon *et al.* 2005). It is unknown whether CfDefNPV may be a competent virus in other, as yet undocumented, host species.

Our objective was to provide a more comprehensive description of the host range of the two known *C. fumiferana* NPVs. Because CfMNPV and CfDefNPV co-occur in wild populations and reared colonies of *C. fumiferana*, we investigated the host range of a mixture of the two viruses, rather than each virus separately. As recommended in the Canadian regulatory guidelines for registration of microbial pest control agents (Pest Management Regulatory Agency 2001), we used a centrifugal approach (phylogenetic extrapolation of Richards *et al.* 1998) to select our test species, working outward from closely related to more distantly related species. This assumes that closely related species are often physiologically and ecologically similar and likely to be susceptible to the same or similar pathogens (but see Goulson 2003).

Materials and methods

The experimental procedures summarized here closely follow Barber *et al.* (1993).

Preparation of virus suspension. Naturally occurring CfMNPV and CfDefNPV were propagated in fifth-instar larvae of *C. fumiferana* and *C. occidentalis* using contaminated artificial diet (McMorran 1965), as per Arif and Brown (1975) and Kreutzweiser *et al.* (1997). Ebling (2004) found no difference in the activity of CfMNPV (Ireland strain) when propagated in these two hosts.

Experimental insects. The 30 species investigated are listed in Table 1. Four species of *Choristoneura* (*C. fumiferana*, *C. occidentalis*, *C. pinus pinus*, and *C. conflictana*), two lymantriine erebids (*Orgyia leucostigma* (Smith) and

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Table 1. Bioassay results for 30 species (29 Lepidoptera, 1 Hymenoptera) challenged with CfMNPV and CfDefNPV.

Taxon	No. alive (dead)		*p* value*	% corrected treatment mortality†	% infected in treated group	No. infected in control group
	Control	Treated				
Lepidoptera						
Tortricoidea						
Tortricidae						
Tortricinae – Archipini						
Choristoneura fumiferana (Clemens)	121 (8)	35 (283)	<0.001	88.3	84.6	0
Choristoneura occidentalis Freeman	48 (1)	9 (138)	<0.001	93.8	87.1	0
Choristoneura pinus pinus Freeman	49 (1)	17 (132)	<0.001	88.4	86.6	0
Choristoneura rosaceana (Harris)	63 (2)	171 (19)	0.054	9.1	54.2	0
Choristoneura fractivittana (Clemens)	139 (2)	389 (30)	0.003	5.8	50.1	6
Choristoneura conflictana (Walker)	49 (1)	145 (5)	0.494	1.4	0.0	0
Archips cerasivorana (Fitch)	172 (16)	23 (530)	<0.001	95.5	96.7	10
Clepsis persicana (Fitch)	69 (0)	205 (2)	0.578	1.0	20.3	0
Clepsis melaleucana (Walker)	104 (17)	333 (32)	0.967	0.0	0.0	0
Olethreutinae – Grapholitini						0
Cydia pomonella (L.)	75 (5)	1 (233)	<0.001	99.5	100.0	3
Pyraloidea						
Crambidae						
Pyraustinae – Pyraustini						
Ostrinia nubilalis (Hübner)	42 (0)	120 (3)	0.433	2.4	10.6	0
Geometroidea						
Geometridae						
Larentiinae – Eupitheciini						
Horisme intestinata (Guenée)	41 (1)	125 (0)	NA	0.0	0.0	0
Noctuoidea‡						
Erebidae						
Lymantriinae – Orgyiini						
Orgyia leucostigma (Smith)	50 (0)	150 (0)	NA	0.0	0.0	0
Lymantriini						
Lymantria dispar (L.)	49 (0)	141 (0)	NA	0.0	0.0	0
Arctiinae – Arctiini						
Estigmene acrea (Drury)	49 (1)	145 (4)	0.644	0.7	1.3	0
Spilosoma congrua Walker	42 (0)	123 (2)	0.557	1.6	0.0	0

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Table 1 (*concluded*).

Taxon	No. alive (dead)		*p* value*	% corrected treatment mortality†	% infected in treated group	No. infected in control group
	Control	Treated				
Noctuidae						
Plusiinae – Plusiini						
Autographa precationis (Guenée)	37 (0)	108 (1)	0.757	0.9	0.0	0
Acronictinae – Acronictini						
Acronicta impleta Walker	37 (0)	1 (115)	<0.001	99.1	0.0	0
Heliothinae						
Pyrrhia exprimens (Walker)	68 (0)	134 (69)	<0.001	34.0	82.8	0
Noctuinae						
Caradrinini						
Caradrina multifera (Walker)	40 (0)	109 (0)	NA	0.0	0.0	0
Xylenini						
Hyppa xylinoides (Guenée.)	38 (1)	122 (2)	0.870	0.0	0.0	0
Oligia illocata (Walker)	45 (0)	139 (1)	0.738	0.8	1.5	0
Orthosiini						
Orthosia revicta (Morrison)	31 (1)	85 (5)	0.518	2.5	0.0	0
Morrisonia latex (Guenée)	44 (0)	134 (0)	NA	0.0	0.0	0
Hadenini						
Melanchra pulverulenta (Smith)	41 (1)	132 (2)	0.878	0.0	0.0	0
Leucaniini						
Mythimna unipuncta (Haworth)	48 (0)	145 (0)	NA	0.0	0.0	0
Noctuini – Agrotina						
Agrotis ipsilon (Hufnagel)	36 (0)	121 (0)	NA	0.0	0.0	0
Noctuini – Noctuina						
Cerastis salicarum (Walker)	39 (1)	124 (4)	0.650	0.6	0.0	0
Diarsia rubifera (Grote)	37 (0)	95 (0)	NA	0.0	0.0	0
Hymenoptera						
Megachilidae						
Megachile rotundata (F.) (males)	29 (0)	81 (0)	NA	0.0	0.0	0

Note: Infection was inferred by positive dot blots using whole-genomic DNA probes.

*Probability that a level of mortality at least as high as that observed in the treated group could have occurred by chance alone, calculated using a permutation test; NA = not applicable, since there was no mortality in the treated group.

†Treatment mortality corrected for control mortality (Abbott 1925).

‡Classification follows Lafontaine and Schmidt (2010).

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Lymantria dispar (L.)), and an arctiine erebid (*E. acrea*) were routinely reared by others at Canadian Forest Service, Sault Ste. Marie, Ontario. The lymantriines were fed Bell diet (Bell *et al.* 1981) while the others were reared on modified (formalin and antifungal spray replaced with Benlate) budworm artificial diet (McMorran 1965).

Eggs of *Cydia pomonella* (L.) were purchased from Okanagan–Kootenay, Sterile Insect Release Program (Osoyoos, British Columbia); caterpillars were reared on the modified budworm diet, with no turnover of generations. Generally, first-instar larvae were transferred to 22 mL creamer cups half-filled with modified budworm diet and maintained at a density of 10–20 larvae per cup during the first three instars.

Most other colonies were initiated from adults collected nocturnally in the vicinity of Sault Ste. Marie, Ontario, and reared on modified budworm diet. Larvae of two species were reared on natural foliage: *Horisme intestinata* (Guenée) (Lepidoptera: Geometridae) on virgin's bower (*Clematis virginiana* L. (Ranunculaceae)) and *Acronicta impleta* Walker (Lepidoptera: Noctuidae) on pin cherry (*Prunus pensylvanica* L. (Rosaceae)). Larvae of *A. cerasivorana* were hatched from egg masses collected from choke cherry (*Prunus virginiana* L.) stems and reared on modified budworm diet. Larvae of *C. rosaceana* were reared on an artificial diet based on pinto beans (Shorey and Hale 1965). The larvae of *Choristoneura fractivittana* (Clemens), *Clepsis persicana* (Fitch), and *Clepsis melaleucana* (Walker) (Lepidoptera: Tortricidae) were reared on modified budworm diet individually in 6 mL glass shell vials to eliminate cannibalism. All adult and larval Lepidoptera were maintained at 20 °C (bioassayed at 25 °C), 60–70% relative humidity, and 16L:8D photoperiod.

Cocoons of *Megachile rotundata* (F.) (Hymenoptera: Megachilidae) were obtained commercially (Northstar Seed Ltd., Neepawa, Manitoba) (see Barber *et al.* 1993 for details of handling and assay).

Bioassay system and procedure. Assays were designed to provide single point estimates of mortality and infection rates, with no intention of statistical comparison of rates among species. Trials were unreplicated with experimental animals recruited as they became available; the treated (virus-fed) group had three times the number of animals as the control (water-fed) group to accommodate potential losses due to food avoidance or feeding interruption.

Viral suspensions were standardized using a stained-film method (Wigley 1980) or a hemacytometer (Bright-Line, Hausser Scientific, Horsham, Pennsylvania) to determine the concentration of occlusion bodies (OBs); counts were consistent between the two methods. The generalized protocol for dosing third-instar larvae was a sequential presentation of four doses of 3×10^4 OBs/dose for a total of 1.2×10^5 OBs over a 4–8 day period (24–48 h per dose). The single-dose level of 3×10^4 OBs represents about 66× the published LD_{50} for third-instar *C. fumiferana* (455 OBs for a mixture of CfMNPV and CfDefNPV; Kaupp and Ebling 1990). Each dose was delivered in 2 µL water to a cylindrical diet plug (approximately 4 mg; in a Beem embedding capsule) or natural foliage (predrilled Plexiglas plates with a circular foliar membrane exposed; see Barber *et al.* 1993); or in 1 µL water to an approximately 2 mg diet plug. Those animals not completely feeding on all four doses were discarded (but see below). Mortality was assessed daily and experimental animals individually frozen by the 11th to 15th day until probing was initiated (Barber *et al.* 1993). Nonparametric permutation tests (Resampling Stats for Excel, Statistics.com, Arlington, Virginia) with 1000 iterations were used to estimate the probabilities of achieving the observed treated-group mortality rates by chance alone.

The assays involving five species (*A. cerasivorana*, *C. fumiferana*, *C. occidentalis*, *C. pinus pinus*, and *C. pomonella*) could not be completed by strictly adhering to a four-dose protocol, as fewer than 25% of individuals in each treated group fed on all four doses. This was attributed to their relatively small size, infection development, and feeding cessation. Consequently, results for those assays include animals that fed on four or fewer doses (*C. pinus pinus* $\leq$ 1 dose; *C. occidentalis* $\leq$ 3 doses; *C. fumiferana* $\leq$ 4 doses; *C. pomonella* $\leq$ 4 doses; and *A. cerasivorana* $\leq$ 4 doses). Another group of seven assays had relatively good feeding rates (52–95% of the treated group, in increasing

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order of *C. persicana*, *E. acrea*, *C. rosaceana*, *C. conflictana*, *C. fractivittana*, *C. melaleucana*, and *Pyrrhia exprimens* (Walker) (Lepidoptera: Noctuidae), but included animals that did not feed on all doses to enable screening for additional infections.

Dot-blot hybridization assay (probing). Detection of CfMNPV and CfDefNPV was accomplished by a nonradioactive, enhanced chemiluminescence (ECL) hybridization assay (Whitehead *et al.* 1983), following protocols outlined by Amersham Biosciences (Baie d'Urfé, Quebec) as modified by Kaupp and Ebling (1993). The viral DNA was extracted and a whole-genomic probe was constructed and used, following Barber *et al.* (1993) as modified by Ebling and Holmes (2002). The DNA in 20 μL subsamples of whole animal macerates was bound to nylon membranes (Hybond-N^+, Amersham Biosciences) by heating at 80 °C for 1 h under vacuum or by using a crosslinker to deliver 30 mJ (GS Gene Linker, Bio-Rad Laboratories, Hercules, California). The remainder of each macerate was frozen for PCR analysis. Using a similar procedure, Kaupp and Ebling (1993) reported a detection threshold of 8000 OBs for a mixture of these two viruses. The detection threshold in this study varied but was modal at the level of approximately 10 000 OBs.

PCR analysis. Some larvae were analyzed by PCR to corroborate that one or both of the viruses administered were present in the insects at the conclusion of the bioassay. With one exception, individuals were chosen nonrandomly to address ambiguous probing results or to represent all four cells of the control: treated × positive:negative matrix. The subsample of individuals of *E. acrea* that probed negative was selected randomly.

DNA for PCR was extracted as outlined in Holmes *et al.* (2008) except that the 37 °C and 80 °C incubation times were reduced from 60 to 30 min. Primers HL03 (5′-TGATTATGAGCGTGGCTCAAGT-3′) and HL04 (5′-ATACTCGCGCGTATCTTATC-3′) were designed to PCR-amplify target sequences in CfMNPV (nucleotides 96 289 – 96 811), CfDefNPV (nucleotides 96 955 – 98 300) and *Anticarsia gemmatalis* MNPV (nucleotides 101 422 – 102 171). The primer set has 100% homology with CfDefNPV, but there are three and four mismatches between HL03 and HL04, respectively, and CfMNPV. The primer set had a detection threshold 10× lower for CfDefNPV than for CfMNPV. Primers LE09 (5′-TCTTGACACGCCCACCATTC-3′) and LE10 (5′-TCGCCAATTGCCAGTGTGAG-3′) (Holmes *et al.* 2008) have 100% homology with CfMNPV and amplify the targeted region of nucleotides 8909–9502. The LE primer set has a detection threshold at least 100× lower than the HL primers. Thus, the HL primers were used primarily to detect CfDefNPV, but had the added convenience of acting as an initial screening tool for CfMNPV. If a visible amount of DNA was not amplified for the latter virus, PCR was repeated on a second sample of the extract using the LE primers and protocol.

To increase confidence that PCR-amplified DNA was from the viruses fed in the bioassays, the uniqueness of the two primer sets was assessed using discontiguous megablast (National Center for Biotechnology Information, Bethesda, Maryland). BLASTs were run separately for Lepidoptera, bacteria, viruses, and Baculoviridae. Two baculoviruses other than the targeted NPVs contain sequences that could be amplified using the HL primer set with a product less than 100 nucleotides different in length from one of the budworm NPVs. The potential PCR products from *Spodoptera frugiperda* MNPV and *Hyphantria cunea* NPV would be similar in length to those from CfDefNPV and CfMNPV, respectively. However, neither of these viruses nor their hosts have been cultured in our laboratory.

DNA amplifications were carried out using the Takara *LA Taq* kit (Takara Bio, Madison, Wisconsin). The template (DNA extract) was added to a reaction mix consisting of 0.02 U/μL *Taq*, 200 μM of each dNTP (Takara Bio), 2 μM of each primer in a set (Mobix Lab, McMaster University, Hamilton, Ontario), and 3 mM BSA (New England Biolabs, Ipswich, Massachusetts) in a 1× buffer with 2.5 mM magnesium chloride. All PCRs were carried out in an Eppendorf Mastercycler gradient thermocycler. Beginning with a preheated block and lid, the thermocycling regimes continued as follows: for HL primers, 4 min at 94 °C and then 30 cycles of 1 min at 95 °C, 1 min at 45 °C, and 2 min at

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72 °C; for LE primers, 3.5 min at 94 °C before 35 cycles of 30 s at 94 °C, 30 s at 62 °C, and 1 min at 72 °C; and both programs concluded with 10 min at 72 °C followed by a 4 °C hold.

The PCR products were electrophoresed in 1.2% agarose gels and either stained with GelRed (Biotium, Hayward, California) and visualized using 302 nm UV transillumination, or stained with GelGreen (Biotium) and visualized at 488 nm using a Typhoon Trio Plus (Amersham Biosciences). Low-end positive controls were detected equally well with both methods.

DNA sequencing. Ten PCR amplicons (five from each primer set) from NPV-infected larvae from five species of Lepidoptera were selected for sequencing to provide evidence that both primer sets were amplifying the intended target DNA(s). Bands were excised from 0.8% agarose gels. The DNA was purified using QIAEX II Gel Extraction Kit (QIAGEN Inc., Mississauga, Ontario), diluted to 20 ng/μL, and sent for sequencing to Laboratory Services, University of Guelph, Guelph, Ontario. The sequences were compared with published ones using Megablast (NCBI).

Results

Table 1 summarizes the results of the assays including accumulated corrected mortality (Abbott 1925) and positive signalling (for NPV DNA) from the probing assessment. Table 2 summarizes the results of PCR and indicates from which groups the amplicon was sequenced.

The data for *C. fumiferana* are summarized first to aid in interpreting the tables. There were 129 control (121 survived and 8 died) and 318 virus-fed (35 survived and 283 died) *C. fumiferana* larvae (Table 1). The permutation test indicated a highly significant treatment mortality with $p < 0.001$. The treatment mortality rate corrected for control mortality was 88.3%. The infection rate in the treatment group was 84.6% and no individuals in the control group probed positive for virus. Only treated animals probed positive for baculovirus, 10 of which were subjected to PCR (Table 2). Of these, all were positive for CfMNPV and two were also positive for CfDefNPV. A subsample of 12 control animals and two treated animals that probed negative for virus were analyzed with PCR. Of the 12 control animals, there were eight *de novo* positive for CfMNPV but none were positive for CfDefNPV. Likewise, one of the two treated animals was *de novo* positive for CfMNPV, but neither was positive for CfDefNPV.

Among Lepidoptera, mortality and infection were generally more prevalent and the rates were higher in the tortricids than elsewhere, although the ranges were similar (0–99.5% mortality and 0–100% infection in Tortricidae versus 0–99.1% mortality and 0–82.8% infection in other Lepidoptera) (Table 1).

Among Tortricidae, the caterpillars of *C. occidentalis, C. pinus pinus, A. cerasivorana,* and *C. pomonella* exhibited mortality (>88%) and infection (>86%) rates similar to those of the nominal host, *C. fumiferana*. Caterpillars of *C. rosaceana* and *C. fractivittana* had much lower mortality (<10%) and infection (approximately 50%) rates. Caterpillars of *C. conflictana, C. persicana*, and *C. melaleucana* had no significant mortality and had low infection rates (0–20%).

Of the 942 probed tortricid controls, there were 19 positive signals obtained among three species (Table 1). Five of the 10 probed-positive *A. cerasivorana* controls were PCR-positive for CfDefNPV, and two were also positive for CfMNPV (Table 2). The identities of both viruses were confirmed by sequencing. None of the six probed-positive *C. fractivittana* controls were confirmed positive by PCR (Table 2). Of the three controls of *C. pomonella* that probed positive, only one was subjected to PCR (negative for both viruses). All 64 treated tortricids in the subsample that probed positive for NPV were confirmed positive by PCR for CfMNPV, CfDefNPV, or both (Table 2).

All but 9 of the 33 tortricid controls in the subsample that probed negative for NPV were verified negative by PCR; a single *C. fractivittana* and eight *C. fumiferana* larvae (Table 2) represented *de novo* CfMNPV-positives. Within the subsample of 19 treated animals that probed negative, there were 14 *de novo* CfMNPV-positive results with PCR, most of which were in *C. rosaceana* and *C. persicana*.

Among the 19 species of non-tortricid Lepidoptera, there were indications of treatment effects

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Table 2. Positive polymerase chain reaction (PCR) results for selected insect species, summarized in groupings based on initial DNA dot-blot hybridization assay results.

	Number	Probed +		Probed −	
Taxon	PCR primer	Control	Treated	Control	Treated
Tortricidae					
Choristoneura fumiferana	n	—	10	12	2
	CfMNPV	—	10	8	1
	CfDefNPV	—	2	0	0
Choristoneura rosaceana	n	—	3	2	7
	CfMNPV	—	3	0	6
	CfDefNPV	—	0	0	0
Choristoneura fractivittana	n	6	6	2	5
	CfMNPV	0	6	1	2
	CfDefNPV	0	1	0	0
Archips cerasivorana	n	10	11	3	—
	CfMNPV	2[‡,¶,§]	11	0	—
	CfDefNPV	5[¶]	11	0	—
Clepsis persicana	n	—	5	2	5
	CfMNPV	—	5	0	5
	CfDefNPV	—	4	0	0
Cydia pomonella	n	1	29	12	—
	CfMNPV	0	28[¶,§]	0	—
	CfDefNPV	0	29[¶]	0	—
Other Lepidoptera					
Ostrinia nubilalis	n	—	6	2	4
	CfMNPV	—	0	0	0
	CfDefNPV	—	2	0	0
Estigmene acrea	n	—	2	2	14
	CfMNPV	—	2[¶]	0	12
	CfDefNPV	—	2[¶]	0	1[‡]
Acronicta impleta	n	—	—	6	22
	CfMNPV	—	—	0	14
	CfDefNPV	—	—	0	0
Pyrrhia exprimens	n	—	29	5	3
	CfMNPV	—	6	0	0
	CfDefNPV	—	29[¶]	0	2
Oligia illocata	n	—	2	2	2
	CfMNPV	—	1	0	0
	CfDefNPV	—	2[¶]	0	0

Note: Probed +, individuals determined with DNA probing to be positive for virus; Probed −, individuals determined with DNA probing to be negative for virus; —, no animals in this category.

[‡]Individuals positive for both viruses were included in companion count.

[¶]CfMNPV and (or) CfDefNPV were confirmed by sequencing DNA amplified using the HL primer set.

[§]CfMNPV was confirmed by sequencing DNA amplified using the LE primer set.

(infection and (or) significant treatment mortality) in only five species: *Ostrinia nubilalis* (Hübner) (Crambidae), *E. acrea*, *P. exprimens*, *A. impleta*, and *Oligia illocata* (Walker) (Noctuidae) (Table 1). There were no positives within the subset of controls subjected to PCR. There were *de novo* positives in the treatment groups for the species listed above, except for *O. nubilalis* and *O. illocata* (Table 2).

Virtually complete mortality occurred in the treated *A. impleta* larvae. No virus was found with probing, but 14 of 22 individuals subjected to PCR were positive for CfMNPV (Tables 1 and 2). *Pyrrhia exprimens* larvae incurred lower

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treatment mortality but had a high infection rate (Table 1). For this species, CfDefNPV was detected by PCR in all 29 individuals in the subsample and CfMNPV was detected in 6 individuals (Table 2).

The remaining three species had nonsignificant treatment mortality and low rates of positive blots (Table 1). By PCR, CfMNPV was more common (14 of 16) than CfDefNPV (3 of 16) in treated *E. acrea* larvae (Table 2). Both viruses were detected by PCR in treated *O. illocata* larvae; whereas, only CfDefNPV was found in treated *O. nubilalis* larvae. However, for these two species, PCR was done on only a very small number of specimens. *Ostrinia nubilalis* was the only species with individuals that probed positive but were negative by PCR (Table 2).

There was no treatment mortality or infection of male leafcutter bees, *M. rotundata* (Table 1).

Discussion

The observed distribution pattern of mortalities and positive tests for infection by CfMNPV and CfDefNPV suggest that susceptibility to these viruses is concentrated within Tortricidae. It is generally accepted that CfMNPV infects the three budworm species (*C. pinus pinus*, *C. occidentalis*, and *C. fumiferana*) (Stairs 1960; Cunningham *et al.* 1983*a*, 1983*b*), and *C. occidentalis* has been used as an alternative host for experimental replication of CfMNPV (Holmes *et al.* 2008; Ebling 2004). CfMNPV has also been shown to infect *A. cerasivorana* (Valéro 1990) and *C. rosaceana* (Lucarotti and Morin 1997). In the latter study, CfMNPV induced 42.0% mortality in a mixture of third- and fourth-instar *C. rosaceana* after dosing with only 1000 OBs, compared with 9% mortality after dosing with 1.2×10^5 OBs in the present study. Such a difference might be attributed at least partly to the relatively short post-exposure holding time in our study (Cory *et al.* 2000).

Of the six species of *Choristoneura* tested, the three budworm species have conifer-feeding larvae and are closely related (Dang 1992; Sperling and Hickey 1994; Harvey 1996). They incurred similarly high mortality and infection rates. Larvae of the other three *Choristoneura* species, *C. rosaceana*, *C. fractivittana*, and *C. conflictana*, all feed on broadleaved deciduous plants and had lower mortality and infection rates. This difference between conifer- and deciduous-feeding species is not surprising given that CfMNPV and CfDefNPV are presumed to have developed in a conifer-feeding species. The difference in feeding habits suggests a distinct evolutionary history for these two groups; this is supported by morphology (Dang 1992), isozymes (Harvey 1996), and mitochondrial DNA (Sperling and Hickey 1994).

In assessing potential susceptibility to a virus, it would be convenient if a host-relatedness threshold could be established over which a viral pathogen would be unlikely to cross. However, the high mortality and infection rates in *A. cerasivorana* and *C. pomonella* provide a clear refutation of this as a strict general principle. Infection was also induced in *C. persicana* though at a modest rate compared with most *Choristoneura* species. But, as in *Choristoneura*, there was a range of infection rates within *Clepsis,* with *C. melaleucana* showing no susceptibility to either virus. Only at a much coarser level is there a higher probability of finding alternative hosts of CfMNPV and CfDefNPV within the Tortricidae than within other families of Lepidoptera.

Bioassays and DNA probing of larvae of five non-tortricid species indicated treatment mortality and (or) infection. A very high mortality was achieved with the noctuid, *A. impleta*, and infection with CfMNPV (but not CfDefNPV) was detected by PCR. No signal was obtained by probing, suggesting either the absence of NPVs or their presence below the detection threshold of about 10 000 OBs. If NPV infection was indeed the causative agent of mortality in *A. impleta*, this species must be extremely sensitive to the virus. However, high mortality in the breeding colony just prior to pupation suggests that the bioassayed animals may have been under stress, possibly involving an entirely different pathogen.

In contrast, the noctuid *P. exprimens* clearly appears to be a permissive species. It had relatively low to moderate mortality, but a high infection rate. Caterpillars of *P. exprimens* are large in comparison to tortricid larvae and perhaps a longer post-exposure holding time for

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this species would result in higher mortality rates. Larvae of this species appear to be more susceptible to CfDefNPV than to CfMNPV.

Oligia illocata, *O. nubilalis*, and *E. acrea* represent a third class of susceptibility, having low mortality rates and low to moderate infection rates. In *O. illocata*, there is evidence of both viruses being involved; whereas, only CfDefNPV was detected in *O. nubilalis* larvae. Shapiro *et al.* (1982) suggested that *E. acrea* was permissive to several baculoviruses including CfMNPV. Our PCR analysis revealed a high proportion of probed-negative animals that contained CfMNPV DNA in their tissues, although presumably at concentrations below the detection limit by probing. Full-blown infections never developed and this species may only be semi-permissive to CfMNPV. A longer post-exposure holding time would be necessary to confirm this.

In several species, notably *E. acrea*, *A. impleta*, *C. rosaceana*, and *C. persicana* but also *P. exprimens*, *C. fumiferana*, and *C. fractivittana*, PCR revealed infections that were missed by whole-genomic probing. This confirms that PCR is a more sensitive detection tool than DNA-probing, at least as applied here. The detection threshold for DNA-probing in this study was approximately 10 000 OBs, compared with <100 OBs for PCR. However, the question remains of whether the lower-level infections detected by PCR necessarily represent permissiveness. For example, although PCR identified infections in a large proportion of the subsamples of treated *E. acrea* and *C. persicana* larvae that probed negative for NPV, there was virtually no treatment-related mortality in these species. Although it might be preferable to subject all experimental animals to two levels of screening (probing to identify permissiveness and PCR to identify low-level infections), the high cost of PCR made this impractical for our study. Our approach of using PCR to shed some light on equivocal results from probing seems reasonable.

Three species had positive probing results for NPV in the controls. Generally, this indicates the presence of a virus in the rearing stock. In *A. cerasivorana*, PCR analysis (and the sequencing of the DNA from selected bands) revealed the presence of CfDefNPV and CfMNPV in some of the controls. This species was reared and bioassayed using larvae hatched directly from field-collected egg masses, and thus, the possibility of contamination within the laboratory was much less than that for species reared through more than one generation. These positive probing results likely indicate naturally occurring infections in this species. In *C. fractivittana*, there was a single positive PCR result for CfMNPV in the controls, probably indicating a low level of infection in wild populations of this species. The cause of the positive probing results in control *C. pomonella* is unknown: no budworm NPVs were detected by PCR.

Resident pathogens are a common problem with short-term colonies of experimental animals initiated from field collections. We made no attempt to "clean" or to "survey" the pathogen load of any of the colonies in our study. The presence of CfMNPV in the *C. fumiferana* controls also demonstrates the vulnerability of long-standing colonies to infection. These infected controls were not detected by DNA-probing but were discovered with the more sensitive PCR analysis.

CfDefNPV was rarely detected in *C. fumiferana* in our study, even though it was originally discovered in this species (Arif *et al.* 1994; Barrett *et al.* 1995). In contrast, the frequency of detection of the two viruses was similar in *A. cerasivorana* and *C. pomonella*, and in *P. exprimens* CfDefNPV was predominant. Specimens of *A. cerasivorana* and *P. exprimens* that tested positive for only CfDefNPV provide additional evidence that this virus is not defective. It may be that this virus evolved in a more susceptible host.

The lack of any detectable infection in *M. rotundata* was expected. Goerzen *et al.* (1990) directly challenged *M. rotundata* with an NPV isolated from *Mamestra configurata* Walker (Lepidoptera: Noctuidae) with no effect demonstrated. Other lepidopteran NPVs have been tested by exposing honey bees, *Apis mellifera* L. (Hymenoptera: Apidae), again with no discernible deleterious effects (Morton *et al.* 1975; Kingsbury *et al.* 1978; Barber *et al.* 1993; Heinz *et al.* 1995). In fact, *Autographa californica* MNPV has been used successfully for control of the greater wax moth, *Galleria mellonella* (L.) (Lepidoptera: Pyralidae), in

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honey bee hives (Dougherty *et al.* 1982, reported as GmMNPV; see Theilmann *et al.* 2005) and foraging honey bees have been used to disseminate Helicoverpa zea SNPV (Gross *et al.* 1994).

Conclusions

Our data point to the Tortricidae as the most likely place to find additional permissive hosts of CfMNPV and CfDefNPV, and generally support the principle of a centrifugal approach to selecting candidate non-target species for potential alternative-host testing (Pest Management Regulatory Agency 2001). Until the genetic basis of host range or host recognition by baculoviruses is firmly established (Miller and Lu 1997), providing another suite of characteristics to survey, further refinement of this approach is unlikely (Goulson 2003; Richards *et al*. 1998). Our results clearly show, however, that the host range of baculoviruses, and particularly the mixture of CfMNPV and CfDefNPV, cannot be predicted with a high degree of certainty based solely on the taxonomic relatedness of potential hosts.

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