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# Pathogenesis of *Junonia coenia* densovirus in *Spodoptera frugiperda*: A route of infection that leads to hypoxia

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ABSTRACT

To evaluate densovirus potential against lepidopteran pests and their capacity to invade new hosts, we have characterised *in vivo* the infection and pathogenesis of the *Junonia coenia* densovirus (*Jc*DNV) in the noctuid pest *Spodoptera frugiperda*. Here we show that infection starts with the ingestion of viral particles that cross the midgut epithelium without replicating. By quantitative PCR we established the kinetic and the route of infection, from virus ingestion to replication in visceral tracheae and hemocytes. *Jc*DNV has a high particle-to-infection ratio mostly due to the barrier function of the midgut. Pathology and cytopathology suggested that infection of tracheal cells impairs oxygen delivery to demanding tissues leading to cytopathic effects in all the tissues. Finally, larval death results from several physiological shocks, including molting arrest and anoxia.



## Introduction

Densoviruses (DNVs) were first discovered as the causative agents of natural epizootics in populations of the greater waxmoth, *Galleria mellonella* (Meynadier et al., 1964). The pathology associated with the disease described a "cellular dense nucleosis", giving their first name to these viruses. Since then, similar epizootics have been discovered in other insects, lepidoptera, mosquitoes, and coackroaches (Hu et al., 1994; Meynadier et al., 1964; O'Neill et al., 1995; Rivers and Longworth, 1972). Because of their high virulence and efficient transmission among insect pests and disease-vectors, DNVs were considered as biological control agents. However their use for this purpose has been under-considered mainly due to the overpromotion of chemical pesticides and the lack of investments. As a consequence, the knowledge on DNV pathogenesis and host range remained globally poor. Nowadays, the development of insect resistance and eco-aware societal demand further complicate pest management. Alternative strategies, including the use of viral agents, have to be constantly renewed and investigated; in this context DNVs are reconsidered with interest.

DNVs are small icosaedral and non-enveloped viral particles (25 nm), packaging a 6 kb linear single-stranded DNA genome ended by two hairpin structures. These characteristics make these insect viruses belong to *Parvoviruses*. Due to their arthropod host range, they have been grouped further in the *Densovirinae* subfamily, including *Densovirus*, *Pefudensovirus*, *Iteravirus* and *Brevidensovirus* genera. Today, more than thirty *Densovirinae* have been discovered, infecting at least 5 insect orders. They reveal a great diversity not only in genomic structures and sequences but also in the biology of infection and host range (Bergoin and Tijssen, 2008). Although transmission mechanisms are poorly known, infection proceeds through oral contamination; the virus spread horizontally in the population and infection is often associated with a high virulence as illustrated by epizootics (Rivers and Longworth, 1972; Meynadier et al., 1964). Interestingly, vertical transmission has been also observed for aphid and mosquito densoviruses associated with low virulence and high replication (van Munster et al., 2003). Vertical transmission

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allowed exploring an alternative use as expression or transducing vectors (Carlson et al., 2006; Ren et al., 2008).

To evaluate the suitability of using DNVs in lepidopteran pest control, and assess for their capacity and their risks of invading new hosts, mechanisms underlying specificity and pathogenesis need to be deciphered carefully. Densoviruses are named according to the first host name they have been discovered on, which does not account for their host range. Among closely related DNVs, host spectrum can be restricted to one host, as described for the *G. mellonella* (*Gm*) DNV, or be rather large and includes several host species, as mentioned for the *Junonia coenia* (*Jc*) DNV or the maize worm *Mythimna loreyi* (*Ml*) DNV (Fediere et al., 2004). Although few data exist, a diversity of tissue tropism has also been described. Some densoviruses infect only the midgut whereas others have a broad tissue tropism but except the midgut, as described for *Gm*DNV (Bergoin and Bres, 1968).

The densovirus prototype, *J. coenia* (*Jc*) DNV was first discovered on a nymphalid and had been propagated on different lepidopteran species including the noctuid *Mamestra brassicae* (Rivers and Longworth, 1972). More recently, *Jc*DNV was shown to be lethal for *Spodoptera littoralis* following intrahemocoelic injections although virus injections might only partially reflect the host range (Abd-Alla et al., 2004; Barat-Houari et al., 2006; Vendeville et al., 2009).

In this work, we analysed the *in vivo* *Jc*DNV infection and pathogenesis in the agronomical pest, the fall armyworm *Spodoptera frugiperda*, from oral contamination to death. We established that *Jc*DNV is pathogenic for *Spodoptera* species and that viral replication occurred in different tissues but excluded the midgut. We showed that the *Jc*DNV pathogenesis severely affects epidermis and tracheae inducing molting failure and oxidative stress. Infection finally leads to anoxia and death of the larvae.

## Results

### *S. frugiperda is susceptible to JcDNV oral infection: effects on development and growth*

Larval stages are a period of intense feeding and rapid growth. In the rearing conditions, *S. frugiperda* caterpillars undergo 6 larval stages, growing in about 2 weeks, from 1 mm and 0.5 mg to more than 3 cm and 0.8 g. To assess for the *S. frugiperda* larval susceptibility to *Jc*DNV we conducted oral infection bioassays. Cohorts of 24 individuals at the second larval instars (L2) were fed with food contaminated with serial dilutions of purified *Jc*DNV. Initial virus concentration was estimated by quantitative PCR according to a *Jc*DNV standard curve at $2.5\times10^{14}$ viral genomes (vg)/ml. Mortality of virus fed L2 increased linearly with increasing viral concentration, showing that infection was dose dependent (Fig. 1A). The lethal dose (LD) required to kill 50% of the cohorts ($LD_{50}$) at L2 was estimated at $5\times10^{9}$ viral genomes (vg) and defined one $LD_{50}$ as the unit used in this study. Decreasing the viral dose to 0.05 $LD_{50}$ did not affect metamorphosis but strongly impaired the pupal development since only 20% of infected larvae developed until the adult stage. This delayed mortality showed that infection and pathogenesis were also time-dependent. In order to analyse whether *Jc*DNV could cause subliminal infections in *S. frugiperda*, we performed quantitative PCR analysis of chrysalids or adults that survived to infection. We were not able to detect any JcDNV suggesting that infection is probably cleared during metamorphosis (data not shown).

Similar results were obtained after *per os* contamination of *S. littoralis*. Interestingly this species was 100-fold more resistant than *S. frugiperda* to *Jc*DNV infection (unpublished data).

To further characterise *Jc*DNV virulence, two phenotypic traits of infection were analysed, time to death and larval weight uptake. L2 to L5 larval cohorts were infected with 100 $LD_{50}$, an over dose previously estimated to kill 100% of infected L2 at larval stage. All larvae were weighted every day to death. Results presented in Fig. 1B showed that for L2 infected larvae, the effect of infection on weight uptake was observed within a day of death meaning that infected larvae maintained their feeding until late in infection. Similar results were obtained with L3 except that infection time was increased and larval death occurred between 6 and 9 days. Concerning L4 and L5 infected with the same dose, death occurred between 7 and 12 days, before or at metamorphosis; larvae often displayed a half-pupa phenotype (data not shown) with no effect on larval weight uptake due to the natural cessation of feeding preceding metamorphosis in control larvae (Fig. 1C).

To estimate whether the lethal dose depends on the developmental stage at the time of infection we next compared the *Jc*DNV lethal doses required at early (L2) and late (L5) larval instars to cause death either at larval stage (Larval) or during metamorphosis, impairing the adult development (Adult). As shown in Fig. 1C, 100 $LD_{50}$ were required to kill 50% of L5 larvae before metamorphosis and might be correlated with the larval mass increase between L2 and L5. This 100-fold difference was balanced by extending the time of infection: considering mortality at pupal stage, 1 $LD_{50}$ and 10 $LD_{50}$ respectively killed 80% of L2 and L5.

Since *Jc*DNV infection affected the development depending on the dose, we next assessed whether infection affected larval molting. A cohort of newly molted third instar (L3) larvae were orally infected with different *Jc*DNV doses, from low (0.01 $LD_{50}$) to high doses (100 $LD_{50}$). Molts were estimated by counting the head capsules in each cohort every day to pupal molt (1 capsule per molt). As shown in Fig. 1D, the number of molts decreased when *Jc*DNV concentration increased. Most L3 larvae infected with 100 $LD_{50}$ molted to the 4th instar and then stopped molting. At low viral dose (0.01 $LD_{50}$), no significant effect on molting was observed, compared to the control cohort. These results showed that molting failure was also dose dependent.

### *JcDNV pathogenesis and time course of infection in S. frugiperda*

To investigate the pathogenesis of *Jc*DNV, we next explored the tissue tropism and the cytopathology induced by *Jc*DNV ingestion. Caterpillars infected at L2 stage with 100 $LD_{50}$ were harvested 5 days pi and analysed by whole mount immuno-labelling with an anti-*Jc*DNV antibody (Fig. 2). Microscopic observations showed a strong labelling in visceral muscles, epidermis and tracheal cells (Fig. 2A, panels 1–3′); all cells displayed “dense nuclei”, hallmark of densonucleosis. Among hemocytes populations, labelled and spread on a coverslip, a productive infection was only observed in one cell type, morphologically identified as granulocytes by phase contrast (Fig. 2A, panels 4–5′). Densonucleosis was also observed in hindgut and foregut cells (data not shown) but not in midgut cells (Fig. 2A, panels 6–6′). No densonucleosis was observed in fat body or in malpighian tubules where only the tracheae branching on these tissues were labelled (Fig. 2A, panels 7–7′). Electron microscopy (TEM) analysis confirmed the presence of viral particles in the hypertrophied nucleus of these cells (Fig. 2B, panels 1–5). Severe defects in epidermis sections were observed, with cuticle disorganisation, numerous autophagic-like vacuoles and pseudo-crystalline viral arrangements in the cytoplasm (Fig. 2B, panels 1–2). Similar disorganisation affected tracheal cells, viral particles were observed within vesicles and within the basal lamina (Fig. 2B, panels 3–4). No pseudo-crystalline viral arrangement was observed. In some tracheae, electron-dense material together with virus-like aggregates obstructed the lumina (panel 5), that might be accumulated chitin that have not been eliminated before hatching. No *Jc*DNV was detected in midgut cells nuclei or in nervous system cells and locomotion muscles. In malpighian tubules and fat body, very seldom viral foci could be observed, closely associated with tracheae, although cytopathies were observed in all these cells (Fig. 2C). In fat body, cells presented shrunk nucleus and numerous autophagic- and apoptic-like bodies were observed in the