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# Host permissiveness to baculovirus influences time-dependent immune responses and fitness costs

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This is an Accepted Article that has been peer-reviewed and approved for publication in the Insect Science but has yet to undergo copy-editing and proof correction. Please cite this article as doi: 10.1111/1744-7917.12755.

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**Abstract**

Insects possess specific immune responses to protect themselves from different types of pathogens. Activation of immune cascades can inflict significant developmental costs to the surviving host. To characterize infection kinetics in a surviving host that experiences baculovirus inoculation, it is crucial to determine the timing of immune responses. Here, we investigated time-dependent immune responses and developmental costs elicited by inoculations from each of two wild-type baculoviruses, Autographa californica multiple nucleopolyhedrovirus (AcMNPV) and Helicoverpa zea single nucleopolyhedrovirus (HzSNPV), in their common host *H. zea*. As *H. zea* is a semi-permissive host of AcMNPV and fully permissive to HzSNPV, we hypothesized that there are differential immune responses and fitness costs associated with resisting infection by each virus species. Newly molted fourth-instar larvae that were inoculated with a low dose ($LD_{15}$) of either virus showed significantly higher hemolymph FAD-glucose dehydrogenase (GLD) activities compared to the corresponding control larvae. Hemolymph phenoloxidase (PO) activity, protein concentration and total hemocyte numbers were not increased, but instead were lower than in control larvae at some time points post-inoculation. Larvae that survived from either virus inoculation exhibited reduced pupal weight; survivors inoculated with AcMNPV grew slower than the control larvae, while survivors of HzSNPV pupated earlier than control larvae. Our results highlight the complexity of immune responses and fitness costs associated with combating different baculoviruses.

**Keywords** ecological immunology; FAD-glucose dehydrogenase; hemocytes; host-pathogen interactions; nucleopolyhedrovirus; phenoloxidase

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## Introduction

Insects have evolved innate immune systems and physical barriers as they are constantly at risk of infection due to the ubiquity of pathogens in their environments. However, immune systems are costly to maintain; like other animals, insects must balance their allocation of resources between investment in the immune system and other life-history traits (Lochmiller & Deernberg, 2000; Zuk & Stoehr, 2002). Baculoviruses are a key group of viruses specific to insects. Members of the genus *Alphabaculovirus* infect only lepidopteran species. Baculoviruses can regulate host population dynamics by causing epizootics in some outbreak populations (Cory & Myers, 2003; Myers & Cory, 2013). Lepidopteran hosts are at risk of infection through both horizontal and vertical transmission, and the virus can persist covertly (i.e., asymptomatic infection) in many populations (Cory, 2015; Williams *et al.*, 2017). Baculoviruses have also been developed and used as biological control agents for numerous lepidopteran pests (Moscardi, 1999; Lacey *et al.*, 2015).

Baculovirus virions are protected from environmental factors in proteinaceous structures called occlusion bodies (OBs). When an insect host consumes a food source that is contaminated with viral OBs, the OBs dissolve in the alkaline midgut, releasing infective virions that establish primary infections in midgut epithelial cells. Viral replication in the midgut cells produces another viral phenotype (budded virus) that is responsible for cell-to-cell transmission within the host (i.e., secondary infection); the infection spreads from the midgut cells to the tracheal epidermal cells, which act as a conduit for the virus to spread from the gut to other body tissues (Engelhard *et al.*, 1994).

In general, the susceptibility of an insect or the suitability of an insect for viral replication is often classified along a gradient from (1) fully permissive, where the host supports full virus replication, (2) semi-permissive, where the host supports partial

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replication, to (3) non-permissive, where the virus is unable to bind to and enter midgut cells, or virus entry occurs but replication is blocked, leading to failure to produce progeny virus (Mcintosh *et al.*, 2005). In semi-permissive and non-permissive insect hosts, successful midgut infection foci can be surrounded quickly by hemocytes and cleared by encapsulation and melanization processes (Trudeau *et al.*, 2003; Washburn *et al.*, 2000). While antiviral immune responses to secondary infection (systemic resistance) to the baculovirus Lymantria dispar nucleopolyhedrovirus (LdMNPV) occurs beyond the midgut (site of primary infection) in the permissive host *L. dispar* (Eribidae) (McNeil *et al.*, 2010a), most baculoviruses that are pathogenic to noctuid hosts cause secondary infection in host tissues without notable systemic resistance to the spread of viral infection (Engelhard & Volkman, 1995; Haas-Stapleton *et al.*, 2003). In semi- and non-permissive hosts resistance mechanisms can include failure to pass through the peritrophic matrix (Wang & Granados, 1997, 1998, 2000), inability to bind and enter midgut epithelial cells, apoptosis (Dougherty *et al.*, 2006) and sloughing of infected midgut cells prior to establishment of secondary infection (Hoover *et al.*, 2000; Washburn *et al.*, 1998), and encapsulation or apoptosis of tracheal infections as the virus attempts to spread beyond the midgut to establish a systemic infection (McNeil *et al.*, 2010a).

Importantly, both cellular and humoral components of insect innate immunity often work in concert to limit pathogen replication in insect hemolymph. Higher numbers of hemocytes, used as a proxy for greater cellular immunity, and higher hemolymph phenoloxidase (PO) activity, indicative of greater humoral immunity, have been associated with higher insect resistance to baculoviruses in some host-baculovirus systems (Lee *et al.*, 2006; Povey *et al.*, 2014), but not in others [i.e., one factor is correlated and the other is not (Shikano *et al.*, 2010, 2015a), or there is no correlation at all with these immune responses (Myers *et al.*, 2011; Saejeng *et al.*, 2010; Shikano *et al.*, 2015b). FAD-glucose dehydrogenase

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(GLD) can generate free radicals and reactive oxygen species derived from quinones during melanization and is hypothesized to actively kill invaders (Lee *et al.*, 2005) as well as strengthen melanized capsules (Cox-Foster & Stehr, 1994). Heightened GLD activity was triggered during encapsulation responses in *Manduca sexta* larvae following an immune inoculation by a parasitoid-polydnavirus complex or by hemocoelic injection of purified polydnavirus (Cox-Foster & Stehr, 1994; Lovallo & Cox-Foster, 1999), suggesting a potential role in systemic anti-viral resistance.

Activation of the insect immune system is associated with fitness costs (Armitage *et al.*, 2003; Sadd & Siva-Jothy, 2006). Costs associated with surviving baculovirus inoculation include reduced survival, prolonged development time, reduced pupal weight and lower fecundity (Milks *et al.*, 1998; Myers *et al.*, 2000, 2011; Monobrullah & Shankar, 2008; Shikano & Cory, 2015; Shikano *et al.*, 2016). However, Shikano and Cory (2015) demonstrated that *T. ni*, which is permissive to two baculovirus species (TnSNPV and AcMNPV), only exhibited significant fitness costs (reduced pupal weight and prolonged development) following TnSNPV-inoculation and not to AcMNPV-inoculation. The differential effects of these two viruses on *T. ni* development might stem from differences in host immune responses to the two baculoviruses.

In this study, we compared the temporal changes of immune responses and associated fitness costs of survivors of *Helicoverpa zea* larvae that were orally inoculated with a low dose of one of two baculoviruses: HzSNPV or AcMNPV. *H. zea* larvae are permissive to HzSNPV (Washburn *et al.*, 2001) and semi-permissive to AcMNPV (Trudeau *et al.*, 2001). Importantly, because baculoviruses are known to block the syntheses of many proteins in their hosts (Ikeda et al., 2013), we assessed temporal changes in hemolymph protein concentration as a measure of insect condition/health. PO and GLD activities per hemolymph

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volume were used as immune response measures. We then used PO and GLD activities relative to hemolymph protein concentration to determine how much protein was allocated to these two humoral defenses, which served as an indicator of the cost of immune responses.

## Materials and Methods

### *Study insects*

*H. zea* eggs were purchased from Frontier Agricultural Sciences (Newark, DE, USA). Larvae were raised individually on a wheat-germ based artificial diet and maintained at 25 ± 1°C and 16:8 (L:D) photoperiod. All third-instar larvae that were preparing to molt to the fourth instar, as indicated by head capsule slippage, were placed individually into an empty plastic cup (30 ml) and allowed to complete molting overnight (approximately 12 h). Newly molted fourth-instar larvae were weighed and inoculated with virus as described below.

### *Virus preparation and inoculation*

Wild-type occlusion bodies (OBs) of AcMNPV (strain C6) and HzSNPV (Gemstar) were obtained from Dr. Robert Harrison (USDA, ARS, Beltsiville, MD, USA). Both viruses were amplified in their fully permissive hosts: *Trichoplusia ni* larvae for AcMNPV and *H. zea* larvae for HzSNPV. Virus-killed cadavers were semi-purified by multiple rounds of centrifugation at 1000 *g* to remove debris (Scholefield, 2015). Aliquots of stock OB suspensions in distilled water were stored at -20°C. The concentration of OBs was estimated using an improved Neubauer brightline hemocytometer (0.1 mm deep; Hausser Scientific) at 400X magnification.

We used two different oral virus inoculation methods, diet plug feeding and delivery of inoculum suspension *per os* using a blunt-tipped needle attached to a tuberculin syringe (i.e., oral inoculation). Dose response assays were performed to determine the $LD_{15}$ to use in

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the experiments. For the diet plug method, a single piece of artificial diet (2 × 3 mm; d × l) was placed in individual wells of a 12-well cell culture plate. A 1 µl suspension containing viral OBs in distilled water was applied to each diet plug and a newly molted 4$^{th}$ instar larva was placed in each well. Control larvae were provided with diet plugs treated with distilled water. All larvae consumed the entire diet plug within 30 min once they started feeding, but since some larvae took longer to start feeding, all larvae were maintained in their individual wells for 2 h to ensure that all larvae consumed the entire diet plug. Individual larvae were then transferred to a plastic cup (30 ml) with *ad libitum* artificial diet.

Direct oral inoculation was performed using a Pax-100 microapplicator (Burkhard Scientific, Uxbridge, UK) equipped with a syringe fitted with a 32-gauge stainless steel blunt tip needle (Popper & Sons, New Hyde Park, NY). OBs were diluted in a high viscosity glycerol solution to keep the OBs from sinking to the bottom of the syringe. A 1 µl suspension containing OBs in 60% glycerol was delivered through the mouth to the anterior midgut of newly molted 4$^{th}$ instar larvae. Control larvae were inoculated with 60% glycerol only. Larvae were immediately transferred to a new plastic cup (30 ml) with *ad libitum* diet.

Larvae are more susceptible to OBs when inoculated directly into the midgut than through ingestion of diet plugs. Thus, oral inoculation of HzSNPV directly into the midgut resulted in nearly 100% mortality even at very low doses, such that a reliable $LD_{15}$ could not be determined using this method. Similarly, ingestion of AcMNPV OBs on diet plugs did not kill a high enough proportion of larvae at extremely high doses to produce a reliable $LD_{15}$. Therefore, we used the diet plug method for HzSNPV ($LD_{15}$ = 130 OBs per larva) and the oral inoculation method for AcMNPV ($LD_{15}$ = 520 OBs per larva). Given the 2 h time difference in full dose delivery using the two inoculation methods, we did not make direct comparisons of host responses to the two viruses at specific time points post-inoculation.

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*Hemolymph collection*

To examine immune responses to baculovirus inoculation, hemolymph was collected at 24 h intervals from 0 to 96 h post-inoculation (hpi); 96 h was the final time point for this study because by 120 hpi some control larvae and survivors of virus inoculation had initiated pupation. A separate group of larvae from the same cohort (i.e., inoculated at the same time) was sampled at each time point so that the same larva was not bled more than once. In total, 14 µl of hemolymph was collected from each larva and allocated as follows: 2 µl for hemocyte counts, 6 µl for GLD activity assays and 6 µl for both PO activity and protein concentration assays. Hemocyte counts were conducted immediately after hemolymph collection. Hemolymph samples for GLD, PO and protein assays were immediately frozen in liquid nitrogen and stored at -80°C until each assay was performed. The number of insects processed at each time point varied from 18 to 28 for both AcMNPV-inoculated and the corresponding control group. For HzSNPV-inoculated and the corresponding control group, the number of insects processed at each time point varied from 17 to 27. To collect hemolymph, larvae were cooled on ice, an anal proleg was excised with dissecting scissors, and the hemolymph was harvested with a pipette. Larvae were euthanized after hemolymph was collected. Each larva was weighed immediately prior to sampling to include larval weight as a covariate for statistical analyses.

*Glucose dehydrogenase (GLD) activity*

GLD activity was measured according to Lovallo and Cox-Foster (1999) with some modifications. Six µl of hemolymph was mixed with 50 µl of ice-cold PBS (pH 7.4) and centrifuged to remove cell debris (10,000g, 5 min, 4°C). Fifty µl of the mixture was placed into individual wells of a 96-well microplate, followed by 150 µl of Tris-HCl (0.1 mol/L, 48

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μmol/L 2, 6-dichlorophenolindophenol, 76 mmol/L β-D-glucose, pH 7.2). The microplate was then placed in a spectrophotometer (SpectraMax 190, USA), and the absorbance was read at 600 nm every 45 s for 40 min at 25°C. The enzymatic rate of GLD was represented by the change in optical density per minute (slope) during the linear phase of the reaction. GLD activity was assessed as the activity of GLD per volume of hemolymph (mOD/min/μl hemolymph) and as the investment in GLD relative to hemolymph protein content (mOD/min/mg protein).

*Phenoloxidase (PO) activity*

Baseline PO (activated PO) activity was measured as described by Shikano et al. (2010) with minor modifications. Six μl of hemolymph sample was thawed and combined with 55 μl of ice-cold PBS (pH 7.4), then centrifuged to remove cell debris (10,000 g, 5 min, 4°C). Twenty-five μl of the mixture was combined with 175 μl of 11.3 mmol/L dopamine hydrochloride solution in individual wells of a 96-well microplate. Absorbance was read at 492 nm every 30 s for 40 min at 25°C. The enzymatic rate of PO was represented by the change in optical density per minute (slope) during the linear phase of the reaction. PO activity was assessed as the activity of PO per volume of hemolymph (mOD/min/μl hemolymph) and as the investment in PO relative to hemolymph protein content (mOD/min/mg protein).

*Protein concentration*

The protein concentration of hemolymph was measured by the Bradford assay (Bradford, 1976). Five μl of hemolymph-PBS sample was obtained from the remaining sample from the PO assay. Each sample was placed in an individual well of a 96-well microplate with 200 μl of Coomassie blue stain. The plate was incubated at room temperature for 5 min, and an endpoint reading was taken at 595 nm. A dilution series of bovine serum

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albumin (BSA), ranging from 0 to 2 mg/ml, was used to produce a standard curve. Hemolymph protein concentration also served as a measure of insect condition (Handke *et al.*, 2013; Shikano *et al.*, 2015a).

*Hemocyte numbers*

Total hemocyte numbers were counted according to Shikano *et al.* (2010). Two microliters of hemolymph were gently mixed with 12 µl of cold phosphate buffered saline (PBS, pH 7.4). The entire mixture was pipetted into an improved Neubauer brightline hemocytometer, and the hemocytes were counted using a compound microscope at 400X magnification.

*Fitness cost measures*

In a separate experiment, newly molted 4$^{th}$ instar larvae were weighed and inoculated with each virus as described above and supplied with diet *ad libitum*; control larvae were treated with distilled water or 60% glycerol and fed on diet *ad libitum*. We used 70 larvae for AcMNPV-inoculation and 56 larvae for their corresponding controls (58 AcMNPV-inoculated larvae and all 56 control larvae survived until pupation). For HzSNPV-inoculation treatments, 62 larvae were inoculated with virus and 38 larvae were used as untreated controls (53 of HzSNPV-inoculated larvae and 38 control larvae survived until pupation). Each larva was defined as a replicate. To estimate fitness costs associated with surviving virus inoculation, we compared daily larval weight, development time (from time of virus inoculation until initiation of pupation) and pupal weight between control larvae and larvae that survived virus-inoculation.

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*Statistical analyses*

Differences in insect condition and immune responses (protein concentration, GLD activity, PO activity and hemocyte numbers) were analyzed separately for each virus using two-way analysis of covariance (ANCOVA). If there was a significant ($P < 0.05$) or marginally significant ($P < 0.1$) interaction between treatments (control or virus-inoculated) and time post-inoculation, a post-hoc analysis was conducted using Tukey's honestly significant difference test to detect significant differences between treatments at each time point. As immune measures can vary significantly with larval weight (Vogelweith et al., 2013), we accounted for differences in larval weight at each time point by including weight as a covariate for all data analyses. GLD and PO activities relative to protein content in the hemolymph of HzSNPV-inoculated and control larvae were $\log_{10}$ transformed to meet the assumptions of normality. PO activity per volume of hemolymph, PO activity relative to protein content, and protein concentration in hemolymph of AcMNPV-inoculated and control larvae were square-root transformed to meet the assumptions of normality. Larval weight as a measure of fitness costs were analyzed with repeated measures ANOVA on the same cohort of larvae from each treatment and the Greenhouse-Geisser correction was applied for sphericity. Fisher's least significant difference (LSD) test was employed as a post-hoc test to compare control and virus-inoculated larvae. Pupal weight and development time were analyzed using Student's *t*-test.

**Results**

*Mortality from each treatment group post virus inoculation*

Inoculation of *H. zea* larvae with an $LD_{15}$ of AcMNPV by the oral inoculation (blunt-needle syringe) method resulted in mortality of 17.1% within 4-19 days post-inoculation;

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inoculation with an $LD_{15}$ of HzSNPV by the diet plug inoculation method produced 14.5% mortality within 3-5 days post-inoculation.

*Insect condition/health: hemolymph protein concentration following virus inoculation*

*AcMNPV*. The hemolymph protein concentration of AcMNPV-inoculated larvae varied over time (Fig. 1A, virus inoculation by time interaction: $F_{3,191} = 6.84$, $P = 0.0001$). Larvae exposed to AcMNPV had similar protein concentrations in their hemolymph relative to control larvae at 24 and 48 hpi, but had significantly lower protein concentrations in their hemolymph than control larvae at 72 and 96 hpi. Smaller larvae at the time of hemolymph collection had lower concentrations of protein in their hemolymph (covariate: $F_{1,197} = 6.75$, $P = 0.01$).

*HzSNPV*. HzSNPV inoculation did not significantly influence hemolymph protein concentration at any time point post-inoculation (Fig. 1B, HzSNPV-inoculation: $F_{1,180} = 1.01$, $P = 0.32$; virus inoculation by time interaction: $F_{1,174} = 1.36$, $P = 0.26$), but protein concentrations fluctuated over time ($F_{3,178} = 33.24$, $P = 0.0001$). Larval weight was positively associated with protein concentration in the hemolymph (covariate: $F_{1,180} = 20.47$, $P = 0.0001$). Protein concentration was lowest at 72 hpi when most larvae were preparing to molt (i.e., undergoing head capsule slippage).

*Humoral defense response: GLD activity post virus inoculation*

*AcMNPV*. GLD activity per volume of hemolymph was significantly higher in AcMNPV-inoculated larvae than in control larvae ($F_{1,180} = 6.91$, $P = 0.009$; Fig. 2A) with fluctuation over time ($F_{3,178} = 44.60$, $P = 0.0001$). However, significant interaction was not observed between AcMNPV-inoculation and time post-inoculation ($F_{3,174} = 1.11$, $P = 0.35$).

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Larval weight did not influence GLD activity per volume of hemolymph (covariate: $F_{1,180}$ = 0.03, $P$ = 0.86).

Interestingly, when GLD activity was examined as a function of hemolymph protein content, it showed that there was a significant interaction between AcMNPV-inoculation and time post-inoculation ($F_{3, 168}$ = 4.33, $P$ = 0.006; Fig. 2B). Post-hoc analysis showed that larval investment in GLD activity relative to total protein content did not differ significantly between AcMNPV-inoculated larvae and control larvae at 24 and 48 hpi, but was significantly higher in AcMNPV-inoculated larvae relative to control larvae at 72 and 96 hpi. GLD activity relative to protein content was not influenced by larval weight (covariate: $F_{1,174}$ = 0.03, $P$ = 0.87).

*HzSNPV*. HzSNPV inoculation significantly elevated GLD activity per volume of hemolymph compared to control larvae ($F_{1,193}$ = 6.53, $P$ = 0.011; Fig. 2C), The levels of GLD activity varied at different time points ($F_{3,191}$ = 143.77, $P$ = 0.0001) and was marginally influenced by larval weight (covariate: $F_{1,193}$ = 2.91, $P$ = 0.09), but there was no significant interaction between HzSNPV-inoculation and time post HzSNPV inoculation ($F_{3,187}$ = 1.82, $P$ = 0.15).

When GLD activity was examined as a function of hemolymph protein content, it showed that GLD activity varied marginally between HzSNPV-inoculated and control larvae at different time points post virus inoculation (virus inoculation by time interaction: $F_{3,185}$ = 2.59, $P$ = 0.06; Fig. 2D). Notably, GLD activity per mg protein was significantly lower in control larvae than HzSNPV inoculated larvae at 72 hpi. GLD activity per mg protein increased with increasing larval weight (covariate: $F_{1,191}$ = 3.91, $P$ = 0.05).

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*Humoral defense response: PO activity post virus inoculation*

*AcMNPV*. PO activity per volume of hemolymph was not significantly influenced by AcMNPV-inoculation ($F_{1,198} = 0.19$, $P = 0.67$), but increased significantly over time ($F_{3,196} = 14.51$, $P = 0.0001$; Fig. 3A). There was no significant interaction between AcMNPV inoculation and time post-inoculation ($F_{3,192} = 1.96$, $P = 0.12$). Larval weight did not significantly influence PO activity per volume of hemolymph (covariate: $F_{1,198} = 1.83$, $P = 0.18$).

When PO activity was assessed as a function of hemolymph protein content, there was a significant interaction between AcMNPV-inoculation and time post-inoculation ($F_{3,191} = 5.37$, $P = 0.001$; Fig. 3B). Larval investment in PO activity relative to protein content did not differ significantly between AcMNPV-inoculated and control larvae at 24, 48 and 96 hpi, but was significantly higher in AcMNPV-inoculated larvae than controls at 72 hpi. Larval weight had no effect on PO activity relative to protein content (covariate: $F_{1,198} = 0.03$, $P = 0.86$).

*HzSNPV*. PO activity per volume of hemolymph exhibited an increasing trend over time ($F_{3,194} = 44.78$, $P = 0.0001$; Fig. 3C), but HzSNPV-inoculation did not significantly alter PO activity ($F_{1,196} = 3.41$, $P = 0.07$). There was no significant interaction between virus inoculation and time post-inoculation ($F_{3,190} = 0.26$, $P = 0.86$).

When PO activity was examined as a function of protein content, there was a prominent interaction between virus inoculation and time post inoculation ($F_{3,189} = 3.15$, $P = 0.03$; Fig. 3D). Post-hoc analysis showed that larval investment in PO activity relative to protein content did not differ significantly between HzSNPV-inoculated larvae and control

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larvae at 24, 48 and 96 hpi, but was markedly higher in HzSNPV-inoculated larvae relative to control larvae at 72 hpi. Smaller virus-inoculated larvae had higher PO activity per mg protein compared to control larvae (covariate: $F_{1,195} = 13.97$, $P = 0.0001$).

*Cellular defense response: number of hemocytes post virus inoculation*

*AcMNPV*. Although the number of hemocytes per volume of hemolymph varied over time ($F_{3,198} = 8.48$, $P = 0.0001$), AcMNPV-inoculated and control larvae had comparable hemocyte numbers across time points post inoculation (virus inoculation, $F_{1,200} = 0.59$, $P = 0.45$; virus inoculation by time interaction, $F_{3,194} = 0.97$, $P = 0.41$) (Fig. 4A). Larval weight did not significantly alter hemocyte numbers (covariate: $F_{1,200} = 0.65$, $P = 0.42$).

*HzSNPV*. Total hemocyte numbers per volume of hemolymph did not differ significantly between HzSNPV inoculated larvae and controls (virus inoculation, $F_{1,197} = 0.10$, $P = 0.92$; virus inoculation by time interaction, $F_{3,191} = 0.49$, $P = 0.69$) (Fig. 4B). It fluctuated over time ($F_{3,195} = 6.63$, $P = 0.0001$) and was not significantly affected by larval weight (covariate: $F_{1,197} = 0.22$, $P = 0.64$).

*Fitness costs of survivors post virus inoculation*

*AcMNPV*. The daily weight of larvae that survived from AcMNPV inoculation was influenced by an interaction between AcMNPV inoculation and time post-inoculation ($F_{4, 560} = 1300.47$, $P = 0.0001$). Control larvae were heavier than AcMNPV-inoculated larvae at 72 and 96 hpi. This suggests that AcMNPV-inoculated larvae grew at a similar rate as control larvae for the first 48 h post-inoculation, but growth slowed for virus-inoculated larvae by 72 hpi (Fig. 5A). The survivors of AcMNPV-inoculation took significantly longer to reach the prepupal stage (Fig. 6A: $t_{1,112} = -4.506$, $P = 0.0001$) and the resulting pupae weighed significantly less than their control counterparts (Fig. 6C: $t_{1,112} = 3.936$, $P = 0.0001$).

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*HzSNPV*. HzSNPV-inoculation did not significantly influence the growth of larvae (HzSNPV-inoculation: $F_{1,\ 453} = 1.199$, $P = 0.28$; HzSNPV-inoculation by time interaction: $F_{4,445} = 1.743$, $P = 0.14$) (Fig. 5B). Unlike the survivors of AcMNPV inoculation, survivors of HzSNPV inoculation pupated significantly earlier than control larvae (Fig. 6B: $t_{1,89} = 3.854$, $P = 0.0001$), and they produced lighter pupae than control larvae (Fig. 6D: $t_{1,\ 60.396} = 2.919$, $P = 0.008$).

**Discussion**

Baculovirus-inoculation triggered changes to some measured immune responses and these effects varied with virus identity. AcMNPV and HzSNPV-inoculated *H. zea* larvae both exhibited a generally elevated level of GLD activity per volume of hemolymph and elevated levels of GLD activity relative to protein content at one or more time points post virus-inoculation compared to control larvae. These results indicated that GLD is likely involved in the larval response to virus-inoculation. In contrast, AcMNPV and HzSNPV inoculation did not impact PO activity per volume of hemolymph. When PO activity was assessed relative to the protein content of the hemolymph, larvae inoculated with either virus showed about a two-fold higher level of activity at 72 hpi than controls. GLD activity per unit protein of AcMNPV and HzSNPV-inoculated larvae was also two-fold higher than control larvae at 72 hpi, which occurred when most virus-inoculated and control larvae were undergoing head capsule slippage (i.e., beginning the molting process). Since timing of the onset of molting did not differ between virus-inoculated and control larvae, this suggests that the observed differences in GLD and PO activity were likely related to infection, not the molting process. Reduced hemolymph protein concentration associated with virus inoculation appeared to be the driver of higher activity of GLD and PO per unit protein at 72 hpi compared to control larvae. This suggests that virus-inoculated larvae had less hemolymph protein during molting

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than mock-inoculated larvae, allocating a higher proportion of total protein to maintain baseline levels of GLD and PO activities.

GLD is involved in the encapsulation and melanization of infected cells and direct killing of pathogens, through enzymatic cascades that lead to the production of free radicals and reactive oxygen species (Cox-Foster & Stehr, 1994). The increased levels of GLD activity in AcMNPV-inoculated *H. zea* larvae is in line with the encapsulation and melanization of infected tracheal cells previously reported in this system (Trudeau *et al.*, 2001). However, induction of GLD in response to baculovirus infection may not be universal or it may depend on viral dose. GLD activity was not increased following LdMNPV-inoculation of fourth instar *L. dispar* larvae at the $LD_{50}$ dose (McNeil *et al.*, 2010a). In *T. ni* that were 18 h post inoculation with TnSNPV, GLD activity was higher than in control larvae at doses that caused 62% mortality and above, but not at a dose that caused 20% mortality (Scholefield *et al*., 2019).

In a previous study, the encapsulation and melanization of AcMNPV-infected tracheal cells in *H. zea* was associated with a dramatic induction of PO activity per volume of hemolymph 72 h after oral inoculation with AcMNPV (using the $LD_{60}$ dose) (Trudeau *et al.*, 2001). However, we found no induction of PO activity after AcMNPV-inoculation using the $LD_{15}$ dose. In fact, PO activity was lower in AcMNPV-inoculated larvae at 96 hpi than in control larvae. Although PO can have virucidal effects (Shelby & Popham, 2006) and has been associated with higher resistance of some lepidopteran hosts to baculovirus-inoculation (Lee *et al.*, 2006; Povey *et al.*, 2014), the role of PO in host resistance against baculoviruses is highly variable. Levels of PO activity are known to fluctuate considerably with the food plant or nutritional quality of the host's diet and among individual insects (Lee *et al.*, 2006; Povey *et al.*, 2014; Shikano *et al.*, 2010; 2015a). It is difficult to ascertain why our results

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differ from those of Trudeau *et al.* (2001). Possibly, the $LD_{15}$ dose used in our study was not high enough for AcMNPV to establish in the midgut epithelium and spread systemically, and thus may not have induced a strong systemic immune response involving PO. Using a range of viral doses and time points post virus-inoculation, Scholefield *et al.* (2019) showed that PO activity following TnSNPV-inoculation in *T. ni* (permissive host of TnSNPV) varied depending on the dose and time post inoculation. However, PO activity in this system was never higher in TnSNPV-inoculated larvae than in control larvae at any virus dose or time post inoculation. In fact, PO activity tended to be lower at higher virus doses compared to a low dose or control.

Hemocytes are critical components of insect immune systems. However, in our study, total hemocyte numbers in *H. zea* larvae were not affected by AcMNPV or HzSNPV inoculation. Hemocytes can encapsulate baculovirus-infected tissues and cells, and clear virus from the hemolymph in *H. zea*, *M. sexta*, *H. virescens* and *L. dispar* (McNeil *et al.*, 2010a; Trudeau *et al.*, 2001; Washburn *et al.*, 2000). In some host-baculovirus systems, a proportion of host hemocytes can be resistant to baculovirus infection (Hori *et al.*, 2013; McNeil *et al.*, 2010b; Rivkin *et al.*, 2006). However, because several different types of hemocytes have been identified in Lepidoptera that have different or no involvement in immune responses to viruses (Cox-Foster & Stehr, 1994; Lavine & Strand, 2002), there may have been changes in the proportion of types of hemocytes in response to infection without changing total hemocyte numbers, which was not accounted for in our study. Some studies have documented changes in the total hemocyte numbers of baculovirus-inoculated caterpillars, but this effect may depend on environmental conditions. For example, *T. ni* larvae inoculated with AcMNPV ($LD_{20}$) had reduced total hemocyte numbers compared to controls when fed a low protein, high carbohydrate diet, but maintained the same number of hemocytes when fed a high protein, low carbohydrate diet (Shikano *et al.*, 2016). In contrast, *S. exempta* larvae

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inoculated with the $LD_{10}$ of SpexNPV had the same numbers of hemocytes as controls when fed a low protein, high carbohydrate diet, but SpexNPV inoculation resulted in increasingly higher hemocyte numbers in their host as the protein content of the host diet increased relative to carbohydrate (Povey *et al.*, 2014). It is possible that under different environmental (dietary) conditions, we may have seen an induction or suppression of total numbers or types of hemocytes associated with baculovirus-inoculation.

Surviving a baculovirus infection can come at the cost of other life-history traits. These survival costs include prolonged development time, reduced pupal weight and lower fecundity (Milks *et al.*, 1998; Myers *et al.*, 2000, 2011; Shikano & Cory, 2015; Shikano *et al.*, 2016). We used hemolymph protein concentration as a measure of insect condition (e.g., Handke *et al.*, 2013; Shikano *et al.*, 2015a), and found that it was positively associated with larval weight gain post inoculation. The *H. zea* larvae that survived AcMNPV inoculation gained weight at the same rate as controls for the first 48 hpi, but by 72 hpi, which was at the beginning of head capsule slippage, their growth rate slowed (Fig 5A). This resulted in a longer development time to pupation and lighter pupae compared with the controls. This slower growth was associated with reduced protein concentration in the hemolymph, and an increased allocation of that reduced protein to GLD and PO activity. In contrast, the *H. zea* larvae that were inoculated with HzSNPV gained weight at the same rate as their corresponding control larvae, which resulted in a shorter development time to pupation, but lighter pupae. These results suggest that resistance of *H. zea* to both viruses was costly, but may have been more costly in response to AcMNPV-inoculation since developmental time was significantly slower.

Importantly, the two viruses were introduced into the hosts using different methods. Since there were no significant differences in pupal weight and development time between

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control insects that ingested a blank diet plug or were orally injected with a blank suspension (pupal weight, $F_{1,92} = 0.51$, $P = 0.80$; development time, $F_{1,85.642} = 9.34$, $P = 0.75$), we think that the virus delivery method itself had no impact on larval development. Also, though the susceptibility of larvae to viruses differs depending on the delivery method, if we had used diet plugs for oral delivery of AcMNPV, we would have required an extremely high dose to obtain the same level of mortality (15%). Nonetheless, since we cannot rule out potential differences in host responses to the virus delivery methods, we exercised caution to not directly compare the timing of immune responses elicited by the two viruses.

Here, we presented the dynamic responses of a host to virus inoculation, using low doses of AcMNPV and HzSNPV on their common host, *H. zea*, which is semi-permissive and fully permissive to the viruses, respectively. *H. zea* that survived virus inoculation exhibited different developmental responses depending on the identity of the invading pathogen, but both were costly and resulted in the production of pupae with reduced weights. Our study highlights the complexity of host-pathogen interactions, whereby the identity of the pathogen can differentially impact immune responses and fitness of surviving hosts.

**Acknowledgments**

Financial support was provided in part by the United States Department of Agriculture (AFRI 2017-67013-26596) awarded to GWF and KH; National Science Foundation (IOS-1645548) awarded to GWF, IS, and KH; and Hatch Project PEN04576 (GWF and KH). QP thanks the financial support from China Scholarship Council (grant 201506300111). IS thanks the financial support from Natural Sciences and Engineering Research Council of Canada Postdoctoral Fellowship (NSERC PDF-488105-2016). We thank Dr. Robert Harrison (United States Department of Agriculture, Agricultural Research

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Service, Beltsville, Maryland, USA) for providing baculovirus OBs used in this study. We gratefully acknowledge Francine McCullough for her technical assistance.

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**Disclosure**

All authors declare no conflicts of interest.

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**Figure legends**

**Fig. 1 Protein concentration (mean + SE) in hemolymph of *H. zea* larvae.** (A) Control and AcMNPV-inoculated *H. zea* larvae, (B) control and HzSNPV-inoculated *H. zea* larvae. The asterisk indicates a significant effect (pairwise comparison; $P < 0.05$) of treatment relative to the control at that time point (n = 17-28 insects for each treatment).

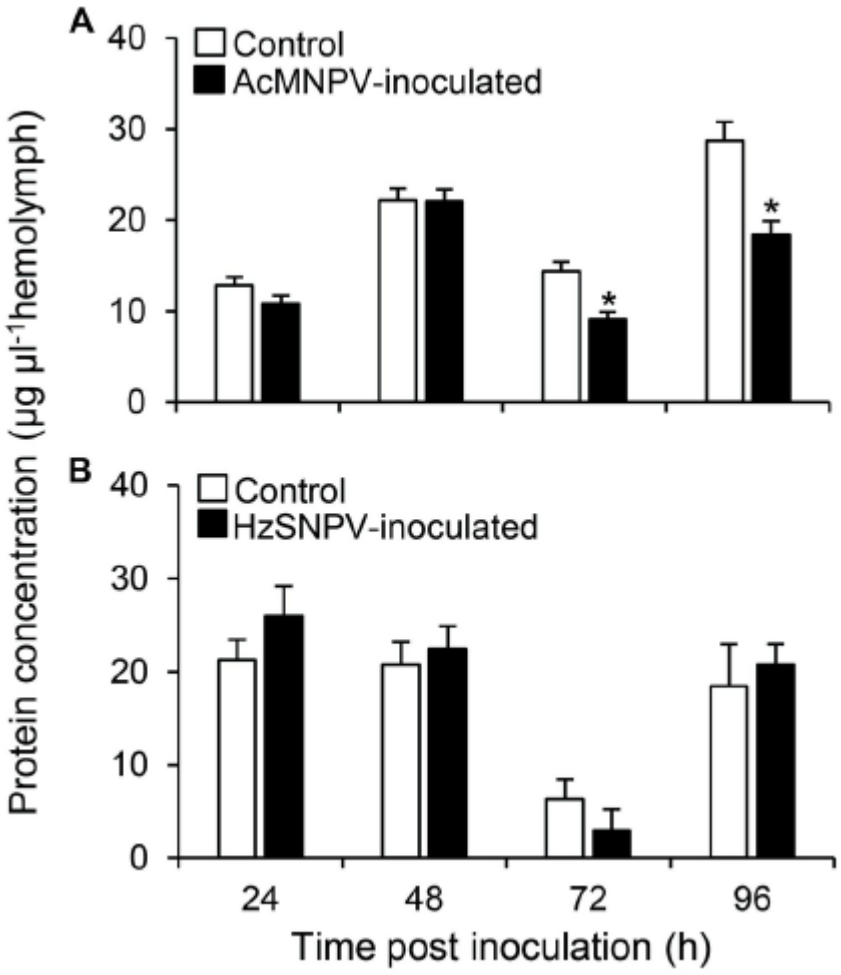


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**Fig. 2 GLD activity (mean + SE) in hemolymph of *H. zea* larvae.** (A) GLD activity per volume of hemolymph and (B) GLD activity relative to protein content in hemolymph of control and AcMNPV-inoculated *H. zea* larvae, (C) GLD activity per volume of hemolymph and (D) GLD activity relative to protein content in hemolymph of control and HzSNPV-inoculated *H. zea* larvae. The asterisk indicates a significant effect (pairwise comparison; $P < 0.05$) of treatment relative to the control at that time point (n = 18-25 insects for each treatment).

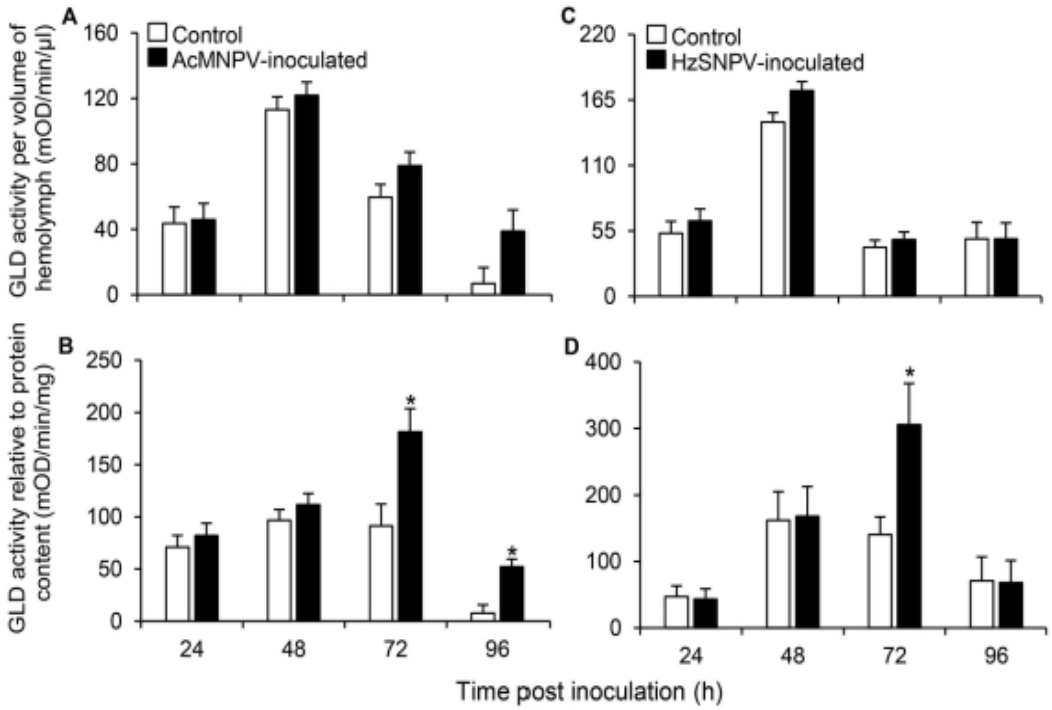


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**Fig. 3 PO activity (mean + SE) in hemolymph of *H. zea* larvae.** (A) PO activity per volume of hemolymph and (B) PO activity relative to protein content in hemolymph of control and AcMNPV-inoculated *H. zea* larvae, (C) PO activity per volume of hemolymph and PO activity relative to protein content in hemolymph of control and HzSNPV-inoculated *H. zea* larvae. The asterisk indicates a significant effect (pairwise comparison; $P < 0.05$) of treatment relative to the control at that time point (n = 24-28 insects for each treatment).

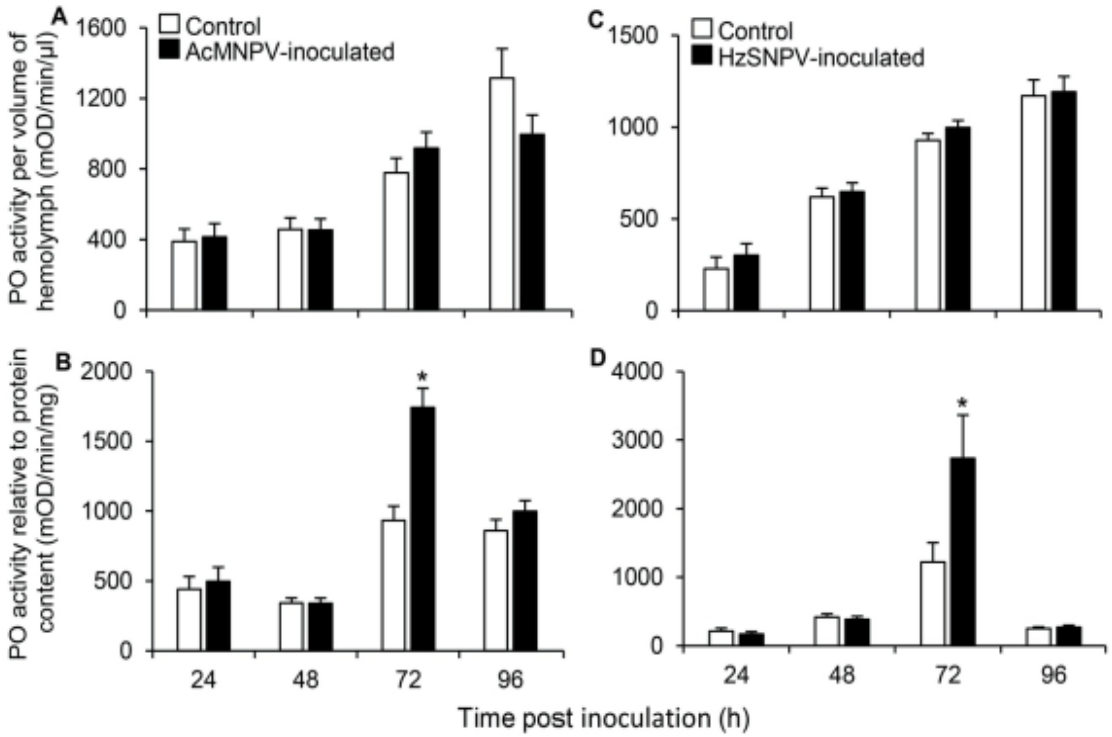


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**Fig. 4 Total hemocyte numbers ($10^3$) in hemolymph (mean + SE) of *H. zea* larvae.** (A) Control and AcMNPV-inoculated *H. zea* larvae, (B) control and HzSNPV-inoculated *H. zea* larvae. The asterisk indicates a significant effect (pairwise comparison; $P < 0.05$) of treatment relative to the control at that time point (n = 23-28 insects for each treatment).

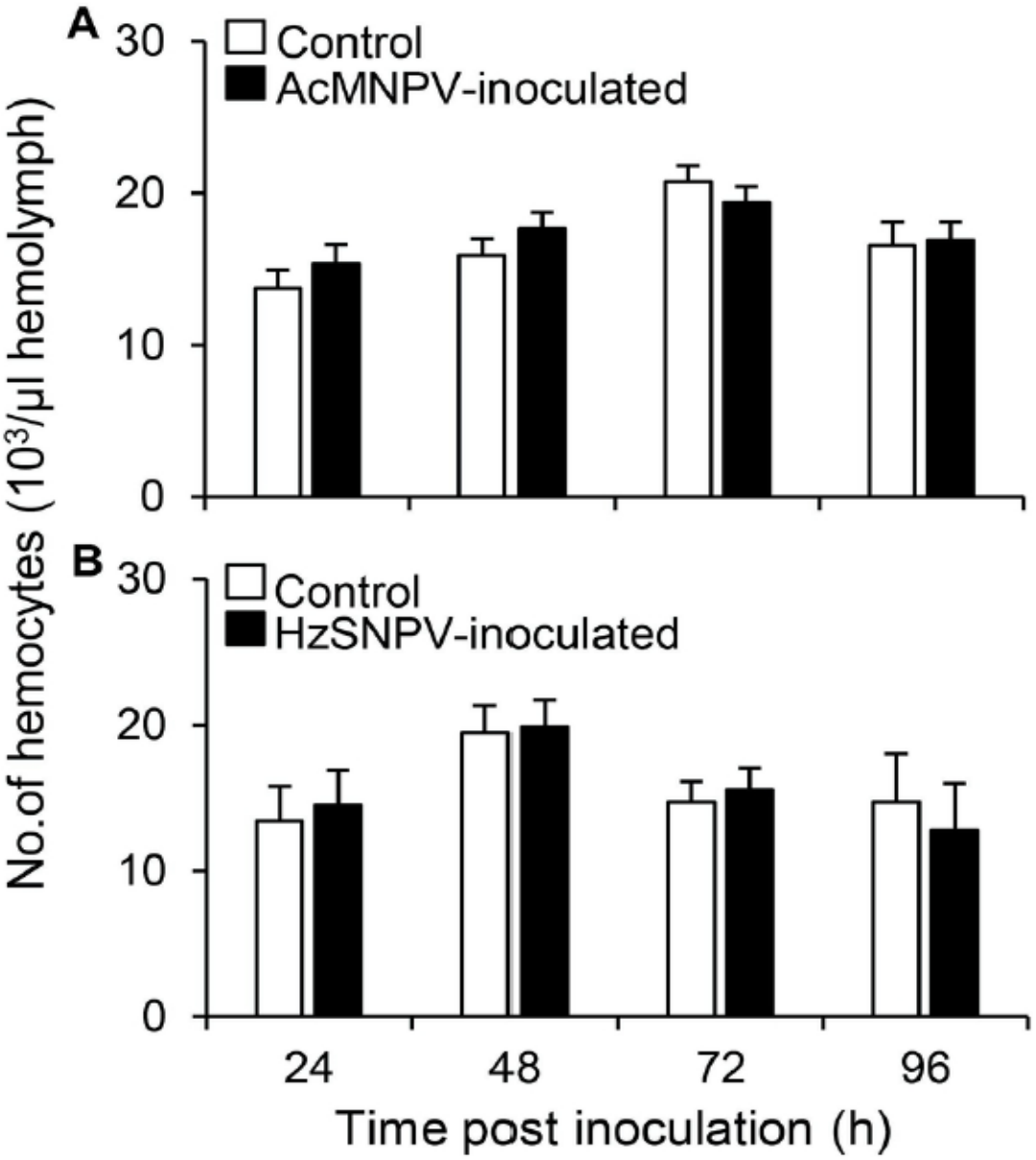


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**Fig. 5 Larval weight (mean + SE) of *H. zea* larvae.** (A) Control and AcMNPV-inoculated *H. zea* larvae, (B) control and HzSNPV-inoculated *H. zea* larvae. The asterisk indicates a significant effect (pairwise comparison; $P < 0.05$) of treatment relative to the control at that time point (n = 38-58 insects for each treatment).

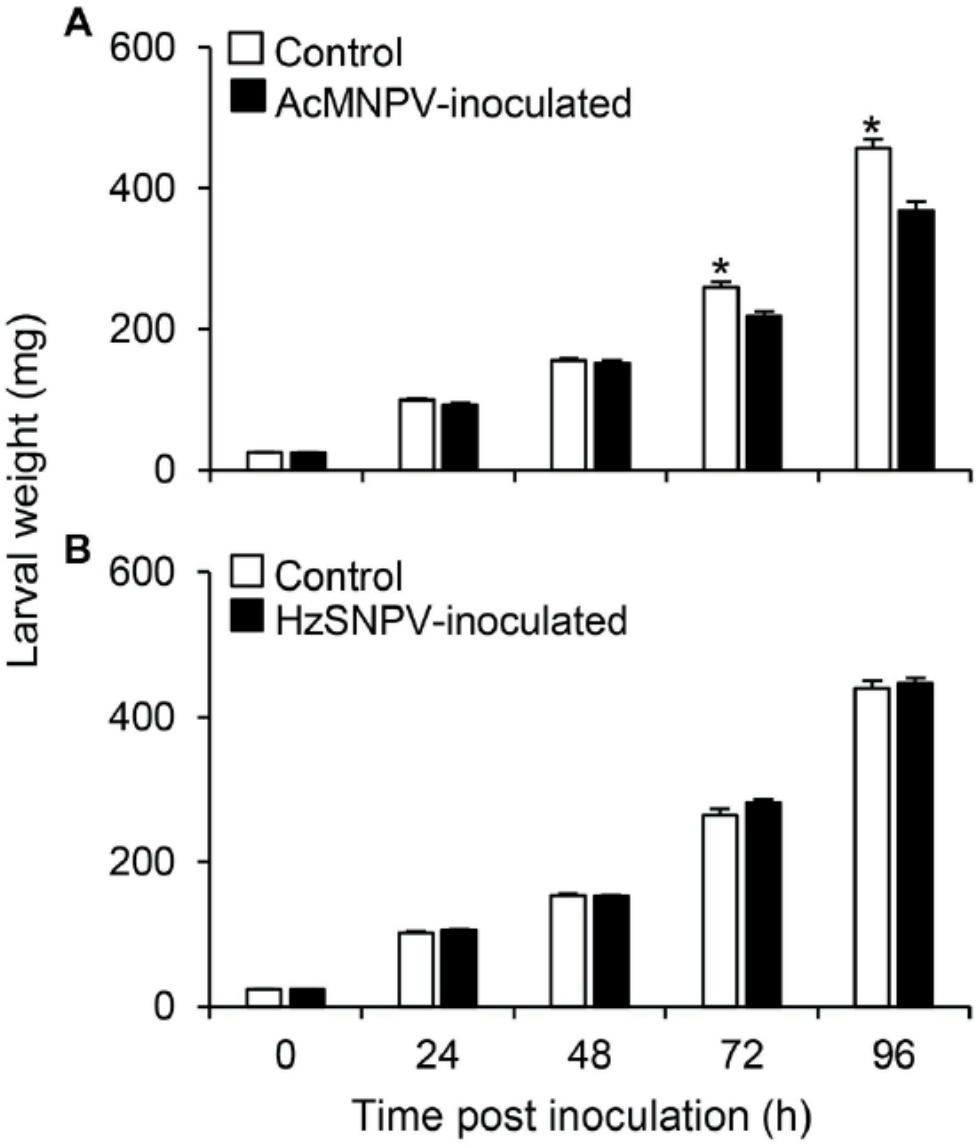


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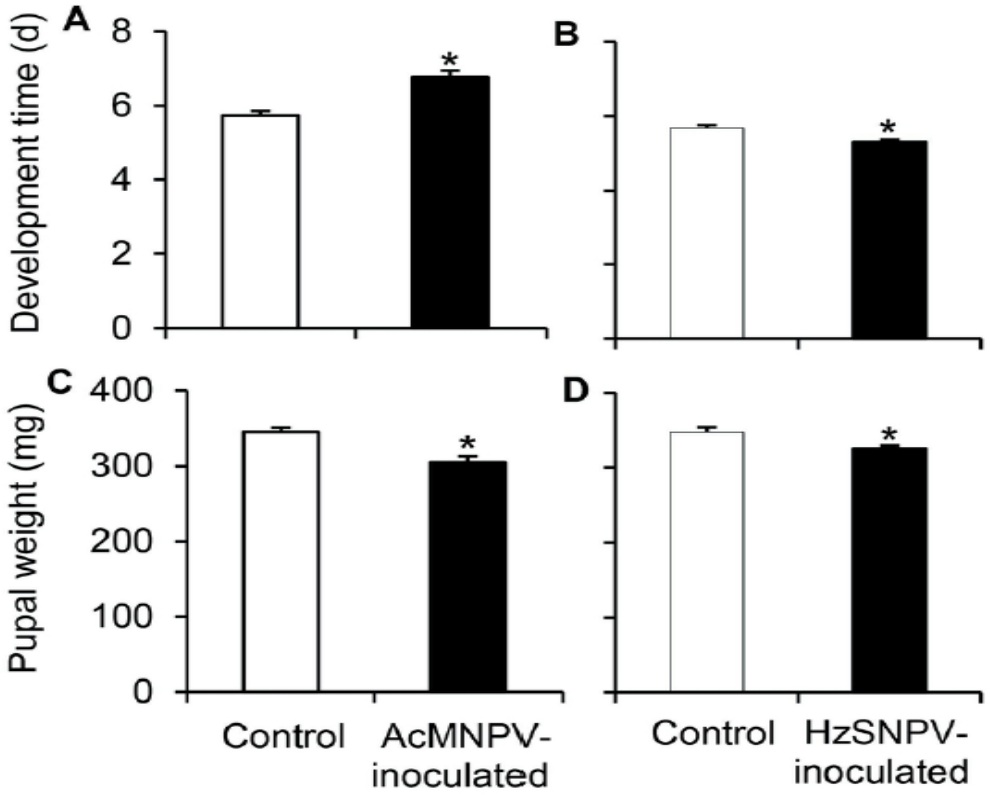
**Fig. 6 Developmental time and pupal weight of *H. zea* larvae at the $LD_{15}$ dose.** (A) Control and AcMNPV-inoculated *H. zea* larvae, (C) control and HzSNPV- inoculated *H. zea* larvae from fourth instar to initiation of pupation. Pupal weight of (B) control and AcMNPV-inoculated *H. zea* larvae, (D) control and HzSNPV-inoculated *H. zea* larvae. The asterisk indicates a significant effect (pairwise comparison; $P < 0.05$) of treatment relative to the control. Values are means (+ SE) (n = 38-58 insects for each treatment).

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